Elsevier Editorial System(tm) for Fish and Shellfish Immunology
Manuscript Draft

Manuscript Number: FSIM-D-16-00938R1

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

Article Type: Full Length Article

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

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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.

8th November, 2016

Ms. Ottavia Benedicenti

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Editors of Fish and Shellfish Immunology,

Prof Ikuo Hirono, Tokyo University of Marine Science and Technology, Tokyo, Japan
Prof Chris Secombes, University of Aberdeen, Aberdeen, UK

Dear Editors,

Please find attached our paper entitled "Characterisation of *arginase* paralogues in salmonids and their modulation by immune stimulation/ infection" that we are submitting for publication in your journal Fish and Shellfish Immunology. We hope the referees will find it suitable for publication in this journal.

Looking forward to hearing from you in due course.

Yours sincerely,

Ottavia Benedicenti

University of Aberdeen

Reviewer #1: The manuscript is a straightforward paper with clear information on the constitutive expression of different arginase isoforms in salmonids and also their modulation after stimuli.

Thank you very much.

Only some suggestions:

- The paper is long and could be reduced.

This has been done in lines 30-41; 98-99; 198-200; 229-230.

- The section of Material and Methods 2.5 includes many details about the pathogen that are not appropriate in this section.

The sentence: "Transmission of *Y. ruckeri* into the host normally occurs by direct contact with infected fish or carriers. The bacteria are thought to adhere to the gill mucus and then invade the branchial vascular system allowing colonisation of internal organs, including the spleen and kidney [41]" has been deleted.

-Abstract: the following sentences "However, arg1a was often higher than arg1b, with highest expression seen in the posterior kidney. The lowest expression of all isoforms was for arg2a in liver" is not relevant in the abstract section. I recommend the following sentence: "However, different constitutive expression patterns were observed for the different isoforms"

The sentence has been changed as follow: "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".

- In many cases the gene and protein nomenclature does not follow the ZFIN Zebrafish Nomenclature Guidelines (https://wiki.zfin.org/display/general/ZFIN+Zebrafish+Nomenclature+Guidelines), which is being extended to the other teleost species. Examples: the name of the recombinant proteins; elongation factor (page 10, line 283); page 10, lines 287-288, the gene symbols included in the tables.

The gene and protein names have been changed in the text and figures accordingly to the ZFIN Zebrafish Nomenclature Guidelines.

Reviewer #2: Manuscript Number: FSIM-D-16-00938

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

The work describes four arginase isoforms (*arg1a, arg1b, arg2a, arg2b*) in rainbow trout and Atlantic salmon, characterised by 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 *Y. ruckeri*, e) *Paramoeba perurans*, and *Tetracapsuloides bryosalmonae*.

The arginase gene is conserved and constitutively expressed, with the exclusion of little isoform variation between different tissues. When stimulating trout and salmon with bacteria and parasites, significant variations between isoforms have been observed, raising the hypothesis of employing arginase isoforms expression as a marker of macrophage type II responses. Authors have analysed a high number of samples deriving from *in vitro* and *in vivo* stimulation of fish with molecules, a vaccine, and with bacterial and parasite cells. Together, the work has been aimed to investigate the involvement of arginases genes by employing a comprehensive experimental approach and, in this respect, it represent a piece of work establishing clearly the involvement of Arg1a/b in the immune response of rainbow trout.

From the basal expression analysis appears evident that Arg1b behaves differently from the Arg family. The *in vitro* experiments included stimulation of kidney macrophages with a group of recombinant cytokines (PAMPS) shows high expression values and significance for Arg1, clearly different from the other isoforms. Same consideration applies in exps of Fig. 5, where Arg1 had the highest expression.

Although arginases are constitutively expressed, and thus it can be difficult to associate quantitative gene expression values to actual biological/physiological features, it is important that authors have employed a panel of recombinant cytokines given *in vitro* to investigate the modulation of arginases genes. This approach has been applied by the proposing group and give an additional value to the work, since authors come from the only group having the quantity and quality of recombinant factors employed.

It is my opinion that the work could be accepted for publication when authors will modify the manuscript accordingly with the minor remarks reported below.

Minor remarks:

-Given the similarity of arginases among vertebrates, the phylogenetic tree (Figure 1) should be given as accessory figure.

We have moved the phylogenetic tree analysis to the Supplementary figures, but have now included the synteny analysis which was previously a Suppl. Figure. This is because we accept the tree is not so informative but we want to confirm to the reader that the paralogues are from

a WGD event, and so believe it is important to verify this one way or another in the main body of the paper.

-In the Discussion, stress that vaccination/stimulation induced Arg isoforms predominantly in non-mucosal tissues.

In the discussion, we have now clarified that "In the vaccination experiment (Figure 5), the trout arginase isoforms were more highly induced at the systemic level in spleen, at day 1 (all isoforms) and day 3 (*arg1a*, *arg2b*), than in the mucosal tissue studied. Interestingly *arg1a* showed the highest up regulation in spleen and was not modulated in gills, in contrast to the other isoforms, which showed a predominant expression in the spleen but were also up regulated in the gills."

-In the Discussion, comment on the different expression pattern between stimulation with the same antigen given in two different forms, namely with ERM (Fig. 6) and an ERM vaccine.

We added this sentence to the text: "The results to live vs dead ERM also differed, as seen in the first few days post-vaccination or challenge (of unvaccinated fish). The most obvious differences were the later peak in *arg1b* expression in spleen, and the more pronounced (relative to the spleen response) increases of *arg2a/b* in the gills following challenge.

-When using eukaryotic pathogens (AGD, PKD) I see a significance only in the last column of Fig. 7 because, to my reading, a claimed statistical difference of samples in ranges +/- 1 expression units might not represent an actual biological difference. Authors should clearly comment this point in the discussion.

In Figure 7 and 8, we expressed the data as a fold change. However, this reviewer is not correct regarding the relative values of +/- 1 as a fold change. A fold change of - 0.5 represents a 2 fold decrease (1 / 0.5 = 2). Most of the AGD and PKD results are lower than - 0.5 and in some cases even lower than – 0.2 which means they have a >4-fold decrease. So we do not believe there is anything to comment on in the discussion.

Highlights

- Four arginase isoforms exist in rainbow trout and Atlantic salmon;
- Synteny analysis revealed they are paralogues of arginase type I and II;
- In macrophages type I were modulated by PAMPs/cytokines but type II by Il-4/13;
- Vaccination/ infection with *Y. ruckeri* also revealed isoform specific responses;
- Parasite infection (AGD in salmon, PKD in trout) down regulated both type I genes.

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

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29 **Abstract**

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

50 .

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

53 # 1. Introduction

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

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

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

86 activity 3-4 fold but these same cells do not increase nitrite production after
87 lipopolysaccharide (LPS, 50 mg/ml) stimulation [7]. Similar findings were obtained with
88 macrophage cultures from uninfected fish stimulated with cAMP but now LPS induced iNOS
89 expression and nitrite production [9, 10]. Modulation of arginase gene expression has also
90 been shown in salmonids during parasite infection. Arginase type I is up regulated in skin of
91 Atlantic salmon (*Salmo salar*) infected with sea lice (*Lepeophtheirus salmonis*) [6] but is
92 down regulated in posterior kidney after *Tetracapsuloides bryosalmonae* infection in rainbow
93 trout (*Oncorhynchus mykiss*) and in gills of salmon after *Paramoeba perurans* infection [11,
94 12]. Functional divergence of arginase type I and arginase type II in fish vs mammals has
95 been recently hypothesized in common carp, where it is suggested that arginase type II is a
96 better marker for alternatively activated macrophages in teleost fish rather than arginase type
97 I [5].
98 A whole-genome duplication (WGD) event occurred at the base of the teleost fish during
99 evolution, and a further WGD (the Ss4R salmonid-specific autotetraploidization event)
100 occurred in the common ancestor of salmonids about 80 Mya after their divergence from
101 Esociformes [13, 14]. Following genome duplication events, duplicated genes can either be
102 lost by pseudogenization or retained as two copies that can diverge by the partitioning of the
103 ancestral gene functions (i.e. subfunctionalization) or by the acquisition of a novel function
104 (i.e. neofunctionalization) [15]. In rainbow trout and Atlantic salmon four different isoforms
105 of arginase (*arg1a*, *arg1b*, *arg2a*, *arg2b*) have been found in this study, which has
106 characterised these molecules in terms of sequence analysis, constitutive expression in
107 different tissues, and modulated expression following stimulation of cultured head kidney
108 macrophages *in vitro* or after vaccination and/ or infection *in vivo*.

