Supplementation of arginine, ornithine and citrulline in rainbow trout (Oncorhynchus mykiss)

Supplementation of arginine, ornithine and citrulline in rainbow trout (*Oncorhynchus mykiss*): effects on growth, amino acid levels in plasma and gene expression responses in liver tissue

Clark, T. C.[1], Tinsley J[2], Sigholt T.[3], Macqueen D.J.[4] and Martin S.A.M.[1*]

[1]School of Biological Sciences, University of Aberdeen, Tillydrone Avenue, Aberdeen, UK

[2]BioMar AS, Grangemouth Docks, Grangemouth, UK

[3]BioMar AS, Trondheim, Norway

[4]The Roslin Institute and Royal (Dick) School of Veterinary Studies, University of Edinburgh, Edinburgh, UK

*Corresponding author:

Prof Samuel A.M. Martin

School of Biological Sciences,

University of Aberdeen,

Tillydrone Avenue,

Aberdeen,

AB24 2TZ

UK

Email address: sam.martin@abdn.ac.uk

Abstract

Functional amino acids (FAA) regulate metabolic pathways directly linked to health, survival, growth and development. Arginine is a FAA with crucial roles in protein deposition and the immune response. In mammals, supplementation of arginine's precursor amino acid, citrulline, is known to increase circulating arginine to levels beyond direct arginine supplementation, however, citrulline supplementation is poorly studied in fish. To address this knowledge gap, we supplemented the diet of rainbow trout with arginine and its precursor amino acids, ornithine and citrulline, at 3 levels (0.5%, 1% and 2% of the total diet) during a 14-week experiment. We sampled fish at 3h and 24h post-feeding to investigate immediate and steady-state effects, respectively. There were no differences in fish growth for any of the diets across a range of indicators. In blood plasma, out of 26 amino acids detected, 11 and 6 displayed significant changes 24h and 3h post-prandial, respectively. Arginine, ornithine and citrulline levels were all significantly increased by the citrulline supplemented diets. In muscle, 8 amino acids were significantly altered by supplemented diets, while there were no significant changes in liver. Arginine was increased by 2% citrulline supplementation in muscle tissue. We also investigated the transcriptional responses of urea cycle, nitric oxide cycle and rate-limiting polyamine synthesis enzymes, related to arginine's metabolism, in liver. At both time points, only 2 enzymes were significantly altered by the supplemented diets, however several significant changes were observed comparing 3h and 24h post-prandial expression levels. Of these, the paralogous polyamine synthesis enzyme encoding genes *ODC1* and *ODC2* displayed the largest increases in 3h post-prandial fish. These findings demonstrate that endogenous synthesis of arginine is possible from a citrulline supplemented diet and improve our understanding of arginine metabolism in fish.

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

1. Introduction

Traditionally amino acids are classed as essential or non-essential based on an organism's ability to endogenously synthesise them. Functional amino acids (FAA) can be essential or non-essential and have roles beyond protein synthesis, including the regulation of metabolic pathways impacting health, survival, growth and development (Wu 2010). FAA supplementation beyond nutritional requirements is of substantial interest to the aquaculture industry, with several studies providing evidence for a wide variety of benefits. For instance, improved growth and health were observed following dietary supplementation of arginine, methionine, tryptophan, glutamate, histidine, proline and taurine in rainbow trout *Oncoryhnchus mykiss* (Lepage *et al*. 2002; Fournier *et al*. 2003; Gaylord *et al*. 2007), sea bass *Dicentrarchus labrax* (Tulli *et al.* 2007), channel catfish *Ictalurus punctatus* (Pohlenz *et al*. 2014) and Atlantic salmon *Salmo salar* (Aksnes *et al*. 2008; Waagbø *et al.* 2010).

Arginine is an essential amino acid with great potential as a FAA. It is involved in numerous metabolic processes including protein deposition, the synthesis of ornithine (used for polyamine synthesis), immune responses (via nitric oxide production) and the removal of nitrogenous waste as urea (Figure 1) (Li *et al.* 2009). Arginine also stimulates the release of growth promoting hormones such as insulin, glucagon and growth hormone in fish (Baños *et al.* 1999; Mommsen *et al.* 2001). In rainbow trout there is generally a high arginine requirement (1.5-2% of the diet) (Walton *et al.* 1986; NRC 1993), reflecting the lack of *de novo* synthesis due to an inefficient urea cycle (Kajimura *et al.* 2004). In ureotelic species, proline, glutamate and glutamine can be synthesised into ornithine from Pyrroline-5-carboxylic acid (P5C) as an intermediate (Wu *et al.* 2009). Carbamoylphosphate synthetase (CPS) catalyses the formation of the co-substrate carbamoylphosphate, which combines with ornithine through the action of ornithine transcarbamylase (OTC) to generate citrulline. Citrulline can be used to synthesise arginine through the action of two further enzymes in the urea cycle, argininosuccinate synthase (ASS) and argininosuccinate lyase (ASL). In rainbow trout, CPS was reported to be expressed at early life stages, but not in adult liver (Korte *et al.* 1997) and at low levels in adult muscle (Todgham *et al.* 2001). The lack of hepatic CPS activity in salmonids is likely the reason for an incomplete urea cycle, as other ureotelic fishes including the toadfish *Opsanus beta* (Laberge *et al.* 2009), catfish *Clarias batrachus*

78 (Saha *et al.* 2007), and lungfish *Protopterus aethiopicus* (Loong *et al.* 2005), were shown to have
79 detectable CPS activity – matched to a functional urea cycle.

80 **FIGURE 1**

81 The potential roles of arginine in growth and health enhancement has created a demand for fish diets
82 with arginine levels exceeding nutritional requirements. Indeed, evidence for the benefits of direct
83 arginine supplementation exist from studies of several farmed fish species, namely Atlantic salmon
84 (Oehme *et al.* 2010), rainbow trout (Fournier *et al.* 2003), hybrid grouper *Epinephelus fuscoguttatus*
85 ♀× *Epinephelus lanceolatus* ♂ (Wu *et al.* 2018), sea bass (Tulli *et al.* 2007), channel catfish (Pohlenz
86 *et al.* 2014), yellow catfish *Pelteobagrus fulvidraco* (Zhou *et al.* 2015) and grass carp
87 *Ctenopharyngodon idella* (Wang *et al.* 2014). An alternative strategy to promote fish health is
88 supplementation with arginine's precursor amino acids ornithine and citrulline. In mammals,
89 considerable attention has been given to citrulline supplementation, which led to circulating arginine
90 levels higher than achieved by direct arginine supplementation (Lassala *et al.* 2009; Elwafi *et al.*
91 2011; Osowska *et al.* 2004; Wijnands 2012). The same approach is yet to be tested in teleosts. The
92 explanation for increased arginine levels following citrulline supplementation, beyond that achieved
93 by direct arginine supplementation, is due to the fact that ingested arginine is readily metabolised by
94 liver arginase, meaning substantial amounts of dietary arginine is excreted as nitrogenous waste
95 (Osowska *et al.* 2004; Wu *et al*. 2007; Wijnands *et al.* 2015). Citrulline bypasses the liver and is
96 instead used in the endogenous synthesis of arginine via the intestinal-renal-axis, where citrulline is
97 formed in the intestine and then uptaken by the kidney for arginine production through the ASS and
98 ASL enzymes (Marini *et al.* 2017).

99 The aim of this study was to determine the effects of arginine supplementation in comparison to its
100 precursors ornithine and citrulline on growth, circulating amino acid levels and the mRNA expression
101 of urea cycle enzymes in rainbow trout during a long-term feeding experiment. The first objective was
102 to test whether the effects of arginine supplementation are replicated by ornithine and citrulline. The
103 second objective was to identify the optimal level of FAA supplementation through graded levels of
104 dietary inclusion. The final objective was to examine the expression of mRNAs encoding urea cycle

and polyamine synthesis enzymes under different dietary regimes. The findings offer novel insights into free amino acid dynamics and the potential for endogenous synthesis of arginine in rainbow trout.

2. Materials and Methods

2.1 Diet formulation

Ten plant protein-based diets were formulated with a basal inclusion of 43% protein (15% from fishmeal) and a blend of fish oil (9%) and rapeseed oil (17%) (Table 1). The control diet was formulated to meet the essential amino acid requirements for rainbow trout, while the nine experimental diets were identical to the control except for the addition of either arginine, ornithine or citrulline. Experimental diets were supplemented with three levels of each amino acid; 0.5%, 1% and 2% (5 g/kg, 10 g/kg and 20 g/kg of feed) referred to hereafter as ARG-0.5, ARG-1, ARG-2, ORN-0.5, ORN-1, ORN-2, CIT-0.5, CIT-1 and CIT-2. Analysis of amino acid content of the diets was performed by Biomar, minus the arginine, ornithine and citrulline content, which were sent to Ansynth Service B.V. (Netherlands) for analysis. The amino acid profiles of the diets are presented in Table 2.

TABLE 1

TABLE 2

2.2 Feeding trial using supplemented diets

The feeding trial was performed at the recirculating aquaculture system (RAS) research facilities of BioMar in Hirtshals, Denmark, and conducted in accordance with laws regulating experimentation using live animals in Denmark, as overseen by the Danish Animal Experiments Inspectorate. Fish of 144 ± 1g average weight were randomly distributed into 30 tanks (400 L) each containing 35 fish. Fish were exposed to a 12-h light : 12-h dark cycle and kept in freshwater at a temperature of 12 ^{o}C. Dietary treatments were randomly assigned to triplicate tanks. Fish were acclimatised for 2 weeks on the control diet before being fed *ad libitum* for 96 days on their respective experimental diets. Uneaten pellets were registered daily from each tank to estimate feed intake.

