

1 **Arginine, ornithine and citrulline supplementation in rainbow trout: free amino acid dynamics**
2 **and gene expression responses to bacterial infection**

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28 **Abstract**

29 Supplementing the diet with functional ingredients is a key strategy to improve fish performance and
30 health in aquaculture. The amino acids of the urea and nitric oxide (NO) cycles - arginine, ornithine and
31 citrulline - perform crucial roles in the immune response through the generation of NO and the synthesis
32 of polyamine used for tissue repair. We previously found that citrulline supplementation improves and
33 maintains circulating free arginine levels in rainbow trout more effectively than arginine
34 supplementation. Here, to test whether supplementation of urea cycle amino acids modulates the
35 immune response in rainbow trout (*Oncorhynchus mykiss*), we supplemented a commercial diet with
36 high levels (2% of total diet) of either arginine, ornithine or citrulline during a 7-week feeding trial,
37 before challenging fish with the bacterium *Aeromonas salmonicida*. We carried out two separate
38 experiments to investigate fish survival and 24h post-infection to investigate the immediate response of
39 free amino acid levels, and transcriptional changes in genes encoding urea cycle, NO cycle and
40 polyamine synthesis enzymes. There were no differences in percentage fish mortality between diets,
41 however there were numerous highly significant changes in free amino acid levels and gene expression
42 to both dietary supplementation and infection. Out of 26 amino acids detected in blood plasma, 8 were
43 significantly changed by infection and 9 by dietary supplementation of either arginine, ornithine or
44 citrulline. Taurine, glycine and aspartic acid displayed the largest decreases in circulating levels in
45 infected fish, while ornithine and isoleucine were the only amino acids that increased in concentration.
46 We investigated transcriptional responses of the enzymes involved in arginine metabolism in liver and
47 head kidney; transcripts for polyamine synthesis enzymes showed highly significant increases in both
48 tissues across all diets following infection. The paralogous arginase-encoding genes, *Arg1a*, *Arg1b*,
49 *Arg2a* and *Arg2b*, displayed complex responses across tissues and also due to diet and infection.
50 Overall, these findings improve our understanding of amino acid metabolism following infection and
51 suggests new potential amino acid targets for improving the immune response in salmonids.

52 **Key words:** Arginine, ornithine, citrulline, functional amino acids, urea cycle, health, polyamine,
53 salmonids.

54

55 **1. Introduction**

56 Maintenance of fish health is a central requirement for efficient and economically feasible aquaculture.

57 A challenge to this goal is that fish are continually exposed to pathogens in the aquatic environment [1].

58 The first line of defence to pathogens is from physical external barriers (e.g. skin and gill mucous)

59 followed by the innate immune system, which is believed to be more important in fish than endotherms

60 due to the longer time required to mount an adaptive response [2]. In all cases, eliciting an immune

61 response is highly energy demanding and a balance between immune response and other physiological

62 and metabolic processes occurs [3].

63 Salmonids use protein as a major energy source, utilising amino acids in gluconeogenesis [4, 5]. The liver

64 is a central organ in the metabolism of amino acids, but under an inflammatory response, its metabolic

65 state is altered to produce large volumes of acute phase proteins [6]. Thus, there may be a trade-off

66 between growth and the immune response in which growth is hindered until the infection is resolved.

67 The synthesis of large volumes of immune proteins during the inflammatory response and the

68 subsequent healing and recovery following infection requires a supply of free amino acids, obtained

69 from the diet or by remobilisation of proteins stored in the skeletal muscle [6, 7]. Supplementing fish

70 diets with functional amino acids (FAAs) offers a strategy to supply a source of useful amino acids to

71 support immune function and more generally improve performance.

72 FAAs can be nutritionally essential, non-essential, or may become conditionally essential (e.g. at

73 different developmental stages or under distinct health, reproductive or stress states) if available

74 quantities are unable to meet the body's demand [8]. FAA supplementation has the potential to improve

75 fish health due to their key roles in the immune response, such as increased gluconeogenesis of alanine as

76 an energy substrate for leukocytes [9], or the antioxidant properties of taurine and glycine [10].

77 Arginine is an FAA attracting considerable attention due its impact on many metabolic systems,

78 including the immune response.

79 During infection, the availability of free arginine decreases for general metabolic processes, as it is

80 preferentially directed towards lymphocyte proliferation and macrophage dependent production of NO

81 and polyamines used in the immune response [11, 12]. Inflammatory responses are associated with

82 polarising T helper cells, specifically T_H1 cells, which secrete proinflammatory cytokines including IL-
83 1 β , TNF α and IFN- γ , activating M1 macrophages (kill macrophages), whereas anti-inflammatory
84 processes activate M2 macrophages (healing macrophages) associated with the T helper cell subtype
85 T_H2, which secrete cytokines such as interleukin 4 or 10 (IL-4, IL-10) [13]. M1 macrophages are
86 believed to metabolise arginine into NO through the action of inducible NO synthase (iNOS) resulting
87 in a macrophage population with increased microbicidal activity [14]. On the other hand, anti-
88 inflammatory responses and healing are associated with M2 cells where arginine is converted to
89 ornithine and subsequently metabolised to polyamines through the action of ornithine decarboxylase
90 (ODC) and s-adenosylmethionine decarboxylase (SAMdc) for tissue repair [15, 16]. Within the immune
91 response, high polyamine levels can be found in rapidly proliferating cells and tissues [17, 18], playing
92 a key role in wound and tissue healing following infection or injury [19, 20]. As M1 and M2
93 macrophages compete for the same substrate, arginine, iNOS and arginase expression have a regulatory
94 effect on each other, where there is a balance between inflammatory response and subsequent cellular
95 repair [21]. This competition for the same substrate may deplete the arginine pool, increasing
96 susceptibility to disease [22]. In channel catfish fed arginine deficient diets, impaired immune function
97 is seen through reduced phagocyte superoxide anion production and neutrophil respiratory burst [23].
98 Additional arginine within the diet has the potential to negate this deficit during stressful conditions
99 including under disease and parasite burden.

100 Supplementing arginine above the nutritional requirement has the potential to enhance the immune
101 response, as demonstrated already in several species of fish and mammals. In tumour-bearing mice,
102 supplemented arginine enhanced survival time and expression of key inflammatory markers (IFN- γ ,
103 TNF- α , NO levels) in splenocytes [24]. Improved immune parameters such as increased production of
104 neutrophil oxidative radicals and superoxide anions along with higher serum lysozyme activity has been
105 seen in both red drum and striped bass [25, 26]. Supplemented arginine was also seen to offset the
106 immunosuppressive effects of repeated handling in both Senegalese sole and turbot [27, 28]. While the
107 supplementation of arginine has been shown to improve the immune response in several aquaculture
108 species, little is known about the other amino acids of the urea cycle, ornithine or citrulline. In mammals,

109 citrulline supplementation increases circulating arginine concentrations more effectively than by direct
110 arginine supplementation [29, 30], with arginine derived from citrulline supplementation also
111 increasing NO production during endotoxemia [29]. We previously demonstrated that citrulline
112 supplementation increased arginine levels in rainbow trout in a similar fashion as in mammals [31],
113 however the impact of citrulline and ornithine supplementation on the immune system remains
114 uncharacterised in fish.

115 The overall objective of this study was to investigate the effects of supplementing the urea cycle amino
116 acids, arginine, ornithine and citrulline on the immune response following a bacterial challenge in
117 rainbow trout. These amino acids have a key role in immune function of an organism namely through
118 arginine's role in NO production, synthesis of polyamines from ornithine and the potential for citrulline
119 to increase circulating arginine greater than arginine itself [31]. *Aeromonas salmonicida*, the causative
120 agent of furunculosis, was chosen as a bacterial pathogen model due to its worldwide spread and
121 lethality in farmed fish [32], as well as, the well understood dynamics of the host response to infection.
122 The effects on health from the amino acid supplementations and disease challenge were investigated by
123 i) a survival study, ii) levels of free amino acids in blood plasma, and iii) mRNA expression of both
124 immune and arginine related metabolic genes in liver and head kidney. The resultant data gives insight
125 into the change in free amino acid profiles following infection and the role of arginine, ornithine and
126 citrulline supplementation on the health of farmed fish.

127

128 **2. Materials and Methods**

129 **2.1 Diet formulation**

130 A commercial rainbow trout diet was used a basal/control diet, enhanced by addition of either arginine,
131 citrulline or ornithine. The basal diet meets the essential amino acid requirements for rainbow trout and
132 contained a protein source derived from fish meal (15%) and plant protein (28%); a blend of fish oil
133 (9%) and rapeseed oil (17%) were used as the dietary lipid source, with additional micro ingredients
134 and minerals added (full details in Table 1). The experimental diets were identical to the basal/control
135 diet except for the supplementation of either arginine (ARG-2), ornithine (ORN-2) or citrulline (CIT-
136 2) at a level of 2% (20g / kg) of the total diet. Supplementation levels of amino acids were decided from
137 a previous study performed by ourselves [31]. All diets were formulated and manufactured by Biomar
138 and identical to the trial in [31]. Analysis of amino acid content of the diets was performed by Biomar.
139 Additional confirmation of the arginine, ornithine and citrulline content were performed by Ansynth
140 Service B.V. The amino acid profiles of the diets are presented in Table 2.

141 **Table 1**

142 **Table 2**

143 **2.2 Rainbow trout feeding trial**

144 All procedures described were carried out in compliance with the Animals (Scientific Procedures) Act
145 1986 under UK Home Office license PPL number 70/8071 and approved by the ethics committee at the
146 University of Aberdeen, UK. Juvenile rainbow trout were maintained at the University of Aberdeen
147 aquarium facility (School of Biological Sciences). Tanks were supplied with recirculating freshwater
148 with a flow 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
149 light:dark. A computerised control system was used to monitor pH, ammonia concentration and oxygen
150 levels. Fish were fed twice daily (9 am and 5 pm) with commercial pellets of respective diets at 3%
151 body weight per day.

152 Fish of average weight \pm SEM (84 ± 1 g) were pit tagged for later identification and distributed into one
153 of twelve 400L tanks, each containing 50 fish. Dietary treatments were randomly assigned to triplicate
154 tanks. Fish were acclimatised on the control diet for 2 weeks before being fed for 49 days (7 weeks) on

155 their respective experimental diets. Fish were fed *ad libitum* and uneaten pellets were weighed at the
156 end of each day to estimate feed intake. Following the conclusion of the feeding trial, growth parameters
157 (final weight, gutted weight, hepatosomatic index, visceral somatic index, condition factor, feed
158 conversion ratio and the specific growth rate) were collected from fish not used in the bacterial
159 immunological stimulations.

160 **2.3 Bacterial challenge following feeding trial**

161 For the survival challenge, n=30 fish per diet were randomly selected (n=10 per triplicate tank) then
162 anaesthetised by immersion in 2-phenoxyethanol, followed by intraperitoneal (i.p.) injection with the
163 live Gram-negative bacterium *Aeromonas salmonicida* (AS), pathogenic Hooke strain ($1.6 \times 10^6 \text{ ml}^{-1}$
164 cells, 0.5 ml/fish). Fish were then randomly but equally divided (relating to their previous diet) between
165 three infection tanks (avoiding any tank effects) and were monitored over twelve days. The pit-tags
166 were used to assign fish back to their original diet. During the challenge, fish were monitored twice
167 daily until mortality started, then every four hours during peak mortality days. Fish showing clinical
168 symptoms of AS infection (i.e. listless, ulcers, or general abnormal behaviour) were removed from the
169 tank and killed by an overdose of anaesthetic followed by destruction of the brain (Schedule 1 Killing
170 method).

171 For the gene expression and free amino acid studies, fish were again randomly selected and either
172 injected with AS (n=6 per diet), as described above, or 0.5 ml of phosphate buffered saline (PBS) (n=6
173 per diet). Fish were then maintained in two separate tanks, infected and uninfected, based on the AS or
174 PBS injection. Fish were sampled 24 h after the stimulation to assess the early immune response of fish
175 before progression of disease. Fish were killed as described previously and samples of liver and head
176 kidney tissue (100 mg) were collected (within 5 minutes of death) and stored in 1.5 ml RNA later at
177 4°C for 24 h. followed by long term storage at -80°C prior to RNA extraction. An aliquot of blood was
178 collected through the ventral blood vessel from the underside of each fish using heparinised syringes,
179 before centrifugation to separate the plasma for free amino acid analysis.

180 **2.4 Gene expression analysis following infection.**

181 The expression of transcripts encoding enzymes of the urea cycle, along with rate limiting enzymes of
182 polyamine synthesis (characterised previously in [33]) were investigated in liver and head kidney
183 tissues using qPCR. Liver was chosen for investigation as it shows a well-established response to
184 infection, while also acting as the main site for the urea cycle and amino acid metabolism [33, 34].
185 While head kidney represents the primary immune organ in teleost fish and site of lymphocyte
186 differentiation, proliferation, and maturation [35, 36]. RNA extractions, cDNA synthesis and qPCR
187 were performed as previously described [33]. Briefly, RNA was extracted from 100 mg of tissue
188 homogenised in 1 ml of TRI Reagent (Sigma-Aldrich) following the manufacturer's instructions. First-
189 strand cDNA was synthesised from 1 µg total RNA using a QuantiTech Reverse Transcription kit
190 (QIAGEN), with an integrated genomic DNA elimination step followed by a 20-fold dilution with
191 RNase/DNase free water (Sigma-Aldrich). qPCR analyses were performed with SYBR Green I dye
192 chemistry using an Mx3005P System (Agilent Technologies). All assays were carried out in duplicate
193 within 96 well plates using 15 µl reactions containing 5 µl of the 1:20-diluted cDNA (corresponding to
194 2.5 ng of reverse-transcribed total RNA), 500 nM sense/antisense primers and 7.5 µl Brilliant III Ultra-
195 Fast SYBR Green (Agilent Technologies). The PCR cycling conditions were 1 cycle of 95 °C for 3 min,
196 followed by 40 cycles of 95 °C for 20 s then 64 °C for 20 s (two step PCR). Candidate gene expression
197 was normalised to three reference genes (*EF-1α*, *ACTB* and *HPRT*). All gene primers used in the study
198 are presented in Table 3.

