# Molecular characterisation of the underlying mechanisms of pathogen-associated molecular pattern (PAMP) recognition in fish

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**Thesis**

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# Chapter I

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# General background

**Abstract**

**The innate immune response is based upon the activation of a restricted number of genotypic encoded receptors, the pathogen recognition receptors (PRRs). PRRs can be soluble proteins such as plasmatic PGRPs or cell membrane-anchored TLRs able to recognize pathogens or their pathogen-associated molecular patterns (PAMPs). PAMP-PRR interaction results in the activation of target genes and promotes the production of pro- and inflammatory mediators. The main goal of this dissertation was to characterise the responses of rainbow trout, *Oncorhynchus mykiss*, and gilthead seabream, *Sparus aurata*, macrophages treated with different PAMPs and to explore subsequent changes in the expression of immune related genes or global shifts in the macrophage transcriptome. A specific goal of this study was to register changes in macrophages activated toward an inflammatory phenotype after treatments with crude gram negative bacterial lipopolysaccharide (LPS) preparations, highlighting that peptidoglycan (PGN) is a contaminant within crude LPS. PGN is able to induce the mRNA expression of IL-1β and IL-6 and release inflammatory products such as prostaglandins. Microarray analyses were made to describe concentration and time-dependent transcriptional modulations both in trout and seabream macrophages treated with PGN or LPS. In the case of sea bream, a specific oligonucleotide microarray was designed and validated for these studies. Results reveal up-regulation of specific mRNA transcripts that are closely related to prostaglandin synthesis and TLR signalling pathways. Thus PGN recognition in fish is a result of recognition mechanisms including non-TLR PRRs such as PGRPs and NODs. These mechanisms appear to be conserved throughout the vertebrate innate immune response.**

## 1. Immune response in vertebrates

All organisms have protective mechanisms that perceive and act against potentially harmful agents that are eliminated by a set of biodestructive mechanisms resulting in survival of the individual. Recognition mechanisms are conserved throughout the animal kingdom whether the agent be a viral, bacterial or fungal pathogen (PAMPs), or an autologous agents that are collectively named as danger associated molecular patterns (DAMPS) [1]. However the use of protective biodestructive mechanisms requires efficient communication to regulate the magnitude of the immune response that can be potentially harmful to the host itself, such as septic shock in mammals [1-4]. The molecular origins including both recognition and effector mechanisms of the vertebrate innate immune response, also named as the non-specific immune response, can be observed throughout the invertebrates [5,6].

A fundamental requirement is the capacity to recognize pathogens (targets) with considerable specificity at the molecular level. In both the invertebrates and vertebrates, target recognition is thought to be activated via germ line encoded pathogen pattern recognition receptors (PRRs) that initiate and orchestrate the innate immune response. These PRRs include toll-like receptors (TLRs) and nucleotide oligomerisation domain molecules (NODs) [5,6] in animals, and receptor-like kinases (RLKs) and R proteins in plants [7,8]. Germline evolution favours the rapid replication of prokaryotic pathogens over the slower eukaryotes. Host-pathogen coevolution promotes that random selection promotes recognition sites with a broad specificity for essential non-variable molecular structures on the pathogen (e.g. lipopolysaccharide or other pathogen-associated molecular patterns, PAMPs).

In the vertebrates another recognition system emerged that is based upon random somatic selection and is defined as the adaptive immune system. The primordial molecular component of this system firstly emerged in the early-jawed fishes [9] and is present only in vertebrates. Germline selection favours that encoded recognition pathogen patterns are genetically transmitted across invertebrates and vertebrates, while with somatic selection this does not happen. Somatic selection is characterised by the generation of immunoglobulin-like molecules by somatic DNA diversification that is driven by recombination activating gene (RAG)-dependent recombination during microbial host-interactions. This generates a large and random repertoire of PAMP specific-signatures that are expressed in different cells. This process is a prerequisite for the development of immune memory on B and T-lymphocytes that can potentially recognize an enormous array of antigens molecules. Thus a combination of PRR and somatic-derived recognition systems makes it virtually impossible for a pathogen to escape recognition [1-2].

The emergence of the adaptive immune system suggests an evolutionary pressure possibly due to the inadequacy of the innate response in the vertebrates. However the underlying reasons for this significant evolutionary leap remain unknown. Many factors can be contributed to this, the vertebrates are long-lived, and occupy varied water and land habitats, with a high structural complexity, varied reproduction mechanisms, providing novel pathogen niches, promoting the develop of the adaptive immune response to facilitate a specific and effective host defence. However, the quick activation of the innate defence system is important for the control of infections at their initial stages and prior to the initiation of a complete immune system response.

The initiation of the immune response requires the previous activation of innate defence cells and the success of the vertebrate immune reaction depends on the complex cellular interactions between both branches of immune system (innate and adaptive). The communication

between leukocytes includes processes such as antigen presentation, co-stimulation and release of helper cytokines that display a high system complexity that is reflected by a very dynamic gene regulation among cells of the immune system [10,11]. Activation of the innate immune response directly triggers cytokines and chemokines that activate acute inflammatory responses [12-14]. Subsequent events, such as recruitment of neutrophils and activation of macrophages, lead to direct killing the microbes by induction of rudimentary protective mechanisms such as phagocytosis, coagulation, encapsulation and generation of reactive oxygen and nitrogen metabolites (also known as respiratory burst) [12] (Figure 1). In parallel, indirect activation of antigen-presenting cells (APC) by microbial products stimulates these cells to activate antigen-specific lymphocytes and turn on the adaptive immune response (Figure 1).

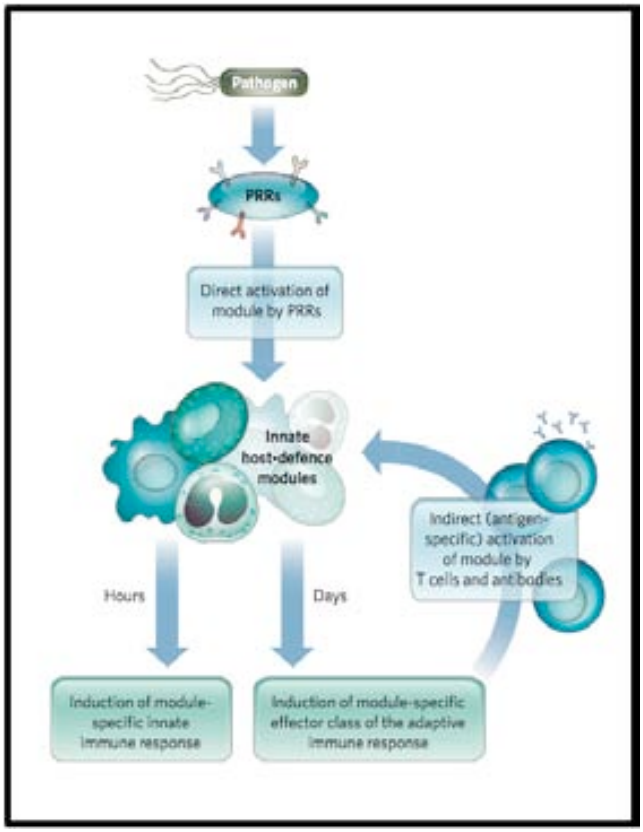


**Figure 1. Activation of host-defence mechanisms.** Host-defence mechanisms can be induced directly, by engagement of PRRs, or indirectly, by T cells and/or antibodies. Each module is characterised by distinct antimicrobial defence mechanisms and can instruct the adaptive immune system to mount a response involving a module-specific effector class. After an adaptive immune response has been initiated, it results in antigen- specific activation of the same innate immune module that instructed the adaptive immune response. For example, macrophages can be activated either directly by TLRs or indirectly by TH1 cells, through IFN-γ, CD40 ligand and other signals. Eosinophils can be activated either directly by an unidentified PRR or indirectly by TH2 cells. And the classical pathway of complement activation can be induced either directly by pentraxins or indirectly by antibodies. Antigen-specific activation of the innate host-defence modules is more efficient than direct activation and is often required for pathogen clearance.

PRRs are primary receptors of PAMPs and are responsible for orchestrating the innate responses and contribute significantly to the activation of adaptive immune responses [15-17]. The primary function of PRRs and the innate immune response is to provide immediate protection from pathogens [12,18]. These tasks include activation of the complement pathway and phagocytosis. In addition, anti-microbial proteins and peptides are induced by TLR engagement in various cell types, especially those of myeloid origin. TLRs also induce activation of cytokines such as IL-1β, IL-6, TNF-α and chemokines (for example, CC-1 and MCP-1) that collectively induce acute inflammatory responses to pathogens (17,19-21). PAMP-PRR activated cells such as macrophages can assume two distinct activation phenotypes (polarization). Phenotype 1; in presence of interferon-γ (IFN-γ), produced by natural killer (NK) cells and T-lymphocytes, macrophages trigger the up-expression of pro-inflammatory cytokines such as tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and IL-12 [14, 22], generating a local autocrine/paracrine response. The stimulation of inflammatory and bactericidal activity of local leukocytes promotes the direct differentiation of T-lymphocytes into an activated phenotype enhancing the expression of IFN-γ. The other macrophage phenotype 2 is capable of expressing anti-inflammatory cytokines such as IL-10, resulting in a down-regulation of pro-inflammatory mRNA expression, inhibition of bactericidal activity and activation of the Th2-type lymphocytes [23-25]. The divergence of the defence response into Th1 or Th2 type are in part directed by the initial activation of macrophages and other antigen presentation cells (APCs) by pathogen-associated molecular patterns (PAMPs) that can specifically drive transcriptomic remodelling [26-28].

**2. PAMPs and pattern recognition receptors (PRRs)**

The host-defence system is activated by pathogen associated molecular patterns (PAMPs), which are endogenous components of pathogens that are vital for their structure and biological integrity. PAMPs are exogenous molecules (antigens) produced only by potential pathogens and not by their multicellular hosts [28] and include peptidoglycan (PGN), lipoprotein, double-stranded RNA (dsRNA), beta-glucans, etc. The host defence system is able to detect PAMPs via a restricted number of germline-encoded receptors named PRRs (pathogen recognition receptors) [14]. These include TLRs, beta2-integrins, and members of the nuclear oligomerisation domain (NODs). The nucleotide-binding oligomerisation domain (NOD)-like receptor family (NLRs) is composed by cytosolic receptors involved in bacterial or viral responses [29,30]. The NLRs sense pathogens through two different structural domains: central nucleotide binding domain and C-terminal leucine rich domain (LRR). The LRR domain resembles the LRR domain found in TLR and is responsible for intracellular pathogen recognition [31]. The most characterised group of PRRs are the toll-like receptors (TLRs), which are type I transmembrane proteins with an ectodomain containing combined leucine-rich repeat

(LRR) motifs involved in recognition of PAMPs. Their cytoplasmic domain is characterised by a Toll/IL-1 receptor (TIR) motif that is involved in signal transduction [12,32]. In mammalian vertebrates, TLR2 is able to recognize microbial molecular patterns such as bacterial lipopeptides [33-35] and lipoteichoic acids (LTAs) [36,37], using a homodimer configuration [38] or forming heterodimers with TLR1 or TLR6 [39,40]. Multiples types of TLR sense different pathogen molecular patterns, TLR5 recognises flagellin [41]; TLR3 dsRNA [42], or TLR4 recognises LPS [43]. The interaction ligand-TLR trigger specifics intracellular signalling cascades that result in the activation (or repression) of transcription factors including NF-κβ, AP1, C/EBP-β, etc. [6,44]. PRR-Ligand interaction generates a homo- or heterotypical dimerisation of PRRs, or a clustering of multiple molecules, as in the case of beta-integrins [45], which is followed by the recruitment and activation of specific adapter molecules or different protein kinases resulting in a dynamic protein phosphorylation [5,6,44] and promoting of specific expression pathogen response-associated genes. For example, dsRNA-TLR3 activates the up-regulation of retinoic acid-inducible gene I (RIG-I) mRNA, and PKR activity that induces IRF- dependent expression of antiviral genes [6].

### 3. Fish PRRs and PAMPs responses

In fish, the major types of myeloid cells involved in the defence response include different types of polymorphonuclear granulocytes and mononuclear phagocytes. These cells are induced by pathogens or PAMPs via PRRs to produce inflammatory cytokines or reactive oxygen and nitrogen species (ROS) [53-57]. Like mammals, the ability of fish macrophages to respond to PAMPs is mostly driven by genotypically encoded PRRs. Since distinct pathogens express different PAMPs, a combination of specific sets of PRRs is essential for integrating an immune response against a specific pathogen. PRRs generally seem to be structurally and functionally well conserved throughout vertebrates [53,54]. Four main types of PRRs have been described in fish: Toll-like receptors (TLRs), NOD-like receptors (NLR), C-type lectin receptors (CLRs) and peptidoglycan recognition proteins (PGRPs). The TLR are well characterised receptor family (in mammals) able to recognize a widespread range of PAMPs with different origin, composition and structure [6]. The members of TLR family have been described throughout all phylogenetic fish groups from ancient Agnathes such as the lamprey to the modern Teleosts as gilthead seabream a member of the Perciformes. Teleost fish possess orthologs of the different mammalian TLR families [55-57], although only the TLR3 ortholog in zebrafish and rainbow trout [57,58] and TLR5 in rainbow trout [59] have been tested and have shown a functional analogy to the mammalian counterparts. The PGRPs recognise peptidoglycans of both Gram-positive and -negative bacteria [60]. 10 different PGRPs have been identified in fishes, zebrafish have 3 PGRPs genes identified that encode proteins with amidase and bactericidal activity and as in mammals are soluble [61]. The CLR family includes proteins

that have at least one domain homologous to a carbohydrate recognition domain but do not always bind carbohydrate structures and may exist both as soluble and as transmembrane proteins [62]. In fish, only 7 transmembrane CLRs have been described in comparison to high vertebrates and are expressed in specific tissues related to the immune system such as hematopoietic head kidney or peripheral blood leukocytes in response to pathogenic stimuli both *in vivo* and *in vitro* [63-65]. In teleost fish, the NODs form a large family of receptors that have been divided into 3 subfamilies (1, 2 and 3) [23]. NOD subfamilies are activated by specifics peptides from different types of bacterial: NOD1 is activated by peptides derived from DAP-PGN (diaminopimelic acid, DAP) present in almost all G-negative bacteria [30]. In contrast, NOD2 is activated by muramyl peptides (MDP) present in both G-positive and negative bacteria [30]. The NOD-3 family has some homology with human NOD3 but zebrafish NOD3 has been shown to be clearly different [31]. However despite the phylogenetic homology of the vertebrate PRR, significant differences are found between fish and mammals in PAMP recognition. TLR activation in mammals is often measured via radical production (ROS) and quantification of NF-κβ activation or downstream expression of pro- and inflammatory genes as TNF-α, IL-1 or IL-6 [6,44,66]. The observed differences in regulation of these cytokines in fish [67-69], indicate that TLR activation and subsequent cytokine induction does not necessarily have homologous functions between mammals and teleost vertebrates. For example in fish, the induction and activation of pro-inflammatory genes require higher doses of LPS (micrograms/millilitre) than human cells (nanograms/millilitre) [52,67,70]. The collective data suggest that fish loses functionality of TLR4 receptor-mediated LPS recognition present in mammals [52,67], probably due a paralogs TLR4 speciation [71], where the phylogenetic homology is likely to cause false premises of their functional conservation [52,67,71].

**4. Functional genomics**

Functional genomics use transcriptome performance for inferring with considerable success the inner working of a cell through analysis of its response to perturbations. On the past decade genomic technologies advances including microarray and RNA-Seq make it possible to examine gene expression function on a genome-wide scale [72]. mRNA transcription represents the first observable and quantifiable phenotype of the organism (phenotype is any observable characteristic of an organism). Observed changes in transcriptomic phenotypes can reveal which mRNA transcripts are essential for an organism, or work in a particular signalling pathway, or specific cellular function. Combining high-throughput screening techniques with experimentally enriched phenotypes enables the observation of detailed reactions during experimental perturbations on a transcriptomic scale [72,73]. Thus knowledge derived from functional genomic studies is indispensable for finding new therapeutic targets to attack the drivers of a disease and not only the symptoms.

## 5. The innate immune system and functional genomics in fish

In fish the mechanisms that support pathogen recognition and regulation is an area of significant importance in comparative immunology and in the same time promotes the management of health in aquaculture. Functional genomics has significantly contributed to transcriptional studies and early descriptions of PAMP-PRR allowed to describe potential gene-specific cassettes in response to pathogens. In general, most studies reported similar effects of PAMPs in fish as to those observed in mammals, reflecting a limited and comprehensive set of actors involved in fish PAMPs recognition. From a phylogenetic perspective, the high degree of conservation of the TLR family has speculatively been associated with the similarities in the immune response between vertebrate classes as phylogenetically distant as fishes and mammals [45,46]. However, with the exception of TLR3-5 [57-59], no other members of the TLR family have been functionally characterised in fish. In addition, there appears to be considerable differences in the function of certain TLR members that, in the case of TLR4, may explain the profound differences in the immune response to LPS of lower vertebrates, and fish in particular, as compared to mammals [52]. Functional genomics studies in fish has identified a significant increase over the last decade where a number of microarrays have been developed for fish and cover an extensive number of species that are representatives of the Pleuronectiformes, Salmoniformes, Cypriniformes and the Siluriformes [74,75] (see figures 2a,b). Functional genomics studies in fish immunology (in the majority of cases in species linked to the intensive aquaculture) have been able to begin to identify distinct gene expression profiles and specific cassettes of responsive genes whose regulatory patterns are conserved across different fish species in response to specific groups of pathogens. In order to address the complexity and peculiarities of pathogen/PAMP specific signalling pathways in fish, the continually increasing coverage (transcriptome) and associated bioinformatic tools represent an attractive option to identify PAMP-PRR specific gene cassettes.

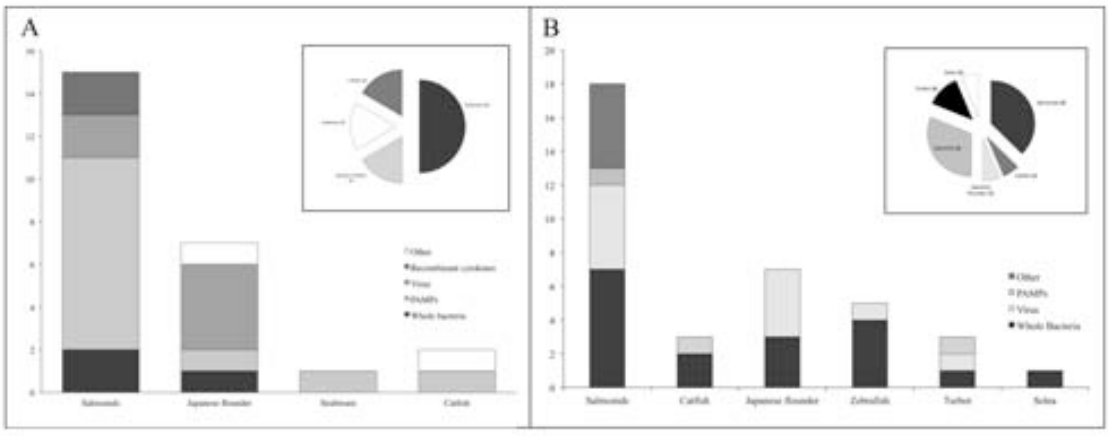


**Figure 2. Summary of transcriptomic studies in fish.** A) Show transcriptomic studies in fish under *in vitro* conditions. The bars show the different fish species and the pathogen used in different transcriptomic studies. The graph-pie describes

the platforms used in the studies with the fish species under *in vitro* conditions. B) Show transcriptomic studies in fish under *in vivo* conditions. The bars show the different fish species and the pathogen used in different transcriptomic studies. The graph-pie describes the platforms used in the studies with the fish species under *in vivo* conditions.

## 6. Phenotypes and transcriptome

The phenotype is a set of observable features of an organism, thus experimental analysis strongly depends on how rich and informative phenotype descriptions are used. For example, the distinction between basic- and complex-phenotypes may sound technical but it is crucial for choosing accurate analysis methods. A basic phenotype description results from a single reporter (or a small number of reporters) [76], such as cell viability or cell death [77], growth rates [78], activity of reporter constructs, e.g. a luciferase, gene expression [79], which provide a "snapshot" of a cells reaction to a gene perturbation. The description of basic phenotypes allows identification of candidate genes on a genome-wide scale that are often used as a first step for bottom-up analysis. In this study basic phenotype descriptors were used to characterise trans-membrane NADPH oxidase complex regulation under treatments with different PAMPs. Further studies addressed the identification of PGN as a component of crude LPS preparations and its role as an inducer of inflammatory responses in trout macrophages. The second part is dedicated to characterise complex phenotypes in trout and seabream macrophages (this strategy allow evaluation of a large number of cellular features at the same time). The use of complex phenotypes can include changes in cell morphology [80-83], or growth rates under a wide range of conditions [84], or transcriptional changes measured on microarrays [80-83], or metabolic and proteomic changes [84] measured by mass spectrometry [85] or flow cytometry [86,87]. Morphological and growth phenotypes can be obtained on a genome-wide scale [75,83], while transcriptional and proteomic phenotypes are often restricted to individual pathways or processes [86,87-91]. In this dissertation two independent experiments were assessed using this strategy, transcriptional changes of macrophages reaction over time after PGN and LPS treatments in two different fish species (trout and seabream).

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## 8. Objectives

The objectives of the research are described as the following specific objectives:

1. To review the state of the art on PAMPs, PRRs and the genomics of gram negative bacterial recognition in fish. (**Chapter II**)

2. To study the phylogeny and regulatory gene expression of the NADPH oxidase complex in trout macrophages treated with LPS, Zymosan, and Poly (I:C). The results described in this chapter provide information about nucleotide sequence conservation of the individual NADPH oxidase components and their regulation after treatments with different PAMPs. (**Chapter III**)

3. To explore the trout/macrophage inflammatory response after treatments with LPS and PGN preparations. The results described in this chapter provide information about the strong inflammatory activity induced by PGN in macrophages in contrast to purified LPS preparations. (**Chapter IV**)

4. To explore the transcriptional modulations of trout/macrophages treated with PGN from different *E. coli* serotypes (O111:B4 and K12) using a cDNA microarray. The results described in this chapter provide information about the specificity of macrophage activation, indicating that a differential immune response is induced by PGN from different *E. coli* serotypes. (**Chapter V)**

5. To develop an oligonucleotide microarray for *Sparus aurata* and to validate its performance in gilthead seabream macrophages. (**Chapter VI**)

# Chapter II

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## PAMPs, PRRs and the genomics of gram negative bacterial recognition in fish

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Chapter II

# PAMPs, PRRs and the genomics of gram negative bacterial recognition in fish

**Abstract**

**Understanding the mechanisms that underpin pathogen recognition and subsequent orchestration of the immune response in fish is an area of significant importance for both basic research and management of health in aquaculture. In recent years much attention has been given to the identification of pattern recognition receptors (PRRs) in fish however characterisation of interactions with specific pathogen-associated molecular patterns (PAMPs) is still incomplete. Microarray studies have significantly contributed to functional studies and early descriptions of PAMP-PRR driven activation of specific response cassettes in the genome have been obtained although much is left to be done. In this review we will address gram negative bacterial recognition in fish addressing contributing factors such as structure-function relationships between G-negative PAMPs, current knowledge of fish PRRs and the input achieved by microarray-based studies ranging from *in vivo* infection studies to directed in vitro PAMP-cell studies. Finally we revisit the endotoxic recognition paradigm in fish and suggest a series of future perspectives that could contribute toward the further elucidation of G-negative bacterial recognition across the highly diverse group of vertebrates that encompass the fishes.**

## 1. Background

The vertebrate innate immune system recognises pathogenic and non-pathogenic micro-organisms via germ line encoded pathogen pattern recognition receptors (PRRs) that sense particular structures of the microorganisms (pathogen-associated molecular patterns, PAMPs) and initiate a well orchestrated immune response [1,2]. The immune response has been proposed to form a modular structure represented by different cellular interactions that are activated initially by PRRs followed by an inflammatory response including recruitment of leukocytes to the site of infection, activation of antimicrobial effector systems and stimulation of adaptive immunity [3]. Throughout vertebrates, a diverse range of receptors and mechanisms, involving both innate and adaptive immunity, result in rapid and efficient responses that lead to pathogen clearance [4]. Continuous interactions with both pathogenic and non-pathogenic bacteria require the co-ordination of multiple PRR - signalling pathways that dictate the outcome of microbial colonisation whether that be symbiotic coexistence, asymptomatic infection or virulent disease [5]. Furthermore these host-microbe relationships are strongly influenced by host species, bacterial virulence factors (genome), and environmental and ecological settings (i.e. aqueous versus terrestrial).

In mammals, gram negative (G-negative) bacterial recognition systems such as the TLR and NOD-like receptor families, have been shown to play a key role in bacterial recognition and associated pathologies including detailed studies for multiple G-negative bacterial pathogens [5,6]. However in fish such detailed functional studies are lacking and although a significant number of PRRs potentially involved in G-negative recognition have been identified in various species, there remains a startling uncertainty as to how these systems function. This is highlighted by the striking difference between the development of TLR-4 mediated endotoxic shock in mammals and its absence in non-mammalian vertebrates even in the presence of a TLR4 receptor [7,8].

Over the past few years a number of microarrays have been developed for fish and cover an extensive number of species that are representatives of the Pleuronectiformes, Salmoniformes, Cypriniformes and the Siluriformes (review [9]). In the majority of cases this development is tightly linked to the intensive aquaculture of these species where understanding the etiology of major bacterial diseases is a key objective toward effective disease control management. Selected examples of published research using microarrays relevant to G- negative infection or PAMPs are highlighted in Table 1. Although in its infancy, microarray studies in fish immunology have been able to begin to identify distinct gene expression profiles and specific cassettes of responsive genes whose regulatory patterns are conserved across different fish species in response to specific groups of pathogens. For example, C-type lectin 2-1, a gene whose product is involved in the C/EBP-driven inflammatory response has been identified in almost all reports in which bacterial preparations have been used to challenge live fish [10-14] suggesting potential as a biomarker for bacterial infection. In order to address the complexity and peculiarities of pathogen/PAMP specific signalling pathways in fish, the continually increasing coverage (transcriptome) of species-specific microarrays represents an attractive option to identify PAMP-PRR specific gene cohorts.

In this review we will address G-negative bacterial recognition in fish addressing contributing factors such as structure-function relationships between G-negative PAMPs, current knowledge of fish PRRs and the input achieved by microarray-based studies ranging from *in vivo* infection studies to directed in vitro PAMP-cell studies. Finally we revisit the endotoxic recognition paradigm in fish and suggest a series of future perspectives that could contribute toward the further elucidation of G-negative bacterial recognition across the highly diverse group of vertebrates that encompass the fishes.

## 2. Structure-function relationship between G-negative bacteria and host types of bacteria, relationship with pathogenesis

Gram-negative and Gram-positive bacteria differ fundamentally in that G-negative bacteria

contain an outer membrane (OM) that is absent in G-positive bacteria. This outer membrane is composed of phospholipids, proteins, lipoproteins and lipopolysaccharide (LPS) while the inner membrane is mainly composed of phospholipid and proteins. The periplasmic space contains peptidolgycans (PGN), periplasmic proteins and enzymes involved in nutrient acquisition, PGN synthesis and modification of toxins such as penicillin [15,16]. LPS consists of three parts: lipid A, a core oligosaccharide, and an O-specific polysaccharide (O-antigen) whose synthesis gives rise to the smooth form of this structure. Rough type LPS lacks the O-polysaccharide chain due to the non functionality of the O-antigen gene cluster (*rfa* and *rfb*) as a result of several frame-shift mutations [17,18]. The active component of LPS (lipid A) is highly conserved in its chemical structure and antigenic cross-reactivity [19], thus each individual organism maintains its LPS structural and immunological identity on the basis of the saccharides (core and O-antigen) attached to lipid A. However, individual LPS molecules may differ in their degree of acylation and glycosylation, and other less common structural modifications. G-negative bacteria have the ability to sense environmental changes in pH, salt concentration, and temperature by two-component regulatory systems such as PhoP/PhoQ and PmrA/ PmrB, which generate structural modifications in LPS [20-25]. Thus the G-negative LPS must be considered a highly heterogeneous group of PAMPs. Furthermore the contribution of extraction methods to the biochemical composition of the resulting LPS molecule and the presence of non-LPS contaminants should be considered in view of PRR-PAMP interactions. Across all vertebrates the immune response to LPS typically involves the release of pro- inflammatory mediators, such as TNF-α, IL-6 and IL-1β which act at local sites of infection to promote inflammation and orchestrate further host response. LPS stimulated cytokine expression has been reported in *P. olivaceus*, *D. rerio* and *C. carpio* leukocytes [26,27] and in *O. mykiss* macrophages along with IL-6 and TNF-α [11,28-31]. Similarly the expression of cyclooxygenase 2 (COX-2), a key mediator of prostaglandin mediated inflammatory responses, has been reported after LPS challenge in *C. auratus* and *O. mykiss* [27,31].

Peptidoglycan (PGN) may account for approximately one-half of the cell wall mass in G-positive bacteria whereas in G-negative bacteria only a relatively thin PGN layer in the periplasmic space is present [32,33]. PGN is a polymer of β (1-4)-linked N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc). G-negative peptidoglycan contains meso-diaminopimelic acid (DAP) as the major cross-linked peptide group whereas most G-positive bacteria have L-lysine as the third amino acid (Lys-type) where Lys-type peptides are cross-linked through an inter-peptide bridge that varies in length and amino acid composition in different bacteria [32,34,35]. In mammals, PGN-mediated activation of macrophages results in TNF-α [36,37] IL-1β and IL-6 [37,38] production

similar to LPS. Peptidoglycans in fish induce biological effects that include the expression and activity of peptidoglycan recognition proteins (*PGRPs*) which have an essential role in defence during bacterial infections [39] and can stimulate specific PGN-recognition-pathways in trout macrophages [40]. PGNs, also stimulate pro-inflammatory cytokine (IL-1β and IL-6) and cyclooxygenase 2 expression in trout macrophages as well as the release of prostaglandin E2 and D2 [31,40].

Porins are a component of the outer membrane protein (OMP) of G-negative bacteria. They are channels that form a pore that allows the passage of hydro-soluble compounds [41,42]. In mammals, porins are recognised by TLR2 [41,42] and stimulate the production of the potent inflammatory mediator, platelet activating factor (PAF) in neutrophils [43], as well as the production of pro- and anti-inflammatory cytokines including TNF-α, IL-1, IL-6, or IL-4 [44,45]. In fish the few available studies suggest both protective and damaging effects during *in vivo* challenges with bacteria (see e.g. [46-49]).

The asymmetric outer membrane of G-negative bacteria contains lipoproteins (BLP) which are anchored via a lipid moiety attached using a unique $NH_3$-terminal lipo-amino acid, *N*- acyl-*S*-diacylglycerol cysteine [50,51]. BLP is known to activate monocytes/macrophages or lymphocytes to produce inflammatory cytokines as TNF-α, IL-1, or IL-6, initiating a defence response via TLR2 [2,41,52-55]. In fish current data suggests a low reactivity of trout macrophages to lipoproteins in comparison to other bacterial structures such as PGN [31].

Motile bacteria display complex surface organelles, known as flagella. The bacterial flagellum is composed of a filament that is attached to a molecular motor (the basal body and hook complex), made up of three functional regions: the basal body or motor, the hook and the filament [56,57]. Flagellar filaments are composed of 11 protofilaments composed almost entirely of flagellin monomers [58,59]. The flagellin is a ligand for TLR5 [60]. Little is known about fish immune responses to flagella and few studies are available. In salmonids flagella has been shown to induce NF-κB activity via TLR5 in *O. mykiss* [61] and facilitate chemotactic motility during infection [62,63]. In Japanese flounder, peripheral blood cells and liver cells expressed pro-inflammatory cytokine and TLR5 is overexpressed in several tissues after *Edwardsiella tarda* infection [64].

These studies suggest a significant although relatively unexplored role for TLR5-flagella recognition in fish. Other microbial products that induce inflammatory responses are bacterial DNA or unmethylated Cytidine-phosphate-Guanosine (CpG) motifs [65]. The host defence mechanism

recognises CpG dinucleotides flanked by specific bases in bacterial DNA as a danger signal [66, 67]. In fish, plasmidic DNA and synthetic CpG induced the production of an IFN-like cytokine and IL-1β mRNAs in Atlantic salmon, *S. salar,* and *O. mykiss* leukocytes [68,69]. In addition, *C. carpio* head-kidney cells were reported to respond to CpG-ODNs up-regulating Th type 1 transcript expression [70]. CpGs have also been reported to regulate an increase in complement, lysozyme respiratory burst and bactericidal activities in *C.carpio* macrophages, *P.olivaceus* phagocytes and *S.aurata* leukocytes [71-75].

**3. Signalling and components of innate immunity involved in pathogen recognition: from receptors to cells.**

Since distinct pathogens express different PAMPs, a combination of specific sets of PRRs is essential for integrating an immune response against a specific pathogen. Teleost fish possess a wide range of PRRs that are involved in the immune response against pathogens. Four main types of *pathogen pattern recognition receptors* (PRRs) have been described to date in fish: Toll-like receptors (TLRs), NOD-like receptors (NLR), C-type lectin receptors (CLRs) and peptidoglycan recognition proteins (PGRPs) (Table 2). The PGRPs recognise peptidoglycans (both from G-positive and -negative bacteria) and have a well conserved structure between invertebrates and vertebrates. In invertebrates such as *Drosophila*, PGRPs trigger the Imd and the Toll signal transduction pathways stimulating the synthesis of anti-microbial peptides and phagocytosis. In mammals, PGRPs seem to play a more direct role on bacterial recognition and destruction, being direct bactericidal agents except for PGRP2 that digests PGN through its amidase activity (for review see [76]. In fish, 10 different PGRPs have been identified to date (Table 2). In zebrafish, the 3 PGRPs genes show amidase activity and, as their mammalian counterparts, are all secreted [39]. *In silico* analysis indicates the existence of two *T. negroviridis* PGRPs and one *T. rubipres* gene [77]. The yellow croaker PGRP2 sequence has a putative signal peptide, indicating that it probably is secreted, and a putative amidase domain [78]. Both rockfish PGRPs show a putative signal peptide and have *in vitro* amidase and bactericidal activity against *E. coli* and *S. typhimurium* [79]. Concerning the salmoniformes, one partial PGRP sequence from salmon is available in public databases and one partial trout PGRP has been described in macrophage cultures (Goetz et al., unpublished results) but no functional data on PGRPs has been reported in salmonids.

**Table 1. Immune-related Fish Microarı**

| Fish species | Pathogen | Stimulus | Tissue/Cell Type | Reference |
|---|---|---|---|---|
| Salmon Atlantic | *Piscirickettsia salmonis* | *in vitro* and *in vivo* | Head Kideny and HK leukocytes | Rise et al., 2004 |
| | *Aeromonas salmonicida* | *in vivo* | Head Kidney | Ewart et al., 2005 |
| | *Neoparamoeba* | *in vivo* | Head Kidney, liver and | Wynne et al., 2007 |

| | | | | |
|---|---|---|---|---|
| | *perurans* | | gills | |
| | *Aeromonas salmonicida* | *in vitro* | Head Kidney macrophages | Ewart et al., 2008 |
| | *Lepeophtheirus salmonis* | *in vivo* | Spleen, head kidney and liver | Skugor et al., 2008 |
| | Virus (ISAV) | *in vitro* | Head Kidney cells | Schiotz et al., 2008 |
| | *Neoparamoeba perurans* | *in vivo* | Gills | Wynne et al., 2008 |
| | Virus (ISAV) | *in vivo* | Spleen, gills, heart and liver | Jorgensen et al., 2008 |
| | *Aeromonas salmonicida* | *in vivo* | Liver and Spleen | Skugor et al., 2009 |
| | Virus (ISAV) | *in vivo* | Head Kidney | Leblanc et al., 2010 |
| Salmon Chinook | *Vibrio anguillarum* | *in vivo* | Head Kidney | Ching et al., 2010 |
| Rainbow Trout | LPS (E.coli 0111:B4) | *in vitro* | Head Kidney macrophages | MacKenzie et al., 2006 |
| | *Vibrio anguillarum* | *in vivo* | Liver | Gerwick et al., 2007 |
| | LPS (E.coli 026:B6) | *in vivo* | Spleen, head kidney and liver | Djorjevic et al., 2008 |
| Channel catfish | LPS (E.coli 0127:B8 ) | *in vivo* | Spleen | Li et al., 2006 |
| | *Edwardsiella ictaluri* | *in vivo* | Spleen, Head kidney, gills, liver, skin | Peatman et al., 2007 |
| Japanese flounder | LPS | *in vitro* | Head Kidney cells | Kurobe et al., 2005 |
| | Virus (VHSV) | *in vivo* | Head Kidney cells | Byon et al., 2005 |
| | Virus (VHSV) | *in vivo* | Head Kidney cells | Byon et al., 2006 |
| | *Streptococcus iniae* | *in vivo* | Head Kidney cells | Dumrongphol et al., 2008 |
| Zebrafish | *Mycobacterium marinum* | *in vivo* | Whole fish | Meijer et al., 2005 |
| | *Mycobacterium tuberculosis* | *in vivo* | Whole fish | Van der Sar et al., 2009 |
| | *Streptococcus suis* | *in vivo* | Whole fish | Wu et al., 2010 |
| Turbot | Nodavirus and poli I:C | *in vivo* | Kidney | Park et al., 2009 |
| Others | Trout recombinant cytokines | *in vitro* | Trout macrophage cell line (RTS-11) | Martin et al., 2006 |
| | Trout recombinant cytokines | *in vitro* | Salmon cell line (SHK-1) | Martin et al., 2007 |
| | Virus (ISAV) | *in vitro* | Salmon macrophage/denditic cell line (TO) | Workenhe et al., 2009 |
| | *Vibrio alginolyticus* | *in vitro* | Carp cell line (EPC) | Cao et al., 2010 |

The CLR family includes proteins that have at least one domain homologous to a carbohydrate recognition domain but do not always bind carbohydrate structures and may exist both as soluble and as transmembrane proteins [80]. The binding of ligand to its receptor leads to the activation of proinflammatory cytokine expression through direct activation of the NF-κB signalling pathway or modulating the TLR signalling pathway [80]. In fish, only 7 transmembrane CLRs have been described to date (Table 2) and different studies indicate that fish CLRs are expressed in immune relevant tissues (head kidney cells or peripheral blood leukocytes) in response to pathogenic stimuli both *in vivo* and *in vitro* [28,81,82]. Further characterisation is necessary to address their precise functional role in the fish immune response.

The NLRs are cytosolic receptors involved in autoimmunity, bacterial and viral responses, as well as apoptosis [83]. The NLRs sense pathogens through two different structural domains: a central nucleotide binding domain and a C-terminal leucine rich domain (LRR). In addition, the N-terminal domain contains protein-protein interaction motifs mostly involved in apoptosis and NF-κB activation [2]. The LRR domain resembles the LRR domain found in TLR and is responsible for intracellular pathogen recognition [84]. In teleost fish, NLRs form a large family of receptors that have been divided in 3 subfamilies (A, B and C) [84]. As is shown in Table 2, zebrafish have a wide NLR diversity with more than 70 members. The NLR-A family, composed of 5 members, contains orthologs of mammalian NOD1-5, while the NLR-B family (6 members) contains the NALP mammalian orthologs. NOD1 is activated by peptides derived from PGN (diaminopimelic acid, DAP) present in almost all G-negative bacteria [83]. In contrast, NOD2 is activated by muramyl peptides (MDP) present both in G-positive and negative bacteria [83]. The NLR-C family has some homology with human NOD3 but zebrafish NOD3 is clearly different [84]. In channel catfish, 2 NLR-C members (3 and 5) have also been described and are expressed in immune relevant tissues and an NLR-X1 gene similar to human and zebrafish NLR-X1 but with less LRR was described [85]. NOD members have been predicted in fugu, stickleback, medaka, grass carp and channel catfish but detailed functional studies are only available in grass carp and channel catfish [85,86]. In both species NOD1 and NOD2 genes are expressed in head kidney, gills, spleen, intestine or leukocytes and are overexpressed in response to *in vitro* (LPS and PGN) or *in vivo* (*E. ictaluri*) challenges [85,86].

**Table 2.** ***Pattern recognition receptors (PRRs)*** **in fish species.**

| Order | Species | Number of Receptors | Name | Reference |
|---|---|---|---|---|
| **TLR** ***(Toll-like Receptors)*** | | | | |
| Cypriniformes | Zebrafish *(Danio rerio)** | 17 | TLR1–3, 4a, b, 5a, b, 7, 8a, b, 9, 18, 20a, b, 22 | Roach et al., 2005 |
| | Japanese puffer *(Takifugu rubripres)** | 12 | TLR1–3, 5, 7–9, 14, 21–23, TLR5-like | Roach et al., 2005 |
| | Green spotted puffer fish *(Tetraodon negroviridis)** | 10 | TLR1a, b, 2, 3, 5, 8, 9, 21–23 | Roach et al., 2005 |
| | Common carp *(Cyprinius carpio)* | 2 | TLR2, 3 | Ribeiro et al.; Rebl et al., 2010 |
| | Goldfish *(Carassius auratus)* | 1 | TLR(?) | Stafford et al., 2003 |
| Petromyzontiformes | Sea lamprey *(Petromyzus marinum)** | 16 | TLR3, 5, 7/8a, 7/8b, 14a-d, 21a-c, 22, 24a-d, | Kasamatsu et al., 2010 |
| | Lamprey *(Lethenteron japonicum)* | 2 | TLR14a, 14b | Ishii et al., 2007 |
| Salmoniformes | Atlantic salmon *(Salmo salar)* | 6 | TLR8, 9, 13, 22a, 22b, 5soluble, | Skaveland et al., 2009: Skaveland et al., 2008; Tsoi et al., 2005; Rebl et al., 2010 |
| | Rainbow trout *(Onchorynchus mykiss)* | 8 | TLR3, 5, 7, 8a, 8b, 9, 22-1, 22-2 | Matsuo et al., 2008; Rebl et al., 2007; Ortega-Villaizan et al., 2009 |
| Pleuronectiformes | Japanese flounder *(Paralycthis olivaceus)* | 3 | TLR2, 3, 9 | Hirono et al., 2004; Takano et al., 2007 |
| | Half-smooth tongue sole *(Cynoglossus semilaevis)* | 1 | TLR9 | Yu et al., Fish and Shellfish Immunol 2009 |
| Perciformes | Sea bream *(Sparus aurata)* | 3 | TLR5, 9a, 9b | Franch et al., 2006; Sepulcre et al., 2007 |
| | Japanese sea bass *(Lateolabrax japonicus)* | 1 | TLR(?) | Shao et al., 2009 |
| | *Larimichthys crocea* | 1 | TLR9 | Rebl et al., 2010 |
| Siluriformes | Catfish *(Ictalurus punctatus)* | 5 | TLR2, 3, 5soluble, 20, 21 | Baoprasertkul et al., 2007a and 2007b; Baoprasertkul et al., 2006 |
| | Minnow *(Gobiocypris rarus)* | 3 | TLR3, 4a, 4b | Rebl et al., 2010; Su et al., 2009 |
| **NLR** ***(NOD-like Receptors)*** | | | | |
| Cypriniformes | Zebrafish *(Danio rerio)** | 73 | NLR-A family (5), NLR-B family (6), NLR-C family (62) | Laing et al., 2008 |
| | Japanese puffer *(Takifugu rubripres)** | 5 | NOD1, 2, 3, 4, 5 | Laing et al., Chen et al., 2010 |
| | Grass carp *(Ctenopharyngodon idella)* | 2 | NOD1, 2 | Chen et al., 2010 |
| Gasterosteiformes | Stickelback | 2 | NOD1, 2 | Chen et al., 2010 |
| Beloniformes | Medaka *(Oryzias latipes)** | 2 | NOD1, 2 | Chen et al., 2010 |
| Siluriformes | Channel catfish *(Ictalurus punctatus)* | 5 | NOD1, 2, NLRC3, 5 and NLRX1 | Sha et al., 2010 |
| **CLR** ***(C-type lectin Receptors)*** | | | | |
| Cypriniformes | Zebrafish *(Danio rerio)** | 1 | zCLR | Chen et al., 2010 |
| Salmoniformes | Atlantic salmon *(Salmo salar)* | 3 | CLR A, B, C | Soanes et al., 2004 and 2008 |
| | Rainbow trout *(Onchorynchus mykiss)* | 1 | rtCLR (CD209-like protein) | Goetz et al., 2004 |
| Osmeriformes | Ayu *(Plecoglossus activellis)* | 1 | aCLR | Chen et al., 2010 |
| Esociformes | Northern pike *(Esox lucius)* | 1 | eCLR | Chen et al., 2010 |
| **PGRP** ***(Peptidoglycan recognition proteins)*** | | | | |
| Cypriniformes | Zebrafish *(Danio rerio)** | 3 | PGLYRP-2, 5, 6 | Li et al., 2007 |
| | Green spotted puffer fish *(Tetraodon negroviridis)** | 2 | tnPGRP-L1, 2 | Chang et al., 2007 |
| | Japanese puffer *(Takifugu rubripres)** | 1 | frPGRP | Chang et al., 2007 |
| Salmoniformes | Atlantic salmon *(Salmo salar)* | 1 | PGRP | NP_001135366.1 |
| | Yellow croacker *(Pseudosciaena crocea)* | 1 | PGRP2 | Mao et al., 2010 |
| Scorpaeniformes | Rockfish *(Sebastes schlegeli)* | 2 | SsPGRP-L1, 2 | Kim et al., 2010 |

** Organisms with available genome.*

The TLR group is a well characterised family that comprises membrane receptors and endolysosomal receptors [1]. The general structure of TLRs consists of a LRR extracellular domain, a transmembrane domain, and a C-terminal toll/IL-1 receptor homology domain (TIR). In fish, members of this group of receptors have been described in ancient fishes such as lamprey, as well as in more modern fish such as sea bream (Table 2). Up to 17 TLRs have been found in zebrafish and up to 16 TLRs have

been described in sea lamprey. In the human genome, 10 TLR members have been identified: the TLR2 subfamily recognises bacterial cell wall PGN and acylated lipopeptides, TLR4 recognises G-negative bacterial LPS, TLR5 recognises bacterial flagellin, while TLR3, 7, 8, and 9 recognise microbial nucleic acids. In fish, all members of the TLRs described in mammals have been reported with conserved functions but also with some remarkable differences (e.g TLR4, discussed in section 5). Some fish species possess additional TLRs such as TLR11, 14, 20, 21, 22 and 24 [87,88]. For example, lamprey TLR14 is involved in sensing of an unknown ligand [89] while TLR22 recognises foreign RNA and TLR24 probably is a pseudogene [90].