Sampling occurred at two time points, 24h following the last meal and 3h post-prandial to identify immediate changes following feeding. The sampling point at 24 h following a meal was considered

representative of the fish's basal levels (Ok *et al.* 2009). Fish (n=3 per tank per time point) were randomly selected and humanely killed by lethal overdose with immersion in the anaesthetic 2-phenoxyethanol followed by destruction of the brain with a scalpel. Growth parameters: end weight, gutted weight, condition factor (K= total body weight *100 / length ^ 3), hepatosomatic index (HSI = liver weight / total body weight *100) and visceral somatic index (VSI = weight of viscera / body weight *100) were recorded and blood (2 ml) was collected through the ventral blood vessel using heparinised syringes for free amino acid analysis in plasma. Samples of liver tissue (~100 mg) were collected (within 5 minutes of death) and stored in 1.5 ml RNA later (Invitrogen) at 4^{o}C for 24 h followed by long-term storage at -80^{o}C for gene expression analysis.

2.3 Free Amino acid analysis

Free circulating amino acid concentrations were determined from sampling the blood plasma of fish. Blood (2 ml per fish) was centrifuged at 1,500g for 15 minutes to separate the plasma from erythrocytes. Plasma supernatant (0.5ml) was aliquoted from each vial and stored in 1.5 ml Eppendorf tubes at -80^{o}C.

At the conclusion of the trial, n=2 fish from each tank (separate from those used for gene expression and plasma analysis) were sampled for free amino acids in liver and muscle (200mg per fish) and pooled (n=3 replicates per diet per tissue). Muscle and liver tissues were homogenised with 3ml of 0.1M HCL using a tissue lyser to free the amino acids from the tissue. Supernatant (0.5ml) was aliquoted from each vial and stored in Eppendorf tubes at -80^{o}C until analysis. Free amino acids from both blood plasma and tissues were shipped on dry ice for amino acid analysis to Ansynth Service B.V. (Netherlands).

2.4 Transcriptional analysis of urea cycle genes

The expression of genes encoding urea cycle enzymes and rate limiting enzymes of polyamine synthesis, namely arginase 1a (*ARG1a*), arginase 1b (*ARG1b*), arginase 2a (*ARG2a*), arginase 2b (*ARG2b*), ornithine transcarbamylase (*OTC*), argininosuccinate synthase (*ASS*), argininosuccinate lyase (*ASL*), ornithine decarboxylase 1 (*ODC1*), ornithine decarboxylase 2 (*ODC2*), s-adenosylmethionine decarboxylase 1 (*SAMdc1*), s-adenosylmethionine decarboxylase 2 (*SAMdc2*) (characterised previously by Clark *et al.* 2019) and inducible nitric oxide synthase (*iNOS*) were investigated using real-time

quantitative PCR (qPCR). RNA extractions, cDNA synthesis and qPCR reactions were performed as previously described (Clark *et al.* 2019). Briefly, RNA was extracted from 100 mg of liver 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). The efficiency of each qPCR assay was assessed using LinRegPCR quantitative PCR data analysis program (download: http://LinRegPCR.HFRC.nl) following Ruijter *et al.* 2009 recommendations. Expression data was then imported and analysed in Genex 5.4.3 (MultiD Analysis). Candidate gene expression was normalised to the expression of two reference genes (*EF-1α* and *HPRT*). All gene primers used in the study are presented in Table 3.

TABLE 3

2.5 Statistical Analysis

All statistical analysis of growth parameters, RT-PCR data and free amino acid concentrations were performed in R (v3.4.0). Differences between diets were assessed with one-way ANOVA followed by Tukey's test to identify significant among group differences. A further comparison from the ANOVA output was examined between the control diet and the other 9 experimental diets where "*" is used to signify significance from the control diet. For the 3h post-prandial to baseline (24h post-feeding) gene expression comparison, a two-way ANOVA was used to compare the effect of diet and sampling time point, using Tukey's test to identify significant among group differences. Diagnostic plots (qq plot and residuals versus fitted values) were visually assessed to test for normality and equal variance. If data met these ANOVA assumptions, the results from R's lm function were interpreted. If not, a log

184 transformation was performed, and the diagnostics plots were reassessed. When data still did not
185 conform to ANOVA assumptions, a nonparametric Kruskal-Wallis test was performed.

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3. Results

3.1 Growth performance of fish

Growth parameters collected at the conclusion of the trial are displayed in Table 4. For all diets, fish more than tripled their weight from an initial mean of 144 ± 1g to 480 ± 7g. Wet weight gain, gutted weight, condition factor, hepatosomatic index (HSI) and visceral somatic index (VSI) showed no significant differences between any of the diets. Tank statistics (n=3) were collected for the growth rate (SGR) and the feed conversion ratios (FCR) of the fish. SGR ranged from 1.06 (% body weight/day^{-1}) in the ORN-2 diet to 1.18 (% body weight/day^{-1}) in the CIT-2 diet, while FCR ranged from 0.99 ± 0.04 in ARG-1 to 1.14 ± 0.05 in ORN-2; however there were no significant differences between any diet.

Table 4

3.2 Basal levels of amino acids in the plasma

Free amino acids were examined in the blood plasma of fish 24 h after feeding. This was expected to closely reflect the baseline amino acid levels, providing a representation of long term changes caused by dietary supplementation. In total, 26 amino acids were detected in the blood and used for analysis (Table 5). Total amino acid (TAA) levels were calculated for all diets and ranged from 7,889 μmol/L in CIT-0.5 to 8,691 μmol/L in ORN-1; however, there were no significant differences in TAA between any of the diets. Both the total essential and non-essential amino acids were compared across diets; total essential amino acids (EAA) ranged from 1,872 ± 154 μmol/L in ARG-2 to 2,298 ± 240 μmol/L in ORN-2, while total non-essential amino acids (NEAA) ranged from 5748 ± 154 μmol/l in CIT-0.5 to 6705± 503 μmol/l in ORN-1; however, there were no significant differences between diets. Taurine was found to have the highest circulating concentration of all the amino acids analysed, but there were no differences in concentration between diets.

The levels for arginine, ornithine and citrulline are also in Figure 2. For arginine, CIT-1 and CIT-2 had significantly higher levels of circulating arginine at 176 ± 11 μmol/L and 333 ± 39 μmol/L respectively. Arginine levels for the rest of the diets were relatively unchanged ranging from 102 ± 8 μmol/L in

ORN-1 to 123 ± 10 μmol/L in ARG-1. Ornithine levels were also significantly increased by CIT-2 (62μmol/L) compared to the control (19 μmol/L) and ORN-2 (29 μmol/l), while there were no significant differences between the other diets. Circulating citrulline levels were low for all diets apart from the citrulline supplemented diets, which showed significantly increased plasma concentrations of citrulline compared to all other diets (Figure 2). Several other plasma amino acids were altered by the CIT-2 diet with leucine, isoleucine, threonine, valine, alanine and cystine all being significantly decreased compared to the control (Table 4). Alanine was also significantly decreased in ARG-2 and CIT-0.5 compared to the control. Phenylalanine was significantly increased in ORN-0.5 and ORN-2 compared to the control.

Table 5

Figure 2

3.3 3-h Post prandial amino acids in the plasma

Free amino acid levels were examined in the blood plasma of fish 3-h post-prandial (Table 6). The total concentrations of circulating amino acids ranged from 9,956 μmol/L in ORN-1 to 15154 μmol/L in CIT-2, which was significantly higher than all barring except CIT-1. The total concentration of EAA ranged from 2,955 μmol/L in CIT-2 to 3688 μmol/L in ARG-2, but there were no significant differences across diets. For the total non-essential amino acids, concentrations ranged from 6,680 μmol/L in ORN-2 to 12199 μmol/L in CIT-2. CIT-2 had significantly higher levels of NEAA than other diets, similar to the levels found for the total amino acids, which is most likely a reflection of the very high levels of circulating citrulline. Of all the amino acids analysed, taurine had the highest concentration in all diets apart from CIT-2, where citrulline levels were greater; however there were no significant differences in taurine concentrations between any diet.

Arginine, ornithine and citrulline showed significantly increasing plasma concentrations matching the level of dietary supplementation (Figure 3). CIT-1 and CIT-2 also increased circulating arginine levels to the same extent as the arginine supplemented diets (Figure 3). Phenylalanine was significantly increased in ORN-2 compared to the other diets and was significantly increased in ORN-0.5 and ORN-

1 compared to the control (Table 6). Alanine also significantly increased in ORN-2 compared to the control, while hydroxyproline was significantly decreased in ORN-2 and ARG-1 compared to the control.

Table 6

Figure 3

3.4 Free amino acids levels in liver and muscle tissue

Twenty-seven amino acids were detected in muscle, with tryptophan the only EAA below the detectable levels (Table 7). Total amino acid concentration ranged from 4,905 μmol/L in ARG-1 (significantly lower than control) to 6,118 μmol/L in ORN-2, while total EAA ranged from 949 μmol/L in ARG-2 to 1,288 μmol/L in ORN-0.5 and NEAA ranged from 3,917 μmol/L in ARG-1 to 4,780 μmol/L in ORN-2. Anserine, a dipeptide of β-alanine and 1-methylhistidine, was found to be most abundant in muscle tissue, ranging from 1,600 μmol/L in ARG-1 to 2088 μmol/L in CIT-1, with significantly higher levels in CIT-1. There were several amino acids altered in the muscle as a result of the different diets. Arginine was significantly higher in CIT-2 (89μmol/L) compared to all other diets, and double the control level (41μmol/L) (Table 7). Compared to the control, ornithine was significantly increased in ORN-1 and CIT-2, whereas citrulline was significantly increased in ORN-1. Lysine levels were significantly decreased in ARG-1, CIT-1 and CIT-2 = compared to the control, while phenylalanine decreased in ARG-1, ARG-2, CIT-0.5 and CIT-2. Threonine was also lower in ARG-1, ARG-2 and CIT-1 than the control. As with the plasma, alanine was significantly increased in ORN-2 compared to the control.