199 **Table 3**

200 **2.5 Plasma free amino acid analysis following bacterial infection**

201 Free circulating plasma amino acid concentrations were determined in the blood plasma samples. Blood
202 (2 ml per fish) was centrifuged at 1,500g for 15 minutes. to separate the plasma from erythrocytes.
203 Plasma supernatant (0.5 ml) was aliquoted from each vial and stored in Eppendorf tubes at -80°C. Blood
204 plasma samples were shipped on dry ice for amino acid analysis to Ansynth Service B.V.

205 **2.6 Statistical Analysis**

206 All statistical analysis of growth parameters, gene expression data, free amino acid concentrations and
207 survival data were performed in R (v3.4.0). Dietary and infection groups were assessed with two-way
208 ANOVA, initially testing for an interaction between diet and infection. If there was no interaction, the
209 ANOVA was repeated without the interaction term. A *post hoc* TUKEY test was performed if the
210 ANOVA result was significant. Diagnostic plots (qq plot and residuals versus fitted values) were
211 visually assessed in order to ensure both normality and equal variance. If data met the assumptions, the
212 ANOVA results from R's lm function were interpreted. If data was not normal, a log transformation
213 was first performed, and the diagnostics plots then reassessed. When data still did not conform to
214 ANOVA assumptions, general least squares regression was performed. Survival data was converted to
215 percentage survival over the course of the ten days and analysed using the Kaplan-Meier estimate. Non-
216 metric multidimensional scaling (nMDS) analysis was used to identify any possible groupings in
217 combined gene expression and free amino acid data based on the 'Gower' index using the 'metaMDS'
218 function in the 'vegan' package in R (v3.4.0). Ordinance plots were created for free amino acid data
219 combined with either liver or head kidney gene expression. The 'envfit' function in 'vegan' was used
220 to illustrate the factors with the largest significant effects on the model, overlaid as vectors on the
221 ordnance plots.

222

223 **3. Results**

224 For clarity within the results, AS and PBS have been added onto the end of diets and gene names have
225 been kept in italics, e.g. infected ARG-2 fed fish are named as ARG-2-AS.

226 **3.1 Growth parameters in control or supplemented amino acid diets**

227 All fish survived the feeding trial and approximately doubled their weight during the trial (growth data
228 presented in Table 4). There was no significant difference in whole body final weight between diets,
229 but fish fed the ORN-2 diet had significantly higher gutted weight than ARG-2 fish (206 ± 4 g to $185 \pm$
230 4 g respectively) but neither were significantly different to the control or CIT-2 fish. There was no
231 significant difference between different diets for HSI or VSI, however for condition factor (K), there
232 was a significant decrease in CIT-2 (1.34 ± 0.01) relative to the control diet (1.39 ± 0.01). Feed
233 conversion ratio (FCR) and specific growth rate (SGR) were calculated for individual fish based on
234 uneaten feed in their respective tanks; however, no significant differences in FCR or SGR were found
235 between any diet.

236 **Table 4**

237

238 **3.2 Mortality following bacterial challenge in fish fed different supplemented diets.**

239 We investigated the effect of amino acid supplementation on fish survival following a bacterial infection
240 with AS over a 12-day challenge (Figure 1). Mortality started at day 4 and peak mortality between days
241 5 and 6, continuing until day 10. Fish were monitored for a further two days where no more mortalities
242 occurred, and the challenge ended. The CIT-2 diet had the lowest survival percentage of any diet
243 followed by ORN-2 supplemented fish while ARG-2 fed fish had the highest percentage survival.
244 However, the Kaplan–Meier estimate test revealed there were no significant differences between diets
245 on survival ($p=0.49$).

246 **Figure 1**

247 **3.3 Free amino acids in blood plasma following AS infection**

248 Free circulating amino acids were examined in the plasma of fish 24 h. after i.p. injection with AS or
249 PBS (control). A total of 26 amino acids were detected and analysed using two-way ANOVA
250 investigating the effects of diet and infection (Table 5). Amino acids that were significantly altered by
251 either diet or infection, are plotted on Figures 2-5. Of the 26 amino acids detected, two essential amino
252 acids (EAA) and six non-essential amino acids (NEAA) were significantly affected by infection (EAA:
253 isoleucine, phenylalanine; NEAA: ornithine, taurine, aspartic acid, glutamic acid, glycine and tyrosine)
254 and 9 amino acids were affected by diet (EAA: arginine, histidine, methionine, phenylalanine, NEAA:
255 ornithine, citrulline, hydroxyproline, asparagine and proline). The total amino acid (TAA)
256 concentration, total EAA and total NEAA was estimated for all individual fish (Table 5). Of these,
257 infection effects were detected for TAA and NEAA with concentrations significantly decreasing in AS
258 fish relative to controls (Figure 2), however no dietary effect was detected (Table 5).

259 **EAA**

260 Arginine levels were significantly affected by diet with increased levels in CIT-2 compared to all other
261 diets (Figure 3). Histidine and methionine were both significantly affected by diet (Figure 3; Table 5).
262 Histidine levels were significantly higher in ARG-2 compared to ORN-2. Methionine levels were
263 decreased in all supplemented diets relative to the control diet, but only CIT-2 displayed a significant
264 decrease (Figure 3). Phenylalanine levels were significantly higher in ORN-2 compared to ARG-2 and
265 CIT-2 diets (Figure 3). Phenylalanine was also significantly affected by infection with levels decreasing
266 in all diets (Figure 4; Table 5). The magnitude of decrease of phenylalanine following infection appears
267 to be diet dependent, with fish fed the control diet displaying the largest decrease from 137 to 94 $\mu\text{mol/l}$,
268 whereas the supplemented diets displayed a decrease of 12-15 $\mu\text{mol/l}$ (Table 5). Isoleucine was the only
269 other essential amino acid affected by infection, where levels increased in infected fish (Figure 4).

270 **NEAA**

271 Ornithine and citrulline levels were both significantly altered by diet with increases observed in CIT-2
272 (Figure 5). A significant diet effect was also detected for proline, hydroxyproline and asparagine (Table
273 5). Proline levels increased in all supplemented diets relative to the control diet, however this was only

274 significant in ARG-2 (Figure 5). Hydroxyproline and asparagine displayed a similar response, with
275 highest levels observed in ARG-2 and lowest levels in ORN-2, which were both significantly different
276 (Figure 5). Taurine and glycine were the most abundant amino acids detected in plasma, apart from
277 citrulline in CIT-2 supplemented fish, and displayed large significant decreases following infection
278 (Figure 4). Glutamic acid, tyrosine, and aspartic acid were all significantly affected by infection with
279 each showing significant decreases in infected fish (Table 5; Figure 4).

280 **Table 5**

281 **Figure 2, 3, 4, 5**

282

283 **3.4 Transcriptional response of immune genes in liver following bacterial challenge.**

284 To confirm the inflammatory responses to infection, the mRNA expression of two key marker genes
285 for the acute phase response, serum amyloid A (*SAA*) and hepcidin (*HAMP*), were examined in infected
286 and control liver tissue (Figure 6). For all the diets, both marker genes significantly increased in
287 expression following AS infection compared to the control (PBS injected) fish, confirming the fish were
288 undergoing a proinflammatory acute phase response. There was no significant difference in the
289 expression of the same genes across the diets.

290 **Figure 6**

291 **3.5 Liver expression response of urea cycle and polyamine synthesis genes**

292 The mRNA expression levels of the urea cycle (*Arg1a*, *Arg1b*, *Arg2a*, *Arg2b*, *OTC*, *ASS* and *ASL*),
293 *iNOS* and rate limiting enzymes of polyamine synthesis (*ODC1*, *ODC2*, *SAMdc1* and *SAMdc2*) were
294 quantified in the liver of control and AS infected fish for all diets (Figure 7; Supplementary Table 1).

295 For the four genes encoding the arginase paralogues (Figure 7), *Arg1a* and *Arg2a* expression was
296 significantly impacted by infection and an interaction effect was detected for both genes
297 (Supplementary Table 1). *Arg1a* expression was significantly increased in ARG-2-AS compared to
298 ARG-2-PBS and no other diet displayed a change from infection. *Arg2a* was significantly increased in

299 control-AS, ORN-2-AS and CIT-2-AS relative to the control (PBS-injected) fish for each respective
300 diet, with no significant difference in *Arg2a* expression between ARG-2-AS and ARG-2-PBS fish.
301 While two-way ANOVA detected a significant effect of infection on *Arg2b* expression (Supplementary
302 Table 1), no differences were detected between diets. *Arg1b* expression was unaffected by both diet and
303 infection.

304 Among the genes encoding the urea cycle enzymes (*OTC*, *ASS*, *ASL*) and *iNOS* (Figure 7), only *ASS*
305 and *iNOS* were significantly altered by AS infection, while an interaction effect between infection and
306 diet was detected in *ASL* and *iNOS*. There was a general decrease in *ASS* expression following infection
307 in fish fed supplemented diets, but a significant difference was only found between ARG-2-AS and
308 ARG-2-PBS. Although there was a significant interaction between diet and infection for *ASL*
309 expression, there were no significant changes between diets. There was a significant increase in *iNOS*
310 expression in control-AS vs. control-PBS; while no significant response was observed in supplemented
311 diets, there was a large non-significant increase in CIT-2-AS relative to CIT-2-PBS. No significant
312 differences were detected for either diet or infection in *OTC* expression.

313 All genes encoding rate-limiting polyamine synthesis enzymes (*ODC1*, *ODC2*, *SAMdc1*, and *SAMdc2*)
314 showed significant responses to AS infection (Figure 7). *ODC1* and *ODC2* increased significantly in
315 expression following infection in all diets except ORN-2 for *ODC1* and ARG-2/ORN-2 for *ODC2*, with
316 no significant differences observed between diets for either gene. *SAMdc1* expression was significantly
317 increased by AS infection in all diets apart from ORN-2, and again no effect of diet was detected.
318 Infection had a significant effect on *SAMdc2* expression (Supplementary Table 1), and increases could
319 be seen in control-AS, ORN-2-AS and CIT-2-AS relative to each diets PBS control, however only CIT-
320 2-AS was significantly higher than control-PBS as determined by the Tukey test (Figure 7). Overall
321 there were major impact on gene expression of urea and polyamine pathway genes resulting from
322 bacterial infection with an interaction caused by diet for *ARG1a* and *iNOS*.

323 **Figure 7**

324 **3.6 Head kidney expression response of urea cycle and polyamine synthesis genes**

325 The relative mRNA expression levels of the same genes considered in section 3.5 were examined in
326 head kidney (Figure 8; Supplementary Table 2). AS infection significantly increased *Arg1a* expression
327 in all diets relative to each diets control, while ARG-2-PBS also showed a significantly higher
328 expression of *Arg1a* than control-PBS. *Arg2b* expression increased following infection in control-AS,
329 ARG-2-AS and CIT-2-AS fish relative to each diets control (PBS), while there was a decrease in
330 expression in ORN-2-AS vs. ORN-2-PBS; however, there were no significant changes detected
331 between diets. There were no significant differences in *Arg2a* expression *Arg1b* expression was not
332 detected.

333 A significant effect of AS infection was detected for both *ASS* and *iNOS* (Figure 8; Supplementary
334 Table 2). *ASS* expression was significantly increased following bacterial infection in CIT-2-AS
335 compared to CIT-2-PBS but was unaffected in the other diets. Although there was a significant overall
336 effect of AS infection on *iNOS* (Supplementary Table 2), the Tukey test revealed no differences between
337 groups (Figure 8). Neither AS infection nor diet had a significant effect on *OTC* and *ASL* expression.

338 Expression of the rate-limiting enzymes of polyamine synthesis was generally increased following
339 infection in head kidney. *ODC1* expression significantly increased following infection in the control
340 diet, but not for the supplemented diets. For *ODC2* expression there was a non-significant increase
341 following infection for ARG-2-AS and CIT-2-AS. *SAMdc1* expression was significantly increased in
342 ORN-2-AS and CIT-2-AS compared to the respective diets controls. For *SAMdc2*, only ARG-2-AS
343 showed a significant increase compared to its respective diets control. For the kidney, the infection
344 resulted in significant changes in expression for both urea cycle and polymamine synthesis genes, unlike
345 there was no interaction between diet and infection observed (Supplemental Table 2.).

346 **Figure 8**

347 **3.7 Non-metric multidimensional scaling analyses**

348 *3.7.1 Liver gene expression and free amino acid responses*

349 To visualize which components were influencing differences in immunological response between diets,
350 nMDS was performed on the amino acid data from blood plasma combined separately with gene

351 expression data from the two tissues. In the liver analysis (Figure 9), there was a clear separation
352 between the infected and uninfected fish, with non-overlapping 95% confidence intervals (Figure 9).
353 The vectors explaining the response to infection were the polyamine synthesis genes (*ODC1*, *ODC2*,
354 *SAMdc1* and *SAMdc2*) and *Arg2a*, with the free amino acid ornithine also contributing a strong vector
355 influence. The factors with the largest impact on the uninfected (PBS) fish were principally taurine,
356 aspartic acid, glycine and glutamic acid. For the infected fish, there was little difference between the
357 diet, with all 95% confidence intervals overlapping. However, for the uninfected fish, ARG-2 is clearly
358 separated from the control and ORN-2 diets.

359 **Figure 9**

360 *3.7.2 Head kidney gene expression and free amino acid responses*

361 There was still separation between the uninfected and infected groups in head kidney (Figure 10), but
362 not as apparent as for liver (Figure 9). Control-PBS, ARG-2-PBS and CIT-2-PBS had non-overlapping
363 95% confidence intervals with the infected fish, while ORN-2-PBS displayed a high degree of
364 individual variation and overlapped with all other groups (Figure 10). The components having the
365 largest impact on the infected groups were *Arg1a*, *Arg2a*, *Arg2b*, *ODC2*, *SAMdc2* and *ASS* expression
366 and ornithine levels, whereas serine, tyrosine and glycine had the largest impact on the uninfected
367 groups. As with the liver analysis, the uninfected ARG-2-PBS was significantly different to the control
368 diet.

369 **Figure 10**

370

371

372

373 **4. Discussion**

374 The physiological effects of functional amino acid supplementation to fish diets is still a largely
375 unexplored field. Here, we attempt to bridge this knowledge gap by investigating arginine, ornithine
376 and citrulline supplementation on immunological response and survival following a controlled bacterial
377 challenge in rainbow trout. We also examined both free amino acids and the changes in gene expression
378 related to arginine metabolism. This study, to the best of our knowledge, is the first to examine the
379 changes in free amino acid concentrations in fish following a bacterial infection and improves our
380 understanding of interactions between the immune and metabolic systems of fish.

