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1 **Rainbow trout (*Oncorhynchus mykiss*) urea cycle and polyamine synthesis gene**  
2 **families show dynamic expression responses to inflammation**

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26 **Abstract**

27 The urea cycle is an endogenous source of arginine that also supports removal of  
28 nitrogenous waste following protein metabolism. This cycle is considered inefficient in  
29 salmonids, where only 10-15 percent of nitrogenous waste is excreted as urea. In rainbow  
30 trout, arginine is an essential amino acid that has attracted attention due to its many  
31 functional roles. These roles include the regulation of protein deposition, immune responses  
32 and polyamine synthesis; the latter is directly linked to the urea cycle and involved in tissue  
33 repair. The key enzymes used in the urea cycle, namely arginase, ornithine  
34 transcarbamylase, argininosuccinate synthase and argininosuccinate lyase, in addition to  
35 two rate limiting enzymes required for polyamine synthesis (ornithine decarboxylase and s-  
36 adenosylmethionine decarboxylase) are poorly studied in fishes, and their responses to  
37 inflammation remain unknown. To address this knowledge gap, we characterised these gene  
38 families using phylogenetics and comparative genomics, investigated their mRNA  
39 distribution among a panel of tissues and established their transcriptional responses to an  
40 acute inflammatory response caused by bacterial infection in liver and muscle. Gene  
41 duplicates (paralogues) were identified for arginase (*ARG1a*, *1b*, *2a* and *2b*), ornithine  
42 decarboxylase (*ODC1* and *2*) and s-adenosylmethionine decarboxylase (*SAMdc1* and *2*),  
43 including paralogues retained from an ancestral salmonid-specific whole genome  
44 duplication. *ARG2a* and *2b* were highly upregulated following bacterial infection in liver,  
45 whereas *ARG1b* was downregulated, while both paralogues of *SAMdc* and *ODC* were  
46 upregulated in both liver and muscle. Overall, these findings improve our understanding of  
47 the molecules supporting the urea cycle and polyamine synthesis in fish, highlighting major  
48 changes in the regulation of these systems during inflammation.

49

50 Key words: Urea cycle, polyamine, salmonids, genome duplication, immune, arginase

51

52

53 **1. Introduction**

54 The ornithine-urea cycle, first discovered by Krebs and Henseleit [1], is central to the  
 55 metabolism of arginine and the excretion of nitrogenous waste. In fish, most nitrogenous  
 56 waste is excreted as ammonia through the gills, with <10% excreted as urea in rainbow trout  
 57 [2]. There are four main enzymes directly involved in the urea cycle, arginase, ornithine  
 58 transcarbamylase, argininosuccinate synthase and argininosuccinate lyase, which  
 59 metabolise and recycle arginine, ornithine, citrulline and argininosuccinate [3]. Arginine is a  
 60 versatile amino acid with functional roles including the modulation of protein deposition,  
 61 production of ornithine for polyamine synthesis, regulation of immune responses through  
 62 nitric oxide (NO) production, and removal of nitrogenous waste [4]. Arginine also stimulates  
 63 the release of insulin, glucagon and growth hormone in fishes, which may regulate  
 64 metabolism and growth [5].

65

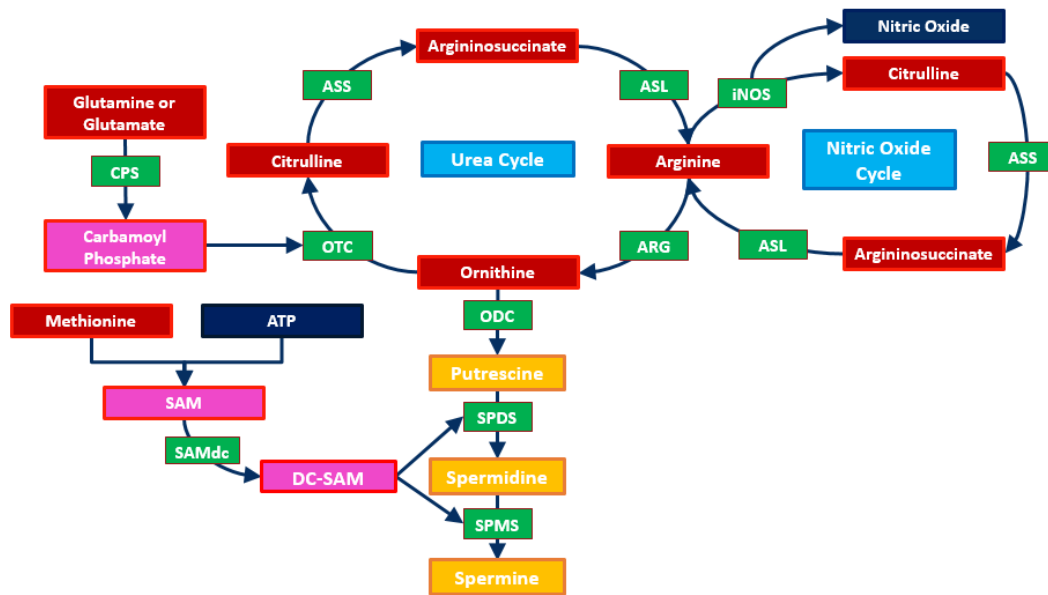


Figure 1. Pathway diagram of urea cycle, nitric oxide cycle and polyamine synthesis. Amino acids are coloured in red boxes, enzymes in green, polyamines in orange, co-substrates in pink and molecules as dark blue. Enzyme acronyms are as follows: ARG, arginase; OTC, ornithine transcarbamylase; ASS, argininosuccinate synthase; ASL, argininosuccinate synthase; CPS, carbamoyl phosphate synthase; iNOS, nitric oxide synthase; SAMdc, S-adenylosylmethionine decarboxylase; ODC, ornithine decarboxylase; SPDS, spermidine synthase; SPMS, spermine synthase. Co-substrate acronyms are as follows: SAM, s-adenosylmethionine; DC-SAM, decarboxylated s-adenosylmethionine.

66 During the urea cycle (Fig. 1), arginine is converted to ornithine by the arginase (ARG)  
 67 enzymes, resulting in urea as the by-product. Ornithine can then be converted into citrulline  
 68 by ornithine transcarbamylase (OTC), used in proline synthesis by the action of ornithine  
 69 aminotransferase, or for polyamine synthesis, where ornithine decarboxylase (ODC)  
 70 converts ornithine into the polyamine putrescine [6]. Recycling citrulline back into arginine is

71 a two-step process that involves two further enzymes (*ASS* and *ASL*), as well as  
72 argininosuccinate as an intermediate [7]. Citrulline and argininosuccinate also form part of  
73 the NO cycle (Fig. 1), as citrulline is generated as a by-product following conversion of  
74 arginine to NO through the action of the NO synthase enzymes [8, 9].

75 An important set of molecules derived from the urea cycle are the polyamines, containing  
76 two or more amine groups (-NH<sub>2</sub>). The diverse functions of polyamines include regulation of  
77 protein synthesis [10], modulation of ion channels [11] and DNA and RNA binding [12]. They  
78 are also crucial for cellular proliferation and inflammatory responses [13], acting as anti-  
79 oxidants and offering cellular protection through inhibition of inflammatory mediators [14].  
80 Polyamines are derived from ornithine by the activity of *ODC* and s-adenylosylmethionine  
81 decarboxylase (*SAMdc*), with the simplest polyamine being putrescine, which can be further  
82 processed to spermidine and spermine (Fig. 1) [7]. During the immune response, high  
83 polyamine levels are found in rapidly proliferating cells and tissues [15, 16] supporting  
84 wound and tissue healing following infection or injury [6, 17].