Table 7

Twenty-five amino acids were detected in liver (Table 8). Total amino acids ranged from 10,111 μmol/L in CIT-2 to 12,835 μmol/L in the control while total EAA ranged from 2,416 μmol/L in CIT-2 to 3,657 μmol/L in the control and NEAA ranged from 7,696 μmol/L in CIT-2 to 9,178 μmol/L in the control (Table 8). As with the samples, taurine was found at the highest levels, ranging from 2,849 μmol/L in CIT-2 to 3,014 μmol/L in ORN-0.5; however, there were no significant differences observed for any of the amino acids examined.

Table 8

3.5 Expression responses of genes involved in the urea cycle and polyamine synthesis

3.5.1 Baseline gene expression

The relative mRNA expression levels of genes encoding enzymes genes involved in the urea cycle and polyamine synthesis were then quantified in liver at the two time points. For fish sampled at the baseline time point (24-h post feeding) there were no significant changes in i) the urea cycle enzymes *arginase 1a*, *1b*, *2a*, *2b*, *OTC*, *ASS* and *ASL*, ii) *iNOS*, which is part of the nitric oxide cycle, or iii) the rate limiting enzymes of polyamine synthesis *ODC1*, *SAMdc1* and *SAMdc2* (Supplementary Table 1). However, the expression of *ODC2* was increased in ORN-2 compared to the control and CIT-2 diets. Considering the lack of significant changes in the urea cycle and polyamine synthesis enzymes between diets in baseline fish, alongside the absence of negative effects from 2% dietary supplementation in terms of growth (Table 3), the 0.5% and 1% diets were not included in further gene expression studies.

3.5.2 Post-prandial gene expression

The same genes examined in baseline fish were tested in the 3-h post prandial fish comparing the control, ARG-2, ORN-2 and CIT-2 diets (Supplementary Table 2). In post-prandial fish fed supplemented diets, *ARG1b* expression was significantly increased in all supplemented diets when compared to the control. However, as with the baseline expression, there were no more significant differences between the rest of the genes examined.

3.6 Changes to gene expression levels in post-prandial fish compared to the baseline fish

Differences in gene expression were examined between the 3-h and 24-h post prandial fish (Figures 4-6.). Differences in these time points should capture phenotypic modulations resulting from changes in free amino acid levels in the blood plasma immediately post-feeding compared to the baseline level. There was a general increase in expression of *ARG1a* and *ARG2b* in 3-h post-prandial fish compared to baseline fish (Figure 4). However, this was only significant in the ORN-2 and CIT-2 diets for *ARG1a* and in the ARG-2 diet for *ARG2b* when compared to ORN-2 and CIT-2 baseline fish. The other two

arginase encoding paralogues, *ARG1b* and *ARG2a*, expression was unchanged between the two time points (Figure 4). Both *ASS* and *ASL* showed decreased expression in 3-h post-prandial fish (Figure 5). *iNOS* expression was significantly decreased in control fish between the two points, but not for any of the supplemented diets (Figure 5). Both ODC paralogues showed significantly changed expression between the two time points (Figure 6). For *ODC1* expression, there was a significant increase in fish fed the CIT-2 diet at 3-h post-prandial relative to baseline expression. *ODC2* was significantly increased in all diets at the 3-h post-prandial time point barring ORN-2 (Figure 6). There were no significant changes in expression of *SAMdc1* or *SAMdc2* paralogues between time points (Figure 6).

Figure 4

Figure 5

Figure 6

4. Discussion

Despite great recent scientific interest in arginine supplementation, there remains a lack of knowledge on the associated metabolic impacts, including on the arginine precursors ornithine and citrulline. This study is the first to investigate the effects of supplementing arginine, ornithine and citrulline on free amino acid levels in both plasma and tissue (liver and muscle) in fish and associated pathway gene expression in liver tissue. We also documented changes in free amino acid levels immediately following feeding and demonstrated that rainbow trout can endogenously synthesise arginine from citrulline supplemented diets.

While there are numerous reports of potential growth benefits from arginine supplementation (see Oehme *et al.*2010; Pohlenz *et al.* 2014; Zhou *et al.* 2015; Wu *et al.* 2018), within this experiment, the dietary supplementation of arginine, ornithine or citrulline had no significant effects on any of the growth parameters measured. This finding is similar to a study in sea bass, where diets were supplemented with arginine at 1% or 2%, and no significant alterations in growth were observed (Azeredo *et al.* 2015). However, in this past study several immune parameters, such as respiratory burst and immune related gene expression, were decreased in fish on the supplemented arginine diets, suggesting an inhibitory effect on immune function (Azeredo *et al.* 2015). In gilthead seabream (Olivia-Teles *et al.* 2017), Atlantic salmon (Andersen *et al.* 2015) and common carp (Hoseini *et al.* 2019), arginine supplementation also resulted in no improvements to growth. Contrasting these results, other work has suggested that arginine supplementation can lead to negative impacts on growth. For example, rainbow trout fed diets with up to 4% (per kg of feed) arginine inclusion displayed negative effects on growth performance compared to animals supplemented with a lower concentration (1.6%) (Fournier *et al.* 2003). In several aquaculture species supplemented with arginine, namely hybrid grouper (1.9% - 4.7 % of diet) (Wu *et al.* 2018), yellow catfish (2.44 – 3.33 % of diet) (Zhou *et al.* 2014) and grass carp (0.7 – 2.4 % of diet) (Wang *et al.* 2014), it was found that while lower levels of supplemented arginine increased growth, this effect plateaued at higher levels of supplementation, which in several cases induced negative growth performance. The decrease in growth induced by high levels of dietary arginine is likely due to an imbalance in the arginine/lysine ratio (Zhou *et al.* 2011). Lysine, another

EAA in salmonids, competes for the same transporter proteins as arginine and is a potent inhibitor of arginase (Luiking and Deutz 2007; Zhou *et al.* 2011). Imbalanced concentrations of arginine and lysine can inhibit each other's uptake; resulting in reduced growth, as seen in pigs (Edmonds and Baker 1987), cobia (Nguyen *et al.* 2013) and Atlantic salmon (Berge *et al.* 2002). As there were no decreases in growth parameters for the fish fed supplemented diets in the present study, it is unlikely that any severe imbalances in these EAAs occurred. It is possible that fish, in the present study, were already growing at a maximal rate and unable to utilise the excess arginine for growth. However, the increased baseline plasma levels of arginine observed in the supplemented citrulline diets could have implications for an improved immune status due to arginine's central role in nitric oxide production and tissue repair. In mammals, improved nitric oxide production/availability has been observed both in mice (Wijnands *et al.* 2012) and humans (Schwedhelm *et al.* 2008; El-Hattab *et al.* 2012; Wijnands *et al.* 2015) resultant from enhanced arginine availability derived from citrulline supplementation in these studies.

Significant changes were seen in the plasma amino acid profiles of the supplemented diet fish at both basal levels and 3-h post prandial. This narrow window of sampling allows time for post-prandial peaks to settle, before a fasting state sets in and provides a useful measure of long-term changes induced by the supplemented diets. In post prandial fish, arginine, ornithine and citrulline were incrementally increased by their respective supplemented diets according to the level of supplementation. However, only the citrulline supplemented diets retained a higher circulating level of all three amino acids following the post-prandial peak at the basal time point. An increase of arginine levels following citrulline supplementation has been shown by several studies in mammals (Osowska *et al.* 2004; Schwedhelm *et al.* 2008; Lassala *et al.* 2009), but to the best of our knowledge, this is the first study to demonstrate such as increase in fish. There are very few studies documenting the urea cycle amino acid dynamics of fish; one such study of rainbow trout demonstrated that replacing half of the dietary arginine content with an equimolar amount of citrulline resulted in no reduction of growth at juvenile stages (Chiu *et al.* 1986). In channel catfish, diets deficient in arginine were supplemented with glutamic acid and resulted in similar growth to the non-deficient diets (Buentello and Gatlin 2000). Plasma levels of arginine, ornithine and citrulline were also increased in these fish, suggesting that *de*

novo synthesis of arginine was occurring through the intestine-renal axis of glutamine ➞ glutamate ➞ P5C ➞ ornithine ➞ citrulline ➞ arginine. The enzymes responsible for this endogenous synthesis of arginine, P5C synthase, CPS and OTC, are expressed at low levels in adult rainbow trout and generally only detectable in muscle (Korte *et al.* 1997; Todgham *et al.* 2001). The present study also demonstrated that supplementing with ornithine does not increase plasma arginine or citrulline levels, likely due to the low observed hepatic expression of *OTC*, which would facilitate the conversion (Wright *et al.* 1995). Interestingly, citrulline, but not arginine or ornithine, supplementation increased basal plasma levels of ornithine. This is likely due to the increased availability of circulating arginine in the citrulline supplemented diets, allowing conversion to ornithine. High basal levels of arginine were only observed in fish fed the CIT-1 and CIT-2 diets, even though post-prandial levels of arginine were comparable in both arginine and citrulline supplemented diets. The ability of citrulline, but not arginine, supplemented fish to maintain a high level of circulating arginine may be linked to the tissues that uptake and metabolise these amino acids. Orally ingested arginine is subject to high rates of first pass metabolism by the liver due to its high endogenous arginase activity (Allerton *et al.* 2018). Arginase is a major component of the urea cycle and hydrolyses arginine into urea and ornithine, meaning much of the arginine that reaches the liver is used to excrete nitrogenous waste (Allerton *et al.* 2018). Citrulline in the liver is mainly compartmentalised to the urea cycle, meaning orally ingested citrulline bypasses hepatic metabolism, and is instead taken up by the proximal tubular cells of the kidney, where it can be converted to arginine and released into circulation (Curis *et al.* 2005; Bahri *et al.* 2012).