381 **Arginine supplementation and growth**

382 Arginine is an important functional amino acid in both terrestrial and aquatic farmed vertebrate, and its
383 dietary supplementation was reported to lead to improvements in growth [37], protein deposition [38],
384 and the immune response [39]. However, arginine supplementation has been associated with many
385 contradictory results in the literature [40], while the effects of ornithine and citrulline supplementation
386 in fish remains largely unknown. In the current study, growth parameters were largely unaltered by the
387 supplemented diets, although fish on the ARG-2 diet had significantly lower gutted weight than those
388 on the ORN-2 diet. Gutted weight is more indicative of the filet yield and profitability than overall
389 weight, as significant inedible portions such as visceral fat deposits and organs are discarded [41]. The
390 significant increase in gutted weight, but not overall weight may indicate an increase in protein
391 deposition from ornithine supplementation, or a decrease in protein deposition following arginine
392 supplementation. Studies in blunt snout bream and gibel carp [38, 42] have shown that arginine
393 supplementation can induce mTOR signalling activity, a central regulator of protein synthesis, cellular
394 growth and proliferation [43]. As the diets used in this study contained high levels of supplemented
395 amino acids, it is possible that the excess arginine in ARG-2 hindered uptake of lysine, another essential
396 amino acid in salmonids that competes for the same transporter proteins [44]. Unbalanced dietary lysine
397 and arginine ratios can inhibit uptake of the other, resulting in reduced growth and health performance
398 [45, 46]; however, lysine levels were unchanged in the present study. Ornithine is a non-proteogenic
399 amino acid, formed as a result of arginine metabolism and is used in polyamine synthesis. Polyamines

400 are essential in cellular proliferation and are able to regulate protein synthesis [47]. The supplemented
401 ornithine in ORN-2 may have increased polyamine levels allowing a higher gutted weight. Fultons
402 condition factor (K) is often used to describe the weight/length relationship of fish to give an indication
403 of energy reserves and general condition [48, 49]. The significantly lowered K in fish fed ARG-2 and
404 CIT-2 diets could indicate lowered lipid content in the tissue of fish fed supplemented diets.

405 **AS challenge and effects on rainbow trout survival**

406 There were no significant differences in survival detected between the diets. The influence of dietary
407 inclusion of ornithine and citrulline on mortality has not been investigated in any organism previously,
408 though the effects of arginine supplementation are well documented. In mice fed arginine supplemented
409 diets, decreased mortality was seen following challenges with bacterial [50] and parasitic pathogens
410 [51]. Similar studies in fish also demonstrated decreased mortality following feeding with arginine
411 supplemented diets, including for Jian carp [52] and channel catfish [53]. In sea bass, arginine
412 supplementation led to decreased respiratory burst and decreased plasma NO, which led to higher
413 disease susceptibility and mortality [54]. As suggested by Azeredo *et al* [54] and supported by findings
414 in this paper, the varying results observed following arginine supplementation are likely due to diverse
415 and complex factors, e.g. pathogen, species, developmental stage, and environmental conditions. An
416 alternative challenge method for future research could have included a bath challenge model where a
417 more natural route of infection may be able to highlight differences in response to infection by diet.

418 **Metabolism of the urea cycle amino acids in response to infection**

419 There were significant modifications to the urea cycle amino acids (arginine, ornithine and citrulline)
420 in the blood plasma due to both AS infection and diet. Circulating arginine levels were significantly
421 increased in the CIT-2 diet, as documented in mammalian studies [29, 30, 55]. In mammals, citrulline
422 supplementation increases arginine levels to a greater extent than direct arginine supplementation and
423 has been linked to improvements in immune function due to greater arginine availability [12]. Citrulline,
424 and not arginine supplementation, is able to bolster arginine levels due to a difference in how the two
425 amino acids are metabolised. Arginine is susceptible to high levels of first pass metabolism from the

426 liver, where arginase is highly active, meaning large amounts of ingested arginine are excreted as
427 nitrogenous waste [56]. Citrulline, on the other hand, is absorbed in the kidney and converted to arginine
428 through the action of *ASS* and *ASL* before being released into the blood as arginine [57]. Fish on the
429 CIT-2 diet also showed significantly increased circulating ornithine levels relative to fish on the control
430 and ARG-2 diets, potentially due to metabolism of the excess circulating arginine. Circulating ornithine
431 was also significantly altered by AS infection and was one of only two amino acids that increased in
432 concentration following treatment. This increase in ornithine could be related to the activation of
433 different macrophage subtypes. M2 (healing) macrophages convert arginine into ornithine for use in
434 polyamine synthesis and subsequent tissue repair [58], whereas M1 (killing) macrophages compete with
435 M2 macrophages for arginine for use in NO synthesis via the action of *iNOS* [59]. Transcripts for all
436 the polyamine synthesis enzymes (*ODC1*, *ODC2*, *SAMdc1* and *SAMdc2*) and *iNOS* were significantly
437 increased by infection in both liver and head kidney, consistent with both M1 and M2 macrophage
438 activation during an immune response. However, as arginine levels were not significantly affected 24
439 h post-infection, this suggests either that a significant recycling of arginine was occurring, or that the
440 sampling timepoint was too early to see a change in arginine levels. Future research could include
441 additional time points to observe any dietary impact in a temporal manner.

442 **Essential amino acid metabolism in response to infection**

443 Histidine, methionine and phenylalanine were significantly affected by diet, while isoleucine and
444 phenylalanine were significantly affected by infection. The significant decrease of methionine in CIT-
445 2 fish could be explained by the observed increase in ornithine levels. Ornithine can be converted to
446 putrescine - the simplest polyamine - through the action of *ODC*; however in order to synthesise the
447 more complex polyamines, spermidine and spermine, a methyl group must be donated from s-
448 adenosylmethionine (SAM), which itself is formed from methionine and ATP [60]. Assuming the high
449 levels of *SAMdc* mRNA expression in the CIT-2 diet is matched to an increase in S-adenosylmethionine
450 decarboxylase activity, the fish may have been utilising more methionine for synthesis of the higher
451 polyamines.

452 The branched chain amino acids (BCAAs) isoleucine, leucine and valine account for 35% of the total
453 composition of EAA in body protein and 14% of the EAAs in muscle tissue [61]. BCAAs have several
454 physiological roles including in protein synthesis, intracellular signalling, lymphocyte proliferation and
455 can be oxidised for energy generation [62, 63]. Isoleucine is incorporated into the proteins of immune
456 cells such as lymphocytes, eosinophils and neutrophils, and the absence of any of the BCAAs vastly
457 reduces leukocyte proliferation [64]. During an immune response, sufficient nutrients and energy are
458 required for an effective immune response. In this respect, skeletal muscle can be catabolised to provide
459 both energy and free amino acids for the synthesis of new proteins and cells [65]. The increase in
460 isoleucine levels in the plasma observed in this study, potentially reflects such increased muscle
461 catabolism for the immune response.

462 Phenylalanine is mainly metabolised into tyrosine through the action of phenylalanine hydroxylase and
463 the cofactor tetrahydrobiopterin (BH4), with the synthesis of BH4 itself limited by the action of GTP-
464 cyclohydrolase I (GCH) [66]. In humans, inflammatory conditions associated with Th1-type responses
465 are known to create a BH4 deficiency, as IFN γ stimulates GCH to produce neopterin over BH4, thus
466 inhibiting the conversion of phenylalanine to tyrosine [67]. This leads to an accumulation of
467 phenylalanine and decrease of tyrosine in plasma, which is a common symptom in patients with chronic
468 diseases such as phenylketonuria or cancer [67]. However, our results show a decrease in both
469 phenylalanine and tyrosine concentrations, suggesting another role for phenylalanine in the immune
470 response. *In vitro* experiments demonstrated that activated mice CD8⁺ cells have significant uptake of
471 phenylalanine compared to naïve cells [68], however phenylalanine's exact role is unknown.

472 **Non-essential amino acid metabolism in response to infection**

473 There were proportionally more non-essential than essential amino acids affected by AS infection, while
474 the opposite was true for dietary effects. Proline, hydroxyproline and asparagine were all significantly
475 affected by diet, while glutamic acid, tyrosine, aspartic acid, taurine and glycine were all significantly
476 affected by treatment.

477 Proline and its metabolite hydroxyproline can synthesise polyamines as well as being responsible for
478 one third of the amino acids in collagen, which constitutes 30% of whole-body protein [69]. In
479 mammals, proline is required for endogenous arginine synthesis, which occurs through the intestine-
480 renal axis of proline or glutamate > P5C > ornithine > citrulline > arginine [70]. The enzymes
481 responsible for this endogenous synthesis of arginine (P5C synthase, CPS and OTC) are all expressed
482 at low levels in most adult teleost species and is one reason that arginine is regarded as an essential
483 nutrient in fish [71, 72]. Both glutamate and proline can synthesise P5C (and each other using P5C as
484 an intermediate molecule), however it has been suggested that the conversion of proline to arginine is
485 the preferred pathway in mammals [70]. The increased proline and hydroxyproline levels observed in
486 fish on the ARG-2 diet may indicate proline synthesis from arginine, or a potentially sparing effect.

487 Taurine and glycine both displayed highly significant decreases in plasma levels following infection.
488 Taurine is a non-proteogenic amino acid with major roles in oxidative defence and the anti-
489 inflammatory response [9, 73, 74]. Leukocytes possess high concentrations of taurine, which allow an
490 increased respiratory burst while decreasing tissue injury without comprising antimicrobial function
491 [73]. Even over the course of an immune response, when plasma taurine levels can become deficient,
492 leukocytes maintain a high taurine concentration, emphasising this amino acid's importance in
493 preventing oxidative damage [75]. Glycine has similar roles in oxidative defence, as well as potential
494 tissue repair and is a particularly abundant amino acid, accounting for >30% of the amino acid
495 composition of collagen and elastin [76, 77], and forming an essential component of glutathione.
496 Glutathione is composed of glutamate, cysteine, and glycine and has an essential role in antioxidant
497 defence to prevent tissue damage following an inflammatory response, as well as the scavenging of free
498 radicals [78]. The observed decreases in glutamic acid (deprotonated glutamate) and glycine in fish
499 following AS infection likely represents the increased oxidative stress from infection and depletion of
500 glutathione. The larger decreases in glycine could also indicate an increase in collagen synthesis for
501 tissue repair following infection. Aspartic acid (deprotonated aspartate) was decreased following
502 infection in this study. Aspartic acid has no direct role in the immune response, but studies on the teleost
503 meagre and chicken have suggested that supplementary aspartate can reduce stress in farmed animals

504 [79, 80]. Aspartate does have direct roles in gluconeogenesis and the urea cycle, where it acts as a substrate
505 to form arginosuccinate from citrulline. The decreases observed in this study could be related to arginine
506 recycling from the additional citrulline generated from *iNOS* and the NO cycle, however both arginine
507 and citrulline plasma levels were unchanged following infection.

508 **Transcriptional responses of arginine metabolism genes to infection**

509 Many vertebrate species possess two distinct arginase paralogues, *Arg1* and *Arg2*, however due to the
510 salmonid-specific whole genome duplication that occurred ~88-103 MYA [81], some salmonids
511 possess a further two copies of each [82]. The two vertebrate arginases each catalyse the same reaction,
512 arginine to ornithine and urea, however they differ in expression levels [33]. *Arg1* is primarily expressed
513 in liver, whereas *Arg2* is expressed in most tissues, with lowest levels in liver [83]. In mammals, *Arg1*
514 is commonly used as a marker for M2 (healing) macrophages [58]. In contrast, there is evidence that
515 *Arg2* is a better marker for M2 macrophages in teleost fish, while *Arg1* is more involved in hepatic
516 metabolism of arginine [14, 84]. In the current experiment, *Arg1* and *Arg2* paralogues displayed
517 differential expression to both infection, diet, and between tissues. In liver, infection and diet had a
518 significant interaction on the expression of *Arg1a* and *Arg2a*. There was a significant increase in
519 expression of *Arg1a* in fish on the ARG-2 diet following infection, whereas *Arg2a* expression was
520 increased in AS infected fish on the control, ORN-2 and CIT-2 diets. In contrast *Arg2a* expression was
521 suppressed following infection in fish on the ARG-2 diet. While it is known that arginase and *iNOS* can
522 regulate each other's expression due to arginine competition [59], it may also be possible that *Arg1a*
523 and *Arg2a* also regulate each other, which may be the case in the ARG-2 group. If *Arg1a* is more
524 involved with the hepatic metabolism of arginine in fish, the higher expression seen in the ARG-2 fed
525 fish, could be related back to where orally ingested arginine is initially metabolised in liver [56].
526 Differential expression of arginase paralogues was also observed in head kidney. *Arg1a* expression was
527 increased in fish fed all diets following AS infection. The CIT-2 group displayed the highest *Arg1a*
528 expression levels post-infection, possibly reflecting the greater availability of arginine in the blood
529 plasma.

530 Following infection, M1 (kill) macrophages are activated by polarising T_H1 cytokines such as IFN- γ -
531 or TNF α [13]. M1 macrophages are characterised by increased *iNOS* expression and bring arginine
532 into the NO cycle for cytotoxic activity, producing both NO and citrulline [59]. The urea cycle enzymes
533 *ASS* and *ASL* also participate in the NO cycle, recycling the citrulline by-product, first into
534 arginosuccinate and then arginine [85]. Both *ASS* and *ASL* have important roles in maintaining arginine
535 levels and sustaining *iNOS* activity. M1 macrophage activity depends on extracellular arginine levels;
536 when there is a sufficient supply, macrophages export citrulline, but under depleted arginine conditions,
537 macrophages import citrulline and show increased expression of *ASS* to sustain NO output [86]. *ASS*,
538 *ASL*, and *iNOS* genes all displayed differential expression dependent on diet, infection and tissue. *iNOS*
539 expression seemed to be suppressed in the infected fish from ARG-2 and ORN-2 diets at varying
540 degrees in both liver and head kidney. In liver, *ASS* expression was decreased in all supplemented diets
541 following AS infection, while in head kidney AS infection caused increased expression in all diets, with
542 a higher magnitude of increase in supplemented diets. *ASL* displayed a similar expression pattern to
543 *ASS* in liver, with only the control diet AS infected fish increasing expression relative to control fish.
544 Increased expression of *ASS* in head kidney following AS infection is likely to be contributing to the
545 similar arginine levels observed in PBS and AS infected fish from the free amino acid analysis. It is
546 also likely that the higher arginine and citrulline levels observed from fish on the CIT-2 diet were
547 contributing to the greater expression of *iNOS* and *ASS* in head kidney.