85 The role of arginine and urea cycle products is attracting attention due to their roles in  
86 mediating immune functions. Inflammatory responses are associated with polarising T<sub>H</sub>1  
87 cytokines including IFN- $\gamma$ - or TNF $\alpha$ -activating M1 macrophages ('kill' macrophages),  
88 whereas anti-inflammatory processes activate M2 macrophages ('healing' macrophages)  
89 associated with T<sub>H</sub>2 cytokines including interleukin 4 and 10 (*IL-4*, *IL-10*) [18]. M1  
90 macrophages metabolise arginine into NO via inducible nitric oxide synthase (*iNOS*),  
91 resulting in a macrophage population with increased microbicidal activity [19]. On the other  
92 hand, anti-inflammatory responses and healing is associated with M2 cells, where arginine is  
93 converted to ornithine and subsequently metabolised to polyamines through *ODC* [20]. As  
94 M1 and M2 macrophages compete for arginine, the expression of either *iNOS* or arginase  
95 has a reciprocal regulatory effect [21]. Arginine is the sole precursor of NO and  
96 supplementation is known to increase NO synthesis in mammals [22].

97 Arginine is a functional feed that can modulate health and performance parameters in  
98 farmed fish. For example, arginine supplementation may enhance growth in Atlantic salmon  
99 during the transition from fresh to seawater [23], improve immune status in carp [24] and  
100 when combined with glutamine, enhance growth and feeding efficiency in Nile tilapia [25].  
101 Despite such recent interest, little is known about the gene families encoding the enzymes  
102 involved in arginine metabolism and their response to disease and inflammation in fishes.

103 The first objective of this study was to identify and characterise the genes encoding the main  
104 urea cycle enzymes and the two rate limiting enzymes in polyamine synthesis in rainbow  
105 trout, including any paralogues retained during salmonid evolutionary history. The second

106 objective was to establish the mRNA expression of these genes in rainbow trout, performed  
107 in a panel of tissues under control conditions, and following a bacterial pathogen challenge  
108 in liver and muscle. The resultant data implies an important role for arginine in both  
109 inflammation and tissue repair.

110

## 111 **2. Materials and Methods**

### 112 **2.1. Animal work**

113 All procedures described hereafter were carried out in compliance with the Animals  
114 (Scientific Procedures) Act 1986 under UK Home Office license PPL number 70/8071 and  
115 approved by the ethics committee at the University of Aberdeen. Juvenile rainbow trout were  
116 purchased from College Mill Trout Farm (Perthshire, U.K.). The fish were kept at the  
117 University of Aberdeen aquarium facility (School of Biological Sciences) in 400 L tanks at a  
118 stocking density of <math><20\text{ kg/m}^3</math>. Tanks were supplied with recirculating freshwater with a flow  
119 rate of 1.5 L/s. Fish were kept at a temperature of  $14 \pm 1^\circ\text{C}$  and a photoperiod of 12:12  
120 light:dark. A computerised control system was used to monitor pH, ammonia concentration  
121 and oxygen levels. Fish were fed *ad libitum* daily with commercial pellets.

122 To assess candidate gene expression responses across tissues,  $n = 4$  adult rainbow trout  
123 ( $499 \pm 54\text{ g}$  mean  $\pm$  SEM) were used to sample a standard panel of tissues; gill, distal  
124 intestine, heart, head kidney, liver, fast-twitch skeletal muscle and spleen (within 5 minutes  
125 of death). Tissues were stored in 1.5 ml RNA later at  $4^\circ\text{C}$  for 24 h followed by long term  
126 storage at  $-80^\circ\text{C}$ .

127 For the bacterial immunological stimulation, fish (as described above) were anaesthetised by  
128 immersion in 2-phenoxyethanol and then injected intraperitoneally (ip) with either phosphate  
129 buffered saline (PBS) (0.5 ml/fish) or the pathogenic Hooke strain of the live Gram-negative  
130 bacterium *Aeromonas salmonicida* (AS) ( $1.6 \times 10^6\text{ ml}^{-1}$  cells, 0.5 ml/fish). After ip injection,  
131 the fish were maintained in 400 L tanks in the University of Aberdeen's freshwater challenge  
132 facility for 48 h. After 48 h,  $n=10$  fish from both PBS and AS groups were randomly sampled  
133 and killed as previously described and both liver and fast-twitch skeletal muscle tissue  
134 sampled and stored in RNA later as described above.

135

### 136 **2.2. Sequence, phylogenetic and genomic analysis**

137 Putative protein-coding nucleotide sequences (cds) for candidate genes were originally  
138 obtained from the rainbow trout genome hosted at <https://www.genoscope.cns.fr/trout/> (NCBI

139 accession: GCA\_900005705.1) [26]. This was achieved using BLASTn searches with human  
140 orthologues downloaded from NCBI as the query: *ODC* (AH002917.2), *SAMdc*  
141 (NM\_001634.5), *ARG1* (NM\_001244438.1), *ARG2* (NM\_001172.3), *OTC* (NM\_0005315),  
142 *ASS* (AH002610.2) and *ASL* (M14218.1). These sequences were also consistent when  
143 compared to the rainbow trout genome deposited on NCBI (GCF\_002163505.1). For  
144 additional phylogenetic analysis, further vertebrate (coding) CDS sequences were retrieved  
145 from NCBI and/or Ensembl [27] databases. Protein sequences from Atlantic salmon, rainbow  
146 trout, northern pike, zebrafish, spotted gar, chicken, mouse and human were retrieved and  
147 accession numbers for all protein sequences are displayed in Supplementary Table 1.  
148 MatGat [28] was used to predict amino acid identity/similarity between all vertebrate proteins  
149 for each gene. Protein sequences were aligned using ClustalW in the MEGA7 software [29].  
150 A phylogenetic tree was constructed using the Jones-Taylor-Thornton model in the  
151 maximum likelihood method in MEGA 7, bootstrapped 500 times.

152 Intron-exon structure and gene synteny analysis was carried out for all candidate gene  
153 families. To determine the genomic neighbourhood around candidate genes and the  
154 conservation of gene order across the same species mentioned earlier, genes were  
155 manually examined in NCBI's genomic region browser. Intron-exon structures were  
156 determined from the same databases used to retrieve cds sequences.

157

### 158 **2.3. Primer design for quantitative PCR (qPCR)**

159 Due to the duplicated nature of salmonid genomes, care was taken to design paralogue-  
160 specific primers for genes with more than one copy within the genome (details of all primers  
161 in Table 1). Nucleotide mRNA sequences were therefore aligned with ClustalOmega [30] in  
162 order to compare paralogues and identify distinguishing regions of sequence. To avoid  
163 amplification of genomic DNA, primers were designed to span an intron-exon junction or  
164 were placed in different exons. Primers were also designed to have an annealing  
165 temperature of ~64°C judged from OligoCalc [31] and a product length of between 100 and  
166 330 bps. Prior to qPCR analysis (section 2.5), confirmation of PCR products generated using  
167 the paralogue-specific primers was carried out by cloning and sequencing. For confirmatory  
168 sequencing the PCR products were ligated into pGEM-T easy cloning vector (Promega) and  
169 then transformed into competent *Escherichia coli* cells (JM109). Plasmid DNA was isolated  
170 by Qiagen mini prep kits as described by manufacturer. A minimum of 5 clones per  
171 paralogue were sent for Sanger sequencing, carried out by Eurofins.

