Characterisation of *arginase* paralogues in salmonids and their modulation by immune stimulation/ infection

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Abstract

In this study we show that four arginase isoforms (*arg1a*, *arg1b*, *arg2a*, *arg2b*) exist in rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar*). We have characterised these molecules in terms of a) sequence analysis, b) constitutive expression in different tissues, and modulated expression following c) stimulation of head kidney macrophages *in vitro*, or d) vaccination/ infection with *Yersinia ruckeri* and e) parasite infection (AGD caused by *Paramoeba perurans* and PKD caused by *Tetracapsuloides bryosalmonae*). Synteny analysis suggested that these arginase genes are paralogues likely from the Ss4R duplication event, and amino acid identity/ similarity analyses showed that the proteins are relatively well conserved across species. In rainbow trout constitutive expression of one or both paralogues was seen in most tissues but different constitutive expression patterns were observed for the different isoforms. Stimulation of rainbow trout head kidney macrophages with PAMPs and cytokines also revealed isoform specific responses and kinetics, with *arg1a* being particularly highly modulated by the PAMPs and pro-inflammatory cytokines. In contrast the type II arginase paralogues were induced by rIL-4/13, albeit to a lesser degree. Vaccination and infection with *Y. ruckeri* also revealed isoform specific responses, with variation in tissue expression level and kinetics. Lastly, the impact of parasite infection was studied, where down regulation of *arg1a* and *arg1b* was seen in two different models (AGD in salmon and PKD in trout) and of *arg2a* in AGD. The differential responses seen are discussed in the context of markers of type II responses in fish and paralogue subfunctionalisation.

Keywords: arginase paralogues; *Oncorhynchus mykiss*; *Salmo salar*; macrophages; vaccination; parasite infections.

1. Introduction

Arginase (amidinohydrolase, EC3.5.3.1) is an ureohydrolase enzyme widely distributed in living organisms, from bacteria and yeast to plants and animals [1, 2]. It catalyses the conversion of L-arginine into L-ornithine plus urea in the Krebs-Henselheit urea cycle. Most studied microorganisms and invertebrates have only one type of arginase, localized in the mitochondria [2, 3]. Arginase gene duplication occurred after the separation of vertebrates and invertebrates with the appearance of a cytosolic arginase in ureotelic animals [1-3]. These two isoforms have been studied extensively in mammals and are termed arginase type I and type II. Type I is the cytoplasmic form and is expressed in liver as part of the urea cycle whilst type II is the mitochondrial associated enzyme which is expressed in several peripheral tissues but primarily in the kidney, prostate, small intestine and lactating glands [4]. Hence the two enzymes catalyse the same biochemical reaction but differ in cellular expression, regulation and subcellular localization [1, 4].

Within the immune system arginase is known to be a marker of type II responses that are broadly anti-inflammatory and associated with tissue healing, as seen in parasite infections. In the classical polarisation model, activated macrophages can either 1) convert L-arginine to L-citrulline and produce nitric oxide (NO)/ reactive nitrogen species by the action of inducible nitric oxide synthase (iNOS) after stimulation by T helper 1 (Th1) cytokines such as interferon (IFN)- γ , or 2) they can express arginase after activation with Th2 cytokines including interleukin (IL)-4, IL-10 and IL-13 [4] thereby generating the “repair” molecule ornithine that is involved in polyamine and collagen biosynthesis, the latter an important extracellular matrix component that promotes tissue remodelling/ fibrosis during healing. These polarized macrophage populations are referred to as classically activated (M1) or alternatively activated (M2) cells respectively. More recently it has become apparent that M1 and M2 may represent extremes of a large array of activation states and that polarization of macrophages first during an innate immune response likely directs T cells to produce Th1 or Th2 adaptive responses, where their secreted cytokines serve to amplify the macrophage dichotomy [5].

M2 macrophages with elevated levels of arginase activity have also been found in fish [5-8]. Common carp (*Cyprinus carpio*) infected with *Trypanosoma carassii* show elevated levels of arginase enzyme activity during the later phase of infection and lack a prominent NO response. Moreover, stimulation of head kidney leukocytes from *T. carassii* infected carp with dibutyryl cyclic adenosine mono phosphate (cAMP, 0.5 mg/ml) increases arginase

activity 3-4 fold but these same cells do not increase nitrite production after lipopolysaccharide (LPS, 50 mg/ml) stimulation [7]. Similar findings were obtained with macrophage cultures from uninfected fish stimulated with cAMP but now LPS induced iNOS expression and nitrite production [9, 10]. Modulation of arginase gene expression has also been shown in salmonids during parasite infection. Arginase type I is up regulated in skin of Atlantic salmon (*Salmo salar*) infected with sea lice (*Lepeophtheirus salmonis*) [6] but is down regulated in posterior kidney after *Tetracapsuloides bryosalmonae* infection in rainbow trout (*Oncorhynchus mykiss*) and in gills of salmon after *Paramoeba perurans* infection [11, 12]. Functional divergence of arginase type I and arginase type II in fish vs mammals has been recently hypothesized in common carp, where it is suggested that arginase type II is a better marker for alternatively activated macrophages in teleost fish rather than arginase type I [5].

A whole-genome duplication (WGD) event occurred at the base of the teleost fish during evolution, and a further WGD (the Ss4R salmonid-specific autotetraploidization event) occurred in the common ancestor of salmonids about 80 Mya after their divergence from Esociformes [13, 14]. Following genome duplication events, duplicated genes can either be lost by pseudogenization or retained as two copies that can diverge by the partitioning of the ancestral gene functions (i.e. subfunctionalization) or by the acquisition of a novel function (i.e. neofunctionalization) [15]. In rainbow trout and Atlantic salmon four different isoforms of arginase (*arg1a*, *arg1b*, *arg2a*, *arg2b*) have been found in this study, which has characterised these molecules in terms of sequence analysis, constitutive expression in different tissues, and modulated expression following stimulation of cultured head kidney macrophages *in vitro* or after vaccination and/ or infection *in vivo*.

2. Materials and methods

2.1 Cloning of arginase isoforms and sequence analysis

The rainbow trout whole genome shotgun (WGS) sequence was searched with tBLASTn [16], using fish Arginase I and II protein sequences. Candidate WGS contigs (contigs 25562, *arginase 1a*; 44798, *arginase 1b*; 119897, *arginase 2a*; and 23604, *arginase 2b*) were identified and exons predicted as described previously [17, 18]. Primers (Table 1) were designed to the 5'- and 3'- untranslated regions (UTR) and used for PCR amplification and cloning of the complete coding region using a mixed tissue cDNA sample. The cloning, DNA and protein sequence analysis was as described previously [19, 20]. Briefly, the nucleotide sequences generated were assembled and analysed using the AlignIR™ Software (LI-COR,

Inc.). The translated trout protein sequences were used to find the four different isoforms in Atlantic salmon in SalmoBase (<http://salmobase.org>) using BLAST search (tBLASTn). The Atlantic salmon amplified products obtained using newly designed primers were confirmed by cloning and sequence analysis. Briefly, the PCR products were cloned into pGEM®-T Easy Cloning Vector (Promega) and transformed into competent *Escherichia coli* cells (RapidTrans™ TAM1; Active Motif). The competent cells were grown on MacConkey agar plates (Sigma-Aldrich, UK) with ampicillin (100 µg/ml) at 37°C for 45 min and colonies with the correct insert size were grown overnight in 4 ml of Luria Bertani (LB) broth (Melford Laboratories Ltd., UK) with ampicillin (100 µg/ml) in a shaking incubator at 37°C. Plasmid DNA purification was performed using a QIAprep®spin DNA miniprep kit (QIAGEN, UK) according to the manufacturer's instructions and purified plasmids were then sent to be sequenced by Eurofins MWG Operon. Protein sequences were then aligned with MAFFT v7 [21].