109 ## 2. Materials and methods

110 ### 2.1 Cloning of arginase isoforms and sequence analysis

111 The rainbow trout whole genome shotgun (WGS) sequence was searched with tBLASTn
112 [16], using fish Arginase I and II protein sequences. Candidate WGS contigs (contigs 25562,
113 *arginase 1a*; 44798, *arginase 1b*; 119897, *arginase 2a*; and 23604, *arginase 2b*) were
114 identified and exons predicted as described previously [17, 18]. Primers (Table 1) were
115 designed to the 5'- and 3'- untranslated regions (UTR) and used for PCR amplification and
116 cloning of the complete coding region using a mixed tissue cDNA sample. The cloning, DNA
117 and protein sequence analysis was as described previously [19, 20]. Briefly, the nucleotide
118 sequences generated were assembled and analysed using the AlignIR™ Software (LI-COR,

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

132 For the phylogenetic tree, protein sequences from other species were predicted from the
133 ENSEMBL (http://www.ensembl.org/index.html) or NCBI websites
134 (http://www.ncbi.nlm.nih.gov/) and verified in UniProt (http://www.uniprot.org/blast/).
135 Agmatine ureohydrolase or agmatinase was used as the outgroup for the phylogenetic tree as
136 it is an important evolutionary related enzyme also involved in arginine and proline
137 metabolism [2]. The software BioEdit [22] was used to align all protein sequences from
138 different species and generate a file for the Guidance2 server [23-25], where sequence
139 alignment was performed using Fast Fourier Transform, MAFFT v7 [21], as an algorithm for
140 Multiple Sequence Alignment (MSA) with a final score of 0.92 from the original alignment
141 and a statistical confidence cut-off score of 0.93 after the removal of unreliable columns [26].
142 A final resulting alignment of 239 amino acids was uploaded to MEGA v6 software [27] to
143 predict the best-fitting amino acid substitution model which was the LG [28] and Gamma site
144 heterogeneity model for arginase with the smallest Akaike information criterion (AIC) of
145 5177.901 and the lowest Bayesian information criterion (BIC) of 5591.264. Bayesian
146 phylogenetic analysis was performed using Bayesian Evolutionary Analysis, by means of the
147 Sampling Trees (BEAST) software package v1.7 [29] employing the best-fitting substitution
148 model (LG+G), an uncorrelated lognormal relaxed clock model [30], a Yule speciation
149 process, and a Unweighted Pair Group Method with Arithmetic Mean (UPGMA) starting
150 tree. Two runs of BEAST were performed, each with a Markov chain Monte Carlo (MCMC)
151 algorithm of 10,000,000 generations for Bayesian phylogenetic inference. Bayesian

152 Evolutionary Analysis Utility (BEAUti), Tracer v1.6 (http://beast.bio.ed.ac.uk/tracer) [31],
153 LogCombiner (http://beast.bio.ed.ac.uk/logcombiner) with 10% of burn-in, TreeAnnotator
154 (http://beast.bio.ed.ac.uk/treeannotator) and FigTree (http://beast.bio.ed.ac.uk/figtree) were
155 used to construct the phylogenetic tree. The software MatGAT (Matrix Global Alignment
156 Tool) was used to predict amino acid identity/ similarity between sequences using the
157 BLOcks amino acid SUbstitution 62 (BLOSUM62) Matrix [32]. The exon-intron structures
158 of arginase genes were determined in Salmobase for *S. salar*, ENSEMBL for *Danio rerio* and
159 *Homo sapiens*, and by aligning the mRNAs with the corresponding genomic DNA using
160 Splign (https://www.ncbi.nlm.nih.gov/sutils/splign/splign.cgi) for *Esox lucius* and *O. mykiss*
161 [33]. The exception was for *arg2b* in *O. mykiss* where the exons had to be inferred using the
162 genomic sequence for *O. mykiss arg2a* (accession number CCAF010119897.1) as it had the
163 highest query cover (99%) allowed for alignment in Splign (58% identity) and hence only
164 exon phase could be determined and not intron size. 100% identity of *arg2b* in *O. mykiss*
165 matched the accession number CCAF010123604.1, supporting the presence of this gene in
166 contig 23604. The synteny of the arginase loci was analysed in Salmobase using BLAST
167 search (tBLASTn).

168 **2.2 Fish maintenance and rearing condition**

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

175 Apparently healthy Atlantic salmon were held as presmolts and smolted at the Ellis aquarium
176 facility at the Marine Scotland Science Marine Laboratory, UK, in tanks containing 350 l of
177 34-35 ppt seawater with a flow-through of 180 l/h. The fish were acclimatized to 12°C for
178 two weeks prior to challenge, and fed daily with Skretting Atlantic Smolt diet at 1% body
179 weight/day.

180 **2.3 Constitutive expression of rainbow trout arginase isoforms in tissues**

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

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

186 **2.4 Modulation of rainbow trout arginase isoform expression in macrophages**

187 Head kidney macrophages were prepared from four individual fish as described previously
188 [34, 35]. At day 4 the primary macrophages were stimulated with polyinosinic:polycytidylic
189 acid (polyI:C, 50 μg/ml, Sigma-Aldrich, UK), peptidoglycan (PGN, 5 μg/ml, Invivogen),
190 trout recombinant (r) Il-1β (20 ng/ml, [36]), rIfn-γ (20 ng/ml, [37]), rIl-6 (100 ng/ml, [34]),
191 rTnf-α (isoform 3) (10 ng/ml, [38]), rIl-12A (p35a1/p40c, 1 μl/ml, [39]), rIl-12B
192 (p35a1/p40b1, 1 μl/ml, [39]), rIl-4/13A and rIl-4/13B (200 ng/ml, [18] for 4, 8 and 24 h). The
193 rTnf-α (isoform 3), rIl-12A and rIl-12B were produced in Chinese hamster ovary (CHO)
194 cells. The concentration chosen for each stimulant was deemed optimal from previous
195 studies. RNA extraction and real time RT-PCR analysis of gene expression in head kidney
196 macrophages was conducted as described below.

197 **2.5 Expression of rainbow trout arginase isoforms during *Y. ruckeri* vaccination and**
198 **challenge**

199 Enteric redmouth disease (ERM) caused by *Yersinia ruckeri* is responsible for significant
200 economic losses in salmonid aquaculture worldwide but can be prevented by vaccination
201 [40]. The modulated expression of trout arginase paralogues *in vivo* was first examined in
202 response to vaccination using a commercial ERM vaccine (AQUAVAC® ERM, MSD
203 Animal Health, UK). A group of 24 fish (approximately 50 g) were vaccinated by
204 intraperitoneal (i.p.) injection of 0.1 ml of vaccine, following the manufacturer's instructions.
205 The same number of fish was injected with phosphate buffer saline (PBS) as control. Fish
206 handling and experimental protocols complied with the Guidelines of the European Union
207 Council (2010/63/EU) for the use of laboratory animals, and were carried out under UK
208 Home Office project licence PPL 60/4013, approved by the ethics committee at the
209 University of Aberdeen. Six fish from both the vaccinated and control groups were killed at
210 1, 3, 7 and 14 days post vaccination. Spleen and gills were taken from each fish and
211 homogenised separately in TRI Reagent® (Sigma-Aldrich, UK). RNA extraction and cDNA
212 synthesis was as described previously [41] and samples were stored at -20°C ready for real
213 time RT-PCR analysis as described below.