**4. Transcriptomic responses to G-negative bacterial PAMPs in fish**

Transcriptome alterations underlying complex processes such as infectious disorders are characterised not only by large scale induction and repression responses, but also by subtle changes in transcript levels that produce slight differences in transcriptomic stability that affect the performance of the immune response [40,91]. cDNA microarrays have played a significant role in assessing transcriptional changes during bacterial infection in the Teleostei mainly concentrating upon species with a strong commercial interest. Whole bacterial challenges using several major causative agents of G-negative bacterial infection, *P. salmonis*, *E.ictaluri*, *V. anguillarum* and *A. salmonicida,* have been used to explore tissue and time-dependent responses in the Atlantic and Chinook salmon, Channel catfish and the Rainbow trout identifying tissue-specific regulation of immunity likely reflecting different phases of activation of the immune response in distinct tissue compartments (Table 1). Many of the targets identified are common across platforms i.e. C-type lectin 2-1, however the complexity of the immune response has not been completely captured probably due to two major factors; 1. variation in coverage of mRNA directly related to immunity represented within and between different cDNA platforms and 2. the complex temporal activation in species-specific recognition-effector systems. A recent study in the zebrafish reported upon a RNA-Seq strategy combining both Agilent microarray technology and Solexa/Illumina sequencing to study of the vertebrate host response to infectious disease. As expected major advantages were obtained in respect to transcript coverage related to transcriptional responses during infectious process [92]. Thus although this heterogeneous set of *in vivo* studies addressing bacterial infection in fish have provided substantial and important information, interpretations of the underlying biological processes and indeed tools for interrogation of data (i.e. molecular interaction databases) are currently limited and will require further development to gain a clearer picture of the immune response in a species-specific fashion.

Surprisingly little attention has been paid to how fishes deal with infection in terms of PAMP-specific bacterial recognition, only LPS and CpG responses have been studied, whereas a number of studies have been published addressing viral infection (for review [9]). Targeted PAMP studies require the use of cell culture systems in order to obtain and enrich for specific cell types including the monocyte/macrophages which orchestrate recognition pathways and subsequent responses. Within this group of studies, differentiated macrophages have been used in both salmon and trout whereas head kidney leukocytes were used in the Japanese flounder (Table 1). Interestingly when comparing both CpG and PGN expression profiles to those obtained with LPS stimulation a divergent picture emerges. Using the same microarray platform (SFA2.0, immunochip) CpG-B treatment on adherent salmon monocyte/macrophage cultures induced a more pronounced response with higher numbers of genes up-regulated [93]. However, pro-inflammatory cytokine production was higher in the LPS-treated cultures after 24 hours stimulation suggesting a time-dependent divergent response to each PAMP. Recent studies in cultures identified PGN as a major pro-inflammatory component of crude LPS preparations characterised by an increase in cytokine mRNAs, IL-1β and IL-6, and release of inflammatory products as prostaglandin $E_2$ ($PGE_2$) [31]. Further studies using microarrays have identified early, medium and late stages of PGN-dependent activation of adherent trout in which PGNs from different strains of *E.coli* exhibit differential activation profiles suggesting that recognition of PAMPs is structure and even bacterial strain-specific in fish [40]. A comparison of activation profiles obtained from LPS and PGN (*E.coli* 0111:B4) stimulated trout monocyte/macrophages is shown in Figure 1. The pronounced intensity of the response observed with PGN stimulation is highlighted by the significantly stronger response (higher percentage of transcripts >2 fold increase; 58% vs 30%) in transcripts after 24 hours of stimulation (Figure 1a). On the other hand Gene ontology analyses identify a more divergent activation of biological functions in respect to LPS transcript-related processes (Figure 1a). This is reinforced by the larger number of LPS-regulated transcripts (723; $p<0.01$) albeit at low intensities identified in the array analysis. These analyses and our previous studies suggest that PGNs activate prostaglandin-dependent pro-inflammatory responses by activating a specific cohort of transcripts that is likely dependent upon specific PGN-PRR recognition. In support of this CpG-B stimulation also induced a specific and divergent response in comparison with LPS that was suggested to activate cellular differentiation rather than pro-inflammatory responses [93]. Thus microarray studies have tentatively uncovered differential responses in fish monocyte/macrophages that reflect PAMP diversity and PRR specificity highlighting the potential of this approach and importantly PAMP specificity and purity is shown to be a key consideration.

**5. Re-evaluation of endotoxin recognition in fish**

In mammals, TLR4 is a key receptor for LPS signalling. Besides mammals, a TLR4 gene has been observed in amphibians (*Xenopus laevis*) (BAF57489), birds [94] and in some fish species such as *Danio rerio* and *Gobiocypris rarus* [8,95] (Table 2), but not in others including the genomes of the pufferfish, *Takifugu rubripes,* and *Tetraodon nigroviridis*. *In silico* studies have demonstrated that some components of the TLR4 signalling machinery are conserved in fish [50,96] yet others such as LBP (lipopolysaccharide binding protein), CD14, MD-2 and TICAM2 appear to be absent [7]. Fish, amphibia and birds are much more resistant to the toxic effects of LPS as compared to mammals [7, 94,97]. In addition, trout macrophages are relatively insensitive to the effects of ultrapure LPS and high concentrations (ug/ml) of crude (phenol-extracted) LPS are necessary to stimulate cytokine transcription [7,50]. The collective data suggest that fish lack the TLR4 receptor-mediated recognition of LPS present in mammals [7], but given the robust cytokine response that can be stimulated in fish immune cells by crude LPS preparation, some other component of the LPS is likely to be responsible for this stimulation. At least in trout, it appears that peptidoglycans may be the component of G-negative bacteria being recognized by macrophages [31,40], similar to what is observed in invertebrates such as *Drosophila* [35,98].

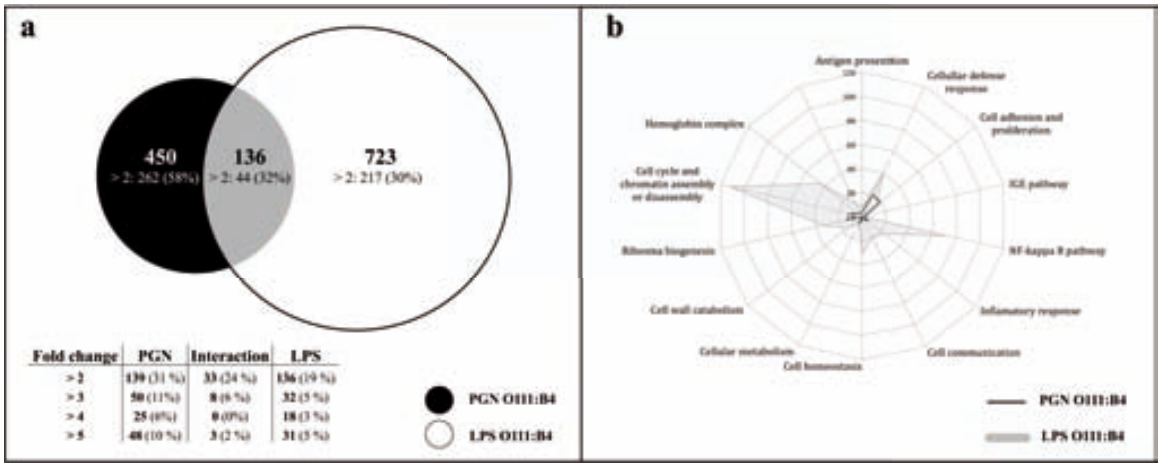


Figure 1: Quantitative and qualitative representation of the transcriptomic response in trout adherent monocyte/macrophage cultures 24 hours after LPS or PGN (E.coli 0111:B4) challenge. a; Venn diagram of differentially expressed transcripts showing PGN(black), LPS(white) and common(grey fill) regulated transcripts ($p<0.01$). Inserted table shows breakdown of transcripts with >2 fold changes highlighting the reduction in common transcripts relative to increased intensity (divergence). b; Over expressed GO categories ($p<0.05$) after LPS and PGN stimulation. The spider-web map, PGN (black line) and LPS (grey line) shows transcript numbers (0-120) relevant to enriched biological process after challenge.

Yet the recognition of LPS in fish may be more involved and exhibit phylogenetic specificities that are just being uncovered. For example, trout macrophages possess different kinases and enzymes such as TACE/ADAM17 involved in TNFα processing, and while ultrapure LPS does not stimulate TNFα

transcription, it does stimulate the release of TNFα from trout macrophages [99]. Further, in higher order fish species such as *Sparus aurata* (MacKenzie et al, unpublished results) and *Perca flavescens* (Olsen and Goetz, unpublished results), ultrapure LPS preparations can stimulate the transcription of cytokines such as TNFα and IL-1β in adherent monocyte/macrophage cells. Thus, there appears to be some type of LPS recognition involving at least cytokine release in lower teleosts and transcription in Perciforms that does not involve other contaminating PAMPs.

## 6. Future Perspectives

Understanding the mechanisms that underpin pathogen recognition and subsequent orchestration of the immune response in fish is an area of significant importance for both basic aspects of research in comparative immunology and in the promotion and management of health in aquaculture. In recent years much attention has been given to the identification of PRRs in fish and this had lead to both expected and unexpected results i.e. number of TLRs present in fish in comparison to mammals. Microarray studies have significantly contributed to functional studies and early descriptions of PAMP-PRR driven activation of specific response cassettes in the genome have been obtained although much is left to be done. In general, most studies so far have reported similar effects of G-negative PAMPs in fish as to those observed in mammals. This may reflect a conserved set of immune effector responses that are activated by diverse sets of vertebrate group-specific PRRs i.e. different TLR genes with different degrees of PAMP specificity. However the reported discrepancies concerning activities or presence of LPS specific PRRs remains unanswered. In view of future studies to address such questions using genomic technologies we would like to highlight two major areas of development; 1. careful characterisation of both cellular tools (cell cultures) and PAMP ligands (structure, purity and strain) is essential to dissect the specific pathways activated. Here the availability of purified PAMPs from gram-negative bacterial pathogens directly relevant to infectious processes in fish would represent a major breakthrough and 2 arrays are only as good as the genes they contain whereas RNA-Seq studies will provide gene discovery and quantitative expression data simultaneously. Thus representing a distinct advantage in the development of transcriptomic studies in other fish species lacking sequenced genomes. However microarrays will still play an important role in gene expression studies as efficient and cost effective tools to analyze large numbers of samples. The combination of genomic tools including whole genomes sequencing, RNA-Seq and microarrays will provide an exemplary set of investigative tools to further elucidate the molecular mechanisms underpinning pathogen recognition and the immune response in fish.

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# Chapter III

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## Characterization and expression of NADPH oxidase in LPS-, poly(I:C)- and zymosan-stimulated trout (*Oncorhynchus mykiss* W.) macrophages

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# Characterisation and expression of NADPH oxidase in LPS-,poly (I:C)- and zymosan-stimulated trout (*Oncorhynchus mykiss* W) macrophages.

**Abstract**

**In vertebrates, the generation of superoxide reactive oxygen species (ROS) via activation of the Nox/Duox family of NADPH oxidases is a prototypical feature of the pathogen-induced defensive responses of activated professional phagocytes. To understand the role of the rainbow trout (*Oncorhynchus mykiss*) Phox oxidase from a phylogenetic and functional perspective we describe the cloning, sequencing and expression analysis of multiple NADPH components in cultured macrophages. Phylogenetic analyses support the notion of the emergence of Phox-related components before the diversification of basal euteleosts and add to the limited collection of Teleost NADPH oxidase's. Expression studies using lipopolysaccharide, polyinosine-polycytidylic acid and zymosan to mimic the onset of inflammatory responses in trout macrophages suggest differences in regulation of the NADPH complex throughout the maturation/differentiation period of culture and between different PAMP treatments**.

## 1. Background

In vertebrates, the generation of superoxide reactive oxygen species (ROS) through the activation of the Nox/Duox family of NADPH oxidases is a prototypical feature of the pathogen-induced defensive responses of activated professional phagocytes (neutrophils and monocyte/macrophages). In mammals, the best studied oxidases is the phagocyte NADPH-oxidase (*Phox*), a multicomponent enzymatic complex responsible for the production of large amounts of superoxide anions during oxidative and/or pathological conditions, both in cultured cells and in infected organisms.

The structure, mechanism of activation and functional relationships between Phox components has been extensively described [1-3]. Upon stimulation, the Phox-indued microbicidal activity of phagocytes is exerted through the sequential assembly of several cytosolic and membrane subunits. The formers include the regulatory components p40*phox*, p47*phox*, p67*phox* and the Rac GTPases. The membrane components, usually located on phagocytic vacuoles, consist of a catalytic protein, Nox2 (aka gp91*phox*) which forms a stable heterodimer (known as flavocytochrome *b*558) with the membrane subunit p22*phox*. The Nox2 subunit contains FAD and NADPH-binding sites along with several histidine residues, highly conserved across taxa [4], which act as ligands for two dissimilar heme groups.

In non-activated cells, the dormant Phox holds each component in its respective cellular compartments. In activated phagocytes, the phosphorylation of the adapter protein p47*phox*, which abolishes autoinhibitory internal interactions, allows its simultaneous binding to p67*phox* and p22*phox*. The phosphorylated p40*phox* collaborates in the assembly of p47*phox* and p67*phox* and also helps to anchor the cytosolic Phox components to the membrane [5]. The coincident conversion of cytosolic Rac-GDP to Rac-GTP and its translocation to the membrane, where it interacts with p67*phox* and flavocytochrome *b*558, facilitates the hydride transfer from NADPH to FAD and the cross-membrane transit of electrons via the heme groups of the catalytic subunit that triggers the reduction of molecular oxygen to form superoxide [1]. The superoxide anion is subsequently converted to hydrogen peroxide, hydroxyl radicals and hypoclorous acid. These and others ROS by-products generated by activated oxidative enzyme complexes generate the so-called 'respiratory burst' characteristic of phagocytic immune responses [6].

Recently, several exhaustive phylogenomic and functional analysis of eukaryotic oxidases identified structurally conserved domains and patterns of activation of the calcium-regulated and subunit-dependent Nox/Duox enzymes, both in phagocytic and non-phagocytic cells [1,4,7-9]. In fish, the characterisation of NADPH oxidases has been described in carp (*Cyprinus carpio*) neutrophils [10], Japanese pufferfish (*Takifugu rubripes*) granulocytes and tissues [11] along with the phylogenetic analysis of Nox/Duox family of oxidases in several genome-mined model species [4,7], such as zebrafish (*Danio rerio*), medaka (*Oryzias latipes*), pufferfishes (*Tetraodon nigroviridis*).

Herein we describe the cloning, sequencing and phylogenetic analysis of cDNAS for catalytic and several regulatory NADPH components, Nox2, p22*phox*, p47*phox*, p67*phox* and p40*phox*, in rainbow trout (*Oncorhynchus mykiss*) cultured macrophages, as well as a series of expression studies, mimicking the onset of inflammatory responses in trout macrophages stimulated with with Lipopolysaccharide (LPS, the major constituent of the external layer of the membrane of Gram-negative bacteria), Polyinosine-polycytidylic acid (poly(I:C), synthetic analog of double-stranded RNA(dsRNA) and Zymosan, polysaccharide prepared from the cell wall of *Saccharomyces cerevisiae*.

## 2. Materials and Methods

### 2.1. Animals

Healthy adult specimens (160 g mean weight) of rainbow trout (*O. mykiss*) were purchased from a commercial hatchery (Piscifactoria Andrés, St Privat, Girona) and held in recirculating freshwater stock tanks (300 L) in the aquarium facilities at the Universitat Autònoma de Barcelona.

Fish were kept at 15º C with a 12 h light/12 h dark photoperiod cycle, and were fed with a maintenance ratio of about 0.5% body weight per day. Water quality indicators (dissolved oxygen, ammonia, nitrite, pH) were analyzed periodically.

**2.2. Cell culture and stimulation**

Rainbow trout monocyte/macrophages were isolated and cultured according to methods that have been previously described [12,13]. Briefly, fish were killed by overdose of MS222 (Sigma) and the head kidneys were dissected and placed in sterile 100 μm nylon mesh cell-strainers. The tissue was squeezed through the bag using sterile spatula in presence of Dulbecco's Modified Eagle Medium (Life Technologies) containing high glucose, 10% heat inactivated fetal calf serum (Gibco) and the antibiotic Primocin (100 μg/ml) (Invivogen). The cells were centrifuged 1 min at 1000 rpm, resuspended in 12 ml of medium and plated on 6 cell culture dishes previously treated with poly-D-lysine (Sigma), 2 ml per well. The cells were held in an incubator at 15º C under 5% CO2. Nonadhering cells were removed at 24 h and new medium (as above) was added. The adherent cells were incubated for another 4 days before stimulation, changing the medium every two days. We have previously shown that head kidney cells incubated for 5 days under these conditions have typical cytological characteristics of macrophages and an increasing phagocytic capacity and cytokine expression [13]. Cell stimulation was carried out with LPS from *Escherichia coli* (Sigma), zymosan A from *Saccharomyces cerevisiae* (Sigma) and polyinosine-polycytidylic acid (poly (I:C) (InvivoGen).

Three of the culture dishes for each trout were stimulated with LPS and one served as unstimulated controls. For stimulation, the medium was decanted from each plate and new medium alone for controls (2 ml) or containing the agents (5, 10, and 50 μg/ml) or not (control), was added back to the plate. Cultures were maintained for an additional 12 h at 15º C under 5% CO2. The same procedures were used to test the effect of zymosan and poly (I:C) on cultured cells.

**2.3. DNA sequencing of trout NADPH oxidase genes**

Trout NADPH oxidase cDNAs from cultured macrophages were obtained from a previously reported annotated cDNA libraries [12]. In brief, five-day cultured trout monocyte/macrophages were stimulated with LPS from *E. coli* (serotype 026:B6, Sigma) maintained for an additional 12 h period at 15º C under 5% CO2. The medium was decanted and replaced with 5 ml of Tri Reagent (Molecular Research Center, Inc.)/plate. Total RNA was extracted from the Tri Reagent according to the manufacturer's protocol [14] and polyA+ RNA was isolated using the PolyAtract mRNA isolation system (Promega). A small portion of the control and LPS stimulated mRNA was reverse transcribed

using AMV reverse transcriptase (Promega) and used to amplify TNF alpha by PCR to test the cell stimulation. mRNA (5.0 μg) from LPS stimulated cells obtained from 8 trout, was used to construct a cDNA library in Zap Express (Stratagene). Complementary DNA produced for library construction was size-fractionated using sephacryl SF500, and two cDNA size classes were ligated in separate reactions with the Zap Express vector. The ligations were packaged separately to produce two cDNA libraries of different average sizes. Both libraries were mass excised to pBK-CMV phagemids and plated at low density. Individual colonies were randomly picked and plasmid preparations made using the RevPrep Orbit (GeneMachines). Plasmid preparations were sequenced from the 5′ end using the dideoxy chain termination method with "Big Dye Terminator" (Applied Biosystems) and the BK reverse vector primer. The reactions were precipitated and resuspended in "Hi-Di Formamide with EDTA" (Applied Biosystems) and run on an ABI Prism 3730 automated sequencer (Applied Biosystems). For some of the genes that were sequenced initially from the 5′ end, the cDNA was subsequently fully sequenced using 3' vector and internal primers.

Sequence chromatogram files were trimmed for quality using phred (http://www.phrap.org/phrap.docs/phred.html), vector screened using cross match (http://www.phrap.org/phrap.docs/phrap.html) and analyzed locally using (1) blastx against the NCBI nonredundant (nr) protein database, (2) blastn against the NCBI nucleotide (nt) database and (3) blastn against the NCBI EST (dbEST) database. A total of 1048 sequences from both libraries were annotated. All sequences were grouped by category and tentative identification was based initially on a blastx similarity score of $<10^{-3}$ or, in the case of blastx scores of $>10^{-3}$, a blastn score of $<10^{-5}$. All sequences were analyzed for redundancy using CAP3 [15].

Both membrane-bound and cytoslic compornents of Phox oxidase were identified from the annotated sequences. Subunits, p22*phox*, p40*phox* and p67*phox* where submitted to the GenBank repository (accession nos. **AY597051**, **AY597050** and **AY597049**, respectively), whereas the components Nox2 and p47*phox* shared 99-100% sequence identity with the trout Nox2 and p47*phox* sequences previously submitted by Hoshiko et al. (GeneBank accession nos. **AB192465** and **AY597049**).

### 2.4. Phylogenetic analysis

Basic Local Alignment Search Tool (BLASTX) at the NCBI (http://www.ncbi.nlm.nih.gov/) was used in order to search similarities with known genes. Sequence and motif analysis were performed using ExPASy (http://www.expasy.org) SMART (http://smart.embl-heidelberg.de) and Pfam 22.0 (http://pfam.sanger.ac.uk) web tools [16]. Multiple sequence alignments were generated with Clustal X2

Multiple Sequence Alignment software [17], and identities and similarities were analyzed with BioEdit software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). A Neighbour Joining (NJ) analysis was conducted on selected vertebrate Phox sequences (see figures 1-5 for a complete list of GenBank accession numbers) using the JTT algorithm available in the MEGA 4.0.1 phylogenetic package [18]. Confidence in estimated relationships of ML tree topologies was evaluated by a bootstrap analysis with 2000 replicate

### 2.5. Gene expression studies

Following stimulation, medium was decanted and replaced with 1ml of Tri Reagent (Molecular Research Center, Inc.)/dish. Total RNA was extracted from the Tri Reagent according to the manufacturer's protocol. Total RNA from cells stimulated/no-stimulated with the different PAMPs (***P***athogen ***A***ssociated ***M***olecular ***P***atterns) were reversing transcribed to cDNA with Super Script III (RNase transcriptase) (Invitrogen) and an anchored oligo-dT primer (Promega). To examine the expression of Phox oxidase subunits in trout PAMP-stimulated macrophages, the patterns of single/dose-response stimulation and time course experiments were analysed by PCR. Amplification was performed in G-Storm thermocycler using primers designed for each Phox component (Table 1), with a step of 94º C 5 min, 35-30-25 cycles to 94º C 45 sec, 56º C 45 sec and 72º C 1 min, followed by 1 cycle of 72º C for 7 min, using 18S as a control. Products were visualized on a 1% agarose gel containing 1 μg/ml of ethidium bromide under UV light.

## 3. Results

### 3.1. Sequence analysis of trout Phox genes

The deduced amino acid sequences of full-length cDNAs of rainbow trout Phox components (Nox2, p22*phox*, p40*phox*, p47*phox* and p67*phox*) were compared with those of human (*Homo sapiens*) and of several teleost fish: Ayu (*Plecoglossus altivelis*), Fugu (*Takifugu rubripes*), Chinese perch (*Siniperca chuatsi*), Carp (*Cyprinus carpio*) and Zebrafish (*Danio rerio*). When compared to other species, the deduced amino acid sequences of the trout Phox components were most similar to those of teleosts (Table 1). Overall, the highest identity was observed with the catalytic subunits of trout Phox, Nox2 (68-89%) and p22*phox* (61-87%). Among the regulatory components, p47*phox* and p40*phox* attained the highest identities (51/53-71/74%) and the lower identity was observed with p67*phox* (44-68%). As stated below, the functional domains and motifs of Phox components seem to be also well conserved among teleosts.

**Table 1.** Primers used in expression studies

| **Phox genes** | | **Nucleotide sequence** |
|---|---|---|
| Nox2 | Nox-Fw | CATCGCCCACCTGTTTAACT |
| | Nox-Rw | GTATGACCTGCGGATGACCT |
| p22*phox* | 22-Fw | TGGGCTAAGCTCACTCAGGT |
| | 22-Rw | CATCAGCAGCAGTGTGTCCT |
| P40*phox* | 40-Fw | GAGCTGAGGTGGTCTTCGAC |
| | 40-Rw | TCAACACACACATCCCTGGT |
| P47*phox* | 47-Fw | CCACCCAGACGGTCTACAAT |
| | 47-Rw | CTGGCTTTGGGATGTTGTCT |
| P67*phox* | 67-Fw | CTCTGGCTTTGCTCCGTTAC |
| | 67-Rw | GGTTATTTCCAAACGCTCCA |

### 3.1.1. Nox2

The catalytic component of Human Phox Nox2 anchors to the membrane by six N-terminal transmembrane domains (TMR) and contain two pairs of invariant histidines (arrowheads in Figure 1A), each in TMR3 ($H^{101}$ and $H^{115}$) and TMR5 ($H^{209}$ and $H^{222}$) regions, which act as coordinating binding sites for heme groups. The loss of both TMR3 and TMR5 conserved histidines has been show to abolish totally or partially the ligation of the two hemes [19]. The C-terminal region contains cytoplasmatic FAD- and NADPH-binding domains homologous to the ferredoxin NADPH reductase family of flavocytochromes, capable of moving electrons to the plasma membrane. In mammals as the deduced trout protein Nox2, the FAD-binding domain includes a critical residue ($His^{338}$) for incorporation of FAD in Nox2 [20]. The binding sites for the pyrophosphate ($^{405}$MLVGAGIGVTPF), adenine ($^{504}$GLKQ) and nicotinamide ($^{535}$FLCGPE) components of NADPH are also well conserved [2]; the binding site for NADPH ribose in teleosts differs from the human sequence ($^{442}$YWLCR) only in the substitution of $Arg^{446}$ by Proline. The putative cytoplasmic binding sites for p47*phox* [21,22] are also well conserved in rainbow trout Nox2 as in other teleosts (Figure 1A, open boxes). Human Nox2 is a heavily glycosylated protein [23], and the putative consensus sites for N-linked glycosylation (N-X-S/T) are also present in trout Nox2 (Figure 1A, gray circles).

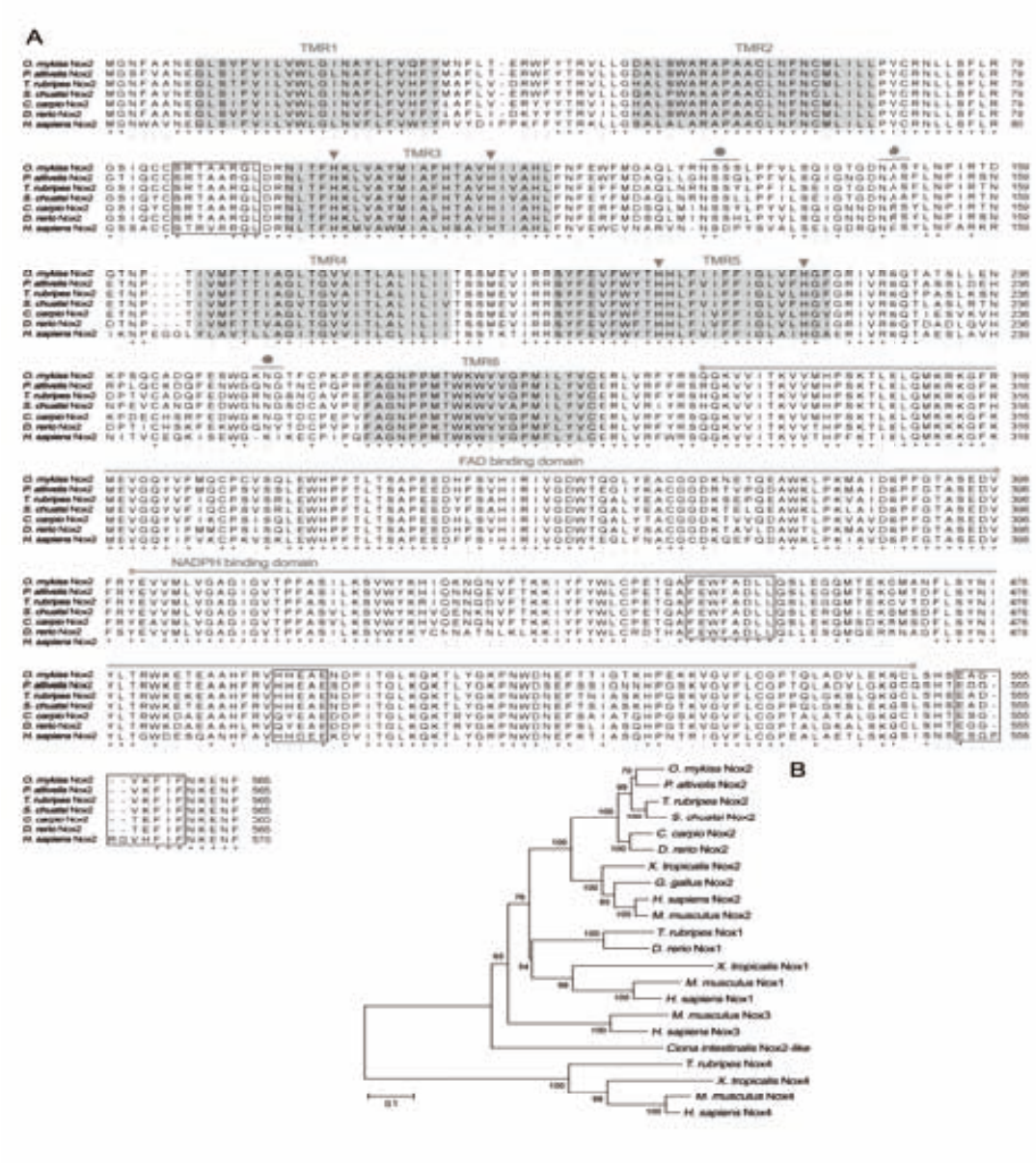


**Figure 1. A:** Alignment of the predicted amino acid sequence of rainbow trout Nox2 with those of selected teleost species and *Homo sapiens*. Conserved amino acids shared by all the sequences are indicated by an asterisk The putative p47phox binding sites and the six predicted transmembrane (TMR) regions are indicated by open and gray boxes, respectively. The FAD and NADPH-binding domains are also represented. The putative heme-coordinating histidines are indicated by arrowheads. Putative consensus sites for N-linked glycosylation (N-X-S/T) are represented by gray circles. GenBank accession numbers for aligned Nox2 sequences are as follows: Rainbow trout (*Oncorhynchus mykiss*) **BAD60779**, Ayu (*Plecoglossus altivelis*) **BAF45308**, Fugu (*Takifugu rubripes*) **NP_001027904**, Chinese perch (*Siniperca chuatsi*) **ABC72118**, Carp (*Cyprinus carpio*) **BAF73664**, Zebrafish (*Danio rerio*) **NP_956708** and human (*Homo sapiens*) **NP_000388**. **B:** Bootstrap consensus tree for Nox homologues in vertebrates inferred using the NJ method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (2000 replicates) are shown next to the branches. The evolutionary distances were computed using the JTT matrix-based method and are in the units of the number of amino acid substitutions per site. In addition to the aforementioned Nox2 sequences, GenBank accession nos. for Nox2 are: Frog (*Xenopus tropicalis*) **NP_001025689**, Chicken (*Gallus gallus*) **NP_001093756**, Mouse (*Mus musculus*) **NP_031833** and an Urochordate Nox2-like (*Ciona intestinalis*) **BR000272**. GenBank accession nos. for Nox1

are: Fugu (*takifugu rubripes*) **BR000268**, Zebrafish (*Danio rerio*) **BR000266**, Mouse (*Mus musculus*) **NM_172203** and Human (*Homo sapiens*) **NM_007052**. GenBank accession nos. for Nox3 are: Mouse (*Mus musculus*) **AY573240** and Human (*Homo sapiens*) **NM_015718**. GenBank accession nos. for Nox4 are: Fugu (*Takifugu rubripes*) **BR000275**, Mouse (*Mus musculus*) **NM_015760** and Human (*Homo sapiens*) **NM_016931**. Ensembl gene numbers for Frog (*Xenopus tropicalis*) Nox1 and Nox4 are **ENSXETP00000021243** and **ENSXETP00000025676** respectively.

### 3.1.2. p22*phox*

Human p22*phox* acts as a stabilizer of and forms together with Nox2 the catalytic Phox cytochrome b558 complex. It contains two transmembrane regions (TMR) and a cytosolic C-terminal Proline-rich region (PRR) involved in the interaction with the SH3 domains of cytosolic p47*phox* [24]. Both the TMR and PRR regions, along with several residues crucial for high affinity binding of human p22*phox* with p47*phox* are well conserved in trout p22*phox* (Figure 2A, arrowheads). The alpha-helix in the C-terminal half of human p22*phox* includes a consensus sequence ($^{161}$AEAR) that appears to enhance the affinity of p22*phox* for p47*phox* [25]. In trout p22*phox*, the deduced sequence is highly similar to the human counterpart, with the substitution of Ala163 by Methionine.

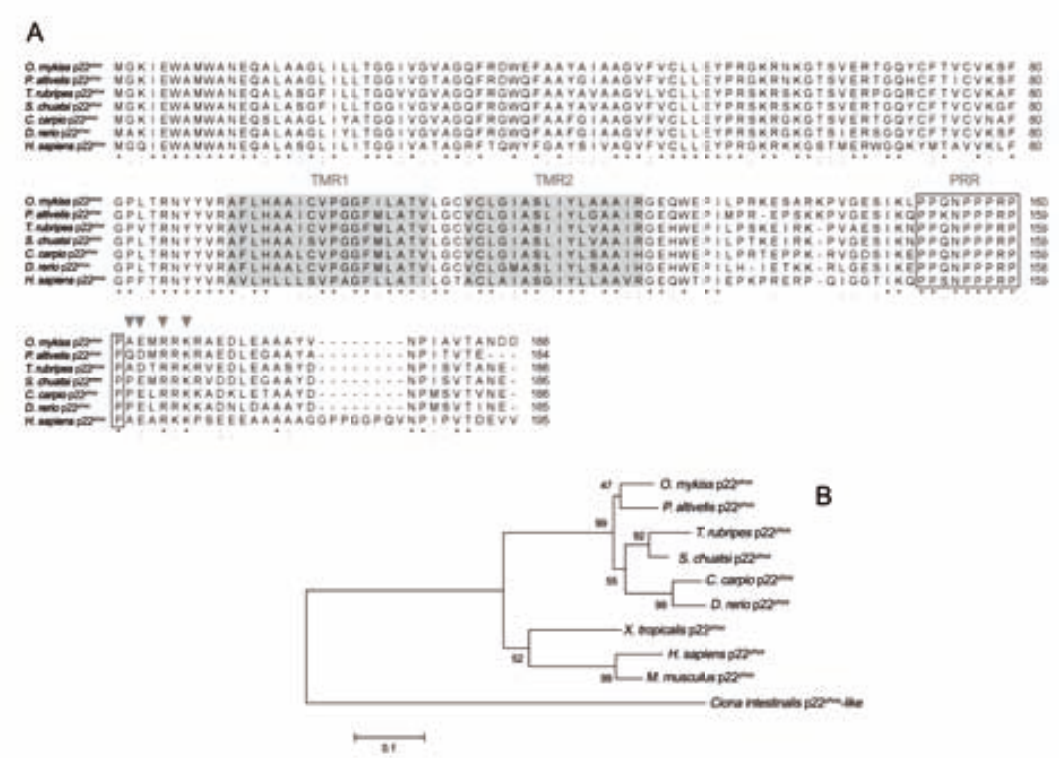


**Figure 2.** Alignment of the predicted amino acid sequence of rainbow trout p22*phox* with those of selected teleost species and *Homo sapiens*. Conserved amino acids shared by all the sequences are indicated by an asterisk. The predicted transmembrane regions (TMR) are indicated with gray boxes and the Proline-rich region (PRR) with open boxes. The residues crucial for high affinity binding with human p47*phox* are indicated by arrowheads. GenBank accession numbers

for aligned p22*phox* sequences are as follows: Rainbow trout (*Oncorhynchus mykiss*) **AAU04547**, Ayu (*Plecoglossus altivelis*) **BAF45309**, Fugu (*Takifugu rubripes*) **NP_001027717**, Chinese perch (*Siniperca chuatsi*) **ABC72118**, Carp (*Cyprinus carpio*) **BAF73665**, Zebrafish (*Danio rerio*) **NP_956873** and Human (*Homo sapiens*) **AAH06465**. **B**: Bootstrap consensus tree for p22*phox* homologues in vertebrates inferred using the NJ method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (2000 replicates) are shown next to the branches. The evolutionary distances were computed using the JTT matrix-based method and are in the units of the number of amino acid substitutions per site. In addition to the aforementioned p22*phox* sequences, GenBank accession nos. for p22*phox* are: Frog (*Xenopus tropicalis*) **AAI21218**, Mouse (*Mus musculus*) **NP_031832** and and an Urochordate p22*phox*-like (*Ciona intestinalis*) **AK114374**.

### 3.1.3. p47*phox*

By far the most studied component of mammalian Phox, the p47*phox* subunit seems to act as an adapter of the p67*phox*-Nox2 complex [1], as suggests by the absence of membrane attachment signal in p67*phox* and by the fact that in cell-free NADPH oxidase essays p47*phox* is not required for the assembly of the oxidase under high concentrations of p67*phox* [26]. In the inactive state, the serine residues in the C-terminal autoinhibitory region (AIR) of human p47*phox* constitute the targets for phosphorylation, whereas the SH3 domains block the activation by binding with the Proline-rich region in AIR [27]. Upon phosphorylation, the binding unblocks and the SH3 domain binds with the PRR region of p22*phox*. The deduced amino acid sequence of trout p47*phox* contains the SH3 and AIR domains, and also highly conserved residues necessary for the activation/inhibition of p47*phox* (Figure 3A), including the residues ($Pro^{363}$, $Pro^{366}$ and $Arg^{368}$) necessary for binding in human p47*phox* [28], and several of the residues 370-390 in the PxxP motif (Figure 3A, gray arrowheads) required for the stable interaction with p67*phox*. In the interaction with Nox participates the Phox homology (PX) domain, a N-terminal region of p47*phox* that binds to phosphatidylinositol (3,4)$P_2$ and phosphatidic acid [29-31]. In trout p47*phox* the PxxP motif ($^{70}$RIIPHLPAP) in the PX domain that regulates the phosphoinositide binding in mammals [32], is also highly conserved, with the minor substitution of $His^{74}$ by Glutamate. Several of the residues (Figure 3, open arrowheads) necessary for the activation and binding to human Nox2 [25] are also present in the PX and SH3-A domains of trout p47*phox*.

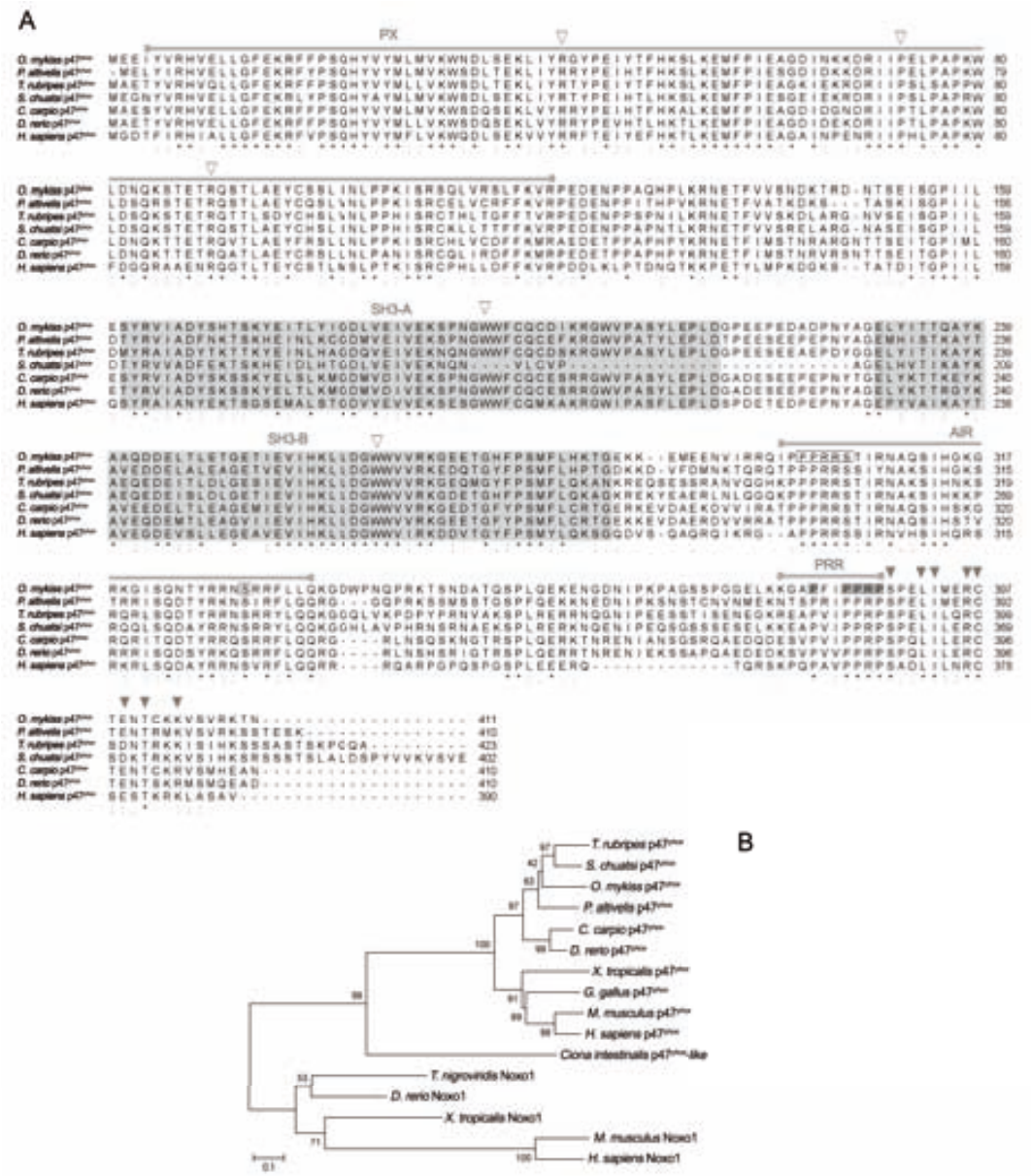


**Figure 3. A:** Alignment of the predicted amino acid sequence of rainbow trout p47*phox* with those of selected teleost species and *Homo sapiens*. Conserved amino acids shared by all the sequences are indicated by an asterisk. The predicted SH3-A and -B binding domains are indicated with gray boxes. The putative PX and autoinhibitory region (AIR) domains and the proline-rich region (PRR) are shown underlined. The residues necessary for the interaction with human p67*phox* and for activation oh human Nox2 are indicated by gray and open arrowheads, respectively. Also shown are the residues required for inhibition/activation of human p47*phox* in the AIR domain (tiny open boxes) and the conserved binding residues in the PRR domain (gray-coloured). GenBank accession numbers for aligned p47*phox* sequences are as follows: Rainbow trout (*Oncorhynchus mykiss*) **BAD60781**, Ayu (*Plecoglossus altivelis*) **BAF45310**, Fugu (*Takifugu rubripes*) **NP_001027718**, Chinese perch (*Siniperca chuatsi*) **ABC72118**, Carp (*Cyprinus carpio*) **BAF73666**, Zebrafish (*Danio rerio*) **CAM16641** and Human (*Homo sapiens*) **AAF34737**. **B:** Bootstrap consensus tree for Noxo1 homologues in vertebrates inferred using the NJ method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (2000 replicates) are shown next to the branches. The evolutionary distances were computed using the JTT matrix-based method and are in the units of the number of amino acid substitutions per site. In addition to the

aforementioned p47*phox* sequences, GenBank accession nos. for p47*phox* are: Frog (*Xenopus tropicalis*) **NP_001106375**, Chicken (*Gallus gallus*) **NP_001025880**, Mouse (*Mus musculus*) **BAE29979** and an Urochordate Nox2-like (*Ciona intestinalis*) **NM_001033828**. GenBank accession nos. for Noxo1 are: Fugu (*Tetraodon nigroviridis*) **BR000291**, Zebrafish (*Danio rerio*) **BR000290**, Frog (*Xenopus tropicalis*), Mouse (*Mus musculus*) **NM_027988** and Human (*Homo sapiens*) **NM_172167**. Ensembl gene number for Noxo1 is: **ENSXETT00000015702**.