172

Gene	Sense	Primer 5'-3'	Product size	Annealing temperature	Accession
<i>EF-1<math>\alpha</math></i> <sup>1</sup>	Forward	CAAGGATATCCGTCGTGGCA	327	64	NM_001124339.1
	Reverse	ACAGCGAAACGACCAAGAGG			
$\beta$ - <i>actin</i> <sup>2</sup>	Forward	ATGGAAGATGAAATCGCCCC	260	64	XM_021595779.1
	Reverse	TGCCAGATCTTCTCCATGTCG			
<i>HPRT</i> <sup>3</sup>	Forward	CCGCCTCAAGAGCTAGTGTAAT	237	64	XM_021583468.1
	Reverse	GTCTGGAACCTCAAACCCTATG			
<i>RPS29</i> <sup>1</sup>	Forward	GGGTCATCAGCAGCTCTATTGG	167	64	XM_021612450.1
	Reverse	AGTCCAGCTTAACAAAGCCGATG			
<i>SAA</i>	Forward	TATGATGCTGCCAGGAGAGGAC	137	64	NM_001124436.1
	Reverse	CGTCCCCAGTGGTTAGCCTT			
<i>HAMP</i>	Forward	AGGAGGTTGGAAGCATTGACAG	101	64	XM_021595153.1
	Reverse	GTGGCTCTGACGCTTGAACCT			
<i>ODC1</i>	Forward	CGTGTGCCAGCTCAGTGTC	179	64	XM_021574142.1
	Reverse	CCATGTCAAAGACACAGCGG			
<i>ODC2</i>	Forward	TGGTGCCACCCTGAAGGCC	128	64	XM_021585068.1
	Reverse	AGATGGCCTGGCTGTAGGTG			
<i>SAMdc1</i>	Forward	GCAAGGACAAGCTAATTAAG	185	64	XM_021600286.1
	Reverse	AACCTTGGGATGGTACGGAG			
<i>SAMdc2</i>	Forward	AACTCACGATGGAAGCGAAC	121	64	XM_021611778.1
	Reverse	AACCTTGGGATGGTACGGAG			
<i>ARG 1A</i>	Forward	AGCACCATATCCTGACGTTG	147	64	XM_021564871.1
	Reverse	CATCGATGTCATAGCTCAGG			
<i>ARG 1B</i>	Forward	GGTGGATCGCCTTGAATCG	179	64	KX998966.1
	Reverse	CTGTGATGTAGATTCCCTCC			
<i>ARG 2A</i>	Forward	TCCAGAGAGTCATGGAAGTCACTTCC	198	64	KX998967.1
	Reverse	CCATCACTGACAACAACCCTGTGTT			
<i>ARG 2B</i>	Forward	CTTGTTGAGGTCAACCCAGC	163	64	KX998968.1
	Reverse	GTCGAAGCTGTTCCGTGTCG			
<i>OTC</i>	Forward	CACAGCCAGGGTTCTCTCTG	116	64	XM_021597830.1
	Reverse	CAGACAGGCCGTTGATGATG			
<i>ASS</i>	Forward	TGAGATTGGAGGGAGGCATG	172	64	XM_021590913.1
	Reverse	GCCCTGTTTGATCCTCCTGA			
<i>ASL</i>	Forward	ACGCTCTCCAACCTCATCACA	129	64	XM_021563243.1
	Reverse	ACCGCATGACTCAGAATCCA			

173 Table 1. Rainbow trout primer sequences used for qPCR with NCBI accession numbers  
174 References <sup>1</sup>[32], <sup>2</sup>[33], <sup>3</sup>[34]



## 176 **2.4. RNA extraction and reverse transcription**

177 Total RNA was extracted from 100mg of tissue homogenised in 1ml of TRI Reagent (Sigma-  
178 Aldrich) following the manufacturer's instructions. The concentration and purity of RNA was  
179 estimated using a NanoDrop 1000 Spectrophotometer (Thermo Scientific). The integrity of  
180 RNA was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies). First-strand  
181 cDNA was synthesized from 1 µg total RNA using a QuantiTech Reverse Transcription kit  
182 (QIAGEN), with an integrated genomic DNA elimination step, as per the manufacturer's  
183 guidelines in a total volume of 20 µl. First strand cDNA samples were diluted 20-fold  
184 (working stock) with RNase/ DNase free water (Sigma-Aldrich) and stored at -20°C until use.

185

## 186 **2.5. Quantitative gene expression analysis**

187 qPCR analyses were performed with SYBR Green I dye chemistry using an Mx3005P  
188 System (Agilent Technologies). All assays were carried out in duplicate within 96 well plates  
189 using 15 µl reactions containing 5 µl of the 1:20-diluted cDNA (corresponding to 2.5 ng of  
190 reverse-transcribed total RNA), 500 nM sense/antisense primers and 7.5 µl Brilliant III Ultra-  
191 Fast SYBR Green (Agilent Technologies). The PCR cycling conditions were 1 cycle of 95°C  
192 for 3 minutes, followed by 40 cycles of 95°C for 20 seconds then 64°C for 20 seconds (two  
193 step PCR). Melting curve analysis (thermal gradient from 55°C to 95°C) was used to confirm  
194 the amplification of a single product. Each plate also included no-template controls in  
195 duplicate (cDNA replaced with water). Threshold fluorescence was set at 2500 during the  
196 linear phase of amplification. The efficiency of each qPCR assay was assessed using  
197 LinRegPCR quantitative PCR data analysis program (download: <http://LinRegPCR.HFRC.nl>)  
198 following Ruijter *et al* [35] recommendations. Expression data was then imported and  
199 analysed in Genex 5.4.3 (MultiD Analysis). Candidate gene expression was normalised to  
200 two reference genes for the tissue distribution (*EF-1α* and *HPRT*) and three genes for the  
201 infection study on muscle (*EF-1α*, *HPRT* and *RPS29*) and liver (*EF-1α*, *ACTB*, and *HPRT*).  
202 *ACTB* was replaced with *RPS29* in muscle due to *ACTB*'s instability in muscle determined  
203 from the dissociation graph following qPCR. All reference gene primers used in the study are  
204 presented in Table 1.

205

## 206 **2.6. Statistical analysis**

207 Statistical analysis of qPCR data was performed in R (v3.4.0). A linear model (lm) was first  
208 constructed in R and the diagnostic plots (qq plot and residuals versus fitted values) were  
209 assessed in order to ensure both normality and equal variance. If data met the assumptions,

210 the one-way ANOVA results from R's lm function could then be interpreted. If data was not  
211 normal, a log transformation was first performed and the diagnostics plots then reassessed.  
212 If the data still did not meet the models assumptions following the transformation, a non-  
213 parametric test (Kruskal wallis) was then performed on the data.

214

215

216

217 **3. Results**

218 **3.1 Comparative analysis of the urea cycle genes**

219 The phylogenetic relationships of all genes were carried out in comparison to a range of  
220 representative vertebrate lineages. Two copies of *ARG1*, *ARG2*, *SAMdc* and *ODC* were  
221 identified, along with single copies of *OTC*, *ASS* and *ASL*. Gene intron-exon structure, amino  
222 acid sequence analysis and synteny were performed on *ODC* as a representative example.

223 Two *ODC* paralogues were identified in the rainbow trout genome (*ODC1* accession:  
224 XM\_021574142, Chr19, LOC110498573, *ODC2* accession: XM\_021585068.1, Chr25,  
225 LOC110505682). The open reading frame for *ODC1* and *ODC2* encoded 457 amino acids  
226 (aa) and 456 aa respectively, and a conserved gene structure of 8 exons and 7 introns (Fig.  
227 2) was evident between the paralogues. Across species, exons 2 and 3 were identical  
228 lengths for all species examined, while exon 4 was conserved in all teleosts, whereas in the  
229 tetrapods an additional intron is present resulting in tetrapod exons 4 and 5 being the same  
230 length as teleost exon 4 (Fig. 2). Similarly, within salmonid exon 5, an additional intron can  
231 be found in the remaining species analysed (Fig. 2). Exons 6 and 7 in salmonids and  
232 corresponding exons in the rest of the vertebrate species are highly conserved with  
233 tetrapods having 3 nucleotides less in relation to salmonid exon 6 and all species having  
234 identical length in salmonid exon 7 (Fig. 2).