For the phylogenetic tree, protein sequences from other species were predicted from the ENSEMBL (<http://www.ensembl.org/index.html>) or NCBI websites (<http://www.ncbi.nlm.nih.gov/>) and verified in UniProt (<http://www.uniprot.org/blast/>). Agmatine ureohydrolase or agmatinase was used as the outgroup for the phylogenetic tree as it is an important evolutionary related enzyme also involved in arginine and proline metabolism [2]. The software BioEdit [22] was used to align all protein sequences from different species and generate a file for the Guidance2 server [23-25], where sequence alignment was performed using Fast Fourier Transform, MAFFT v7 [21], as an algorithm for Multiple Sequence Alignment (MSA) with a final score of 0.92 from the original alignment and a statistical confidence cut-off score of 0.93 after the removal of unreliable columns [26]. A final resulting alignment of 239 amino acids was uploaded to MEGA v6 software [27] to predict the best-fitting amino acid substitution model which was the LG [28] and Gamma site heterogeneity model for arginase with the smallest Akaike information criterion (AIC) of 5177.901 and the lowest Bayesian information criterion (BIC) of 5591.264. Bayesian phylogenetic analysis was performed using Bayesian Evolutionary Analysis, by means of the Sampling Trees (BEAST) software package v1.7 [29] employing the best-fitting substitution model (LG+G), an uncorrelated lognormal relaxed clock model [30], a Yule speciation process, and a Unweighted Pair Group Method with Arithmetic Mean (UPGMA) starting tree. Two runs of BEAST were performed, each with a Markov chain Monte Carlo (MCMC) algorithm of 10,000,000 generations for Bayesian phylogenetic inference. Bayesian

Evolutionary Analysis Utility (BEAUti), Tracer v1.6 (<http://beast.bio.ed.ac.uk/tracer>) [31], LogCombiner (<http://beast.bio.ed.ac.uk/logcombiner>) with 10% of burn-in, TreeAnnotator (<http://beast.bio.ed.ac.uk/treeannotator>) and FigTree (<http://beast.bio.ed.ac.uk/figtree>) were used to construct the phylogenetic tree. The software MatGAT (Matrix Global Alignment Tool) was used to predict amino acid identity/ similarity between sequences using the BLOcks amino acid SUBstitution 62 (BLOSUM62) Matrix [32]. The exon-intron structures of arginase genes were determined in Salmobase for *S. salar*, ENSEMBL for *Danio rerio* and *Homo sapiens*, and by aligning the mRNAs with the corresponding genomic DNA using Splign (<https://www.ncbi.nlm.nih.gov/sutils/splign/splign.cgi>) for *Esox lucius* and *O. mykiss* [33]. The exception was for *arg2b* in *O. mykiss* where the exons had to be inferred using the genomic sequence for *O. mykiss arg2a* (accession number CCAF010119897.1) as it had the highest query cover (99%) allowed for alignment in Splign (58% identity) and hence only exon phase could be determined and not intron size. 100% identity of *arg2b* in *O. mykiss* matched the accession number CCAF010123604.1, supporting the presence of this gene in contig 23604. The synteny of the arginase loci was analysed in Salmobase using BLAST search (tBLASTn).

2.2 Fish maintenance and rearing condition

Apparent healthy rainbow trout with no history of infection were purchased from the Mill of Elrich Trout Fishery (Aberdeenshire, Scotland, UK) and maintained in 1-m-diameter fibreglass tanks with recirculating freshwater at 14±1°C at the Scottish fish immunology research centre, the University of Aberdeen, UK. The fish were acclimatised to the system for two weeks prior to use and were fed twice daily with a commercial diet (EWOS) at 2% body weight/day.

Apparently healthy Atlantic salmon were held as presmolts and smolted at the Ellis aquarium facility at the Marine Scotland Science Marine Laboratory, UK, in tanks containing 350 l of 34-35 ppt seawater with a flow-through of 180 l/h. The fish were acclimatized to 12°C for two weeks prior to challenge, and fed daily with Skretting Atlantic Smolt diet at 1% body weight/day.

2.3 Constitutive expression of rainbow trout arginase isoforms in tissues

Six rainbow trout (average weight, 142 g) were anaesthetised using 2-phenoxyethanol (Sigma-Aldrich, UK), killed and 17 tissues (tail fin, adipose fin, gills, thymus, brain, scales, skin, muscle, adipose tissue, liver, spleen, gonad, head kidney, posterior kidney, intestine,

heart and blood) were collected. RNA extraction and complementary (c) DNA synthesis was as described previously [19, 20].

2.4 Modulation of rainbow trout arginase isoform expression in macrophages

Head kidney macrophages were prepared from four individual fish as described previously [34, 35]. At day 4 the primary macrophages were stimulated with polyinosinic:polycytidylic acid (polyI:C, 50 µg/ml, Sigma-Aldrich, UK), peptidoglycan (PGN, 5 µg/ml, Invivogen), trout recombinant (r) Il-1β (20 ng/ml, [36]), rIfn-γ (20 ng/ml, [37]), rIl-6 (100 ng/ml, [34]), rTnf-α (isoform 3) (10 ng/ml, [38]), rIl-12A (p35a1/p40c, 1 µl/ml, [39]), rIl-12B (p35a1/p40b1, 1 µl/ml, [39]), rIl-4/13A and rIl-4/13B (200 ng/ml, [18] for 4, 8 and 24 h). The rTnf-α (isoform 3), rIl-12A and rIl-12B were produced in Chinese hamster ovary (CHO) cells. The concentration chosen for each stimulant was deemed optimal from previous studies. RNA extraction and real time RT-PCR analysis of gene expression in head kidney macrophages was conducted as described below.

2.5 Expression of rainbow trout arginase isoforms during *Y. ruckeri* vaccination and challenge

Enteric redmouth disease (ERM) caused by *Yersinia ruckeri* is responsible for significant economic losses in salmonid aquaculture worldwide but can be prevented by vaccination [40]. The modulated expression of trout arginase paralogues *in vivo* was first examined in response to vaccination using a commercial ERM vaccine (AQUAVAC® ERM, MSD Animal Health, UK). A group of 24 fish (approximately 50 g) were vaccinated by intraperitoneal (i.p.) injection of 0.1 ml of vaccine, following the manufacturer's instructions. The same number of fish was injected with phosphate buffer saline (PBS) as control. Fish handling and experimental protocols complied with the Guidelines of the European Union Council (2010/63/EU) for the use of laboratory animals, and were carried out under UK Home Office project licence PPL 60/4013, approved by the ethics committee at the University of Aberdeen. Six fish from both the vaccinated and control groups were killed at 1, 3, 7 and 14 days post vaccination. Spleen and gills were taken from each fish and homogenised separately in TRI Reagent® (Sigma-Aldrich, UK). RNA extraction and cDNA synthesis was as described previously [41] and samples were stored at -20°C ready for real time RT-PCR analysis as described below.

We next studied the expression of the arginase paralogues following pathogen challenge of vaccinated and control fish and the challenge procedure and tissue sampling was as described

previously [41] but with some modifications. Briefly, 32 rainbow trout were vaccinated with AQUAVAC® ERM as above and an additional 32 trout were i.p. injected with 0.1 ml of Hanks' Balanced Salt Solution (HBSS, Sigma-Aldrich, UK) as controls. Ten weeks later, the fish were challenged by i.p. injection with *Y. ruckeri* strain MT3072 at 0.5 ml/fish (1×10^6 cfu/ml) or 0.5 ml of HBSS as control. The fish were sampled at day 1 and day 2, before naïve fish show signs of disease (day 3) under the same challenge dose. Four fish from each group were killed at each sampling point, with gills, head kidney and spleen collected from each fish, and homogenised in TRI Reagent® (Sigma-Aldrich, UK). RNA extraction and cDNA synthesis was as described previously [41] and samples were stored at -20°C ready for real time RT-PCR analysis as described below.

2.6 Expression of Atlantic salmon arginase isoforms during *P. perurans* infection

Amoebic gill disease (AGD) is an ectoparasitic infection caused by the amoeba *P. perurans*. A polyclonal and a derived clonal amoebae line, named B8, recently characterized by [42] were cultured and counted as described previously [12] to reach a concentration of 500 cells/l and 5,000 cells/l for an *in vivo* challenge. Two groups of 5 fish (ca. 400 g) were exposed to the two doses of amoebae as described previously [12], in a total volume of 120 l of seawater. Five control fish were exposed to the medium used for amoeba culture. After 4 h the water volume was increased to 350 l and exchanged in a flow through system at 3 l/min. At 3 weeks post-exposure, fish were anaesthetised with 0.3 g/l of ethyl 3-aminobenzoate methanesulfonate (Sigma-Aldrich, UK) and killed. RNA extraction and cDNA synthesis was as described previously [12]. Real-time RT-PCR analysis was conducted as described below.

2.7 Expression of rainbow trout arginase isoforms during *T. bryosalmonae* infection

Proliferative Kidney Disease (PKD) is a parasitic disease caused by the myxozoan parasite *T. bryosalmonae*. Approximately 100 mg of posterior kidney tissue was removed from the area associated with the onset of clinical disease in fish during a natural outbreak at a commercial trout farm in Southern England. *T. bryosalmonae* presence was confirmed as published previously [11] and kidney swabs from infected and uninfected fish taken at the time of sampling were negative for the presence of common bacterial pathogens, implying the host gene expression profiles seen were as a consequence of the *T. bryosalmonae* infection. A kidney swelling grade from 0 (control fish) to 3 (higher swelling grade) was assigned to each fish according to the kidney swelling index previously published [43]. RNA extraction and cDNA synthesis was as described previously [11] and real-time RT-PCR analysis was conducted as described below.