### 3.1.4. p67*phox*

Like p47*phox*, the human and deduced amino acid sequence of trout p67*phox* contains tandem C-terminal SH3 domains one of which (SH3-B) is involved in the interaction with p47*phox* and other regions mediating protein-protein interactions, such as the tetratricopeptide repeats (TPR) and the protein binding module PB1, located in the C-terminus and responsible for the interaction with the PCCR motif of p40*phox* [33]. Several residues in the PB1 domain involved in the interaction with human p40*phox* are also conserved in trout (Figure 4A, gray arrowheads). In human, the TPR regions of p67*phox* act as binding sites for the Rac-GTPase [34] and thus contribute, together with the activation domain (AD) that controls the transfer of hydride from NADPH to FAD [35], to the tethering of p67*phox* to the membrane and subsequent activation of Nox2, thus a similar function can be inferred in trout p67*phox* owing to the conservation of several residues, most notably $Val^{204}$ crucial for the activation of Nox2 in humans, [35] in those domains. Trout p67*phox* contains, as in the rest of vertebrates NADPH oxidases studied so far, exclusive SH3-A and ADSIS (AD-SH3 Intervening Sequence) domains of unknown function [7], the last with a highly conserved PRR region ($^{227}$PRP in humans).

### 3.1.5. p40*phox*

The human p40*phox* acts as an enhancer in the activation of Nox2. Upon phosphorylation, p40*phox*, along with p67*phox*, binds to phosphorylated lipids in the membrane by means of the PX domain. In human, and probably in trout p40*phox*, several conserved residues in the PX domain ($Phe^{35}$, $Arg^{57}$, $Tyr^{59}$, $Leu^{65}$ and $Arg^{105}$) are essential in the phospholipid binding [29]. A head-to-tail PX-PB1 domain intramolecular interaction in inactive p40*phox* has been suggested as a blockade to the anchoring of p40*phox* to the membrane [36], and the residues described in human crucial for the union between the PX and PB1 domains ($Phe^{35}$, $Val^{257}$, $Pro^{265}$ and $Phe^{320}$) and the activation of Phox (residues 318-328) are also conserved in trout, with the exception of the variable residue ($Leu^{273}$) already reported in other vertebrate species [7]. The residues Asp289, Glu291, Asp293 and Asp302 in the human OPCA (OPR/PC/AID) motif in the PB1 domain of human p40*phox*, required for the binding to p67*phox* [37] as mentioned above are also conserved in trout (Figure 5, gray arrowheads). To date, no putative function has been ascribed to the SH3 domain of p40*phox*.

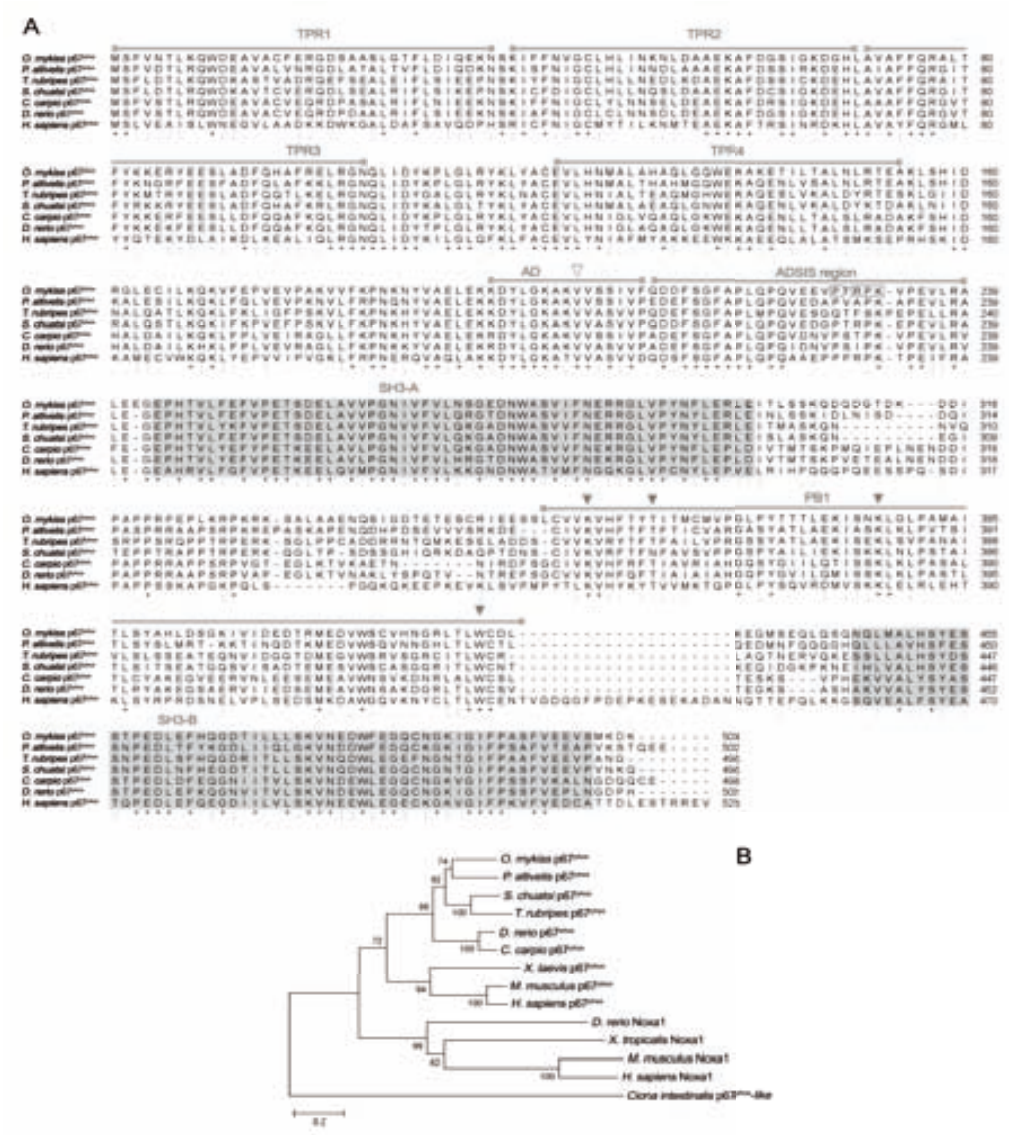


**Figure 4. A:** Alignment of the predicted amino acid sequence of rainbow trout p67*phox* with those of selected teleost species and *Homo sapiens*. Conserved amino acids shared by all the sequences are indicated by an asterisk. The predicted SH3-A and -B binding domains are indicated with gray boxes. The four putative tetratricopeptide repeat motifs (TPR), the PB1 domain and the AD and ADSIS regions are shown underlined. The residues involved in the interaction with human p40*phox* (gray arrowheads) and activation of human Nox2 (open arrowheads) are also shown. The PRR-like conserved residues in the ADSIS region are represented with tiny open boxes. GenBank accession numbers for aligned p67*phox* sequences are as follows: Rainbow trout (*Oncorhynchus mykiss*) **AAU04545**, Ayu (*Plecoglossus altivelis*) **BAF45311**, Fugu (*Takifugu rubripes*) **NP_001027854**, Chinese perch (*Siniperca chuatsi*) **ABC72120**, Carp (*Cyprinus carpio*) **BAF73667**, Zebrafish (*Danio rerio*) **FAA00363** and human (*Homo sapiens*) **AAA36379. B:** Bootstrap consensus tree for Noxa1 homologues in vertebrates inferred using the NJ method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (2000 replicates) are shown next to the branches. The evolutionary distances were computed using the JTT matrix-based method and are in the units of the number of amino acid substitutions per site. In addition to the aforementioned p67*phox* sequences, GenBank accession nos. for p67*phox* are: Frog (*Xenopus laevis*) **NP_001086058,** (*Mus musculus*) **ABE02824** and an Urochordate p67*phox*-like (*Ciona intestinalis*) **NM_001033828** GenBank accession nos. for Noxa1 are: Zebrafish (*Danio rerio*) **XM_679087**, Frog (*Xenopus tropicalis*) **BC075351**, Mouse (*Mus musculus*) **NM_172204** and Human (*Homo sapiens*) **NM_006647**.

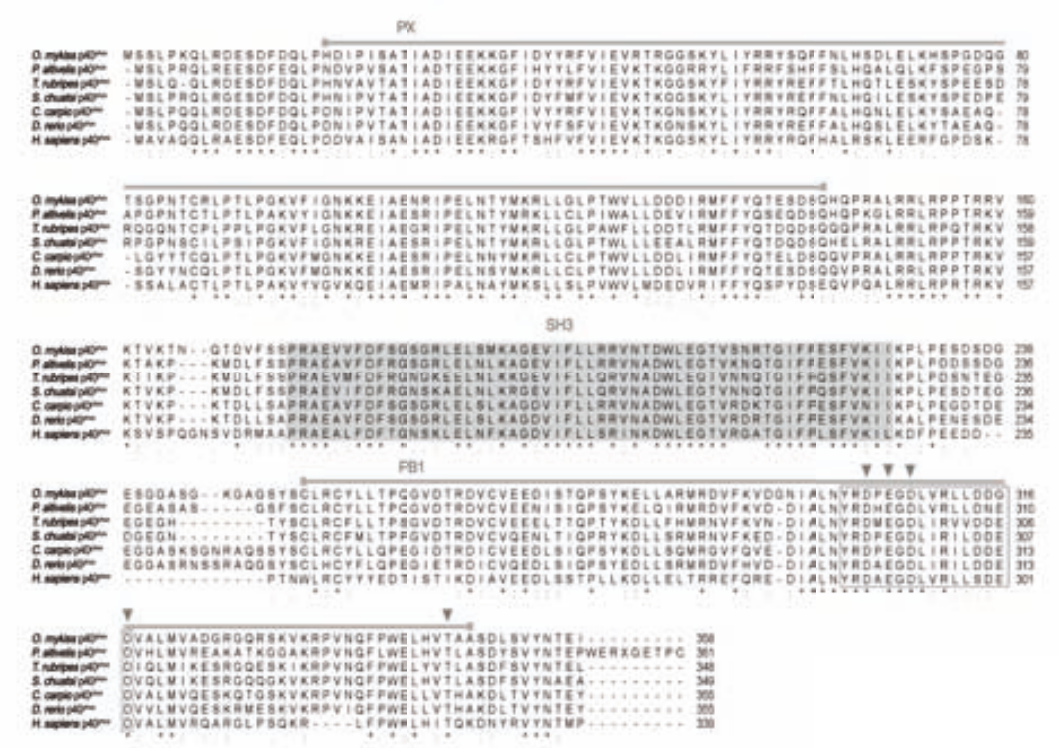


**Figure 5.** Alignment of the predicted amino acid sequence of rainbow trout p40*phox* with those of selected teleost species and *Homo sapiens*. Conserved amino acids shared by all the sequences are indicated by an asterisk. The predicted SH3 binding domain (gray box) and the PX and PB1domains (underlined) are shown. The OPCA motif (open box) required for the binding with human p67*phox* PB1 domain are also represented. The residues involved in the interaction with p67*phox* are indicated by arrowheads. GenBank accession numbers for aligned p40*phox* sequences are as follows: Rainbow Trout (*Oncorhynchus mykiss*) **AAU04546**, Ayu (*Plecoglossus altivelis*) **BAF45312**, Fugu (*Takifugu rubripes*) **NP_001027719**, Chinese perch (*Siniperca chuatsi*) **ABC72119**, Carp (*Cyprinus carpio*) **BAF73668**, Zebrafish (*Danio rerio*) **NP_001076354** and Human (*Homo sapiens*) **CAG46875**.

### 3.2. Phylogenetic relationships of teleost Phox genes

The phylogenetic tress depicted in Figures 1B, 2B, 3B and 4B show the molecular taxonomic relationships of vertebrate homologues of the Nox1/2/3/4, p47*phox*/Noxo1 and p67*phox*/Noxa1 families, respectively. As in the representative tree of vertebrate Phox components discussed below, each trout Phox subunit analyzed clusters with the correspondent homologue in teleosts. Trout Nox2, p22*phox*, p47*phox* and p67*phox* amino acid sequences are closely related with those of the ayu (*Plecoglocssus altivelis*), another basal euteleostean [38].

### 3.3. Induction of Phox components in LPS-, poly(I:C)- and zymosan-primed macrophages

No remarkable differences were observed in the induction of trout Phox in differentiated macrophages upon incremental doses (5, 10 and 50 μg/ml) of LPS, poly(I:C) and the fungal PAMP zymosan (Figure 6). Albeit being similar across treatments, low expression levels were detected in

LPS-stimulated p40*phox* transcripts. Low induction of p47*phox* and, most notably, p67*phox* transcripts defined the response to increasing doses of poly(I:C) (Figure 6).

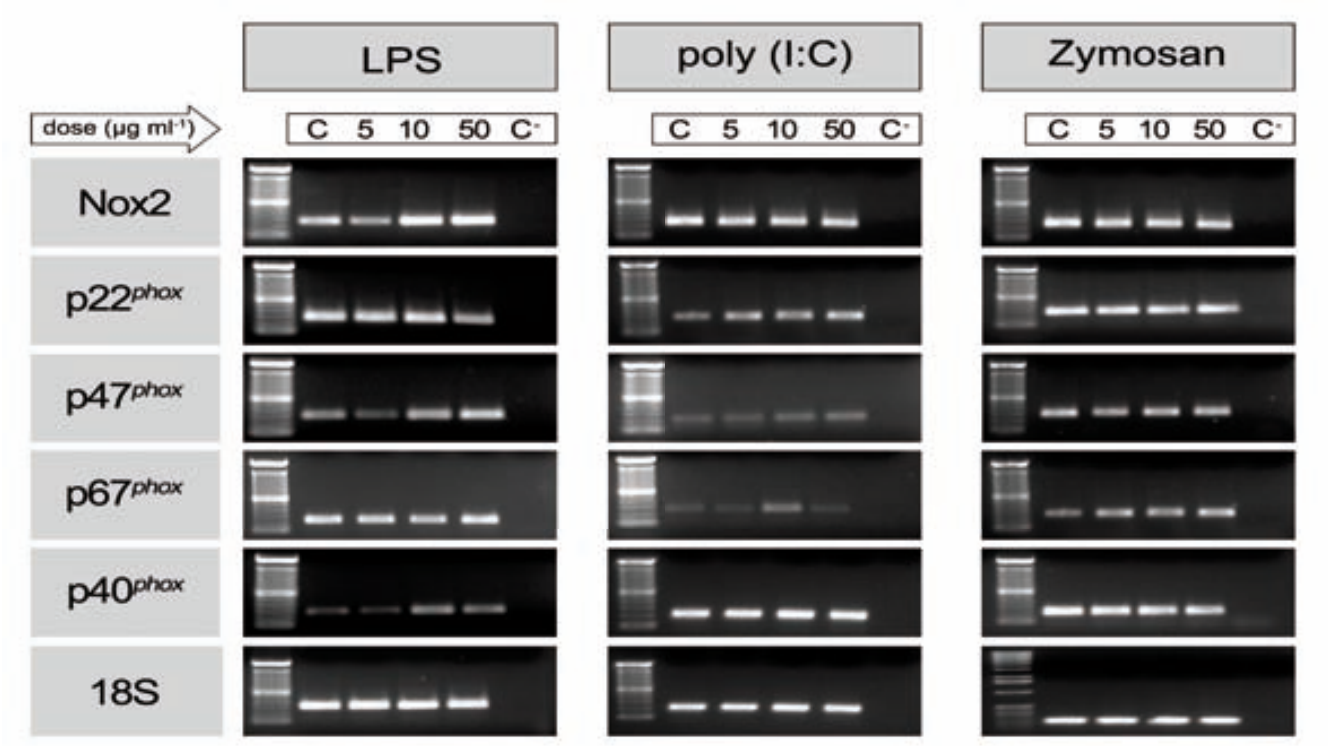


**Figure 6.** Dose-response expression of rainbow trout Nox2, p22*phox*, p47*phox*, p67*phox* and p40*phox* in macrophages stimulated with 5, 10 and 50µg/ml of *E. coli* LPS, poly (I:C) and zymosan. Amplification of 18s was used as a control. C: unstimulated (control); C-: negative control.

### 3.4. Time-course expression of Phox components over differentiation in cultured monocyte/macrophages

A repetitive PAMP stimulation (LPS, poly(I:C) and zymosan) with maximal doses (50 µg/ml) of differentiating monocyte/macrophages was performed to elucidate the cell response over a period of five days. As previously described, differentiated $4^{th}$-$5^{th}$ day macrophages are capable of respond to stimulation and/or secrete cytokines [13], thus the time-course stimulation outlines the PAMP-responsiveness during the transition/differentiation of head kidney-derived monocyte populations. Only the p47*phox* component showed a consistent and highly similar expression during the experimental period, regardless of the stimulus (Figure 7). The expression of p22*phox* showed a similar pattern, but the level of transcripts in LPS-stimulated macrophages diminished in the $4^{th}$ and $5^{th}$ days, as in LPS-stimulated Nox2 and p49*phox*. The expression of p67*phox* seemed not to be affected by LPS stimulation during the experimental period. By contrast, the repetitive stimulation by poly(I:C) induced marked expression of p67*phox* and Nox2 on days 1-4 and 1-3, respectively. The p40*phox*

component followed a variable pattern of expression, although with higher responsiveness to poly(I:C) stimulation. P40*Phox* and p67*Phox* were stimulated on the 5th day by zymosan, but no expression was detected for zymosan-stimulated Nox2 subunit on days 2-5. Overall, this results suggest a more pronounced and sustained effect of poly(I:C) treatment on the expression of Phox subunits following repeated monocyte/macrophage stimulation.

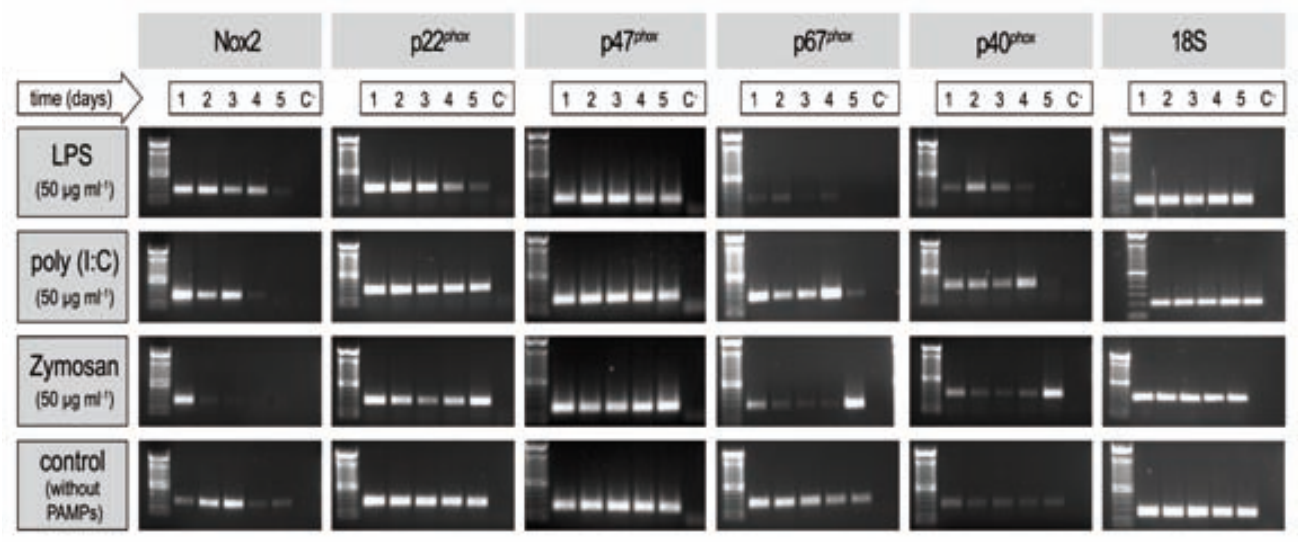


**Figure 7.** Time course expression of rainbow trout Nox2, p22*phox*, p47*phox*, p67*phox* and p40*phox* in monocyte/macrophages exposed to 50µg/ml of *E. coli* LPS, poly (I:C) and zymosan. Amplification of 18s was used as a control. C-: negative control.

## 4. Discussion

The degree of shared homologies among vertebrates in the predicted amino acid sequences of Phox components depicted in Figures 1to5 suggests that, in trout, as in mammals, all the Phox subunits of trout macrophages contain the essential domains and interaction modules required for the correct activation of the enzymatic complex. To date, the characterised Phox components in fish, either in terms of structure and function are scarce and include those of basal teleosts [39] (Ostariophysi: *Danio rerio*, *Cyprinus carpio*) and those belonging to the so-called "bushy top" [40] species (Percomorpha: Aterinomorpha: *Oryzas latipes*; Tetraodontifomres: *Takifugu rubripes*, *Tetraodon nigroviridis*). Therefore, the description of Phox components in a primitive species, the rainbow trout, representative of one of the main groups of basal teleosts (Protacanthopterygii), contributes to the overall phylogenetic definition of Nox enzymes, as shown by the grouping of Phox components with the basal Osmeriforme *Plecoglossus altivelis*.

As indicated in Figures 1B and 4B, the Nox2-like, and P67*phox*-like components in *C. intestinalis* clearly predates the diversification of Nox1/2 and p67*phox*/Noxa1 respectively in vertebrates [41]. The p22*phox*-like and p47*phox*-like components of *C. intestinalis* constitutes the basal taxa for the diversification of vertebrate p22*phox* (Figure 2B) and p47*phox* (Figure 3B) components, and thus shares a common ancestor with the p47*pho*/Noxo1 homologues in vertebrates as have been previously suggested [4,7]. Therefore, the branching pattern of Nox2, p47*phox* and p67*phox* in fish as in the rest of vertebrates suggests the emergence of Phox-related components before the diversification of basal euteleosts. As has been reported [4], the Nox3 and Nox4 proteins seems to be restricted to vertebrates other than fishes, whereas Nox1 and Nox2 are widespread in vertebrates (Figures 1B).

The regulation of NADPH oxidase gene expression and intracellular signalling either in mature (activated) neutrophils and tissue macrophages (reviewed in [42]) are quite similar, nevertheless both cellular types accomplish different, though complementary, functions during acute inflammation (e.g. the gradual, long-lasting and iterative onset of respiratory burst of macrophages upon continuous stimulation, in sharp contrast to the stepped, short-lived and non-iterative production of ROS observed on stimulated neutrophils [43,44]). We have previously demonstrated the acquisition of a mature reactive macrophage phenotype, in terms of PAMP responsiveness, phagocytic capacity and cytokine secretion in head kidney-derived cells from trout incubated for 5 days in primary culture [13,45,46]. Thus a natural extension of the model concerns the expression of Trout Phox subunits described herein, during the 5-day incubation period and at its terminus.

The single or dose-response stimulation with zymosan did not show significative differences in the expression of trout Phox subunits (Figure 6). Maximal doses of LPS (50 μg/ml) did not seem to enhance the expression of the catalytic and activator components (Nox2/p22*phox* and p67*phox*, respectively), the organizer subunit (p47*phox*) or the modulatory component (p40*phox*) of trout Phox, albeit the later showed a low expression. In this respect, several evidences [47,48] ascribe a minor collaborative role of p40*phox* in the activation of Phox monocyte/macrophages, as shown by its lack of response following stimulation with INF-γ and TNF-α in human monocytes [49]. Therefore, the relative dispensability of p40*phox* related to the onset of Phox-mediated ROS response, could explain the low expression of its transcripts either in immature and differentiated LPS-stimulated trout (Figures 6 and 7). Thus, our results suggest an in vitro constitutive expression of trout Phox components in mature macrophages stimulated with maximal doses (up to 50 μg/ml) of LPS or zymosan.

In mammals, LPS is well-known as a primer for enhancing ROS responses in phagocytes, by means of up-regulation of NADPH oxidase assembly and activation of its catalytic components [50-52]. It has also been demonstrated a Phox-dependent transcriptional modulation of NF-κB through participation in events upstream the Toll-like receptor 4 (TLR4) signalling pathway [53]. However, the effective dose for inducing a measurable expression of all Phox components in trout macrophages (10 to 50 μg/ml) is several orders of magnitude higher than the commonly used in its mammalian counterparts [53,54].

The stimulation of trout monocyte/macrophages with zymosan had no noticeable effects in the expression of p22*phox* and p47*phox* during the maturation period, but affected the expression of Nox2, p67*phox* and p4o*phox* at the beginning (day 1) and final (day 5) of the differentiation process. By contrast, the repeated stimulation with maximal doses of LPS elicited a diminished time-dependent pattern of expression in the catalytic, activator and modulator components of trout Phox. Moreover, maximal doses of LPS failed to regulate the expression of p67*phox* during the incubation period. By contrast, the organizer component, p47*phox*, was up-regulated regardless of the intensity or frequency of the stimulus.

As stated above, in mammals Nox2 and p22*phox* are believed to act as a unique catalytic unity (flavocytochrome b588), necessary for the activation of Phox in macrophages, and the similar pattern of expression in its trout counterpart seems to state for the coordinate expression/activation of Phox in response to the LPS stimulation. However, the lack of LPS-mediated induction of p67*phox* transcripts during all the maturation process in trout monocyte/macrophages suggests a lack of Phox-mediated oxidative response in those cells undergoing repeated stimulation with LPS. In mammals, the regulation of p67*phox* is delayed in maturing myeloid cells and thus is considered a rate-limiting cofactor in Phox activation [55]. Therefore, the aforementioned gradual, long-lasting and iterative onset of respiratory burst typical of macrophages upon continuous stimulation must depend of the activation of other ROS-generating enzymatic complexes, such as nitric oxide synthases (NOS). This, in turn, suggests an alternate non-oxidative role of Nox2 and p22*phox*, extensive to the constantly up-regulated p47*phox* (Figure 7), in the intracellular signalling pathways. In mammals, Nox2 is considered the most ubiquitous NOX isoform its cell and tissue distribution suggesting an extended, albeit not fully understood, role in regulation of proinflammatory genes, cell proliferation and apoptosis (reviewed in [56]). A similar role has also suggested for p47*phox* [57]. In this respect, our results follow those of Inoue et al. [68] relative to the fast and sustained expression of p47*phox* and the low and slow expression of Nox2 and p22*phox* in differentiating myeloid HL-60 and U397 cell lines stimulated repeatedly with PMA.

In trout mature macrophages, the exposition to single (10 μg/ml) or incremental doses (5 to 50 μg/ml) of poly(I:C) had no effect on the expression of catalytic Phox subunit as well as the p40*phox* component. However, a lower transcript expression of p47*phox* and most notably, p67*phox* was detected (Figure 6). The last may be attributable to the collaborative/organisative role of the p47*phox* in the activation of Phox discussed above. Besides, it has been shown that low levels of p67*phox* can produce an effective Phox-mediated ROS response [58]. Therefore, the activation of Phox in trout mature macrophages does not seem to be impaired upon stimulation with poly(I:C), albeit no clear differences exist between the doses tested.

In contrast to LPS stimulation, our result suggest a constitutive expression of all trout Phox upon repeat stimulation with poly(I:C). The expression of p22*phox, p47phox*, p67*phox* and p40*phox* transcripts lasted until the $4^{th}$-$5^{th}$ day of maturation, but the expression of Nox2 seemed to be related to the early differentiation process (Figure 7). However, we cannot rule out an inactivation of Phox induced by repeated PAMP stimulation during the differentiation period. Interestingly, two of the catalytic/activator components (Nox2/p22*phox* and p67*phox*, respectively) remained highly expressed during differentiation even though high levels of catalytic components are not required to sustain a ROS response [58].

Overall, the transcript response of Phox components to repeated simulation with poly(I:C) of trout macrophages during the maturation/differentiation period appeared stronger than that of LPS or zymosan. This suggests an enhanced responsiveness of ROS response to viral rather than bacterial insults in trout macrophages. The recently described TLR3 in rainbow trout phagocytes [59], together with the uncoupling of IFN and bacterial-related responses in trout macrophages stimulated with poly(I:C) [60] may imply that the differences observed in the single and repeated poly(I:C)-mediated stimulation of trout macrophages can be due to the differential stimulation pathways endured by maturing macrophages. In fact, the onset of respiratory burst in granulocytes of the gilthead sea bream (*Sparus aurata*) stimulated with poly(I:C) has been attributed to the presence of poly(I:C)-responsive macrophages in the stimulation assay [61].

**Acknowledgments**

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# Chapter IV

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## Peptidoglycan, not endotoxin, is the key mediator of cytokine gene expression induced in rainbow trout macrophages by crude LPS☆

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# Peptidoglycan, not endotoxin, is the key mediator of cytokine gene expression induced in rainbow trout macrophages by crude LPS

**Abstract**

**In trout macrophages, phenol-extracted LPS preparations stimulate proinflammatory cytokine expression but ultrapure preparations of endotoxin are inactive. Crude LPS preparations could potentially have a number of contaminants including PGNs, nucleic acids and lipoproteins. Thus, in the current study we individually tested potentially contaminating pathogen associated molecular patterns (PAMPs) on trout macrophages to determine which ones could induce proinflammatory cytokine expression. We found that PGNs derived from Gram-negative bacteria (*E. coli* 0111:B4 and K12), are potent inducers of IL-1β and IL-6 expression and were equal to, or more potent than, crude LPS. On the otherhand, PGNs of Gram-positive bacteria, DNA, RNA and lipoteichoic acid were weak stimulators, and lipid A, lipoprotein (Pam3CSK4) and ultrapure LPS were nonstimulatory. More importantly, crude LPS treated with lysozyme to degrade PGNs, exhibited greatly reduced activity in stimulating IL-1β and IL-6, indicating that PGNs in the crude LPS are responsible for a significant amount of the proinflammatory activity. Finally, we showed that PGN treatment induces expression of COX-2 and the subsequent synthesis and release of prostaglandin $E_2$ ($PGE_2$), an important mediator of inflammatory processes. The strong stimulatory effect of *E. coli* PGNs by themselves on trout macrophages suggests that the recognition of Gram-negative bacteria in trout is through PGNs in the bacterial wall, and indicates that the systems responsible for bacterial recognition in invertebrates (e.g., *Drosophila*) may also be conserved in some vertebrates.**

## 1. Background

Pathogens are recognized by the immune system through specific components referred to as pathogen associated molecular patterns (PAMPs), including lipopolysaccharides (LPS) of Gram-negative bacteria, peptidoglycans (PGNs) found in Gram-positive and Gram-negative bacteria, β-glucans of fungi, and viral nucleic acids. Various pathogen recognition receptors (PRRs) are involved in this recognition including toll-like receptors (TLRs), PGN recognition proteins (PGRPs), and nucleotide-binding oligomerization domain-like (NODs) receptors. Each of these receptors or recognition proteins contains a number of subtypes with differing pathogen recognition characteristics. The sequencing of genomes across vertebrates and invertebrates has facilitated studies on the evolution of various PRRs. These receptors are represented throughout vertebrates [1,2,3], suggesting that the recognition of PAMPs would be evolutionary conserved within the Vertebrata. However, there have

been relatively few studies that have specifically investigated PAMP recognition and function in nonmammalian vertebrates.

The study of LPS and the mechanism by which it stimulates an immune response has been a central focus in vertebrate immunology, particularly in view of the potential lethal effects of LPS over-stimulation. LPS is the major constituent of the external layer of the outer membrane of Gram-negative bacteria. It is composed of a polysaccharide portion consisting of a carbohydrate O-antigen and an oligosaccharide core region, and a lipid portion termed "lipid A" that is responsible for the innate immune response in mammals and confers the endotoxic properties of LPS [4,5]. Mammalian cells are extremely sensitive to the effects of LPS, in part because of the facilitatory action of a serum protein called lipopolysaccharide binding protein (LBP) [6]. In mammals, LPS aggregates are initially recognized by LBP [7] that facilitates the transfer of LPS to the co-stimulatory molecule CD14 [8] and then in monomeric form to LY96 [9]. LY96 is associated with TLR4 and specifically binds the endotoxin moiety of LPS [10]. The activation of TLR4 by LPS/LY96 is followed by the recruitment of intracellular adaptor molecules including a pathway involving MyD88 and TIRAP [11,12], and another pathway including TICAM1 and TICAM2. The Myd88 pathway leads to the early activation of NFκB while the TICAM1/2 pathway leads to the later activation of NFκB and also the induction of the antiviral genes [13].

It has been known for some time that nonmammalian vertebrates, and particularly fish, are immune to the toxic effects of LPS that cause septic shock in mammals [14]. Further, fish leukocytes are orders of magnitude less sensitive than mammalian cells to LPS-stimulated cytokine induction either in the presence or absence of fish serum [15]. We hypothesized that the differences observed between fish and mammals in the response to LPS are most likely a result of differences in the receptor-mediated recognition of LPS [16]. Namely, it appears that fish lack several of the co-stimulatory molecules and intracellular mediators that are involved in the action of LPS in mammals. Fish do not appear to have CD14, LY96 and TICAM2 molecules or a LBP-like factor in the serum [16]. Further, while zebrafish appear to have a TLR4 ortholog, other fish such as the pufferfish (*T. rubripes*) and tetraodon (*T. negroviridis*) do not [1]. Finally, recent studies using TLR4 in zebrafish have shown no response in the NFκB signalling pathway after *Vibrio angillarum* challenge [17]. Regardless, LPS stimulates a strong inflammatory cytokine response in fish macrophages *in vitro* at μg/ml concentrations [15], but how that is mediated is unknown.

We and others have shown that ultrapure LPS preparations have little stimulatory action on proinflammatory or antiviral gene expression in trout macrophages [16,18,19], further supporting the hypothesis that some fish may not recognize pure endotoxin. Based on studies with *Drosophila* using repurified LPS, we can hypothesize that ultrapure LPS preparations would be free of contaminating agents [20]. The decreased effect of ultrapure LPS on antiviral gene expression in trout macrophages suggests that unpurified LPS contains nucleic acid contamination that is responsible for antiviral gene expression [16]. In support of this, it was recently reported that the stimulation of cathelicidins in chinook salmon by crude LPS was reversed by DNase treatment, and it was hypothesized that the stimulatory activity of LPS on cathelicidins was a result of DNA contamination (Maier et al., 2008). In *Drosophila*, it was shown that the immunostimulatory action of crude LPS preparations was, to a significant extent, a result of contaminating PGNs [20]. This led to the further elucidation of a number of PGRPs that bind specific types of PGNs from Gram-negative and -positive bacteria and activate specific immune signal transduction pathways in *Drosophila* [21].

In the current study, we used primary trout macrophages to test the effects on gene expression of pure preparations of various PAMPs that might be present in crude LPS. Surprisingly, we found that PGN was a very potent stimulator of proinflammatory gene expression and, aside from crude LPS itself, was the most potent PAMP tested. Further, the stimulatory effects of LPS could be greatly reduced by incubation with chicken lysozyme, a muramidase responsible for PGN degradation. These results indicate that the PGN that is present in the LPS preparation, is the primary agent responsible for stimulating cytokine expression. We also found that PGN, as well as LPS, induces cyclooxygenase-2 (COX-2; prostaglandin endoperoxide synthetase 2) expression and an increase in prostaglandin $E_2$ ($PGE_2$) levels which are also observed in mammals during PGN stimulation. Based on these results, we hypothesize that the recognition of Gram-negative bacteria has in fact been evolutionary conserved within animals and that the recognition in trout, at least, is similar to that described for *Drosophila* involving bacterial PGNs [21]. Further, these results support the idea that PGNs have more than just bactericidal roles in vertebrates.

## 2. Materials and Methods

### 2.1. Materials

DMEM and FBS were purchased from PAA Laboratories (Spain). Lysozyme and poly-D-lysine were purchased from Sigma (Tres Cantos, Madrid). Primocin, lipopolysaccharide (LPS: *E. coli* 0111:B4), ultrapure LPS (upLPS: *E. coli* 0111:B4), ribonucleic acid (RNA: *E. coli* K12), deoxyribonucleic acid (DNA: *E. coli* 0111:B4), synthetic lipoprotein (palmitoyl-3-cysteine-serine-

lysine-4; Pam3CSK4), lipid A (synthetic monophosphoryl lipid A: *E. coli*), lipoteichoic acid (LTA: *B. subtilis*) and all PGN preparations (PGN *E.coli* K12, PGN *E. coli* 0111:B4, PGN *S. aureus*, PGN *B. subtilis*) were purchased from Invivogen (Nucliber, Spain). Cell strainers and plasticware were from BD Biosciences (Madrid, Spain). GelGreen was purchased from Biotium (Labnet, Spain). Amicon Ultra-4 filters (3 kDa cut-off) were purchased from Millipore Iberica (Madrid, Spain). Prostaglandin $E_2$ enzyme immunoassay (EIA) kit was from Cayman (Vitro, Spain).

**2.2. Cell culture and stimulation**

The experimental protocols used for head kidney isolation have been reviewed and approved by the Ethics and Animal Welfare Committee of the Universitat Autonoma de Barcelona, Spain. After anesthetizing the fish in 3-aminobenzoic acid ethyl ester (0.1 g/l), animals were sacrificed by a blow to the head and the head kidney was dissected. Trout macrophages were isolated as previously described [22]. Before stimulation, differentiated macrophages were incubated in serum free medium for 3 h. For stimulation, the medium of each well was removed and fresh medium containing the indicated concentrations of LPS, PGN, DNA, RNA, LTA, lipid A and lipoprotein were added and the cultures were incubated for the indicated times.

**2.3. RNA extraction and complementary DNA (cDNA) synthesis**

Total RNA was extracted from cell cultures using 1 ml of TriReagent (Molecular Research Center) per well, following the manufacturer's instructions. Quantification was carried out with a Nanodrop1000 (Thermo Scientific) and the quality of the RNA was checked with a Bioanalyzer (Agilent technologies). Total RNA (2 μg) was used to synthesize cDNA with SuperScript III Transcriptase (Invitrogen) and oligo-dT primer (Promega).

**2.4. Real-Time quantitative PCR**

Complimentary DNA (1 μl) was used as a template for real-time quantitative PCR (QPCR) with specific primers for IL-1β and IL-6 [23]. As a reference gene, gas7 was amplified from the same cDNA samples [24]. For COX-2 gene expression analysis the following specific primers were used: For-TACCAAGCAGATCGCTGGAC, Rev-GCGTATGGCTTCATGGAGAA and 18S was used as a reference gene [19]. Real-time PCR reactions were carried out in a 25 μl reaction with SYBR Green I (Stratagene) using a 1:25 dilution of the cDNA and 250 nM of primers. Quantitative PCR was performed using a Mx 3000P System (Stratagene) and quantification was done according to the Pfaffl method corrected for efficiency for each primer set [25].

**2.5. Peptidoglycan and LPS lysozyme digestion.**

Lipopolysaccharide or PGN were dissolved in LPS-free water. Chicken lysozyme was dissolved at 1 mg/ml in PBS. Peptidoglycan and LPS solutions were mixed with an equal volume of the lysozyme solution, or buffer without enzyme as a control, and incubated at 37ºC for 16–18 h. After digestion, LPS or PGN solutions were purified using 3 kDa Amicon Ultra-4 filters to separate small PGN-digested fragments.

**2.6. Measurement of $PGE_2$ levels**

Supernatants from stimulated cell cultures from 3 different fish were recovered, centrifuged and stored at -20ºC until use. Measurement of $PGE_2$ levels was completed with a monoclonal EIA according to the manufacturer's instructions. The prostaglandin kit detection limit was 8 pg/ml. Prior to $PGE_2$ determination, supernatants were diluted five times in EIA assay buffer. The same macrophage cells were used to obtain total RNA for the determination of COX-2 gene expression as well as the supernatants for $PGE_2$ determination.

**3. Results**

**3.1. Peptidoglycan and LPS are the major inducers of the inflammatory response in trout macrophages.**

Using real-time PCR, we tested the ability of different PAMPs to stimulate the expression of IL-1β and IL-6 in differentiated rainbow trout macrophages after a 12 h stimulation (Fig. 1). Lipopolysaccharide and PGN from *E. coli* (PGN 0111:B4) were able to induce, in a dose dependent manner, the expression of IL-1β (Fig. 1A) and IL-6 (Fig. 1B). At low doses (1 μg/ml), PGN induced a response similar to LPS for the induction of IL-1β expression (Fig. 1A), but a stronger increase than LPS for IL-6 expression (Fig. 1B); indicating a specific induction by PGN. Deoxyribonucleic acid and RNA were also able to stimulate the expression of IL-1β and IL-6, but to a much lesser extent than LPS or PGN (Fig. 1). We did not detect any changes in IL-1β or IL-6 expression when primary macrophage cell cultures were stimulated with upLPS, lipid A or lipoprotein (Pam3CSK4) (Fig. 1).

Figure 1

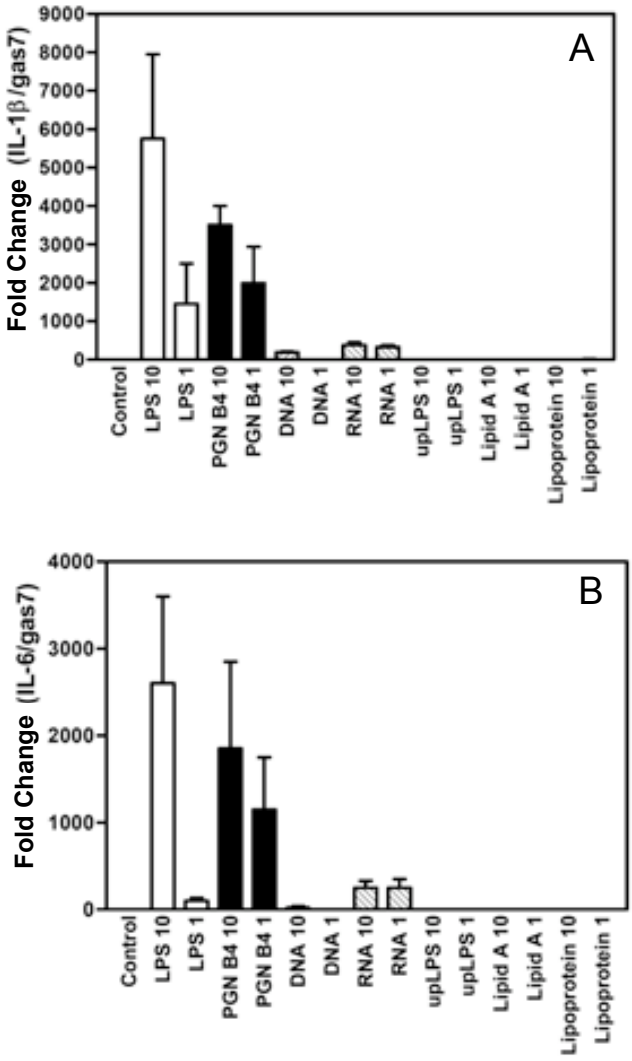


**Figure 1.** Il-1β (A) and IL-6 (B) expression in primary macrophage cell cultures stimulated for 12 h with LPS (1 and 10μg/ml), ultrapure LPS (upLPS) (1 and 10μg/ml), PGN (1 and 10μg/ml) from *E. coli* 0111:B4 (PGN B4), DNA (1 and 10μg/ml), RNA (1 and 10μg/ml), Lipid A (1 and 10μg/ml) and lipoprotein (1 and 10μg/ml). Results (mean ± S.D; n=3) from three independent experiments expressed as fold change with respect to control.

To further explore the PGN response, we performed dose-response and time-course experiments. As shown in Figure 2A and 2B, IL-1β as well as IL-6 expression, increased in a dose-dependent manner when stimulated with PGN from *E. coli* 0111:B4. Both cytokines had similar temporal and fold change expression patterns (Fig. 2A and 2B). The time-course of IL-1β and IL-6 showed a rapid induction since we could detect expression at 1 h that reached a maximum level of expression at 12 h (Fig. 2C and 2D). In contrast, the induction of IL-1β and IL-6 expression by LPS was slightly delayed compared to PGN, with cytokine expression starting after 1 h (Fig. 2E and 2F).