214 We next studied the expression of the arginase paralogues following pathogen challenge of
215 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.

249 **2.8 Real time RT-PCR**

250 Real time RT-PCR was carried out using a 384 LightCycler® 480 (Roche Applied Science)
251 in a 10 µl reaction using SYBR® Green I (Invitrogen™, Carlsbad, USA) and IMMOLASE
252 ™ DNA Polymerase (Bioline, UK). 4 µl cDNA were used in each reaction and the real time
253 analysis program consisted of 1 cycle of denaturation (95 °C for 10 min), 40 cycles of
254 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
255 for 5 s and 75°C for 1 min. Program profiles differed for annealing temperature and time for
256 elongation (Tables 1 & 2). At least one of each real time RT-PCR primer pair was designed
257 to cross an exon-intron boundary to avoid amplification of genomic (g) DNA (Tables 1 & 2).
258 Primer efficiency was tested using 4 fold serial dilutions of cDNA from pooled RNA samples
259 and calculated by the 'LightCycler® 480 software version 1.5.1.62' (Roche Applied Science)
260 as $E = 10^{(-1/s)}$, where s is the slope generated from the Log dilution of cDNA plotted against
261 Cp (cycle number of crossing point) [44].

262 **2.9 Statistical analysis**

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

280 # 3. Results

281 ## 3.1 Cloning and sequence analysis

282 Four rainbow trout arginase cDNA sequences (accession numbers KX998965 for Arginase
283 1a, KX998966 for Arginase 1b, KX998967 for Arginase 2a and KX998968 for Arginase 2b)
284 have been cloned and sequence analysed. Each cDNA sequence had an in frame stop codon
285 before the main open reading frame (ORF), a complete ORF and a partial 3'-UTR. The main
286 ORF encoded 338 amino acids (aa), 337 aa, 347 aa and 347 aa for Arginase 1a, 1b, 2a and 2b,
287 respectively (see Suppl. Figures S1-S4). Using the trout arginase protein sequence as bait, the
288 Atlantic salmon counterparts were identified in Salmobase. The salmonid orthologues
289 between trout and salmon share higher identities of 95.9%, 97.9%, 96.0% and 97.7% for
290 Arginase 1a, 1b, 2a and 2b, respectively, than paralogues, i.e. 93.2-93.8% identities between
291 Arginase 1a and 1b, and 93.7-95.4% identities between 2a and 2b (Table 3). The salmonid
292 arginase type I paralogues have lower identities (51.9-53.4%) to salmonid arginase type II
293 paralogues, similar to those to arginase type II from third round (3R) whole genome
294 duplication (WGD) event fish species (eg. *E. lucius*, *D. rerio*, *Takifugu rubripes* and
295 *Oreochromis niloticus*, 50.6-53.8% identities) and tetrapods (52.8-55.1% identities) (Table
296 3). As expected, salmonid arginase type I paralogues shared the highest identities to northern
297 pike (*E. lucius*) Arginase 1 (87.9-91.4%), high identities to 3R fish Arginase 1 (67.5-77.1%),
298 medium identities to 2R spotted gar (*Lepisosteus oculatus*) Arginase 1 (62.8-63.3%), and
299 lowest identities to tetrapod arginase type I (57.2-60.2%) (Table 3). Similarly, salmonid
300 arginase type II paralogues share the highest identities to northern pike Arginase 2 (88.5-
301 90.2%), have relatively high identities to other 3R fish arginase type II (73.0-81.0%), medium
302 identities to 2R spotted gar arginase type II (64.3-65.5%), and lowest identities to tetrapod
303 arginase type II (61.7-64.1%) (Table 3).

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

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

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

323 **3.2 Constitutive expression of rainbow trout arginase isoforms in tissues**

324 The constitutive expression of the four arginase isoforms was studied in 17 different tissues
325 from healthy rainbow trout by real time RT-PCR (Figure 3). Statistical analysis was
326 performed for each gene among the different tissues, starting with a general `aov` model (R
327 software, v3.0.1), and then grouping together the most similar parameter estimates in a
328 stepwise *a posteriori* procedure to combine non-significant factor levels until the models'
329 comparison was significant ($p < 0.05$). Diagnostic plots of the final model were always
330 performed to validate that linear model assumptions were met (results not shown). The final
331 model of *arg1a* analysis showed that the tissues grouped based on their expression as
332 follows, from lowest to highest: 1) intestine; 2) adipose tissue, spleen, blood, head kidney,
333 scales and tail fin; 3) adipose fin, brain, heart, gonad, muscle and thymus; 4) skin; 5) gills and
334 liver; 6) posterior kidney. For *arg1b* the groups were: 1) tail fin, intestine and scales; 2)
335 adipose tissue, spleen and head kidney; 3) adipose fin and posterior kidney; 4) blood, muscle
336 and thymus; 5) gills; 6) brain, skin, heart, gonad and liver. For *arg2a* the tissues were
337 grouped as: 1) liver; 2) tail fin; 3) adipose tissue, intestine, posterior kidney, head kidney,
338 heart and skin; 4) blood, gonad, scales, gills, spleen, brain and thymus; 5) adipose fin and
339 muscle. Lastly, for *arg2b* the groups were: 1) liver; 2) intestine and tail fin; 3) adipose tissue,
340 spleen, gills and scales; 4) adipose fin, skin, blood, posterior kidney, gonad, head kidney and
341 thymus; 5) heart; 6) brain and muscle. Overall, constitutive expression of one or both
342 paralogues was seen in most tissues. However, *arg1a* was often higher than *arg1b*, with
343 highest expression seen in the posterior kidney. As in mammals, the expression of type I
344 arginase was much higher in liver in comparison with type II, with almost no expression for
345 *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-1β, rIl-6, rIfn-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-1β (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 rIfn-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 *arg*2a 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

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

414 4. Discussion

415 Arginase, a ubiquitous enzyme found in prokaryotic and eukaryotic organisms, is responsible
416 for cleaving the guanidine group from arginine into ornithine and urea [1, 2]. Invertebrates
417 have only one type of arginase, localized in mitochondria, while most vertebrates have two
418 types as a consequence of a gene duplication that occurred after the separation of vertebrates
419 and invertebrates [2, 3]. These two arginase genes have diverged in terms of where and when
420 they are expressed. For example, type I arginase is cytoplasmic and expressed in liver as part
421 of the urea cycle whilst type II is a mitochondrial enzyme, likely the surviving form of the
422 ancestral gene, and is expressed in a variety of peripheral tissues [4]. Similarly within the
423 immune system the roles of these two isoforms differ, as seen in mammalian macrophages
424 which can express both types [48]. Classically type I arginase is expressed in M2
425 macrophages, associated with anti-inflammatory responses and wound healing. In contrast
426 type II plays a role in pro-inflammatory responses of macrophages and is expressed upon
427 activation of M1 cells by LPS, during monocyte maturation to macrophages and in some
428 chronic disease states [49]. It is not clear that the above mammalian paradigm will hold true
429 in all vertebrate groups that express these two arginase isoforms, and indeed in fish there is
430 evidence to suggest that type II may be a good marker of M2 macrophages in carp [5-8]. In
431 salmonid fish, that have undergone a further round of WGD (Ss4R), the situation may be
432 even more complex with the potential for further paralogues of arginase to be present. Hence
433 this study initially characterised the arginase genes found in trout and salmon, and then
434 examined their constitutive expression in a range of tissues, and after immune stimulation *in*
435 *vitro* and *in vivo*, to assess whether there was evidence of functional divergence between the
436 genes present.
437

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

443 to form a sub-clade separate from lobe-finned fish and tetrapods. Interestingly only a single
444 type of arginase, type II, appears present in the coelacanth (*L. chalumnae*), lizards (*A.*
445 *carolinensis*) and birds (*F. albicollis*), with gene loss of type I in the latter (reptiles and birds)
446 hypothesised to be linked to the transition to land and a further adaptation to dispose the
447 excess of ammonium nitrogen into uric acid (uricotelic) [1, 50]. Occurrence of the duplicated
448 genes in salmonids is most likely a result of the known ancestral WGD event that occurred in
449 this fish lineage, as suggested by the homology and gene synteny analysis (Table 3, Figure 1)
450 [13].