2.8 Real time RT-PCR

Real time RT-PCR was carried out using a 384 LightCycler® 480 (Roche Applied Science) in a 10 µl reaction using SYBR® Green I (Invitrogen™, Carlsbad, USA) and IMMOLASE™ DNA Polymerase (Bioline, UK). 4 µl cDNA were used in each reaction and the real time analysis program consisted of 1 cycle of denaturation (95 °C for 10 min), 40 cycles of amplification (95°C for 30 s, 66°C for 20 s, 72°C for 20 s, 86°C for 5 s), followed by 95°C for 5 s and 75°C for 1 min. Program profiles differed for annealing temperature and time for elongation (Tables 1 & 2). At least one of each real time RT-PCR primer pair was designed to cross an exon-intron boundary to avoid amplification of genomic (g) DNA (Tables 1 & 2). Primer efficiency was tested using 4 fold serial dilutions of cDNA from pooled RNA samples and calculated by the 'LightCycler® 480 software version 1.5.1.62' (Roche Applied Science) as $E = 10^{(-1/s)}$, where s is the slope generated from the Log dilution of cDNA plotted against Cp (cycle number of crossing point) [44].

2.9 Statistical analysis

Normality of data was tested with a Shapiro-Wilk's W-Test (R software, v3.0.1), and data not normally distributed ($p < 0.05$) were Log transformed. Different tests have been used for the homogeneity of variances ($p > 0.05$) based on the type of data: Bartlett's test was used if the data were normally distributed, Levene's test was used in the case of small departures from normality as it is more robust than Bartlett's test, and the Fligner-Killeen test was used as a non-parametric test which is very robust against departures from normality (R software, v3.0.1). To test for transcript constitutive expression in tissues, modulation in rainbow trout macrophages, expression kinetics following *Y. ruckeri* vaccination and infection, and *T. bryosalmonae* infection experiments either a factorial anova (aov) or a linear model (lm) was used, the latter in the case of missing data, with a stepwise deletion method to simplify models using the R software v3.0.1 [45]. Briefly, the most similar parameter estimates were aggregated together in a stepwise *a posteriori* procedure to combine non-significant factor levels. For multiple comparisons the Holm-Bonferroni correction method [46] was used. Expression levels of the gene of interest (GOI) in the AGD experiment were analysed as reported previously [12]. Briefly, the expression level of the GOI relative to that of the reference gene elongation factor 1α (*ef1α*) was calculated by the 'Relative expression software tool' (REST©) [44, 47].

3. Results

3.1 Cloning and sequence analysis

Four rainbow trout arginase cDNA sequences (accession numbers KX998965 for Arginase 1a, KX998966 for Arginase 1b, KX998967 for Arginase 2a and KX998968 for Arginase 2b) have been cloned and sequence analysed. Each cDNA sequence had an in frame stop codon before the main open reading frame (ORF), a complete ORF and a partial 3'-UTR. The main ORF encoded 338 amino acids (aa), 337 aa, 347 aa and 347 aa for Arginase 1a, 1b, 2a and 2b, respectively (see Suppl. Figures S1-S4). Using the trout arginase protein sequence as bait, the Atlantic salmon counterparts were identified in Salmobase. The salmonid orthologues between trout and salmon share higher identities of 95.9%, 97.9%, 96.0% and 97.7% for Arginase 1a, 1b, 2a and 2b, respectively, than paralogues, i.e. 93.2-93.8% identities between Arginase 1a and 1b, and 93.7-95.4% identities between 2a and 2b (Table 3). The salmonid arginase type I paralogues have lower identities (51.9-53.4%) to salmonid arginase type II paralogues, similar to those to arginase type II from third round (3R) whole genome duplication (WGD) event fish species (eg. *E. lucius*, *D. rerio*, *Takifugu rubripes* and *Oreochromis niloticus*, 50.6-53.8% identities) and tetrapods (52.8-55.1% identities) (Table 3). As expected, salmonid arginase type I paralogues shared the highest identities to northern pike (*E. lucius*) Arginase 1 (87.9-91.4%), high identities to 3R fish Arginase 1 (67.5-77.1%), medium identities to 2R spotted gar (*Lepisosteus oculatus*) Arginase 1 (62.8-63.3%), and lowest identities to tetrapod arginase type I (57.2-60.2%) (Table 3). Similarly, salmonid arginase type II paralogues share the highest identities to northern pike Arginase 2 (88.5-90.2%), have relatively high identities to other 3R fish arginase type II (73.0-81.0%), medium identities to 2R spotted gar arginase type II (64.3-65.5%), and lowest identities to tetrapod arginase type II (61.7-64.1%) (Table 3).

The amino acid sequences were further studied by phylogenetic analysis (Suppl. Figure S5) using the BEAST software package v1.7. [29]. Arginase type I and II are clearly two independent clades. Within both the arginase type I and II clades, the ray-finned fish (Actinopterygii) molecules group together to form a sub-clade separate from the tetrapod and lobe-finned fish (coelacanth) species. In the subclades containing arginase sequences from salmonids and pike, their closest 3R relative, the salmonid orthologues group first, a typical scenario where the two paralogues have risen from the 4R WGD in salmonids. Furthermore, the duplicated salmonid paralogues are located on different chromosomes (in Atlantic salmon, Figure 1) or contigs (in rainbow trout) (Figure 2). Although syntenic analysis is not

possible in rainbow trout because of short contig length, syntenic conservation in the paralogue loci was apparent in Atlantic salmon (Figure 1). Such evidence suggests that the salmonid arginase paralogues are the result of the Ss4R duplication event. Interestingly, in the coelacanth (*Latimeria chalumnae*), Carolina anole (*Anolis carolinensis* - class Reptilia), and collared flycatcher (*Ficedula albicollis* - class Aves) only a type II sequence was found.

All the salmonid arginase genes have an 8 exon/ 7 intron structure, with highly conserved exon length although some variability is seen for the first and last exon. The introns are more variable in size but have a conserved intron phase (Figure 2). Conserved exon length is also present in the other fish and the human genes, although the latter has a different length for the third exon in both arginase I and II (Figure 2).

3.2 Constitutive expression of rainbow trout arginase isoforms in tissues

The constitutive expression of the four arginase isoforms was studied in 17 different tissues from healthy rainbow trout by real time RT-PCR (Figure 3). Statistical analysis was performed for each gene among the different tissues, starting with a general aov model (R software, v3.0.1), and then grouping together the most similar parameter estimates in a stepwise *a posteriori* procedure to combine non-significant factor levels until the models' comparison was significant ($p < 0.05$). Diagnostic plots of the final model were always performed to validate that linear model assumptions were met (results not shown). The final model of *arg1a* analysis showed that the tissues grouped based on their expression as follows, from lowest to highest: 1) intestine; 2) adipose tissue, spleen, blood, head kidney, scales and tail fin; 3) adipose fin, brain, heart, gonad, muscle and thymus; 4) skin; 5) gills and liver; 6) posterior kidney. For *arg1b* the groups were: 1) tail fin, intestine and scales; 2) adipose tissue, spleen and head kidney; 3) adipose fin and posterior kidney; 4) blood, muscle and thymus; 5) gills; 6) brain, skin, heart, gonad and liver. For *arg2a* the tissues were grouped as: 1) liver; 2) tail fin; 3) adipose tissue, intestine, posterior kidney, head kidney, heart and skin; 4) blood, gonad, scales, gills, spleen, brain and thymus; 5) adipose fin and muscle. Lastly, for *arg2b* the groups were: 1) liver; 2) intestine and tail fin; 3) adipose tissue, spleen, gills and scales; 4) adipose fin, skin, blood, posterior kidney, gonad, head kidney and thymus; 5) heart; 6) brain and muscle. Overall, constitutive expression of one or both paralogues was seen in most tissues. However, *arg1a* was often higher than *arg1b*, with highest expression seen in the posterior kidney. As in mammals, the expression of type I arginase was much higher in liver in comparison with type II, with almost no expression for *arg2a* seen in this tissue.

3.3 Modulation of arginase isoform expression in rainbow trout macrophages

Macrophages represent a first line of defence *in vivo* and they are important for arginase enzyme activity after polarization along the M2 pathway. Therefore, we examined the expression and modulation of rainbow trout arginase isoforms in primary head kidney macrophages stimulated by a viral and bacterial PAMP, namely polyI:C and PGN, and a variety of recombinant (r) trout cytokines (rIL-1 β , rIL-6, rIL-1 γ , rIL-12A, rIL-12B, rTnf- α , rIL-4/13A, and rIL-4/13B). A separate linear model (lm) was used for the analysis of each gene and time point (R software, v3.0.1). The expression of *arg1a* was more highly induced in comparison to the other isoforms, while *arg1b* was the least inducible. *arg1a* was mainly found up regulated in comparison to control fish at 24 h after stimulation with polyI:C (28-fold, $p < 0.001$, $n = 4$), PGN (66-fold, $p < 0.001$, $n = 4$), rIL-1 β (45-fold, $p < 0.001$, $n = 3$), rIL-6 (3-fold, $p < 0.01$, $n = 3$), and rTnf- α (isoform 3) (16-fold, $p < 0.001$, $n = 4$). The expression of *arg1a* and *arg1b* was also found to be significantly down regulated in comparison to control fish in three cases: *arg1a* after stimulation with rIL-4/13A at 8 h ($p < 0.05$, $n = 3$) and rIL-1 γ at 24 h ($p < 0.001$, $n = 3$) and *arg1b* after stimulation with rIL-4/13A at 24 h ($p < 0.01$, $n = 3$). *arg2a* was mostly induced at 4 h by the different stimulants in comparison to *arg2b* but both isoforms were induced significantly by rIL-4/13A in comparison to control fish: *arg2a* at 4 h (2-fold, $p < 0.05$, $n = 3$) and *arg2b* at all three timings (2-fold, $p < 0.01$, $n = 3$, at 4 and 8 h; 2-fold, $p < 0.05$, $n = 4$, at 24 h).

