Arginine, ornithine and citrulline supplementation in rainbow trout

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

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Abstract

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

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

1. Introduction

Maintenance of fish health is a central requirement for efficient and economically feasible aquaculture. A challenge to this goal is that fish are continually exposed to pathogens in the aquatic environment [1]. The first line of defence to pathogens is from physical external barriers (e.g. skin and gill mucous) followed by the innate immune system, which is believed to be more important in fish than endotherms due to the longer time required to mount an adaptive response [2]. In all cases, eliciting an immune response is highly energy demanding and a balance between immune response and other physiological and metabolic processes occurs [3].

Salmonids use protein as a major energy source, utilising amino acids in glucogenesis [4, 5]. The liver is a central organ in the metabolism of amino acids, but under an inflammatory response, its metabolic state is altered to produce large volumes of acute phase proteins [6]. Thus, there may be a trade-off between growth and the immune response in which growth is hindered until the infection is resolved. The synthesis of large volumes of immune proteins during the inflammatory response and the subsequent healing and recovery following infection requires a supply of free amino acids, obtained from the diet or by remobilisation of proteins stored in the skeletal muscle [6, 7]. Supplementing fish diets with functional amino acids (FAAs) offers a strategy to supply a source of useful amino acids to support immune function and more generally improve performance.

FAAs can be nutritionally essential, non-essential, or may become conditionally essential (e.g. at different developmental stages or under distinct health, reproductive or stress states) if available quantities are unable to meet the body's demand [8]. FAA supplementation has the potential to improve fish health due to their key roles in the immune response, such as increased glucogenesis of alanine as an energy substrate for leukocytes [9], or the antioxidant properties of taurine and glycine [10]. Arginine is an FAA attracting considerable attention due its impact on many metabolic systems, including the immune response.

During infection, the availability of free arginine decreases for general metabolic processes, as it is preferentially directed towards lymphocyte proliferation and macrophage dependent production of NO and polyamines used in the immune response [11, 12]. Inflammatory responses are associated with

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

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

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

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

2. Materials and Methods

2.1 Diet formulation

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

Table1

Table 2

2.2 Rainbow trout feeding trial

All procedures described were carried out in compliance with the Animals (Scientific Procedures) Act 1986 under UK Home Office license PPL number 70/8071 and approved by the ethics committee at the University of Aberdeen, UK. Juvenile rainbow trout were maintained at the University of Aberdeen aquarium facility (School of Biological Sciences). Tanks were supplied with recirculating freshwater with a flow rate of 1.5 L/s. Fish were kept at a temperature of 14 ± 1^{o}C and a photoperiod of 12:12 light:dark. A computerised control system was used to monitor pH, ammonia concentration and oxygen levels. Fish were fed twice daily (9 am and 5 pm) with commercial pellets of respective diets at 3% body weight per day.

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

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

2.3 Bacterial challenge following feeding trial

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

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

2.4 Gene expression analysis following infection.

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

Table 3

2.5 Plasma free amino acid analysis following bacterial infection

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

2.6 Statistical Analysis

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

3. Results

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

3.1 Growth parameters in control or supplemented amino acid diets

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

Table 4

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

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

Figure 1

3.3 Free amino acids in blood plasma following AS infection

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

EAA

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

NEAA

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

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

Table 5

Figure 2, 3, 4, 5

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

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

Figure 6

3.5 Liver expression response of urea cycle and polyamine synthesis genes

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

For the four genes encoding the arginase paralogues (Figure 7), *Arg1a* and *Arg2a* expression was significantly impacted by infection and an interaction effect was detected for both genes (Supplementary Table 1). *Arg1a* expression was significantly increased in ARG-2-AS compared to 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**