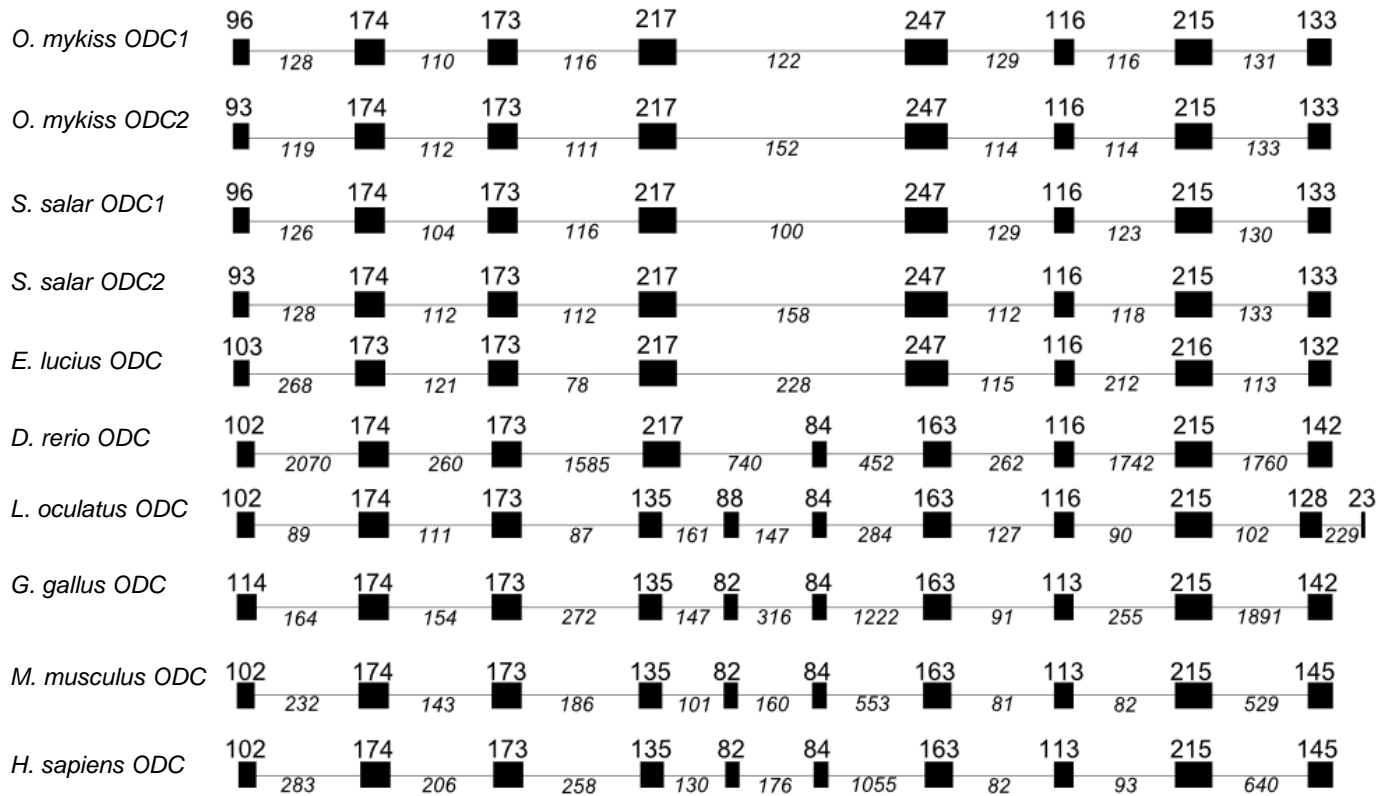


Figure 2. Intron and exon structure of ODC coding regions across vertebrates, 5' and 3' UTRs not shown. Black boxes represent exons and are drawn to scale with nucleotide base pair sizes indicated above. Black lines connecting exons and are not drawn to scale with sizes indicated below the line in italics. The sequences used for the intron exon analysis are as follows: *O. mykiss ODC1* (XM\_021574142.1), *O. mykiss ODC2* (XM\_021585068.1), *S. salar ODC1* (XM\_014192087.1), *S. salar ODC2* (XM\_014211026.1), *E. lucius ODC* (XM\_010892375.3), *D. rerio ODC* (ENSDARG00000007377), *L. oculatus ODC* (ENSLOCT00000020613.1), *G. gallus ODC* (ENSGALT00000026527.5), *M. musculus ODC* (ENSMUST00000171737.1), *H. sapiens ODC* (ENST00000234111.8)

235

236 Phylogenetic analysis of the *ODC1* and 2 proteins revealed that each molecule has an  
 237 orthologue in both Atlantic salmon and rainbow trout that shared ~98% aa identity (Table 2).  
 238 Within species, both rainbow trout and salmon *ODC1* compared to *ODC2* has 93.4% and  
 239 94.7% identity respectively (Table 2). The presence of the genes on distinct chromosomes  
 240 and the branching of northern pike as a sister group to both *ODC1* and 2 suggests these  
 241 genes are products of the salmonid specific WGD (ssWGD) (Fig. 3). *ODC1* and 2 are highly  
 242 conserved with other vertebrate *ODC* orthologues (Table 2), for example sharing ~73% aa  
 243 level identity each with human.

244

245

	<i>O. mykiss</i> ODC1	<i>S. salar</i> ODC1	<i>O. mykiss</i> ODC2	<i>S. salar</i> ODC2	<i>E. Lucius</i> ODC	<i>D. rerio</i> ODC	<i>L. oculatus</i> ODC	<i>G. gallus</i> ODC	<i>M. musculus</i> ODC	<i>H. sapiens</i> ODC
<i>O. mykiss</i> ODC1		98.2	93.2	94.5	92.4	85	77.5	71.4	71.2	72.5
<i>S. salar</i> ODC1	99.3		93.4	94.7	92.8	84.6	77.9	70.8	71	72.3
<i>O. mykiss</i> ODC2	96.1	96.5		98.2	92.6	85.5	79.4	71	72.1	72.5
<i>S. salar</i> ODC2	96.9	97.1	98.9		93.4	85.9	79	71.4	72.1	72.9
<i>E. lucius</i> ODC	95.4	96.1	95.2	95.9		87	79.2	71.7	72.5	72.7
<i>D. rerio</i> ODC	90.7	91.8	92	92	93.1		80.1	73.7	73.9	74.1
<i>L. oculatus</i> ODC	87.6	88	88.2	87.3	87.6	89.7		79.4	76.8	78.3
<i>G. gallus</i> ODC	83.8	83.6	82.5	82.5	82.6	86.4	89.3		81.5	83.9
<i>M. musculus</i> ODC	84.6	85.2	85.2	84.8	84.2	87	88.4	91.2		90.7
<i>H. sapiens</i> ODC	83.9	84.6	83.5	83.7	83.3	85.5	89.5	92.9	94.1	

Identity	Similarity
----------	------------

246 Table 2. Comparison of amino acid identities and similarities (%) for ODC using the similarity matrix in MatGat  
247 2.02 software. Accession numbers for all proteins can be found on Supplementary Table 1

248 Phylogenetic analysis further confirmed *ODC1*, *ODC2* (Fig. 3) and *SAMdc1*, *SAMdc2*  
249 (Supplementary Fig. 1) as products of ssWGD with higher relatedness to their salmonid  
250 relative than to their own species duplicate. In the case of the *ARG1* and *ARG2*, these genes  
251 are present in all vertebrates and likely diverged before the evolution of vertebrates.  
252 However as there are 4 copies of arginase present in salmonids (*ARG1a*, *ARG1b*, *ARG2a*  
253 and *ARG2b*), it can be seen that the two paralogues for arginase 1 and 2 are likely products  
254 of the ssWGD (Supplementary Fig. 2). All other genes characterised in this study (*OTC*,  
255 *ASS*, *ASL*) have not retained a duplicate copy (Supplementary Figures 3-5).