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

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

475 6, rI-12A, rIl-12B, rIfn-γ, rTnf-α – isoform 3). The expression of *arg1a* was much more
476 highly induced in comparison to the other isoforms, with *arg1b* the least inducible. Moreover,
477 the highest transcript levels of *arg1a* were seen at 24 h after stimulation (with polyI:C, PGN,
478 rIl-1β, rIl-6 and rTnf-α), although a degree of up regulation was also apparent at 8 h.
479 Interestingly, *arg1a* and *arg1b* were significantly down regulated after stimulation with rIl-
480 4/13A (at 8 h for *arg1a* and 24 h for *arg1b*). The two type II arginases differed in their
481 kinetics of induction with *arg2a* being up regulated at 4 h by a range of stimulants in
482 comparison to *arg2b* but both isoforms were significantly induced by rIl-4/13A (*arg2a* at 4 h,
483 *arg2b* at 4 h, 8 h and 12 h). These results hint that in relation to trout macrophage expression
484 type I arginase may play a role in pro inflammatory responses (M1) whilst type II appears
485 induced by type II cytokines (i.e. Il-4/13) and is potentially a marker of M2 cells (M2a) as in
486 carp [5].

487 We next studied the expression of the arginase isoforms *in vivo* in trout after vaccination and/
488 or challenge with *Y. ruckeri*, the causative agent of enteric redmouth disease (ERM) in
489 salmonid fish species, and in trout and salmon after parasitic infection. In the vaccination
490 experiment, the trout arginase isoforms were more highly induced at the systemic level in
491 spleen, at day 1 (all isoforms) and day 3 (*arg1a*, *arg2b*), than in the mucosal tissue studied.
492 Interestingly *arg1a* showed the highest up regulation in spleen and was not modulated in
493 gills, in contrast to the other isoforms, showing a predominant expression in the spleen but
494 were also up regulated in the gills. In the vaccination/ challenge experiment, the different
495 isoforms also had different expression profiles in trout after infection, in terms of tissue, level
496 and kinetics of expression. The *arg1a* transcript was more highly induced by *Y. ruckeri* than
497 *arg1b* in unvaccinated fish and up regulation of these paralogues was highest in the spleen,
498 especially at day 2. In contrast *arg2a* and *arg2b* showed highest up regulation in the gills of
499 challenged/ unvaccinated fish, with *arg2a* induction highest at day 1 but *arg2b* at day 2. As
500 seen with pro-inflammatory gene expression in this host-pathogen model [41], vaccinated
501 fish exposed to *Y. ruckeri* typically showed lower induction levels of the arginase isoforms in
502 comparison to the challenged/ unvaccinated fish. The results to live vs dead ERM also
503 differed, as seen in the first few days post-vaccination or challenge (of unvaccinated fish).
504 The most obvious differences were the later peak in *arg1b* expression in spleen, and the more
505 pronounced (relative to the spleen response) increases of *arg2a/b* in the gills following
506 challenge. Clearly the isoforms have diverged in their tissue expression pattern when
507 comparing type I and type II genes, and also in their level of induction (as seen with the type

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

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

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

537 Ethics statement

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

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

542 Acknowledgements

543 OB was supported by a PhD studentship from the Marine Collaboration Research Forum
544 (MarCRF), which is a collaboration between the University of Aberdeen and Marine
545 Scotland Science, Marine Laboratory (MSS), and through Scottish Government project
546 AQ0080. EW was supported by a PhD studentship from the Ministry of Science and
547 Technology of Thailand and Mahasarakham University. TW received funding from the
548 Marine Alliance for Science and Technology for Scotland (MASTS), a pooling initiative
549 funded by the Scottish Funding Council (grant reference HR09011), and JWH was supported
550 by the Swiss National Science Foundation (grant reference CRSII3_147649-1).

551

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 *ef1α*.

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 *ef1α*. 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

582 control fish, normalized to *ef1α*. A linear model (`lm`) with the Holm-Bonferroni correction
583 method for multiple comparisons were used for statistical analysis (R software, v3.0.1). * = p
584 < 0.05, ** = p < 0.01, *** = p < 0.001.

585 **Figure 7.** Relative expression of Atlantic salmon *arg1a*, *arg1b*, *arg2a*, *arg2b* (mean ± SEM,
586 n = 5) in gill samples infected with a polyclonal or clonal culture of *P. perurans* at two
587 trophozoite doses. Expression was determined using REST© 2009 (relative expression
588 software tool), and the fold change calculated as the relative expression in comparison to gills
589 from control fish, normalized to *ef1α*. * = p < 0.05, ** = p < 0.01, *** = p < 0.001.

590 **Figure 8.** Relative expression of rainbow trout *arg1a*, *arg1b*, *arg2a*, *arg2b* in kidney samples
591 infected with *T. bryosalmonae*, presented as a fold change relative to control kidney samples,
592 after normalizing to *ef1α*. A kidney swelling grade from 0 (control fish) to 3 (higher swelling
593 grade) was assigned to each fish according to the kidney swelling index of [43]. A linear
594 model (`lm`) with the Holm-Bonferroni correction method for multiple comparisons were used
595 for statistical analysis (R software, v3.0.1). * = p < 0.05, ** = p < 0.01, *** = p < 0.001, n =
596 10 for uninfected fish; n = 5 for fish exhibiting grade 1; n = 9 for fish exhibiting grade from 1
597 to 2; n = 10 for fish exhibiting grade 2; n = 9 for fish exhibiting grade 3.

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

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

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

604

605 ## 6. References

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Table

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 (%)
ef1α	Forward Reverse	Real time RT-PCR	CAAGGATATCCGTCGTGGCA ACAGCGAAACGACCAAGAGG	AF321836	63 – 30	> 99
arg1a	Forward Reverse	PCR cloning	ATTCTGAGCCGCTAACCCTTG CACTGTAATCGAAAGGCTCTGTGG			
arg1a	Forward Reverse	Real time RT-PCR	CAGAGGTGGATCGCCTTGGAATA GCAGACAGCATCCCTGTCTGACA	KX998965	66 – 20	> 94
arg1b	Forward Reverse	PCR cloning	GGCAAAGATGAGTTATGCAATTTTAGTG TAATACAAAATATTGCGTTTGATGGC			
arg1b	Forward Reverse	Real time RT-PCR	AGGTGGATCGCCTTGGAATCG GCAGACAGCAGCCCTGTCTGACT	KX998966	66 – 20	> 96
arg2a	Forward Reverse	PCR cloning	TCTCAGCCTTGGTCGTTAAAC TGCCAAGTGGTCACATGTTGAAAG			
arg2a	Forward Reverse	Real time RT-PCR	TCCAGAGAGTCATGGAAGTCACTTTCC CCATCACTGACAACAACCCTGTGTT	KX998967	66 – 20	> 96
arg2b	Forward Reverse	PCR cloning	GCAGCCTTGGTCGTTAAACGG GCCAAGTGGTTACATGTTGAGTC			
arg2b	Forward Reverse	Real time RT-PCR	TCCAGAGAGTCATGGAAGTCTCTTTCG CATCACCGACAACAACCCTGTGTT	KX998968	66 – 20	> 94