548 Polyamines regulate the inflammatory response through the inhibition of inflammatory mediators, their
549 antioxidant properties, as well as their roles in cell proliferation [87, 88]. During an immune response,
550 M2 (healing) macrophages direct the conversion of arginine to ornithine for polyamine synthesis and
551 subsequent wound healing and tissue repair [19, 20]. The significant increases seen in all of the
552 polyamine synthesis enzymes (*ODC1*, *ODC2*, *SAMdc1*, and *SAMdc2*) in response to infection in both
553 liver and head kidney illustrates the importance of polyamines in the immune response.

554 nMDS analysis is a powerful tool that can analyse distinct datasets from the same experiment, here gene
555 expression data and free amino acid concentrations, to identify similarities between individuals and
556 non-trivial patterns in large data sets. Our nMDS plots displayed a clear separation between uninfected

557 and infected groups, but more importantly highlighted the possible role that the arginase and polyamine
558 synthesis enzymes have in the immune response, due to their large effects on the nMDS results. Several
559 amino acids, namely glycine, taurine, aspartic acid and ornithine were also identified, likely reflecting
560 large changes in the concentration of these amino acids due to infection.

561 **5. Conclusion**

562 In conclusion we show that the citrulline supplementation significantly increased circulating
563 arginine levels, however this had little effect on improving the immune response in rainbow trout
564 within this study or survival to pathogen challenge. The amino acids taurine, glycine and aspartic
565 acid showed the largest significant decreases in circulating plasma levels in response to infection
566 and could be key targets for immune enhancing diets, due to their essential roles in antioxidation
567 and cellular energy. The arginase paralogues displayed differing responses between liver and
568 head kidney and both diet and infection had complex impacts on their expression while the rate-
569 limiting enzymes of polyamine synthesis were all altered in expression following infection in
570 liver and head kidney, highlighting an important role for this pathway in the immune response.
571 Overall, these findings highlight potential functional amino acid targets for dietary
572 supplementation to bolster the immune response of salmonids.

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577 References

- 578 [1] S.A.M. Martin, E. Król, Nutrigenomics and immune function in fish: new insights from omics
579 technologies, *Dev. Comp. Immunol.* 75 (2017) 86–98. doi:10.1016/j.dci.2017.02.024.
580
- 581 [2] S.K. Whyte, The innate immune response of finfish – A review of current knowledge, *Fish*
582 *Shellfish Immunol.* 23 (2007) 1127–1151. doi:10.1016/j.fsi.2007.06.005.
583
- 584 [3] A. Alzaid, J.-H. Kim, R.H. Devlin, S.A.M. Martin, D.J. Macqueen, Growth hormone transgenesis
585 in coho salmon disrupts muscle immune function impacting cross-talk with growth systems, (2018).
586 doi:10.1242/jeb.173146.
587
- 588 [4] S. Kirchner, S. Kaushik, S. Panserat, Effect of partial substitution of dietary protein by a single
589 gluconeogenic dispensable amino acid on hepatic glucose metabolism in rainbow trout
590 (*Oncorhynchus mykiss*)., *Comp. Biochem. Physiol. A. Mol. Integr. Physiol.* 134 (2003) 337–47.
591
- 592 [5] R. Weihe, J.-E. Dessen, R. Arge, M.S. Thomassen, B. Hatlen, K.-A. Rørvik, Increased protein-to-
593 lipid ratio in energy dense diets improves slaughter yields and muscle thickness of different weight
594 classes of farmed Atlantic salmon (*Salmo salar* L.), *Aquac. Reports.* 13 (2019) 100173.
595 doi:10.1016/j.aqrep.2018.10.001.
596
- 597 [6] S.A.M. Martin, A. Douglas, D.F. Houlihan, C.J. Secombes, Starvation alters the liver
598 transcriptome of the innate immune response in Atlantic salmon (*Salmo salar*), *BMC Genomics.* 11
599 (2010) 418. doi:10.1186/1471-2164-11-418.
600
- 601 [7] S. Martin, S. Blaney, A. Bowman, D. Houlihan, Ubiquitin-proteasome-dependent proteolysis in
602 rainbow trout (*Oncorhynchus mykiss*): effect of food deprivation, *Pflugers Arch. Eur. J. Physiol.* 445
603 (2002) 257–266. doi:10.1007/s00424-002-0916-8.
604
- 605 [8] G. Wu, Functional amino acids in nutrition and health, *Amino Acids.* 45 (2013) 407–411.
606 doi:10.1007/s00726-013-1500-6.
607
- 608 [9] P. Li, Y.-L. Yin, D. Li, S.W. Kim, G. Wu, Amino acids and immune function, (2007).
609 doi:10.1017/S000711450769936X.
610
- 611 [10] Y.-Z. Fang, S. Yang, G. Wu, Free radicals, antioxidants, and nutrition., *Nutrition.* 18 (2002) 872–
612 9. <http://www.ncbi.nlm.nih.gov/pubmed/12361782>.
613
- 614 [11] G. Wu, A.E. Fuller, W.B. Ae, T.A.D. Ae, S. Woo, K. Ae, P. Li, A.J.M. Rhoads, A.M. Carey, S.
615 Ae, S.B. Smith, A.E. Thomas, E.S. Ae, Y. Yin, Arginine metabolism and nutrition in growth, health
616 and disease, *Amino acids*, 37(1), (2009). doi:10.1007/s00726-008-0210-y.
617
- 618 [12] K. Wijnands, T. Castermans, M. Hommen, D. Meesters, M. Poeze, Arginine and Citrulline and
619 the Immune Response in Sepsis, *Nutrients.* 7 (2015) 1426–1463. doi:10.3390/nu7031426.
620
- 621 [13] P.J. Murray, T.A. Wynn, Protective and pathogenic functions of macrophage subsets, *Nat. Rev.*
622 *Immunol.* 11 (2011) 723–737. doi:10.1038/nri3073.

623
624 [14] G.F. Wiegertjes, A.S. Wentzel, H.P. Spaijk, P.M. Elks, I.R. Fink, Polarization of immune
625 responses in fish: The ‘macrophages first’ point of view, *Mol. Immunol.* 69 (2016) 146–156.
626 doi:10.1016/j.molimm.2015.09.026.
627
628 [15] D.M. Mosser, J.P. Edwards, Exploring the full spectrum of macrophage activation, *Nat. Rev.*
629 *Immunol.* 8 (2008) 958–969. doi:10.1038/nri2448.
630
631 [16] R. Balaña-Fouce, E. Calvo-Álvarez, R. Álvarez-Velilla, C.F. Prada, Y. Pérez-Pertejo, R.M.
632 Reguera, Role of trypanosomatid’s arginase in polyamine biosynthesis and pathogenesis, *Mol.*
633 *Biochem. Parasitol.* 181 (2012) 85–93. doi:10.1016/J.MOLBIOPARA.2011.10.007.
634
635 [17] K. Nishimura, K. Murozumi, A. Shirahata, M.H. Park, K. Kashiwagi, K. Igarashi, Independent
636 roles of eIF5A and polyamines in cell proliferation, *Biochem. J.* 385 (2005) 779–785.
637 doi:10.1042/BJ20041477.
638
639 [18] A.J. Michael, Biosynthesis of polyamines and polyamine-containing molecules, *Biochem. J.* 473
640 (2016) 2315–2329. doi:10.1042/BCJ20160185.
641
642 [19] L. Ozer, S. Elgun, B. Ozdemir, B. Pervane, N. Ozmeric, Arginine–Nitric Oxide–Polyamine
643 Metabolism in Periodontal Disease, *J. Periodontol.* 82 (2011) 320–328. doi:10.1902/jop.2010.100199.
644
645 [20] C. Moinard, L. Cynober, J. Debandt, Polyamines: metabolism and implications in human
646 diseases, *Clin. Nutr.* 24 (2005) 184–197. doi:10.1016/j.clnu.2004.11.001.
647
648 [21] D.M. Hardbower, M. Asim, P.B. Luis, K. Singh, D.P. Barry, C. Yang, M.A. Steeves, J.L.
649 Cleveland, C. Schneider, M.B. Piazuelo, A.P. Gobert, K.T. Wilson, Ornithine decarboxylase regulates
650 M1 macrophage activation and mucosal inflammation via histone modifications., *Proc. Natl. Acad.*
651 *Sci. U. S. A.* 114 (2017) E751–E760. doi:10.1073/pnas.1614958114.
652
653 [22] S. Badurdeen, M. Mulongo, J.A. Berkley, Arginine depletion increases susceptibility to serious
654 infections in preterm newborns, (2014). doi:10.1038/pr.2014.177.
655
656 [23] C. Pohlenz, A. Buentello, S. le J Helland, D.M. Gatlin, Effects of dietary arginine
657 supplementation on growth, protein optimization and innate immune response of channel catfish
658 *Ictalurus punctatus* (Rafinesque 1818), *Aquac. Res.* 45 (2014) 491–500. doi:10.1111/j.1365-
659 2109.2012.03252.x.
660
661 [24] Y. Cao, Y. Feng, Y. Zhang, X. Zhu, F. Jin, L-Arginine supplementation inhibits the growth of
662 breast cancer by enhancing innate and adaptive immune responses mediated by suppression of
663 MDSCs in vivo, *BMC Cancer.* 16 (2016) 343. doi:10.1186/s12885-016-2376-0.
664
665 [25] Z. Cheng, A. Buentello, D.M. Gatlin, Effects of dietary arginine and glutamine on growth
666 performance, immune responses and intestinal structure of red drum, *Sciaenops ocellatus*,
667 *Aquaculture.* 319 (2011) 247–252. doi:10.1016/J.AQUACULTURE.2011.06.025.
668
669 [26] Z. Cheng, D.M. Gatlin, A. Buentello, Dietary supplementation of arginine and/or glutamine
670 influences growth performance, immune responses and intestinal morphology of hybrid striped bass

671 (Morone chrysops × Morone saxatilis), *Aquaculture*, 362–363 (2012), pp. 39–43.
672 doi:10.1016/j.aquaculture.2012.07.015.
673

674 [27] B. Costas, L.E.C. Conceição, J. Dias, B. Novoa, A. Figueras, A. Afonso, Dietary arginine and
675 repeated handling increase disease resistance and modulate innate immune mechanisms of Senegalese
676 sole (*Solea senegalensis* Kaup, 1858), *Fish Shellfish Immunol.* 31 (2011) 838–847.
677 doi:10.1016/j.fsi.2011.07.024.
678

679 [28] B. Costas, P.C.N.P. Rêgo, L.E.C. Conceição, J. Dias, A. Afonso, Dietary arginine
680 supplementation decreases plasma cortisol levels and modulates immune mechanisms in chronically
681 stressed turbot (*Scophthalmus maximus*), *Aquac. Nutr.* 19 (2013) 25–38. doi:10.1111/anu.12086.
682

683 [29] K.A.P. Wijnands, H. Vink, J.J. Briedé, E.E. van Faassen, W.H. Lamers, W.A. Buurman, M.
684 Poeze, Citrulline a More Suitable Substrate than Arginine to Restore NO Production and the
685 Microcirculation during Endotoxemia, *PLoS One.* 7 (2012) e37439.
686 doi:10.1371/journal.pone.0037439.
687

688 [30] S. Osowska, C. Moinard, N. Neveux, C. Loï, L. Cynober, Citrulline increases arginine pools and
689 restores nitrogen balance after massive intestinal resection, *Gut.* 53 (2004) 1781–1786.
690 doi:10.1136/gut.2004.042317.
691

692 [31] T.C. Clark, T. Sigholt, J. Tinsely, D.J. Macqueen, S.A.M. Martin, Supplementation of arginine,
693 ornithine and citrulline in rainbow trout (*Oncorhynchus mykiss*): Effects on growth, amino acid levels
694 and gene expression responses in plasma and tissue (2019). Manuscript submitted for publication.
695

696 [32] S. Dallaire-Dufresne, K.H. Tanaka, M. V. Trudel, A. Lafaille, S.J. Charette, Virulence, genomic
697 features, and plasticity of *Aeromonas salmonicida* subsp. *salmonicida*, the causative agent of fish
698 furunculosis, *Vet. Microbiol.* 169 (2014) 1–7. doi:10.1016/j.vetmic.2013.06.025.
699

700 [33] T.C. Clark, J. Tinsley, D.J. Macqueen, S.A.M. Martin, Rainbow trout (*Oncorhynchus mykiss*)
701 urea cycle and polyamine synthesis gene families show dynamic expression responses to
702 inflammation, *Fish Shellfish Immunol.* 89 (2019) 290–300. doi:10.1016/j.fsi.2019.03.075.
703

704 [34] D.R. Causey, M.A.N. Pohl, D.A. Stead, S.A.M. Martin, C.J. Secombes, D.J. Macqueen, High-
705 throughput proteomic profiling of the fish liver following bacterial infection, *BMC Genomics.* 19
706 (2018) 719. doi:10.1186/s12864-018-5092-0.
707

708 [35] A. Zapata, B. Diez, T. Cejalvo, C. Gutiérrez-de Frías, A. Cortés, Ontogeny of the immune system
709 of fish, *Fish Shellfish Immunol.* 20 (2006) 126–136. doi:10.1016/j.fsi.2004.09.005.
710

711 [36] S. Datta, D. Ghosh, D.R. Saha, S. Bhattacharaya, S. Mazumder, Chronic exposure to low
712 concentration of arsenic is immunotoxic to fish: Role of head kidney macrophages as biomarkers of
713 arsenic toxicity to *Clarias batrachus*, *Aquat. Toxicol.* 92 (2009) 86–94.
714 doi:10.1016/j.aquatox.2009.01.002.
715

716 [37] Q. Chen, H. Zhao, Y. Huang, J. Cao, G. Wang, Y. Sun, Y. Li, Effects of dietary arginine levels
717 on growth performance, body composition, serum biochemical indices and resistance ability against
718 ammonia-nitrogen stress in juvenile yellow catfish (*Pelteobagrus fulvidraco*), *Anim. Nutr.* 2 (2016)