3.4 Expression of rainbow trout arginase isoforms during *Y. ruckeri* vaccination and challenge

The expression of all trout arginase genes except *arg1a* in gills was modulated in the spleen and gills by ERM injection vaccination (Figure 5). In the spleen, *arg1a* expression was increased 6.9-fold at 1 day post vaccination (dpv) and increased further to 50.9-fold at 3 dpv but had come back to control levels at 7 and 14 dpv. *arg2b* expression was also relatively highly induced (11.3-fold at 1 dpv and 2.9-fold at 3 dpv). A modest up regulation by ERM vaccination was also seen at 1 dpv for *arg1b* and *arg2a* (about 4-fold) but *arg2a* expression subsequently decreased to 0.42-fold of the time matched control at 7 dpv (Figure 5). Although *arg1a* expression was refractory in the gills, a modest increase (up to 2-fold) was seen after vaccination at 1-14 dpv for *arg1b*, and at 1, 3 and 14 dpv for *arg2a* and *arg2b* (Figure 5).

We next investigated trout arginase gene expression after challenge with *Y. ruckeri* in ERM vaccinated and control fish. Appropriate control groups (unvaccinated fish – HBSS_V; non-challenged fish – HBSS_C) were included in the analysis. Expression of the four isoforms was studied in the gills, spleen and head kidney at days 1 and 2 post challenge. For statistical analysis, linear models (lm) for each gene, time point, and tissue were applied within R software, v3.0.1, and for multiple comparisons the Holm-Bonferroni correction method [46] was used. In comparison to control fish (HBSS_V - HBSS_C), in unvaccinated fish challenged with *Y. ruckeri* (HBSS_V - YR_C) *arg1a* was more highly induced than *arg1b* and was up regulated mainly in spleen and head kidney at days 1 and 2 ($p < 0.001$, $n = 4$). Almost no up regulation was found in head kidney for *arg1b* and *arg2a*, with only a small increase in *arg1b* at day 1 ($p < 0.05$, $n = 4$). Significant up regulation was also found in spleen for *arg1b* at day 2 ($p < 0.001$, $n = 4$). In contrast, the type II arginase isoforms showed highest induction in gills after challenge with *Y. ruckeri*, as seen with *arg2a* in both vaccinated and unvaccinated fish at day 1 ($p < 0.001$, $n = 4$), and with *arg2b* in unvaccinated fish at day 2 ($p < 0.001$, $n = 4$). However, up regulation of the arginase II isoforms was also seen in the spleen of unvaccinated fish challenged with *Y. ruckeri* (HBSS_V - YR_C) in comparison to control fish (HBSS_V - HBSS_C) at both days, for *arg2a* at day 1 ($p < 0.001$, $n = 4$) and day 2 ($p < 0.01$, $n = 4$) and for *arg2b* at both days ($p < 0.001$, $n = 4$), with a low level of induction in head kidney for *arg2b*. Curiously, injection of *Y. ruckeri* vaccinated fish with HBSS (YR_V – HBSS_C) caused a significant down regulation of 1) *arg1a* at day 1 in spleen ($p < 0.01$, $n = 4$) and head kidney ($p < 0.001$, $n = 4$); 2) *arg2a* at day 1 in spleen ($p < 0.05$, $n = 4$); and 3) *arg2b* at day 1 in head kidney ($p < 0.001$, $n = 4$). Significant up regulation of vaccinated fish challenged with *Y. ruckeri* (YR_V – YR_C) was found only at day 1 in spleen ($p < 0.01$, $n = 4$) and head kidney ($p < 0.001$, $n = 4$) for *arg1a*; in gills ($p < 0.001$, $n = 4$) for *arg2a*; and in gills ($p < 0.05$, $n = 4$), spleen ($p < 0.05$, $n = 4$) and head kidney ($p < 0.001$, $n = 4$) for *arg2b*.

3.4 Expression of arginase isoforms during parasitic infection

The expression of Atlantic salmon arginase transcripts was examined during *P. perurans* (AGD) infection. *arg1a*, *arg1b*, *arg2a* were significantly down regulated in comparison to control fish in gills from salmon exposed to both concentrations of cloned B8 trophozoites. *arg1a* and *arg1b* were also down regulated when using the higher concentration (5,000 cells/l) of the polyclonal culture. In contrast, *arg2b* was significantly up regulated by exposure to the higher concentration of the clonal culture ($p < 0.05$, $n = 5$). Similar results

were found for the expression of rainbow trout *arg1a* and *arg1b* transcripts during *T. bryosalmonae* (PKD) infection, where the transcripts in kidney were down regulated with increasing swelling index. The type II arginases were not modulated by the presence of the parasite.

4. Discussion

Arginase, a ubiquitous enzyme found in prokaryotic and eukaryotic organisms, is responsible for cleaving the guanidine group from arginine into ornithine and urea [1, 2]. Invertebrates have only one type of arginase, localized in mitochondria, while most vertebrates have two types as a consequence of a gene duplication that occurred after the separation of vertebrates and invertebrates [2, 3]. These two arginase genes have diverged in terms of where and when they are expressed. For example, type I arginase is cytoplasmic and expressed in liver as part of the urea cycle whilst type II is a mitochondrial enzyme, likely the surviving form of the ancestral gene, and is expressed in a variety of peripheral tissues [4]. Similarly within the immune system the roles of these two isoforms differ, as seen in mammalian macrophages which can express both types [48]. Classically type I arginase is expressed in M2 macrophages, associated with anti-inflammatory responses and wound healing. In contrast type II plays a role in pro-inflammatory responses of macrophages and is expressed upon activation of M1 cells by LPS, during monocyte maturation to macrophages and in some chronic disease states [49]. It is not clear that the above mammalian paradigm will hold true in all vertebrate groups that express these two arginase isoforms, and indeed in fish there is evidence to suggest that type II may be a good marker of M2 macrophages in carp [5-8]. In salmonid fish, that have undergone a further round of WGD (Ss4R), the situation may be even more complex with the potential for further paralogues of arginase to be present. Hence this study initially characterised the arginase genes found in trout and salmon, and then examined their constitutive expression in a range of tissues, and after immune stimulation *in vitro* and *in vivo*, to assess whether there was evidence of functional divergence between the genes present.

Our bioinformatics analysis revealed that four different arginase isoforms are present in rainbow trout and Atlantic salmon, two related to type I (*arg1a*, *arg1b*) and two related to type II (*arg2a*, *arg2b*) arginase, as evidenced by the amino acid homology analysis (Table 3) and phylogenetic analysis (Suppl. Figure S5). In the phylogenetic tree type I and II molecules form two independent clades, and in both cases the ray-finned fish clade is grouped together

to form a sub-clade separate from lobe-finned fish and tetrapods. Interestingly only a single type of arginase, type II, appears present in the coelacanth (*L. chalumnae*), lizards (*A. carolinensis*) and birds (*F. albicollis*), with gene loss of type I in the latter (reptiles and birds) hypothesised to be linked to the transition to land and a further adaptation to dispose the excess of ammonium nitrogen into uric acid (uricotelic) [1, 50]. Occurrence of the duplicated genes in salmonids is most likely a result of the known ancestral WGD event that occurred in this fish lineage, as suggested by the homology and gene synteny analysis (Table 3, Figure 1) [13].

Constitutive expression of the four arginase isoforms was next studied in 17 different tissues from healthy rainbow trout. The findings demonstrated that in general one or both paralogues of type I and type II arginase were expressed in most tissues. However, *arg1a* was often higher than *arg1b*, with highest expression seen in the posterior kidney. Interestingly, both type I paralogues were much more highly expressed in liver compared with the type II paralogues, with almost no expression of *arg2a* apparent. Similarly, in mammals arginase type I is mainly expressed in liver where it is involved in the hepatic urea cycle [4].