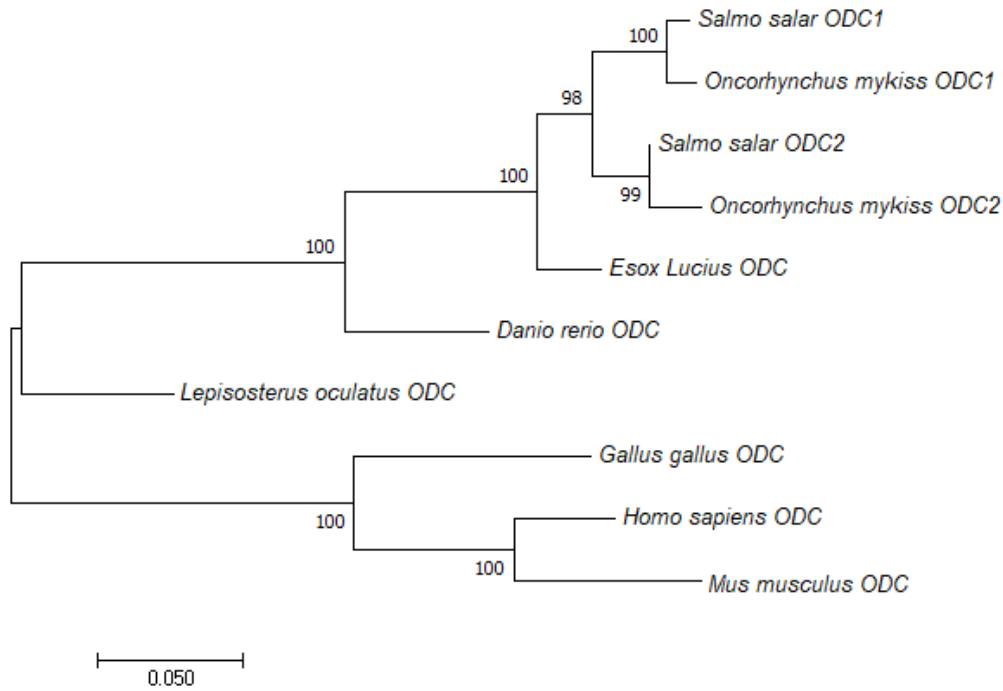


Figure 3. Maximum likelihood phylogenetic tree showing the evolutionary relationship of ODC amino acid sequences in vertebrates. The branch support values were gained by non-parametric bootstrapping (500 replicates). The scale bar represents the number of modelled substitutions per site. Accession numbers from NCBI are as follows: *O. mykiss* ODC1 (XP\_021429817.1), *O. mykiss* ODC2 (XP\_021440743.1), *S. salar* ODC1 (XP\_014047562.1), *S. salar* ODC2 (XP\_014066501.1), *E. lucius* ODC (XP\_010890677.1), *L. oculatus* ODC (XP\_006626107.1), *D. rerio* ODC (NP\_571876.1), *G. gallus* ODC (NP\_001161238.1), *M. musculus* ODC (NP\_038642.2), *H. sapiens* ODC (AAA59968.1)

267 Synteny analysis revealed highly conserved gene order in the genomic regions containing  
 268 *ODC1* and 2, both in relation to each other and also in comparison to single copy *ODC*  
 269 orthologues in non-salmonid teleosts and tetrapod vertebrates (Fig. 4). At the 5' of *ODC*, the  
 270 most proximal neighbouring gene (*NOL10*) was retained in the same location in all  
 271 vertebrates. We also identified several other annotated genes with high synteny located near  
 272 *ODC* including *KCNA1* and *ROCK2*. Towards the 3' end of *ODC*, salmonid genes shared  
 273 similarity of gene order with the northern pike, but with no other vertebrates including  
 274 zebrafish, suggesting a chromosomal rearrangement prior to the salmonid / pike divergence  
 275 from other teleosts. As for the genes downstream of *ODC*, we identified some annotated  
 276 genes in two copies on both the *ODC1* and 2 chromosomes, barring the *YIPF* family of  
 277 genes. Towards the 3' of rainbow trout *ODC1* there was no *YIPF* gene annotated, but in  
 278 Atlantic salmon *YIPF* was identified downstream of both *ODC1* and 2.



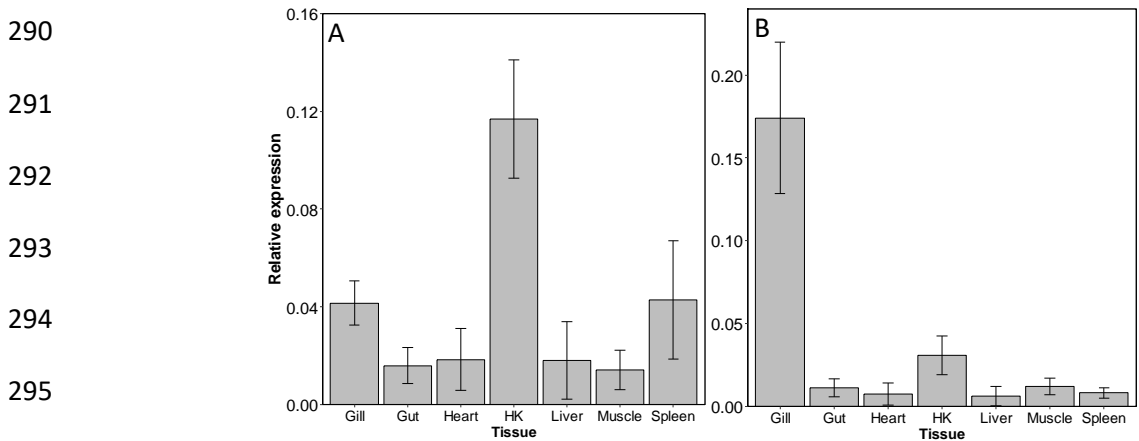
Figure 4. Phylogenetic tree and gene synteny of *ODC* in vertebrates. The tree was constructed using the maximum likelihood method in MEGA7 and bootstrapped 500 times. The syntenically conserved gene blocks are shown in matching colours. The arrows represent transcriptional direction. Gene synteny was compiled from up and downstream locations relative to each species *ODC* taken from NCBI, *ODC* protein accession numbers on supplementary table 1, chromosome number and range (from left of the diagram to right) as follows: *O. mykiss ODC1* (Chr 19, 34,937,046 > 35,209,567), *O. mykiss ODC2* (Chr 25, 63,402,440 > 63,152,107), *S. salar ODC1* (Chr ssa01, 29,382,254 > 29,167,678), *S. salar ODC2* (Chr ssa09, 27,309,511 > 27,043,274), *E. lucius ODC* (Chr LG15, 14,186,061 > 14,321,347), *D. rerio ODC* (Chr 17, 51,655,239 > 51,757,548), *L. oculatus ODC* (Chr LG1, 43,496,860 > 43,340,082), *G. gallus ODC* (Chr 3, 97,175,730 > 96,773,203), *M. musculus ODC* (Chr 12, 16,894,978 > 17,791,944), *H. sapiens ODC* (Chr 2, 11,179,759 > 10,427,617).

279

### 280 3.2. Urea cycle and polyamine synthesis gene expression

281 3.2.1 Tissue distribution

282 The relative mRNA expression levels of the characterized urea cycle (*ARG1a*, *ARG1b*,  
283 *ARG2a*, *ARG2b*, *OTC*, *ASS* and *ASL*) and rate limiting enzymes of polyamine synthesis  
284 (*ODC1*, *ODC2*, *SAMdc1* and *SAMdc2*) were quantified by qPCR in seven tissues in healthy  
285 rainbow trout under control conditions (Fig. 5 for *ODC1* and 2; all other genes:  
286 supplementary figures 6-8). Both *ODC* paralogues were expressed in all tissues examined,  
287 with *ODC2* less abundant than *ODC1* in all tissues barring gill (Fig. 5). *ODC1* was most  
288 highly expressed in head kidney followed by spleen and gill. *ODC2* had the highest  
289 expression in gill followed by head kidney.