Figure 2

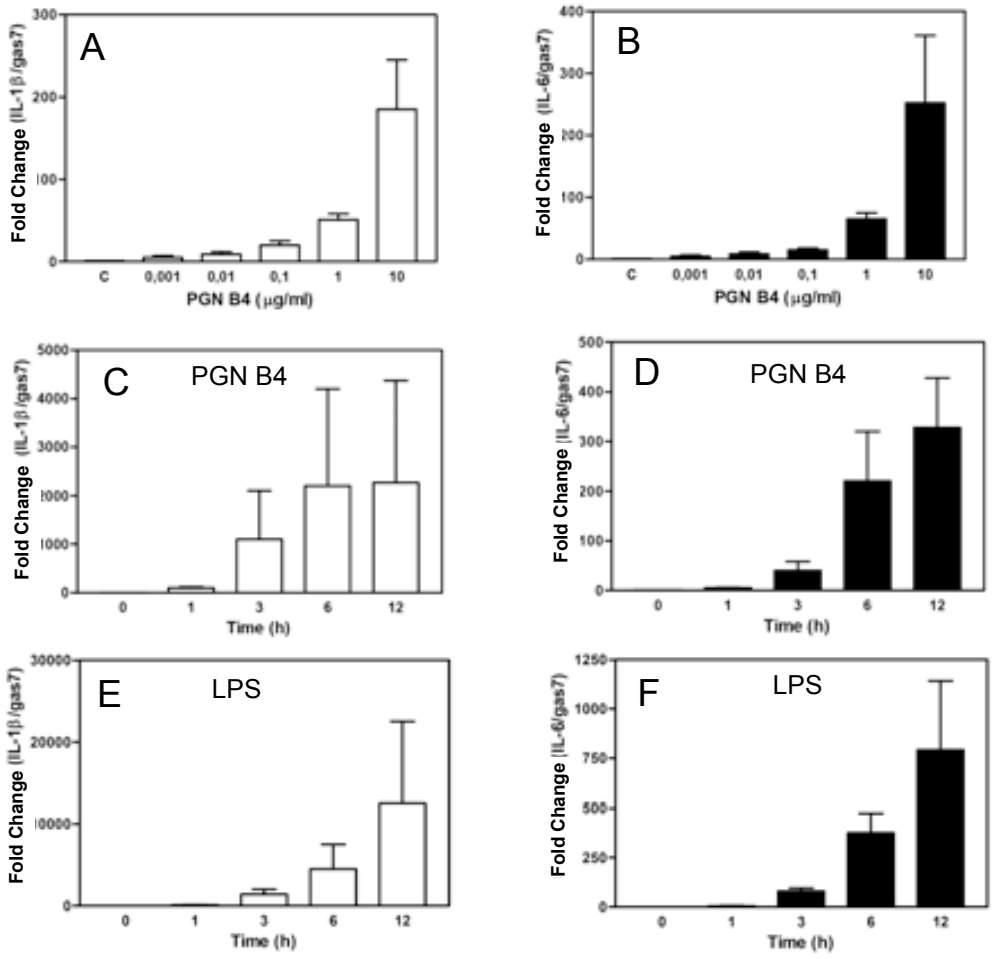


**Figure 2.** Dose response of Il-1β (A) and IL-6 (B) expression in primary macrophage cell cultures stimulated with PGN from *E. coli* 0111:B4 (PGN B4: 10μg/ml). Time-course of Il-1β and IL-6 expression in primary macrophage cell cultures stimulated with PGN B4 (10μg/ml) (C and D) and LPS (10ug/l) (E and F). Results (mean ± S.D; n=3) from three independent experiments expressed as fold change with respect to control.

Four commercially available purified PGN preparations were used to evaluate the expression of IL1-β (Fig. 3A) and IL-6 (Fig. 3B) after PGN treatment. Peptidoglycan 0111:B4 and PGN K12 from *E. coli* strongly induced the expression of IL-1β and IL-6, while *B. subtilis* and *S. aureus* PGNs induced a weak response in trout macrophages after 12 h (Fig. 3). Lipoteichoic acid (from *B. subtilis*), the major immuno-stimulatory component of Gram-positive bacteria, induced the same level of IL-1β and IL-6 expression as did Gram-positive PGN (Fig. 3).

Figure 3

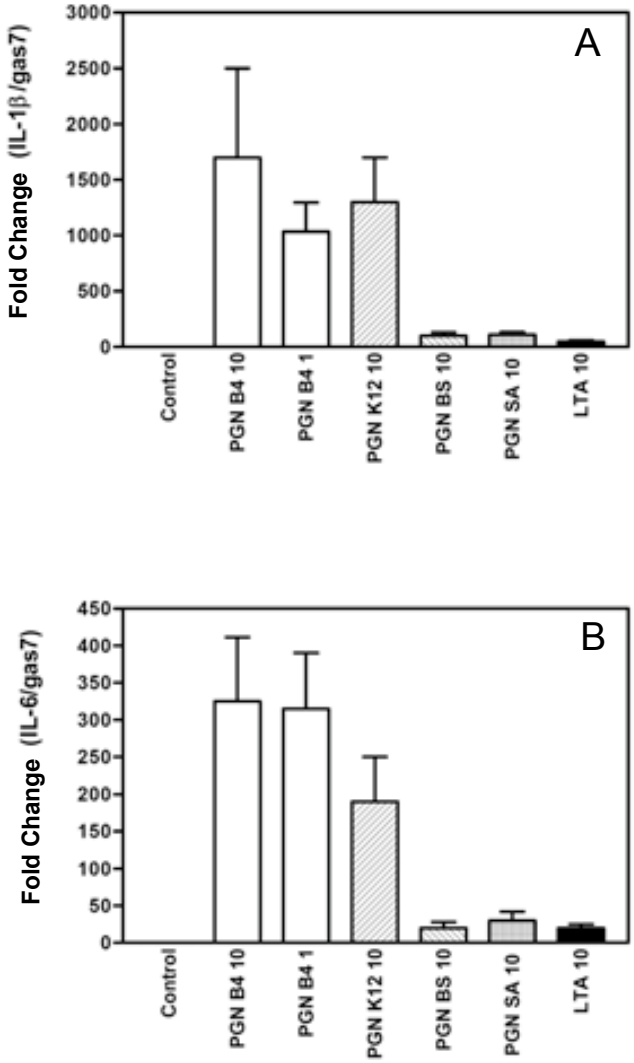


**Figure 3.** Il-1β (A) and IL-6 (B) expression in primary macrophage cell cultures stimulated for 12 h with PGN (1 and 10µg/ml) from *E. coli* 0111:B4 (PGN B4), PGN (10µg/ml) from *E. coli* K12 (PGN K12), PGN (10µg/ml) from *B. subtilis* (PGN BS), PGN (10µg/ml) from *S. aureus* (PGN SA) and lipoteichoic acid (10µg/ml) from *B. subtilis* (LTA). Results (mean ± S.D; n=3) from three independent experiments expressed as fold change with respect to control.

### 3.2. Gram-negative PGNs, not endotoxin, are the agents in crude LPS responsible for gene activation in trout macrophages.

We previously suggested that commercial LPS preparations contained other molecules such as nucleic acids, that could stimulate an immune response in trout macrophages (Iliev et al., 2005b). In addition, in it was shown that contaminating PGNs were responsible for a large portion of the stimulatory action of crude LPS (Kaneko et al., 2004). To test for the presence of PGN in our LPS preparation, we

treated the preparation with chicken lysozyme before using it to stimulate macrophages. Clearly, there was an inhibition of IL-1β (~85%: Fig. 4A) and IL-6 (~60%: Fig. 4B) expression with lysozyme-digested LPS. A PGN lysozyme control was conducted in parallel to demonstrate that lysozyme was able to degrade PGN and inhibit the cytokine expression induced by untreated PGN. Further, the lysozyme digested PGN preparation was subjected to molecular weight fractionation (3 kDa) to evaluate if small PGN fractions were able to induce a pro-inflammatory response. As is shown in Fig. 5, we did not observe any activity in the small molecular weight eluates suggesting that smaller fragments of PGN containing, for example, small peptide chains containing diaminopimelic acid (DAP) (492,5 Da) are not active agents. In addition, treatment of macrophages with diaminopimelic acid (DAP) did not induce cytokine expression (data not shown). On the other hand, the digested high molecular PGN fraction lost activity, indicating that integrity of the glycosidic bonds between *N*-acetylglucosamine and *N*-acetylmuramic are necessary for PGN activity. Nnnnnnn

Figure 4

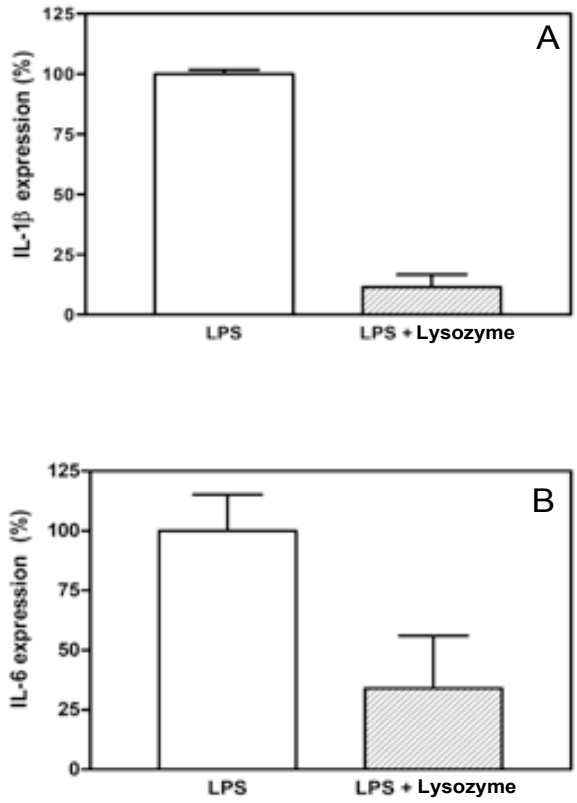


**Figure 4.** Il-1β (A) and IL-6 (B) expression in primary macrophage cell cultures stimulated for 12 h with LPS (open bars) or LPS digested with lysozyme (stripped bars). Results (mean ± S.D; n=3) from three independent experiments expressed as percent of control (LPS) expression.

Figure 5

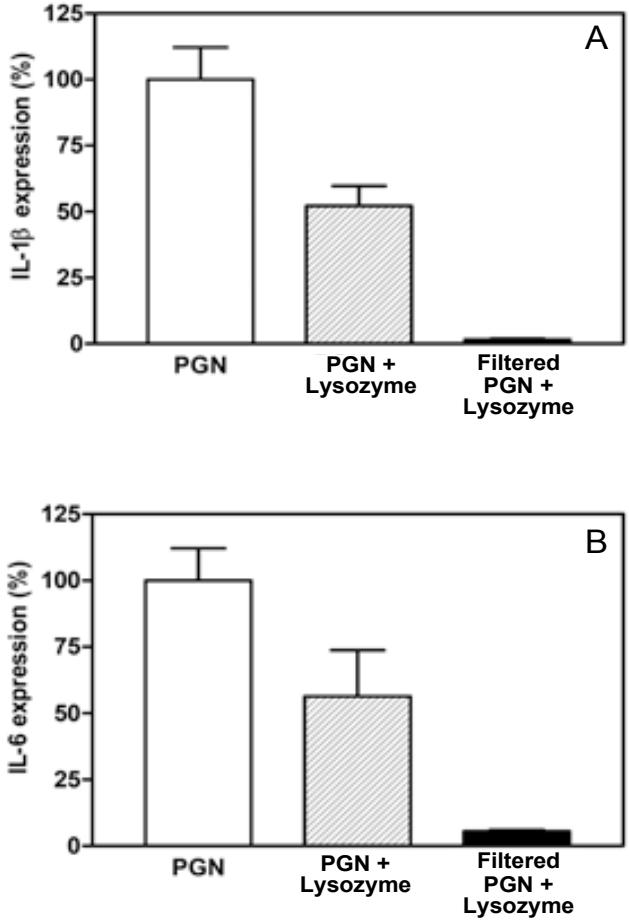


**Figure 5.** Il-1β (A) and IL-6 (B) expression in primary macrophage cell cultures stimulated for 12 h with PGN (open bars) or PGN digested with lysozyme (stripped bars) or PGN digested and ultrafiltrated (dark bars). Results (mean ± S.D; n=3) from three independent experiments expressed as percentage of control (PGN) expression.

### 3.3. Peptidoglycan treatment induces COX-2 expression and $PGE_2$ formation.

Using a salmonid cDNA microarray platform (SFA2.0) [26,27, 28], we obtained preliminary data on the transcriptomic response of macrophages to PGN and LPS (data not shown). We found that one of the genes induced at 12 h post stimulation, was COX-2 (3.8 ±0.8 fold change in response to PGN *E. coli* 0111:B4 and 5 ±2 fold change in response to LPS; mean ± SD; n=3). Thus, using QPCR, we determined the time course in COX-2 mRNA expression in macrophages stimulated with *E. coli* 0111:B4 LPS and PGN. As is shown in Figure 6A, LPS induced a transient but potent increase in COX-2 expression reaching a maximum 30 min after stimulation and being almost undetectable after 3 h. In contrast, there was a slightly delayed expression of COX-2 with PGN stimulation, increasing at 1 h, reaching a maximum at 3 h, and decreasing drastically after 12 h. Both PGN and LPS induced the release of $PGE_2$ to the same extent into the cell culture medium (Fig. 6B). The highest $PGE_2$ levels (200 pg/ml) were observed by 12 h and are similar to that described in mammalian macrophages in response to PGN [29,30]. The

production of $PGE_2$, induced by LPS, started earlier than that induced by PGN in agreement with the earlier expression of COX-2 mRNA under LPS induction (Fig. 6A).

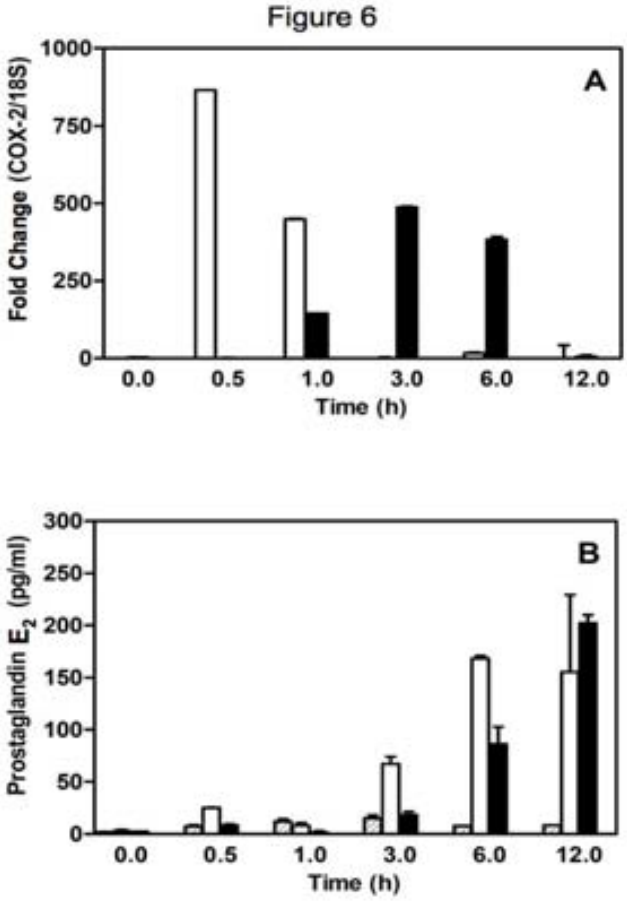


**Figure 6.** Time course of COX-2 expression (A) and $PGE_2$ levels (B) in primary macrophage cell cultures stimulated with LPS (10μg/ml) from *E. coli* 0111:B4 (LPS B4) or with PGN (10μg/ml) from *E. coli* 0111:B4 (PGN B4). Results (mean ± S.D; n=3) from three independent experiments expressed as fold change with respect to control (COX-2) or pg/ml ($PGE_2$).

## 4. Discussion

The results of this study demonstrate that the induction of proinflammatory cytokine expression in trout macrophages by crude LPS is primarily a result of PGN contamination. Previously, we and others demonstrated that upLPS was incapable of inducing the expression of inflammatory cytokines in trout macrophage cultures even though phenol-extracted preparations of *E. coli* and *P. aeruginosa* LPS were potent inducers at ug/ml concentrations [15,16,18,19]. Our *in silico* analysis [15] of fish genomes indicated that fish may be lacking several mediators such as CD14, LY96, TICAM2 and LBP that are involved in the recognition and action of endotoxin in mammals [31] and might explain why upLPS was ineffective in trout. However, if upLPS is not capable of inducing gene expression in trout macrophages, it was unclear how LPS preparations induce proinflammatory cytokine expression.

Crude LPS preparations could potentially have a number of contaminants including PGNs, nucleic acids and lipoproteins. Thus, in the current study we sought to test potential contaminants individually on trout macrophages to determine if any of them could induce proinflammatory cytokine expression. As shown before, upLPS and lipid A, the lipid portion of endotoxin that is responsible for activity in mammals, were unable to induce IL-6 or IL-1β expression in the current study. Ribonucleic acid, and to a lesser extent, DNA, were able to induce a small increase in IL-6 and IL-1β expression though this was extremely low compared to LPS. Previously, we showed that crude LPS stimulated antiviral gene expression in trout macrophages [16]. We speculated that since upLPS could not stimulate gene expression, that nucleic acid contamination was responsible for the antiviral gene expression observed following crude LPS stimulation [16]. That LPS preparations have active nucleic acid contamination, is supported by a recent study in which stimulation of cathelicidins in chinook salmon by crude LPS was reversed by DNase treatment [32].

In contrast, our results clearly demonstrate that PGNs by themselves are potent inducers of IL-6 and IL-1β expression in trout macrophages. Interestingly, crude LPS at 10 ug/ml was more potent than the most potent PGN we tested, *E. coli* PGN 0111:B4; while at 1 ug/ml, PGN 0111:B4 was equal to, or more potent than LPS. Since these cultures were conducted on macrophages taken from the same fish, these results suggests that there could be some interaction of components in the crude LPS preparation that enhance the stimulation particularly at higher levels. The results indicate that PGNs are potent stimulators of cytokine expression in trout macrophages. However, that PGNs are the active agents in the crude LPS preparation is demonstrated by the fact that lysozyme treatment greatly reduced the ability of LPS to stimulate IL-6 and IL-1β expression. Lysozyme is a muramidase that hydrolyzes PGN leading to its degradation. It has been reported that a deficiency in lysozyme activity results in an impaired response to infection [33], and transgenic zebrafish expressing lysozyme had enhanced survival to bacterial challenge [34], suggesting that lysozyme is naturally involved in bacterial PGN degradation. In the current study, lysozyme treatment reduced both LPS and PGN activity showing that PGN can be degraded by lysozyme, and that the LPS preparation had PGN contamination that is responsible for the majority of the cytokine induction. In addition, the inactivity of low molecular weight fractions from lysozyme-digested PGN, suggest that polymeric PGN is the active agent. Overall, the results presented here are similar to those reported for *Drosophila* in which muramidase treatment of crude LPS preparations greatly reduced the stimulation of the immune deficiency (IMD) pathway while PGN from *E. coli* was a very potent inducer [20].

PGNs are polymers of alternating *N*-acetylglucosamine and *N*-acetylmuramic acid that are cross-linked by short peptide stems. These stems confer the specificity of PGNs and divide them into two primary groups: lysine type (L-type) of Gram-positive bacteria, and *meso*-diaminopimelic acid type (D-type) of Gram-negative bacteria and Gram-positive bacilli [35]. In the L-type PGN, crosslinking at the lysine residue involves a peptide bridge of 1-5 glycines depending on bacteria while in the D-type PGN, the lysine is replaced by *meso*-diaminopimelic acid and the peptide stems are usually directly connected [36,35]. In the current study, we tested the effects of PGNs from 4 different bacterial sources, *E. coli* K12, *E. coli* 0111:B4, *S. aureus* and *B. subtilis*. PGN from both strains of Gram-negative *E. coli* were potent stimulators of IL-6 and IL-1β expression, whereas the PGN from two Gram-positive bacteria, *S. aureus* and *B. subtilis,* were weakly stimulatory. From these results, it initially appears that only the D-type PGN is effective in inducing a significant amount of cytokine expression in trout macrophages. However, the relationship between induction and the type of PGN may be more complex since PGN from *B. subtilis* contains a stem peptide with *meso*-diaminopimelic acid even though it is a Gram-positive bacteria [37]. This conundrum between the activities of L- and D-type PGN was also observed in human polymorphonuclear leukocytes in which PGN from both *B. subtilis and S. aureus* stimulated the release of arachidonic acid from cell membranes [29].

To our knowledge, there has been only one other investigation, aside from the current study, that has investigated the direct effects of PGNs (not PGRPs) on immune function in fish. In that study, Chen et al, [38] showed that PGN injection in grass carp resulted in a significant increase in interferon gamma expression, particularly in the spleen. In *Drosophila*, in which the mechanism of PGN action has been well studied, PGNs are involved in immunity in 3 primary ways [21,35]. D-type PGNs stimulate immune responses through the IMD pathway; L-type PGNs stimulate an immune response through a Toll receptor pathway; and PGNs stimulate the prophenoloxidase cascade (PPO) in the hemolymph. In the case of the IMD and Toll pathways, PGNs act indirectly through various PGRPs to activate signal transduction pathways that resemble PAMP-modulated pathways in mammals. In mammals and fish, various PGRPs, with sequence similarities to those in *Drosophila*, have been identified [35,40] however, PGRPs in fish and mammals are thought to be directly bactericidal [39,41,42], whereas in *Drosophila* only one PGRP has been shown to act directly [43]. Downregulation of specific PGRPs in zebrafish embryos or eggs have demonstrated increased susceptibility to bacterial infections [39,44] but the mechanism for this action is not clear. Presumably, these PGRPs would be activated by, or interact with, PGNs. However, is the result of this activation or interaction more than just a direct action of the PGRP on the bacteria? Recent papers have indicated that the expression of various signal transduction pathway genes were altered in

zebrafish when specific PGRPs were down-regulated using RNA interference approaches [44,40]. While this is not a direct effect of PGNs, it suggests that the PGRPs may be linked in some way to signal transduction pathways as is likely the case for the induction of IL-1β and IL-6 by PGN in trout macrophages in the current study. Whether the effects of PGN on trout macrophage gene expression involve PGRPs is unknown. However, recent data from a transcriptomic screen of PGN treated trout macrophages show that a PGRP mRNA is upregulated in response to different gram negative PGNs (Boltaña and MacKenzie, unpublished data). PGNs could act via several receptors in addition to PGRPs including TLR2 and NOD receptors [45,46]. As far as we know, TLR2 has not been described in trout but is present in *D. rerio*, *T. rubripes, I. punctatus*, *S. salar* and *T. nigroviridis* [1, 47] and Nod receptors have been identified in *D. rerio*, *T. rubripes* and *C. idella* [48,38].

With regard to TLR2 signaling, we showed that lipoprotein (Pam3CSK4) did not stimulate expression of pro-inflammatory cytokines, suggesting that the canonical TLR2 response to lipoproteins may not be conserved in trout macrophages. In mammals, sensing of PGN by TLR2 has been controversial [49], however, recent studies looking at binding to TLR2 and TNF activation have shown that TLR2 binds both D- and L-type PGN muropeptides and that a wider range of D-type muropeptides were recognized with higher affinity [45]. The activation of TNF correlated with the binding data but, interestingly, *E. coli* PGN did not induce TNF expression nor did this D-type PGN bind to TLR2 [45] so this particular type of PGN may not activate via TLR2. Nucleotide binding oligomerization domain receptors bind to small fragments of PGN such as the dipeptide, D-γ-glutamyl-*meso*-diaminopimelic acid (NOD1), or muramyl dipeptide (NOD2) [50]. While NOD receptors might be involved in the recognition of PGN by trout macrophages, the current study showed that small fragments from lysozyme-degraded PGN were not active, and we have previously shown [15] that muramyl dipeptide is a relatively weak and transient stimulator of proinflammatory cytokine expression in trout macrophages; so the role of NOD receptors in this case is questionable.

We also found that LPS and PGN from Gram-negative bacteria are potent inducers of COX-2 expression, and that one eicosanoid product that is elevated is $PGE_2$. COX-2 catalyzes the conversion of arachidonic acid to the endoperoxide, $PGH_2$, which can be further metabolized to various prostaglandins, prostacyclin, and thromboxanes, all of which are important immune mediators. Curiously, in mammalian cell models, such as the RAW264.7 mouse macrophage cell line or human polymorphonuclear leukocytes, PGN from Gram-positive bacteria induces COX-2 expression [51,29 52,53] and $PGE_2$ formation [51,29]. We have previously demonstrated that COX-2 expression is upregulated by LPS from both *E. coli* and *P. aeruginosa,* another Gram-negative bacteria [15]. Since we did not test the effects of PGN from a Gram-

positive bacteria on COX-2 expression or $PGE_2$, and since there do not appear to be any published reports on the effects of PGNs from Gram-negative bacteria on COX-2 expression or $PGE_2$ in mammals, it is unclear whether the situation in mammals and trout are the opposite in terms of the stimulation of COX-2 and $PGE_2$.

In summary, we propose that crude Gram-negative LPS preparations stimulate trout macrophages not through endotoxin but primarily through the presence of contaminating PGNs. It is also likely that the complete response of trout macrophages to crude LPS is the result of a combination of several contaminants that also include nucleic acids. The strong stimulatory effect of *E. coli* PGNs by themselves on trout macrophages suggests that the recognition of Gram-negative bacteria in trout is through PGNs in the bacterial wall, and indicates that the systems responsible for bacterial recognition in invertebrates may also be conserved in some vertebrates.

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# Chapter V

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RESEARCH ARTICLE Open Access

## Divergent responses to peptidoglycans derived from different *E. coli* serotypes influence inflammatory outcome in trout, *Oncorhynchus mykiss*, macrophages

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# Divergent response to peptidoglycan derived from different *E. coli* serotypes influence inflammatory outcome in trout, *Oncorhynchus mykiss*, macrophages

**Abstract**

**Pathogen-associated molecular patterns (PAMPs) are structural components of pathogens, such as lipopolysaccharide (LPS) and peptidoglycan (PGN) from bacterial cell walls. PAMP-recognition by the host results an induction of defence-related genes and often the generation of an inflammatory response. We evaluated both the transcriptomic and inflammatory response in trout (*O. mykiss*) macrophages in primary cell culture stimulated with DAP-PGN (DAP; meso-diaminopimelic acid, PGN; peptidoglycan) from two strains of *Escherichia coli* (PGN-K12 and PGN-O111:B4) over time. Transcript profiling was assessed using function-targeted cDNA microarrays hybridisation (n = 36) and results show differential responses to both PGNs that are both time and treatment dependent. Wild type *E. coli* (K12) generated an increase in transcript number/diversity over time whereas PGN-O111:B4 stimulation resulted in a more specific and intense response. In line with this gene Ontology analysis (GO) highlights a specific transcriptomic remodelling for PGN-O111:B4 whereas results obtained for PGN-K12 show a high similarity to a generalised inflammatory priming response where multiple functional classes are related to ribosome biogenesis or cellular metabolism. Prostaglandin release was induced by both PGNs and macrophages were significantly more sensitive to PGN-O111:B4 as suggested from microarray data. Responses at the level of the transcriptome and the inflammatory outcome (prostaglandin synthesis) highlight the different sensitivity of the macrophage to slight differences (serotype) in peptidoglycan structure. Such divergent responses are likely to involve differential receptor sensitivity to ligands or indeed different receptor types. Such changes in biological response will likely reflect upon pathogenicity of certain serotypes and the development of disease.**

## 1. Background

Detection of pathogens by host organisms requires direct contact between host PRRs (pattern recognition receptors) and pathogen-associated molecular patterns (PAMPs) where PAMP-PRR interactions subsequently dictate the development of the host immune response [1,2]. PAMPs such as the lipopolysaccharides (LPS) and peptidoglycans (PGN) both bacterial cell wall components have been directly implicated in the induction of the host immune response across the vertebrata [3-9]. Peptidoglycan and related fragments are recognised by the host and induce diverse biological effects, including inflammation, leukocytosis, or enhanced immune responses [10-13]. Like LPS, peptidoglycan, including its

minimal immunomodulatory subunit, muramyl dipeptide, can bind to the CD14 receptor of target cells in mammals [14-16] although peptidoglycan does not bind to LBP or BPI [17,18]. PGN's do not activate TLR4-mediated signal transduction but do activate both the TLR2 and NOD pathways [19-24].

In Drosophila PGN recognition is achieved by the Toll or Immune deficiency (Imd) pathways, at least in part, through peptidoglycan recognition proteins (PGRPs) [25,26]. Both pathways share common features with mammalian Toll-like receptor (TLR) and tumour necrosis factor-α (TNF-α) receptor signalling cascades that regulate NF-kappaβ activation [27-29]. In vivo studies in the zebrafish have shown that the PGRP response is essential for successful responses to bacterial infection [30] and recently in trout macrophages PGN has been shown to be the major stimulatory component in crude LPS preparations characterised by an increase in cytokine mRNAs, IL-1β and IL-6, and release of inflammatory products as prostaglandin $E_2$ ($PGE_2$) [9]. However studies addressing different responses to serotype-specific PGNs are scarce throughout the vertebrata including mammals.

PGN may account for approximately one-half of the cell wall mass in gram-positive bacteria whereas in gram-negative bacteria only a relatively thin PGN layer in the periplasmic space is present [31,32]. Gram-negative peptidoglycan contains meso-diaminopimelic acid (DAP) as the major peptide group that is directly cross-linked whereas most gram-positive bacteria have L-lysine as the third amino acid (Lys-type) where Lys-type peptides are cross-linked through an inter-peptide bridge that varies in length and amino acid composition in different bacteria [32-34].

As the structure and composition of the microbial motif has an important role in host sensing and minor modifications in structure can influence the immune response [35-38] we explored the response of differentiated trout macrophages in cell culture to different PGNs from *E. coli* of different strains (K12 and O111:B4). Our results show that trout macrophages differentially respond to different PGNs at the level of the transcriptome by either differentially activating RNA transcripts related to prostaglandin synthesis resulting in the liberation of prostaglandins (PGN-O111:B4) or by generating a non-defined inflammatory response,(PGN-K12).

## 2. Materials and methods

### 2.1. Animals and Materials

Healthy adult specimens (160g mean weight) of rainbow trout (*O. mykiss*) were purchased from a commercial hatchery (Piscifactoria Andrés, St Privat, Girona) and held in recirculating freshwater stock tanks (300L) in the aquarium facilities at the Universitat Autònoma de Barcelona. Fish were kept at 15°C

with a 12 hours light/12 hours dark photoperiod cycle, and were fed with a maintenance ratio of about 0.5% body weight per day. Water quality indicators (dissolved oxygen, ammonia, nitrite, pH) were analysed periodically. DMEM and FBS were purchased from PAA Laboratories (Spain). Poly-D-lysine was purchased from Sigma (Tres Cantos, Madrid). Primocin, and PGN preparations (PGN *E. coli* K12, O111:B4) were purchased from Invivogen (Nucliber, Spain). Cell strainers and plasticware were from BD Biosciences (Madrid, Spain). GelGreen was purchased from Biotium (Labnet, Spain). Prostaglandin $E_2$ and $D_2$ enzyme immunoassay (EIA) kit was from Cayman (Scharlab, Spain).

### 2.2. Cell culture and stimulation

The experimental protocols used for head kidney isolation have been reviewed and approved by the Ethics and Animal Welfare Committee of the Universitat Autonoma de Barcelona, Spain. After anaesthetising the fish in 3-aminobenzoic acid ethyl ester (0.1g/L), animals were sacrificed and the head kidney was dissected. Trout macrophages were isolated as previously described [39]. Before stimulation, differentiated macrophages were incubated in serum free medium for 3 hours. For stimulation, the medium of each well was removed and fresh medium containing the indicated concentrations of PGN were added and the cultures were incubated for the indicated times.

### 2.3. RNA extraction and complementary DNA (cDNA) synthesis

Total RNA was extracted from cell cultures using 1mL of TriReagent (Molecular Research Center) per well cell culture, following the manufacturer's instructions. Quantification was carried out with a Nanodrop1000 (Thermo Scientific) and the quality of the RNA was checked with a Bioanalyzer (Agilent technologies). All RNA samples had a RIN value > 7. Total RNA (2µg) was used to synthesize cDNA with SuperScript III Transcriptase (Invitrogen) and oligo-dT primer (Promega).

### 2.4. Measurement of $PGE_2$ and $PGD_2$ levels

Supernatants from stimulated cell cultures (triplicates) from 3 different fish were recovered, centrifuged and stored at -80°C until use. Measurement of $PGE_2$ and $PGD_2$ levels was completed with a monoclonal EIA according to the manufacturer's instructions. The prostaglandin kit detection limit was 8pg/mL. Prior to prostaglandin determination; supernatants were diluted five times in EIA assay buffer. The same macrophage cells were used to obtain total RNA for the determination of COX-2 and Prostaglandin D synthase gene expression as well as the supernatants for $PGE_2$-$PGD_2$ determination.

### 2.5. Microarray analysis

The design of the microarray is described in detail elsewhere [40,41] and a full description of

the platform and data presented in this manuscript are accessible through the public GEO depositories (accession number GPL6154 and GSE22330). The genes were selected by their functional classes; random clones from common and subtracted cDNA libraries 1800 genes printed in six replicates each were compared with the known vertebrate proteins using blastx; Overall, the platform was enriched in a number of functional classes, such as immune response (236 genes), signal transduction (245 genes), receptor activity (126 genes), apoptosis (120 genes), cell cycle (70 genes), protein catabolism (90 genes), folding (70 genes), response to oxidative stress (39 genes), stress and defence response (145 and 105 genes, respectively), and chaperone activity (41 genes). Total RNA was extracted from cell cultures using 1mL of TriReagent (Molecular Research Centre) per well, following the manufacturer's instructions, the quantity and integrity was analysed by Experion RNA StdSens Analysis Kit (Bio-Rad). Microarray analyses were conduced in pooled samples (see experimental design of microarray assay). A dye swap design of hybridisation was applied. In analyses of infected immune cells, the non-infected cells were used as a control. Each sample was analysed with two slides. Scanning was performed with Alphascan (High Performance Dual-Laser Scanner for Microarray Slides from Alpha Innotech and images were processed with VisionLite (ThermoSpectronic). The measurements in spots were filtered by criteria $I/B \geq 3$ and $(I-B)/(SI + SB) \geq 0.6$, where I and B are the mean signal and background intensities and SI, SB are the standard deviations. After subtraction of mean background, locally weighted non-linear regression (Lowess) normalisation [42] was performed separately for each slide. To assess differential expression of genes, the normalised log intensity ratios were analysed with Student's t-test ($p < 0.01$). The Bayesian modification to the false discovery rate (FDR) was used to correct for multiple comparison tests, estimating the q-value for the set of differentially expressed genes [43]. The functional categories of Gene Ontology [44] were compared with regulated genes ($p < 0.01$) by the sums of ranks (Student t-test $p < 0.05$). The statistical significance of over-represented functional categories, showing the differential expression in the experiment grouped by functional classes compared with all genes an GO categories from the chip, was assessed using the Chi square test with Yates correction ($p < 0.05$).

### 2.6. Real-Time quantitative PCR and validation

In order to verify microarray results, real-time PCR (qRT-PCR) was carried out. Two micrograms of the individuals RNA was used to synthesize cDNA with SuperSript III RNase Transcriptase (Invitrogen) and oligo-dT primer (Promega). As a house-keeping gene, 18s was amplified from the same cDNA samples. For different gene expression analysis specific primers were used (Additional file 1). Real-time PCR reactions were carried out in a 25µL reaction with SYBR Green I (Stratagene) using a 1:25 dilution of the cDNA and 250nM of primers. Quantitative qRT-PCR

was performed using a Mx 3000P System (Stratagene) and quantification was done according to the Pfaffl method corrected for efficiency for each primer set [45]. Values for each sample were expressed as "fold differences", calculated relative to controls group and normalised for each gene against those obtained for the house keeping gene 18S.

**2.7. Experimental design**

***Microarray analysis***: macrophage cell cultures isolated from 84 animals were stimulated with PGNs from E.coli O111:B4 and K12 strains and compared to parallel control cultures (without stimulation). Cell cultures were stimulated individually with both peptidoglycans for 1, 6 and 12 hours (12 by PGN and time, n=72), and 12 control cultures (N=84). Individuals RNAs were grouped into three pools from 4 cell cultures for each time point (1, 6, and 12 hours). The transcriptomic response was analysed by microarray assay, and divided in three experimental time points named early (1h), median (6h) and late stage (12h). The analysis was carried out with common genes expressed within three replicate pools over the control (GDE one way ANOVA $p > 0.01$). The qRT-PCR validation assay was conducted with total RNA from late stage cell cultures.

***Time Course***: macrophage cell cultures isolated from 9 animals were stimulated with PGN O111:B4 and K12 during 0, 30 min, 1, 3, 6, and 12 hours. The mRNA abundance of COX-2 (or prostaglandin endoperoxide synthase 2) and PTGDS was measured by qRT-PCR, prostaglandin release ($PGE_2$-$PGD_2$) were measured using a prostaglandin EIA assay (Cayman). Three individual replicates were made for each peptidoglycan stimulation. The control group was non-stimulated cell cultures (n = 3).

***Dose-Response***: macrophage cell cultures isolated from 9 animals were stimulated with PGN from the *E. coli* strains 0111:B4 and K12. The treatment was conducted overnight (12h) with different concentrations, 0, 0.1 and 10g/mL, of PGNs. Expression of COX-2 and PTGDS mRNAs was measured by qRT-PCR, prostaglandin release ($PGE_2$-$PGD_2$) were measured using a prostaglandin EIA assay (Cayman). Three individual replicates were made for each peptidoglycan stimulation. The control group was non-stimulated cell cultures (n = 3).

***Statistical analysis:*** All statistical analysis was conducted with the software SPSS Statistic 17.0. The relationship between intensity of expression and time was examined, and tested for significant differences between the PGNs with covariance analysis (ANCOVA) using the transcriptomic magnitude as co-variable, followed by one-way ANOVA analysis for up- or down

regulated transcripts. The Student t-test was made to explore the difference between the expression registered in the microarray assay and the qRT-PCR (Additional file 2). two-way ANOVAs were made to compare the differences between COX-2 and PTGDS expression and prostaglandin release in the time-course and dose-response assay.

## 3. Results

### 3.1. Global comparisons of the transcriptomic response to PGN (microarray analysis at 1, 3, and 12 hours)

Microarray analyses were evaluated using a salmonid-specific target cDNA platform of 1800 cDNAs enriched with immune-related genes (SFA 2.0). Gene expression profiles obtained highlighted a marked contrast in the macrophage response to PGN purified from *E. coli* (PGN-O111:B4 and K12). Samples were taken over time early (1h), median (6h) and late stage (12h) and separate one-way ANOVAs ($p > 0.01$) were conducted to identify differentially expressed transcripts over the control (GDE). Transcripts expressed within all three biological replicates were used to analyse changes for both treatment (PGN) and time stage (Additional files 3a-f). The kinetics of the response obtained from peptidoglycans derived from K12 or O111:B4 were significantly different in both transcript number (total number of differentially expressed transcript over the control, one-way ANOVA $p < 0.01$) and intensity (fold change FC > 2) (Figure 1). In total 819 transcripts were differentially expressed (GDE) in both treatments over the control (all cDNAs expressed on the array), with 270, 221 and 328 in the early stage, median and late stages respectively (Figure 1, and Additional file 3a-f, and 4). Stimulation with PGN-O111:B4 revealed a significant peak in intensity at the median stage (130 transcripts one-way ANOVA $p < 0.01$ and FC > 2; 92) and a strong and intense response was maintained throughout (FC > 2; 51, 92 and 72 at 1, 6 and 12 hours respectively). In contrast PGN-K12 induced a significant diversity of transcripts (magnitude) over time, note a decrease at 6h, where the response intensity although high at 1h (FC > 2; 134 transcripts) significantly decreased through time where late stage transcripts with FC > 2 represent only 17% of the early stage total (Figure 1, Additional file 4). Regression analysis (up regulated genes ANCOVA, $F_{5, 68} = 1.178$ $p > 0.05$, followed by two-way ANOVA, $F_{2, 68} = 27.124$: $p < 0.05$; down regulated genes ANCOVA, $F_{5, 68} = 2.303$: $p > 0.05$, followed by two-way ANOVA, $F_{2, 68} = 37.124$: $p < 0.05$) (Additional file 5, and 6) highlights that a stronger induction of gene expression and likely more directed response is obtained with PGN-O111:B4 challenge.

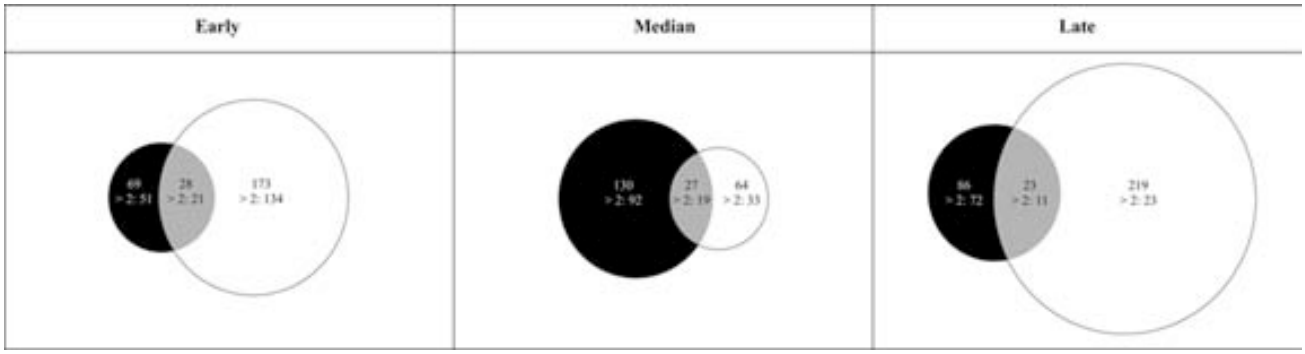


**Figure 1**: **Characterisation of the transcriptomic response.**A; Venn diagram representing mRNA transcripts differentially expressed over control during PGN-O111:B4 and PGN-K12 challenges throughout the time (early, median and late stage). The area of the circles is scaled to the number of transcripts (one way ANOVA $p<0.01$) and the fold change (FC>2) expressed in each stage. Black circles: 69, 130, 86 number of transcripts differentially expressed under PGN-O111:B4 treatment. White circles: 173, 64, 219 number of transcripts differentially expressed under PGN-K12 challenge.

### 3.2. Qualitative comparisons of the transcriptomic response to PGN: Differentially expressed transcripts in early, median, and late stages of activation

**Early stage**: A higher number of induced transcripts were observed with PGN-K12 treatment in respect to B4 highlighting a common down-regulation of inflammatory processes by both (Table 1a,b). Major differences could also be identified in ligand recognition where macrophages-PGN K12 up-regulated BPI binding protein (BPI). In fish BPI has been suggested to be involved in LPS binding and recognition [46] whereas PGN-B4 stimulation led to up-regulation of antigen-processing including MHC I, and MARCO. The alternative spliced form of MARCO, Cysteine-rich protein 1, that also recognises bacterial cell wall ligands was co-ordinately down-regulated [47]. Transcripts related to the inflammatory response were down regulated under both PGN challenges including for PGN-B4; N-acetylmuramoyl-L-alanine amidase (bactericidal activity), PGLYPR6 and peroxiredoxin (Table 1a) and for PGN-K12; NF-kappaβ inhibitor alpha-1 and arachidonate 5-lipoxygenase (Table 1b). Microsomal glutathione S-transferase, a precursor for leukotriene and prostaglandin production [48] was down-regulated by both treatments. Interestingly, Annexin A1-1 was strongly up-regulated (FC; 9.8) in response to PGN-K12. This transcript has been suggested to have anti-inflammatory activity due to its phospholipase A2 (essential for inflammatory prostaglandin production) inhibitory activity [49] (Table 1b and Additional file 3d).