Macrophages represent an important innate defence against various pathogens, and in addition to undergoing phagocytosis, bacterial killing, and defence against protozoan and metazoan parasites, they aid wound healing [2]. Two main types of macrophage populations are known that differ in terms of activation triggers and effector function: 1) the classically activated M1 macrophages induced by Th1 cytokines that convert L-arginine to L-citrulline, producing NO and reactive nitrogen species, and 2) the alternatively activated M2 macrophages that express arginase after activation with Th2 cytokines [4, 8]. Recently this polarisation model has been considered an oversimplification in mammals, and the M2 macrophages can now be subdivided into: i) M2a macrophages activated by IL-4 or IL-13, ii) M2b macrophages induced by the combined exposure to immune complexes and toll-like receptor (TLR) or IL-1R agonists, and iii) M2c macrophages deactivated by glucocorticoids or by cytokines such as transforming growth factor (TGF)- β or IL-10 [5, 51, 52]. Both types of activated macrophages (M1 and M2) have been demonstrated in fish, with the latter showing elevated levels of arginase activity [5-8]. In this study we examined the expression and modulation of rainbow trout arginase isoforms in primary head kidney macrophages stimulated by two PAMPs (polyI:C, PGN) and a range of trout cytokines relevant to proinflammatory responses and type-I and -II immunity (e.g. rIL-1 β , rIL-4/13A, rIL-4/13B, rIL-

I paralogues) and kinetics of induction (as seen with the type II paralogues), similar to the *in vitro* findings above.

Two parasite infection models were also studied: AGD in the gills of Atlantic salmon and PKD in the kidney of trout. During AGD *arg1a*, *arg1b* and *arg2a* were significantly down regulated in gill tissue in comparison to control fish, although with *arg1a* and *arg1b* only at the higher concentration of trophozoites in the case of the polyclonal culture used for infection and with *arg2a* only using the clonal B8 culture, considered a relatively virulent clone [42]. The down regulation of arginase type I was as seen previously [12]. Curiously, *arg2b* was significantly up regulated with the higher concentration of trophozoites for this clone. Similar results were found for PKD in trout. *arg1a* and *arg1b* were down regulated in kidneys with the highest swelling index (grade 3), with a clear correlation to increasing pathology in the case of *arg1a*. This is in line with a previous study of arginase type I, where the primers used amplified both paralogues [11]. However, the two arginase type II paralogues were not modulated by the presence of the parasite. In a previous study, *il-4/13A* and *il-4/13B2*, believed to be related to the Th2 pathway in fish (M2a type) [18, 53, 54] were significantly up regulated during AGD [12]. Whether this accounts for the increase in *arg2b* transcript levels during AGD and whether this is beneficial to the host or to *P. perurans* remains to be determined. However, once again it is clear that differences exist between the type I and type II arginase expression patterns, in this case after parasite infection, with *arg1* and *arg2* expression potentially induced by different stimuli as in the trypanosome-carp model [7].

In summary, four arginase isoforms have been characterised in trout and salmon. Analysis of their constitutive and modulated expression has shown that the type I and II genes have different responses and that the paralogues also vary in their magnitude and kinetics of expression. The data support the contention that arginase type II may be a more relevant marker of M2a cells in teleost fish, with arginase type I induced by proinflammatory stimuli and down regulated during parasite infections. However, in salmonids there is an added complexity with the presence of additional paralogues which also show varied expression patterns.

Ethics statement

All handling of fish was conducted in accordance with the Animals (Scientific Procedures) Act 1986 and all proposed experiments were first subject to detailed statistical review to

ensure that a minimum number of fish was used, which would allow statistically meaningful results to be obtained.

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5. Figures and Tables

Figure 1. Gene synteny of Atlantic salmon arginase. The synteny was analysed using Salmobase (<http://salmobase.org>). The syntenically conserved gene blocks are shown in matching colours. The arrows indicate the transcriptional direction.

Figure 2. Inferred exon-intron structures of arginase I (A) and arginase II (B). Quantitative information on the lengths of exon coding sequence (black boxes, to scale) and introns (white boxes, not to scale), and intron phase are presented. Inferred exons and intron phase for *arg2b* in *O. mykiss* were characterised using the *arg2a* genomic sequence (accession number CCAF010119897.1).

Figure 3. Expression profiles of *arg1a*, *arg1b*, *arg2a*, *arg2b* in 17 different tissues of healthy rainbow trout determined by real-time RT-PCR. Transcript levels were first calculated using a serial dilution of references in the same PCR run. Relative expression levels (mean + SEM, n = 6) are expressed as arbitrary units after normalisation to *ef1a*.

Figure 4. Relative expression of rainbow trout *arg1a*, *arg1b*, *arg2a*, *arg2b* in primary head kidney macrophages following stimulation with PAMPs and cytokines. The fold change (mean ± SEM, n = 4) was calculated as the relative expression in comparison to control cells, after normalizing to *ef1a*. A linear model (lm) was used for statistical analysis (R software, v3.0.1). * = p < 0.05, ** = p < 0.01, *** = p < 0.001.

Figure 5. Relative expression of rainbow trout *arg1a*, *arg1b*, *arg2a*, *arg2b* in spleen and gills after vaccination with AQUAVAC® ERM. Two groups of rainbow trout were vaccinated by i.p. injection of AQUAVAC® ERM or PBS as control, and spleen/gill tissue sampled at day 1, 3, 7 and 14. The fold change (means ± SEM, n = 6) was calculated as the mean expression levels in vaccinated fish normalized to time-matched controls in the same tissue. The relative significance of a LSD post hoc test after a significant one way-ANOVA between the vaccinated and control groups at the same time point is shown above the bars as: * = p < 0.05, ** = p < 0.01 and *** = p < 0.001.

Figure 6. Relative expression of rainbow trout *arg1a*, *arg1b*, *arg2a*, *arg2b* in gill, spleen, head kidney samples of control (HBSS_V) and ERM vaccinated (YR_V) fish subsequently injected with HBSS (HBSS_C) or *Yersinia ruckeri* (YR_C) and sampled 1 and 2 days later. The fold change (mean ± SEM, n = 4) was calculated as relative expression in comparison to

control fish, normalized to *eflα*. A linear model (lm) with the Holm-Bonferroni correction method for multiple comparisons were used for statistical analysis (R software, v3.0.1). * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

Figure 7. Relative expression of Atlantic salmon *arg1a*, *arg1b*, *arg2a*, *arg2b* (mean $\pm$ SEM, $n = 5$) in gill samples infected with a polyclonal or clonal culture of *P. perurans* at two trophozoite doses. Expression was determined using REST© 2009 (relative expression software tool), and the fold change calculated as the relative expression in comparison to gills from control fish, normalized to *eflα*. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

Figure 8. Relative expression of rainbow trout *arg1a*, *arg1b*, *arg2a*, *arg2b* in kidney samples infected with *T. bryosalmonae*, presented as a fold change relative to control kidney samples, after normalizing to *eflα*. A kidney swelling grade from 0 (control fish) to 3 (higher swelling grade) was assigned to each fish according to the kidney swelling index of [43]. A linear model (lm) with the Holm-Bonferroni correction method for multiple comparisons were used for statistical analysis (R software, v3.0.1). * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, $n = 10$ for uninfected fish; $n = 5$ for fish exhibiting grade 1; $n = 9$ for fish exhibiting grade from 1 to 2; $n = 10$ for fish exhibiting grade 2; $n = 9$ for fish exhibiting grade 3.

Table 1. Rainbow trout primer sequences used for PCR cloning and expression analysis (real time RT-PCR).

Table 2. Atlantic salmon primer sequences used for gene expression analysis (real time RT-PCR).

Table 3. Comparison of the amino acid identities/ similarities for arginase I and II using the Similarity Matrix BLOSUM62 within MatGat 2.02 software.