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 (%)
ef1α	Forward Reverse	CAAGGATATCCGTCGTGGCA ACAGCGAAACGACCAAGAGG	AF321836	63 – 30	> 99
arg1a	Forward Reverse	CAGAGGTGGATCGCCTTGGAATA GCAGACAGCATCCCTGTCTGACA	XP_014013843.1	66 – 25	> 99
arg1b	Forward Reverse	AGGTGGATCGCCTTGGAATCG CAGACAGCAGCCCTGTCTGACA	NP_001134788.1	66 – 25	> 86
arg2a	Forward Reverse	GACCACCTCTTGTCAAGGAAGCA CTCACGGGTCTGTCCTAGGGC	XP_014045709.1	66 – 20	> 97
arg2b	Forward Reverse	GACCACCTCTTGTCAAGGAAGCA CCATGGAAGCGGTGCTCG	XP_014067199.1	66 – 20	> 88

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

	O. mykiss **1a** (KX998965)	*S. salar* **1a** (XP_014013843.1)	*O. mykiss* **1b** (KX998966)	*S. salar* **1b** (NP_001134788.1)	*E. lucius* **1** (XP_010863350.1)	*D. rerio* **1** (E7F8R4)	*O. niloticus* **1** (I3KSD9)	*T. rubripes* **1** (H2RY07)	*L. oculatus* **1** (W5NIT3)	*B. taurus* **1** (Q2KJ64)	*H. sapiens* **1** (P05089-2)	*X. tropicalis* **1** (F7CN24)	*O. mykiss* **2a** (KX998967)	*S. salar* **2a** (XP_014045709.1)	*O. mykiss* **2b** (KX998968)	*S. salar* **2b** (XP_014067199.1)	*E. lucius* **2** (XP_010877736.1)	*D. rerio* **2** (Q6PH54)	*O. niloticus* **2** (I3KUB9)	*T. rubripes* **2** (H2SAE9)	*L. oculatus* **2** (W5NA23)	*B. taurus* **2** (F1N1Z5)	*H. sapiens* **2** (P78540)	*X. tropicalis* **2** (Q05AR1)
O. mykiss **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**
S. salar **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**
O. mykiss **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**
S. salar **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**
E. lucius **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**
D. rerio **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**
O. niloticus **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**
T. rubripes **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**
L. oculatus **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**
B. taurus **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**
H. sapiens **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**
X. tropicalis **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**
O. mykiss **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**
S. salar **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**
O. mykiss **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**
S. salar **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**
E. lucius **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**
D. rerio **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**
O. niloticus **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**
T. rubripes **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**
L. oculatus **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**
B. taurus **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**
H. sapiens **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**	**79.4**	**74.0**	**96.9**		**71.7**
X. tropicalis **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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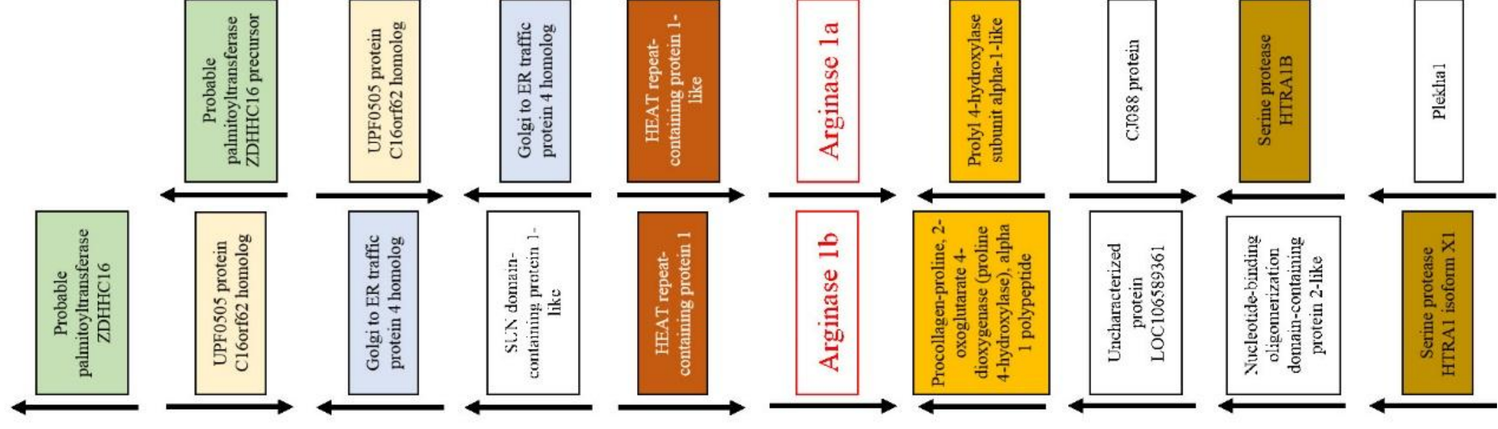

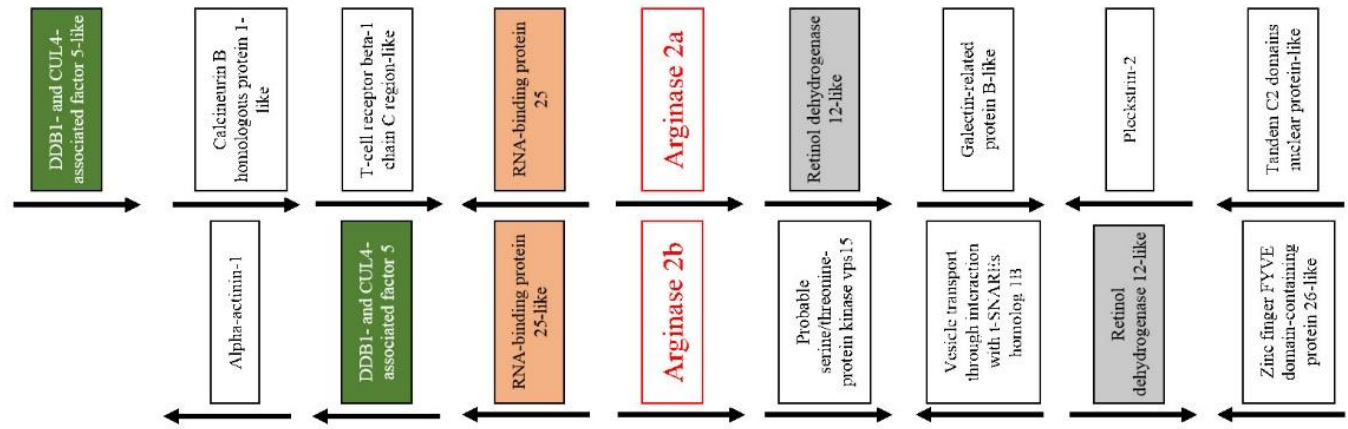

Figure 2.