**Median stage**: Of note at the median stage PGN-B4 induces a co-ordinated increase in pro-inflammatory and cellular defence activity with increased intensity (Table 1a,b). Mediators of inflammatory prostaglandin production are up-regulated highlighted by increased Arachidonate 5 lipoxygenase mRNA synthesis. In parallel, Cathepsin transcripts (protease activity) (n=6), PGLYPR6

(amidase) and the Interleukin enhancer 3 mRNA (regulates Interleukin production during the infectious processes (e.g., [50]), were also up-regulated. PGN-K12 stimulation at this point is highlighted by a strong down-regulation of transcript diversity, including cell adhesion, defence response, cell homeostasis and metabolism, with almost all observed early stage transcripts returning to base-line conditions (Table 1a,b). Potentially of importance is the up-regulation of the transcription factor, CCAAT/enhancer binding protein beta (C/EBP-beta) mRNA by PGN-K12. C/EBP-beta has been shown to be intimately linked to immune and inflammatory processes and regulates the transcription of the pro-inflammatory cytokine, Interleukin-6. On the other hand the tumour necrosis factor (TNF) decoy receptor, which inhibits apoptosis and NF-kappaβ inhibitor alpha-3 were strongly up-regulated in addition to an abrupt increase in BPI with PGN-B4.

**Table 1. Summary of selected transcripts expressed after challenges with PGN-O111:B4**

| | Early | | Median | | Late | |
|---|---|---|---|---|---|---|
| Antigen presenttion | Mean | SD | Mean | SD | Mean | SD |
| MHC class I heavy chain-1 | 4.92 | 2.35 | 3.27 | 1.67 | n/s | n/s |
| Macrophage receptor MARCO | 2.07 | 0.56 | 5.02 | 1.75 | n/s | n/s |
| Cysteine-rich protein 1 | -3.59 | 0.74 | n/s | n/s | n/s | n/s |
| BPI binding protein | n/s | n/s | 11.93 | 5.78 | n/s | n/s |
| Cell adhesion and proliferation | | | | | | |
| CD166 | 1.79 | 0.35 | 4.99 | 2.97 | 3.06 | 1.48 |
| Cytokines and Chemokines | | | | | | |
| C-C chemokine receptor type 3 | 4.25 | 3.47 | n/s | n/s | n/s | n/s |
| Chemokine receptor CXCR4 | n/s | n/s | -4.24 | 1.01 | n/s | n/s |
| Cellullar defense response | | | | | | |
| N-acetylmuramoyl-L-alanine amidase | -1.60 | 0.05 | 1.46 | 0.03 | 1.46 | 0.17 |
| Peroxiredoxin 1-1 | -2.98 | 1.42 | 1.30 | 0.76 | 1.74 | 0.30 |
| Interleukin enhancer-binding factor 3 | n/s | n/s | 2.39 | 1.47 | n/s | n/s |
| TNF decoy receptor | n/s | n/s | 11.42 | 3.86 | 12.09 | 10.98 |
| NF-kappaB inhibitor alpha-3 | n/s | n/s | 9.24 | 6.05 | n/s | n/s |
| Myeloid differentiation primary response | n/s | n/s | n/s | n/s | 1.56 | 0.29 |
| Phosphotyrosine SH2 domain | n/s | n/s | n/s | n/s | 2.86 | 1.17 |
| Procathepsin L-1 | n/s | n/s | 4.11 | 1.67 | n/s | n/s |
| Procathepsin L-2 | n/s | n/s | 3.47 | 1.28 | n/s | n/s |
| Cathepsin B-2 | n/s | n/s | 3.36 | 2.29 | n/s | n/s |
| Cathepsin D-2 | n/s | n/s | 3.99 | 0.25 | n/s | n/s |
| Cathepsin C-1 | n/s | n/s | 3.28 | 1.16 | n/s | n/s |
| Cathepsin C-2 | n/s | n/s | 5.14 | 5.30 | n/s | n/s |
| MAPK/ERK | | | | | | |
| Serine/threonine-protein kinase 2 | n/s | n/s | 5.03 | 2.65 | n/s | n/s |
| MAPK/ERK kinase kinase 5-1 | n/s | n/s | -1.68 | 0.33 | n/s | n/s |
| C-Jun protein | n/s | n/s | n/s | n/s | 3.99 | 1.62 |
| MAPK/ERK kinase kinase 1-2 | n/s | n/s | n/s | n/s | 1.87 | 0.46 |

| | | | | | | |
|---|---|---|---|---|---|---|
| MAPK kinase 9-2 | n/s | n/s | n/s | n/s | 5.78 | 3.73 |
| FYVE phosphoinositide kinase | n/s | n/s | n/s | n/s | 2.16 | 0.55 |
| Inflammatory response | | | | | | |
| Microsomal glutathione S-transferase 3 | -1.86 | 0.19 | n/s | n/s | n/s | n/s |
| Annexin A1-2 | n/s | n/s | 3.72 | 0.84 | 1.32 | 0.19 |
| Microsomal glutathione S-transferase 3 | n/s | n/s | 1.56 | 0.05 | n/s | n/s |
| Arachidonate 5-lipoxygenase-2 | n/s | n/s | 3.13 | 2.12 | n/s | n/s |
| Prostaglandine D synthase | n/s | n/s | n/s | n/s | 7.91 | 6.09 |
| Prostaglandin endoperoxide synthase-2 | n/s | n/s | n/s | n/s | 5.11 | 3.04 |
| Cell homeostasis | | | | | | |
| Glutathione S-transferase P-2 | -4.28 | 3.35 | n/s | n/s | n/s | n/s |
| Glutathione peroxidase 4 | n/s | n/s | 2.56 | 0.52 | n/s | n/s |
| Transcription | | | | | | |
| Transcription factor jun-B-1 | 3.07 | 0.79 | 1.78 | 0.56 | 3.99 | 1.42 |

Transcripts represented were firstly selected for expression level ($p$<0.01) and then implication in biological processes related to PGN stimulation (immune/inflammatory responses) during PGN-O111:B4. n/s: not signal, Mean: Fold expression average (n=3), SD: standard deviation.

## Table 2. Summary of selected transcripts expressed after challenges with PGN-K12.

| | Early | | Median | | Late | |
|---|---|---|---|---|---|---|
| Antigen presenttion | Mean | SD | Mean | SD | Mean | SD |
| MHC class I heavy chain-1 | 1.6 | 0.3 | 3.8 | 1.1 | 4.1 | 3.9 |
| BPI binding protein | 3.4 | 2.5 | n/s | n/s | 1.5 | 0.6 |
| Macrophage receptor MARCO | n/s | n/s | n/s | n/s | 0.4 | 1.8 |
| Cell adhesion and proliferation | | | | | | |
| Fibronectin receptor beta | 11.6 | 11.3 | n/s | n/s | n/s | n/s |
| CD2 binding protein 1-1 | 2.9 | 1.1 | n/s | n/s | n/s | n/s |
| Matrix metalloproteinase 9 | 2.0 | 0.7 | n/s | n/s | -4.7 | 1.8 |
| Cytokines and Chemokines | | | | | | |
| Cytokine receptor gamma chain | 1.7 | 0.5 | n/s | n/s | n/s | n/s |
| CC chemokine SCYA110-1 | n/s | n/s | n/s | n/s | 1.2 | 0.1 |
| Cellullar defense response | | | | | | |
| TNF receptor associated factor 1 | n/s | n/s | 4.6 | 4.1 | n/s | n/s |
| NF-kappaB inhibitor alpha-1 | 4.6 | 4.3 | n/s | n/s | n/s | n/s |
| Cathepsin C-3 | 2.8 | 2.3 | n/s | n/s | n/s | n/s |
| Cathepsin D-1 | 3.2 | 2.8 | n/s | n/s | 1.6 | 0.5 |
| Cathepsin D-2 | 3.7 | 3.6 | n/s | n/s | n/s | n/s |
| MAPK/ERK | | | | | | |
| MAPK/ERK kinase kinase 6 | 1.7 | 0.3 | 2.1 | 0.5 | n/s | n/s |
| Serine/threonine-protein kinase 2 | 2.9 | 2.2 | n/s | n/s | -1.2 | 0.1 |
| Tyrosine-protein kinase FRK | n/s | n/s | n/s | n/s | 1.7 | 0.5 |
| Tyrosine-protein kinase SYK | n/s | n/s | n/s | n/s | -2.0 | 1.4 |
| Inflammatory response | | | | | | |
| Annexin A1-1 | 9.8 | 9.4 | n/s | n/s | 1.4 | 0.3 |
| Microsomal glutathione S-transferase 3 | -1.6 | 0.2 | -1.4 | 0.2 | 1.2 | 0.1 |

| | | | | | | |
|---|---|---|---|---|---|---|
| Arachidonate 5-lipoxygenase-1 | -3.4 | 0.4 | n/s | n/s | n/s | n/s |
| Prostaglandine D synthase | 1.3 | 0.1 | n/s | n/s | n/s | n/s |
| Angiotensin I converting enzyme | 0.0 | n/s | n/s | n/s | 1.2 | n/s |
| Cell homeostasis | | | | | | |
| Metallothionein A | -4.4 | 1.1 | n/s | n/s | 1.4 | 0.2 |
| Heat shock 27 kDa protein-1 | 2.8 | 0.8 | n/s | n/s | -1.8 | 0.6 |
| Heat shock 70 kDa protein 1 | 3.0 | 1.4 | n/s | n/s | -1.7 | 0.4 |
| Glutathione reductase | 2.0 | 0.8 | n/s | n/s | -2.2 | 1.0 |
| Cellular metabolism | | | | | | |
| Malate dehydrogenase, cytoplasmic | 2.1 | 0.6 | n/s | n/s | n/s | n/s |
| Glucose-6-phosphate isomerase-1 | n/s | n/s | n/s | n/s | 2.4 | 1.7 |
| ATP synthase factor 6 | 2.4 | 1.7 | n/s | n/s | n/s | n/s |
| Transcription | | | | | | |
| Reverse transcriptase-like-2 | 2.4 | 1.7 | -2.7 | 0.6 | n/s | n/s |
| CCAAT/enhancer binding protein beta | n/s | n/s | 7.0 | 2.6 | 1.4 | 0.5 |
| Chromatin dis-assembly | | | | | | |
| Transposase-15 | -4.0 | 3.4 | n/s | n/s | -1.3 | 0.3 |
| Transposase-56 | n/s | n/s | -3.4 | 0.5 | n/s | n/s |
| G1/S-specific cyclin D2 | -3.1 | 2.0 | -3.6 | 1.4 | 1.5 | 0.4 |

Transcripts represented were selected for expression level ($p<0.01$) and then implication in biological processes related to PGN stimulation (immune/inflammatory responses) during PGN-K12. n/s: no signal. Mean: Fold expression average (n=3), SD: standard deviation.

**Late stage**: For PGN-B4 a defined response was observed after 12 hours of stimulation where the Prostaglandin endoperoxide synthase-2 (COX-2), and Prostaglandin D synthase, both linked to the synthesis of inflammatory prostaglandins were strongly up-regulated (Table 1a). COX-2 (Prostaglandin endoperoxide synthase-2) catalyzes the conversion of arachidonic acid to prostaglandin ($PGH_2$) [51,52], and Prostaglandin D synthase (PTGDS) catalyzes the conversion of $PGH_2$ to prostaglandin $D_2$ ($PGD_2$) [53,54]. Signalling components for TLR (Toll-like receptor) pathways are also up-regulated by PGN-B4 including the MAPK pathways and myeloid differentiation primary response (MyD88) mRNA, an adapter protein between TLR and the transcription factor NF-kappaβ. Interestingly these components plus the serine/threonine-protein kinase 2 are required to respond to microbial ligands [55]. TNF decoy receptor is maintained up-regulated highlighting the anti-apoptotic response of PGN-B4 activated macrophages. TNF-α is secreted into the culture medium as soon as 1 hour after PGN treatment (MacKenzie et al, unpublished results). In contrast to the strong inflammatory profile obtained for PGN-B4 the PGN-K12 response at 12 hours appears related to biological themes associated with energy, protein metabolism and cellular homeostasis at a low level of intensity (Table 1b and Additional file 3f). These results imply close similarities with those previously obtained for trout macrophages activated with crude LPS [56-59] suggesting a common

recognition mechanism distinct to that observed for PGN-B4. From transcripts identified as differentially expressed and significantly up or down regulated (one-way ANOVA $p < 0.01$) we selected sixteen transcripts from the late stage for qRT-PCR validation. All sixteen transcripts were significantly expressed between the two PGNs and significantly correlated when tested by qRT-PCR and Students-T test ($p < 0.05$); thereby confirming the microarray results. FC values obtain by microarray and qRT-PCR analyses are listed in the additional file 2 (Student T tests $p > 0.05$).

### 3.3. Functional categories are associated with combinations of PGN and time parameters

Analysis of function using GO annotations revealed that most over-expressed transcripts were related to the immune response and GO functional categories are specifically influenced by a combinatorial PGN-Time effect (Chi-square with Yates correction, $p < 0.01$, Figure 2). In the early stage, different GO categories expressed were PGN-dependent and include MHC class I receptor, Lysozome, NF-kappaβ cascade, peptidase activity, cell adhesion, Ribosome, or Chromatin assembly or disassembly (Figure 2). At the median stage the intensity of the PGN-B4 response is highlighted by a set of biological processes specifically associated to the immune response whereas only two GO classes, cell adhesion and negative regulation of cell proliferation, were represented with PGN-K12 (Figure 2). At the late stage an inverse correlation was observed where peptidase activity, complement activation, cell homeostasis, and mitochondrial electron transport were highly represented with PGN-K12 and NF-kappaβ cascade, Protein-MAPKinase cascade, and Ribosome related to the PGN-O111:B4 response (Figure 2). Remarkably, cell wall catabolism was only observed with PGN-K12 and not during PGN-O111:B4 challenge (Figure 2).

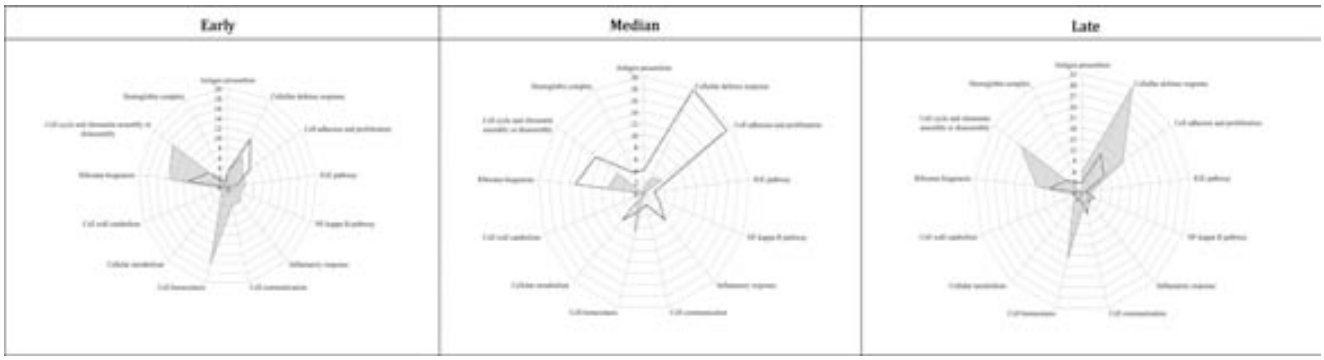


**Figure 2**: **Qualitative and quantitative representations of biological processes (GO) over represented during challenges.** Qualitative and quantitative representations of over expressed GO categories (Chi-square with Yates correction $p<0.05$). The corners of the spider-web maps represent biological processes identified in the GO analysis. Different numbers of transcripts were grouped in each biological process. The continuous mark lines (black or grey) represent the different number of transcripts in each biological process. The differences in the shape of the GO pattern (continuous mark line) are due to divergence in the number of transcripts grouped to each Gene Class (biological process) under both PGN challenges; the black line shows the GO pattern for PGN-O111:B4 and the grey line shows the GO pattern for PGN-K12 treatment.

### 3.4. Characterisation of the prostaglandin response (time course and dose response of PGN challenges)

Both COX-2 and PTGDS were identified by microarray analyses as differentially expressed between the two PGNs therefore we measured both $PGE_2$ and $PGD_2$ release into the culture supernatant and in parallel COX-2 and PTGDS mRNA abundance by qRT-PCR. Analyses were done both in respect to response to PGN-B4 and PGN-K12 over time (30 min, 1, 3, 6 and 12 hours; Figure 3) and subsequently as a dose response (0.1, 1 and 10µg/mL; Figure 4).

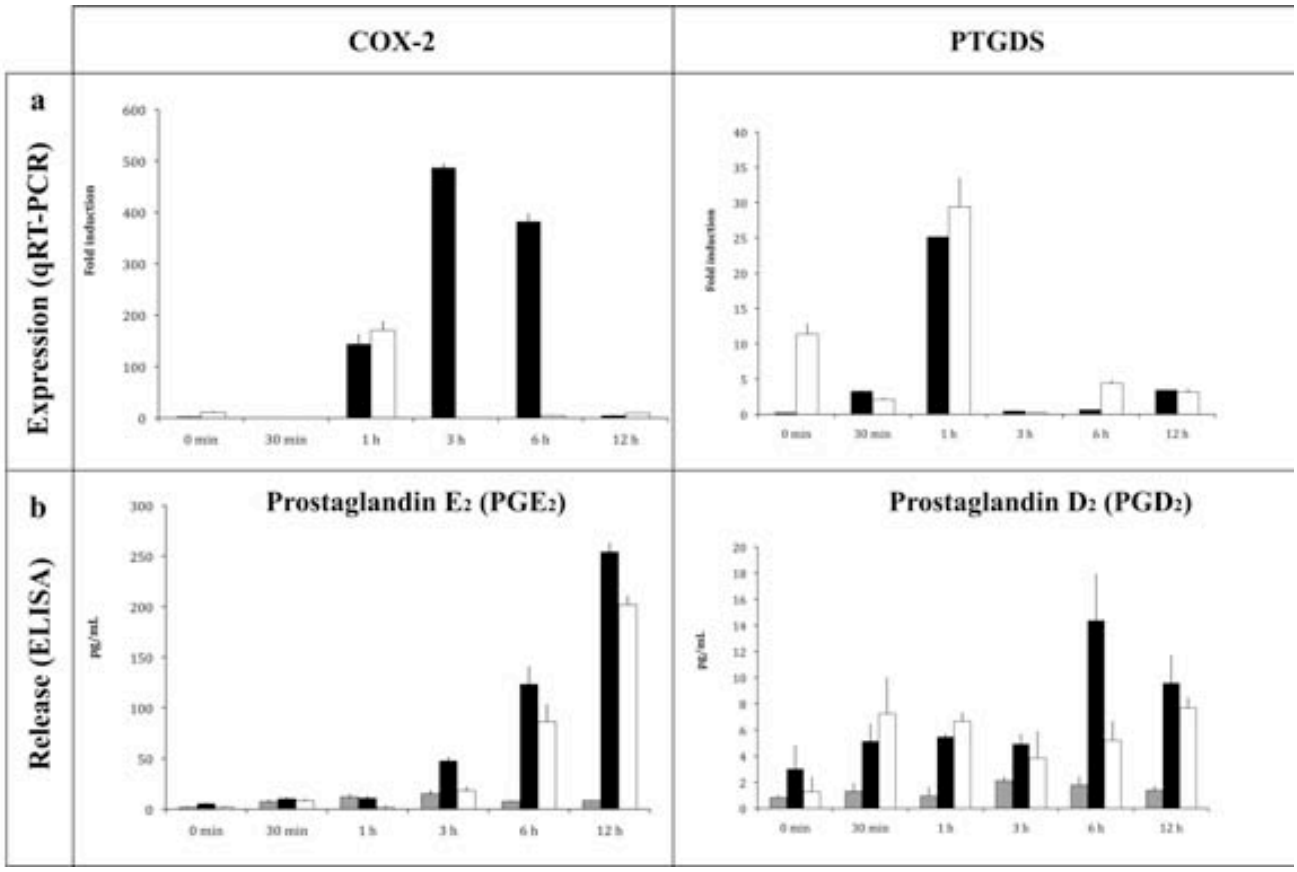


**Figure 3**: **Temporal characterisation of the prostaglandin response.**Time course response to macrophages stimulated during 0, 30 min, 1, 3, 6, and 12 hours with 10 ìg/mL of PGN O111:B4 and K12. Experiments were performed in independent groups of PGN-stimulated (n=3) or control macrophage cultures (n=9). a) COX-2 and PTGDS mRNA abundance over time in response to PGN-B4 (black bar) or PGN-K12 (white bar). Were observed significative differences in the mRNA abundance between the times and treatments (PGNs) in both genes (two way ANOVA $p<0.01$). b) $PGE_2$ and $PGD_2$ release (pg/mL) stimulated by PGN-O111:B4 (black bars), PGN-K12 (white bars) and control (grey bars) into the culture medium (n=3/treatment). Were observed significative differences in the release between the times and treatments (PGNs or control) by both prostaglandin (two way ANOVAs $p<0,01$). The results are presents as fold change relative to 18S abundance and ± std deviation.

### 3.5. Time course response assay (0, 30 min, 1, 3, 6, and 12hrs of PGN challenges)

COX-2 mRNA expression is strongly regulated by PGN-B4 over time followed by a significant increase in $PGE_2$ secretion into the culture medium. Stimulation with PGN-K12 results in an increase of mRNA abundance at 1 hour (two way ANOVA, $F_{5, 35} = 8.678$, $p < 0.05$, Figure 3a) and a more gradual accumulation of $PGE_2$ in the culture medium in comparison with PGN-B4. The dynamics of PTGDS mRNA expression was time dependent (two way ANOVA, $F_{5, 35} = 4.584$, $p < 0.05$, Figure 3a) showing changes a few minutes after stimulation with both PGNs (30 minutes) and a strong increase 1 hour post-treatment (Figure 3a). The release of $PGD_2$ was significantly different (increasing) in PGN-B4 treated macrophages 6 hours after stimulation. Differences observed between $PGE_2$ and $PGD_2$ release are correlated to both time and treatment (two way ANOVA, $F_{10, 54} = 4.553$, $p < 0.05$, Figure 3b) where $PGD_2$ has a low response, concentrations in the range of 1-14pg/mL, when compared with the $PGE_2$ secretion, > 200pg/mL. $PGE_2$ and $PGD_2$ liberation patterns were strongly influenced by the interaction between PGN and time (two way ANOVA, $F_{10, 54} = 2.522$, $p < 0.05$, Figure 3b).

### 3.6. Dose response assay (0.1, 1, and 10 μg/mL of PGN O111:B4 and K12)

In dose response assays the expression pattern of COX-2 mRNA induction was both dose and PGN-dependent (two way ANOVA, $F_{5, 18} = 5.824$, $p < 0.05$, Figure 4a). In figure 4a, a peak of COX-2 expression was registered at 10μg/mL of PGN-O111:B4. Interestingly, PGN-K12 stimulation generated a lower expression of COX-2 mRNA (10μg/mL; > 50 fold) when compared to PGN-B4 although at a dose of 1μg/mL fold changes are similar for both PGNs (Figure 4a). This is reflected in $PGE_2$ liberation where 10μg/mL of PGN-B4 generated a strong response (> 600 fold increase; PGN-interaction, two way ANOVA, $F_{2, 48} = 182.588$, $p < 0.05$) that correlated to increased COX-2 mRNA abundance and all other concentrations for both PGNs induced similar responses (> 50 fold). The liberation pattern of $PGD_2$ was significantly dependent upon PGN type, and showed a single increase at 10μg/mL with PGN-B4 (Two way ANOVA, $F_{2, 48} = 4.588$, $p < 0.05$). Surprisingly this is not mirrored in PTGDS mRNA abundance levels where PTGDS mRNA is significantly up-regulated by PGN-K12 at 0.1 and 1g/mL and PGN-B4 at 1g/mL (Figure 4b).

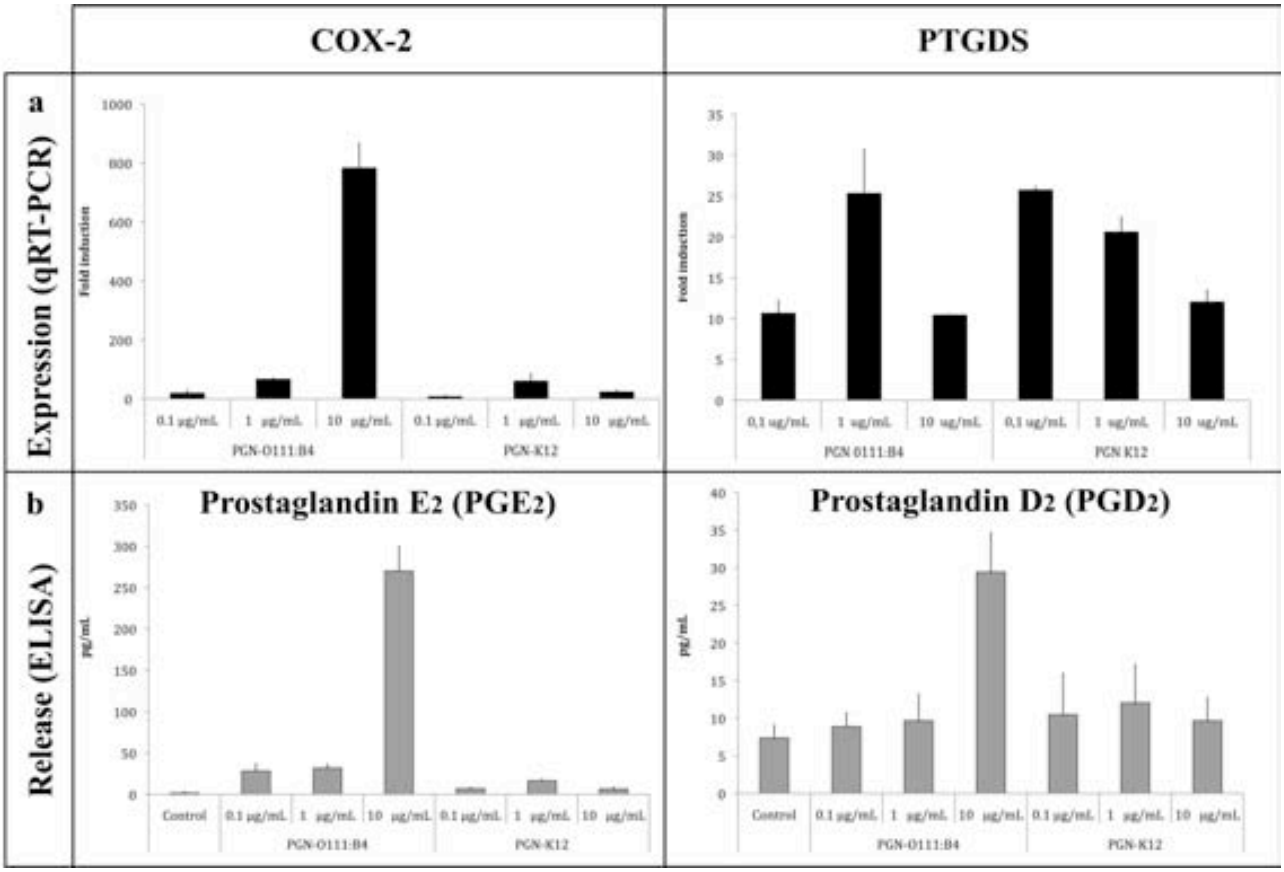


**Figure 4. Concentration dependence of the prostaglandin response.** Dose response (0.1, 1, and 10 µg/mL) of trout macrophages to PGN O111:B4 and K12 challenge. Experiments were performed overnight in independent macrophage cultures (n=3). a) COX-2 and PTGDS mRNA abundance (black bar) in response to different doses of PGN-O111:B4 or PGN-K12 (0.1, 1, 10 µg/mL). Were observed significative differences in the mRNA abundance between different doses and treatments (PGNs) in both genes (two way ANOVAs $p<0.01$). b) $PGE_2$ and $PGD_2$ release (pg/mL) into the culture medium (grey bars). Were observed significative differences in the release between doses and treatments (PGNs or control) by both prostaglandins (two way ANOVAs $p<0.01$). The results are presented as fold change relative to 18S abundance and mean ± std deviation.

## 4. Discussion

In recent studies in trout macrophages peptidoglycan (PGN-O111:B4) was identified as a major pro-inflammatory component of crude LPS preparations in which TLR4 and canonical TLR2 signalling pathways were discarded as potential recognition systems for peptidoglycans [9]. As structural differences in PGN peptide moieties from different bacterial-strains have been shown to modulate host responses in both Drosophila and mammals [32,36,38] we investigated, a priori with targeted microarray analysis, the effects of two different PGNs from different strains of *E. coli*, O111:B4 and K12, with different serological features that have been shown to affect the host immune response [60]. A systematic dissection of the impact of (combinations of) culture parameters (time and

treatment) revealed a significant re-modelling of the trout macrophage transcriptome highlighting the divergence of the response to the two different PGNs (PGN-B4 vs. PGN-K12). As there were no other known variables, the differences in the transcriptomic profile are assumed to be solely due to the structure of the different PGNs and therefore differential recognition of those by the macrophages. This assumption is supported by the variation in transcript number (Figure 1a, 2), their intensities (Figure 1a,b), and diversity (Table 1a,b). In fish, modifications in the transcriptomic profile have been observed in response to environmental changes, stress and maintenance of the steady state of transcriptional activity [61,62], or bidirectional transcriptomic remodelling to inflammatory stimuli in fishes [56,63-67]; however, our data emphasises that macrophages respond differentially to highly similar bacterial PGNs resulting in a directed response i.e. prostaglandin release or a more generalised 'state of activation'.

In fish, the shift from a steady state to a functional inflammatory state i.e. secretion of pro-inflammatory cytokines or $PGE_2$ in trout macrophages stimulated with crude LPS preparations has been shown to be driven mainly by gram negative PGN, DNA and RNA and ultra-pure LPS preparations are unable to induce mRNA expression of pro-inflammatory cytokines [9,68]. Our microarray analysis identified differential regulation of both Prostaglandin D-synthase (PTGDS), and Prostaglandin endoperoxide synthase-2 (COX-2) that are directly involved in eicosanoid production; $PGD_2$ and $PGE_2$ respectively [51,53] (Table 1a,b). COX-2 is regulated in macrophage/monocyte cell types and is responsible for inflammatory prostaglandin, $PGE_2$, synthesis from arachidonic acid and is involved in cellular or tissue damage generated in acute and/or chronic inflammatory states [69]. PTGDS metabolises $PGH_2$ to $PGD_2$, [53,54,70,73] where $PGD_2$ plays a role during the injury process as vasodilator/constrictor or as potent inflammatory mediator [72,73]. However the action of $PGD_2$ in fish as a mediator of the immune response is undefined. Downstream analyses, qRT-PCR and prostaglandin release, of both COX-2 and PTGDS mRNA regulation and $PGH_2$ and $PGD_2$ concentration in supernatants reveals a strong correlation, both time and dose-dependent, between PGN-type (B4 vs K12), mRNA abundance and inflammatory outcome as measured by $PGE_2$ and $PGD_2$ release (Figure 4). PGN-B4 is clearly a more potent regulation of the COX-2 mRNA/$PGE_2$ pathway where the activation threshold for de novo synthesis of COX-2 is marked at 10g/mL. Interestingly this threshold concentration has also been observed on numerous occasions for pro-inflammatory cytokine mRNA synthesis in trout macrophages [9,39,46,68]. On the other hand PTGDS mRNA synthesis appears as more dose sensitive for both PGNs with a similar temporal expression pattern, suggestive of a different signal transduction mechanism, however PGN-B4 stimulation at 10g/mL results in higher $PGD_2$ secretion. The regulation and biological effects of $PGE_2$ and $PGD_2$ secretion in inflammatory responses in fish clearly warrant more investigation.

In Drosophila the biological activity of a large panel of natural and synthetic DAP-PGN, showed significant variability in their stimulatory capacity and immune response [74] and PGRP (peptidoglycan recognition protein) deficient *Drosophila* are more susceptible to bacterial infections [75]. In human monocytes exposed to synthetic muropeptides (peptide moiety of PGNs), TNF-α mRNA expression and release was highly dependent upon structural modifications between peptides [38]. Thus inflammatory outcomes are modified in accordance to sensitivity to peptidoglycan structure. Such sensitivity is likely conferred by the participation of different PRRs, PAMP-PRR interactions or the accumulative signalling intensity (i.e. threshold) of the group PRRs involved in recognition.

Peptidoglycan recognition in mammals is mainly facilitated by three different PRR families; TLR2 (gram positive peptidoglycan), NOD2 and PGRPs all of which can bind peptidoglycans [35,75,76]. TLR2 has been described in fish species [77] although stimulation with lipoprotein (Pam3CSK4) a classical TLR2-ligand does not stimulate an inflammatory response in our macrophage model [9]. However MyD88, an adaptor molecule involved in the classical Drosophila or mammalian Toll signalling cascades, which together with the receptor associated kinase (IRAK) and TNF activated factor (TRAF6) allow NF-kappaβ translocation to the nucleus (promoting expression of inducible inflammatory cytokines such as TNF-α) during gram-negative bacterial infection [46,78-80] was specifically up-regulated during PGN-B4 stimulation. This suggests TLR involvement in the PGN-mediated inflammatory response in trout macrophages. Concerning PGRPs, PGRP-2, -5 and -6, have been shown in the zebrafish to play an essential role in defence during bacterial infections [30] and in the trout PGRP-2 responds to PGN-B4 [9]. In this study we also identified PGLYRP-6 (up-regulated; PGN-B4) suggesting that the PGRPs also play a role in specific-PGN recognition and this may be conserved throughout the fishes.

In contrast to the specific directed response obtained from PGN-B4 stimulation PGN-K12 did not elicit a clear functional response at the level of the macrophage transcriptome or release of inflammatory mediators. A wide diversity of transcripts were activated although at a relatively low level. These results are similar to those previously observed for stimulation with crude LPS preparations in trout macrophages ([56] and Boltaña et al unpublished data) where both preparations can stimulate the release of TNF-α into the culture medium ([68], Roher et al unpublished data). Interestingly, TNF receptor associated factor 1 was specifically induced by PGN-K12. This transcript encodes a receptor-protein involved in the activity of apoptotic pathways mediated by TNF-α [80-82] however we did not detect apoptosis during the experimental period (MacKenzie et al, unpublished

data). Moreover, the gene ontology category cell wall catabolism was consistently over-expressed throughout PGN-K12 treatment (Figure 2a,b) supporting the existence of a strong transduction signal generated by PGN-K12.

In conclusion our data highlights the significant differences observed in macrophages responding to two PGNs derived from different serotypes of the same bacteria. Responses at the level of the transcriptome and the inflammatory outcome (prostaglandin synthesis) highlight the different sensitivity of the macrophage to slight differences (serotype) in peptidoglycan structure. Such divergent responses are likely to involve differential receptor sensitivity to ligands or indeed different receptor types. Such changes in biological response will likely reflect upon pathogenicity of certain serotypes and the development of disease.

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**Additional files**

**Additional file 1.**

Specific primers used for quantitative qRT-PCR (sequence and accession number).

| Gene name | Primer Fw | Primer Rv | Acces number |
|---|---|---|---|
| CCL4 | ACCAGCCAGCAGTGCTCTAA | TACAGGAGTGGCGTCTGCTT | AY561709 |
| Nuclear factor NF-kappa-B | GGAGGGAACACTCCTCTCCA | TGGTCCATCCTCTTGCTCCT | BT058984.1 |
| Glutathione peroxidase | GATTCGTTCCAAACTTCCTGCTA | GCTCCCAGAACAGCCTGTTG | AF281338.1 |
| Tumor necrosis factor receptor 2 (traf2) | ATGGAGCACTTGCGTCTGAT | CTGTGGCTGCAATAGCTGTG | NM_001124393.1 |
| COX-2 | TACCAAGCAGATCGCTGGAC | GCGTATGGCTTCATGGAGAA | NM_001124667.1 |
| Prostaglandine D synthase | GGCTCTTGCTGGAGGATGAC | TCCGTGTTTGGTCTTGATGG | AF281353.1 |
| Peroxiredoxin 6 | ATCTTGTACCCTGCCACCAC | CTCTGCATCGGAGAGAGAGG | BT074031.1 |
| TNF-decoy receptor | CCTGGGAATCTGTCTGTGGA | CAGGAACCCAGTGATCTTGC | NM_001124393.1 |
| C-X-C chemokine receptor type 3 | CATCAGTCTGGACCGCTACC | TCCCTCACAGACTCCAGGAA | NM_001124423.1 |
| A globin | CTGGAGCGGAACCGGGGGCC | CAGCTAAGGACAAAGCCAACGTG | BE859114.1 |
| 18S | CGAGCAATAACAGGTCTGTG | GGGCAGGGACTTAATCAA | AF243428.2 |

**Additional file 2.**

Comparison of expression data for selected transcripts obtained from microarray analyses and qRT-PCR validation. The results are presented as fold change relative to 18S abundance and mean ± std deviation.

| | PGN-O111: B4 | | | | | PGN-K12 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | QPCR | | Microarray | | T student (2,2) | QPCR | | Microarray | | T student (2,2) |
| | Mean | SD | Mean | SD | *p* | Mean | SD | Mean | SD | *p* |
| CCL4 | 6.80 | 1.57 | 13.86 | 9.05 | > 0.05 | 2.26 | 0.50 | 1.51 | 0.15 | > 0.05 |
| Nuclear factor NF-kappa-B | 1.94 | 0.49 | 0.52 | 1.88 | > 0.05 | 1.98 | 0.96 | 0.52 | 1.53 | > 0.05 |
| Glutathione peroxidase | 1.46 | 0.04 | 1.61 | 0.38 | > 0.05 | 1.71 | 0.37 | 1.54 | 0.24 | > 0.05 |
| Traf2 | 6.15 | 0.83 | 11.06 | 16.85 | > 0.05 | 1.93 | 0.76 | 1.29 | 0.29 | > 0.05 |
| COX-2 | 31.39 | 3.10 | 16.60 | 12.82 | > 0.05 | 1.76 | 0.94 | 3.72 | 1.79 | > 0.05 |
| Prostaglandine D synthase | 2.27 | 1.87 | 6.33 | **7.59** | > 0.05 | 2.85 | 2.78 | -0.96 | 3.41 | > 0.05 |
| Peroxiredoxin 6 | -2.54 | 0.31 | -1.90 | 0.37 | > 0.05 | 1.03 | 0.03 | 1.28 | 0.09 | **< 0.05** |
| TNF-decoy receptor | 7.74 | 2.02 | 11.93 | 6.37 | > 0.05 | 4.09 | 1.52 | 2.95 | 2.49 | > 0.05 |
| C-X-C receptor type 3 | 4.56 | 1.00 | 0.24 | 2.54 | > 0.05 | 3.32 | 1.94 | 1.04 | 2.13 | > 0.05 |
| A globin | 1.75 | 0.58 | 4.77 | 2.07 | **< 0.05** | 1.25 | 0.24 | -1.89 | 0.96 | **< 0.05** |

**Additional file 3a-f.**

Description of PGN (0111;B4 or K12) regulated transcripts/genes over the control (all cDNAs on the array) at respective stages (1, 6 and 12 hours) (table a-f). Transcripts represented were selected for expression level ($p$<0.01) and then implication in biological processes related to PGN stimulation (immune/inflammatory responses) during PGN-K12. n/s: no signal. Mean: Fold expression average (n=3), SD: standard deviation.