6. References

1. Jenkinson CP, Grody WW, Cederbaum SD. Comparative properties of arginases. *Comparative Biochemistry and Physiology - B Biochemistry and Molecular Biology* 1996;114(1):107-32.
2. Dzik JM. Evolutionary roots of arginase expression and regulation. *Frontiers in Immunology* 2014;5(544):1-11.
3. Samson M-. *Drosophila* arginase is produced from a nonvital gene that contains the *elav* locus within its third intron. *Journal of Biological Chemistry* 2000;275(40):31107-14.
4. Munder M. Arginase: An emerging key player in the mammalian immune system: REVIEW. *British Journal of Pharmacology* 2009;158(3):638-51.
5. Wiegertjes GF, Wentzel AS, Spaink HP, Elks PM, Fink IR. Polarization of immune responses in fish: The 'macrophages first' point of view. *Molecular Immunology* 2016;69:146-56.
6. Skugor S, Glover KA, Nilsen F, Krasnov A. Local and systemic gene expression responses of Atlantic salmon (*Salmo salar* L.) to infection with the salmon louse (*Lepeophtheirus salmonis*). *BMC Genomics* 2008;9(498):1-18.
7. Joerink M, Forlenza M, Ribeiro CMS, de Vries BJ, Savelkoul HFJ, Wiegertjes GF. Differential macrophage polarisation during parasitic infections in common carp (*Cyprinus carpio* L.). *Fish & Shellfish Immunology* 2006 11;21(5):561-71.
8. Forlenza M, Fink IR, Raes G, Wiegertjes GF. Heterogeneity of macrophage activation in fish. *Developmental and Comparative Immunology* 2011 12;35(12):1246-55.
9. Joerink M, Savelkoul HFJ, Wiegertjes GF. Evolutionary conservation of alternative activation of macrophages: Structural and functional characterization of arginase 1 and 2 in carp (*Cyprinus carpio* L.). *Molecular Immunology* 2006;43(8):1116-28.
10. Joerink M, Ribeiro CMS, Stet RJM, Hermesen T, Savelkoul HFJ, Wiegertjes GF. Head kidney-derived macrophages of common carp (*Cyprinus carpio* L.) show plasticity and functional polarization upon differential stimulation. *Journal of Immunology* 2006;177(1):61-9.
11. Gorgoglione B, Wang T, Secombes CJ, Holland JW. Immune gene expression profiling of proliferative kidney disease in rainbow trout *Oncorhynchus mykiss* reveals a dominance of anti-inflammatory, antibody and T helper cell-like activities. *Veterinary Research* 2013;44(55):1-16.

- 638 12. Benedicenti O, Collins C, Wang T, McCarthy U, Secombes CJ. Which th pathway is
639 involved during late stage amoebic gill disease? Fish & Shellfish Immunology 2015
640 10;46(2):417-25.
- 641 13. Lien S, Koop BF, Sandve SR, Miller JR, Kent MP, Nome T, Hvidsten TR, Leong JS,
642 Minkley DR, Zimin A, Grammes F, Grove H, Gjuvsland A, Walenz B, Hermansen RA,
643 Von Schalburg K, Rondeau EB, Di Genova A, Samy JKA, Olav Vik J, Vigeland MD,
644 Caler L, Grimholt U, Jentoft S, Inge Våge D, De Jong P, Moen T, Baranski M, Palti Y,
645 Smith DR, Yorke JA, Nederbragt AJ, Tooming-Klunderud A, Jakobsen KS, Jiang X,
646 Fan D, Hu Y, Liberles DA, Vidal R, Iturra P, Jones SJM, Jonassen I, Maass A, Omholt
647 SW, Davidson WS. The Atlantic salmon genome provides insights into rediploidization.
648 Nature 2016;533:200-5.
- 649 14. Macqueen DJ, Johnston IA. A well-constrained estimate for the timing of the salmonid
650 whole genome duplication reveals major decoupling from species diversification.
651 Proceedings of the Royal Society B: Biological Sciences 2014;281(20132881):1-8.
- 652 15. Pasquier J, Cabau C, Nguyen T, Jouanno E, Severac D, Braasch I, Journot L, Pontarotti
653 P, Klopp C, Postlethwait JH, Guiguen Y, Bobe J. Gene evolution and gene expression
654 after whole genome duplication in fish: The PhyloFish database. BMC Genomics
655 2016;17(1).
- 656 16. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search
657 tool. Journal of Molecular Biology 1990 5 October 1990;215(3):403-10.
- 658 17. Wang T, Jiang Y, Wang A, Husain M, Xu Q, Secombes CJ. Identification of the
659 salmonid IL-17A/F1a/b, IL-17A/F2b, IL-17A/F3 and IL-17N genes and analysis of their
660 expression following in vitro stimulation and infection. Immunogenetics
661 2015;67(7):395-412.
- 662 18. Wang T, Johansson P, Abós B, Holt A, Tafalla C, Jiang Y, Wang A, Xu Q, Qi Z,
663 Huang W, Costa MM, Diaz-Rosales P, Holland JW, Secombes CJ. First in-depth
664 analysis of the novel Th2-type cytokines in salmonid fish reveals distinct patterns of
665 expression and modulation but overlapping bioactivities. Oncotarget 2016;7(10):10917-
666 46.
- 667 19. Jiang Y, Husain M, Qi Z, Bird S, Wang T. Identification and expression analysis of
668 two interleukin-23 α (p19) isoforms, in rainbow trout *Oncorhynchus mykiss* and Atlantic
669 salmon *Salmo salar*. Molecular Immunology 2015 8;66(2):216-28.
- 670 20. Wang T, Diaz-Rosales P, Costa MM, Campbell S, Snow M, Collet B, Martin SAM,
671 Secombes CJ. Functional characterization of a nonmammalian IL-21: Rainbow trout
672 *Oncorhynchus mykiss* IL-21 upregulates the expression of the Th cell signature
673 cytokines IFN- γ , IL-10, and IL-22. Journal of Immunology 2011;186(2):708-21.
- 674 21. Katoh K, Standley DM. MAFFT multiple sequence alignment software version 7:
675 Improvements in performance and usability. Molecular Biology and Evolution
676 2013;30(4):772-80.

- 677 22. Hall TA. Bioedit: A user-friendly biological sequence alignment editor and analysis
678 program for windows 95/98/NT. Nucleic Acids Symposium Series 1999;41:95-8.
- 679 23. Landan G, Graur, D. Local reliability measures from sets of co-optimal multiple
680 sequence alignments. Pacific symposium on biocomputing 2008, PSB 2008; 2008. 15-24
681 p.
- 682 24. Sela I, Ashkenazy H, Katoh K, Pupko T. GUIDANCE2: Accurate detection of
683 unreliable alignment regions accounting for the uncertainty of multiple parameters.
684 Nucleic Acids Research 2015;43(W1):W7-W14.
- 685 25. Penn O, Privman E, Ashkenazy H, Landan G, Graur D, Pupko T. GUIDANCE: A web
686 server for assessing alignment confidence scores. Nucleic Acids Research
687 2010;38(SUPPL. 2).
- 688 26. Penn O, Privman E, Landan G, Graur D, Pupko T. An alignment confidence score
689 capturing robustness to guide tree uncertainty. Molecular Biology and Evolution
690 2010;27(8):1759-67.
- 691 27. Tamura K, Stecher G, Peterson D, Filipinski A, Kumar S. MEGA6: Molecular
692 evolutionary genetics analysis version 6.0. Molecular Biology and Evolution
693 2013;30(12):2725-9.
- 694 28. Le SQ, Gascuel O. An improved general amino acid replacement matrix. Molecular
695 Biology and Evolution 2008;25(7):1307-20.
- 696 29. Drummond AJ, Suchard MA, Xie D, Rambaut A. Bayesian phylogenetics with
697 BEAUti and the BEAST 1.7. Molecular Biology and Evolution 2012;29(8):1969-73.
- 698 30. Drummond AJ, Ho SYW, Phillips MJ, Rambaut A. Relaxed phylogenetics and dating
699 with confidence. PLoS Biology 2006;4(5):699-710.
- 700 31. Rambaut A, Suchard M, Xie D, Drummond A. Tracer v1.6.
701 <http://beast.bio.ed.ac.uk/software/tracer/> 2014.
- 702 32. Campanella JJ, Bitincka L, Smalley J. MatGAT: An application that generates
703 similarity/identity matrices using protein or DNA sequences. BMC Bioinformatics
704 2003;4(29):1-4.
- 705 33. Kapustin Y, Souvorov A, Tatusova T, Lipman D. Splign: Algorithms for computing
706 spliced alignments with identification of paralogs. Biology Direct 2008;3(20):1-13.
- 707 34. Costa MM, Maehr T, Diaz-Rosales P, Secombes CJ, Wang T. Bioactivity studies of
708 rainbow trout (*Oncorhynchus mykiss*) interleukin-6: Effects on macrophage growth and
709 antimicrobial peptide gene expression. Molecular Immunology 2011 9;48(15–16):1903-
710 16.
- 711 35. Xu Q, Li R, Monte MM, Jiang Y, Nie P, Holland JW, Secombes CJ, Wang T.
712 Sequence and expression analysis of rainbow trout CXCR2, CXCR3a and CXCR3b aids
713 interpretation of lineage-specific conversion, loss and expansion of these receptors

714 during vertebrate evolution. *Developmental and Comparative Immunology* 2014
715 8;45(2):201-13.

716 36. Hong S, Zou J, Crampe M, Peddie S, Scapigliati G, Bols N, Cunningham C, Secombes
717 CJ. The production and bioactivity of rainbow trout (*Oncorhynchus mykiss*) recombinant
718 IL-1 β . *Veterinary Immunology and Immunopathology* 2001 8/30;81(1-2):1-14.

719 37. Wang T, Huang W, Costa MM, Martin SAM, Secombes CJ. Two copies of the genes
720 encoding the subunits of putative interleukin (IL)-4/IL-13 receptors, IL-4Ra, IL-13Ra1
721 and IL-13Ra2, have been identified in rainbow trout (*Oncorhynchus mykiss*) and have
722 complex patterns of expression and modulation. *Immunogenetics* 2011;63(4):235-53.