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

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

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

Figure 8

3.7 Non-metric multidimensional scaling analyses

3.7.1 *Liver gene expression and free amino acid responses*

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

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

Figure 9

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

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

Figure 10

4. Discussion

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

Arginine supplementation and growth

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

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

Essential amino acid metabolism in response to infection

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

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

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

Non-essential amino acid metabolism in response to infection

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

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

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

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

Transcriptional responses of arginine metabolism genes to infection

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

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

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

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

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

5. Conclusion

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

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

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

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

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

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

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

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

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

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

Figure 10. Non-metric multidimensional scaling plot of free amino acid levels in blood plasma and head 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)

Ingredients[1]	**Control**	**ARG-2**	**ORN-2**	**CIT-2**
Fish Meal	150	150	150	150
Soya SPC	135	135	135	135
Wheat Gluten	176.8	176.8	176.8	176.8
Maize Gluten	152	152	152	152
Wheat	110	90	90	90
Fish Oil	89.6	89.6	89.6	89.6
Rapeseed Oil	166.4	166.4	166.4	166.4
Vit + Min premix	32.5	32.5	32.5	32.5
Yttrium	0.5	0.5	0.5	0.5
Proximate composition				
MOISTURE (%)	5.8	5.5	5.5	5.5
PROTEIN - crude (%)	43.6	45.4	45.4	45.4
FAT - crude (%)	29.3	29.3	29.3	29.3
ASH (%)	6.0	6.0	6.0	6.0

[1]Water change of -12.8g

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

	Control	**ARG-2**	**ORN-2**	**CIT-2**
Alanine	23.1	23.5	23.4	23.8
Aspartic Acid	32.1	32.1	32.4	32.6
Cystine	7.18	6.92	6.77	7.4
Glutamic Acid	103.0	105.0	104.0	108
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
Leucine	40.3	40.7	41.1	41.9
Lysine	26.1	26.2	26.1	26.8
Methionine	9.23	9.4.0	9.34	10.0
Phenylalanine	22.9	23.4	23.0	23.5
Proline	34.5	35.0	34.7	38.5
Serine	21.3	21.1	21.1	22.2
Threonine	15.8	15.7	15.7	15.8
Valine	19.5	20.2	20.0	20.2
Arginine[1]	20.2	37	20.7	21
Ornithine	0.2	0.2	13.4	0.3
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
EF-1α	Forward	CAAGGATATCCGTCGTGGCA	327	64	NM_001124339.1
	Reverse	ACAGCGAAACGACCAAGAGG			
HPRT	Forward	CCGCCTCAAGAGCTAGTGTAAT	237	64	XM_021583468.1
	Reverse	GTCTGGAACCTCAAACCCTATG			
β–actin	Forward	ATGGAAGATGAAATCGCCCC	260	64	XM_021595779.1
	Reverse	TGCCAGATCTTCTCCATGTCG			
SAA	Forward	TATGATGCTGCCAGGAGAGGAC	137	64	NM_001124436.1
	Reverse	CGTCCCCAGTGGTTAGCCTT			
HAMP	Forward	AGGAGGTTGGAAGCATTGACAG	101	64	XM_021595153.1
	Reverse	GTGGCTCTGACGCTTGAACCT			
ARG 1A	Forward	AGCACCATATCCTGACGTTG	147	64	XM_021564871.1
	Reverse	CATCGATGTCATAGCTCAGG			
ARG 1B	Forward	GGTGGATCGCCTTGGAATCG	179	64	KX998966.1
	Reverse	CTGTGATGTAGATTCCCTCC			
ARG 2A	Forward	TCCAGAGAGTCATGGAAGTCACTTTCC	198	64	KX998967.1
	Reverse	CCATCACTGACAACAACCCTGTGTT			
ARG 2B	Forward	CTTGTTGAGGTCAACCCAGC	163	64	KX998968.1
	Reverse	GTCGAAGCTGTTCCGTGTCG			
OTC	Forward	CACAGCCAGGGTTCTCTCTG	116	64	XM_021597830.1
	Reverse	CAGACAGGCCGTTGATGATG			
ASS	Forward	TGAGATTGGAGGGAGGCATG	172	64	XM_021590913.1
	Reverse	GCCCTGTTTGATCCTCCTGA			
ASL	Forward	ACGCTCTCCAACTCATCACA	129	64	XM_021563243.1
	Reverse	ACCGCATGACTCAGAATCCA			
ODC1	Forward	CGTGTGCCAGCTCAGTGTC	179	64	XM_021574142.1
	Reverse	CCATGTCAAAGACACAGCGG			
ODC2	Forward	TGGTGCCACCCTGAAGGCC	128	64	XM_021585068.1
	Reverse	AGATGGCCTGGCTGTAGGTG			
SAMdc1	Forward	GCAAGGACAAGCTAATTAAG	185	64	XM_021600286.1
	Reverse	AACCTTGGGATGGTACGGAG			
SAMdc2	Forward	AACTCACGATGGAAGCGAAC	121	64	XM_021611778.1
	Reverse	AACCTTGGGATGGTACGGAG			
iNOS	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 (±SEM, n=45).