719 204–210. doi:10.1016/J.ANINU.2016.07.001.
720
721 [38] H. Liang, M. Ren, H.-M. Habte-Tsion, X. Ge, J. Xie, H. Mi, B. Xi, L. Miao, B. Liu, Q. Zhou, W.
722 Fang, Dietary arginine affects growth performance, plasma amino acid contents and gene expressions
723 of the TOR signaling pathway in juvenile blunt snout bream, *Megalobrama amblycephala*,
724 *Aquaculture*. 461 (2016) 1–8. doi:10.1016/J.AQUACULTURE.2016.04.009.
725
726 [39] Y. Yue, Z. Zou, J. Zhu, D. Li, W. Xiao, J. Han, H. Yang, Effects of dietary arginine on growth
727 performance, feed utilization, haematological parameters and non-specific immune responses of
728 juvenile Nile tilapia (*Oreochromis niloticus* L.), *Aquac. Res.* 46 (2015) 1801–1809.
729 doi:10.1111/are.12333.
730
731 [40] I.A. Fauzi, Y. Haga, H. Kondo, I. Hirono, S. Satoh, Effects of arginine supplementation on
732 growth performance and plasma arginine, ornithine and citrulline dynamics of rainbow trout,
733 *Oncorhynchus mykiss*, *Aquac. Res.* 50 (2019) 1277–1290. doi:10.1111/are.14004.
734
735 [41] J.-E. Dessen, R. Weihe, B. Hatlen, M.S. Thomassen, K.-A. Rørvik, Different growth
736 performance, lipid deposition, and nutrient utilization in in-season (S1) Atlantic salmon post-smolt
737 fed isoenergetic diets differing in protein-to-lipid ratio, *Aquaculture*. 473 (2017) 345–354.
738 doi:10.1016/J.AQUACULTURE.2017.02.006.
739
740 [42] Y. Tu, S. Xie, D. Han, Y. Yang, J. Jin, X. Zhu, Dietary arginine requirement for gibel carp
741 (*Carassis auratus gibelio* var. CAS III) reduces with fish size from 50 g to 150 g associated with
742 modulation of genes involved in TOR signaling pathway, *Aquaculture*. 449 (2015) 37–47.
743 doi:10.1016/J.AQUACULTURE.2015.02.031.
744
745 [43] M. Laplante, D.M. Sabatini, mTOR signaling at a glance., *J. Cell Sci.* 122 (2009) 3589–94.
746 doi:10.1242/jcs.051011.
747
748 [44] F. Zhou, Q. Shao, J. Xiao, X. Peng, B.-O. Ngandzali, Z. Sun, W.-K. Ng, Effects of dietary
749 arginine and lysine levels on growth performance, nutrient utilization and tissue biochemical profile
750 of black sea bream, *Acanthopagrus schlegelii*, fingerlings, *Aquaculture*. 319 (2011) 72–80.
751 doi:10.1016/J.AQUACULTURE.2011.06.001.
752
753 [45] Y.C. Luiking, N.E.P. Deutz, Biomarkers of arginine and lysine excess., *J. Nutr.* 137 (2007)
754 1662S–1668S. doi:10.1093/jn/137.6.1662S.
755
756 [46] G.E. Berge, H. Sveier, E. Lied, Effects of feeding Atlantic salmon (*Salmo salar* L.) imbalanced
757 levels of lysine and arginine, *Aquac. Nutr.* 8 (2002) 239–248. doi:10.1046/j.1365-2095.2002.00211.x.
758
759 [47] K. Igarashi, K. Kashiwagi, Modulation of cellular function by polyamines, *Int. J. Biochem. Cell*
760 *Biol.* 42 (2010) 39–51. doi:10.1016/j.biocel.2009.07.009.
761
762 [48] A. Mozsar, G. Boros, P.S. Aly, L. Antal, S.A. Nagy, Relationship between Fulton’s condition
763 factor and proximate body composition in three freshwater fish species, (2014).
764 doi:10.1111/jai.12658.
765
766 [49] A. Kachari, S. Abujam, D.N. Das, Length- weight relationship (LWR) and condition factor of

767 amblyceps apangi, J. Aquaculture. Engineering and Fisheries Research 3 (2017) 97–107.
768 doi:10.3153/JAEFR17013.
769

770 [50] G. Liu, W. Ren, J. Fang, C.-A.A. Hu, G. Guan, N.A. Al-Dhabi, J. Yin, V. Duraipandiyan, S.
771 Chen, Y. Peng, Y. Yin, l-Glutamine and l-arginine protect against enterotoxigenic Escherichia coli
772 infection via intestinal innate immunity in mice, *Amino Acids*. 49 (2017) 1945–1954.
773 doi:10.1007/s00726-017-2410-9.
774

775 [51] S. Carbajosa, H.O. Rodríguez-Angulo, S. Gea, C. Chillón-Marinas, C. Poveda, M.C. Maza, D.
776 Colombet, M. Fresno, N. Gironès, L-arginine supplementation reduces mortality and improves
777 disease outcome in mice infected with *Trypanosoma cruzi*, *PLoS Negl. Trop. Dis.* 12 (2018)
778 e0006179. doi:10.1371/journal.pntd.0006179.
779

780 [52] G. Chen, Y. Liu, J. Jiang, W. Jiang, S. Kuang, L. Tang, W. Tang, Y.A. Zhang, X. Zhou, L. Feng,
781 Effect of dietary arginine on the immune response and gene expression in head kidney and spleen
782 following infection of Jian carp with *Aeromonas hydrophila*, *Fish Shellfish Immunol.* 44 (2015) 195–
783 202. doi:10.1016/j.fsi.2015.02.027.
784

785 [53] J.A. Buentello, D.M. Gatlin, Effects of Elevated Dietary Arginine on Resistance of Channel
786 Catfish to Exposure to *Edwardsiella ictaluri*, *J. Aquat. Anim. Health.* 13 (2001) 194–201.
787 doi:10.1577/1548-8667(2001)013<0194:EOEDAO>2.0.CO;2.
788

789 [54] R. Azeredo, J. Pérez-Sánchez, A. Sitjà-Bobadilla, B. Fouz, L. Tort, C. Aragão, A. Oliva-Teles, B.
790 Costas, European sea bass (*Dicentrarchus labrax*) immune status and disease resistance are impaired
791 by Arginine dietary supplementation, *PLoS One.* 10 (2015) 1–19. doi:10.1371/journal.pone.0139967.
792

793 [55] A. Lassala, F.W. Bazer, T.A. Cudd, P. Li, X. Li, M.C. Satterfield, T.E. Spencer, G. Wu,
794 Intravenous Administration of L-Citrulline to Pregnant Ewes Is More Effective Than L-Arginine for
795 Increasing Arginine Availability in the Fetus, *J. Nutr.* 139 (2009) 660–665.
796 doi:10.3945/jn.108.102020.
797

798 [56] T. Allerton, D. Proctor, J. Stephens, T. Dugas, G. Spielmann, B. Irving, l-Citrulline
799 Supplementation: Impact on Cardiometabolic Health, *Nutrients.* 10 (2018) 921.
800 doi:10.3390/nu10070921.
801

802 [57] A.W. El-Hattab, L.T. Emrick, W.J. Craigen, F. Scaglia, Citrulline and arginine utility in treating
803 nitric oxide deficiency in mitochondrial disorders, *Mol. Genet. Metab.* 107 (2012) 247–252.
804 doi:10.1016/j.ymgme.2012.06.018.
805

806 [58] Z. Yang, X.-F. Ming, Functions of arginase isoforms in macrophage inflammatory responses:
807 impact on cardiovascular diseases and metabolic disorders., *Front. Immunol.* 5 (2014) 533.
808 doi:10.3389/fimmu.2014.00533.
809

810 [59] M. Rath, I. Muller, P. Kropf, E.I. Closs, M. Munder, Metabolism via Arginase or Nitric Oxide
811 Synthase: Two Competing Arginine Pathways in Macrophages, *Front. Immunol.* 5 (2014) 532.
812 doi:10.3389/fimmu.2014.00532.
813

814 [60] S.M. Andersen, R. Waagbø, M. Espe, Functional amino acids in fish nutrition, health and

815 welfare., *Front. Biosci. (Elite Ed)*. 8 (2016) 143–69. <http://www.ncbi.nlm.nih.gov/pubmed/26709652>
816 (accessed August 26, 2019).
817

818 [61] R. Riazi, L.J. Wykes, R.O. Ball, P.B. Pencharz, The Total Branched-Chain Amino Acid
819 Requirement in Young Healthy Adult Men Determined by Indicator Amino Acid Oxidation by Use of
820 l-[1-13C] Phenylalanine, *J. Nutr.* 133 (2003) 1383–1389. doi:10.1093/jn/133.5.1383.
821

822 [62] M. Monirujjaman, A. Ferdouse, Metabolic and Physiological Roles of Branched-Chain Amino
823 Acids, *Adv. Mol. Biol.* 2014 (2014) 1–6. doi:10.1155/2014/364976.
824

825 [63] R.R. Wolfe, Branched-chain amino acids and muscle protein synthesis in humans: myth or
826 reality?, *J. Int. Soc. Sports Nutr.* 14 (2017) 30. doi:10.1186/s12970-017-0184-9.
827

828 [64] X. Mao, C. Gu, M. Ren, D. Chen, B. Yu, J. He, J. Yu, P. Zheng, J. Luo, Y. Luo, J. Wang, G.
829 Tian, Q. Yang, l-Isoleucine Administration Alleviates Rotavirus Infection and Immune Response in
830 the Weaned Piglet Model, *Front. Immunol.* 9 (2018) 1654. doi:10.3389/fimmu.2018.01654.
831

832 [65] P.C. Calder, Branched-Chain Amino Acids and Immunity, *J. Nutr.* 136 (2006) 288S-293S.
833 doi:10.1093/jn/136.1.288S.
834

835 [66] R.E. Frye, Central tetrahydrobiopterin concentration in neurodevelopmental disorders, *Front.*
836 *Neurosci.* 4 (2010) 52. doi:10.3389/fnins.2010.00052.
837

838 [67] S. Geisler, J.M. Gostner, K. Becker, F. Ueberall, D. Fuchs, Immune activation and inflammation
839 increase the plasma phenylalanine-to-tyrosine ratio, *Pteridines.* 24 (2013) 27–31. doi:10.1515/pterid-
840 2013-0001.
841

842 [68] L. V Sinclair, J. Rolf, E. Emslie, Y.-B. Shi, P.M. Taylor, D.A. Cantrell, Control of amino-acid
843 transport by antigen receptors coordinates the metabolic reprogramming essential for T cell
844 differentiation., *Nat. Immunol.* 14 (2013) 500–8. doi:10.1038/ni.2556.
845

846 [69] G. Wu, F.W. Bazer, R.C. Burghardt, G.A. Johnson, S.W. Kim, D.A. Knabe, P. Li, X. Li, J.R.
847 McKnight, M.C. Satterfield, T.E. Spencer, Proline and hydroxyproline metabolism: implications for
848 animal and human nutrition, *Amino Acids.* 40 (2011) 1053–1063. doi:10.1007/s00726-010-0715-z.
849

850 [70] C. Tomlinson, M. Rafii, M. Sgro, R.O. Ball, P. Pencharz, Arginine is synthesized from proline,
851 not glutamate, in enterally fed human preterm neonates, *Pediatr. Res.* 69 (2011) 46–50.
852 doi:10.1203/PDR.0b013e3181fc6ab7.
853

854 [71] J.J. Korte, W.L. Salo, V.M. Cabrera, P.A. Wright, A.K. Felskie, P.M. Anderson, Expression of
855 Carbamoyl-phosphate Synthetase III mRNA during the Early Stages of Development and in Muscle
856 of Adult Rainbow Trout (*Oncorhynchus mykiss*), *J. Biol. Chem.* 272 (1997) 6270–6277.
857 doi:10.1074/jbc.272.10.6270.
858

859 [72] A.M. Zimmer, P.A. Wright, C.M. Wood, Ammonia and urea handling by early life stages of
860 fishes, (2017). doi:10.1242/jeb.140210.
861

862 [73] M. Ekremoğlu, N. Türközkan, H. Erdamar, Y. Kurt, H. Yaman, Protective effect of taurine on

863 respiratory burst activity of polymorphonuclear leukocytes in endotoxemia, *Amino Acids*. 32 (2007)
864 413–417. doi:10.1007/s00726-006-0382-2.

865

866 [74] L. Su, H. Li, A. Xie, D. Liu, W. Rao, L. Lan, X. Li, F. Li, K. Xiao, H. Wang, P. Yan, X. Li, L.
867 Xie, Dynamic Changes in Amino Acid Concentration Profiles in Patients with Sepsis, *PLoS One*. 10
868 (2015) e0121933. doi:10.1371/journal.pone.0121933.

869

870 [75] C. Chiarla, I. Giovannini, J.H. Siegel, G. Boldrini, M. Castagneto, The Relationship between
871 Plasma Taurine and Other Amino Acid Levels in Human Sepsis, *J. Nutr.* 130 (2000) 2222–2227.
872 doi:10.1093/jn/130.9.2222.

873

874 [76] P. Li, G. Wu, Roles of dietary glycine, proline, and hydroxyproline in collagen synthesis and
875 animal growth, *Amino Acids*. 50 (2018) 29–38. doi:10.1007/s00726-017-2490-6.

876

877 [77] M.D. Shoulders, R.T. Raines, Collagen Structure and Stability, *Annu. Rev. Biochem.* 78 (2009)
878 929–958. doi:10.1146/annurev.biochem.77.032207.120833.

879

880 [78] M. Diotallevi, P. Checconi, A.T. Palamara, I. Celestino, L. Coppo, A. Holmgren, K. Abbas, F.
881 Peyrot, M. Mengozzi, P. Ghezzi, Glutathione Fine-Tunes the Innate Immune Response toward
882 Antiviral Pathways in a Macrophage Cell Line Independently of Its Antioxidant Properties, *Front.*
883 *Immunol.* 8 (2017) 1239. doi:10.3389/fimmu.2017.01239.

884

885 [79] E. Erwan, V.S. Chowdhury, M. Nagasawa, R. Goda, T. Otsuka, S. Yasuo, M. Furuse, Central
886 injection of L- and D-aspartate attenuates isolation-induced stress behavior in chicks possibly through
887 different mechanisms, *Eur. J. Pharmacol.* 736 (2014) 138–142. doi:10.1016/j.ejphar.2014.04.042.