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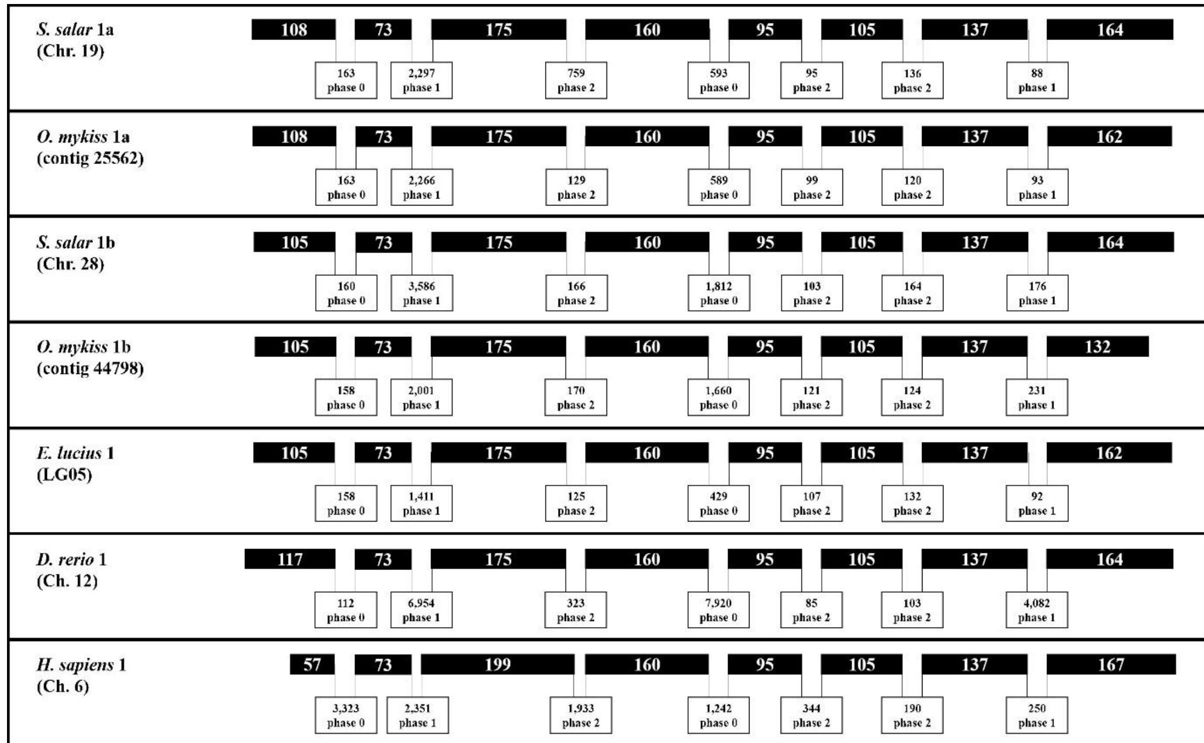

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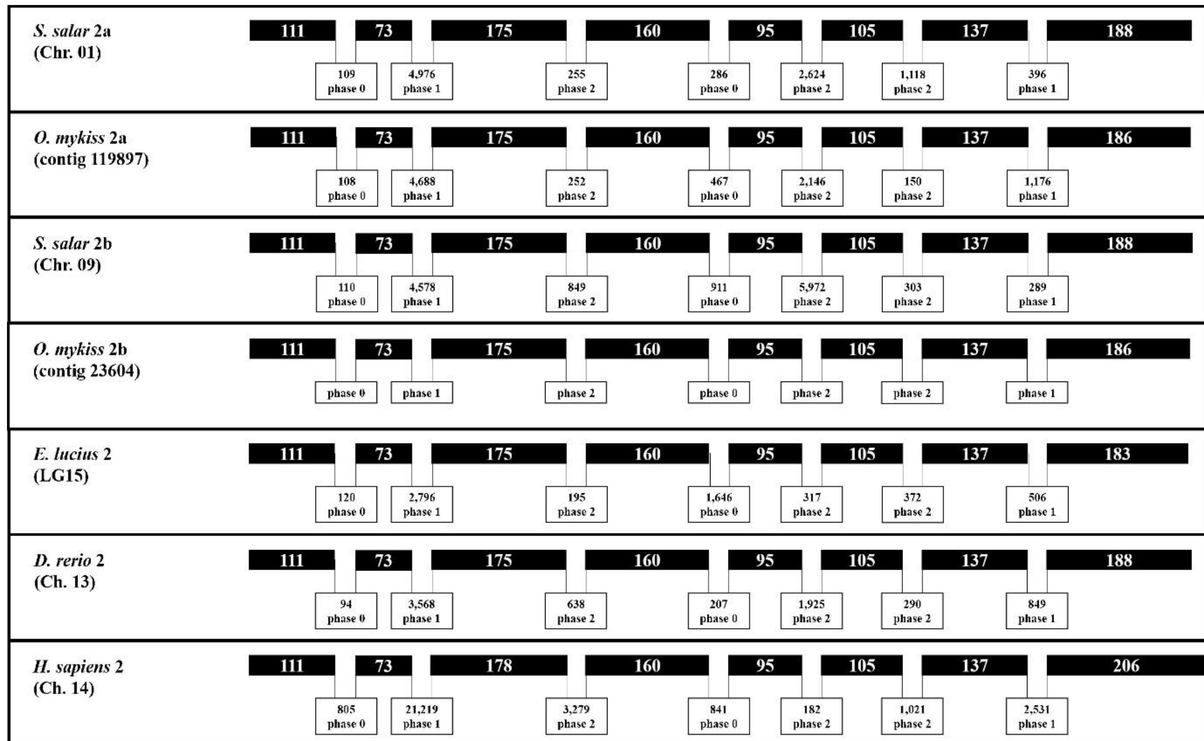

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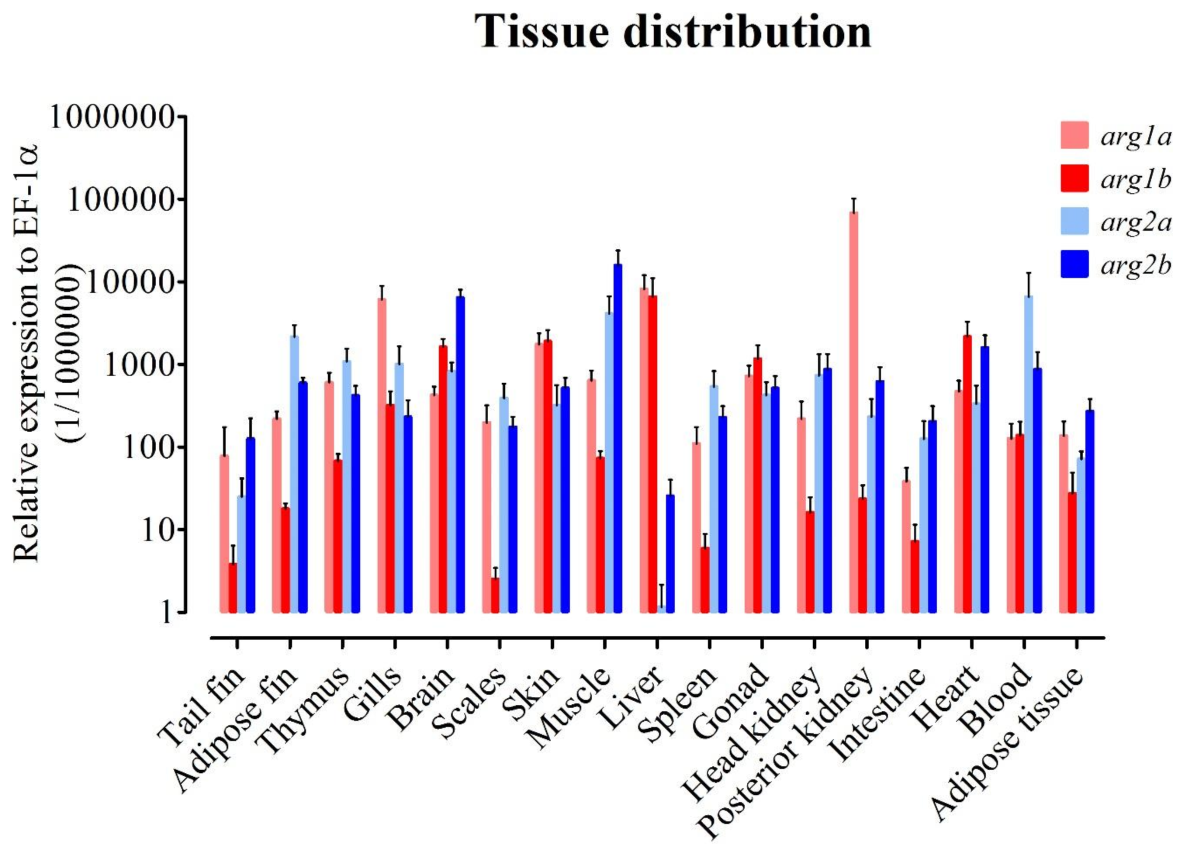