The branched chain amino acids (BCAAs), leucine, isoleucine and valine were all significantly decreased in the CIT-2 diet at basal levels. BCAAs are all EAAs used in protein synthesis and have the capability, particularly leucine, to activate the mTOR pathway (Chen *et al.* 2016; Kawaguchi *et al.* 2011). Following a protein-rich meal there is a post-prandial spike in BCAA plasma concentration as the major enzyme in their catabolism, branched-chain-amino-acid aminotransferase (BCAT), has low hepatic expression, allowing the BCAAs to pass rapidly into circulation (Adeva *et al.* 2012; Holeček 2018). BCAT has high activity levels in skeletal muscle, meaning the initial BCAA catabolism occurs there (Brosnan and Brosnan 2006). The BCAT reaction deaminates BCAAs, providing a source of

nitrogen to synthesise glutamate along with the corresponding branched chain keto acids (BCKAs), α-ketoisocaproate (KIC, ketoleucine), α-keto-β-methylvalerate (KMV, ketoisoleucine), and α-ketoisovalerate (KIV, ketovaline) (Holeček 2018). The rate of BCAA degradation is highly dependent on their availability; in the present study the lower levels of alanine observed in CIT-2 fish is likely due to the lower availability of BCAAs in these fish. The supplementation of BCAAs is common in athletes in order to improve performance, however excess concentrations of BCAAs can enhance ammonia levels through their stimulatory effect on glutamine synthesis after BCAA metabolism to glutamate, causing hyperammonemia (Holecek 2013). One study done in Taekwondo athletes (Chen *et al.* 2016) found that supplementing citrulline with BCAAs reduced this build-up of ammonia through arginine synthesis and increased activation of the urea cycle. It is possible that the increased arginine levels observed in the CIT-2 diet in the present study allowed for a greater turnover of the BCAAs without hyperammonemia.

Phenylalanine was significantly increased in fish fed all of the ornithine supplemented diets 3-h post prandial, and in fish fed the ORN-0.5 and ORN-2 diets at the basal time point. Phenylalanine is an EAA that is converted into the NEAA tyrosine. Phenylalanine hydroxylase catalyses this reaction and is rate limiting to the degradation of excess phenylalanine from dietary proteins (Flydal and Martinez 2013). Tyrosine can be further degraded for use in the citric acid cycle, used in protein synthesis or converted to L-DOPA which in turn, is used for the synthesis of dopamine, norepinephrine, and epinephrine (Flydal and Martinez 2013). The exact mechanism for the observed increased phenylalanine levels in ornithine supplemented diets in this study is unknown, as they do not share any metabolic pathways, and, to the best of our knowledge, this is the first documentation of the phenomena. Endogenous ornithine is either recycled into citrulline or used in polyamine synthesis through the action of ODC, synthesising putrescine. Putrescine can then synthesise the higher polyamines, spermidine, and then spermine, through the action of spermidine synthase and spermine synthase, respectively, and via the donation of a methyl group from the other rate-limiting enzyme in polyamine synthesis, SAMdc (Liao *et al.* 2015). One study on rat liver cells from Fisher *et al.* (1986), demonstrated that high concentrations of polyamines (particularly spermine) antagonized the action of phenylalanine hydroxylase, preventing

phenylalanine's metabolism. We hypothesise that the excess ornithine in the supplemented ORN diets were inhibiting phenylalanine hydroxylase and allowing phenylalanine levels to increase.

No significant changes were observed in the liver samples of fish fed supplemented diets, however several changes occurred in muscle. As with the plasma samples, the CIT-2 diet significantly increased muscle arginine levels, suggesting enhanced arginine synthesis; which may also improve the nutritional quality of the fillet. However, the increased arginine concentration in CIT-2 fed fish correlated with a significant decrease in lysine levels, similar to observations for the ARG-1 and CIT-1 diets. This reduction in muscle lysine is likely due to increased competition with arginine for the arginine/lysine transporter. The significant increase in ornithine (ORN-1 and CIT-2 diets) and citrulline (ORN-1 diet) is likely due to the higher expression of OTC and CPS in rainbow trout muscle in comparison to liver (Todgham *et al.* 2001), which can utilise the extra circulating ornithine and citrulline. Moreover, this observation suggests the conversion of ornithine to citrulline is only possible in the muscle of rainbow trout, as similar changes were not observed in plasma. The concentrations of ornithine and citrulline are relatively low in comparison to the more abundant amino acids in muscle such as glycine, a major component of collagen for structural purposes (Li and Wu 2017), or anserine, an abundant dipeptide utilised as an energy source e.g. to aide burst swimming activity (Ogata and Murai 1994).

Transcriptional responses of the urea cycle enzymes, rate limiting enzymes in polyamine synthesis and *iNOS* were examined in the liver of all diets for baseline fish. The liver was chosen due to its central role in amino acid metabolism and as the main site of the urea cycle (Brosnan 2000). Despite the large phenotypic changes in amino acid levels observed in plasma, there were no significant differences in baseline gene expression between diets for any of the genes examined except *ODC2*, which was higher in fish fed the ORN-2 diet compared to CIT-2 diet. Expression of *ARG1b* was significantly increased in all supplemented diets compared to the control at the post-prandial time point. Both ARG1 and 2 enzymes catalyse the same reaction (arginine to ornithine and urea) but are nonetheless differentially expressed. ARG1 is primarily expressed in the liver and is thought to be the major metaboliser of hepatic arginine for nitrogenous waste secretion, whereas increased ARG2 expression is a marker for M2 (healing) macrophages, and thought to be involved with tissue repair following an immune response

(Rath *et al.* 2014; Forlenza *et al.* 2011). The increased urea cycle amino acid concentrations observed in fish fed the supplemented diets likely generated an increase in nitrogenous waste excretion, reflected by an increase in *ARG1b* expression.

ARG1a expression was significantly increased in 3-h post-prandial fish fed ORN-2 and CIT-2 diets compared to baseline fish fed the same diets. The remaining genes of the urea cycle enzymes were generally decreased in post-prandial fish relative to each diets baseline (apart from *OTC* in ARG-2). This may indicate that the conversion from citrulline to arginine, or general metabolism of the urea cycle amino acids takes place over a longer time as plasma amino acid levels at the 3h post-prandial time point are still relatively high in comparison to baseline levels. *iNOS* expression was also investigated as it competes with arginase for arginine (Rath *et al.* 2014) and may give an indication of surplus arginine on the fish's immune response and general health. *iNOS* was significantly decreased in the control diets post-prandial fish relative to the control diet's baseline, whereas there were no significant differences between the supplemented diets baseline and post-prandial. In the polyamine synthesis enzymes, both ODC1 and ODC2 paralogues were generally increased post-prandially but this was only significant in CIT-2 post-prandial compared to CIT-2 baseline. Polyamines are known to have roles in regulating synthesis rates of nucleic acid and proteins with studies in rats that have shown an increase in *ODC* expression following a meal suggesting *ODC* is crucial in post-absorptive digestion (Iwami *et al.* 1994; Igarashi and Kashiwagi 2015). There were no significant changes in either *SAMdc1* or *SAMdc2.*

In summary, our findings suggest that rainbow trout can endogenously synthesise arginine from dietary citrulline, but not ornithine. Of great interest is the discovery that dietary citrulline can maintain a high level of circulating arginine in the plasma, much more effectively than dietary arginine, in a dose dependant manner. As such citrulline supplementation may be an excellent choice for increasing circulating arginine levels. However, we did not observe improvements in biometric measurements such as growth and feed conversion parameters in the fish fed the supplemented diets compared to a control diet. This potentially reflects a scenario where the fish were already growing at maximal rate on diets meeting their amino acid requirements. The genes encoding the urea cycle enzymes were largely

unchanged in expression between diets in the liver at both post-prandial and baseline time points and it is likely that the conversion of citrulline to arginine is taking place in other tissues. Future research should investigate whether citrulline supplemented diets improve the immune response through enhanced arginine availability.

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705

706 **Figure legends**

707 Figure 1. Arginine's metabolic pathways and associated enzymes: nNOS, neural nitric oxide synthase;
708 IGF/GH, insulin like growth factor / growth hormone; ODC, ornithine decarboxylase; iNOS, inducible
709 nitric oxide synthase; AGAT, arginine:glycine amidinotransferase

710 Figure 2. Baseline free amino acid concentrations (μmol/L) of Arginine (A), Ornithine (B) and
711 Citrulline (C) in the blood plasma of rainbow trout following a 14-week feeding trial with amino acid
712 enriched diets. Fish were fed either the control commercial diet or graded levels (0.5%, 1%, 2%) of
713 supplemented amino acid over nutritional requirements. Bars represent mean (± SEM), n=9.

714 Figure 3. Free amino acid concentrations (μmol/L) of Arginine (A), Ornithine (B) and Citrulline (C) in
715 the blood plasma of rainbow trout 3-h post prandial following a 14-week feeding trial with amino acid
716 enriched diets. Fish were fed either the control commercial diet or graded levels (0.5%, 1%, 2%) of
717 supplemented amino acid over the nutritional requirement. Bars represent mean (± SEM), n=9.

718 Figure 4. Relative gene expression of arginase enzymes (*ARG1a*, *ARG1b*, *ARG2a* and *ARG2b*) in liver
719 tissue, between baseline and 3h post-prandial fish for the control and maximum supplementation diets
720 of arginine (ARG-2), ornithine (ORN-2) and citrulline (CIT-2). Bars represent mean (± SEM), n=9;
721 different superscript letters are significantly different ($p < 0.05$); * represents a significant difference
722 from the respective time points control diet (see Supplemental Tables 1 and 2).

723 Figure 5. Relative gene expression of urea cycle enzymes (*OTC*, *ASS*, *ASL*) and *iNOS* in liver tissue,
724 between baseline and 3-h post-prandial fish for the control and maximum supplementation level diets
725 of arginine (ARG-2), ornithine (ORN-2) and citrulline (CIT-2). Bars represent mean (± SEM), n=9;
726 different superscript letters are significantly different ($p < 0.05$).