888

889 [80] D. Gonzalez-Silvera, M. Herrera, I. Giráldez, M. Esteban, Effects of the Dietary Tryptophan and
890 Aspartate on the Immune Response of Meagre (*Argyrosomus regius*) after Stress, *Fishes*. 3 (2018) 6.
891 doi:10.3390/fishes3010006.

892

893 [81] D.J. Macqueen, I.A. Johnston, A well-constrained estimate for the timing of the salmonid whole
894 genome duplication reveals major decoupling from species diversification, *Proc. R. Soc. B Biol. Sci.*
895 281 (2014) 20132881. doi:10.1098/rspb.2013.2881.

896

897 [82] O. Benedicenti, T. Wang, E. Wangkahart, D.J. Milne, J.W. Holland, C. Collins, C.J. Secombes,
898 Characterisation of arginase paralogues in salmonids and their modulation by immune stimulation/
899 infection, *Fish Shellfish Immunol.* 61 (2017) 138–151. doi:10.1016/j.fsi.2016.12.024.

900

901 [83] B.C. Tennant, S.A. Center, Hepatic Function, in: *Clin. Biochem. Domest. Anim.*, Elsevier, 2008:
902 pp. 379–412. doi:10.1016/B978-0-12-370491-7.00013-1.

903

904 [84] M. Forlenza, I.R. Fink, G. Raes, G.F. Wiegertjes, Heterogeneity of macrophage activation in fish,
905 *Dev. Comp. Immunol.* 35 (2011) 1246–1255. doi:10.1016/J.DCI.2011.03.008.

906

907 [85] A. Husson, C. Brasse-Lagnel, A. Fairand, S. Renouf, A. Lavoinnie, Argininosuccinate synthetase
908 from the urea cycle to the citrulline-NO cycle, *Eur. J. Biochem.* 270 (2003) 1887–1899.
909 doi:10.1046/j.1432-1033.2003.03559.x.

910

- 911 [86] J.E. Qualls, C. Subramanian, W. Rafi, A.M. Smith, L. Balouzian, A.A. DeFreitas, K.A. Shirey,
912 B. Reutterer, E. Kernbauer, S. Stockinger, T. Decker, I. Miyairi, S.N. Vogel, P. Salgame, C.O. Rock,
913 P.J. Murray, Sustained Generation of Nitric Oxide and Control of Mycobacterial Infection Requires
914 Argininosuccinate Synthase 1, *Cell Host Microbe*. 12 (2012) 313–323.
915 doi:10.1016/j.chom.2012.07.012.
916
- 917 [87] L. Messina, A. Arcidiacono, G. Spampinato, L. Malaguarnera, G. Berton, L. Kaczmarek, A.
918 Messina, Accumulation of ornithine decarboxylase mRNA accompanies activation of human and
919 mouse monocytes/macrophages, *FEBS Lett*. 268 (1990) 32–34. doi:10.1016/0014-5793(90)80965-L.
920
- 921 [88] T. Hussain, B. Tan, W. Ren, N. Rahu, R. Dad, D.H. Kalhor, Y. Yin, Polyamines: therapeutic
922 perspectives in oxidative stress and inflammatory diseases, *Amino Acids*. 49 (2017) 1457–1468.
923 doi:10.1007/s00726-017-2447-9.
- 924

925 Figure 1. Timeline of percentage mortality of rainbow trout over a 12 day challenge with *Aeromonas*
926 *salmonicida*. Challenge took place following a 7-week feeding trial with rainbow trout fed one of four
927 diets; control commercial diet, ARG-2, ORN-2 or CIT-2. Kaplan–Meier estimate test was used to
928 analyse the differences between diets for survival (n=30).

929 Figure 2. Boxplots of essential, non-essential and total amino acids. Fish fed control and supplemented
930 diets were grouped together and split between uninfected (PBS, n=24) and infected groups (AS, n=24)
931 and then plotted to illustrate the changes in blood plasma concentration ($\mu\text{mol/l}$) following bacterial
932 infection, full details of individual groups in table 5. Asterisks above boxplots indicate significance
933 level (* = 0.05, ** = 0.01, *** = 0.001), outliers are displayed as small black dots.

934 Figure 3. Bar graphs of essential amino acids where two-way ANOVA detected a significant dietary
935 effect. Fish infected with *Aeromonas salmonicida* (AS) and uninfected (PBS) groups were grouped
936 together and split between diet to illustrate the changes in concentration ($\mu\text{mol/l}$) following dietary
937 amino acid supplementation. Full details of individual groups in Table 5. Bars represent mean (\pm SEM),
938 n=12. Results of the Tukey post hoc test are displayed above the bars. Bars which do not share a letter
939 are significantly different.

940 Figure 4. Boxplots of amino acids where two-way ANOVA detected a significant infection effect. Fish
941 fed control and supplemented diets were grouped together and split between uninfected (PBS, n=24)
942 and infected groups (AS, n=24) and then plotted to illustrate changes in blood plasma concentration
943 ($\mu\text{mol/l}$) following bacterial infection. Full details of individual groups in table 5. Asterisks above
944 boxplots indicate significance level (* = 0.05, ** = 0.01, *** = 0.001), outliers are displayed as small
945 black dots.

946 Figure 5. Bar graphs of non-essential amino acids where two-way ANOVA detected a significant
947 dietary effect. Fish infected with *Aeromonas salmonicida* (AS) and uninfected (PBS) groups were
948 grouped together and split between diet to illustrate the changes in concentration following dietary
949 amino acid supplementation. Other details are as given in the Figure 3 legend.

950 Figure 6. Relative expression of rainbow trout serum amyloid A (SAA) and hepcidin (HAMP) in liver
951 following a 7-week feeding trial with amino acid enriched diets and then subsequent bacterial infection.
952 Fish were injected i.p. with either PBS or *Aeromonas salmonicida* (AS). Expression was normalised to
953 housekeeping genes *EF-1 α* , *ACTB* and *HPRT*. A linear model was used for analysis of both genes. Bars
954 represent mean (\pm SEM), n=6. Results of the Tukey post hoc test are displayed above the bars. Bars
955 which do not share a letter are significantly different.

956 Figure 7. Expression of genes encoding urea cycle, *iNOS* and polyamine synthesis enzymes in liver.
957 Fish were injected i.p. with either PBS or *Aeromonas salmonicida* (AS). Other details are as given in
958 the Figure 6 legend.

959 Figure 8. Expression of genes encoding urea cycle, *iNOS* and polyamine synthesis enzymes in head
960 kidney. Fish were injected intraperitoneally with either phosphate buffered saline (PBS) *Aeromonas*
961 *salmonicida* (AS). *Arg1b* expression was not detectable in head kidney and excluded from the analysis.
962 Other details are as given in the Figure 6 legend.

963 Figure 9. Non-metric multidimensional scaling plot of free amino acid levels in blood plasma and liver
964 gene expression data from rainbow trout. Fish were fed a control commercial diet or amino acid
965 enriched diets for 7 weeks before a subsequent 24 h bacterial challenge. Fish were injected i.p. with
966 either phosphate buffered saline (PBS) or *Aeromonas salmonicida* (AS). Vectors plotted over the 95%
967 confidence intervals indicate factors with the largest effect on the data ($p < 0.001$). Genes are coloured
968 in black and amino acids in purple.

969 Figure 10. Non-metric multidimensional scaling plot of free amino acid levels in blood plasma and head
970 kidney gene expression data from rainbow trout. Other details are as given in the Figure 9 legend.

Table 1. Ingredients and proximal composition of experimental diets (g/kg)

	Ingredients¹	Control	ARG-2	ORN-2	CIT-2
971	Fish Meal	150	150	150	150
	Soya SPC	135	135	135	135
972	Wheat Gluten	176.8	176.8	176.8	176.8
	Maize Gluten	152	152	152	152
973	Wheat	110	90	90	90
	Fish Oil	89.6	89.6	89.6	89.6
974	Rapeseed Oil	166.4	166.4	166.4	166.4
	Vit + Min premix	32.5	32.5	32.5	32.5
975	Yttrium	0.5	0.5	0.5	0.5
	Proximate composition				
976	MOISTURE (%)	5.8	5.5	5.5	5.5
	PROTEIN - crude (%)	43.6	45.4	45.4	45.4
977	FAT - crude (%)	29.3	29.3	29.3	29.3
	ASH (%)	6.0	6.0	6.0	6.0

¹Water change of -12.8g

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Table 2. Amino acid composition of experimental diets (g/kg diet)

	Control	ARG-2	ORN-2	CIT-2	
982	Alanine	23.1	23.5	23.4	23.8
	Aspartic Acid	32.1	32.1	32.4	32.6
983	Cystine	7.18	6.92	6.77	7.4
	Glutamic Acid	103.0	105.0	104.0	108
984	Glycine	17.9	18.1	18.1	18.3
	Histidine	10.1	10.5	10.4	10.5
	Isoleucine	17.0	17.1	17.2	17.9
985	Leucine	40.3	40.7	41.1	41.9
	Lysine	26.1	26.2	26.1	26.8
986	Methionine	9.23	9.4.0	9.34	10.0
	Phenylalanine	22.9	23.4	23.0	23.5
987	Proline	34.5	35.0	34.7	38.5
	Serine	21.3	21.1	21.1	22.2
988	Threonine	15.8	15.7	15.7	15.8
	Valine	19.5	20.2	20.0	20.2
989	Arginine ¹	20.2	37	20.7	21
	Ornithine	0.2	0.2	13.4	0.3
990	Citrulline	0.0	0.0	0.1	19.1

¹ Arginine, ornithine and citrulline were analysed by Ansynth Service B.V.

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Table 3 Rainbow trout primer sequences used for qPCR with NCBI accession numbers

Gene	Sense	Primer 5'-3'	Product size	Annealing temperature	Accession
<i>EF-1α</i>	Forward	CAAGGATATCCGTCGTGGCA	327	64	NM_001124339.1
	Reverse	ACAGCGAAACGACCAAGAGG			
<i>HPRT</i>	Forward	CCGCCTCAAGAGCTAGTGTAAT	237	64	XM_021583468.1
	Reverse	GTCTGGAACCTCAAACCCTATG			
<i>β-actin</i>	Forward	ATGGAAGATGAAATCGCCCC	260	64	XM_021595779.1
	Reverse	TGCCAGATCTTCTCCATGTCG			
<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>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	TCCAGAGAGTCATGGAAGTCACTTTCC	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	ACGCTCTCCAACATCACA	129	64	XM_021563243.1
	Reverse	ACCGCATGACTCAGAATCCA			
<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>iNOS</i>	Forward	CGAATGGAGCTATCGTCAGACC	234	64	AJ300555.1
	Reverse	CGGGAACGTTGTGGTCATAATACC			

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Table 4. Growth performance of adult rainbow trout from a 7 week feeding trial fed diets supplemented with arginine, ornithine or citrulline (\pm SEM, n=45).

	Control¹	ARG-2	ORN-2	CIT-2	ANOVA
Start Weight (g)	84 \pm 1.8	82 \pm 1.5	83 \pm 1.6	85 \pm 1.7	0.46
End Weight (g)	225 \pm 5	220 \pm 4	235 \pm 5	229 \pm 4	0.13
Gutted Weight (g)	192 \pm 5 ^{ab}	185 \pm 4 ^a	206 \pm 4 ^b	195 \pm 4 ^{ab}	0.0089
HSI ²	1.53 \pm 0.03	1.46 \pm 0.03	1.47 \pm 0.03	1.52 \pm 0.03	0.37
VSI ³	13.7 \pm 0.2	13.9 \pm 0.2	13.6 \pm 0.2	14.1 \pm 0.2	0.2
Condition Factor ⁴	1.39 \pm 0.01 ^a	1.35 \pm 0.01 ^{bc}	1.38 \pm 0.01 ^{ab}	1.34 \pm 0.01 ^c	0.0002
FCR ⁵	0.85 \pm 0.1	0.77 \pm 0.02	0.71 \pm 0.02	0.76 \pm 0.03	0.4
SGR (%) ⁶	2.02 \pm 0.04	2.01 \pm 0.03	2.14 \pm 0.04	2.04 \pm 0.04	0.065

¹ Concentration values in the same row with different superscript letters are significantly different (p < 0.05)

² HSI: Hepatosomatic index = liver weight / body weight *100

³ VSI: Visceral fat somatic index = weight of viscera / body weight *100

⁴ Fultons condition factor (K) = (weight *100) / length ^ 3

⁵ FCR: Feed conversion ratio = wet weight gain / dry feed intake

⁶ SGR: Specific growth rate = (Ln end weight – Ln start weight)/days

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Table 5. Free essential amino acid levels ($\mu\text{mol/l}$) in blood plasma of rainbow trout infected with *Aeromonas salmonicida* (AS) or uninfected control fish (PBS) after a 7 week feeding trial with diets supplemented with arginine, ornithine or citrulline (mean \pm SEM, n=6)