	Control[1]	**ARG-2**	**ORN-2**	**CIT-2**	**ANOVA**
Start Weight (g)	84± 1.8	82± 1.5	83± 1.6	85±1.7	0.46
End Weight (g)	225± 5	220± 4	235± 5	229± 4	0.13
Gutted Weight (g)	192± 5^{ab}	185± 4^{a}	206± 4^{b}	195± 4^{ab}	0.0089
HSI[2]	1.53± 0.03	1.46± 0.03	1.47± 0.03	1.52± 0.03	0.37
VSI[3]	13.7± 0.2	13.9± 0.2	13.6± 0.2	14.1± 0.2	0.2
Condition Factor[4]	1.39± 0.01^{a}	1.35± 0.01^{bc}	1.38± 0.01^{ab}	1.34± 0.01^{c}	0.0002
FCR[5]	0.85± 0.1	0.77± 0.02	0.71± 0.02	0.76± 0.03	0.4
SGR (%)[6]	2.02± 0.04	2.01± 0.03	2.14± 0.04	2.04± 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 (µmol/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 ±SEM, n=6)

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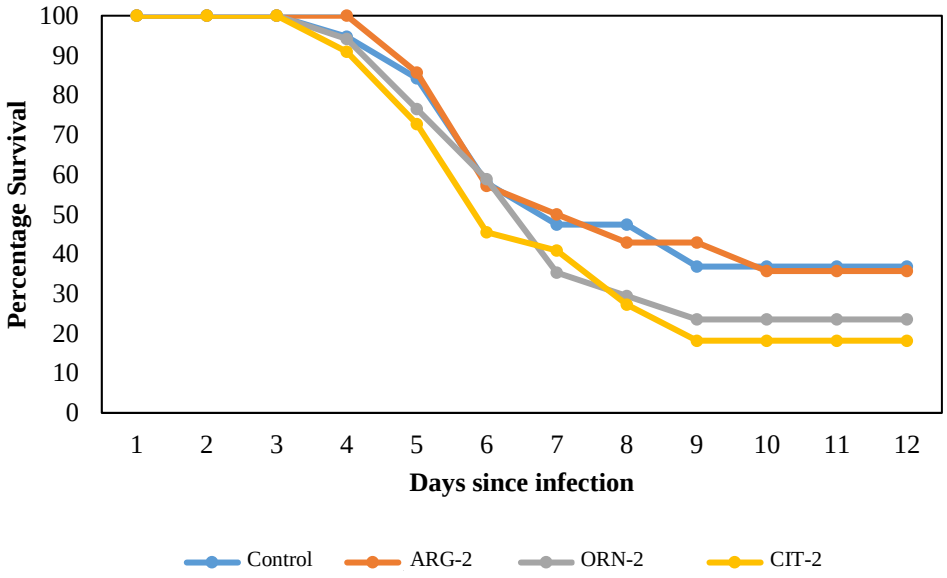