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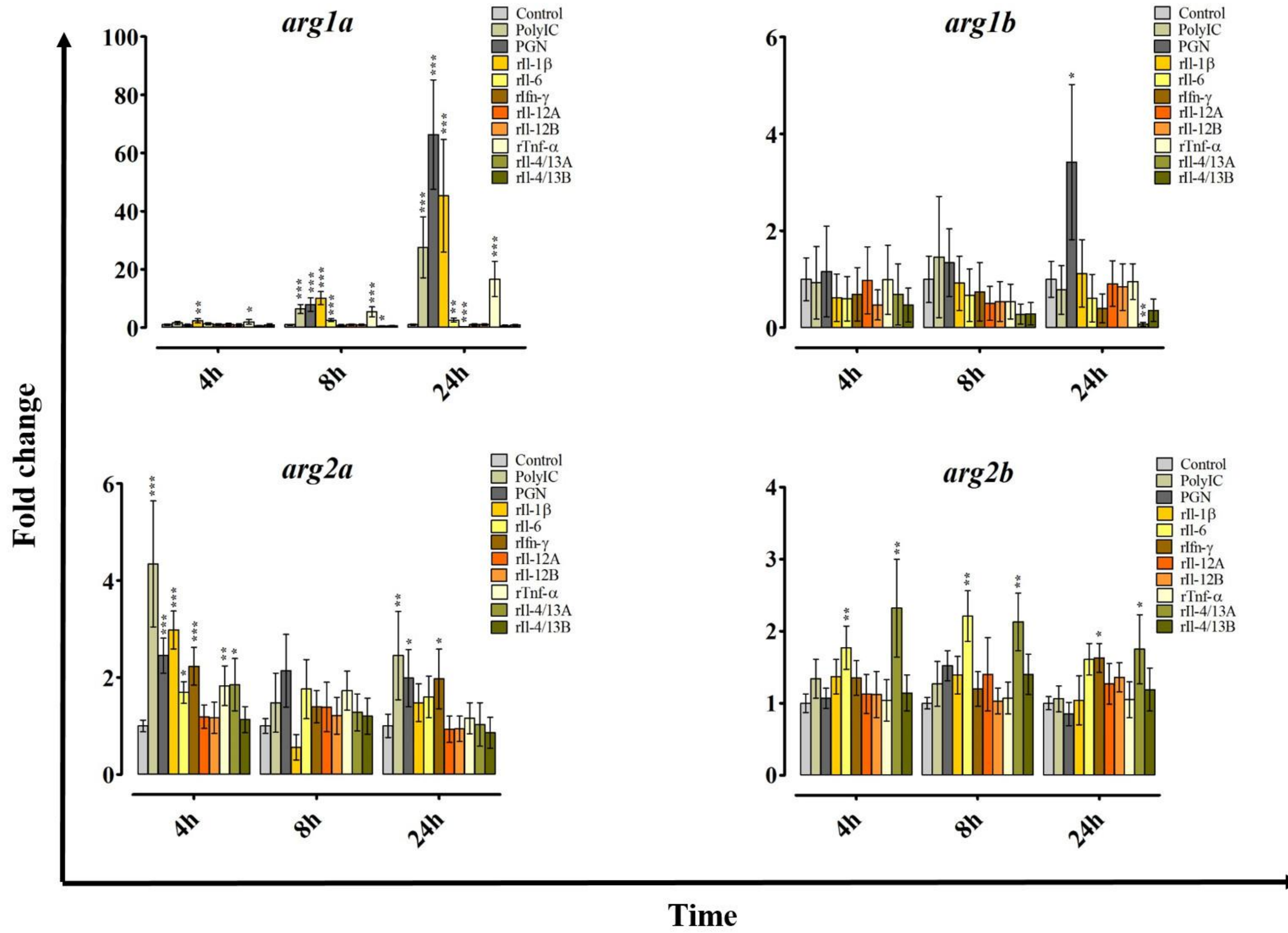
14 **Figure 4.**



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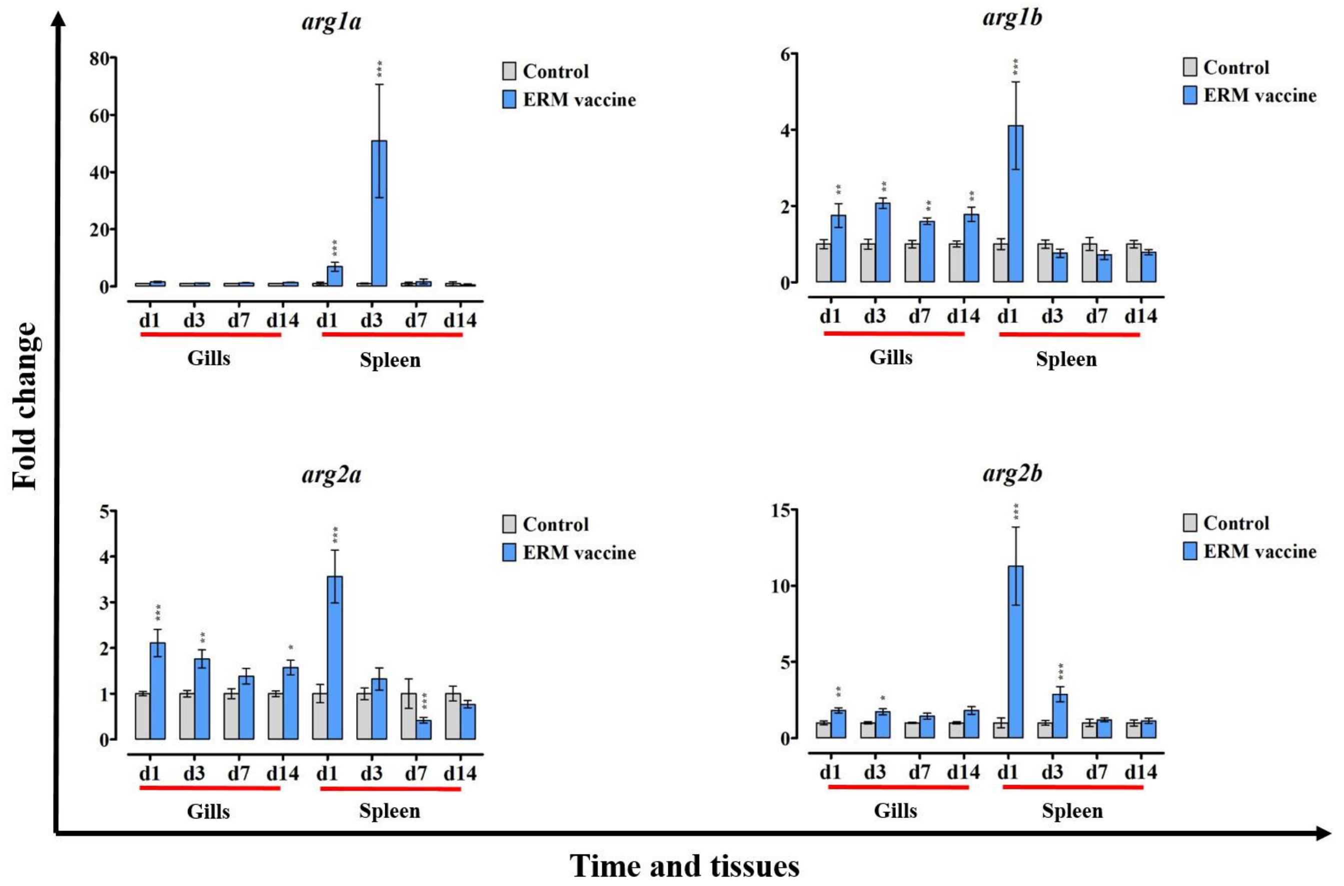
16 **Figure 5.**