Amino Acid ^{1,2}	Control		ARG-2		ORN-2		CIT-2		ANOVA	p
	PBS	AS	PBS	AS	PBS	AS	PBS	AS		
Essential Amino Acids										
Arginine	96 \pm 13 ^a	108 \pm 6 ^{ab}	114 \pm 10 ^{ab}	93 \pm 8 ^a	85 \pm 11 ^a	72 \pm 6 ^a	158 \pm 10 ^{bc}	174 \pm 16 ^c	Diet	0.0001 ***
									Infection	0.71
									Diet	0.044 *
Histidine	115 \pm 5	144 \pm 16	153 \pm 23	129 \pm 14	105 \pm 11	102 \pm 13	116 \pm 12	122 \pm 11	Infection	0.86
									Diet	0.13
Isoleucine	124 \pm 11	134 \pm 10	110 \pm 8	139 \pm 18	110 \pm 13	146 \pm 13	103 \pm 6	107 \pm 9	Infection	0.016 *
									Diet	0.07
Leucine	279 \pm 28	253 \pm 17	230 \pm 16	269 \pm 25	246 \pm 28	297 \pm 42	212 \pm 9	216 \pm 15	Infection	0.31
									Diet	0.07
Lysine	188 \pm 29	182 \pm 12	199 \pm 23	193 \pm 27	142 \pm 13	160 \pm 8	154 \pm 14	173 \pm 19	Infection	0.45
									Diet	0.019 *
Methionine	90 \pm 8 ^a	76 \pm 3 ^{ab}	77 \pm 3 ^{ab}	74 \pm 7 ^{ab}	81 \pm 11 ^{ab}	75 \pm 2 ^{ab}	62 \pm 2 ^b	68 \pm 4 ^{ab}	Infection	0.32
									Diet	0.0009 ***
Phenylalanine	137 \pm 9 ^{ab}	94 \pm 7 ^b	112 \pm 4 ^{ab}	100 \pm 6 ^{ab}	153 \pm 21 ^a	138 \pm 17 ^{ab}	104 \pm 4 ^{ab}	90 \pm 6 ^b	Infection	0.0077 **
									Diet	0.14
Threonine	141 \pm 27	109 \pm 10	118 \pm 16	98 \pm 4	90 \pm 11	109 \pm 15	85 \pm 8	102 \pm 14	Infection	0.93
									Diet	0.36
Tryptophan	31 \pm 3	24 \pm 1	27 \pm 1	27 \pm	25 \pm 2	27 \pm 1	25 \pm 1	24 \pm 1	Infection	0.35
									Diet	0.09
Valine	306 \pm 20	322 \pm 20	300 \pm 18	326 \pm 32	269 \pm 21	330 \pm 16	268 \pm 11	270 \pm 16	Infection	0.07
									Diet	0.27
EAA ³	1509 \pm 120	1448 \pm 74	1442 \pm 82	1447 \pm 101	1307 \pm 105	1456 \pm 83	1288 \pm 48	1347 \pm 95	Infection	0.49
Non-Essential Amino Acids										
Ornithine	19 \pm 3 ^a	53 \pm 11 ^{bc}	26 \pm 4 ^{ab}	40 \pm 5 ^{abc}	38 \pm 6 ^{ab}	45 \pm 5 ^{bc}	36 \pm 4 ^{ab}	71 \pm 8 ^c	Diet	0.01 **
									Infection	0.0001 ***
									Diet	0.0001 ***
Citrulline	46 \pm 7 ^a	40 \pm 2 ^a	32 \pm 5 ^a	27 \pm 4 ^a	36 \pm 7 ^a	25 \pm 5 ^a	285 \pm 85 ^b	399 \pm 86 ^b	Infection	0.84
									Diet	0.72
Taurine	2343 \pm 658 ^{ab}	833 \pm 94 ^a	2219 \pm 383 ^b	1130 \pm 118 ^{ab}	1802 \pm 246 ^{ab}	1101 \pm 176 ^{ab}	2083 \pm 346 ^{ab}	1313 \pm 328 ^{ab}	Infection	0.0001 ***
									Diet	0.3
Aspartic acid	39 \pm 5 ^{ab}	25 \pm 3 ^b	44 \pm 8 ^a	28 \pm 3 ^{ab}	34 \pm 4 ^{ab}	24 \pm 2 ^b	43 \pm 5 ^a	29 \pm 4 ^{ab}	Infection	0.0001 ***
									Diet	0.027 *
Hydroxyproline	80 \pm 14	106 \pm 16	111 \pm 9	78 \pm 12	59 \pm 11	58 \pm 10	70 \pm 9	84 \pm 17	Infection	0.89
									Diet	0.06
Serine	108 \pm 8	129 \pm 18	135 \pm 12	92 \pm 12	98 \pm 12	81 \pm 12	97 \pm 7	90 \pm 8	Infection	0.19
									Diet	0.045 *
Asparagine	62 \pm 15	104 \pm 20	92 \pm 10	85 \pm 12	52 \pm 9	60 \pm 8	69 \pm 7	71 \pm 9	Infection	0.18
									Diet	0.09
Glutamic acid	58 \pm 6	44 \pm 5	70 \pm 11	52 \pm 3	52 \pm 6	44 \pm 7	67 \pm 6	54 \pm 7	Infection	0.0048 **
									Diet	0.47
Glutamine	172 \pm 18	247 \pm 33	246 \pm 24	201 \pm 25	187 \pm 27	184 \pm 15	207 \pm 23	189 \pm 15	Infection	0.88
									Diet	0.033 *
Proline	109 \pm 12 ^a	120 \pm 27 ^{ab}	226 \pm 64 ^{ab}	232 \pm 44 ^b	114 \pm 28 ^{ab}	144 \pm 49 ^{ab}	142 \pm 27 ^{ab}	198 \pm 65 ^{ab}	Infection	0.08
									Diet	0.34
Glycine	1150 \pm 148 ^{ab}	922 \pm 140 ^{ab}	1248 \pm 156 ^a	716 \pm 79 ^{ab}	1017 \pm 121 ^{ab}	627 \pm 109 ^b	1069 \pm 106 ^{ab}	805 \pm 144 ^{ab}	Infection	0.0002 ***
									Diet	0.11
Alanine	601 \pm 43	784 \pm 145	845 \pm 88	664 \pm 91	584 \pm 37	560 \pm 81	609 \pm 57	578 \pm 48	Infection	0.82
α -Aminobutyric acid	16 \pm 1	22 \pm 4	22 \pm 4	16 \pm 3	13 \pm 1	17 \pm 2	14 \pm 1	17 \pm 1	Infection	0.25
									Diet	0.33
Tyrosine	63 \pm 7 ^a	46 \pm 5 ^{ab}	50 \pm 4 ^{ab}	42 \pm 4 ^{ab}	57 \pm 8 ^{ab}	46 \pm 4 ^{ab}	50 \pm 3 ^{ab}	40 \pm 4 ^b	Infection	0.16
									Diet	0.0019 **
β Alanine	58 \pm 14	45 \pm 10	98 \pm 13	83 \pm 21	81 \pm 19	71 \pm 21	77 \pm 19	59 \pm 18	Infection	0.15
									Diet	0.25
1-Methylhistidine	27 \pm 8	23 \pm 7	37 \pm 11	40 \pm 21	26 \pm 10	36 \pm 11	20 \pm 5	25 \pm 8	Infection	0.67
									Diet	0.82
NEAA ⁴	4951 \pm 879 ^{ab}	3539 \pm 362 ^{ab}	5500 \pm 649 ^a	3526 \pm 114 ^{ab}	4251 \pm 424 ^{ab}	3122 \pm 425 ^b	4936 \pm 457 ^{ab}	4012 \pm 588 ^{ab}	Infection	0.27
									Diet	0.0005 ***
TAA ⁵	6459 \pm 973	4987 \pm 397	6942 \pm 710	4973 \pm 156	5558 \pm 495	4578 \pm 433	6224 \pm 488	5359 \pm 616	Infection	0.42
									Diet	0.0019 **

¹ Concentration values in the same row with different superscript letters are significantly different (p < 0.05)

² Asterixis next to p values indicate significance level (* = 0.05, ** = 0.01, *** = 0.001)

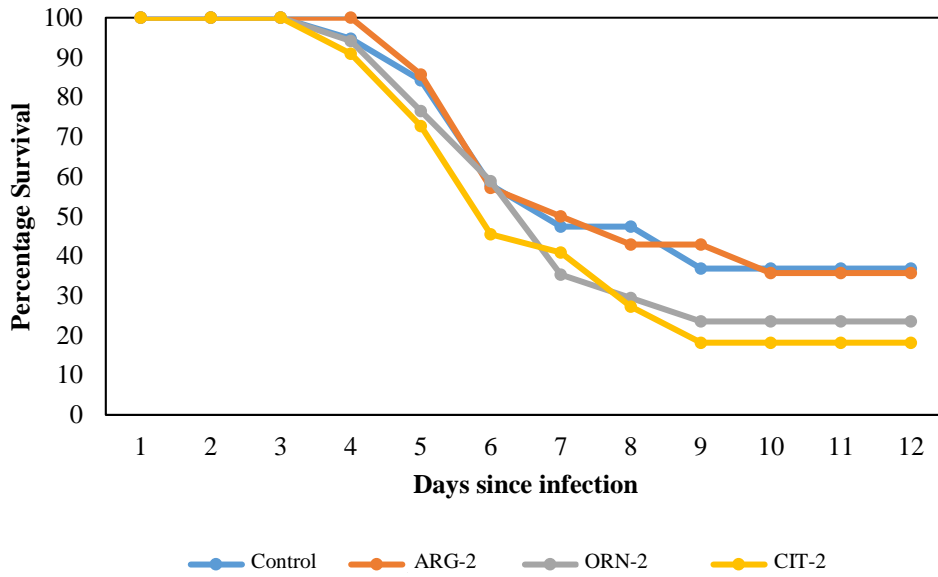
³ EAA: Totalled essential amino acids

⁴ NEAA: Totalled non-essential amino acids

⁵ TAA: Totalled amino acids

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Figure 1



1011 Figure 2

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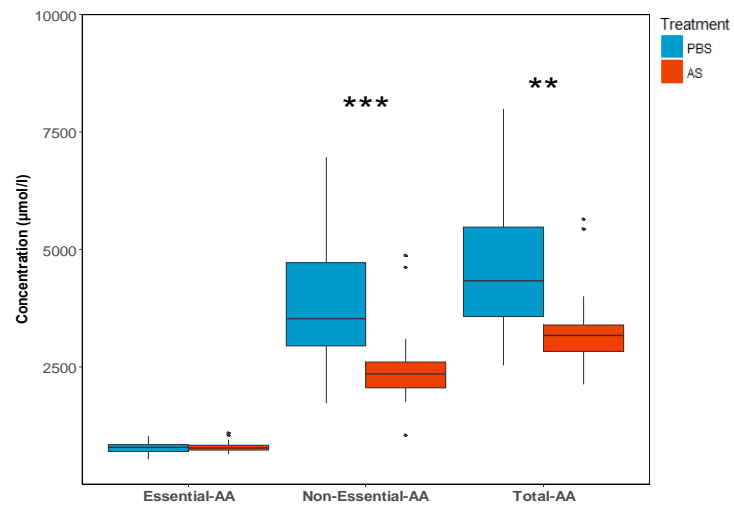
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1025 Figure 3

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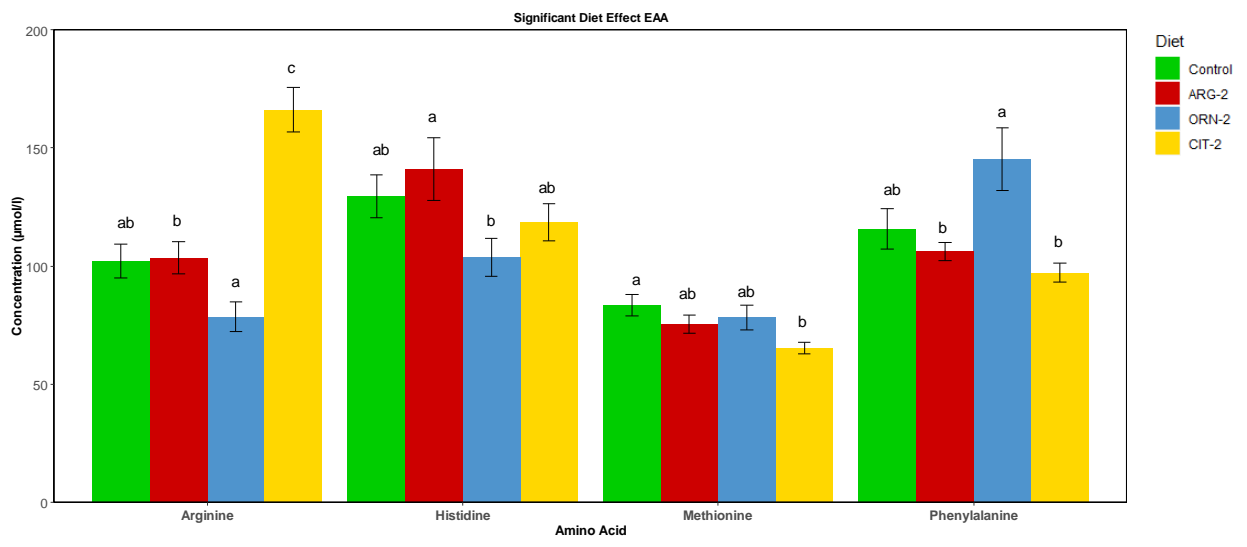
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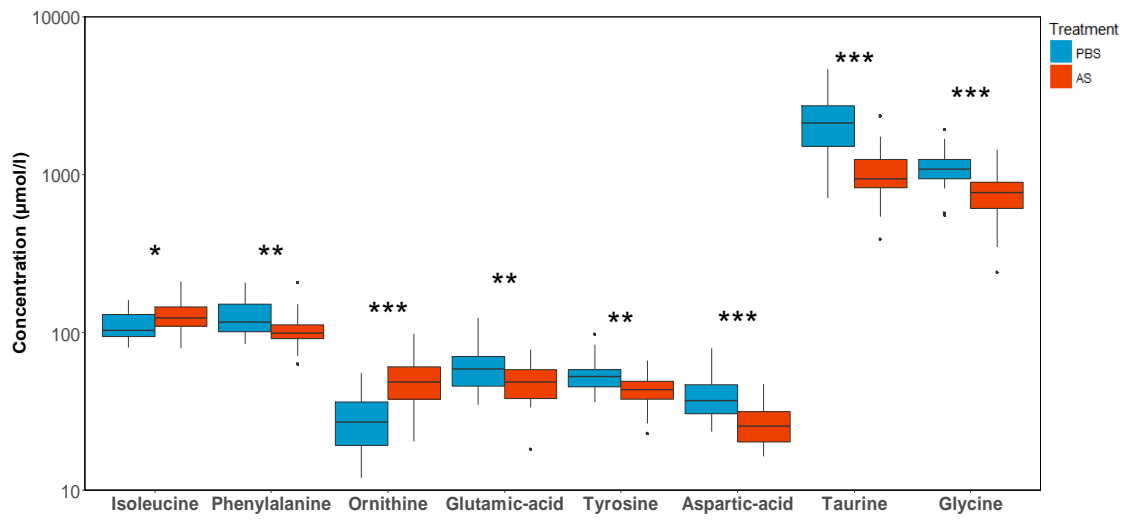
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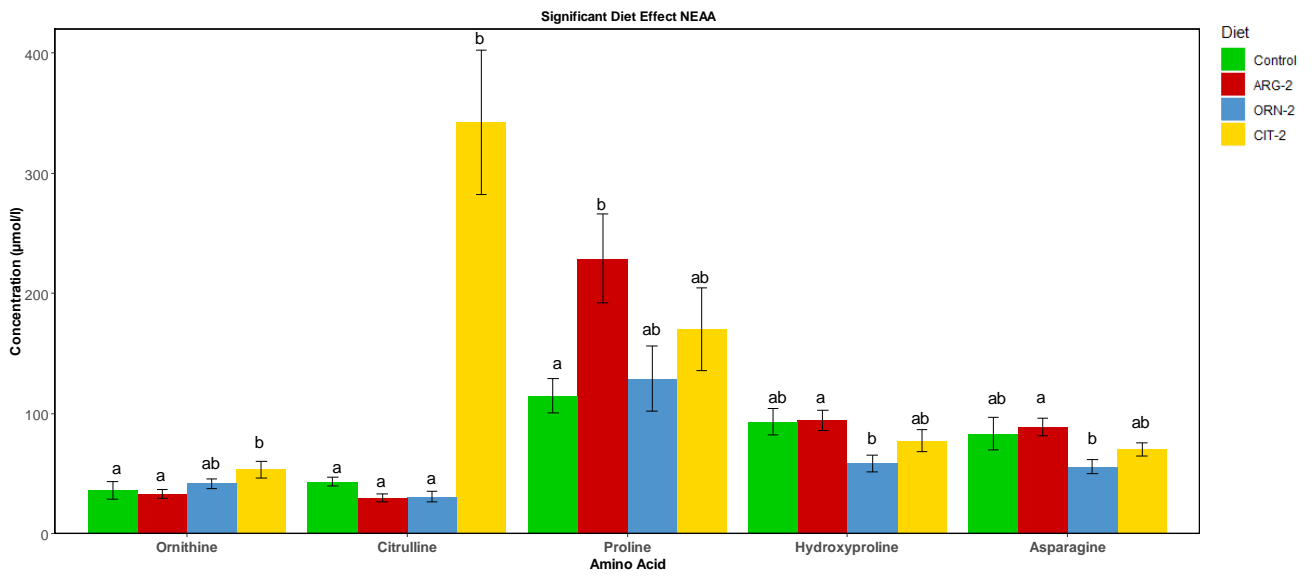