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296 *Figure 5. Tissue distribution of ODC1 (A) and ODC2 (B) in rainbow trout. The relative*  
297 *expression in each tissue was normalised with the expression of two housekeeping genes*  
*EF-1 $\alpha$  and HPRT. Bars represent mean ( $\pm$  SEM), n=4.*

298 The four arginase paralogues showed a more variable expression distribution  
299 (Supplementary Fig. 6). *ARG1a* and *ARG1b* both showed highest expression in the liver  
300 (Supplementary Fig. 6). *ARG1a* was expressed in all tissues examined, with highest  
301 expression in liver and gill. *ARG1b* showed highest expression in liver, with lower expression  
302 levels in gill, heart and muscle, and no detectable expression in gut, head kidney or spleen  
303 expression. *ARG2a* and *ARG2b* were expressed in all tissues, both showing highest  
304 expression in the muscle and lowest expression in liver.

305 The single copy urea cycle genes *OTC*, *ASS* and *ASL* were expressed in all the tissues  
306 examined, with *OTC* and *ASL* having highest expression in muscle and *ASS* in gill  
307 (Supplementary Fig. 7). *OTC* was expressed at a very low level in the liver (Supplementary  
308 Fig. 7). The two *SAMdc* paralogues were expressed in all tissues, with both genes having  
309 highest expression in heart and muscle (Supplementary Fig. 8).

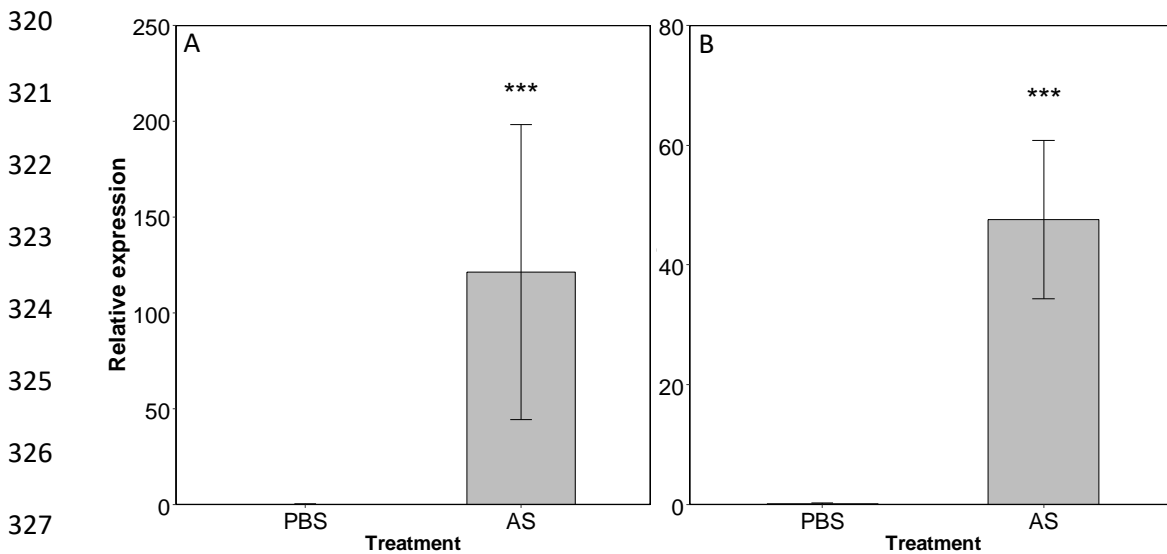
310

311 3.2.2. Modulation of mRNA expression following bacterial infection



312 Expression of the urea cycle enzymes and polyamine synthesis genes was examined in  
 313 adult rainbow trout liver and muscle tissue sampled following an experimental infection with  
 314 a pathogenic strain of the bacterium *Aeromonas salmonicida* (AS). To confirm the fish were  
 315 undergoing an inflammatory response to the infection, the expression of two marker genes  
 316 for the acute phase response (APR), serum amyloid A (SAA) and hepcidin (*HAMP*), were  
 317 examined in control and AS-infected fish. Both genes showed highly significant upregulation  
 318 in infected fish (Fig. 6) indicating a strong immune response.

319

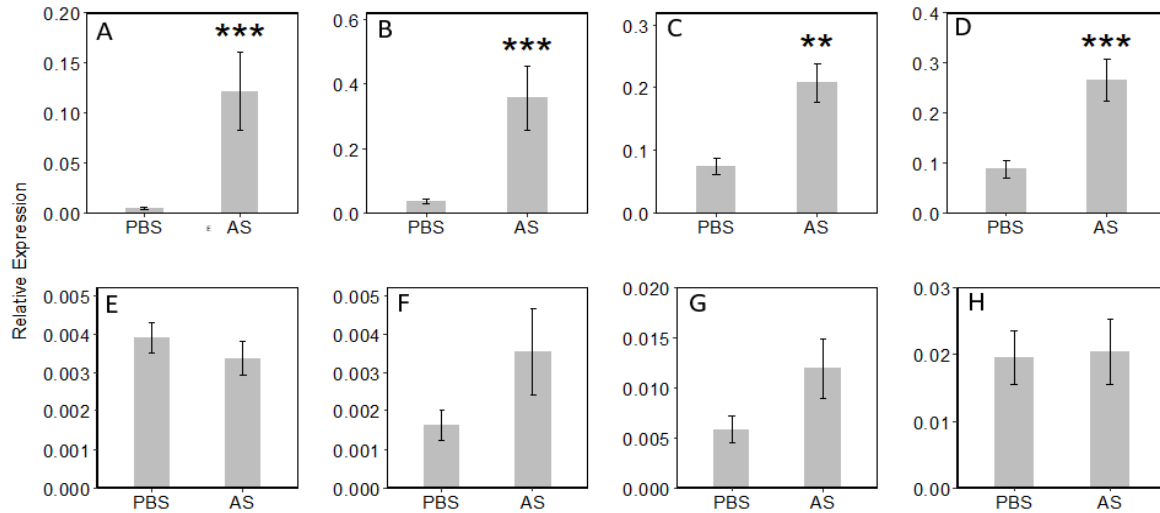


327

Figure 6. Relative expression of rainbow trout SAA (A) and HAMP (B) in liver tissue following a bacterial infection. Fish were injected intraperitoneally with either phosphate buffered saline (PBS) or *Aeromonas salmonicida* (AS). SAA and HAMP expression was normalised to housekeeping genes *EF-1 $\alpha$* , *ACTB* and *HPRT*. Linear model in R was used for analysis of both genes. Bars represent mean ( $\pm$  SEM),  $n=10$ , \*\*\* =  $p < 0.001$