Figure 2

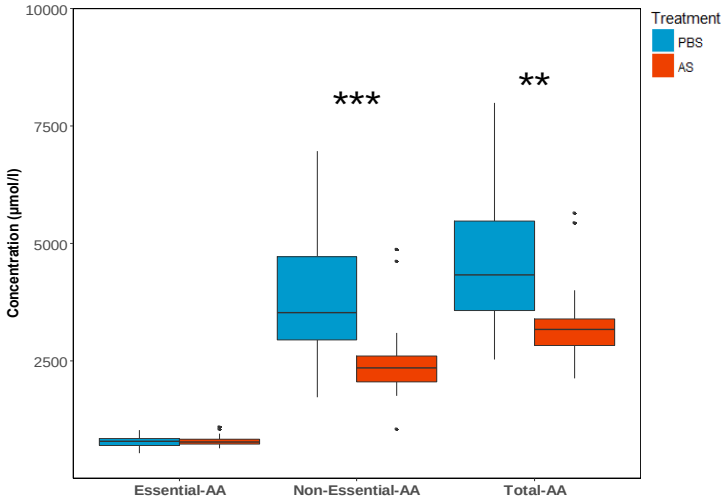
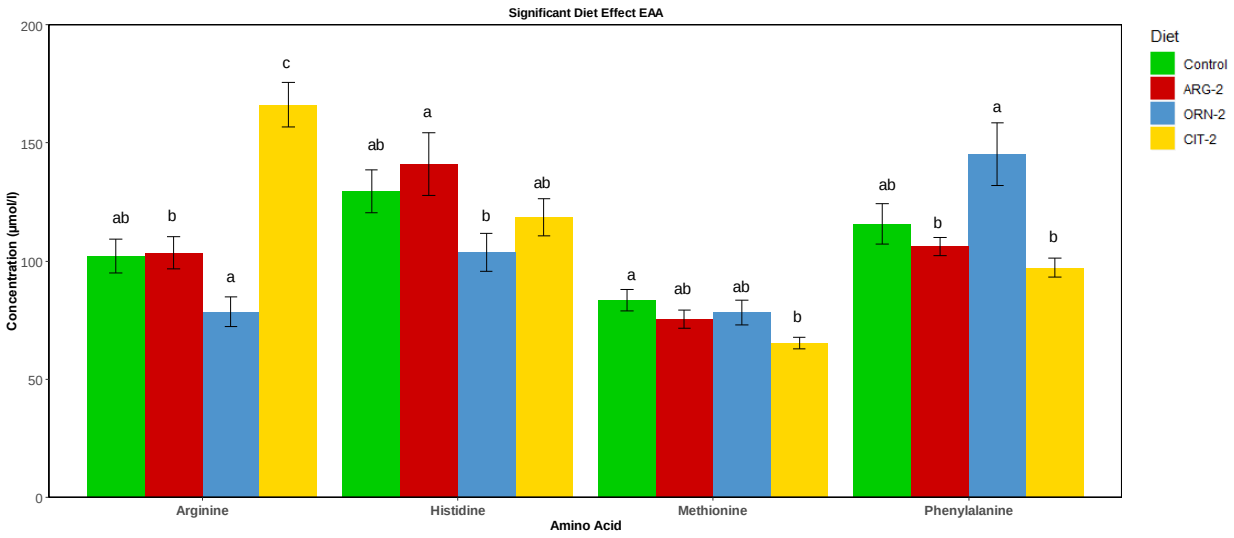
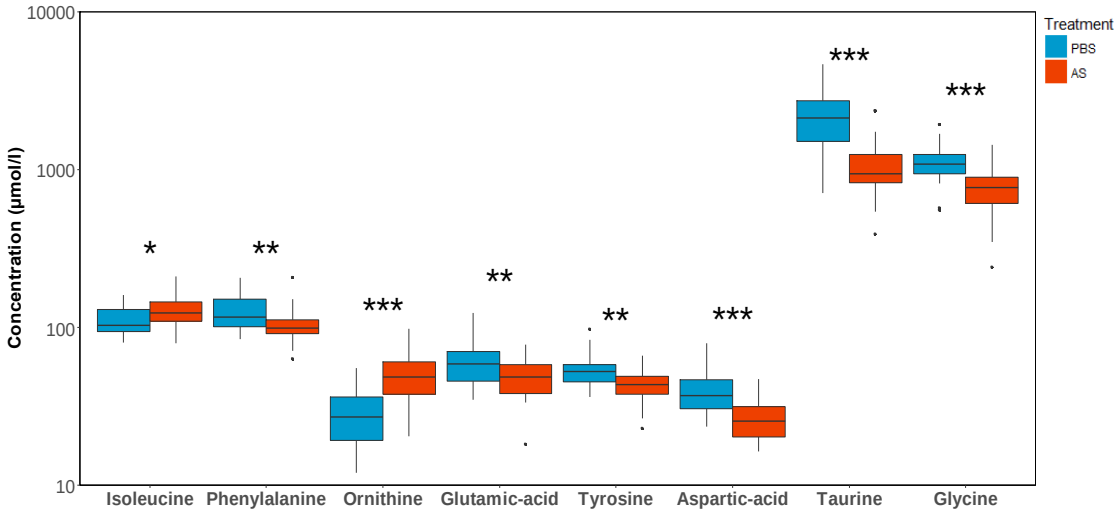