**Additional file 3a**

**PGN:B4 1 hrs**

| Clone ID | Clone Name | Mean (FC) | SD | *p* |
|---|---|---|---|---|
| CA343327 | Transposase -61 | -1.84 | 0.29 | < 0.01 |
| CA349280 | Chemokine-like factor family member 7 | 0.59 | 2.14 | < 0.01 |
| CA350711 | B-cell lymphoma/leukemia 11A | 2.08 | 0.23 | < 0.01 |
| CA350777 | CD166 | 1.79 | 0.35 | < 0.01 |
| CA354559 | CD2 antigen cytoplasmic tail-binding protein 2 | 1.78 | 0.32 | < 0.01 |
| CA355265 | Transposase-52 | -2.10 | 0.67 | < 0.01 |
| CA357820 | Calponin 1 | 1.81 | 0.20 | < 0.01 |
| CA358266 | T-cell, immune regulator 1, isoform a | 1.60 | 0.30 | < 0.01 |
| CA358998 | Cysteine-rich protein 1 | -3.59 | 0.74 | < 0.01 |

| | | | | |
|---|---|---|---|---|
| CA364112 | N-acetylmuramoyl-L-alanine amidase (PGLYP2) | -1.60 | 0.05 | < 0.01 |
| CA373850 | Signal transducer and activator of transcription 1-alpha/beta | 1.91 | 0.42 | < 0.01 |
| CA376536 | VHSV-induced protein | 2.51 | 0.79 | < 0.01 |
| CA377397 | C-C chemokine receptor type 3 | 4.25 | 3.47 | < 0.01 |
| CA379964 | Histone deacetylase 1 | 2.09 | 0.65 | < 0.01 |
| CA385270 | Granulins | 2.75 | 1.15 | < 0.01 |
| ENH2_H02 | Peroxiredoxin 1-1 | -2.98 | 1.42 | < 0.01 |
| ENH2_H06 | Unknown-5 | -1.04 | 2.12 | < 0.01 |
| est01b09 | Tolloid-like 2 protein (nephrosin) | -13.73 | 11.70 | < 0.01 |
| est01e10 | Tolloid-like protein (nephrosin)-1 | -3.51 | 0.59 | < 0.01 |
| est02h12 | Unknown-33 | -2.50 | 0.93 | < 0.01 |
| est03d11 | Unknown-42 | 1.92 | 0.65 | < 0.01 |
| EST1-3A_A01 | Cytosolic nonspecific dipeptidase | -3.00 | 1.87 | < 0.01 |
| EST1-3A_B08 | Unknown-67 | -3.24 | 2.43 | < 0.01 |
| EST1-3A_H06 | Transcription factor jun-B-1 | 3.07 | 0.79 | < 0.01 |
| EXOB1_A05 | Ribosomal protein L6-1 | -1.80 | 0.12 | < 0.01 |
| EXOB1_H01 | Hypothetical-fish 42 | 3.28 | 1.33 | < 0.01 |
| EXOB1_H12 | NADH dehydrogenase subunit 4 | 2.28 | 0.74 | < 0.01 |
| EXOB2_A01 | MHC class II invariant chain-like protein 1 | 4.92 | 2.35 | < 0.01 |
| EXOB2_F08 | CDC10 protein homolog | 2.32 | 0.60 | < 0.01 |
| EXOB2_H07 | WD-repeat containing protein Ciao 1 | 2.70 | 0.88 | < 0.01 |
| EXOB3_D12 | Unknown-113 | 0.41 | 2.38 | < 0.01 |
| EXOB4_B09 | Macrophage receptor MARCO | 2.07 | 0.56 | < 0.01 |
| EXOB4_F03 | Cathepsin S | 0.70 | 1.90 | < 0.01 |
| EXOB4_G09 | Histone H14 | -2.37 | 0.37 | < 0.01 |
| Hete0002_E03 | 60S ribosomal protein L36 | -2.30 | 0.73 | < 0.01 |
| Hete0002_H06 | Ribosomal protein S2 | -1.36 | 0.08 | < 0.01 |
| HK0001_C08 | Galectin-9 (VHSV-induced protein)-3 | 5.19 | 2.93 | < 0.01 |
| HK0002_F06 | Histone 3A-ATP synthase F0 6 | 1.59 | 3.17 | < 0.01 |
| HK0002_G03 | Histone H10 | 3.07 | 2.37 | < 0.01 |
| HK0002_G10 | T-cell receptor alpha chain V region HPB-MLT precursor (Fragment) | -1.94 | 0.56 | < 0.01 |
| HKT0001_H07 | 60S ribosomal protein L37a | -3.79 | 0.77 | < 0.01 |
| HST0001_D08 | Beta-globin | -1.98 | 0.21 | < 0.01 |
| KVkm2_F06 | 60S ribosomal protein L32-1 | -2.09 | 0.35 | < 0.01 |
| utu01f03 | Unknown-238 | -2.50 | 0.61 | < 0.01 |
| utu02h08 | Glutathione S-transferase P-2 | -4.28 | 3.35 | < 0.01 |
| CA358621 | Microsomal glutathione S-transferase 3 | -1.86 | 0.19 | < 0.01 |

**Additional file 3b**

**PGN:B4 6 hrs**

| Clone ID | | Clone Name | Mean (FC) | SD | *p* |
|---|---|---|---|---|---|
| CA342430 | Ig kappa chain V-III region VG | -2.77 | 0.28 | < 0.01 | |
| CA342675 | Heme oxygenase-1 | -7.64 | 6.03 | < 0.01 | |
| CA344428 | Glutathione peroxidase 4 | 2.56 | 0.52 | < 0.01 | |
| CA344488 | Cathepsin B-2 | 3.36 | 2.29 | < 0.01 | |
| CA346979 | MAPK/ERK kinase kinase 5-1 | -1.68 | 0.33 | < 0.01 | |
| CA347342 | Transposase-14 | -2.01 | 0.36 | < 0.01 | |
| CA348325 | BAG-family molecular chaperone regulator-4 | 2.02 | 0.94 | < 0.01 | |
| CA349338 | Unknown-275 | -2.45 | 0.67 | < 0.01 | |
| CA349380 | LPS binding protein | 11.93 | 5.78 | < 0.01 | |
| CA350330 | Unknown-276 | -2.74 | -2.16 | < 0.01 | |
| CA351440 | TNF decoy receptor | 11.42 | 3.86 | < 0.01 | |
| CA351691 | Tissue inhibitor of metalloproteinase 2 | 2.99 | 0.14 | < 0.01 | |
| CA352526 | Transposase-22 | -3.37 | 1.34 | < 0.01 | |
| CA354612 | Cathepsin C-1 | 3.28 | 1.16 | < 0.01 | |
| CA355516 | Transposase-26 | -3.72 | 1.32 | < 0.01 | |
| CA356686 | Receptor-interacting serine/threonine-protein kinase 2 | 5.03 | 2.65 | < 0.01 | |
| CA356762 | Transposase-28 | -2.46 | 0.67 | < 0.01 | |
| CA358107 | Ectonucleoside triphosphate diphosphohydrolase 1 | -1.72 | 0.23 | < 0.01 | |
| CA359009 | Midkine-related growth factor Mdk2 | -2.50 | 1.49 | < 0.01 | |
| CA360060 | T-complex protein 1, subunit 3 | -2.05 | 0.15 | < 0.01 | |
| CA360163 | Tyrosine-protein kinase SYK | -1.47 | 0.16 | < 0.01 | |
| CA361151 | Annexin A1-2 | 3.72 | 0.84 | < 0.01 | |
| CA362806 | Gamma-interferon inducible lysosomal thiol reductase | 1.85 | 0.44 | < 0.01 | |
| CA363064 | C166_BRARE CD166 antigen homolog | 3.99 | 3.45 | < 0.01 | |
| CA363120 | Heme oxygenase-2 | -4.28 | 2.78 | < 0.01 | |
| CA363737 | Transposase-41 | -2.47 | 0.23 | < 0.01 | |
| CA365039 | CD63 | 1.42 | 0.13 | < 0.01 | |
| CA365458 | Cathepsin D-2 | 3.99 | 0.25 | < 0.01 | |
| CA365505 | Retinoblastoma-like protein 1 | 7.01 | 4.20 | < 0.01 | |
| CA366403 | Heat shock 27 kDa protein-1 | -1.79 | 0.35 | < 0.01 | |
| CA366604 | Interleukin enhancer-binding factor 3 (M-phase phosph | 2.39 | 1.47 | < 0.01 | |
| CA366892 | Unc-112 related protein 2 | 2.42 | 1.04 | < 0.01 | |
| CA367195 | Complement factor Bf-1 | -1.87 | 0.53 | < 0.01 | |

| | | | | |
|---|---|---|---|---|
| CA367903 | Procathepsin L-1 | 4.11 | 1.67 | < 0.01 |
| CA369101 | Phospholipid hydroperoxide glutathione peroxidase B | 1.82 | 0.02 | < 0.01 |
| CA370329 | Lysozyme C precursor | -5.49 | 2.36 | < 0.01 |
| CA373291 | Chemokine receptor-2 | 2.22 | 0.32 | < 0.01 |
| CA373770 | Procathepsin L-2 | 3.47 | 1.28 | < 0.01 |
| CA374193 | Chemokine receptor CXCR4 | -4.24 | 1.01 | < 0.01 |
| CA376536 | VHSV-induced protein | -2.32 | 0.18 | < 0.01 |
| CA378399 | Tumor necrosis factor receptor associated factor 2 | 2.89 | 1.17 | < 0.01 |
| CA379576 | Galectin like 2 | 2.37 | 0.43 | < 0.01 |
| CA382877 | Fibronectin receptor beta | 5.28 | 1.96 | < 0.01 |
| CA383435 | Semaphorin 7A | 2.91 | 0.74 | < 0.01 |
| CA387866 | Arachidonate 5-lipoxygenase-2 | 3.13 | 2.12 | < 0.01 |
| CA388461 | NF-kappaB inhibitor alpha-3 | 9.24 | 6.05 | < 0.01 |
| ENH2_H06 | Unknown-5 | -2.79 | 0.67 | < 0.01 |
| est01e10 | Tolloid-like protein (nephrosin)-1 | -15.42 | 9.34 | < 0.01 |
| est02f04 | Tubulin alpha-3 chain | 3.98 | 3.89 | < 0.01 |
| est02g11 | Cytochrome c oxidase subunit I-1 | 1.57 | 0.28 | < 0.01 |
| est03b03 | Cathepsin C-2 | 5.14 | 5.30 | < 0.01 |
| est03c04 | Matrix metalloproteinase-9 | 4.05 | 1.60 | < 0.01 |
| est03d06 | Eukaryotic translation initiation factor 2 subunit 2 | 1.78 | 0.56 | < 0.01 |
| est04b01 | Similar to rRNA (Vangl2) | 2.38 | 0.54 | < 0.01 |
| est04c10 | Unknown-56 | -1.62 | 0.22 | < 0.01 |
| est04d09 | 60S ribosomal protein L37 | -1.76 | 0.15 | < 0.01 |
| EST1-3A_B08 | Unknown-67 | -4.11 | 1.65 | < 0.01 |
| EST1-3A_F01 | Ependymin I | 1.86 | 0.92 | < 0.01 |
| EXOB1_A05 | Ribosomal protein L6-1 | -2.51 | 1.75 | < 0.01 |
| EXOB1_C08 | Eukaryotic initiation factor 4A-I | -3.68 | 0.83 | < 0.01 |
| EXOB1_D12 | Unknown-86 | -3.11 | 0.88 | < 0.01 |
| EXOB1_E03 | Eukaryotic translation initiation factor 3 subunit 5 | -2.81 | 1.34 | < 0.01 |
| EXOB1_H08 | ADP,ATP carrier protein 3 | -3.36 | 0.72 | < 0.01 |
| EXOB2_B10 | Hemoglobin beta chain | -2.66 | 2.19 | < 0.01 |
| EXOB2_C03 | 60S ribosomal protein L10-1 | -2.30 | 0.47 | < 0.01 |
| EXOB2_E03 | 14-3-3B1 | 4.39 | 0.92 | < 0.01 |
| EXOB2_G07 | Septin 10 | -2.07 | 0.18 | < 0.01 |
| EXOB2_H06 | High affinity immunoglobulin epsilon receptor alpha | 7.89 | 4.13 | < 0.01 |
| EXOB3_A04 | Unknown-104 | -2.01 | 0.33 | < 0.01 |

| | | | | |
|---|---|---|---|---|
| EXOB3_A11 | Unknown-107 | 3.41 | 2.52 | < 0.01 |
| EXOB3_B01 | Cathepsin C-3 | -1.97 | 0.11 | < 0.01 |
| EXOB3_E01 | Na/K ATPase alpha subunit-2 | -2.24 | 0.52 | < 0.01 |
| EXOB3_F09 | MHC class I heavy chain-1 | 3.27 | 1.67 | < 0.01 |
| EXOB3_H05 | Glucose-6-phosphate isomerase-2 | -2.95 | 1.96 | < 0.01 |
| EXOB4_B09 | Macrophage receptor MARCO | 5.02 | 1.75 | < 0.01 |
| EXOB4_C05 | Galectin-1 | 1.34 | 0.13 | < 0.01 |
| EXOB4_E01 | Transposase-58 | -1.84 | 0.07 | < 0.01 |
| EXOB4_E09 | Hypoxanthine-guanine phosphoribosyltransferase | -2.38 | 0.80 | < 0.01 |
| EXOB4_E11 | Annexin IV | 2.71 | 2.10 | < 0.01 |
| EXOB4_E12 | Annexin 5 | 3.40 | 1.12 | < 0.01 |
| EXOB4_F03 | Cathepsin S | 1.54 | 0.36 | < 0.01 |
| EXOB4_F11 | 40S ribosomal protein S20 | -2.48 | 0.85 | < 0.01 |
| EXOB4_G02 | Galectin-9 (VHSV-induced protein)-2 | 2.38 | 0.19 | < 0.01 |
| EXOB4_G09 | Histone H14 | -4.07 | 0.85 | < 0.01 |
| EXOB4_H05 | Unknown-133 | 5.14 | 2.24 | < 0.01 |
| Hete0002_A06 | 40S ribosomal protein S11 | -3.33 | 2.58 | < 0.01 |
| Hete0002_B08 | Eukaryotic translation elongation factor 1 alpha 3 | 2.82 | 1.63 | < 0.01 |
| HK0002_B09 | Transgelin | 4.32 | 0.80 | < 0.01 |
| HK0002_C07 | 60S ribosomal protein L17 | -1.85 | 0.60 | < 0.01 |
| HK0002_G03 | Histone H10 | 3.33 | 1.17 | < 0.01 |
| HK0002_G11 | Myristoylated alanine-rich protein kinase C substrate | 5.79 | 2.47 | < 0.01 |
| HKT0001_E06 | Stomatin | 3.73 | 2.54 | < 0.01 |
| HST0001_C04 | Hemoglobin alpha chain | -2.19 | 0.39 | < 0.01 |
| HST0001_D04 | Alpha-globin I-2 | -4.43 | 1.25 | < 0.01 |
| HST0001_E03 | Cyclophilin-3 | 1.53 | 0.11 | < 0.01 |
| KVkm2_D11 | Ubiquinol-cytochrome C reductase complex 11 kDa protein, mitochondrial precursor | -0.41 | 4.43 | < 0.01 |
| KVkm2_F01 | Unknown-224 | 0.15 | 1.83 | < 0.01 |
| utu01b08 | Unknown-231 | 0.78 | 2.07 | < 0.01 |
| utu01g10 | Keratin, type II cytoskeletal 8 | 1.94 | 0.27 | < 0.01 |
| utu01h02 | Cytochrome oxidase subunit III-3 | 1.76 | 0.50 | < 0.01 |
| utu02b08 | High mobility group protein 2 | -1.81 | 0.47 | < 0.01 |
| utu04g05 | 40S ribosomal protein S9-3 | -0.61 | 1.76 | < 0.01 |
| CA358548 | Microsomal glutathione S-transferase 3 | 1.56 | 0.05 | < 0.01 |
| CA364112 | N-acetylmuramoyl-L-alanine amidase | 1.46 | 0.03 | < 0.01 |

**Additional file 3c**

**PGN:B4 12 hrs**

| Clone ID | Clone Name | Mean (FC) | SD | *p* |
|---|---|---|---|---|
| CA341808 | Tax1 binding protein 1 | 1.86 | 0.75 | < 0.01 |
| CA342707 | Unknown-272 | 4.70 | 2.59 | < 0.01 |
| CA343700 | CXC chemokine receptor transcript variant B | 1.82 | 0.37 | < 0.01 |
| CA348284 | CCAAT/enhancer binding protein beta | 3.39 | 1.95 | < 0.01 |
| CA350038 | Transposase-18 | 2.78 | 1.72 | < 0.01 |
| CA351435 | Selenoprotein T-3 | 8.21 | 6.06 | < 0.01 |
| CA351440 | TNF decoy receptor | 152.09 | 230.98 | < 0.01 |
| CA351516 | Reverse transcriptase-like-2 | 3.94 | 2.64 | < 0.01 |
| CA352451 | Transposase-21 | 3.65 | 2.09 | < 0.01 |
| CA352474 | Prostaglandin endoperoxide synthase-2 | 5.11 | 3.04 | < 0.01 |
| CA352760 | Toll-interacting protein | 3.90 | 1.52 | < 0.01 |
| CA352804 | Transposase-23 | 6.83 | 6.25 | < 0.01 |
| CA354559 | CD2 antigen cytoplasmic tail-binding protein 2 | -1.66 | 0.30 | < 0.01 |
| CA355516 | Transposase-26 | 3.84 | 2.20 | < 0.01 |
| CA356429 | NACHT-, LRR- and PYD-containing protein 2 | 4.10 | 2.58 | < 0.01 |
| CA356454 | Guanine nucleotide exchange factor | 1.69 | 0.54 | < 0.01 |
| CA356744 | Apoptosis regulatory protein Siva | 1.56 | 0.29 | < 0.01 |
| CA357014 | Myeloid differentiation primary response | 5.38 | 5.66 | < 0.01 |
| CA357261 | Cathepsin F | 1.81 | 0.75 | < 0.01 |
| CA358202 | Drebrin-like protein | 3.99 | 1.62 | < 0.01 |
| CA361151 | Annexin A1-2 | 1.32 | 0.19 | < 0.01 |
| CA361415 | C-Jun protein | 2.16 | 0.55 | < 0.01 |
| CA363064 | C166_BRARE CD166 antigen homolog | 3.06 | 1.48 | < 0.01 |
| CA366403 | Heat shock 27 kDa protein-1 | 1.89 | 0.23 | < 0.01 |
| CA367667 | FYVE finger-containing phosphoinositide kinase | 3.06 | 1.69 | < 0.01 |
| CA368283 | MAPK/ERK kinase kinase 1-2 | 1.87 | 0.46 | < 0.01 |
| CA369722 | Peptidyl-prolyl cis-trans isomerase 9 | 2.85 | 1.35 | < 0.01 |
| CA369896 | Interferon regulatory factor 4-1 | 2.23 | 1.09 | < 0.01 |
| CA371199 | Lymphoid translocation protein 1 | 2.31 | 0.90 | < 0.01 |
| CA371267 | Transcription factor jun-B-2 | 3.99 | 1.42 | < 0.01 |
| CA371454 | Vitellogenin-2 | 2.86 | 1.17 | < 0.01 |
| CA371809 | Suppressor of initiator codon mutations, related sequence 1 | 2.75 | 1.98 | < 0.01 |
| CA375694 | Phosphotyrosine independent ligand for the Lck SH2 domain p62 | 3.26 | 1.52 | < 0.01 |
| CA376213 | TNF receptor associated factor 1 | 7.89 | 5.69 | < 0.01 |
| CA378399 | Tumor necrosis factor receptor associated factor | 15.12 | 13.29 | < 0.01 |

| | | | | |
|---|---|---|---|---|
| | 2 | | | |
| CA380133 | Complement factor MASP-3 | 3.84 | 2.95 | < 0.01 |
| CA381045 | Aminolevulinate, delta-, synthetase 1-2 | 5.81 | 4.08 | < 0.01 |
| CA381085 | Mitogen-activated protein kinase 9-2 | 5.78 | 3.73 | < 0.01 |
| CA384134 | G1/S-specific cyclin D2 | -1.62 | 0.45 | < 0.01 |
| ENH2 | Beta-2-microglobulin-2 | -1.91 | 0.58 | < 0.01 |
| est01d09 | Selenoprotein T-1 | -2.20 | 0.58 | < 0.01 |
| est01g07 | Proteasome activator complex subunit 2 | -2.56 | 0.41 | < 0.01 |
| est02a09 | Gap junction alpha-3 protein | -1.73 | 0.57 | < 0.01 |
| est02a11 | Ig kappa chain V-IV region B17-2 | -2.80 | 0.59 | < 0.01 |
| est04a08 | Ras-related C3 botulinum toxin substrate 2 | -2.99 | 1.43 | < 0.01 |
| EST1-3A | Coronin-1C | -1.64 | 0.10 | < 0.01 |
| | Ribosomal protein L6-1 | 2.27 | 0.54 | < 0.01 |
| EXOB2_F12 | 60S ribosomal protein L7a-1 | -1.62 | 0.32 | < 0.01 |
| EXOB2_G11 | Stanniocalcin-1 | 2.36 | 1.11 | < 0.01 |
| EXOB2_H04 | Unknown-101 | 3.35 | 1.46 | < 0.01 |
| EXOB3_A01 | Unknown-103 | 1.50 | 2.77 | < 0.01 |
| EXOB3_A11 | Unknown-107 | 0.50 | 2.01 | < 0.01 |
| EXOB3_C04 | Glyceraldehyde-3-phosphate dehydrogenase-1 | -1.87 | 0.61 | < 0.01 |
| EXOB3_E08 | Unknown-116 | -2.17 | 1.06 | < 0.01 |
| EXOB3_E11 | Unknown-118 | -2.01 | 0.68 | < 0.01 |
| EXOB3_F12 | Unknown-121 | -1.77 | 0.43 | < 0.01 |
| EXOB4_D10 | Prosaposin | -1.82 | 0.51 | < 0.01 |
| EXOB4_E04 | Elongation factor 1-beta | -1.87 | 0.33 | < 0.01 |
| Hete0002 | 40S ribosomal protein S8 | -2.02 | 0.87 | < 0.01 |
| HK0001_H12 | Unknown-162 | 0.99 | 3.04 | < 0.01 |
| HK0002_D08 | Unknown-173 | 2.44 | 1.07 | < 0.01 |
| HKT0001_C09 | Malate dehydrogenase, mitochondrial-1 | -0.64 | 1.73 | < 0.01 |
| HST0001_C02 | Alpha-globin I-1 | -3.69 | 1.62 | < 0.01 |
| HST0001_D04 | Alpha-globin I-2 | -4.23 | 1.88 | < 0.01 |
| utu03b02 | Ribosomal protein L6-2 | -1.89 | 0.51 | < 0.01 |
| CA352578 | Prostaglandine D synthase | 7.91 | 6.09 | < 0.01 |
| CA364112 | N-acetylmuramoyl-L-alanine amidase | 1.46 | 0.17 | < 0.01 |

**Additional file 3d**

**PGN:K12 1 hrs**

| Clone ID | Clone Name | Mean (FC) | SD | *p* |
|---|---|---|---|---|
| EST1-3A_F10 | 14-3-3 B1-like | 2.36 | 1.34 | < 0.01 |
| utu03f12 | 26S proteasome non-ATPase regulatory subunit 14 | -1.64 | 0.17 | < 0.01 |

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|---|---|---|---|---|
| Hete0002_H07 | 40S ribosomal protein S10 | -1.52 | 0.53 | < 0.01 |
| Hete0002_A06 | 40S ribosomal protein S11 | -1.48 | 0.47 | < 0.01 |
| HK0001_A12 | 40S ribosomal protein S2 | 1.62 | 0.34 | < 0.01 |
| utu04g05 | 40S ribosomal protein S9-3 | -1.43 | 0.32 | < 0.01 |
| EXOB2_C03 | 60S ribosomal protein L10-1 | 2.17 | 0.96 | < 0.01 |
| EXOB3_E04 | 60S ribosomal protein L4 | 2.07 | 0.24 | < 0.01 |
| KVkm2_D08 | 60S ribosomal protein L14 | -1.77 | 0.91 | < 0.01 |
| HK0001_H03 | 60S ribosomal protein L18a | 1.36 | 0.17 | < 0.01 |
| utu04c03 | 60S ribosomal protein L26 | -1.41 | 0.28 | < 0.01 |
| EXOB3_G05 | Actin, cytoplasmic 2 | 2.38 | 0.55 | < 0.01 |
| EST1-3A_H05 | Adenosine deaminase 3 | 2.30 | 1.03 | < 0.01 |
| CA383795 | Allograft inflammatory factor-1 | 1.87 | 0.85 | < 0.01 |
| EXOB4_H06 | Alpha-globin 1-3 | -2.37 | 1.24 | < 0.01 |
| CA364941 | Annexin A1-1 | 9.81 | 9.42 | < 0.01 |
| CA361151 | Annexin A1-2 | 5.16 | 3.15 | < 0.01 |
| utu02e04 | ATP synthase coupling factor 6, mitochondrial precursor | 2.37 | 1.68 | < 0.01 |
| CA344745 | B-cell receptor CD22-1 | 1.57 | 0.24 | < 0.01 |
| CA370661 | Barrier-to-autointegration factor | 2.10 | 0.92 | < 0.01 |
| CA367787 | Bax inhibitor-1 | 1.35 | 0.14 | < 0.01 |
| EXOB1_B04 | Beta actin-1 | 4.24 | 3.30 | < 0.01 |
| EXOB4_D02 | Beta actin-2 | 2.52 | 0.86 | < 0.01 |
| ENH2_F09 | Beta-2-microglobulin-1 | -1.86 | 0.95 | < 0.01 |
| ENH2_H01 | Beta-2-microglobulin-2 | -1.86 | 0.95 | < 0.01 |
| Hete0002_C05 | Calmodulin-2 | 1.47 | 0.22 | < 0.01 |
| CA344488 | Cathepsin B-2 | 2.56 | 2.04 | < 0.01 |
| EXOB3_B01 | Cathepsin C-3 | 2.76 | 2.30 | < 0.01 |
| CA347041 | Cathepsin D-1 | 3.22 | 2.84 | < 0.01 |
| CA365458 | Cathepsin D-2 | 3.70 | 3.63 | < 0.01 |
| CA370300 | CD2 binding protein 1-1 | 2.87 | 1.14 | < 0.01 |
| CA365039 | CD63 | 1.20 | 0.07 | < 0.01 |
| CA369653 | Cellular nucleic acid binding protein | 1.73 | 0.45 | < 0.01 |
| CA374193 | Chemokine receptor CXCR4 | -1.78 | 0.44 | < 0.01 |
| HK0001_D10 | Coagulation factor X precursor | 1.36 | 0.14 | < 0.01 |
| EXOB2_C01 | Cofilin, muscle isoform | 2.34 | 1.23 | < 0.01 |
| CA362419 | Complement component C6 | -1.55 | 0.42 | < 0.01 |
| CA383775 | Complement receptor 1-1 | 2.29 | 0.65 | < 0.01 |
| CA363481 | Cornichon homolog | 1.91 | 0.52 | < 0.01 |
| est01e06 | Cyclophilin-2 | 1.39 | 0.02 | < 0.01 |
| CA362758 | Cyclophilin-3 | -1.67 | 0.69 | < 0.01 |
| EST1-3A_H07 | Cytochrome b-1 | 0.48 | 1.47 | < 0.01 |

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|---|---|---|---|---|
| CA373525 | Cytochrome B-245 heavy chain-2 | 2.44 | 0.77 | < 0.01 |
| CA373539 | Cytochrome b-245 light chain | 1.76 | 0.29 | < 0.01 |
| HKT0001_H05 | Cytochrome b-3 | 1.49 | 0.19 | < 0.01 |
| est02g11 | Cytochrome c oxidase subunit I-1 | 1.54 | 0.48 | < 0.01 |
| EXOB2_G09 | Cytochrome c oxidase subunit I-2 | 1.44 | 0.57 | < 0.01 |
| est02f05 | Cytochrome c oxidase subunit III-4 | 1.27 | 0.11 | < 0.01 |
| est03a08 | Cytochrome c-1 | -2.06 | 0.94 | < 0.01 |
| utu01g04 | Cytochrome oxidase subunit III-2 | 1.25 | 0.14 | < 0.01 |
| utu01h02 | Cytochrome oxidase subunit III-3 | 1.31 | 0.18 | < 0.01 |
| CA351746 | Cytokine receptor common gamma chain | 1.72 | 0.53 | < 0.01 |
| EXOB2_B11 | D-dopachrome tautomerase | 2.58 | 0.98 | < 0.01 |
| EXOB1_D11 | Dynein light chain 2, cytoplasmic | -1.62 | 0.41 | < 0.01 |
| EXOB2_D07 | Ependymin related protein-1 | 2.31 | 0.14 | < 0.01 |
| est03d06 | Eukaryotic translation initiation factor 2 subunit 2 | 1.33 | 0.15 | < 0.01 |
| est03f04 | F-box/WD-repeat protein 11 | -1.72 | 0.30 | < 0.01 |
| EXOB3_G08 | Fatty acid-binding protein-1 | 3.50 | 2.50 | < 0.01 |
| CA348053 | Ferritin H-3 | 2.07 | 0.22 | < 0.01 |
| est04c05 | Ferritin heavy chain-1 | -1.30 | 0.27 | < 0.01 |
| CA382877 | Fibronectin receptor beta | 11.64 | 11.27 | < 0.01 |
| CA384134 | G1/S-specific cyclin D2 | -3.13 | 1.99 | < 0.01 |
| CA379576 | Galectin like 2 | 2.04 | 0.78 | < 0.01 |
| EXOB4_G02 | Galectin-9 (VHSV-induced protein)-2 | 1.93 | 0.53 | < 0.01 |
| CA362806 | Gamma-interferon inducible lysosomal thiol reductase | 1.63 | 0.20 | < 0.01 |
| EXOB3_H05 | Glucose-6-phosphate isomerase-2 | 1.54 | 0.28 | < 0.01 |
| est04e05 | Glutathione peroxidase-gastrointestinal | -0.59 | 1.57 | < 0.01 |
| CA352456 | Glutathione reductase, mitochondrial-2 | 1.98 | 0.75 | < 0.01 |
| EST1-3A_D08 | Growth arrest and DNA-damage-inducible protein alpha-2 | 1.78 | 0.12 | < 0.01 |
| CA351992 | Heat shock 27 kDa protein-1 | 2.81 | 0.81 | < 0.01 |
| CA366403 | Heat shock 70 kDa protein 1 | 3.02 | 1.40 | < 0.01 |
| EST1-3A_F05 | Heat shock 70kDa protein 8 | 2.87 | 1.02 | < 0.01 |
| HK0002_B07 | Hemoglobin alpha chain | 4.18 | 1.75 | < 0.01 |
| EXOB3_D08 | Histone H33-2 | -2.06 | 1.48 | < 0.01 |
| CA366564 | Huntingtin | 4.71 | 0.91 | < 0.01 |
| EXOB3_B02 | Hyperosmotic protein 21 | -2.06 | 1.05 | < 0.01 |
| EXOB1_B02 | Hypothetical-fish 34 | 2.96 | 1.85 | < 0.01 |
| est04c09 | Hypothetical-fish 41 | -1.69 | 0.57 | < 0.01 |
| EST1-3A_B03 | Hypothetical-fish 44 | 2.26 | 0.37 | < 0.01 |
| Hete0002_E02 | Hypothetical-fish 8 | -3.09 | 2.50 | < 0.01 |

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|---|---|---|---|---|
| EXOB4_E09 | Hypoxanthine-guanine phosphoribosyltransferase | -2.01 | 0.99 | < 0.01 |
| CA342430 | Ig kappa chain V-III region VG | -2.30 | 1.14 | < 0.01 |
| est02a11 | Ig kappa chain V-IV region B17-2 | -5.94 | 10.72 | < 0.01 |
| CA363438 | Interferon-induced protein 44-3 | 3.15 | 1.67 | < 0.01 |
| utu01g10 | Keratin, type II cytoskeletal 8 | 0.18 | 2.64 | < 0.01 |
| CA349380 | LPS binding protein | 3.38 | 2.54 | < 0.01 |
| CA373759 | Lymphocyte antigen 75 | 2.03 | 0.89 | < 0.01 |
| CA369420 | Lymphocyte pore forming protein | 5.86 | 5.77 | < 0.01 |
| CA369597 | Lysosomal acid lipase/cholesteryl ester hydrolase | 4.13 | 2.49 | < 0.01 |
| CA370329 | Lysozyme C precursor | -4.00 | 2.89 | < 0.01 |
| EXOB4_A08 | Lysozyme g-3 | -2.68 | 0.45 | < 0.01 |
| est04e08 | Malate dehydrogenase, cytoplasmic | 2.11 | 0.64 | < 0.01 |
| CA368189 | MAPK/ERK kinase kinase 6 | -1.57 | 0.41 | < 0.01 |
| CA342769 | Matrix metalloproteinase 9-1 | 0.89 | 2.28 | < 0.01 |
| EXOB1_A03 | Metallothionein A | -4.40 | 1.08 | < 0.01 |
| CA359170 | Metallothionein B | -3.22 | 1.30 | < 0.01 |
| Hete0002_A07 | Metallothionein-IL | -4.72 | 1.71 | < 0.01 |
| EXOB3_F09 | MHC class I heavy chain-1 | 1.62 | 0.34 | < 0.01 |
| CA379977 | MHC class II alpha chain | -2.76 | 0.94 | < 0.01 |
| HKT0001_H03 | Microtubule-associated protein RP/EB | -2.01 | 0.73 | < 0.01 |
| CA376758 | Mitogen-activated protein kinase 6 | 1.69 | 0.28 | < 0.01 |
| HK0002_G11 | NAD(P)H menadione oxidoreductase 1, dioxin-inducible | -2.04 | 0.91 | < 0.01 |
| CA369440 | NADH dehydrogenase subunit 2 | 2.20 | 1.70 | < 0.01 |
| CA343143 | NF-kappaB inhibitor alpha-1 | 4.56 | 4.28 | < 0.01 |
| est02c08 | Nicotinamide riboside kinase 2 | 1.67 | 0.35 | < 0.01 |
| est02h09 | Nuclease sensitive element binding protein 1-2 | 1.90 | 0.49 | < 0.01 |
| EXOB3_F10 | Peroxiredoxin 1-2 | -1.49 | 0.34 | < 0.01 |
| est02b08 | PEST-containing nuclear protein | -1.60 | 0.38 | < 0.01 |
| est02b02 | Phospholipid hydroperoxide glutathione peroxidase B | 1.79 | 0.36 | < 0.01 |
| CA369101 | Phosphotyrosine independent ligand for the Lck SH2 domain p62 | 1.69 | 0.39 | < 0.01 |
| CA375694 | Polyposis locus protein 1 | 1.39 | 0.31 | < 0.01 |
| est04f01 | Ras activator RasGRP | 1.80 | 0.67 | < 0.01 |
| CA350333 | Receptor-interacting serine/threonine-protein kinase 2 | 2.85 | 2.16 | < 0.01 |
| CA356686 | Regulator of G-protein signaling 1-2 | 1.47 | 0.23 | < 0.01 |
| CA361101 | Reverse transcriptase-like-2 | 2.41 | 1.73 | < 0.01 |

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|---|---|---|---|---|
| CA342656 | Ribosomal protein L36a-like-2 | -1.49 | 0.44 | < 0.01 |
| CA346623 | Selenium-binding protein 1 | 4.14 | 2.32 | < 0.01 |
| CA383435 | Semaphorin 7A | 2.68 | 0.15 | < 0.01 |
| EXOB2_H02 | Serine/arginine repetitive matrix 1 | 2.00 | 0.40 | < 0.01 |
| HKT0001_E06 | Stomatin | 1.34 | 0.14 | < 0.01 |
| EXOB2_B04 | Syntenin 1 | 1.86 | 0.26 | < 0.01 |
| EXOB1_G12 | Thioredoxin | -6.56 | 3.72 | < 0.01 |
| est01e02 | Thymosin beta-4-1 | 3.28 | 1.43 | < 0.01 |
| HK0003_A03 | Thymosin beta-4-2 | -1.52 | 0.28 | < 0.01 |
| est03f08 | Transaldolase | 2.57 | 1.26 | < 0.01 |
| CA356055 | Transposase -63 | -3.09 | 2.32 | < 0.01 |
| CA347787 | Transposase-15 | -4.00 | 3.41 | < 0.01 |
| CA352804 | Transposase-23 | 1.73 | 0.57 | < 0.01 |
| Hete0002_E09 | Transposase-56 | -2.52 | 1.82 | < 0.01 |
| EXOB4_E01 | Transposase-58 | 1.41 | 0.17 | < 0.01 |
| est02f04 | Tubulin alpha-3 chain | 2.12 | 0.88 | < 0.01 |
| HST0001_B05 | Tumor differentially expressed protein 1 | 1.58 | 0.36 | < 0.01 |
| HK0001_D01 | Ubiquitin | 3.40 | 2.94 | < 0.01 |
| EXOB3_A10 | Unknown-106 | 1.74 | 0.02 | < 0.01 |
| EXOB3_B12 | Unknown-109 | 0.07 | 3.28 | < 0.01 |
| EXOB3_F12 | Unknown-121 | 0.58 | 1.62 | < 0.01 |
| EXOB4_H05 | Unknown-133 | 0.26 | 2.83 | < 0.01 |
| HK0002_H09 | Unknown-181 | -2.36 | 0.94 | < 0.01 |
| HK0003_G05 | Unknown-201 | -0.49 | 1.55 | < 0.01 |
| HK0003_G10 | Unknown-202 | 0.62 | 1.83 | < 0.01 |
| HKT0001_E01 | Unknown-207 | 1.34 | 0.07 | < 0.01 |
| KVkm2_F01 | Unknown-224 | 2.53 | 0.07 | < 0.01 |
| utu01f03 | Unknown-238 | -1.32 | 2.96 | < 0.01 |
| utu04d10 | Unknown-268 | 0.60 | 1.73 | < 0.01 |
| est03d11 | Unknown-42 | 2.63 | 8.21 | < 0.01 |
| ENH2_H06 | Unknown-5 | -1.53 | 2.75 | < 0.01 |
| EST1-3A_B08 | Unknown-67 | -1.24 | 2.11 | < 0.01 |
| EXOB1_C02 | Unknown-83 | 2.18 | 0.55 | < 0.01 |
| CA374472 | X-linked interleukin-1 receptor accessory 2 | 1.92 | 0.51 | < 0.01 |
| CA358621 | Microsomal glutathione S-transferase 3 | -1.61 | 0.15 | < 0.01 |
| CA346166 | Arachidonate 5-lipoxygenase-1 | -3.35 | 0.37 | < 0.01 |

**Additional file 3e**

**PGN:K12 6 hrs**

| Clone ID | Clone Name | Mean (FC) | SD | *p* |
|---|---|---|---|---|
| CA342656 | Reverse transcriptase-like-1 | -2.66 | 0.61 | < 0.01 |
| CA343473 | C3a anaphylatoxin chemotactic receptor | 2.35 | 0.36 | < 0.01 |
| CA346079 | Transposase-48 | -2.27 | 0.64 | < 0.01 |
| CA348053 | Ferritin H-3 | -8.86 | 3.21 | < 0.01 |
| CA348284 | CCAAT/enhancer binding protein beta | 7.02 | 2.60 | < 0.01 |
| CA348983 | Cathepsin Y | -2.32 | 0.89 | < 0.01 |
| CA351516 | Reverse transcriptase-like-2 | -3.27 | 0.97 | < 0.01 |
| CA359170 | Metallothionein B | -3.81 | 1.06 | < 0.01 |
| CA361754 | Glutathione S-transferase theta 1 | 2.37 | 1.62 | < 0.01 |
| CA367787 | Bax inhibitor-1 | 1.96 | 0.62 | < 0.01 |
| CA368203 | Transposase-55 | -3.43 | 0.45 | < 0.01 |
| CA376213 | TNF receptor associated factor 1 | 4.55 | 4.12 | < 0.01 |
| CA376758 | Mitogen-activated protein kinase 6 | 2.11 | 0.54 | < 0.01 |
| CA379977 | MHC class II alpha chain | 3.84 | 1.12 | < 0.01 |
| CA381226 | ATF-like basic leucine zipper transcriptional factor B-ATF | -4.00 | 3.32 | < 0.01 |
| CA381566 | Programmed cell death protein 6 | -1.87 | 0.41 | < 0.01 |
| CA384134 | G1/S-specific cyclin D2 | -3.63 | 1.44 | < 0.01 |
| CA385270 | Granulins | 2.72 | 1.26 | < 0.01 |
| CA386429 | Calcium homeostasis endoplasmic reticulum protein | -3.83 | 3.89 | < 0.01 |
| ENH2_H06 | Unknown-5 | -1.76 | 0.11 | < 0.01 |
| est03f07 | Transposase-4 | -2.16 | 1.16 | < 0.01 |
| est04c01 | ARP2/3 complex 34 kDa subunit | 3.63 | 3.98 | < 0.01 |
| 3A_B08 | Unknown-67 | -2.09 | 0.45 | < 0.01 |
| 3A_D08 | Glyceraldehyde-3-phosphate dehydrogenase-6 | -3.60 | 2.96 | < 0.01 |
| EXOB1_A07 | Unknown-79 | -1.96 | 0.66 | < 0.01 |
| EXOB1_C02 | Unknown-83 | 2.09 | 0.33 | < 0.01 |
| EXOB1_F02 | Transcription regulator protein BACH1 | -4.00 | 3.46 | < 0.01 |
| EXOB1_G12 | Thioredoxin | -8.14 | 4.43 | < 0.01 |
| EXOB2_D07 | Ependymin related protein-1 | 3.59 | 3.31 | < 0.01 |
| EXOB3_C04 | Glyceraldehyde-3-phosphate dehydrogenase-1 | -2.44 | 1.21 | < 0.01 |
| EXOB3_E04 | 60S ribosomal protein L14 | -1.53 | 0.24 | < 0.01 |
| EXOB3_G05 | Actin, cytoplasmic 2 | 1.61 | 0.16 | < 0.01 |
| EXOB4_E04 | Elongation factor 1-beta | -1.53 | 0.39 | < 0.01 |

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|---|---|---|---|---|
| Hete0002_B09 | Unknown-136 | -2.75 | 1.20 | < 0.01 |
| Hete0002_E09 | Transposase-56 | -4.45 | 2.25 | < 0.01 |
| HST0001_D08 | Beta-globin | 2.52 | 0.80 | < 0.01 |
| utu01d02 | Unknown-235 | 2.58 | 1.10 | < 0.01 |
| CA358621 | Microsomal glutathione S-transferase 3 | -1.42 | 0.16 | < 0.01 |

**Additional file 3f**

**PGN:K12 6 hrs**

| Clone ID | Clone Name | Mean (FC) | SD | *p* |
|---|---|---|---|---|
| CA342227 | Protein tyrosine kinase 2 beta | 1.15 | 0.04 | < 0.01 |
| CA342656 | Reverse transcriptase-like-1 | 1.42 | 0.51 | < 0.01 |
| CA343389 | Oxidoreductase UCPA | 1.41 | 0.27 | < 0.01 |
| CA344201 | Peroxisomal targeting signal 2 receptor | 1.18 | 0.05 | < 0.01 |
| CA344488 | Cathepsin B-2 | 1.62 | 0.69 | < 0.01 |
| CA346016 | MHC class I heavy chain-2 | -1.57 | 0.32 | < 0.01 |
| CA347041 | Cathepsin D-1 | -1.12 | 0.02 | < 0.01 |
| CA347141 | Cathepsin B-1 | 1.79 | 0.45 | < 0.01 |
| CA347787 | Transposase-15 | -1.33 | 0.27 | < 0.01 |
| CA348053 | Ferritin H-3 | 2.08 | 0.50 | < 0.01 |
| CA348743 | Sphingosine 1-phosphate receptor Edg-3 | 1.37 | 0.27 | < 0.01 |
| CA349275 | Macrophage capping protein | 1.52 | 0.25 | < 0.01 |
| CA349364 | Cathepsin K-2 | 1.41 | 0.49 | < 0.01 |
| CA349380 | LPS binding protein | 1.53 | 0.56 | < 0.01 |
| CA349577 | Tyrosine-protein kinase FRK | 1.72 | 0.46 | < 0.01 |
| CA350804 | Unknown-277 | 1.15 | 0.05 | < 0.01 |
| CA352381 | Protein kinase C, alpha type | 1.34 | 0.13 | < 0.01 |
| CA352456 | Glutathione reductase, mitochondrial-2 | -2.18 | 1.04 | < 0.01 |
| CA352526 | Transposase-22 | -1.32 | 0.21 | < 0.01 |
| CA354612 | Cathepsin C-1 | 1.54 | 0.50 | < 0.01 |
| CA355065 | Tapasin-1 | -0.50 | 1.46 | < 0.01 |
| CA355527 | Adenosine kinase 2 | -1.77 | 0.31 | < 0.01 |
| CA356752 | Transposase-27 | -1.32 | 0.29 | < 0.01 |
| CA357173 | Peptidyl-prolyl cis-trans isomerase 2-2 | -1.23 | 0.16 | < 0.01 |
| CA357749 | Transposase-30 | -1.38 | 0.36 | < 0.01 |
| CA358266 | T-cell, immune regulator 1, isoform a | 1.28 | 0.20 | < 0.01 |
| CA358887 | FRG1 protein | 1.42 | 0.32 | < 0.01 |
| CA358990 | B-cell lymphoma 6 protein-2 | -2.01 | 1.18 | < 0.01 |
| CA358998 | Cysteine-rich protein 1 | 1.37 | 0.15 | < 0.01 |
| CA359144 | Aldehyde dehydrogenase 1A2 | -3.07 | 1.68 | < 0.01 |
| CA359170 | Metallothionein B | 1.37 | 0.25 | < 0.01 |

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|---|---|---|---|---|
| CA360163 | Tyrosine-protein kinase SYK | -1.97 | 1.39 | < 0.01 |
| CA361817 | Transposase-36 | -1.41 | 0.29 | < 0.01 |
| CA363120 | Heme oxygenase-2 | 3.15 | 1.22 | < 0.01 |
| CA363438 | Interferon-induced protein 44-3 | 1.97 | 1.01 | < 0.01 |
| CA363944 | Nuclear factor NF-kappa-B | 1.35 | 0.14 | < 0.01 |
| CA364325 | Peroxiredoxin 6 | 1.25 | 0.14 | < 0.01 |
| CA364941 | Annexin A1-1 | 1.37 | 0.31 | < 0.01 |
| CA365162 | Transposase-43 | -1.20 | 0.05 | < 0.01 |
| CA365876 | Aminolevulinate, delta-, synthetase 1-2 | -3.01 | 1.83 | < 0.01 |
| CA366512 | Alcohol dehydrogenase [NADP+] | 1.27 | 0.04 | < 0.01 |
| CA366564 | Huntingtin | -1.36 | 0.16 | < 0.01 |
| CA367619 | Programmed cell death protein 8, mitochondrial precursor | 1.30 | 0.28 | < 0.01 |
| CA368189 | MAPK/ERK kinase kinase 6 | -1.16 | 0.06 | < 0.01 |
| CA368203 | Transposase-55 | -1.38 | 0.41 | < 0.01 |
| CA368961 | 78 kDa glucose-regulated protein precursor | -3.43 | 2.20 | < 0.01 |
| CA369000 | Glutathione peroxidase 1 | 1.42 | 0.20 | < 0.01 |
| CA369597 | Lysosomal acid lipase/cholesteryl ester hydrolase | 1.34 | 0.25 | < 0.01 |
| CA369915 | Transposase-46 | -1.34 | 0.24 | < 0.01 |
| CA370333 | Melanoma derived growth regulatory protein | 1.51 | 0.61 | < 0.01 |
| CA370339 | Nucleophosmin 1 | -1.71 | 0.44 | < 0.01 |
| CA371363 | Glucose-6-phosphate isomerase-1 | 2.36 | 1.69 | < 0.01 |
| CA371933 | Adenosine deaminase 2 | 1.70 | 0.24 | < 0.01 |
| CA372588 | Inhibitor of apoptosis protein 2 | 1.19 | 0.02 | < 0.01 |
| CA372952 | Survival of motor neuron-related splicing factor 30 | 1.26 | 0.22 | < 0.01 |
| CA373525 | Cytochrome B-245 heavy chain-2 | 1.41 | 0.26 | < 0.01 |
| CA373890 | Heat shock protein HSP 90-beta-1 | -1.69 | 0.36 | < 0.01 |
| CA374030 | Peroxiredoxin 5, mitochondrial | 1.34 | 0.08 | < 0.01 |
| CA376813 | CC chemokine SCYA110-1 | 1.24 | 0.09 | < 0.01 |
| CA377672 | Defender against cell death 1 | 0.40 | 1.63 | < 0.01 |
| CA378908 | Early growth response protein 2 | -1.01 | 2.20 | < 0.01 |
| CA379787 | Cyclin G1 | 1.55 | 0.39 | < 0.01 |
| CA380218 | Macrophage migration inhibitory factor | 1.64 | 0.29 | < 0.01 |
| CA380457 | Aminopeptidase N | 1.83 | 0.74 | < 0.01 |
| CA381440 | Double-stranded RNA-specific adenosine deaminase | 0.52 | 1.44 | < 0.01 |
| CA382425 | B-cell translocation gene 1-2 | 0.71 | 1.77 | < 0.01 |
| CA382570 | Mitogen-activated protein kinase 13 | -0.58 | 1.57 | < 0.01 |
| CA383089 | Neutrophil cytosol factor 2 | -1.80 | 1.00 | < 0.01 |