723 38. Hong S, Li R, Xu Q, Secombes CJ, Wang T. Two types of TNF- α exist in teleost fish:
724 Phylogeny, expression, and bioactivity analysis of type-II TNF- α 3 in rainbow trout
725 *Oncorhynchus mykiss*. *Journal of Immunology* 2013;191(12):5959-72.

726 39. Wang T, Husain M, Hong S, Holland JW. Differential expression, modulation and
727 bioactivity of distinct fish IL-12 isoforms: Implication towards the evolution of Th1-like
728 immune responses. *European Journal of Immunology* 2014;44(5):1541-51.

729 40. Tobback E, Decostere A, Hermans K, Haesebrouck F, Chiers K. *Yersinia ruckeri*
730 infections in salmonid fish. *Journal of Fish Diseases* 2007;30(5):257-68.

731 41. Harun NO, Wang T, Secombes CJ. Gene expression profiling in naïve and vaccinated
732 rainbow trout after *Yersinia ruckeri* infection: Insights into the mechanisms of protection
733 seen in vaccinated fish. *Vaccine* 2011;29(26):4388-99.

734 42. Collins C, Hall M, Bruno D, Sokolowska J, Duncan L, Yucel R, McCarthy U, Fordyce
735 MJ, Pert CC, McIntosh R, Mackay Z. Generation of *Paramoeba perurans* clonal cultures
736 using flow cytometry and confirmation of virulence. *Journal of Fish Diseases*
737 2016;doi:10.1111/jfd.12517.

738 43. Clifton-Hadley RS, Bucke D, Richards RH. A study of the sequential clinical and
739 pathological changes during proliferative kidney disease in rainbow trout, *Salmo*
740 *gairdneri* Richardson. *Journal of Fish Diseases* 1987;10(5):335-52.

741 44. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-
742 PCR. *Nucleic Acids Research* 2001 MAY 1 2001;29(9):2002-7.

743 45. Crawley MJ. The R book. In: The R book. ; 2007. p 1-942.

744 46. Holm S. A simple sequentially rejective multiple test procedure. *Scandinavian Journal*
745 *of Statistics* 1979;6(2):65-70.

746 47. Pfaffl MW, Horgan GW, Dempfle L. Relative expression software tool (REST) for
747 group-wise comparison and statistical analysis of relative expression results in real-time
748 PCR. *Nucleic Acids Research* 2002;30(9):1-10.

48. Yang Z, Ming XF. Functions of arginase isoforms in macrophage inflammatory responses: Impact on cardiovascular diseases and metabolic disorders. *Frontiers in Immunology* 2014;5(533).
49. Ming XF, Rajapakse AG, Yepuri G, Xiong Y, Carvas JM, Ruffieux J, Scerri I, Wu Z, Popp K, Li J, Sartori C, Scherrer U, Kwak BR, Montani JP, Yang Z. Arginase II promotes macrophage inflammatory responses through mitochondrial reactive oxygen species, contributing to insulin resistance and atherogenesis. *Journal of the American Heart Association* 2012;1(4):e000992.
50. Amemiya CT, Alfoldi J, Lee AP, Fan S, Philippe H, MacCallum I, Braasch I, Manousaki T, Schneider I, Rohner N, Organ C, Chalopin D, Smith JJ, Robinson M, Dorrington RA, Gerdol M, Aken B, Biscotti MA, Barucca M, Baurain D, Berlin AM, Blatch GL, Buonocore F, Burmester T, Campbell MS, Canapa A, Cannon JP, Christoffels A, De Moro G, Edkins AL, Fan L, Fausto AM, Feiner N, Forconi M, Gamielien J, Gnerre S, Gnirke A, Goldstone JV, Haerty W, Hahn ME, Hesse U, Hoffmann S, Johnson J, Karchner SI, Kuraku S, Lara M, Levin JZ, Litman GW, Mauceli E, Miyake T, Mueller MG, Nelson DR, Nitsche A, Olmo E, Ota T, Pallavicini A, Panji S, Picone B, Ponting CP, Prohaska SJ, Przybylski D, Saha NR, Ravi V, Ribeiro FJ, Sauka-Spengler T, Scapigliati G, Searle SMJ, Sharpe T, Simakov O, Stadler PF, Stegeman JJ, Sumiyama K, Tabbaa D, Tafer H, Turner-Maier J, Van Heusden P, White S, Williams L, Yandell M, Brinkmann H, Volff J-, Tabin CJ, Shubin N, Schartl M, Jaffe DB, Postlethwait JH, Venkatesh B, Di Palma F, Lander ES, Meyer A, Lindblad-Toh K. The african coelacanth genome provides insights into tetrapod evolution. *Nature* 2013;496(7445):311-6.
51. Mantovani A, Sica A, Sozzani S, Allavena P, Vecchi A, Locati M. The chemokine system in diverse forms of macrophage activation and polarization. *Trends in Immunology* 2004;25(12):677-86.
52. Martinez FO, Gordon S. The M1 and M2 paradigm of macrophage activation: Time for reassessment. *F1000Prime Reports* 2014;6(13):1-13.
53. Wang T, Secombes CJ. The evolution of IL-4 and IL-13 and their receptor subunits. *Cytokine* 2015;75(1):8-13.
54. Yamaguchi T, Miyata S, Katakura F, Nagasawa T, Shibasaki Y, Yabu T, Fischer U, Nakayasu C, Nakanishi T, Moritomo T. Recombinant carp IL-4/13B stimulates in vitro proliferation of carp IgM+ B cells. *Fish & Shellfish Immunology* 2016;49:225-9.

Table 1. Rainbow trout primer sequences used for PCR cloning and expression analysis (real time RT-PCR).

Gene		Application	Oligonucleotides (5' – 3')	Accession Number	Ta (°C) – Time (s)	Efficiency (%)
<i>ef1a</i>	Forward	Real time RT-PCR	CAAGGATATCCGTCGTGGCA	AF321836	63 – 30	> 99
	Reverse		ACAGCGAAACGACCAAGAGG			
<i>arg1a</i>	Forward	PCR cloning	ATTCTGAGCCGCTAACCCTTG			
	Reverse		CACTGTAATCGAAAGGCTCTGTGG			
<i>arg1a</i>	Forward	Real time RT-PCR	CAGAGGTGGATCGCCTTGGAATA	KX998965	66 – 20	> 94
	Reverse		GCAGACAGCATCCCTGTCTGACA			
<i>arg1b</i>	Forward	PCR cloning	GGCAAAGATGAGTTATGCAATTTTAGTG			
	Reverse		TAATACAAAATATTGCGTTTGATGGC			
<i>arg1b</i>	Forward	Real time RT-PCR	AGGTGGATCGCCTTGGAATCG	KX998966	66 – 20	> 96
	Reverse		GCAGACAGCAGCCCTGTCTGACT			
<i>arg2a</i>	Forward	PCR cloning	TCTCAGCCTTGGTCGTAAAC			
	Reverse		TGCCAAGTGGTCACATGTTGAAAG			
<i>arg2a</i>	Forward	Real time RT-PCR	TCCAGAGAGTCATGGAAGTCACTTTCC	KX998967	66 – 20	> 96
	Reverse		CCATCACTGACAACAACCCTGTGTT			
<i>arg2b</i>	Forward	PCR cloning	GCAGCCTTGGTCGTAAACGG			
	Reverse		GCCAAGTGGTTACATGTTGAGTC			
<i>arg2b</i>	Forward	Real time RT-PCR	TCCAGAGAGTCATGGAAGTCTCTTTCG	KX998968	66 – 20	> 94
	Reverse		CATCACCGACAACAACCCTGTGTT			

Table 2. Atlantic primer sequences used for gene expression analysis (real time RT-PCR).

Gene		Oligonucleotides (5' – 3')	Accession Number	Ta (°C) – Time (s)	Efficiency (%)
<i>ef1a</i>	Forward	CAAGGATATCCGTCGTGGCA	AF321836	63 – 30	> 99
	Reverse	ACAGCGAAACGACCAAGAGG			
<i>arg1a</i>	Forward	CAGAGGTGGATCGCCTTGGAATA	XP_014013843.1	66 – 25	> 99
	Reverse	GCAGACAGCATCCCTGTCTGACA			
<i>arg1b</i>	Forward	AGGTGGATCGCCTTGGAATCG	NP_001134788.1	66 – 25	> 86
	Reverse	CAGACAGCAGCCCTGTCTGACA			
<i>arg2a</i>	Forward	GACCACCTCTGTCAAGGAAGCA	XP_014045709.1	66 – 20	> 97
	Reverse	CTCACGGGTCTGTCCTAGGGC			
<i>arg2b</i>	Forward	GACCACCTCTGTCAAGGAAGCA	XP_014067199.1	66 – 20	> 88
	Reverse	CCATGGAAGCGGTGCTCG			

Table 3. Comparison of the amino acid identities/ similarities for arginase I and II using the Similarity Matrix BLOSUM62 within MatGat 2.02 software.