Concentration (μmol/l)
10000
7500
5000
2500
***
**
Essential-AA
Non-Essential-AA
Total-AA
Treatment
PBS
AS

Figure 3


Significant Diet Effect EAA
Concentration (µmol/l)
200
150
100
50
0
ab
b
a
c
ab
a
b
ab
a
ab
ab
b
ab
b
a
b
Arginine
Histidine
Methionine
Phenylalanine
Amino Acid
Diet
Control
ARG-2
ORN-2
CIT-2

1037 Figure 4.

1038



1039 Figure 5

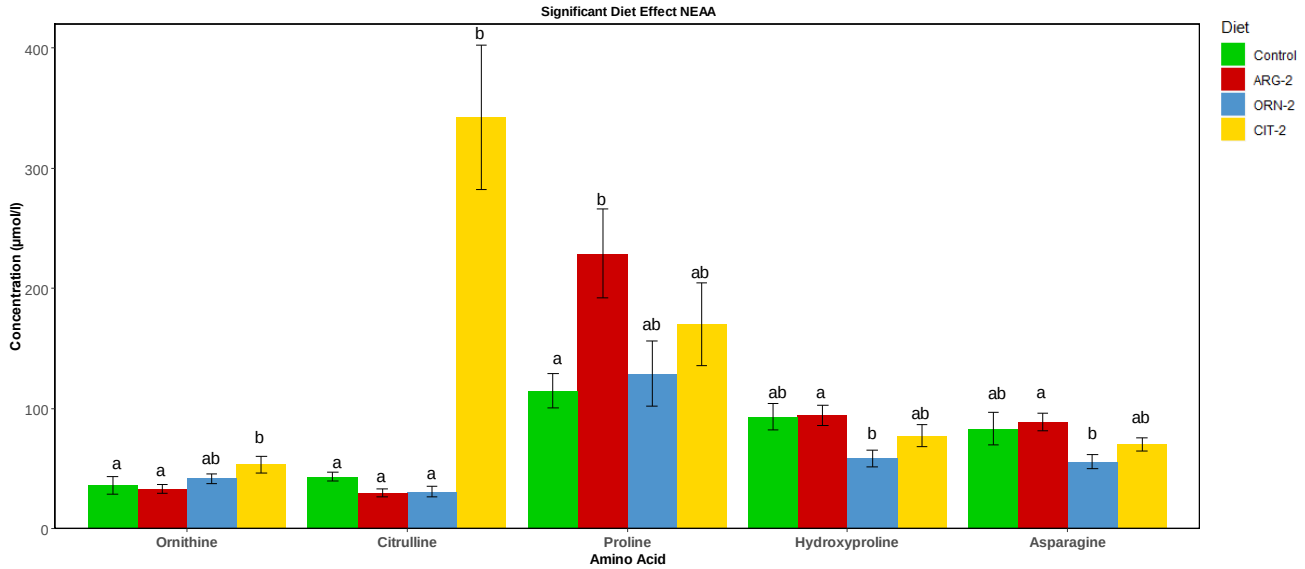


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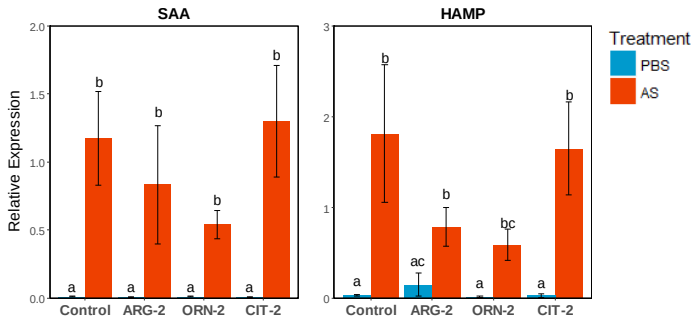
Figure 6


SAA
HAMP
Relative Expression
Treatment
PBS
AS
Control
ARG-2
ORN-2
CIT-2
a
b
ac
bc