727 Figure 6. Relative gene expression of rate-limiting polyamine synthesis enzymes (*ODC1*, *ODC2*,
728 *SAMdc1* and *SAMdc2*) in liver tissue, between baseline and 3h post-prandial fish for the control and
729 maximum supplementation level diets of arginine (ARG-2), ornithine (ORN-2) and citrulline (CIT-2).
730 Bars represent mean (± SEM), n=9; different superscript letters are significantly different ($p < 0.05$); *
731 represents a significant difference from the respective time points control diet (see Supplemental Tables
732 1 and 2).

733

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

		Arginine			Ornithine			Citrulline		
Ingredients[1]	**Control**	**0.5%**	**1%**	**2%**	**0.5%**	**1%**	**2%**	**0.5%**	**1%**	**2%**
Fish Meal	150	150	150	150	150	150	150	150	150	150
Soy Protein Concentrate	135	135	135	135	135	135	135	135	135	135
Wheat Gluten	176.8	176.8	176.8	176.8	176.8	176.8	176.8	176.8	176.8	176.8
Maize Gluten	152	152	152	152	152	152	152	152	152	152
Wheat	110	105	100	90	105	100	90	105	100	90
Fish Oil	89.6	89.6	89.6	89.6	89.6	89.6	89.6	89.6	89.6	89.6
Rapeseed Oil	166.4	166.4	166.4	166.4	166.4	166.4	166.4	166.4	166.4	166.4
Vitamin + Mineral Premix	32.5	32.5	32.5	32.5	32.5	32.5	32.5	32.5	32.5	32.5
Yttrium	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Proximate composition										
Moisture (%)	5.8	5.7	5.7	5.5	5.7	5.7	5.5	5.7	5.7	5.5
Protein - Crude (%)	43.6	44.1	44.5	45.4	44.1	44.5	45.4	44.1	44.5	45.4
Fat - Crude (%)	29.3	29.3	16.6	29.3	29.3	16.6	29.3	29.3	16.6	29.3
Ash (%)	6.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0
Digestible Energy (MJ/kg)	21.9	21.8	21.73	21.6	21.8	21.73	21.6	21.8	21.73	21.6
Digestible Protein (%)	39.3	39.2	39.18	39.1	39.2	39.18	39.1	39.2	39.18	39.1

[1]Predicted water content of 12.8 g/kg

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

		Arginine			Ornithine			Citrulline		
	Control	**0.5%**	**1%**	**2%**	**0.5%**	**1%**	**2%**	**0.5%**	**1%**	**2%**
Alanine	23.1	22.3	22.6	23.5	24.9	23.1	23.4	22	21.9	23.8
Aspartic Acid	32.1	30	31.8	32.1	34.6	31.7	32.4	29.7	30	32.6
Cystine	7.18	6.7	6.83	6.92	6.61	7.16	6.77	6.56	6.79	7.4
Glutamic Acid	103	97.9	100	105	101	106	104	96.9	96.3	108
Glycine	17.9	17.1	17.6	18.1	20.3	18.2	18.1	17.2	16.9	18.3
Histidine	10.1	9.73	10.2	10.5	11.4	10.4	10.4	9.75	9.58	10.5
Isoleucine	17	16.3	16.8	17.1	18.4	17.5	17.2	16.2	16.2	17.9
Leucine	40.3	39.1	39.9	40.7	41	40.3	41.1	38.4	38.3	41.9
Lysine	26.1	25	25.9	26.2	28.7	26.2	26.1	23.9	24.4	26.8
Methionine	9.23	9.26	9.65	9.4	10.4	9.67	9.34	8.97	9.03	10
Phenylalanine	22.9	21.8	22.3	23.4	23	22.6	23	21.6	21.6	23.5
Proline	34.5	33.1	34.3	35	33.1	34.4	34.7	33.7	34.4	38.5
Serine	21.3	19.1	21	21.1	21.4	21.5	21.1	19.8	20	22.2
Threonine	15.8	14.6	15.6	15.7	16.5	15.4	15.7	14.6	15	15.8
Valine	19.5	19.1	19.6	20.2	21.1	20	20	18.6	18.7	20.2
Arginine	20.2	23.2	28.3	37	21.9	19.9	20.7	19.1	18.9	21
Ornithine	0.2	0.2	0.2	0.2	3.5	7.0	13.4	0.5	0.3	0.3
Citrulline	0.0	0.0	0.0	0.0	0.0	0.1	0.1	3.4	9.3	19.1

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

Table 3. Rainbow trout primer sequences used for qPCR with NCBI accession numbers. References: [1]Alzaid *et al.* 2016; [2] Heidari *et al.* 2015

Gene	Sense	Primer 5'-3'	Product size (bp)	Annealing temperature (°C)	Accession Number	Efficiency
EF-1α[1]	Forward	CAAGGATATCCGTCGTGGCA	327	64	**NM_001124339.1**	1.87
	Reverse	ACAGCGAAACGACCAAGAGG				
HPRT[2]	Forward	CCGCCTCAAGAGCTAGTGTAAT	237	64	**XM_021583468.1**	1.90
	Reverse	GTCTGGAACCTCAAACCCTATG				
ARG 1A	Forward	AGCACCATATCCTGACGTTG	147	64	**XM_021564871.1**	1.91
	Reverse	CATCGATGTCATAGCTCAGG				
ARG 1B	Forward	GGTGGATCGCCTTGGAATCG	179	64	**KX998966.1**	1.86
	Reverse	CTGTGATGTAGATTCCCTCC				
ARG 2A	Forward	TCCAGAGAGTCATGGAAGTCACTTTCC	198	64	**KX998967.1**	1.92
	Reverse	CCATCACTGACAACAACCCTGTGTT				
ARG 2B	Forward	CTTGTTGAGGTCAACCCAGC	163	64	**KX998968.1**	1.91
	Reverse	GTCGAAGCTGTTCCGTGTCG				
OTC	Forward	CACAGCCAGGGTTCTCTCTG	116	64	**XM_021597830.1**	1.88
	Reverse	CAGACAGGCCGTTGATGATG				
ASS	Forward	TGAGATTGGAGGGAGGCATG	172	64	**XM_021590913.1**	1.86
	Reverse	GCCCTGTTTGATCCTCCTGA				
ASL	Forward	ACGCTCTCCAACTCATCACA	129	64	**XM_021563243.1**	1.90
	Reverse	ACCGCATGACTCAGAATCCA				
ODC1	Forward	CGTGTGCCAGCTCAGTGTC	179	64	**XM_021574142.1**	1.92
	Reverse	CCATGTCAAAGACACAGCGG				
ODC2	Forward	TGGTGCCACCCTGAAGGCC	128	64	**XM_021585068.1**	1.89
	Reverse	AGATGGCCTGGCTGTAGGTG				
SAMdc1	Forward	GCAAGGACAAGCTAATTAAG	185	64	**XM_021600286.1**	1.80
	Reverse	AACCTTGGGATGGTACGGAG				
SAMdc2	Forward	AACTCACGATGGAAGCGAAC	121	64	**XM_021611778.1**	1.93
	Reverse	AACCTTGGGATGGTACGGAG				
iNOS	Forward	CGAATGGAGCTATCGTCAGACC	234	64	**AJ300555.1**	1.94
	Reverse	CGGGAACGTTGTGGTCATAATACC				

Table 4. Growth performance of adult rainbow trout from a 14 week feeding trial fed diets supplemented with different levels of arginine, ornithine or citrulline (±SEM, n=24 unless superscript states otherwise).

		Arginine			Ornithine			Citrulline			
	Control	**0.5%**	**1%**	**2%**	**0.5%**	**1%**	**2%**	**0.5%**	**1%**	**2%**	**ANOVA**
IBW[1,2] (g)	139± 3	144± 5	142± 8	146± 2	142± 4	140± 8	148± 2	146± 2	150± 6	146± 3	0.84
FBW[3] (g)	495± 24	484± 24	453± 22	447± 16	472± 20	507± 26	472± 18	506± 21	474± 20	483± 24	0.56
WG[4] (g)	356± 24	340± 24	311± 22	301± 16	331± 20	366± 26	324± 18	359± 21	323± 20	337± 24	0.46
GW[5] (g)	412± 19	405± 19	381± 18	376± 14	396± 17	425± 22	395± 16	418± 18	399± 17	408± 20	0.68
HSI[6]	1.75± 0.05	1.62± 0.05	1.63± 0.04	1.70± 0.08	1.73± 0.06	1.70± 0.05	1.69± 0.07	1.74± 0.06	1.74± 0.06	1.70± 0.06	0.85
VSI[7]	16.6± 0.5	16.2± 0.5	15.8± 0.4	16.0± 0.7	16.2± 0.4	15.9± 0.5	16.5± 0.6	17.3± 0.8	15.8± 0.5	15.5± 0.4	0.65
CF[8]	1.73± 0.03	1.69± 0.03	1.69± 0.03	1.68± 0.03	1.70± 0.02	1.72± 0.03	1.73± 0.03	1.73± 0.04	1.75± 0.03	1.66± 0.03	0.58
FCR[1, 9]	1.02± 0.03	1.05± 0.04	0.99± 0.01	1.02± 0.03	1.08± 0.06	1.02± 0.02	1.14± 0.05	1.07± 0.08	1.00± 0.01	0.98± 0.05	0.31
SGR[1, 10] (%)	1.15± 0.08	1.17± 0.04	1.17± 0.01	1.14± 0.02	1.07± 0.06	1.12± 0.05	1.06± 0.07	1.10± 0.09	1.10± 0.02	1.18± 0.02	0.71