330

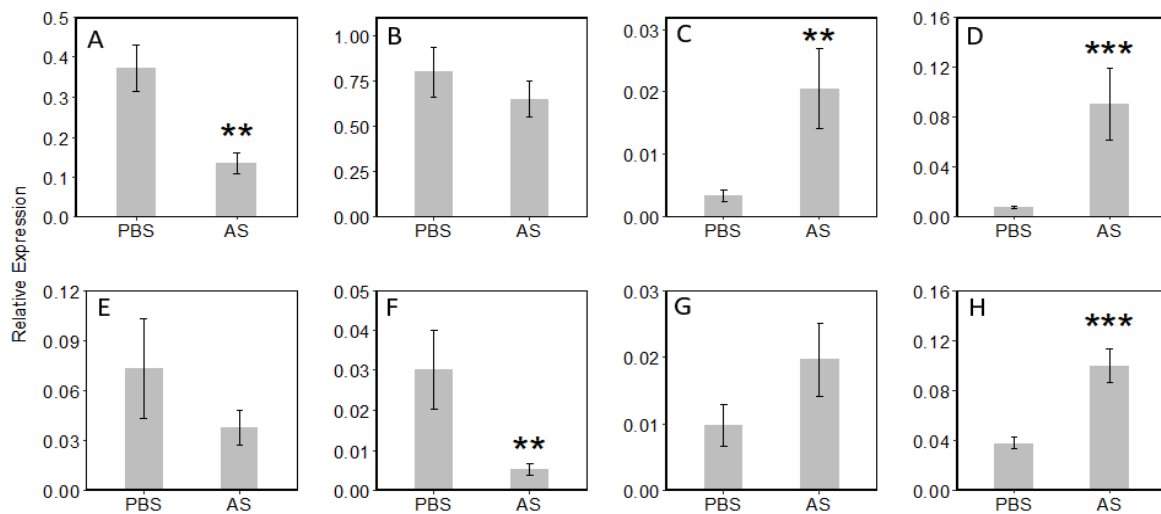
331 Following AS infection both *ODC* paralogues were significantly upregulated in the liver (Fig.  
 332 7). In muscle, *ODC1* and 2 expression did not change following infection (Fig. 7). Both  
 333 *SAMdc1* and 2 were significantly upregulated in liver but not muscle (Fig. 7). *ARG1a* was  
 334 significantly downregulated in liver and unchanged in muscle whereas the opposite pattern  
 335 was observed for *ARG1b* (Fig. 8). *ARG2a* and *ARG2b* expression was significantly  
 336 increased in liver, with *ARG2b* also significantly increased in muscle but not *ARG2a* (Fig. 8).  
 337 Both *OTC* and *ASL*'s expression was significantly increased in muscle, but not liver (Fig. 9),  
 338 whereas *ASL* expression increased significantly in infected liver but not in muscle (Fig. 9).



339

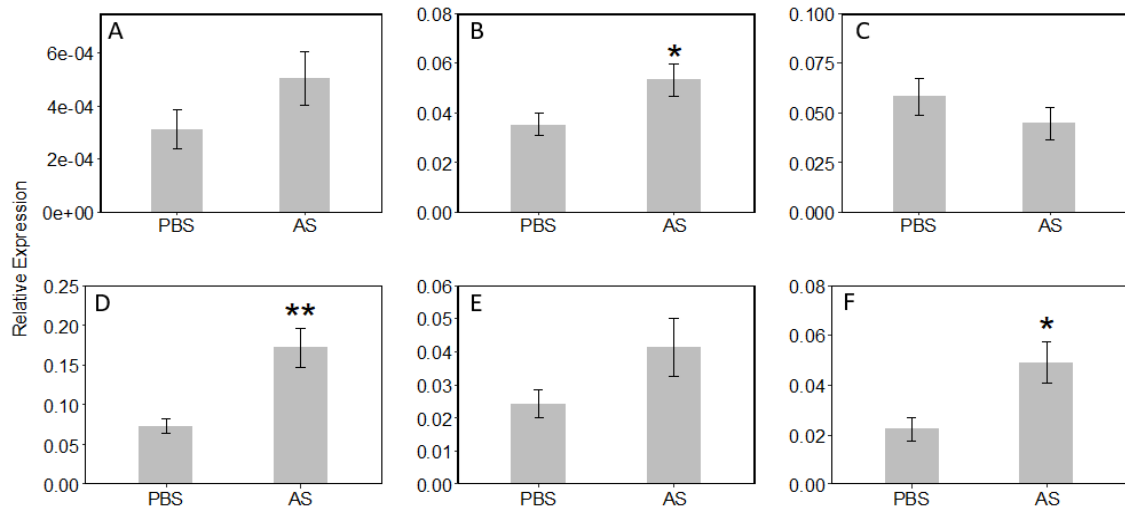
340 *Figure 7. Relative expression of the rate limiting enzymes in polyamine synthesis, SAMdc and ODC, in rainbow*  
 341 *trout muscle and liver tissue following a bacterial infection. Fish were injected intraperitoneally with either*  
 342 *phosphate buffered saline (PBS) or Aeromonas salmonicda (AS). The top row of graphs show gene in liver*  
 343 *tissue: ODC1 (A), ODC2 (B), SAMdc1 (C), SAMdc2 (D), expression was normalised to housekeeping genes EF-*  
 344 *1α, ACTB and HPRT. The bottom row of graphs show gene expression in muscle tissue: ODC1 (E), ODC2 (F),*  
 345 *SAMdc1 (G), SAMdc2 (H), expression was normalised to housekeeping genes EF-1α, RPS29 and HPRT. Bars*  
 346 *represent mean (± SEM), n=10, \* = p < 0.05, \*\* = p < 0.01, \*\*\* = p < 0.001*

347



348

349 *Figure 8. Relative expression of rainbow trout Arginase paralogues following a bacterial infection. Fish were*  
 350 *injected intraperitoneally with either phosphate buffered saline (PBS) or Aeromonas salmonicda (AS). Top row*  
 351 *represents gene expression of ARG1a (A), ARG1b (B), ARG2a (C) and ARG2b (D) in liver tissue, expression*  
 352 *was normalised to the house keeping genes EF-1α, ACTB and HPRT. Bottom row represents gene expression of*  
 353 *ARG1a (E), ARG1b (F), ARG2a (G) and ARG2b (H) in muscle tissue, expression was normalised to the house*  
 354 *keeping genes EF-1α, RPS29 and HPRT. Bars represent mean (± SEM), n=10, \* = p < 0.05, \*\* = p < 0.01, \*\*\* = p*  
 355 *< 0.001*



356

357 *Figure 9. Relative expression of rainbow trout OTC (A), ASS (B) and ASL (C) in liver tissue following a bacterial*  
 358 *infection. Second row represents relative expression of rainbow trout OTC (D), ASS (E) and ASL (F) in muscle*  
 359 *tissue. Fish were injected intraperitoneally with either phosphate buffered saline (PBS) or Aeromonas salmonicida*  
 360 *(AS). For the genes examined in the liver, expression was normalised to housekeeping genes EF-1 $\alpha$ , ACTB and*  
 361 *HPRT. For the bottom panel where muscle tissue was examined, expression was normalised to housekeeping*  
 362 *genes EF-1 $\alpha$ , RPS29 and HPRT. Bars represent mean ( $\pm$  SEM), n=10, \* =  $p < 0.05$ , \*\* =  $p < 0.01$ , \*\*\* =  $p < 0.001$*

363

#### 364 4. Discussion

365 Arginine metabolism and the urea cycle are major components of nitrogen metabolism, the  
366 inflammatory response and subsequent tissue repair. However, there is a lack of information  
367 available in salmonid fish regarding the genes regulating these metabolic pathways. Here we  
368 address this knowledge gap by characterising the key enzymes in the urea pathway and by  
369 documenting their transcriptional responses to a bacterial infection.

370 In fish, arginine is often regarded as an essential amino acid required for efficient protein  
371 synthesis, in addition to other functional processes influencing health status [5, 36]. For  
372 arginine to be synthesised endogenously, carbamoylphosphate, an intermediate molecule in  
373 the urea cycle, is combined with ornithine to form citrulline through the action of *OTC* (Fig.  
374 1). In ureotelic mammals and amphibians, carbamoylphosphate synthetase (*CPS*) catalyses  
375 the formation of carbamoylphosphate, allowing its use in the urea cycle [37, 38]. In rainbow  
376 trout, *CPS* is expressed at early life stages but not in the liver of adults [37] with low levels in  
377 muscle [39]. This lack of hepatic *CPS* activity in salmonids could be the reason for an  
378 incomplete urea cycle and inability to synthesise arginine endogenously. Some teleosts  
379 including the toadfish *Opsanus beta* [40], the catfish *Clarias batrachus* [41], and the lungfish  
380 *Protopterus aethiopicus* [42], have detectable *CPS* activity and a functional urea cycle.  
381 However, our gene expression data suggests that the urea cycle enzymes are functional in  
382 adult rainbow trout and the modulation we observed following AS infection indicates a role  
383 during the inflammatory response.