1049 Figure 7

1050

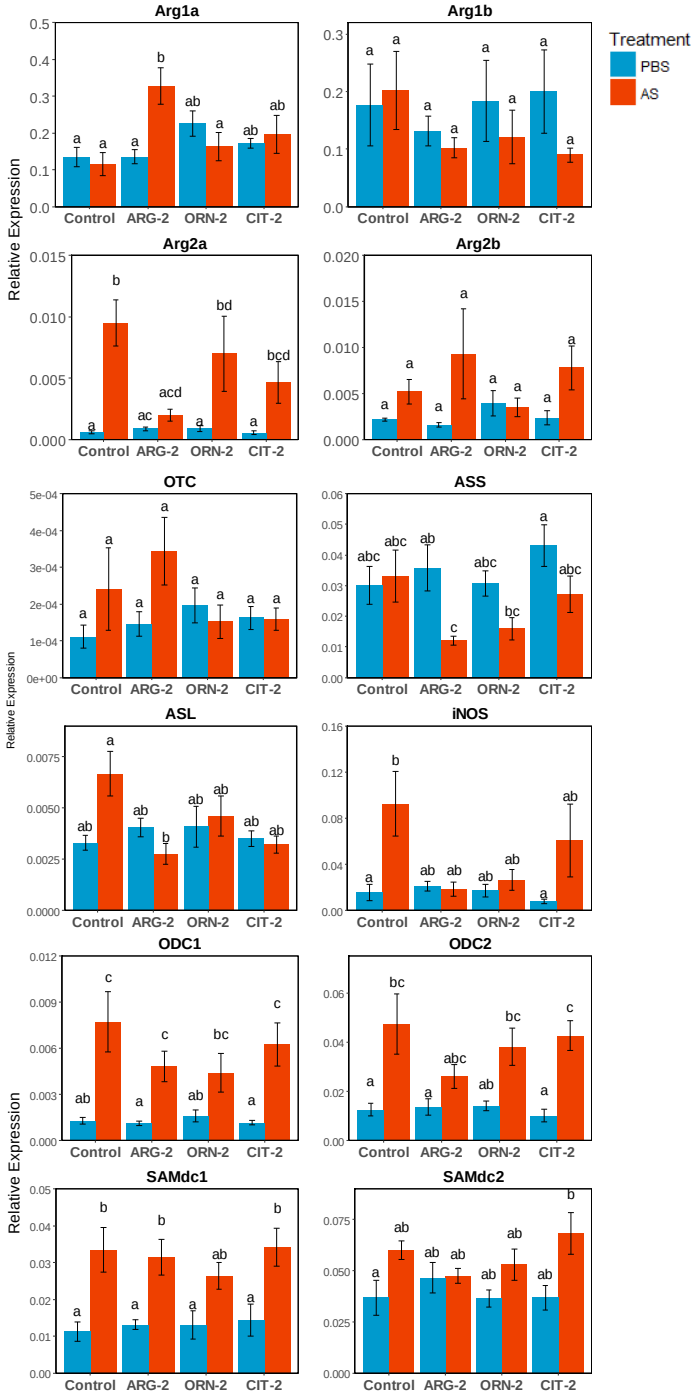