[1] Tank statistics (n=3)
[2] Initial body weight
[3] Final body weight
[4] Weight gain
[5] Gutted weight
[6] Hepatosomatic index = liver weight / body weight *100
[7] Visceral somatic index = weight of viscera / body weight *100
[8] Condition factor
[9] Feed conversion ratio = wet weight gain / dry feed intake
[10] Specific growth rate = (Ln end weight – Ln start weight)/days

Table 5. Basal free essential amino acid levels (µmol/l) in blood plasma of adult rainbow trout fed diets supplemented with different levels of arginine, ornithine or citrulline (mean ± SEM, n=9)

		Arginine			Ornithine			Citrulline			
Amino Acid	**Control**	**0.5%**	**1%**	**2%**	**0.5%**	**1%**	**2%**	**0.5%**	**1%**	**2%**	**ANOVA**
Essential Amino Acids											
Arginine	104± 8^{a}	123± 10^{ab}	113± 12^{a}	109± 8^{a}	113± 7^{a}	102± 8^{a}	109± 7^{a}	104± 8^{a}	176± 11^{b***}	333± 39^{c***}	0.0001
Histidine	142± 6	147± 10	153± 6	142± 6	149± 6	129± 4	139± 7	137± 11	166± 8	140± 11	0.13
Isoleucine	193± 11^{ab}	200± 8^{ab}	180± 13^{abc}	163± 7^{bc*}	209± 10^{ab}	191± 9^{abc}	213± 12^{a}	205± 14^{ab}	201± 12^{ab}	149± 10^{c**}	0.0001
Leucine	350± 18^{ab}	351± 15^{ab}	330± 21^{abc}	298± 13^{bc}	391± 31^{ab}	329± 13^{abc}	392± 27^{a}	363± 23^{ab}	373± 23^{ab}	258± 14^{c***}	0.0001
Lysine	267± 23	335± 32	300± 31	304± 18	285± 18	290± 23	318± 20	292± 18	307± 16	285± 34	0.77
Methionine	80± 4	97± 9	87± 6	75± 6	95± 11	78± 5	94± 8	91± 7	107± 11	74± 6	0.06
Phenylalanine	124± 9	134± 5	132± 7	132± 5	171± 11**	144± 16	180± 30**	132± 6	153± 13	124± 7	0.045
Threonine	300± 18^{ab}	360± 35^{a}	255± 17^{ab}	245± 6^{ab}	306± 21^{ab}	255± 21^{ab}	328± 36^{a}	319± 36^{ab}	329± 27^{a}	213± 20^{b**}	0.0009
Tryptophan	30± 2	32± 1	32± 2	31± 1	35± 3	28± 1	31± 1	32± 2	32± 1	29± 1	0.23
Valine	432± 21^{abc}	456± 17^{abc}	402± 22^{abc}	372± 19^{bc}	486± 22^{a}	440± 20^{abc}	493± 28^{a}	468± 28^{ab}	452± 28^{abc}	352± 22^{c*}	0.0002
EAA[3]	2022± 209	2235± 265	1983± 277	1872± 154	2240± 243	1986± 190	2298± 240	2142± 120	2295± 160	1959± 260	0.018
Non-Essential Amino Acids											
Ornithine	19± 2^{a}	26± 2^{a}	25± 2^{a}	31± 9^{a}	25± 2^{a}	25± 3^{a}	29± 3^{a}	41± 12^{ab**}	36± 7^{ab*}	62± 8^{b***}	0.0001
Citrulline	13± 1^{a}	11± 1^{a}	10± 0^{a}	10± 0^{a}	16± 2^{a}	13± 1^{a}	19± 5^{a}	53± 14^{b***}	643± 221^{c***}	1147± 275^{c***}	0.0001
Taurine	3531± 262	3355± 271	3497± 358	4235± 523	3535± 413	4283± 466	3611± 416	3435± 451	3325± 521	2877± 415	0.453
Aspartic acid	34± 4	38± 4	35± 5	36± 5	35± 6	30± 3	37± 5	33± 6	34± 4	26± 2	0.68
Hydroxylproline	74± 9	75± 6	62± 5	58± 9	75± 6	60± 6	63± 5	67± 7	71± 9	66± 6	0.59
Serine	67± 4	88± 7	71± 5	72± 8	80± 5	70± 4	72± 5	77± 6	81± 4	81± 7	0.247
Asparagine	76± 13	94± 13	85± 14	63± 9	74± 8	66± 8	91± 9	81± 12	86± 14	88± 9	0.55
Glutamic acid	118± 12	129± 13	125± 16	124± 13	113± 11	105± 11	119± 12	113± 21	120± 13	104± 6	0.9
Glutamine	277± 20	330± 20	287± 29	274± 13	311± 19	277± 9	336± 33	298± 24	315± 19	276± 20	0.32
Proline	263± 38^{ab}	444± 138^{ab}	346± 76^{ab}	222± 32^{ab}	419± 57^{a}	289± 38^{ab}	405± 113^{ab}	190± 18^{ab}	303± 40^{ab}	165± 27^{b}	0.013
Glycine	559± 59	623± 46	508± 38	561± 31	604± 45	604± 48	531± 56	493± 44	478± 51	450± 50	0.15
Alanine	806± 35^{a}	780± 38^{a}	761± 36^{ab}	698± 28^{ab*}	809± 43^{a}	731± 39^{ab}	719± 23^{ab}	677± 21^{ab**}	730± 38^{ab}	609± 25^{b***}	0.0013
α-Aminobutric	16± 2	18± 2	14± 1	16± 3	17± 2	14± 1	17± 2	20± 3	13± 2	13± 2	0.302
Cystine	15± 1^{abc}	15± 1^{abc}	17± 1^{a}	14± 1^{abc}	15± 1^{abc}	15± 1^{abc}	16± 1^{ab}	14± 1^{abc}	13± 1^{bc*}	12± 1^{c**}	0.0023
Tyrosine	47± 4	52± 2	54± 5	55± 4	61± 7	45± 2	54± 4	58± 4	63± 7	46± 3	0.095
β Alanine	114± 20	101± 15	90± 12	86± 9	77± 9	77± 8	70± 10	98± 18	85± 18	76± 19	0.51
NEAA[4]	6030± 528	6182± 414	5985± 613	6553± 595	6267± 395	6705± 503	6189± 549	5748± 508	6395± 711	6096± 1482	0.95
TAA[5]	8052± 694	8417± 639	7968± 850	8425± 640	8507± 553	8691± 626	8487± 519	7889± 532	8690± 801	8055± 1693	0.92

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

[2] Concentration values in the same row with a "*" represent a significant difference from the control diet (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$)

[3] EAA: Totalled essential amino acids

[4] NEAA: Totalled non-essential amino acids

[5] TAA: Total amino acids

Table 6. Free essential amino acid levels (µmol/l) in blood plasma of 3 hours post prandial adult rainbow trout fed diets supplemented with different levels of arginine, ornithine or citrulline (mean ±SEM, n=9)

		Arginine			Ornithine			Citrulline			
Amino Acid	**Control**	**0.5%**	**1%**	**2%**	**0.5%**	**1%**	**2%**	**0.5%**	**1%**	**2%**	**ANOVA**
Essential Amino Acids											
Arginine	113± 7^{a}	133± 19^{ab}	150± 20^{abc}	222± 29^{cd***}	124± 12^{ab}	121± 9^{ab}	124± 11^{ab}	132± 6^{ab}	178± 11^{bcd**}	254± 18^{d***}	0.0001
Histidine	212± 11	206± 16	188± 15	223± 6	198± 13	185± 9	188± 14	200± 4	219± 7	203± 19	0.32
Isoleucine	343± 30	303± 26	303± 32	377± 32	341± 30	289± 25	325± 32	318± 15	374± 25	280± 29	0.16
Leucine	724± 58	651± 60	632± 60	778± 48	717± 58	611± 49	673± 66	658± 30	780± 45	506± 81	0.07
Lysine	281± 19	282± 40	298± 35	325± 32	288± 24	315± 19	371± 33	317± 31	316± 31	289± 28	0.57
Methionine	229± 17	214± 18	180± 16	234± 9	204± 12	199± 14	178± 15	226± 15	227± 10	185± 24	0.063
Phenylalanine	241± 14^{ab}	254± 26^{ab}	253± 22^{ab}	275± 10^{ab}	329± 26^{ab**}	333± 19^{b**}	468± 36^{c***}	270± 12^{ab}	286± 14^{ab}	236± 20^{a}	0.0001
Threonine	507± 25	412± 37	397± 41	429± 19	436± 40	461± 42	401± 22	463± 21	465± 16	378± 41	0.147
Tryptophan	45± 3	48± 3	45± 4	52± 2	48± 4	41± 1	45± 3	43± 2	49± 2	43± 3	0.27
Valine	734± 60	648± 52	617± 59	773± 54	720± 64	647± 51	687± 51	677± 28	776± 49	588± 62	0.14
Total EAA[3]	3431± 94	3153± 98	3064± 111	3688± 55	3405± 99	3202± 74	3460± 121	3303± 100	3670± 108	2955± 130	0.26
Non-Essential Amino Acids											
Ornithine	21± 1^{a}	26± 3^{a}	35± 6^{ab}	45± 5^{ab}	67± 11^{bc**}	120± 27^{c***}	293± 78^{d***}	31± 3^{ab}	34± 3^{ab}	44± 3^{ab}	< 0.0001
Citrulline	18± 1^{ab}	14± 1^{bc}	11± 1^{c**}	12± 1^{bc*}	19± 2^{ab}	23± 4^{ab}	25± 2^{a}	759± 81^{d***}	2544± 193^{e***}	5637± 954^{f***}	< 0.0001
Taurine	4074± 408	4108± 257	4064± 426	4126± 544	3784± 328	3232± 348	3074± 482	3431± 407	3275± 507	3434± 537	0.53
Aspartic acid	49± 6	47± 4	38± 4	37± 4	41± 4	39± 4	33± 6	36± 4	34± 4	34± 4	0.16
Hydroxyproline	73± 4	68± 4	58± 4*	60± 4	79± 4	82± 4	59± 6*	74± 5	68± 8	76± 9	0.008
Serine	105± 8	107± 9	90± 7	92± 5	124± 12	106± 9	109± 12	104± 7	101± 9	91± 7	0.19
Asparagine	116± 9	87± 14	95± 5	101± 12	117± 16	138± 12	113± 11	128± 16	118± 20	105± 12	0.27
Glutamic acid	145± 15	148± 11	131± 10	122± 11	132± 12	120± 10	122± 12	129± 12	124± 11	146± 13	0.6
Glutamine	849± 62	758± 112	800± 97	829± 53	802± 76	715± 73	739± 103	901± 65	887± 78	742± 114	0.81
Proline	739± 58	564± 63	527± 80	672± 74	520± 64	681± 92	613± 68	714± 77	707± 115	577± 101	0.29
Glycine	685± 56	650± 52	607± 64	598± 50	729± 69	675± 85	585± 35	684± 83	585± 59	582± 75	0.73
Alanine	573± 15^{abc}	563± 30^{abc}	611± 27^{abc}	527± 36^{abc}	652± 53^{ab}	609± 47^{abc}	672± 39^{a*}	573± 33^{abc}	512± 25^{bc}	484± 23^{c}	0.0036
α-Aminobutric	10± 0	10± 0	10± 0	10± 0	10± 0	10± 0	10± 0	10± 0	11± 1	12± 2	N.A[1]
Cystine	24± 2	26± 1	23± 2	22± 2	26± 3	22± 2	25± 2	24± 2	23± 2	20± 2	0.56
Tyrosine	107± 6	115± 14	123± 13	122± 8	142± 13	101± 8	123± 13	122± 7	127± 11	103± 10	0.22
β Alanine	74± 9	80± 8	87± 12	71± 6	78± 6	67± 5	71± 6	81± 9	63± 6	88± 13	0.45
Total NEAA[4]	7676± 299^{a}	7384± 307^{a}	7322± 369^{a}	7469± 545^{a}	7337± 480^{a}	6754± 516^{a}	6680± 508^{a}	7814± 539^{a}	9226± 533^{ab}	12199± 495^{b***}	0.0004
Total AA[5]	11106± 303^{a}	10537± 314^{a}	10385± 311^{a}	11156± 539^{a}	10741± 488^{a}	9956± 502^{a}	10140± 498^{a}	11118± 601^{a}	12896± 524^{ab}	15154± 500^{b***}	0.0007