1037 Figure 4.

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1039 Figure 5



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1043 Figure 6

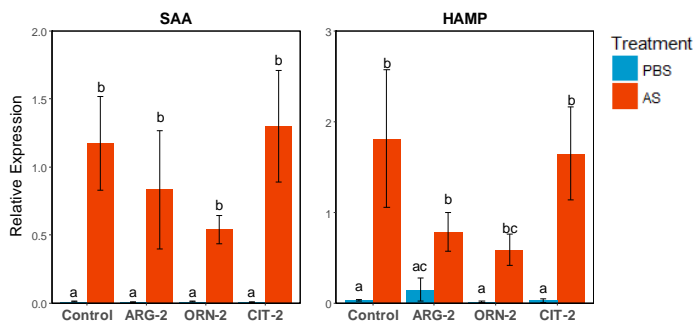
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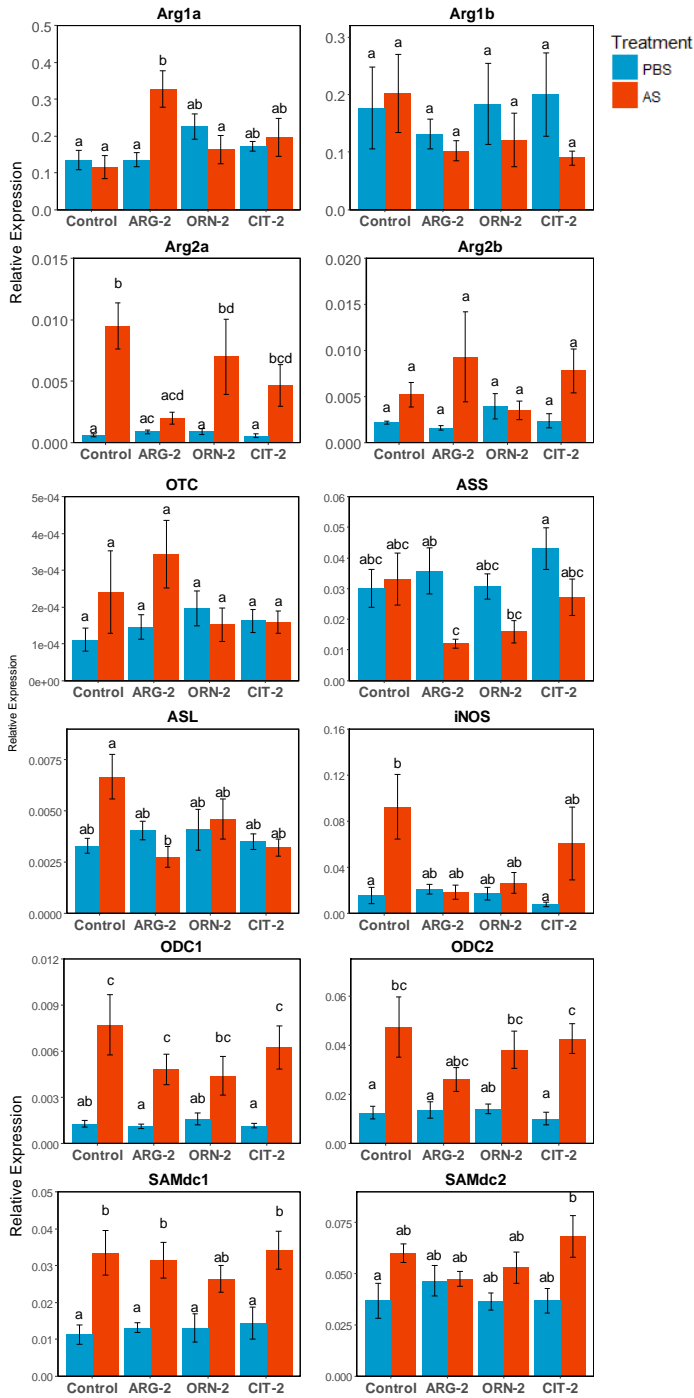
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1051 Figure 8

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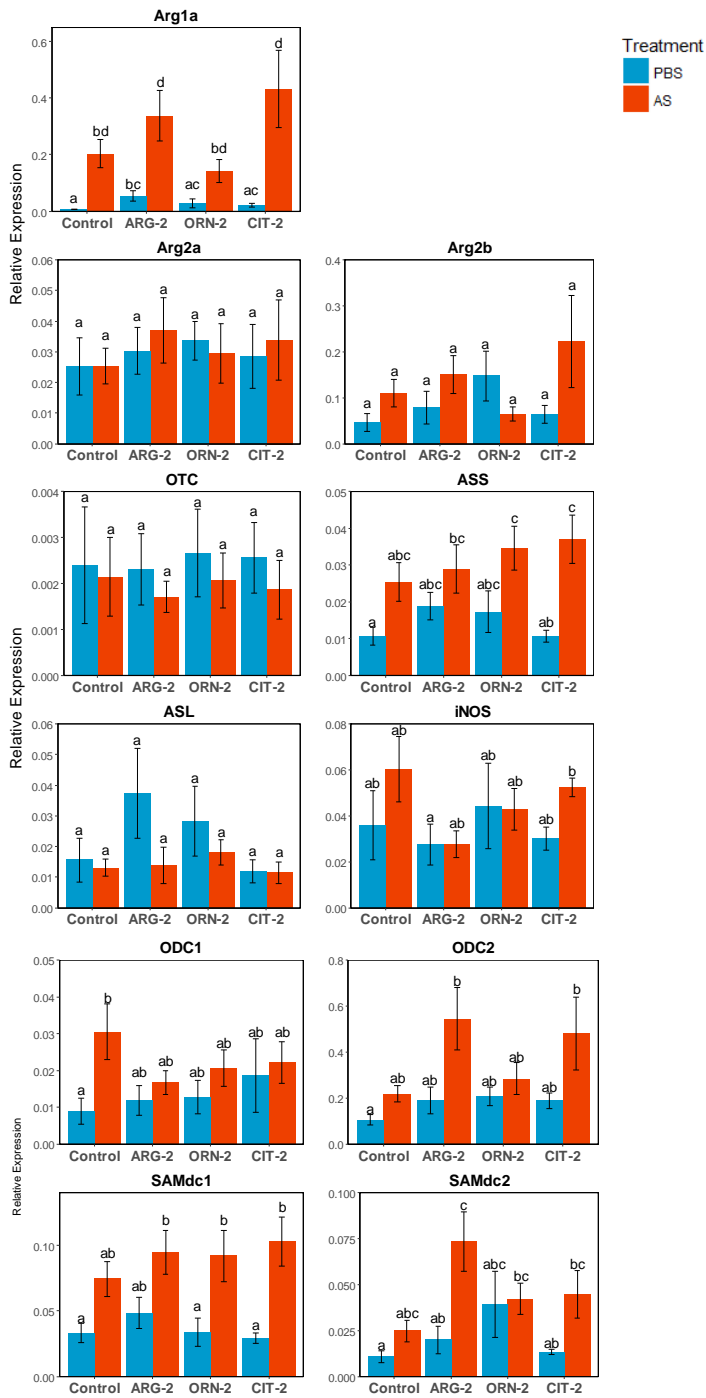
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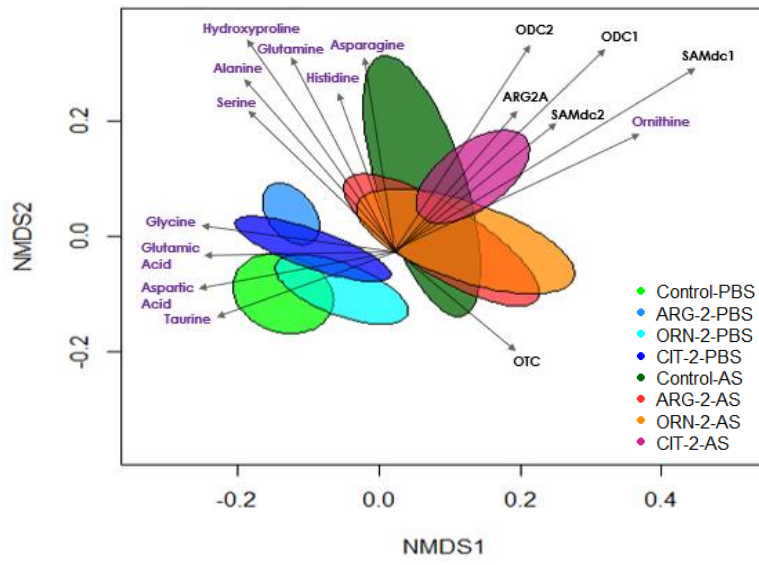
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1076 Figure 9



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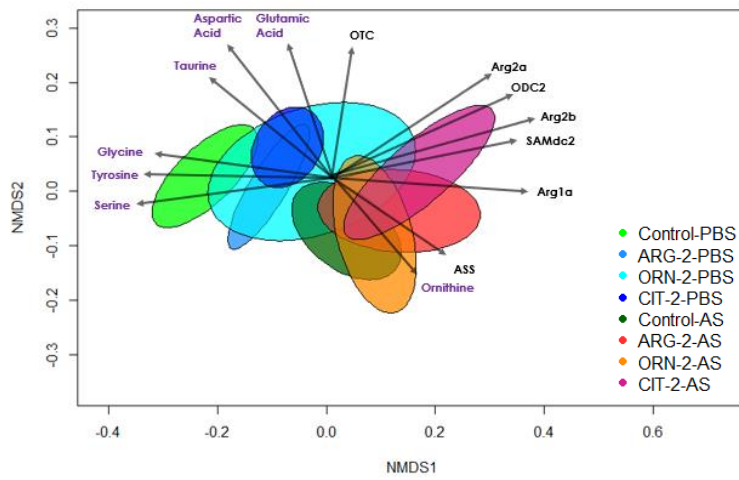
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Supplementary Table 1. ANOVA results in liver gene expression

Gene	ANOVA	p
<i>Arg1a</i>	Infection	0.14
	Diet	0.049 *
	Interaction	0.005 **
<i>Arg1b</i>	Infection	0.35
	Diet	0.85
	Interaction	N.S
<i>Arg2a</i>	Infection	0.0001 ***
	Diet	0.17
	Interaction	0.031 *
<i>Arg2b</i>	Infection	0.001 ***
	Diet	0.95
	Interaction	N.S
<i>OTC</i>	Infection	0.17
	Diet	0.53
	Interaction	N.S
<i>ASS</i>	Infection	0.0006 ***
	Diet	0.08
	Interaction	N.S
<i>ASL</i>	Infection	0.56
	Diet	0.19
	Interaction	0.02 *
<i>iNOS</i>	Infection	0.001***
	Diet	0.57
	Interaction	0.012 *
<i>ODC1</i>	Infection	0.0001 ***
	Diet	0.842
	Interaction	N.S
<i>ODC2</i>	Infection	0.0001 ***
	Diet	0.72
	Interaction	N.S
<i>SAMdc1</i>	Infection	0.0001 ***
	Diet	0.77
	Interaction	N.S
<i>SAMdc2</i>	Infection	0.0002 ***
	Diet	0.874
	Interaction	N.S

¹* = p < 0.05, ** = p < 0.01, *** = p < 0.001

²N.S Not significant

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Supplementary Table 2. ANOVA results in head kidney gene expression

Gene	ANOVA	p
<i>Arg1a</i>	Infection	0.0001 ***
	Diet	0.01**
	Interaction	N.S
<i>Arg2a</i>	Infection	0.56
	Diet	0.668
	Interaction	N.S
<i>Arg2b</i>	Infection	0.07
	Diet	0.63
	Interaction	N.S
<i>OTC</i>	Infection	0.79
	Diet	0.81
	Interaction	N.S
<i>ASS</i>	Infection	0.0001 ***
	Diet	0.3
	Interaction	N.S
<i>ASL</i>	Infection	0.33
	Diet	0.29
	Interaction	N.S
<i>iNOS</i>	Infection	0.009 **
	Diet	0.03 *
	Interaction	N.S
<i>ODC1</i>	Infection	0.0008 ***
	Diet	0.933
	Interaction	N.S
<i>ODC2</i>	Infection	0.0003 ***
	Diet	0.08
	Interaction	N.S
<i>SAMdc1</i>	Infection	0.0001 ***
	Diet	0.626
	Interaction	N.S
<i>SAMdc2</i>	Infection	0.0001 ***
	Diet	0.031 *
	Interaction	N.S

¹* = p < 0.05, ** = p < 0.01, *** = p < 0.001

²N.S Not significant