384 Our phylogenetic analyses revealed that *ODC*, *SAMdc* and *ARG* have multiple paralogues  
385 retained from the salmonid-specific whole genome duplication (ssWGD), which occurred 88-  
386 103 Mya [43, 44]. In the case of *ARG*, we observed four salmonid copies, with two  
387 paralogues retained for both *ARG1* and *ARG2*, which was also recently shown for Atlantic  
388 salmon [20], which are conserved in all vertebrates and presumably the result of an early  
389 gene duplication event, perhaps past WGD events in the vertebrate ancestor [45]. However,  
390 we did not identify any duplicated paralogues of the key urea cycle genes retained from the  
391 teleost-specific WGD event [45]. The duplicated copies that are retained either specialise in  
392 function (subfunctionilization) or develop a novel function (neofunctionilization) [46, 47].  
393 Following a WGD event, the resulting duplicated genome eventually only retains a small  
394 percentage of duplicated genes, as the redundant genes are inactivated by a process  
395 termed gene fractionation [26]. The ssWGD is relatively recent when compared to the teleost  
396 specific WGD (~100Mya compared to ~300Mya) and this is evident from the large number of  
397 duplicated genes still present in the genome (48% of genes with retained ohnologs) [26].

398 The genes encoding *OTC*, *ASS* and *ASL* were found to be present as single copy genes  
399 suggesting one duplicated copy was lost in the ancestor of the trout and salmon lineage.

400 The genes encoding the urea cycle molecules *ARG*, *OTC*, *ASS* and *ASL* were expressed in  
401 all tissues examined, with *OTC* showing negligible levels in liver; this latter observation may  
402 contribute to a low functioning urea cycle in salmonids, and other teleost's [37]. However,  
403 genes encoding the other urea cycle enzymes (*ARG*, *ASS* and *ASL*) were transcribed in the  
404 liver, indicating components of the urea cycle may have other metabolic roles beyond  
405 nitrogenous waste excretion. The expression of *ASS* and *ASL*, may indicate an efficient  
406 conversion of citrulline to arginine in adult trout, but the lack of the carbamoyl substrate is a  
407 limiting factor. If the urea cycle is fully functioning, then citrulline could be used to bolster  
408 arginine levels, as is found in mammalian species [48, 49]. This idea is also supported by the  
409 functioning NOS cycle in salmonids as they are able to produce NO in the innate immune  
410 response against pathogens [50]. Citrulline is generated as a by-product of the *iNOS*  
411 reaction and could be recycled back into arginine by *ASS* and *ASL*. The arginase genes  
412 show an interesting expression profile, *ARG 1a* and *1b* are expressed at high levels in the  
413 liver whereas *ARG 2a* and *2b* are virtually absent in liver tissue. For genes encoding  
414 polyamine enzymes there is low level of expression in non-stimulated liver and small  
415 difference between paralogue expression.

416 To gain information on the expression of the urea cycle genes/paralogues, we quantified  
417 their mRNA expression and transcriptional responses following bacterial infection in muscle  
418 and liver. We sampled liver as a key indicator of the acute phase response [51, 52] that  
419 shows a well-established response to bacterial infection, while also acting as the main site  
420 for amino acid metabolism and the urea cycle. Skeletal muscle was also selected for  
421 analysis, due to high transcript levels identified in many of the genes in the tissue  
422 distributions (see Results section 3.2.1). Fish were sampled 48h after infection to represent  
423 the early immune response before physiological changes due to disease could occur [51].  
424 There were significant increases in liver expression for the polyamine genes *ODC1*, *2* and  
425 *SAMdc1* and *2* suggesting upregulation of the polyamine pathway and subsequent  
426 production of putrescine. This could be related to cellular repair and also potential increased  
427 availability of ornithine from increased *ARG2* activity following infection.

428 Both the *ARG2* paralogues increased in expression following infection, whilst *ARG1a* was  
429 significantly decreased showing potential subfunctionalisation of the duplicated genes.  
430 Recent research has suggested that *ARG1* is involved as a major metaboliser of hepatic  
431 arginine whereas *ARG2* may be more involved with the immune response in the form of  
432 healing M2 macrophages [19]. During an immune response M2 macrophages demonstrate

433 elevated levels of arginase activity and also play an important role in the innate immune  
434 defence against various pathogens in both a bactericidal and healing sense [18]. The two  
435 major types of macrophages, M1 and M2, both depend on the same substrate (arginine) for  
436 either healing (M2) or bactericidal activity (M1). The enzymes *iNOS* and arginase have been  
437 described as useful markers for M1 and M2 macrophages (respectively) in both mammals  
438 [53] and some fish species [54]. As both *iNOS* and arginase compete for arginine they can  
439 regulate each other's expression either driving an inflammatory response via the nitric oxide  
440 cycle or wound healing from polyamine synthesis by *ODC* [53]. The increased expression of  
441 *ARG2* along with *ODC* and *SAMdc* following infection shown in this study suggests that the  
442 conversion of arginine into polyamines is taking place for tissue repair. This is in agreement  
443 with studies in humans where arginine derived from ornithine also plays a role in tissue  
444 remodelling as high levels of arginase can be observed in fibroblasts of patients suffering  
445 from pulmonary fibrosis [55]. Further evidence showing the regulatory effect *ARG* and *iNOS*  
446 have on each other can be seen in mice when infected with *Helicobacter pylori*, arginase 2  
447 knockout lead to increased M1 macrophage activation [56].

448 The transcriptional changes seen in the liver from *ARG2* and the polyamine synthesis genes  
449 *ODC* and *SAMdc* suggests a signature activation of wound healing M2 macrophages [21].  
450 Although macrophages are virtually present in all tissues [20], we find in muscle, that during  
451 inflammation there is a less dramatic response compared to liver. There were no significant  
452 changes in the muscle for genes involved in polyamine synthesis suggesting liver is a major  
453 source for these molecules. For *ARG* genes there was a similar pattern in muscle and liver  
454 indicating conserved regulation between tissues and a conserved inflammatory response.  
455 The urea cycle enzymes (*OTC*, *ASS* and *ASL*) displayed some variation between tissue  
456 where *OTC* and *ASL* were significantly upregulated in muscle but unchanged in liver while  
457 *ASS* was significantly increased in liver but unchanged in muscle.

458

## 459 **5. Conclusion**

460 In summary, the genes encoding the enzymes of the urea cycle and the two rate limiting  
461 enzymes in polyamine synthesis have been characterised and their response to infection  
462 investigated. Our findings demonstrate that *ARG 1* and *2*, *SAMdc* and *ODC* genes have  
463 retained functional paralogues from the salmonid-specific WGD, with several of the  
464 duplicated copies showing different regulation across tissues. The nutritional requirement of  
465 arginine in the diet in salmonids is likely to be due to a lack of activity from *CPS* and *OTC*  
466 enzymes in adult liver. It is likely that half of the urea cycle is functional and the enzymes  
467 responsible for the conversion of citrulline to arginine are active, especially due to the

468 functioning NO cycle. We also observed significant changes in the urea/polyamine pathways  
469 following bacterial challenge, suggesting enhanced recycling and metabolism of arginine for  
470 both inflammatory and tissue healing roles following infection.

471

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476

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