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19 **Figure 6.**

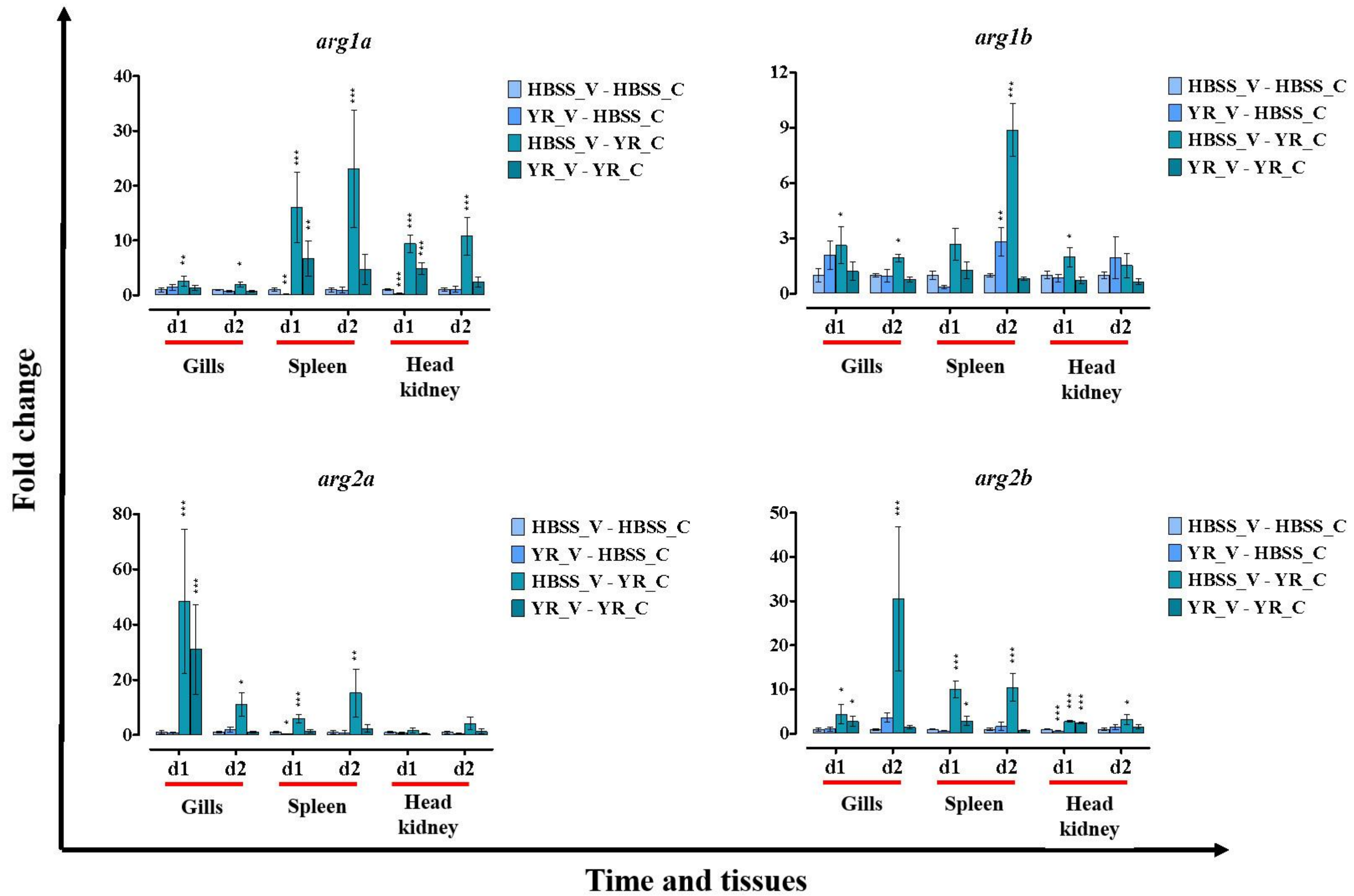

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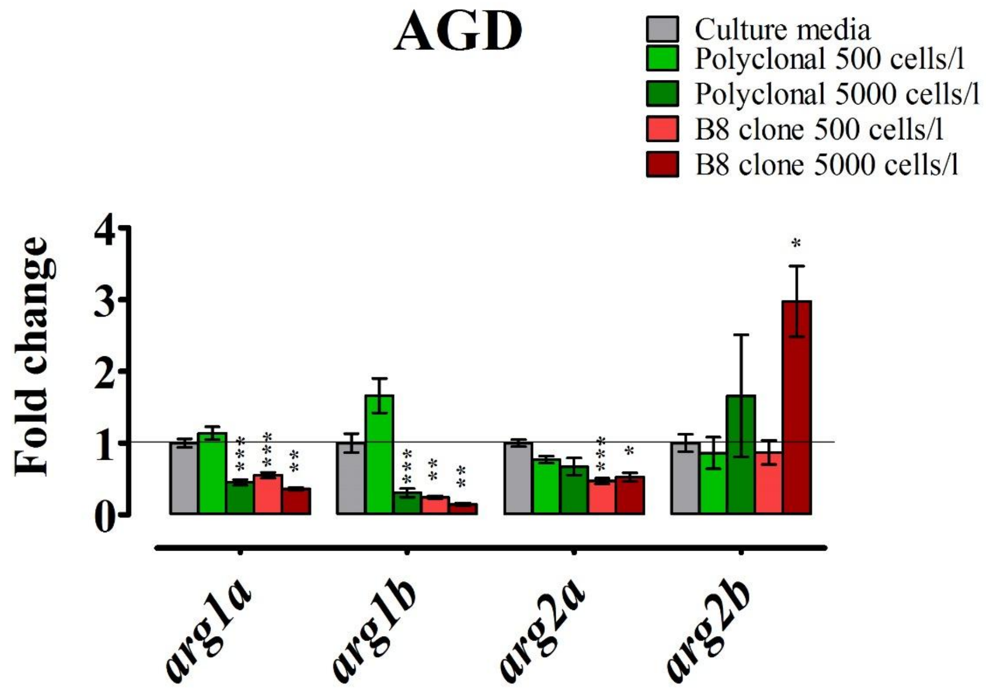
Figure 7.



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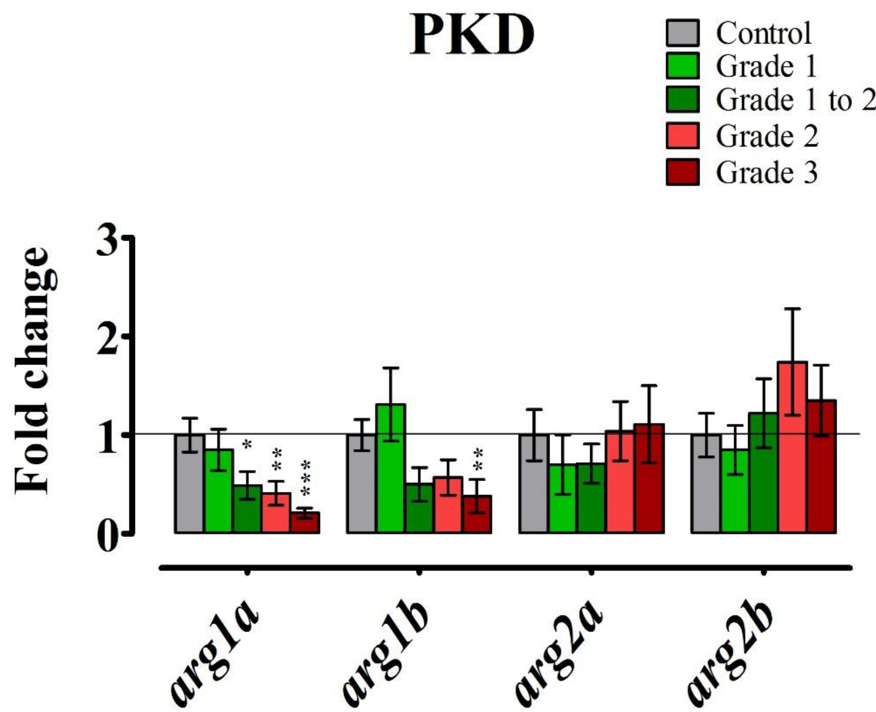

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