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

[2] Concentration values in the same row with a "*" represent a significant difference from the control diet (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$)

[3] EAA: Totalled essential amino acids

[4] NEAA: Totalled non-essential amino acids

[5] TAA: Total amino acids

Table 7. Free essential and non-essential amino acid levels (μmol/l) in muscle tissue of adult rainbow trout fed diets supplemented with different levels of arginine, ornithine or citrulline (mean ±SEM, n=3)

		Arginine			Ornithine			Citrulline			
Amino Acid	**Control**	**0.5%**	**1%**	**2%**	**0.5%**	**1%**	**2%**	**0.5%**	**1%**	**2%**	**ANOVA**
Essential Amino Acids											
Arginine	41± 7[a]	34± 3[a]	34± 1[a]	35± 1[a]	40± 1[a]	45± 5[a]	40± 3[a]	41± 6[a]	36± 11[a]	89± 18[b**]	0.0021
Histidine	691± 109	634± 126	690± 54	600± 18	864± 57	750± 6	758± 73	742± 23	721± 34	680± 50	0.31
Isoleucine	26± 3	25± 4	17± 0	17± 0	21± 2	21± 2	22± 1	18± 2	20± 5	15± 2	0.11
Leucine	50± 9	47± 10	33± 3	33± 1	41± 3	40± 3	41± 5	35± 2	38± 6	30± 1	0.22
Lysine	127± 13[abc]	110± 9[abc]	64± 3[c*]	110± 25[bc]	132± 32[abc]	169± 14[ab]	254± 45[a*]	115± 18[abc]	65± 12[c**]	66± 12[c**]	0.0002
Methionine	15± 3	15±	8± 1	10± 0	11± 1	10± 1	11± 2	10± 1	10± 3	6± 1	0.059
Phenylalanine	22± 4[ab]	20± 4[ab]	15± 2[a*]	14± 1[a*]	18± 1[ab]	18± 1[ab]	27± 2[b]	16± 1[a*]	16± 2[a]	14± 0[a*]	0.005
Threonine	129± 14[ab]	116± 3[ab]	86± 1[ab*]	87± 13[ab*]	113± 10[ab]	100± 5[ab]	132± 17[a]	109± 14[ab]	78± 8[b**]	111± 14[ab]	0.02
Tryptophan	N.D [6]	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.A[7]
Valine	62± 8	55± 9	42± 2	42± 2	50± 4	49± 4	53± 5	43± 5	46± 9	37± 4	0.16
Total EAA[3]	1164± 84	1056± 130	988± 49	949± 27*	1288± 98	1202± 7	1338± 146	1128± 67	1032± 9	1048± 50	0.04
Non-Essential Amino Acids											
Ornithine	9± 1[abc]	9± 1[abc]	7± 1[a]	8± 1[abc]	12± 1[abc]	14± 1[c*]	13± 1[bc]	10± 1[abc]	8± 1[ab]	15± 4[c*]	0.0026
Citrulline	12± 1[ab]	10± 1[ab]	14± 1[ab]	10± 1[a]	17± 5[ab]	28± 9[b**]	22± 3[ab]	18± 2[ab]	10± 2[ab]	8± 2[ab]	0.023
Taurine	716± 134	805± 246	438± 246	462± 134	349± 70	372± 27	322± 26	360± 43	300± 43	483± 71	0.06
AsparticAcid	29± 6	28± 4	22± 4	21± 3	23± 2	24± 1	27± 5	23± 1	19± 1	19± 1	0.45
Hydroxyproline	41± 6	32± 1	37± 1	46± 11	35± 2	33± 2	51± 6	41± 6	38± 6	44± 4	0.41
Serine	36± 12	35± 13	20± 13	19± 4	19± 1	20± 1	32± 15	24± 6	20± 6	18± 2	0.6
Asparagine	5± 2	5± 2	3± 2	5± 2	3± 0	3± 0	4± 1	3± 0	3± 0	3± 0	N.A
GlutamicAcid	118± 34	106± 31	55± 31	91± 8	63± 6	75± 16	123± 36	89± 13	82± 13	136± 10	0.06
Glutamine	9± 2	9± 3	4± 3	6± 2	3± 0	3± 0	4± 1	3± 0	4± 0	8± 3	N.A
Proline	100± 23	156± 68	71± 68	154± 72	122± 14	191± 29	265± 15	133± 26	112± 26	175± 51	0.13
Glycine	922± 149	800± 64	748± 64	787± 107	932± 132	909± 60	1003± 125	861± 112	845± 112	784± 46	0.68
Alanine	472± 11[ab]	440± 18[ab]	392± 18[a]	451± 52[ab]	460± 4[ab]	447± 18[ab]	619± 96[b*]	433± 31[ab]	436± 31[ab]	526± 37[ab]	0.048
α-Aminobutric	12± 1	12± 2	11± 2	12± 1	13± 2	9± 1	15± 3	10± 3	8± 3	9± 1	0.39
Tyrosine	21± 3	20± 3	17± 3	16± 2	13± 1	13± 2	14± 3	14± 2	14± 2	13± 1	0.13
bAlanine	171± 22	135± 11	180± 11	216± 43	184± 17	166± 10	194± 14	206± 27	198± 27	161± 40	0.52
Methylhistidine	26± 12	19± 8	8± 8	13± 6	6± 1	7± 1	8± 1	11± 4	8± 4	7± 0	N.A
Anserine	1760± 42[ab]	1600± 202[a]	1795± 202[ab]	1635± 98[a]	1966± 50[ab]	1821± 43[ab]	1959± 45[ab]	1884± 67[ab]	2088± 67[b*]	1731± 10[ab]	0.013
Carnosine	77± 8	79± 14	95± 14	98± 12	82± 14	87± 20	107± 22	79± 11	76± 11	117± 7	0.42
NEAA[4]	4537± 193	4298± 17	3917± 17	4049± 201	4303± 203	4222± 19	4780± 217	4202± 144	4269± 144	4257± 116	0.067
TAA[5]	5701± 226[ab]	5354± 121[ab]	4905± 233[a*]	4998± 226[a*]	5591± 274[ab]	5424± 19[ab]	6118± 362[b]	5330± 210[ab]	5301± 90[ab]	5306± 160[ab]	0.034

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

[2] Concentration values in the same row with a "*" represent a significant difference from the control diet (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$)

[3] EAA: Totalled essential amino acids

[4] NEAA: Totalled non-essential amino acids

[5] TAA: Total amino acids

[6] N.D: Not detectable

[7] N.A: Not-applicable, not possible to conduct any meaningful analysis

Table 8. Free essential and non-essential amino acid levels (μmol/l) in liver tissue of adult rainbow trout fed diets supplemented with different levels of arginine, ornithine or citrulline (mean ±SEM, n=3)