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|---|---|---|---|---|
| CA383435 | Semaphorin 7A | 1.38 | 0.14 | < 0.01 |
| CA383795 | Allograft inflammatory factor-1 | 2.14 | 1.62 | < 0.01 |
| CA385270 | Granulins | 1.71 | 0.45 | < 0.01 |
| CA387837 | Nucleolar protein NAP57 | -1.21 | 0.02 | < 0.01 |
| ENH2_C01 | Hypothetical-fish 28 | 1.72 | 0.20 | < 0.01 |
| ENH2_C04 | Cytochrome oxidase subunit III-1 | 1.28 | 0.08 | < 0.01 |
| ENH2_F03 | Cathepsin K-1 | 0.55 | 1.57 | < 0.01 |
| ENH2_H01 | Beta-2-microglobulin-2 | -1.46 | 0.57 | < 0.01 |
| ENH2_H02 | Peroxiredoxin 1-1 | 0.32 | 1.45 | < 0.01 |
| ENH2_H06 | Unknown-5 | -1.58 | 0.37 | < 0.01 |
| est01b09 | Tolloid-like 2 protein (nephrosin) | 7.72 | 7.86 | < 0.01 |
| est01c04 | Unknown-11 | 4.57 | 3.15 | < 0.01 |
| est01e06 | Coronin-1B | 2.79 | 1.07 | < 0.01 |
| est01f03 | Deltex protein 1 | 0.45 | 1.92 | < 0.01 |
| est01h03 | Galectin-3 binding protein | -0.41 | 1.82 | < 0.01 |
| est02b11 | Pol polyprotein | -1.80 | 0.45 | < 0.01 |
| est02c08 | Nicotinamide riboside kinase 2 | -1.41 | 0.28 | < 0.01 |
| est02e04 | Membrane associated progesterone receptor component 2 | -0.96 | 1.93 | < 0.01 |
| est03a03 | RER1 protein | -1.32 | 0.05 | < 0.01 |
| est03b03 | Cathepsin C-2 | 1.66 | 0.55 | < 0.01 |
| est03b11 | Acidic leucine-rich nuclear phosphoprotein 32 A-1 | 1.32 | 0.13 | < 0.01 |
| est03c04 | Matrix metalloproteinase-9 | -4.67 | 1.77 | < 0.01 |
| est03c05 | Unknown-37 | -3.90 | 1.46 | < 0.01 |
| est03c11 | Unknown-38 | -1.88 | 0.06 | < 0.01 |
| est03d11 | Unknown-42 | 2.34 | 1.17 | < 0.01 |
| est03e05 | Hypothetical protein LOC122618 | 1.86 | 0.27 | < 0.01 |
| est03f08 | Transaldolase | -1.42 | 0.28 | < 0.01 |
| est03f09 | NADH-ubiquinone oxidoreductase 49 kDa subunit, mitochondrial precursor | -0.48 | 1.44 | < 0.01 |
| est03h05 | Acidic leucine-rich nuclear phosphoprotein 32 A-2 | 1.51 | 0.25 | < 0.01 |
| est04b01 | Similar to rRNA (Vangl2) | 1.63 | 0.30 | < 0.01 |
| est04b04 | Cytokeratin 8 | -1.26 | 0.16 | < 0.01 |
| est04b12 | Leucine-rich repeats and calponin domain containing 4 | -0.40 | 1.49 | < 0.01 |
| est04c05 | Ferritin heavy chain-1 | -0.37 | 2.94 | < 0.01 |
| est04c06 | Unknown-54 | 1.32 | 0.15 | < 0.01 |
| est04c07 | Ferritin heavy chain-2 | -0.37 | 3.01 | < 0.01 |
| est04e04 | MHC class 1b antigen | -1.61 | 0.13 | < 0.01 |

| | | | | |
|---|---|---|---|---|
| est04e08 | Malate dehydrogenase, cytoplasmic | 0.48 | 1.70 | < 0.01 |
| EST1-3A_B03 | Hypothetical-fish 44 | 3.08 | 0.96 | < 0.01 |
| EST1-3A_F05 | Heat shock 70 kDa protein 1 | -1.79 | 0.63 | < 0.01 |
| EST1-3A_F08 | Heat shock protein HSP 90-beta-2 | -1.70 | 0.44 | < 0.01 |
| EST1-3A_G02 | NADH dehydrogenase subunit 2 | 1.18 | 0.07 | < 0.01 |
| EST1-3A_H06 | Transcription factor jun-B-1 | -1.67 | 0.41 | < 0.01 |
| EST1-3A_H07 | Cytochrome b-1 | 1.45 | 0.50 | < 0.01 |
| EXOB1_A07 | Unknown-79 | 1.42 | 0.32 | < 0.01 |
| EXOB1_B02 | Hypothetical-fish 34 | 1.33 | 0.09 | < 0.01 |
| EXOB1_B09 | Ribonucleoside-diphosphate reductase large subunit | -1.31 | 0.12 | < 0.01 |
| EXOB1_D11 | Dynein light chain 2, cytoplasmic | 1.37 | 0.06 | < 0.01 |
| EXOB1_F01 | 60S ribosomal protein L35a-1 | 1.36 | 0.34 | < 0.01 |
| EXOB1_F02 | Transcription regulator protein BACH1 | -1.29 | 0.26 | < 0.01 |
| EXOB1_G12 | Thioredoxin | -1.40 | 2.24 | < 0.01 |
| EXOB2_A01 | MHC class II invariant chain-like protein 1 | 4.08 | 3.95 | < 0.01 |
| EXOB2_A06 | Nucleolar protein Nop56-2 | -1.67 | 0.46 | < 0.01 |
| EXOB2_A09 | 60S ribosomal protein L5-2 | -1.42 | 0.31 | < 0.01 |
| EXOB2_C01 | Cofilin, muscle isoform | 0.07 | 1.99 | < 0.01 |
| EXOB2_D04 | Xaa-Pro dipeptidase | -0.34 | 1.64 | < 0.01 |
| EXOB2_D05 | Matrix metalloproteinase 9-2 | -1.27 | 6.81 | < 0.01 |
| EXOB2_D09 | GWSC6486 | -0.56 | 3.93 | < 0.01 |
| EXOB2_D10 | Unknown-96 | -1.68 | 3.68 | < 0.01 |
| EXOB2_G02 | Profilin-1 | 1.50 | 0.19 | < 0.01 |
| EXOB2_G09 | Cytochrome c oxidase subunit I-2 | -1.24 | 0.04 | < 0.01 |
| EXOB2_G12 | Tolloid-like protein (nephrosin)-2 | 6.83 | 9.21 | < 0.01 |
| EXOB2_H01 | Unknown-100 | 7.92 | 11.39 | < 0.01 |
| EXOB2_H06 | High affinity immunoglobulin epsilon receptor alpha | 1.46 | 0.32 | < 0.01 |
| EXOB3_A01 | Unknown-103 | -1.27 | 0.27 | < 0.01 |
| EXOB3_A03 | SEC13-related protein | 1.25 | 0.17 | < 0.01 |
| EXOB3_A10 | Unknown-106 | -1.33 | 0.11 | < 0.01 |
| EXOB3_B12 | Unknown-109 | -1.59 | 0.43 | < 0.01 |
| EXOB3_C03 | Unknown-110 | 1.21 | 0.04 | < 0.01 |
| EXOB3_F07 | Unknown-120 | -0.49 | 1.64 | < 0.01 |
| EXOB3_F08 | 60S ribosomal protein L10-1 | -0.55 | 1.55 | < 0.01 |
| EXOB3_G05 | Actin, cytoplasmic 2 | -0.34 | 1.36 | < 0.01 |
| EXOB3_G06 | 40S ribosomal protein S15-1 | -0.76 | 1.81 | < 0.01 |
| EXOB3_G08 | Fatty acid-binding protein-1 | -1.35 | 3.24 | < 0.01 |
| EXOB3_G09 | Ornithine decarboxylase antizyme-2 | -1.29 | 0.08 | < 0.01 |
| EXOB3_H02 | Unknown-123 | 1.25 | 0.09 | < 0.01 |

| | | | | |
|---|---|---|---|---|
| EXOB3_H04 | Transposase-1 | -1.37 | 0.45 | < 0.01 |
| EXOB3_H05 | Glucose-6-phosphate isomerase-2 | 2.44 | 2.09 | < 0.01 |
| EXOB4_A08 | Lysozyme g-3 | 1.56 | 0.58 | < 0.01 |
| EXOB4_B09 | Macrophage receptor MARCO | 0.37 | 1.76 | < 0.01 |
| EXOB4_C02 | Unknown-127 | 0.79 | 1.89 | < 0.01 |
| EXOB4_D09 | Hypothetical-fish 7 | 1.32 | 0.12 | < 0.01 |
| EXOB4_D12 | Hpa repeat-1 | -0.60 | 1.59 | < 0.01 |
| EXOB4_G09 | Histone H14 | 1.83 | 0.68 | < 0.01 |
| EXOB4_H04 | Fructose-1,6-bisphosphatase isozyme 2 | 1.59 | 0.79 | < 0.01 |
| EXOB4_H05 | Unknown-133 | -1.53 | 0.34 | < 0.01 |
| EXOB4_H07 | Beta-galactosidase-related protein | 1.14 | 0.06 | < 0.01 |
| EXOB4_H08 | Alanine-glyoxylate aminotransferase 1 | 1.15 | 0.09 | < 0.01 |
| Hete0002_A07 | Metallothionein-IL | 0.71 | 1.98 | < 0.01 |
| Hete0002_C04 | Peroxiredoxin 2 | 1.47 | 0.30 | < 0.01 |
| Hete0002_E02 | Hypothetical-fish 8 | 1.30 | 0.09 | < 0.01 |
| Hete0002_H06 | Ribosomal protein S2 | -1.37 | 0.47 | < 0.01 |
| Hete0002_H07 | 40S ribosomal protein S10 | -0.53 | 1.46 | < 0.01 |
| HK0001_A05 | Unknown-146 | 0.37 | 1.37 | < 0.01 |
| HK0001_B01 | 40S ribosomal protein S15-2 | -1.37 | 0.16 | < 0.01 |
| HK0001_H05 | Angiotensin I converting enzyme | 1.17 | 0.05 | < 0.01 |
| HK0002_B07 | Heat shock 70kDa protein 8 | -1.75 | 0.49 | < 0.01 |
| HK0002_B09 | Transgelin | -1.50 | 0.27 | < 0.01 |
| HK0002_C02 | Chromosome-associated kinesin KIF4A | 1.23 | 0.02 | < 0.01 |
| HK0002_C03 | Unknown-168 | 1.19 | 0.04 | < 0.01 |
| HK0002_C04 | Apolipoprotein E-2 | -0.47 | 2.36 | < 0.01 |
| HK0002_D07 | Unknown-172 | 1.72 | 0.67 | < 0.01 |
| HK0002_G03 | Histone H10 | 2.68 | 0.85 | < 0.01 |
| HK0002_G10 | T-cell receptor alpha chain V region HPB-MLT precursor (Fragment) | 1.14 | 0.10 | < 0.01 |
| HK0002_G11 | Myristoylated alanine-rich protein kinase C substrate | -1.60 | 0.47 | < 0.01 |
| HK0002_H04 | Unknown-179 | 1.26 | 0.04 | < 0.01 |
| HK0002_H09 | Unknown-181 | -0.52 | 1.38 | < 0.01 |
| HK0002_H11 | Creatine kinase, B chain | -0.39 | 2.72 | < 0.01 |
| HK0003_C03 | Unknown-189 | 1.19 | 0.01 | < 0.01 |
| HK0003_C08 | Parvalbumin alpha-2 | 1.17 | 0.05 | < 0.01 |
| HK0003_C10 | Over-expressed breast tumor protein-like | 1.24 | 0.20 | < 0.01 |
| HK0003_F07 | Unknown-198 | 1.15 | 0.06 | < 0.01 |
| HK0003_F10 | Unknown-200 | 1.22 | 0.06 | < 0.01 |
| HK0003_H02 | Cathepsin L2 | 1.22 | 0.09 | < 0.01 |
| HKT0001_A08 | Antifreeze protein LS-12 | 1.18 | 0.06 | < 0.01 |

| | | | | |
|---|---|---|---|---|
| HKT0001_H05 | Cytochrome b-3 | 1.35 | 0.21 | < 0.01 |
| KVkm2_F01 | Unknown-224 | -1.33 | 0.15 | < 0.01 |
| KVkm2_H10 | Unknown-227 | 2.43 | 1.32 | < 0.01 |
| utu01c03 | Unknown-232 | 1.20 | 0.06 | < 0.01 |
| utu01e12 | Zinc finger protein 228 | 1.17 | 0.01 | < 0.01 |
| utu01f04 | Actin, alpha skeletal 2 | 1.42 | 0.06 | < 0.01 |
| utu01g04 | Cytochrome oxidase subunit III-2 | 1.46 | 0.06 | < 0.01 |
| utu01g11 | Actin, alpha skeletal 3 | 1.36 | 0.02 | < 0.01 |
| utu01h02 | Cytochrome oxidase subunit III-3 | 1.40 | 0.16 | < 0.01 |
| utu02a08 | Ubiquitin and ribosomal protein S27a-2 | -1.21 | 0.11 | < 0.01 |
| utu02a12 | Brain protein 44-like protein | 1.57 | 0.18 | < 0.01 |
| utu03b10 | Beta enolase-3 | -1.30 | 0.12 | < 0.01 |
| utu03f12 | 26S proteasome non-ATPase regulatory subunit 14 | -1.25 | 0.18 | < 0.01 |
| utu03g02 | Heat shock cognate 70 kDa | -1.48 | 0.32 | < 0.01 |

**Additional file 4.**

Quantitative summary of transcripts/genes differentially expressed over the control in both treatment and stages.

**Number of genes**

| PGN | Early | Median | Late | Total |
|---|---|---|---|---|
| PGN:B4 | 69 | 130 | 86 | 285 |
| PGN:K12 | 173 | 64 | 219 | 456 |
| Interaction | 28 | 27 | 23 | 78 |

**Number of genes < 2**

| PGN | Early | Median | Late | Total |
|---|---|---|---|---|
| PGN:B4 | 51 | 94 | 72 | 217 |
| PGN:K12 | 134 | 33 | 23 | 190 |
| Interaction | 21 | 19 | 11 | 51 |

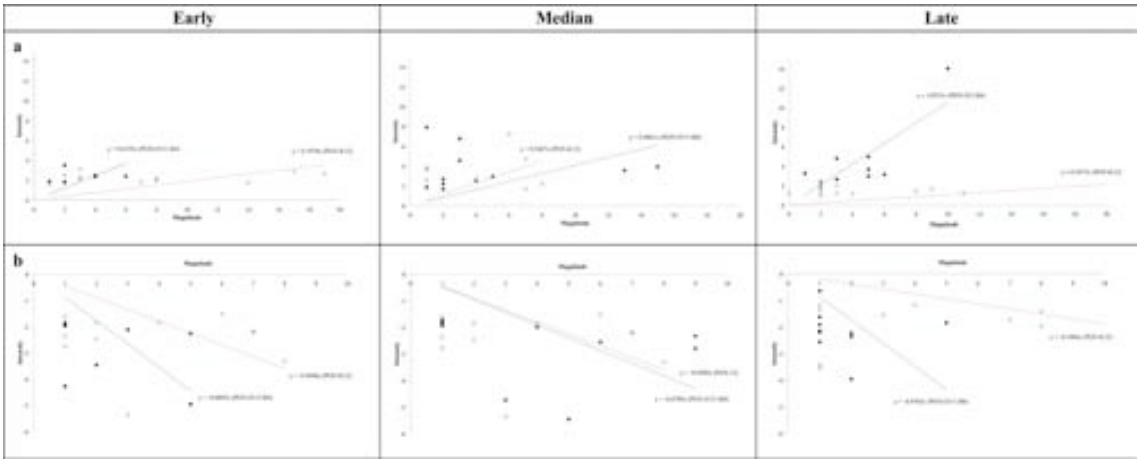
**Porcent of genes < 2**

| PGN | Early | Median | Late | Total |
|---|---|---|---|---|
| PGN:B4 | 74 | 72 | 84 | 76 |
| PGN:K12 | 77 | 52 | 11 | 42 |
| Interaction | 75 | 70 | 48 | 65 |

**Additional file 5.**

Relationship between intensity and magnitude of transcriptomic response in up (a) and down (b) regulated genes at different time stages during the PGNs challenge. The horizontal abscises (magnitude) show the number of transcripts grouped in biological processes expressed in both treatments as: Antigen presentation, Cell adhesion and proliferation, Cytokines and Chemokines, Cellular defense response, MAPK/ERK, Inflammatory response, Cell homeostasis, Transcription. The vertical abscises (intensity) show fold change mean (FC: intensity) of the transcripts grouped in each biological process. The black

circle and the blue slope represented the fit generated by the intensity and magnitude of the transcriptomic response under PGN-O111:B4 treatment. The white circle and the red slope represented the fit generated by the intensity and magnitude of the transcriptomic response under PGN-K12 treatment. Transcriptomic profiles were highly ranked dependent upon PGN-type (two-way ANCOVA on transcriptomic magnitudes of respective intensities α= 0.05; N= 68).




**Additional file 6.**

Summary of ANCOVA analysis for common slope of regression and adjusted means examining differences in intensity and magnitude of the transcriptomic response at different times (1, 6 and 12 hrs, N=68)

**ANCOVA up reguated**

| Common slope | Mean square | DF | F | *p* |
|---|---|---|---|---|
| Treatment (PGNs) | 0.134 | 1 | 0.044 | 0.834 |
| Time stage | 3.27 | 2 | 1.076 | 0.347 |
| Magnitude | 21.521 | 1 | 7.081 | < 0,05 |
| Treatment* time stage * magnitude | 3.579 | 5 | 1.178 | **0.330** |
| Error | 3.039 | 62 | | |
| Adjusted means | | | | |
| Treatment (PGNs) | 64.945 | 1 | 27.124 | **< 0,05** |
| Time stage | 6.209 | 2 | 2.070 | 0.134 |
| Covariable (magnitude) | 6.310 | 1 | 2.104 | 0.152 |
| Error | 2.999 | 65 | | |

**ANCOVA down reguated**

| Common slope | Mean square | DF | F | *p* |
|---|---|---|---|---|
| Treatment (PGNs) | 0.007 | 1 | 0.004 | 0.947 |
| Time stage | 0.197 | 2 | 0.117 | 0.890 |
| Magnitude | 34.302 | 1 | 20.305 | < 0,05 |
| Treatment* time stage * magnitude | 3.891 | 5 | 2.303 | **0.055** |
| Error | 3.039 | 62 | | |
| Adjusted means | | | | |
| Treatment (PGNs) | 70.527 | 1 | 37.124 | **< 0,05** |
| Time stage | 1.781 | 2 | 0.937 | 0.397 |
| Covariable (magnitude) | 20.702 | 1 | 10.897 | < 0,05 |
| Error | 2.999 | 65 | | |

# Chapter VI

Manuscript in preparation

## Development and validation of the oligo-microarray from *Sparus aurata* and its application to the macrophage expression profile during treatments with lipopolysaccharide (LPS) and peptidoglycan (PGN).

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# Development and validation of the oligo-microarray from *Sparus aurata* and its application to the macrophage expression profile during treatments with lipopolysaccharide (LPS) and peptidoglycan (PGN).

**Abstract**

**This study describes the development and validation of Aquagenomic *Sparus aurata* oligonucleotide-microarray (AsONM) based on the Agilent Technology system (eArray) to provide a platform for studies in the gene expression on gilthead seabream. In fish LPS give a robust cytokine response that is stimulated by crude LPS preparations, some component of the LPS complex are responsible for this stimulation.Peptidoglycan is component of G-negative bacteria (found as contaminant of crude LPS preparations) able to be recognized by macrophages inducing depth transcriptional modulations and a strong inflammatory response. For microarray analysis head kidney macrophage cultures were used (N = 36 fish). Each cell culture was stimulated with equal concentration of PGN and LPS from *E. coli* O111:B4 strain (10 μg/mL): non-stimulated cell cultures (control n = 9 fish), stimulated during 6 h with LPS (n = 9), and stimulated during 1 h (n = 9), and 6 h (n = 9) with PGN. A loop microarray design approach was used for the study, all experimental RNA samples were labelled with single colour dye (Cy3) and each stimulated sample was compared to the control sample (pool without stimulation) labelled with the same dye (Cy3). Our microarray analyses identified differential transcriptional modulations on macrophages stimulated with both LPS and PGN at the level of differentially activated RNA transcripts related with the regulation of transcriptional program, prostaglandin synthesis or highlighting the expression of responsive gene-cassettes tightly related to LPS-PGN host recognition.**

## 1. Background

This study describes the development and validation of Aquagenomic *Sparus aurata* oligonucleotide-microarray (AsONM) based on the Agilent Technology system (eArray) to provide a platform for studies in the gene expression on gilthead seabream. The platform developed used all available public EST stored and annotated in the Aquagenomic Consortium seabream library (10K). The expression analysis was based upon the activation of adherent monocyte/macrophage by PAMPs (pathogen associated molecular pattern), which together is a process characterised by directional transcriptomic changes in the cells [2-7]. Transcriptional studies on fish have significantly contributed to functional reports and early descriptions of PAMP-PRR that driven the activation of specific response cassettes in fish genome [3,6,7]. Lipopolysaccharide (LPS) is a PAMP widely used to studies on the immune response. In mammals, TLR4 is key receptor and adaptor for LPS signalling pathway

[8], which has been characterised only in some fish species such as *Danio rerio* and *Gobiocypris rarus* [9,10]. However, despite the phylogenetic homology with vertebrate TLR, significant functional differences are found between fish and mammals during LPS recognition. TLR activation in mammals is often measured via radical production (ROS) and quantification of NF-κβ activation (luciferase, gene expression), or downstream expression of pro- and inflammatory genes such as TNF-α, IL-1β or IL-6 [11,12]. The observed differences in cytokine regulation [9,13,14] indicate that TLR activation and cytokine induction does not necessarily share homologous functions between mammals and teleost. Fish are much more resistant to toxic effects of LPS as compared to mammals [15-19]. In fish the induction and activation of pro- and inflammatory genes are required higher doses of LPS (micrograms/milliliter) than human cells (nanograms/milliliter) [16,18,20]. The collective data suggest that fish loses functionality of TLR4 receptor-mediated LPS recognition present in mammals [9,16,18], probably due a paralogs TLR4 speciation [20], where the phylogenetic homologies is likely to cause false premises of their functional conservation.

In fish LPS give a robust cytokine response that is stimulated by crude LPS preparations, some component of the LPS complex are responsible for this stimulation [21]. In trout, peptidoglycan is component of G-negative bacteria (found as contaminant of crude LPS preparations) able to be recognized by macrophages inducing depth transcriptional modulations and a strong inflammatory response [3,21]. However, trout macrophages have the enzymatic/kinasic mechanisms as TACE/ADAM17 able to process TNF-α in presence of LPS, ultrapure LPS preparation does not stimulate TNF-α transcription, but can it does stimulate the release of TNF-α [22]. In modern Perciforms fish as *S. aurata* (MacKenzie et al, unpublished results), ultrapure LPS preparations can stimulate the transcription of cytokines such as TNF-α and IL-1β in macrophage cells. This comparison of similar treatments at the transcriptomic level between modern and ancient representation of the Teleostei seems to be that not contaminated LPS may be recognised or at least are involving in cytokine transcription in modern fish. However, transcriptomic studies addressing different responses to LPS and PGN are scarce through modern Percifomes group. In considering, we explore the transcriptomic response of adherent *S. aurata* monocyte/macrophages activated with PAMPs such as lipopolysaccharide (LPS) and peptidoglycan (PGN). Our microarray analyses identified differential transcriptional modulations on macrophages stimulated with both LPS and PGN at the level of differentially activated RNA transcripts related with the regulation of transcriptional program, prostaglandin synthesis or highlighting the expression of responsive gene-cassettes tightly related to LPS-PGN recognition.

## 2. Materials and methods

### 2.1. Experimental cell culture setup and materials

Healthy adult specimens (160g mean weight) of gilthead sea bream (*Sparus aurata*) were purchased from a commercial hatchery (Cripesa Ametlla de Mar, Tarragona, Spain) and held in recirculating freshwater stock tanks (300L) in the aquarium facilities at the Universitat Autònoma de Barcelona. Fish were kept at 15°C with a 12 hours light/12 hours dark photoperiod cycle, and were fed with a maintenance ratio of about 0.5% body weight per day. Water quality indicators (dissolved oxygen, ammonia, nitrite, pH) were analysed periodically. The experimental protocols used for head kidney isolation have been reviewed and approved by the Ethics and Animal Welfare Committee of the Universitat Autonoma de Barcelona, Spain. The fishes were sampled from tanks and immediately euthanized with a lethal dose of MS-222 (0.1g/L). After lethal anaesthesia the head kidney was dissected and seabream macrophages were isolated as previously described [23,24]. Before stimulation, differentiated macrophages were incubated in serum free medium for 3 hours. For stimulation, the medium was removed and fresh medium containing the indicated concentrations of PGN and LPS were added and the cultures were incubated for the indicated times. DMEM and FBS were purchased from PAA Laboratories (Spain). Poly-D-lysine and and MS-222 were purchased from Sigma (Tres Cantos, Madrid). Primocin, and LPS or PGN preparations (*Escherichia coli* O111:B4) were purchased from Invivogen (Nucliber, Spain). Cell strainers and plasticware were from BD Biosciences (Madrid, Spain). Prostaglandin $E_2$ and $D_2$ enzyme immunoassay (EIA) kit was from Cayman (Scharlab, Spain).

### 2.2. Measurement of $PGE_2$ and $PGD_2$ levels

Supernatants from stimulated cell cultures (triplicates) from 6 different fish were recovered, centrifuged and stored at -80°C until use. Measurement of $PGE_2$ levels was completed with a monoclonal EIA according to the manufacturer's instructions. The prostaglandin kit detection limit was 8pg/mL. Prior to prostaglandin determination supernatants were diluted three times in cell culture medium DMEM. The same macrophage cells were used to obtain total RNA for the determination of transcriptional response as well as the supernatants for $PGE_2$ determination.

### 2.3. RNA isolation and complementary DNA (cDNA) synthesis

Total RNA was extracted from cell cultures using 1mL of TriReagent (Molecular Research Center) per well of cell culture, following manufacturer's instructions. RNA concentration was quantified using Nanodrop ND-1000 and RNA integrity and quality was assessed using Bioanalyzer 2100 with the RNA 6000 Nano LabChip kit (Agilent Technologies). The RNA integrity number (RIN) was calculated for each sample using the Agilent 2100 Expert software, only RNAs with a RIN number > 7 were processed (to reduce experimental bias). RNA (2µg) was used to synthesize cDNA with SuperScript III Transcriptase (Invitrogen) and oligo-dT primer (Promega).

**2.4. RNA labelling and hybridisation**

For microarray analysis head kidney macrophage cultures were used (N = 36 fish). Each cell culture was stimulated with equal concentration of PGN and LPS from *E. coli* O111:B4 strain (10 μg/mL): non-stimulated cell cultures (control n = 9 fish), stimulated during 6 h with LPS (n = 9), and stimulated during 1 h (n = 9), and 6 h (n = 9) with PGN. RNAs samples were grouped into pools with 3 cell cultures for each PAMPs and time point. A loop microarray design approach was used for the study, all experimental RNA samples were labelled with single colour dye (Cy3) and each stimulated sample was compared to the control sample (pool without stimulation) labelled with the same dye (Cy3). Denatured samples of RNA were reversed transcribed and indirectly labelled with Cy3. RNA labelling, hybridisations, and scanning were performed according to manufacturer's instructions. Briefly, total RNA (500 ng) was amplified and Cy3-labeled with Agilent's One-Color Microarray-Based Gene Expression Analysis (Quick Amp Labelling kit) along with Agilent's One-Color RNA SpikeIn Kit. Each sample (1.65 μg of RNA) was hybridized to *S. aurata* array (ID 024502, Agilent) at 65 °C for 17 hours using Agilent's GE Hybridisation Kit. Washes were conducted as recommended by the manufacturer using Agilent's Gene Expression Wash Pack with stabilisation and drying solution. Microarrays slides were scanned with Agilent Technologies Scanner model G2505B. Spot intensities and other quality control features were extracted with Agilent's Feature Extraction software version 10.4.0.0. One-channel TIFF images were imported into the GeneSpring software GX 11.0. Assessment of spot quality was done by ratio (R) between the difference of signal and background intensities (SI − SB) and sum of their standard deviations (SDI + SDB). Percentile shift normalization was made to adjust all spot intensities in an array. This normalization takes each column in an experiment independently, and computes the median expression values for this array, across all spots, and then subtracts this value from the expression value of each entity [25]. After Percentile normalization, all data were filtered by comparison of the standard deviation expression among groups (filter by expression). The entities that had values lesser or greater than the standard deviation value were retained. This filter procedure allowed selected samples that have outlier entities, and filters out probes that have a high/low variation expression values across the samples. For each annotated transcript three probes (technic bias) at non-overlapping positions as near as possible to the 3'-end were spotted into the slide. To inspect the hybridisation accuracy was randomly selected two technical probes of three. The variability between the probes was evaluated using a Pearson correlation coefficient between Probe_1 and Probe_2 for each transcript within each hybridisation. Pearson correlation coefficients also were conduced to estimate the technical variability of each transcript among the arrays, ensuring the repeatability and accuracy of the results. A non-parametric Spearman rank-correlation test was used to assess correlation between expression values measured respectively with quantitative real-time PCR and microarray. Statistical test were implemented in the GeneSpring software GX 11.0 used to select transcripts differentially expressed between control and treatments ($P <$

0.01). One-way ANOVAs were used to explore differences in the transcriptomic profile between the treatments. Pearson and Spearman correlation coefficients were conduced to estimate the technical variability. The statistical tests were made using SPSS 17.0. The complete design has been submitted to Gene Expression Omnibus (GEO) database.

### 2.6. Microarray hybridisation

A total of 43,398 oligonucleotide probes were used to construct high-density sea bream microarray based on the Agilent 4 × 44 K design format. Microarray hybridisation validation was made analysing the gene expression profile in primary cultures of seabream macrophages (MC). 7,285 transcripts with annotated sequences were spotted in triplicated into the slide (total probes 21,855), as well as 8,377 ESTs without annotation, 183 enriched sequences (gene bank) with 15 replicated probes (total probes 2,745), and finally 1,417 internal control probes of Agilent (N = 43,398). The mRNAs were put independently in equal amounts with the fluorescent cyanine dye Cy3 and hybridised on the microarray, and as expected results similar between samples.

### 2.7. Real-Time quantitative PCR and validation

In order to verify microarray results quantitative real-time PCR (qRT-PCR) was carried out. The primers for Real-time PCR (Additional file 1) were designed with Primer3 version 4.0 based on target sequences obtained from the sea bream database. Primers were designed to target near at 3'- region and we ensured that the primer pair specifically amplifies the target sequence by searching for the nucleotide sequences containing both primer sequences on opposing strands in the NCBI Genbank database using BLAST (http://www.ncbi.nlm.nih.gov/BLAST). Two micrograms of individuals RNA was used to synthesize cDNA with SuperScript III RNase Transcriptase (Invitrogen) and oligo-dT primer (Promega). The copy number of each transcript was analysed using the MyIQ real-time PCR system (Bio-Rad, CA). Each sample was tested in triplicate in a 96-well plate (Bio-Rad, CA). The reaction mix (15 μL final volume) consisted of 7.5 μL of SybGreen mix (Bio-Rad), 0.75 μL of each primer (500 nM final concentration), 2.5 μL of $H_2O$, and 3.75 μL of a 1/10 dilution of the cDNA sample. The thermocycling program consisted of one hold at 95°C for 4 min, followed by 40 cycles of 10 s at 95°C and 45 s at 60°C. After completion of these cycles, melting-curve data were then collected to verify PCR specificity, contamination and the absence of primer dimers. Quantification was done according to the Pfaffl method corrected for efficiency for each primer set [26]. As a house keeping gene, 18s gene was amplified from the same cDNA samples. Values for each sample were expressed as "fold differences", calculated relative to controls group and normalised for each gene against those obtained for the 18s mRNA abundance.

## 3. Results

### 3.1. Quality assessment of the microarray hybridisation

To estimate the hybridisation performance of the ONM total RNA from *S. aurata* macrophages (MC) were used to produce Cy3 labelled amplified RNA (mRNAs). The success of hybridisations was evaluated for each sample by percentage of probes that were positively hybridised. The number of expressed probes including all EST was high, in total 28,793 were hybridised. The percentage binding of MCs probes (3 probes/target) in the AsONM chip was 96%. Analysis of variation between/within biological and technical replicates is important for the evaluation of hybridisation accuracy. This consistency was examined by reviewing both repeatability and reproducibility at two dependent levels: quantitative signal values and qualitative detection calls. The variation of expression ratio was from moderate to low, the largest standard deviation values were found at moderate intensity values (SI). Variation represented by probe standard deviation variation of the intensity decreased substantially at SI above the threshold indicated (Figure 1). Percentile normalization was carried out to adjust spot intensities in the array data. The data were filtered comparing the standard deviations of intensity value (SI) among probe groups. Probes that had values lesser or greater than the observed threshold of the standard deviation were filtered out for further analysis resulting in the removal of 10% of the total probes (4,301). To evaluate probe correction in the expression data for annotated target (3 probes/target) we randomly selected two of three probes present for each target (technical bias). Pearson coefficient analysis was carried out to explore the correlation between the probes within each hybridisation (Additional file 2). The total expression values of Probe_1 and Probe_2 showed a correlation coefficient greater than 0.7 and always were significantly positive $p < 0.001$ (the smallest rank correlation value was 0,67). The correlations among probes throughout the hybridisations also were evaluated by Pearson analysis. The distribution of correlation coefficients indicates that most probes (81%) had a strong positive correlation ($r > 0.7$), 14% moderate ($0.5 < r < 0.7$), and a small proportion of probes were negatively correlated (5%) (Figure 2). Relative correlation between microarray-based and qRT-PCR expression measured target transcript values register a positive Spearman correlation coefficient > 0.7 (Figure 3), highlighting the high reproducibility of seabream ONM using an independent expression of measurement method (qRT-PCR).

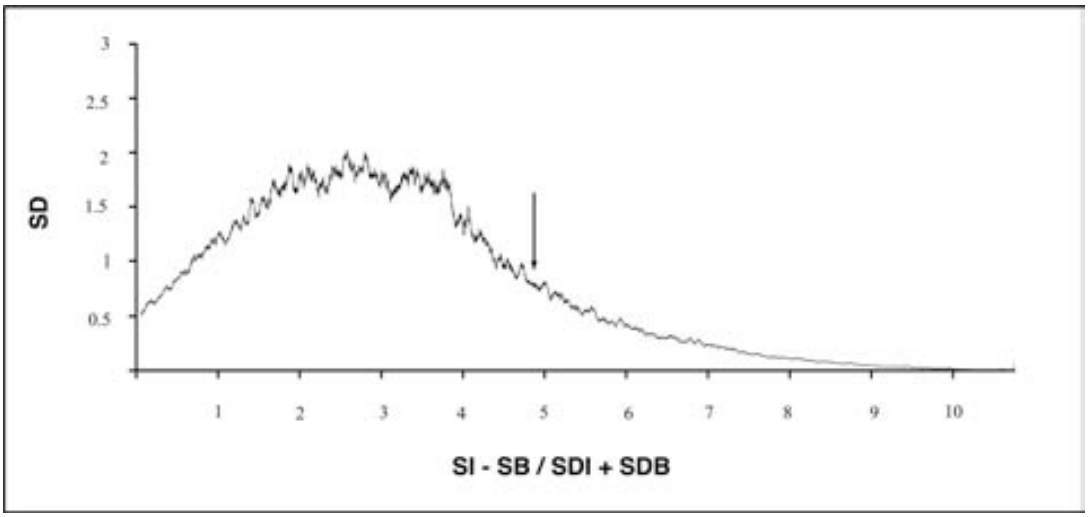


**Figure 1**. **Characterisation the intensity profile.** Standard deviation of log2-expression ratio (SD, y-axis) versus signal intensity (x-axis). Results of all hybridizations were analyzed; spots were filtered with GeneSpring GX 11.0. Lower threshold value for acceptable signal intensity is indicated with an arrow.

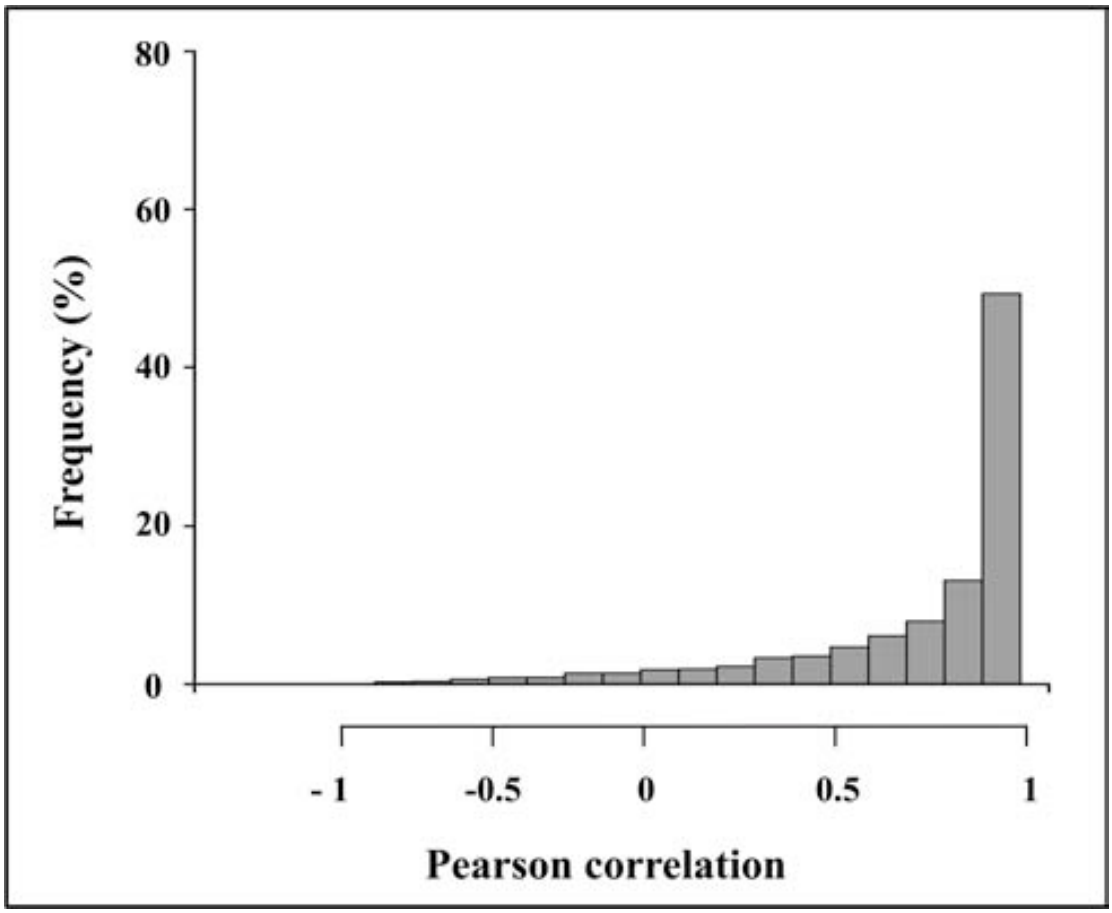


**Figure 2**. **Correlation between levels of gene expression measured by Probe_1 and Probe_2**. For each gene, the Pearson correlation coefficient was calculated within and among arrays.

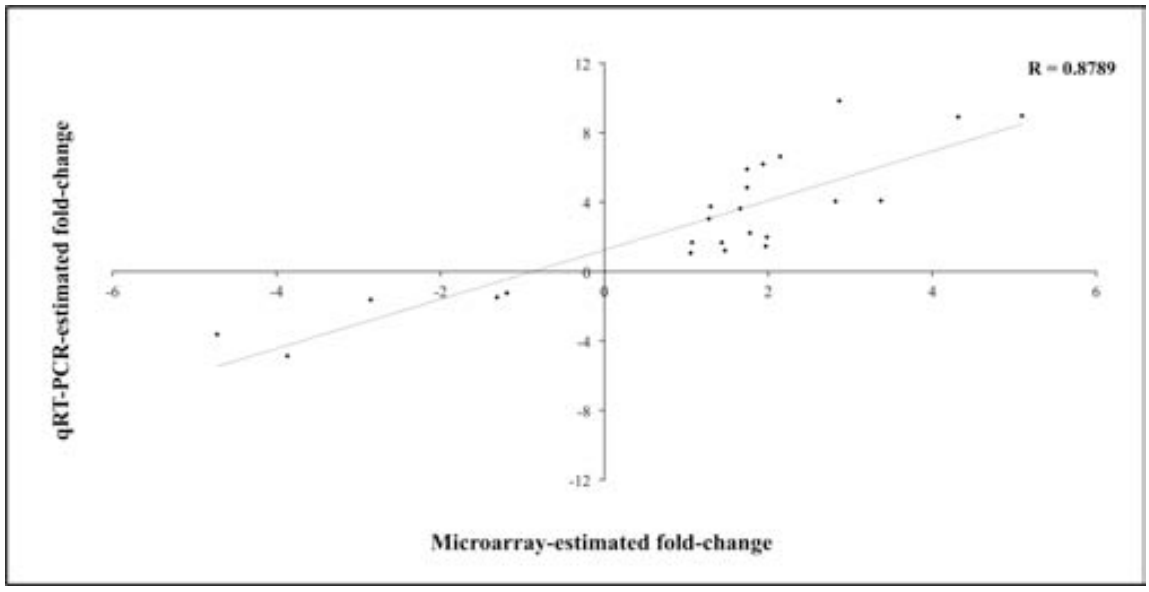


**Figure 3. Comparison between microarray and qPCR results**. Expression values for the eleven target genes were compared between microarray probes and Real-time RT-PCR data. Ratio between micrtoarray expression values and qPCR-estimated fold-changes.

### 3.2. Comparative transcriptome analysis

The *S. aurata* ONM was used to explore transcriptional modulations in macrophages treated with either LPS or PGN, and a time dependent response to PGN was carried out (1 and 6 hours). GeneSpring software allows condense the 3 probes/target (3`technical bias) to 1 probes/target yielding a total of 3,987 transcripts expressed (55% of 7,285 annotated transcripts present on the array), of which 1,168 had a fold change > 2 that represent 30% of the total transcriptomic representation on the array. To identify differentially expressed transcripts over the control all transcripts expressed in each treatment were subjected to separate one-way ANOVAs ($p$ > 0.01). The gene expression profiles highlighted differences in the macrophage response to the different PAMPs and to PGN over time.

Transcriptomic profiles obtained were significantly different between macrophages treated with equal concentrations of LPS or PGN (10 μg/mL), in both transcript number and intensity (fold change FC > 2). In total 3,351 transcripts were differentially expressed in both treatments (LPS or PGN). LPS induced the expression of 1,201 transcripts (p < 0.01) where 184 had a FC > 2 (15%) (Figure 4a). Exclusive regulated transcripts (restricted to one treatment) and common transcripts (regulated under both experimental stimulations) had a low intensity in LPS incubations where 17 (1.4%) and 167 (14%) had a FC > 2 (transcripts exclusive and common respectively). On the other hand PGN induced the expression 2,152 transcripts (p < 0.01), where 928 (43%) had a FC > 2 (Figure 4b). Exclusive and

common transcripts had equal intensity performance in where 43% of each transcriptomic profile had a FC > 2, highlighting the strong intensity of transcriptional modulations in response to PGN in stark contrast to LPS.

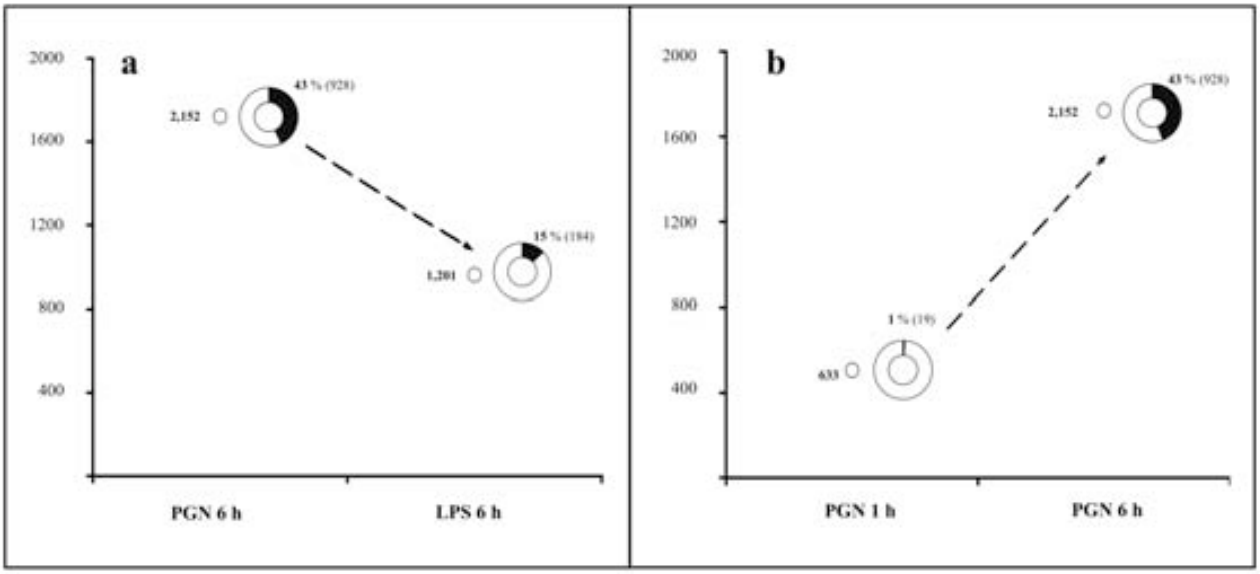


**Figure 4. Characterisation of the transcriptomic response. A**; Diagram representing mRNA transcripts differentially expressed over control during PGN and LPS challenges after 6h of treatment ($p$<0.001). The open circles represent the number of expressed transcripts restricted to one treatment PGN (2,152) or LPS (1,201). The radial diagram represents the intensity profile registered in both treatments with PGN and LPS, where PGN had 928 (43%) transcripts with a FC >2 and LPS had 184 (15%) transcripts with a FC >2..Exclusive transcripts to PGN had an intensity performance of 43%(492) with a FC>2. Exclusive transcripts to LPS had an intensity performance of 1.4%(17) with a FC>2. **B**; Diagram representing mRNA transcripts differentially expressed over control during PGN challenges throughout the time (1 and 6 hours, $p$<0.001). The open circles represent the number of expressed transcripts restricted to PGN 1h (633) and 6h (2,152). The radial diagram represents the intensity profile registered with PGN throughout the time where at 1h 1% had an intensity performance with a FC>2, and at 6h the 43% (928) an intensity performance with a FC>2.