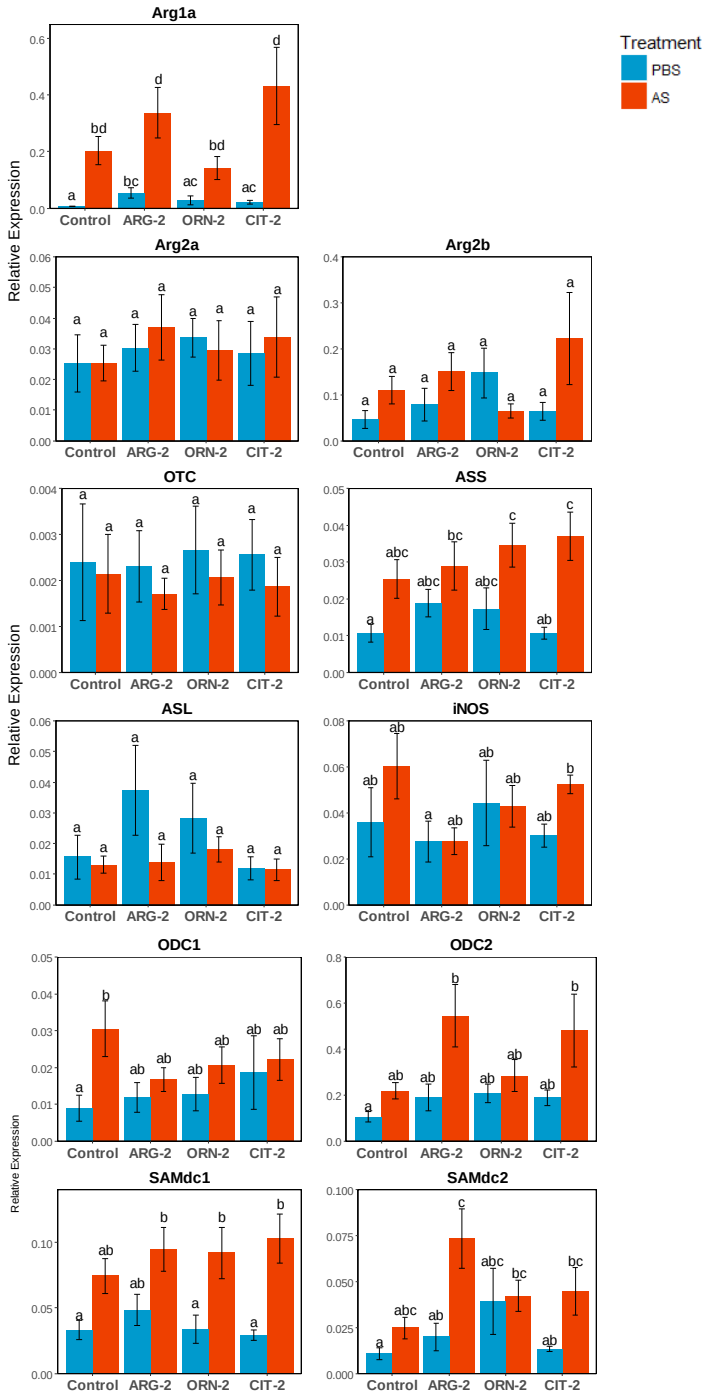
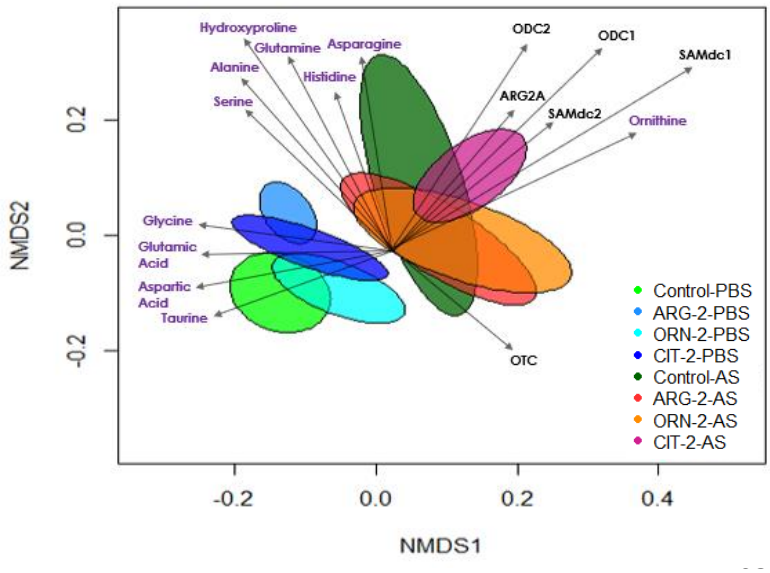