	<i>O. mykiss</i> 1a (KX998965)	<i>S. salar</i> 1a (XP_014013843.1)	<i>O. mykiss</i> 1b (KX998966)	<i>S. salar</i> 1b (NP_001134788.1)	<i>E. lucius</i> 1 (XP_010863350.1)	<i>D. rerio</i> 1 (E7F8R4)	<i>O. niloticus</i> 1 (I3KSD9)	<i>T. rubripes</i> 1 (H2RY07)	<i>L. oculatus</i> 1 (W5NIT3)	<i>B. taurus</i> 1 (Q2KJ64)	<i>H. sapiens</i> 1 (P05089-2)	<i>X. tropicalis</i> 1 (F7CN24)	<i>O. mykiss</i> 2a (KX998967)	<i>S. salar</i> 2a (XP_014045709.1)	<i>O. mykiss</i> 2b (KX998968)	<i>S. salar</i> 2b (XP_014067199.1)	<i>E. lucius</i> 2 (XP_010877736.1)	<i>D. rerio</i> 2 (Q6PH54)	<i>O. niloticus</i> 2 (I3KUB9)	<i>T. rubripes</i> 2 (H2SAE9)	<i>L. oculatus</i> 2 (W5NA23)	<i>B. taurus</i> 2 (F1N1Z5)	<i>H. sapiens</i> 2 (P78540)	<i>X. tropicalis</i> 2 (Q05AR1)
<i>O. mykiss</i> 1a (KX998965)		95.9	93.5	93.8	87.9	74.2	69.4	70.0	63.3	58.4	58.2	60.4	52.7	52.7	53.0	52.7	53.8	53.0	51.1	53.2	46.8	52.8	54.8	53.9
<i>S. salar</i> 1a (XP_014013843.1)	98.5		93.2	93.5	87.9	75.1	68.7	69.1	62.9	57.2	58.2	60.1	51.9	51.9	52.6	52.9	53.5	53.0	50.6	52.9	46.3	52.8	54.2	53.9
<i>O. mykiss</i> 1b (KX998966)	97.0	97.3		97.9	91.1	77.1	67.5	69.1	62.8	58.0	58.4	60.2	52.9	52.9	53.2	53.4	53.6	53.3	51.3	53.0	47.4	53.1	54.5	53.9
<i>S. salar</i> 1b (NP_001134788.1)	97.3	97.6	99.1		91.4	77.1	68.1	69.7	62.8	57.4	57.8	59.6	52.3	52.3	52.6	52.9	53.0	52.7	50.7	52.7	46.9	53.7	55.1	53.6
<i>E. lucius</i> 1 (XP_010863350.1)	95.6	95.9	96.4	96.7		77.1	69.6	68.8	63.6	58.6	58.4	59.9	53.9	53.9	54.3	54.6	54.9	54.5	52.6	52.1	48.5	55.1	55.9	53.6
<i>D. rerio</i> 1 (E7F8R4)	87.1	88.0	87.7	87.7	87.7		67.1	66.5	59.4	56.1	57.1	56.0	50.7	51.3	51.0	52.1	52.0	53.0	51.4	50.1	45.3	52.9	53.6	50.4
<i>O. niloticus</i> 1 (I3KSD9)	84.9	84.3	84.1	84.6	83.8	84.1		71.0	58.4	55.5	55.4	55.7	51.6	51.1	51.6	51.3	51.8	51.6	51.0	51.9	44.9	50.8	52.1	51.1
<i>T. rubripes</i> 1 (H2RY07)	82.2	81.9	82.2	82.5	81.6	79.6	81.7		57.9	56.1	56.8	56.0	50.8	51.1	50.8	51.1	50.7	51.1	51.6	51.0	43.9	48.0	50.1	49.4
<i>L. oculatus</i> 1 (W5NIT3)	77.4	77.4	77.7	77.4	77.7	76.1	75.0	74.5		53.9	55.7	54.3	50.3	50.8	50.5	50.5	50.9	52.4	51.6	51.4	46.4	49.6	51.2	51.9
<i>B. taurus</i> 1 (Q2KJ64)	72.5	72.2	72.4	72.1	73.3	71.6	71.0	72.3	67.7		88.8	68.1	53.9	53.3	53.6	54.2	54.4	53.6	54.0	54.4	46.3	54.0	54.5	53.9
<i>H. sapiens</i> 1 (P05089-2)	74.0	74.6	74.2	73.9	74.2	74.2	73.0	73.5	69.0	92.7		66.8	51.8	51.0	51.5	52.1	52.5	53.8	52.8	52.9	47.5	53.9	53.3	53.3
<i>X. tropicalis</i> 1 (F7CN24)	76.0	75.4	75.1	75.7	75.4	75.4	72.8	73.8	71.2	80.7	80.9		53.0	53.3	52.7	53.3	52.9	53.3	52.9	53.0	45.5	51.4	52.3	53.1
<i>O. mykiss</i> 2a (KX998967)	69.2	69.2	69.5	69.2	68.9	69.5	68.6	68.9	66.8	68.9	69.7	71.2		96.0	95.4	94.8	88.8	79.8	78.9	73.2	64.3	62.4	62.1	61.7
<i>S. salar</i> 2a (XP_014045709.1)	69.5	69.5	69.5	69.2	69.2	69.7	68.9	69.5	67.7	68.9	68.3	71.8	98.0		93.7	95.4	88.5	79.3	79.1	73.2	65.0	63.8	63.8	63.1
<i>O. mykiss</i> 2b (KX998968)	70.0	70.6	70.3	70.0	70.0	69.7	68.9	68.9	67.7	69.2	70.0	71.8	97.4	97.1		97.7	89.9	81.0	80.6	73.0	65.0	62.4	62.4	62.5
<i>S. salar</i> 2b (XP_014067199.1)	70.0	70.6	70.3	70.0	70.0	70.0	68.9	68.9	67.4	69.2	70.0	71.8	98.0	97.7	99.4		90.2	81.0	80.9	73.2	65.5	64.1	64.1	63.6
<i>E. lucius</i> 2 (XP_010877736.1)	71.7	70.8	71.1	70.8	71.4	72.0	70.8	70.2	69.6	69.9	69.9	72.3	94.2	95.1	94.5	95.4		79.9	78.6	72.4	65.0	63.3	63.6	62.8
<i>D. rerio</i> 2 (Q6PH54)	70.9	70.3	69.5	69.2	70.3	71.2	68.0	68.0	67.7	68.6	69.2	70.9	91.4	91.1	91.9	91.9	91.6		79.7	74.4	67.3	60.7	61.0	63.3
<i>O. niloticus</i> 2 (I3KUB9)	70.6	69.7	70.3	70.0	70.3	71.7	68.0	70.0	68.2	69.4	70.3	69.1	87.1	87.4	88.0	88.3	87.7	88.9		76.2	64.4	62.5	63.0	63.2
<i>T. rubripes</i> 2 (H2SAE9)	70.1	69.6	69.6	69.0	69.3	68.2	67.3	67.6	67.4	69.6	70.1	69.3	83.7	83.7	84.5	84.5	83.4	84.2	87.6		58.9	63.2	63.2	63.3
<i>L. oculatus</i> 2 (W5NA23)	61.3	61.0	61.5	60.8	60.5	62.0	59.5	59.0	62.5	57.3	58.8	61.0	75.5	75.5	75.8	76.3	76.8	76.3	76.0	72.5		56.4	56.6	58.8
<i>B. taurus</i> 2 (F1N1Z5)	69.2	69.5	69.2	68.9	70.1	70.3	68.9	65.8	67.9	68.4	69.2	69.8	78.8	79.9	79.4	79.7	79.4	79.4	80.2	79.4	73.0		92.7	71.4
<i>H. sapiens</i> 2 (P78540)	69.8	69.5	69.8	69.5	70.9	71.2	70.6	66.7	68.5	67.8	68.1	69.2	79.4	80.2	79.7	79.9	79.9	79.4	79.4	74.0	96.9			71.7
<i>X. tropicalis</i> 2 (Q05AR1)	66.9	66.7	67.8	67.2	67.2	67.5	66.7	64.4	66.8	65.6	66.7	67.2	75.6	76.4	75.6	76.1	75.0	75.6	74.7	76.9	72.5	85.6	84.4	

Identity	Similarity
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1 Figure 1

2

S. salar 1a
(Chr. 19)

S. salar 1b
(Chr. 28)

S. salar 2a
(Chr. 01)

S. salar 2b
(Chr. 09)

3

4

Figure 2.

(A)

(B)

11 **Figure 3.**

12

13

14 **Figure 4.**

16 **Figure 5.**

17

18

19 **Figure 6.**

Figure 7.

Figure 8.