The complete list of genes with differential expression is found in the additional file 3 and 4. We selected 10 common transcripts (biologically relevant) expressed in both treatments with PGN and LPS (Table 1) that represent common activation steps. We observed the up-expression of the signal transducer/transcription activator STAT3 and the non-receptor-tyrosine protein kinase (TYK2-JAK) mRNAs, both constituting the transcription factor JAK/STAT. The JAK/STAT pathway is a pleiotropic cascade used to transduce a multitude of upstream signals during the inflammatory processes and is a mediator of cytokine induction through activation of the NF-κβ transcription factor [27-30]. The up-expression of NF-κβ inhibitor mRNA that can restrain the activity of dimeric NF-κβ/REL complexes on cellular stimulation by immune and pro-inflammatory process also was observed [31]. An other transcription factor up-regulated is the CCAAT/enhancer binding

protein beta (C/EBP-beta) mRNA. C/EBP-beta is intimately linked to immune and inflammatory processes and regulates the transcription of the pro-inflammatory cytokine, Interleukin-6 [32]. The PRR trans-membrane receptor C-type lectin (CLR) also was up-expressed. CLR in fish is regulated in response to whole or bacterial components [2,33], and is related with activation the NF-κβ transcription factor and gene expression of pro-and inflammatory cytokines [70,71]. Transcripts of extracellular matrix protein (ECM), and other related with cell proliferation, or leukocytes migration was expressed as Matrix metalloproteinases (MMP9) that destroy the extracellular matrix facilitating infiltration of leukocytes. The list also includes transcripts that encoding effector proteins p67phox and myeloperoxidase both required for the production of free oxygen radicals by the NADPH oxidase to directly destroy pathogens (classical innate immune response). The GO search for biological process reflected that the transcripts expressed can be grouped them into functional categories represented by hematopoiesis, cell adhesion, JAK/STAT or NF-κβ pathway (Table 1).

**Table 1.**

**Common selected transcripts expressed in macrophages under LPS and PGN treatment**

| Description | Corrected p-value | FC | Regulation |
|---|---|---|---|
| Stat3 [Danio rerio] | 0.03 | 2.70 | up |
| TNF-α | 0.01 | 1.78 | up |
| Serine/threonineprotein kinase (TBK1) | 0.03 | 2.22 | up |
| NF-kB in ibitor | 0.01 | 1.82 | up |
| Ankyrin repeat an zinc finger | 0.02 | 2 35 | up |
| CCAAT e hancer b nding protei beta | 0.03 | 3.01 | u |
| Extracellular ma rix protein (ECM) | 0.05 | 3.89 | down |
| Matrix etalloproteinase 9 | 0.03 | 3.62 | up |
| Matrix metalloproteinase 1 | 0.00 | 1.50 | up |
| Ctype lectin receptor | 0.03 | 6.28 | up |
| p67phox | 0.04 | 2.67 | up |
| Myeloperoxidase precursor | 0.01 | 2.86 | down |

**Enrichment of functional classes (GO) in the macrophage transcriptome treated with PGN-LPS**

| Gene Ontology | No. Genes | Corrected p-value |
|---|---|---|
| Cellular defense response | 3 | 0.02 |
| Detection of bacteria | 1 | 0.02 |
| C-C chemokine | 2 | 0.01 |
| Activation of JAK/STAT | 7 | 0.01 |

| | | |
|---|---|---|
| Cell homeostasis | 13 | 0.04 |
| NF-kappa B pathway | 4 | 0.05 |

Transcripts represented were selected for expression level ($p$<0.01) and then implication in biological processes related to PGN and LPS stimulation (immune/inflammatory responses).

In order to distinguish transcripts responsible to activation of inflammatory phenotypes we filtered differentially expressed transcripts for each PAMP (PAMP-dependent), and selected those exclusively responsive to LPS or PGN (LPSrt and PGNrt). The list with responsive transcripts to LPS selected by their biological relevance is shown in Table 2. Due to the small size of this data set (only 17 transcripts had a FC > 2) this limited the search of responsive transcript and target identification for each gene class. However, of consideration is the up-expression of interleukin-8 that is tightly related with increases the cell proliferation by regulation of the MM9 synthesis [34-36]. Also, we observed the mRNA expression of macrophage inflammatory protein alpha (MIP1 alpha). This gene encodes a protein expressed by macrophages and other immune cells, and is required by chemoattractive activity during inflammatory events [37,38]. We analysed the enrichment of GO terms between LPS responsive transcripts, however this search did not reveal significant enrichment of functional groups or pathways.

Most of the PGN responsive transcripts identified have known roles in the immune response and are functionally involved in the PGN-host recognition. The list of selected transcripts is shown in Table 2. Of particular interest NLR-3 was a transcript expressed exclusively after PGN treatment. NLR3 is a member of cytosolic receptor family NOD, which through the recognition of bacterial peptides leads the activation of pro-inflammatory cytokine expression by the direct activation of the transcription factor NF-κβ [39-43]. We also observed the expression of TRAF-6, and the adaptor molecule MyD88 both involved in the NF-κβ activation and TLR signalling pathway [44,45]. As well as important inflammatory mediators involved in the prostaglandin synthesis such as COX-2, microsomal glutathione transferase 2, or Prostaglandin E synthase [48-50]. Also, both PGN and LPS induced the release of $PGE_2$ to the same extent into the cell culture medium (Figure 5). The $PGE_2$ levels (50pg/ml) were observed by 12 h of LPS-PGN treatment and are similar to those described previously in trout macrophages in response to PGN [3,21]. This data were reflected in GO analysis for enrichment of biological process, which include eicosanoid synthesis, cell adhesion or NF-κβ pathway (Table 2).

**Table 2.**

**Selected transcripts expressed in macrophages under LPS (6h) treatment**

| Description | p-Value | FC | Regulation |
|---|---|---|---|
| interleukin8 receptor CXCR1 | 0.00 | 2.27 | uo |
| interleukin8like protein | 0.03 | 1.97 | up |
| CC chemokine receptor3 | 0.02 | 3.04 | down |
| chemokine CK1 | 0.01 | 3.03 | up |
| allograft inflammatory factor1 (AIF1) | 0.00 | 3.51 | up |

**Selected transcripts expressed in macrophages under PGN (6h) treatment**

| Description | p-Value | FC | Regulation |
|---|---|---|---|
| NOD receptor C | 0.02 | 2.41 | up |
| Myeloid differentiation factor 88 | 0.02 | 2.48 | up |
| TRAF 6 | 0.02 | 3.75 | up |
| Serine/threonineprotein kinase (TBK1) | 0.03 | 2.22 | up |
| Leukotriene A4 hydrolase | 0.05 | 1.63 | down |
| Prostaglandin E synthase 3 | 0.02 | 1.50 | up |
| prostaglandin F receptor | 0.02 | 2.28 | up |
| prostaglandin transporter | 0.02 | 2.01 | up |
| COX isoform 2 | 0.03 | 2.78 | up |
| Microsomal glutathione Stransferase 2 | 0.04 | 2.28 | up |
| 15hydroxyprostaglandin dehydrogenase | 0.03 | 1.87 | down |
| CC chemokine receptor3 | 0.03 | 4.07 | down |

**Enrichment of functional classes (GO) in the macrophage transcriptome treated with PGN**

| Gene ontology | No. Genes | p-Value |
|---|---|---|
| Cellular defense response | 4 | 0.02 |
| C-C chemokine | 6 | 0.03 |
| Eicosanoid activity | 14 | 0.02 |
| NF-kappaB cacade | 7 | 0.03 |

Transcripts represented were selected for expression level ($p$<0.01) and then implication in biological processes related to PGN and LPS stimulation (immune/inflammatory responses).

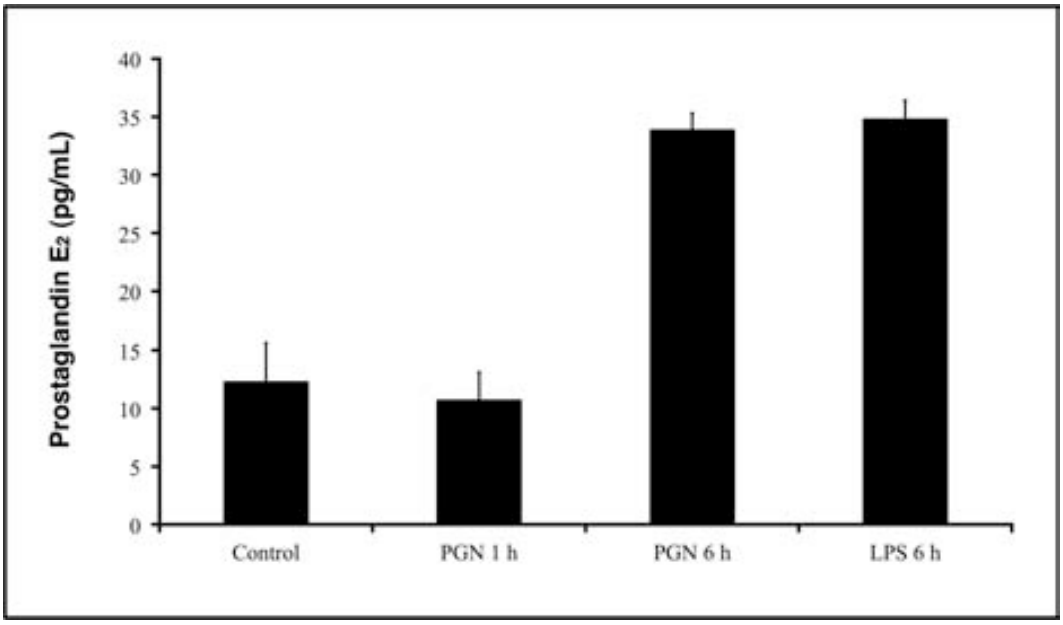


**Figure 5. Characterisation of the prostaglandin response.** $PGE_2$ levels in primary cell cultures stimulated during 12 hours with LPS ad PGN (10μg/mL) from *E. coli* O111:B4. Results (mean±SD; n=6) from six independent experiments expressed as pg/mL ($PGE_2$).

Due to the results obtained above a shift course arising to explore transcriptional induction of the response to PGN allows further explore the PGN-time dependent response (Table 3). The kinetic of the response obtained was significantly different between 1 and 6 hours. In total 2,786 transcripts were differentially expressed, of these 633 (1 h) and 2,152 (6 h), where intensity performance of 1% (19) and 43% (928) transcripts had a FC >2. The number of transcripts exclusive ($p<0.01$) was different 37 and 1,557 (1 and 6 h respectively), as well as the intensity where 19 and 948 had a FC > 2 (1 and 6 h), resulting in an intensity increase from 3 to 52% (Figure 4b). Thus describing an increase in the transcriptomic response throughout the time in response to PGN as the response to PGN shutdown reactive in the cells.

The PGN-time dependent response was different at the level of transcriptome by either differentially activating RNA transcripts related to PGN-host recognition, onset of transcriptional expression program, or inflammatory response (Table 3). Major differences could be identified in ligand recognition where macrophages stimulated with PGN (1 h) up-expressed NOD3 mRNA. The kinetic of transcript abundance show that NOD3 mRNA decreases through the time until 6 h where finally was down-regulated (Table 3). PGN give early transcriptional activation (1 h) triggering the up-expression of several transcription factors mRNAs such as TFIID and CREB both pleiotropically involved in the initiation of transcriptional expression program in all eukaryotes cells [51-54]. The early up-expression of transcription factors (1 h) stark contrast with the bidirectional regulation (up-

and down-regulated) observed at 6 h. The initial shutdowns (1 h) and posterior up-expression of transcripts linked to effector proteins highlight that PGN give gradual transcriptomic activation throughout time to an macrophage inflammatory phenotype. We observed a getting on (6 h) switching-on expression of transcripts encoding effector proteins closely linked to inflammatory response such as COX-2, Prostaglandin E synthase, or interleukins IL-1β, IL-22. As well as the up-expression of transcripts classically involved in the innate immune response as complement response and ROS production (table 3).

**Table 3**

**Selected transcripts expressed in macrophages in PGN-time dependent response treatment**

| Treatment | Description | p-value | FC | Regulation |
|---|---|---|---|---|
| PGN 1 h | NODlike receptor C | 0.02 | 2.41 | up |
| PGN 6 h | NODlike receptor C | 0.04 | 1.30 | down |
| PGN 1 h | activating transcription factor 1 | 0.02 | 2.11 | up |
| PGN 6 h | activating transcription factor 1 | 0.02 | 1.41 | down |
| PGN 1 h | Nuclear factor interleukin3regulated protein | 0.04 | 1.24 | down |
| PGN 6 h | Nuclear factor interleukin3regulated protein | 0.04 | 1.21 | up |
| PGN 1 h | Nuclear transcription factor Y, gamma | 0.03 | 1.12 | up |
| PGN 6 h | Nuclear transcription factor Y, gamma | 0.01 | 1.20 | down |
| PGN 1 h | Osmotic stress transcription factor 1 (OSTF1) | 0.00 | 1.25 | up |
| PGN 6 h | Osmotic stress transcription factor 1 (OSTF1) | 0.02 | 1.68 | down |
| PGN 1 h | Transcription cofactor vestigiallike protein 4 | 0.03 | 1.29 | up |
| PGN 6 h | Transcription cofactor vestigiallike protein 4 | 0.00 | 1.17 | down |
| PGN 1 h | Transcription factor A, mitochondrial | 0.04 | 1.49 | down |
| PGN 6 h | Transcription factor A | 0.04 | 1.16 | up |
| PGN 1 h | Transcription initiation factor TFIID subunit 10 | 0.02 | 1.30 | down |
| PGN 6 h | Transcription initiation factor TFIID subunit 12 | 0.04 | 2.68 | up |
| PGN 1 h | CREBregulated transcription coactivator 3 | 0.05 | 1.11 | up |
| PGN 6 h | CREBregulated transcription coactivator 3 | 0.00 | 2.68 | up |
| PGN 1 h | Prostaglandin E synthase 3 | **n/s** | **n/s** | **n/s** |
| PGN 6 h | Prostaglandin E synthase 3 | 0.02 | 1.50 | up |
| PGN 1 h | Microsomal glutathione Stransferase 2 | **n/s** | **n/s** | **n/s** |
| PGN 6 h | Microsomal glutathione Stransferase 2 | 0.04 | 2.28 | up |
| PGN 1 h | COX isoform 2 | **n/s** | **n/s** | **n/s** |
| PGN 6 h | COX isoform 2 | 0.03 | 2.78 | up |
| PGN 1 h | inter ukin 1β | **n/s** | **n/s** | **n/** |
| PGN 6 h | interleukin 1β | 0.01 | 5.09 | up |
| PGN 1 h | interleukin22 | **n/s** | **n/s** | **n/s** |

| | | | | |
|---|---|---|---|---|
| PGN 6 h | interleukin22 | 0.04 | 3.15 | up |
| PGN 1 h | interleukin16 | **n/s** | **n/s** | **n/s** |
| PGN 6 h | interleukin16 | 0.00 | 2.82 | up |
| PGN 1 h | ADH ehydro enase subu i 5 | **n/s** | **n/s** | **n/s** |
| PGN | NADH dehydrogena e sub nit 5 | 0.00 | 2.56 | up |
| PGN 1 h | complement component C8 gamma | **n/s** | **n/s** | **n/s** |
| PGN 6 h | complement component C8 gamma | 0.03 | 4.13 | up |
| PGN 1 h | complement binding protein | **n/s** | **n/s** | **n/s** |
| PGN 6 h | complement binding protein | 0.05 | 3.95 | down |

Transcripts represented were selected for expression level ($p$<0.01). n/s: no signal.

## 4. Discussion

### 4.1. Development of Sea Bream ONM

We have developed and validated a 44 k *S. aurata* ONM to provide a platform to the study of global gene expression regulation in sebream. In depth quality control analysis shows a robust platform reproducibility and accuracy. The number of annotated transcripts represented by three probes was 7,285, while 8,377 targets EST have only one probe due to a lack of annotation. Multiple spot replicates are recommended for genes expressed at low levels since the probability of error increases substantially at low SI [6]. In concordance with other reports the low level of variation in SI dues not affect data analysis [55]. In our analysis 4,301 probes were removed (10% the total probes represented on the array) with SI values lesser o greater than the intensity variation (intensity expressed as SD), excluding outlier values and maximizing the probability of detecting real differences in gene expression. For most sequences the non-overlapping probes designed (3`bias) for each transcript had a strong correlation between probe-pairs (Figure 2). Only 303 transcripts (5%) Probe_1 and Probe_2 showed a negative correlation possibly due to cross-hybridisation of alternative spliced mRNAs, duplicated loci, or by the difficult to distinguish the background fluorescence signal of low intensity values [56-61]. The repeatability of microarray data across both technical and biological replicates was robust (Pearson correlation coefficient > 0.7). The MicroArray Quality Control (MAQC) project and other authors also have documented the high reproducibility of RNA measurement using the Agilent oligo-array [62-69]. The quality of data set also was confirmed by independent qRT-PCR analysis. The ONM expression values had a significant and positive correlation with qRT-PCR expression values (Spearman correlation coefficient > 0.7), confirming the reliability of the seabream ONM.

### 4.2. Qualitative comparisons of the transcriptomic response to LPS-PGN

Our expression results have identified distinct gene expression profiles and specific cassettes of responsive transcripts whose regulatory patterns are induced in response to lipopolysaccharide (LPS) and peptidoglycan (PGN). The filtering approach represents only a low number of transcripts in response to LPS and a high number in response to PGN (Figure 4). In recent studies in trout macrophages PGN was identified as a major pro-inflammatory component of crude LPS preparations [21], and is able to induce a strong inflammatory activity including PGE release [3]. The ligand-regulated activation of transcription generated by LPS or PGN is represented by specific changes in the macrophage transcription program. This assumption is supported by the variation in the total and responsive transcript number, and their intensities (Figure 4a). Also, we observed that G-positive-negative PAMP-driven the canonical activation of C-type lectin receptor (CLR), JAK/STAT, metalloproteinase (MM-9) and extracellular matrix (ECM) mRNAs, all transcripts largely regulated by macrophages in response to PAMPs treatments [2,3,70,71-78]. Tests based on host responses to PAMPs have relevant value to the knowledge to potential molecular pathways of pathogen recognition. Analyses with AsONM identified a group of genes with similar up-regulation during LPS-PGN stimuli in other fish species, highlighting the accuracy and success of the *S. aurata* oligonucleotide microarray. The search for LPS- and PGN-responsive transcripts showed genes that encode proteins mainly related with peptidoglycan host-recognition. LPS unlike PGN, induce a low diversity of responsive transcripts. The low number of exclusive transcripts forces us to search LPS markers and perform other analyses from stimulated cell cultures with different LPS-purified preparations, which may be causing unlike host responses by differential LPS sensitivity [79-81].

The examination of culture parameters (time/treatment) showed strong transcriptomic modulations and expression of a high diversity of inflammatory target genes in response to PGN. The kinetic of PGN-time depend response show an early up-regulation of transcription factors that induce transcriptional modulations in the cells by activation or inhibits in their gene expression program [51-54]. This assumption is supported by the dramatic increase of transcript and their intensities throughout the time (Figure 4b). In fish macrophages the activation to inflammatory activated phenotype is characterised by the faculty to induce the expression and production of pro-inflammatory cytokines, ROS or $PGE_2$ (prostaglandin $E_2$) that are driven mainly by pathogen or their molecular associated pattern such as PGN, DNA or RNA preparations [21,25,73]. The activated cellular phenotype is tightly regulated by activation the transcription factor NF-κβ that determines the induction of pro-inflammatory genes by interaction with pathogen recognition receptors PPR as cytosolic NODs receptors. The nucleotide-binding oligomerisation domain (NOD)-like receptor is part of the NLRs family receptors largely activated in immune cells by G-positive-negative

peptidoglycans [39-43,82,83]. We observed that PGN is able to induce the up-expression of NLR-2 mRNA, NLR-2 has been described in the grass carp and show specific regulation under bacterial peptidoglycan treatments [84,85]. In mammals immune cells PGN induces the activation of the inflammatory phenotype through the activation of TLR2 a classical PRR [86-90], which linked with universal adapter MyD88, the receptor associated kinase (IRAK) and TNF activated factor (TRAF6) are required by the NF-κβ translocation and promoting of inducible inflammatory cytokines as TNF-α [91]. In our transcritomic expression profile PGN is inducing the up-regulation of TRAF6 and MyD88 transcripts. MyD88 activation also has been observed in Japanese flounder *Paralichthys olivaceus* in response to PGN [92]. In trout and carp macrophages have been suggested the TLR involvement in the PGN-mediated inflammatory response [3,93], although stimulation with the lipoprotein ($Pam_3CSK_4$) a classical TLR2-ligand have a different response. In Salmonids fish, $Pam_3CSK_4$ does not stimulate an inflammatory response [29,94 unlike in the more modern cyprinids in which TLR2 is activated in response to $Pam_3CSK_4$ [93].

We observed the up-regulation of COX-2, prostaglandin E-synthase (PGE) and microsomal glutathione-transferase-2 mRNAs, all transcripts involved in the prostaglandin synthesis. Prostaglandins are synthesized from arachidonic acid (AA) first by cyclooxygenase (COX)-1 or -2, which convert AA into $PGH_2$. This precursor ($PGH_2$) is further processed by PGE and microsomal prostaglandin synthases to become $PGE_2$ [95]. PGN induces IL-6 production in murine/macrophages by a mechanism involving COX-2 induction, $PGE_2$ release, and PKA activation [96-99], suggesting that $PGE_2$ play a vital role in the inflammatory response by regulation of interleukin-6 production [96-103]. The increase of COX-2 mRNA has been widely observed on macrophages in response to peptidoglycan and mediated by the TLR-2 signalling pathway [96-100]. The mRNA expression of COX-2, IL-6 and $PGE_2$ release also has been documented in trout macrophages under PGN stimuli [3,21]. Our microarray analysis identified differential regulation of COX-2, prostaglandin E-synthase and microsomal glutathione transferase-2 mRNAs in response to PGN treatment (Table 3), also were registered by independent qRT-PCR analysis high abundance of IL6 mRNA (Boltaña et al, data not shown). Besides, we found that PGN is an inducer of $PGE_2$ release into the cell culture medium (Figure 6). PGN from G-negative bacteria induce COX-2 expression and $PGE_2$ formation in trout macrophages [3,21]. The collective data suggest that PGN have a strong effect on $PGE_2$ production in fish macrophages, since we did not test the effects of PGN on COX-2 mRNA abundance is unclear whether the situation in trout are similar by seabream in terms of the stimulation of COX-2 expression and $PGE_2$ release by PGN. The activated phenotype described in seabream monocyte/macrophages show that PGN trigger the mRNAs expression of transcripts closely related to prostaglandin and TLR pathway and production of $PGE_2$, it should be noted, that our observations do not exclude the possibility that TLR2 signalling cascade can are involved in the activation of

seabream macrophages by induction of $PGE_2$ under PGN stimuli, however the prostaglandin regulation/syntheses and PGN recognition clearly warrant more investigation. We selected transcripts that responded exclusively to peptidoglycan, the relevant of this selection is that large part of these showed similar induction in trout macrophages stimulated with PGN [3], highlighting the role of PGN as inducer of inflammatory response in fish macrophages. This suggests the presence of a limited cohort of transcripts involved in the downstream response to PGN recognition.

As the main goal of this chapter was the development and validation of Agilent oligonucleotide microarray (ONM), the results provided an enriched platform for the study of gene expression regulation in *Sparus aurata*. The reproducibility of the AsONM was achieved, and microarray data were cross-validated using an independent method to quantify mRNA expression (qRT-PCR). Results of expression analysis confirmed differences in the transcriptomic response to different PAMPs (PGN and LPS). The hybridisation accuracy and suitability of gene screening stressed the exactness of expression profiling registered for Aquagenomics *Sparus aurata* oligonucleotide microarray (AsONM), and promotes their application in expression studies on gilthead seabream.

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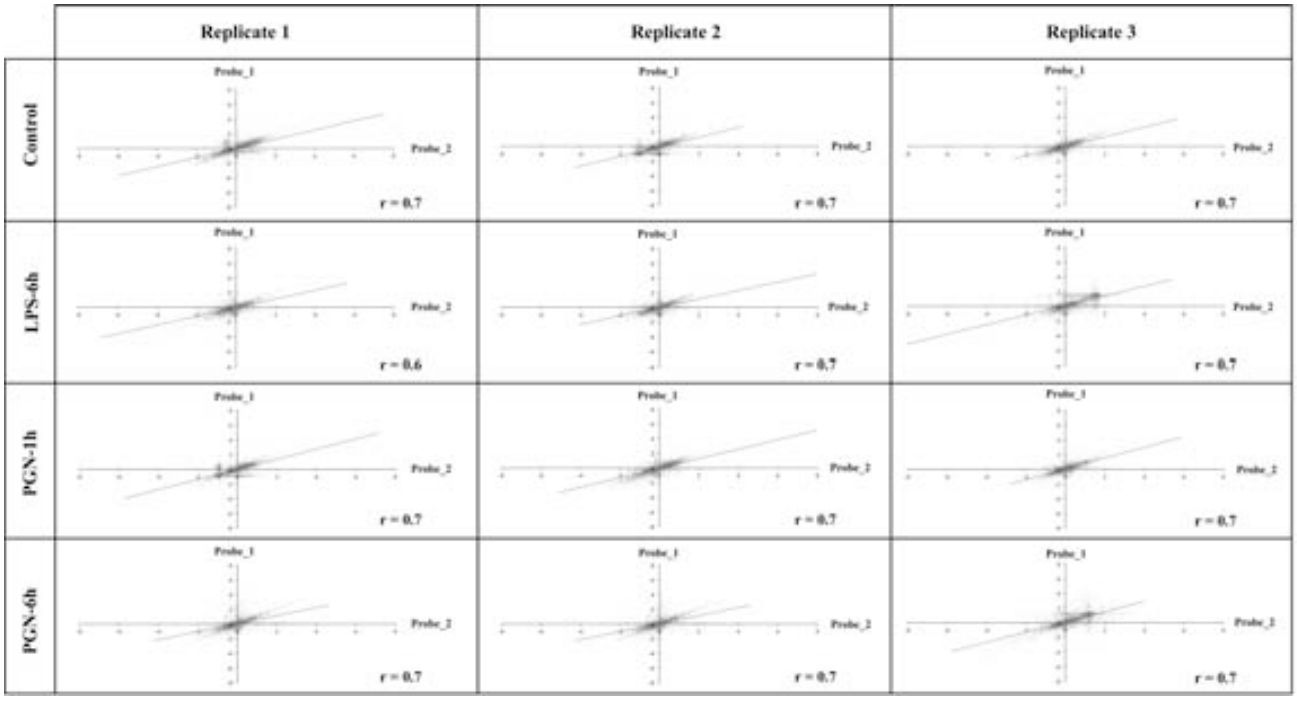
## Additional files

**Additional file 1.** Specific primers used by quantitative qRT-PCR

| Gene name | Primer Fw | Primer Rv |
|---|---|---|
| glucose-6-phosphatase | ACGATGGAGAAAGCCCAGAA | GTTGAGGGGCGAGTGAAGAC |
| cd8 | CACCAGCGTGTACTGCAACA | TCTGGTCGGCATGTGTTTTC |
| interleukin-6 | CCTTCCACCAACTTCAGCAT | TGAATCTTGAGGGGATCTGG |
| lipoprotein lipase | ACGTTGCCAAGTTTGTGACC | TCCAGACCTGTGATCCTGCT |
| MHCI | CGATGGAACCTTCCAGATGA | CCTCGTTCACACCAGAGAGC |
| MHCII | ACAACATGAACGCTGAGCTG | CTCGTCCACAGAGTCATCCA |
| MX | AGGAAAGACAAACGCAATGG | TTCAGGTGCAGCATCAACTC |
| T-cell receptor beta chain | CTTCAATGGGACAGGAACGA | GTAGGAGAGCTTGGCGGTCT |
| troponin T 2 | CCTTGTCAACCGAATCGAGA | TCCTTTTCCTCCGCAAGTCT |
| CD83 | CACCAGCGTACTGCAACA | TCGGTCGGCATGTGTTTTC |
| GR | GTTTGTTGGCTCTGAATGTCTCG | GCTGCAAGGTGTTCTTCAAGAG |
| IL1 | ATGCCCCAGGGGCTGGGC | CAGTTGCTGAAGGGAACAGAC |

## Additional file 2.

Distribution analysis of hybridization success across 12 microarray experiments. To evaluate probe correction in the expression data for annotated target (3 probes/target) we randomly selected two of three probes present for each target (technical bias). For each gene Pearson correlation coefficient was calculated between the Probe withing arrays



# Chapter VII

**General discussion**
**Acknowledgment**

# General discussion

Exploring the recognition of bacterial-PAMPs and subsequent responses of adherent monocyte/macrophages derived from two different fish species allowed us to characterise the cellular response through differential expression of mRNA transcripts related to the immune response. The main goal of this approach is to characterize the evolutionary relationships involved in PAMP-PRR recognition and their subsequent functional responses. The essence of this argument lies in the fact that although PRRs generally have been identified using nucleotide sequence homology (BLAST) and their relationship with downstream functional responses are far from clear. Thus currently it is difficult to assign a specific response to a specific PAMP and therefore describe the mechanisms that have been conserved throughout evolution and those that have arisen and are specific to certain vertebrate groups i.e. septic shock in mammals. Failure to characterise functional responses may lead to false inference of function based upon experimental data from phyogenetically distant vertebrates and limit our understanding of the complex interactions between pathogens and the immune response.

This dissertation presents a set of experiments that explore the immune response of fish macrophages treated with different PAMPs with the main goal of dissecting the signalling pathways involved during PAMP-driven activation. The data obtained supports the expectation that certain specific gram negative PAMP-PRR interactions are conserved throughout vertebrate evolution (peptidoglycans), however significant functional differences can be observed between the fishes and the mammals (lipolysaccharides). The main deductions from the results of each specific experiment are presented in the following conclusions elated to each of the objectives

**Objective 1. PAMPs, PRRs and the genomics of gram negative bacterial recognition in fish.**

Understanding the mechanisms that underpin pathogen recognition and subsequent orchestration of the immune response in fish is an area of significant importance for basic aspects of research in both comparative immunology and in the promotion and management of health in aquaculture. In general, most studies so far have reported similar effects of G-negative PAMPs in fish as to those observed in mammals. This may reflect a conserved set of immune effector responses that are activated by diverse sets of vertebrate group-specific PRRs i.e. different TLR genes with different degrees of PAMP specificity. However the reported discrepancies concerning activities or presence of LPS specific PRRs remains unanswered. In view of future studies to address such questions using genomic technologies we would like to highlight two major areas of development; 1. Careful characterisation of both cellular tools (cell cultures) and PAMP ligands (structure, purity and strain) is essential to dissect the specific activated pathways. 2. The combination of genomic tools including whole genome sequencing, RNA-Seq and

microarrays will provide an exemplary set of investigative tools to further elucidate the molecular mechanisms underpinning pathogen recognition and the immune response in fish.

**Objective 2. Characterisation and expression of NADPH oxidase in LPS-, poly (I:C)- and zymosan-stimulated trout (*Oncorhynchus mykiss* W.) macrophages.**

The degree of shared homologies among vertebrates in the predicted amino acid sequences of Phox components suggests that, in trout, as in mammals, all of the Phox subunits of trout macrophages contain the essential domains and interaction modules required for the correct activation of the enzymatic complex. Nox2-like and P67*phox*-like components in *C. intestinalis* clearly predict the diversification of Nox1/2 and p67*phox*/Noxa1 respectively in vertebrates. The p22*phox*-like and p47*phox*-like components of *C. intestinalis* constitute the basal taxa for the diversification of vertebrate p22*phox* and p47*phox* components, and thus share a common ancestor with the p47*pho*/Noxo1 homologues in vertebrates. Therefore, the branching pattern of Nox2, p47*phox* and p67*phox* in fish as in the rest of vertebrates suggests the emergence of Phox-related components before the diversification of basal euteleosts. The Nox3 and Nox4 proteins seem to be restricted to vertebrates other than fishes, whereas Nox1 and Nox2 are widespread in vertebrates.

The regulation of NADPH oxidase gene expression and intracellular signalling either in mature (activated) neutrophils or in tissue macrophages are quite similar. Nevertheless, both cell types accomplish different, though complementary, functions during acute inflammation. The single or dose-response stimulation with zymosan or LPS did not show significant differences in the mRNA expression of trout Phox subunits.

The stimulation of trout monocyte/macrophages with zymosan had no noticeable effects in the expression of p22*phox* and p47*phox* during the maturation period, but affected the expression of Nox2, p67*phox* and p40*phox* at the beginning (day 1) and end (day 5) of the differentiation process. The repeated stimulation with maximal doses of LPS elicited a diminished time-dependent pattern of expression in the catalytic, activator and modulator components of trout Phox. Moreover, maximal doses of LPS failed to regulate the expression of p67*phox* during the incubation period in contrast to the organizer component, p47*phox*, that was up-regulated regardless of the intensity or frequency of the stimulus.

In trout mature macrophages, the exposure to poly (I:C) had no effect on the expression of the catalytic Phox subunit as well as the p40*phox* component. However, a lower transcript expression of

p47*phox* and most notably, p67*phox* was detected. The latter may be attributable to the collaborative/organisational role of the p47*phox* in the activation of Phox. Besides, it has been shown that low levels of p67*phox* can produce an effective Phox-mediated ROS response. Therefore, the activation of Phox in trout mature macrophages does not seem to be impaired upon stimulation with poly(I:C), albeit no clear differences exist between the doses tested. Interestingly, two of the catalytic/activator components (Nox2/p22*phox* and p67*phox*, respectively) remained highly expressed during differentiation even though high levels of catalytic components are not required to sustain a ROS response. The transcript response of Phox components to repeated stimulation with poly (I:C) during the maturation/differentiation period appeared stronger than that of LPS or zymosan. This suggests an enhanced responsiveness of the ROS response to viral rather than bacterial insults in trout macrophages.

**Objective 3**. **Peptidoglycan and LPS as mediators of cytokine expression induced in rainbow trout macrophages.**

The results of this chapter demonstrate that the induction of proinflammatory cytokine mRNA expression in trout macrophages by crude LPS is primarily a result of PGN contamination. Crude LPS preparations could potentially have a number of contaminants including PGNs, nucleic acids and lipoproteins. upLPS and lipid A, the lipid portion of endotoxin that is responsible for TLR4-induced activity in mammals, were unable to induce IL-6 or IL-1β expression. Ribonucleic acid, and to a lesser extent, DNA, were able to induce a small increase in IL-6 and IL-1β expression though this was extremely low when compared to crude LPS. In contrast, PGNs by themselves are potent inducers of IL-6 and IL-1β expression in trout macrophages. In support of a significant role for PGN contamination in crude LPS lysozyme treatment greatly reduced the ability of LPS to stimulate IL-6 and IL-1β expression.

Our results clearly demonstrate that crude Gram-negative LPS preparations stimulate trout macrophages not through endotoxin but primarily through the presence of contaminating PGNs. It is also likely that the complete response of trout macrophages to crude LPS is the result of a combination of several contaminants that also includes nucleic acids. The strong stimulatory effect of *E. coli* PGNs by themselves on trout macrophages suggests that the recognition of Gram-negative bacteria in trout is primarily through PGNs from the bacterial wall, and indicates that the systems responsible for bacterial recognition in invertebrates may also be conserved in some vertebrates.

**Objective 4. Response to peptidoglycan derived from different *E. coli* serotypes influence inflammatory outcome in trout, *Oncorhynchus mykiss* macrophages.**

This chapter shows that systematic dissection of macrophage culture parameters (time and treatment) reveal a significant re-modelling of the trout macrophage transcriptome highlighting the divergence of the response to the two different PGNs (PGN-B4 vs. PGN-K12). As there were no other known variables, the differences in the transcriptomic profile are assumed to be solely due to the structure of the different PGNs and therefore differential recognition of those by the macrophages. This assumption is supported by the variation in transcript number, including intensities and transcript diversity. These data emphasize that macrophages differentially respond to highly similar bacterial PGNs resulting in a directed response i.e. prostaglandin release or a more generalised 'state of activation'.

The microarray analysis identified a differential regulation of both Prostaglandin D-synthase (PTGDS), and Prostaglandin endoperoxide synthase-2 (COX-2) that are directly involved in eicosanoid production; $PGD_2$ and $PGE_2$ respectively. Downstream analyses, qRT-PCR and prostaglandin release, of both COX-2 and PTGDS mRNA regulation and $PGH_2$ and $PGD_2$ concentration in supernatants reveal a strong correlation, both time and dose-dependent, between PGN-type (B4 vs K12), mRNA abundance and inflammatory outcome as measured by $PGE_2$ and $PGD_2$ release.

The collective data generated in this study suggest TLR involvement in the PGN-mediated inflammatory response in trout macrophages. We also identified PGLYRP-6 (up-regulated; PGN-B4), suggesting that the PGRPs also play a role in specific-PGN recognition and this may be conserved throughout the fishes. Our data highlights the significant differences observed in macrophages responding to two PGNs derived from different serotypes of the same bacteria. Responses at the level of the transcriptome and the inflammatory outcome (prostaglandin synthesis) highlight the different sensitivity of the macrophage to slight differences (serotype) in peptidoglycan structure. Such divergent responses are likely to involve differential receptor sensitivity to ligands or indeed different receptor types. Such changes in the biological response will be likely reflected upon pathogenicity of certain serotypes and the development of disease.

**Objective 5. Development and validation of the oligo-microarray from *Sparus aurata* and its application to the macrophage expression profile after treatments with lipopolysaccharide (LPS) and peptidoglycan (PGN).**

We have developed and validated a 44 k *S. aurata* ONM to provide a platform for the study of global gene expression regulation in the Gilthead seabream. In-depth quality control analysis shows a robust platform in reproducibility and accuracy. The number of annotated transcripts represented by three probes was 7,285, while 8,377 target ESTs had one probe, due to a lack of annotation. For most sequences the non-overlapping probes designed (3`bias) for each transcript had a strong correlation between probe-pairs. In only 303 transcripts (5%) Probe_1 and Probe_2 showed a negative correlation, possibly due to cross-hybridisation of alternative spliced mRNAs, duplicated loci, or because of the difficulty to distinguish the background fluorescence signal of low intensity values. The quality of the data set was also confirmed by independent qRT-PCR analysis. The ONM expression values had a significant and positive correlation with qRT-PCR expression values (Spearman correlation coefficient > 0.7), confirming the reliability of the seabream ONM.

Our expression results have identified distinct gene expression profiles and specific cassettes of responsive transcripts whose regulatory patterns are induced in response to lipopolysaccharide (LPS) and peptidoglycan (PGN). The filtering approach represents only a low number of transcripts in response to LPS and a high number in response to PGN. The ligand-regulated activation of transcription generated by LPS or PGN is represented by specific changes in the macrophage transcription program. This assumption is supported by the variation in the total and responsive transcript number, and their intensities. Also, we observed that G-positive-negative (LPS-PGN) PAMP-driven the canonical activation of C-type lectin receptor (CLR), metalloproteinase (MM-9), and extracellular matrix (ECM) mRNAs, all transcripts largely regulated by macrophages in response to bacterial-PAMPs treatments. Tests based on host responses to PAMPs have a relevant value to uncover potential molecular pathways of pathogen recognition. Analyses with AsONM identified a group of genes with similar up-regulation during LPS-PGN stimuli in other fish species, highlighting the accuracy and success of the *S. aurata* oligonucleotide microarray.

The examination of culture parameters (time/treatment) showed shift in transcriptomic modulation and expression of a high diversity of inflammatory target genes in response to PGN. The kinetics of PGN time-depending response shows an early up-regulation of transcription factors that induce transcriptional modulations in the cells by activation or inhibition of their gene expression program. This assumption is supported by the dramatic increase of transcripts and their intensities

throughout the time. In higher vertebrates PGN induces the activation of the inflammatory phenotype through the activation of TLR2 a classical PRR. We observed in the transcriptomic expression profile that PGN induces the up-regulation of TRAF6 and MyD88 transcripts, both closely related to TLR-2 signalling pathway. MyD88 activation also has been observed in the Japanese flounder *Paralichthys olivaceus* in response to PGN. In trout and carp macrophages the TLR involvement in the PGN-mediated inflammatory response also has been suggested, although stimulation with the lipoprotein ($Pam_3CSK_4$), a classical TLR2-ligand shows a different response. In salmonid fish, $Pam_3CSK_4$ does not stimulate an inflammatory response unlike in the more modern cyprinids in which TLR2 is activated in response to $Pam_3CSK_4$.

We observed in the activated monocyte/macrophages the up-regulation of COX-2, prostaglandin E-synthase (PGE) and microsomal glutathione-transferase-2 mRNAs, all involved in the prostaglandin synthesis. Furthermore, we found that PGN induces $PGE_2$ release into the cell culture medium. It should be noted that our observations do not exclude the possibility that the TLR2 signalling cascade could be involved in the activation of seabream macrophages by induction of $PGE_2$ under PGN stimuli. However prostaglandin regulation/synthesis and PGN recognition clearly warrants more investigation. We selected transcripts that responded exclusively to peptidoglycan, and the relevance of this selection is that a large part of them showed similar induction patterns in trout macrophages stimulated with PGN, highlighting the role of PGN as inducer of inflammatory response in fish macrophages. This suggests the presence of a limited cohort of transcripts involved in the downstream response to PGN recognition.

As the main goal of this chapter was the development and validation of Agilent oligonucleotide microarray (ONM), the results provided an enriched platform for the study of gene expression regulation in *Sparus aurata*. The reproducibility of the AsONM was achieved, and microarray data were cross-validated using an independent method to quantify mRNA expression (qRT-PCR). Results of expression analysis confirmed differences in the transcriptomic response to different PAMPs (PGN and LPS). The hybridisation accuracy and suitability of gene screening stressed the exactness of expression profiling registered for Aquagenomics *Sparus aurata* oligonucleotide microarray (AsONM), and promotes their application in expression studies on gilthead seabream.

**Concluding comments**

PRRs generally seem to be structurally well conserved throughout the vertebrates. This dissertation presents experimental evidence highlighting the fact that inference of function for PRRs

based upon nucleotide homology derived from organisms separated by large phylogenetic distance can lead to erroneous assumptions about their function across vertebrates. In fish, PAMP-PRR driven activation and its relationship with downstream functional responses in mammals are unclear. For example, TLRs recognise a wide range of PAMPs with different origins, composition and structure. Fish possess orthologs of the different mammalian TLR families, although only TLR3 and 5 have functionally characterised in two species (zebrafish and rainbow trout) and show a functional homology with their mammalian counterparts. On the other hand in mammals the interaction between LPS and its receptor (TLR4) triggers a strong inflammatory response (cytokine storm) often resulting in septic shock whereas in all other vertebrate groups septic shock cannot be induced with high doses of LPS. In fish ultrapure LPS treatment is unable to induce IL-6 or IL-1β mRNA expression this a characteristic inflammatory response. This highlights the difficulties associated with identifying specific responses to PAMPs in non-mammalian vertebrates as the conceptual for molecular mechanisms underpinning PAMP recognition frameworks is based mainly upon mammalian studies. In this thesis we have described the contribution of gram-negative peptidoglycans to the inflammatory response in fish. These PAMPs induce a strong inflammatory response in all organisms thus far studied, from Drosophila to man. However if one was to follow functional descriptions in mammalians i.e. LPS-TLR4 the more prominent PGN-induced response would remain undiscovered. Therefore we propose that a comprehensive review of PAMP-PRR interactions in fish based upon adequate experimental design including the examination of low and complex responses and the use of characterised PAMP formulations. This approach can provide access toward identifying responses in the fish immune system assuming its differences with the mammalian system and therefore improve knowledge about PAMP-PRR interactions in fish and the development of downstream applications in aquaculture.

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