Figure 8

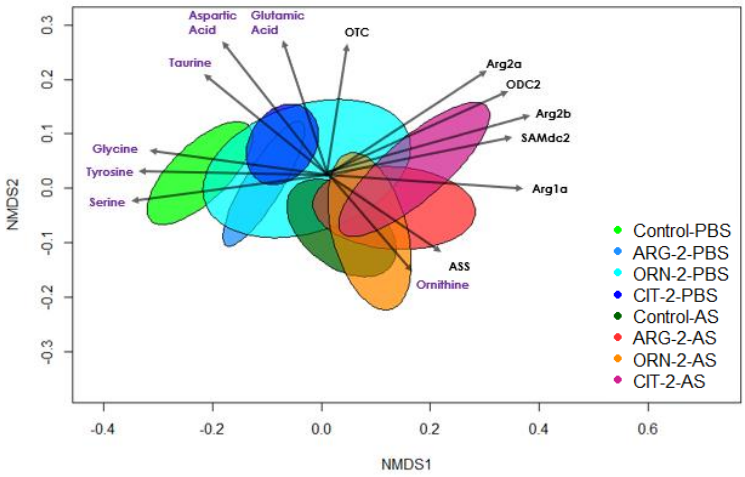

Arg1a
Arg2a
Arg2b
OTC
ASS
ASL
iNOS
ODC1
ODC2
SAMdc1
SAMdc2
Relative Expression
Control
ARG-2
ORN-2
CIT-2
Treatment
PBS
AS

1076 Figure 9



1088

Figure 10


Aspartic Acid
Glutamic Acid
OTC
Taurine
Arg2a
ODC2
Arg2b
SAMdc2
Glycine
Tyrosine
Serine
Arg1a
ASS
Ornithine
Control-PBS
ARG-2-PBS
ORN-2-PBS
CIT-2-PBS
Control-AS
ARG-2-AS
ORN-2-AS
CIT-2-AS
NMDS1
NMDS2
-0.4
-0.2
0.0
0.2
0.4
0.6
0.3
0.2
0.1
0.0
-0.1
-0.2
-0.3

Supplementary Table 1. ANOVA results in liver gene expression

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

[1]* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$

[2] N.S Not significant

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Supplementary Table 2. ANOVA results in head kidney gene expression

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

[1]* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$

[2] N.S Not significant

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