		Arginine			Ornithine			Citrulline			
Amino Acid	**Control**	**0.5%**	**1%**	**2%**	**0.5%**	**1%**	**2%**	**0.5%**	**1%**	**2%**	**ANOVA**
Essential Amino Acids											
Arginine	205± 31	131± 29	205± 63	132± 9	188± 30	154± 13	139± 11	279± 62	125± 36	118± 27	0.11
Histidine	213± 27	183± 26	193± 20	170± 16	194± 16	177± 18	163± 15	185± 33	161± 9	161± 11	0.69
Isoleucine	317± 55	255± 56	306± 65	222± 18	287± 43	259± 40	215± 24	305± 60	205± 24	200± 35	0.47
Leucine	671± 101	547± 107	638± 134	477± 27	624± 90	555± 74	464± 59	678± 116	423± 55	432± 68	0.33
Lysine	656± 116	535± 110	602± 109	481± 46	600± 68	549± 69	475± 52	653± 139	452± 44	441± 65	0.58
Methionine	209± 40	150± 27	170± 35	144± 20	169± 29	155± 28	134± 24	198± 47	116± 19	121± 21	0.46
Phenylalanine	278± 49	214± 41	256± 58	193± 14	247± 42	233± 31	199± 27	269± 48	169± 26	173± 26	0.43
Threonine	528± 79	469± 98	523± 75	400± 19	509± 46	477± 55	409± 20	532± 91	401± 20	387± 47	0.53
Tryptophan	54± 12	39± 10	45± 9	37± 3	43± 7	43± 7	33± 7	49± 13	31± 7	34± 6	0.68
Valine	526± 92	441± 91	498± 91	389± 31	476± 63	441± 65	378± 39	501± 100	360± 37	349± 58	0.61
Total EAA[1]	3657± 596	2964± 590	3437± 657	2646± 202	3336± 429	3043± 397	2610± 273	3648± 707	2442± 273	2416± 361	0.45
Non-Essential Amino Acids											
Ornithine	198± 40	181± 36	169± 17	147± 15	167± 21	172± 33	134± 33	111± 18	137± 5	163± 9	0.4
Citrulline	10± 0	10± 0	10± 0	10± 0	11± 1	10± 0	11± 0	14± 2	11± 1	14± 4	N.A[1]
Taurine	2962± 96	3009± 103	2986± 199	2897± 143	3014± 112	2962± 31	2946± 31	2914± 163	2982± 91	2849± 105	0.922
Aspartic acid	494± 113	387± 98	460± 87	359± 55	437± 56	403± 63	347± 63	464± 105	343± 36	316± 60	0.75
Serine	530± 114	434± 107	494± 94	393± 55	495± 72	441± 75	381± 75	534± 125	378± 41	346± 71	0.74
Asparagine	67± 7	46± 11	70± 23	56± 2	69± 28	60± 8	49± 8	88± 18	31± 2	31± 9	0.059
Glutamic acid	1498± 133	1297± 64	1286± 50	1269± 90	1362± 74	1245± 64	1310± 64	1307± 111	1194± 51	1250± 73	0.43
Glutamine	249± 54	218± 35	219± 48	211± 35	232± 45	221± 37	185± 37	258± 52	169± 36	172± 29	0.81
Proline	422± 78	365± 70	389± 41	332± 27	378± 53	365± 48	335± 48	425± 86	308± 30	296± 42	0.73
Glycine	908± 124	799± 107	846± 44	775± 87	843± 74	789± 73	775± 73	834± 146	746± 37	727± 69	0.93
Alanine	1410± 90	1309± 140	1343± 120	1280± 41	1356± 49	1281± 74	1283± 74	1290± 178	1214± 77	1214± 78	0.93
α-Aminobutric	16± 1	13± 3	14± 1	13± 1	17± 1	14± 2	11± 2	17± 3	11± 1	10± 0	0.09
Cystine	24± 2	17± 3	25± 4	28± 3	24± 2	20± 5	27± 5	26± 3	26± 3	25± 4	0.43
Tyrosine	218± 42	172± 30	203± 48	152± 12	196± 28	184± 26	145± 26	206± 31	134± 22	137± 20	0.4
bAlanine	175± 21	159± 9	157± 9	150± 16	164± 13	146± 16	151± 16	143± 14	143± 13	147± 11	0.82
NEAA[2]	9178± 901	8416± 678	8672± 538	8071± 353	8765± 387	8311± 508	8090± 508	8630± 1035	7825± 92	7696± 349	0.77
TAA[3]	12835± 1498	11380± 1255	12109± 1157	10717± 522	12101± 815	11354± 905	10700± 905	12278± 1742	10267± 365	10111± 707	0.63

[1] EAA: Totalled essential amino acids

[2] NEAA: Totalled non-essential amino acids

[3] TAA: Total amino acids Concentration values in the same row with different superscript letters are significantly different ($p < 0.05$)

[4] N.A: Not-applicable, not possible to conduct any meaningful analysis

Figure 1.

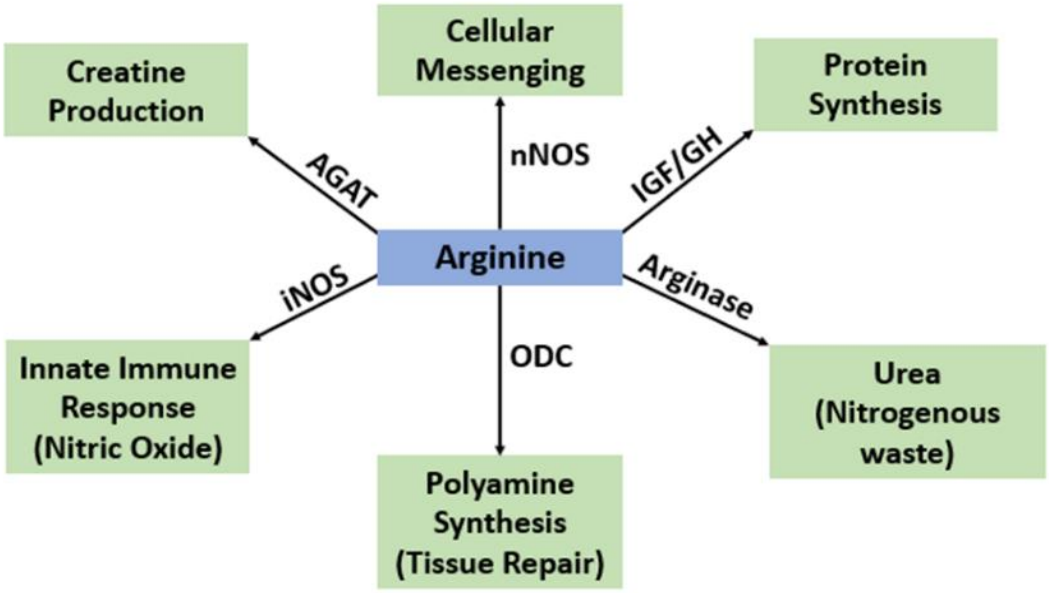

Cellular Messenging
Creatine Production
Protein Synthesis
AGAT
nNOS
IGF/GH
Arginine
iNOS
Arginase
ODC
Innate Immune Response (Nitric Oxide)
Urea (Nitrogenous waste)
Polyamine Synthesis (Tissue Repair)

Figure 2

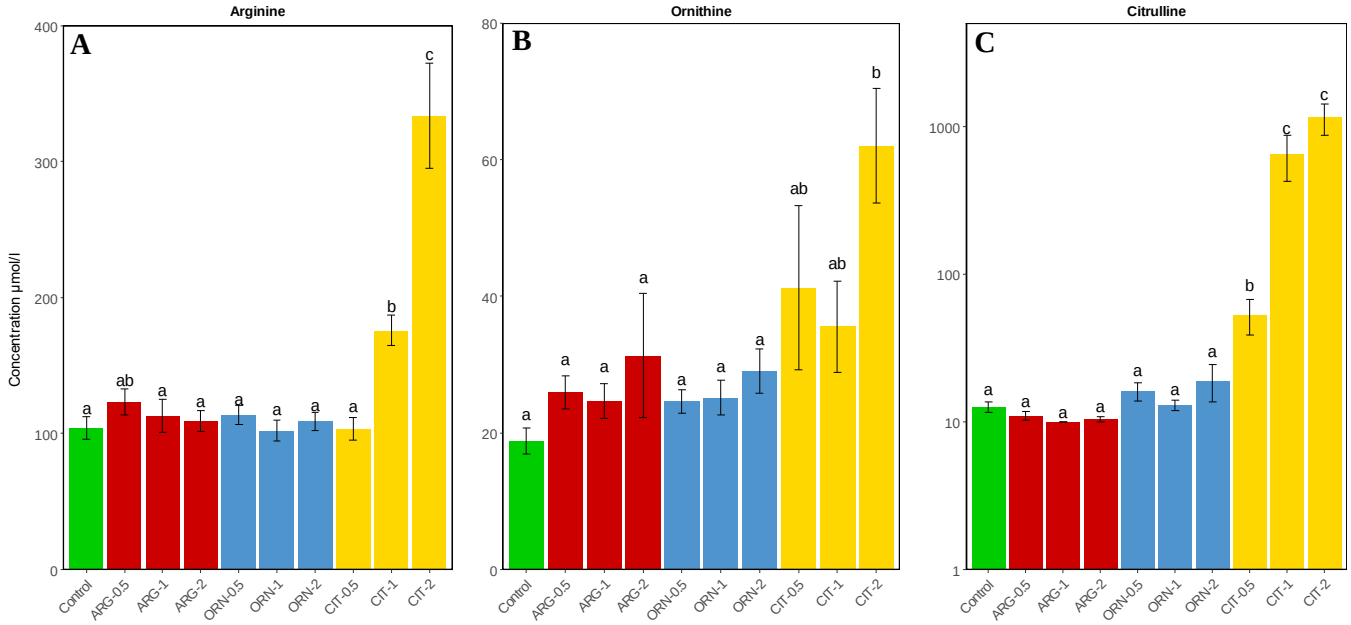

Arginine
A
Concentration µmol/l
400
300
200
100
0
a
ab
a
a
a
a
a
a
b
c
Ornithine
B
80
60
40
20
0
a
a
a
a
a
a
a
ab
ab
b
Citrulline
C
1000
100
10
1
a
a
a
a
a
a
a
b
c
c
Control
ARG-0.5
ARG-1
ARG-2
ORN-0.5
ORN-1
ORN-2
CIT-0.5
CIT-1
CIT-2

Figure 3

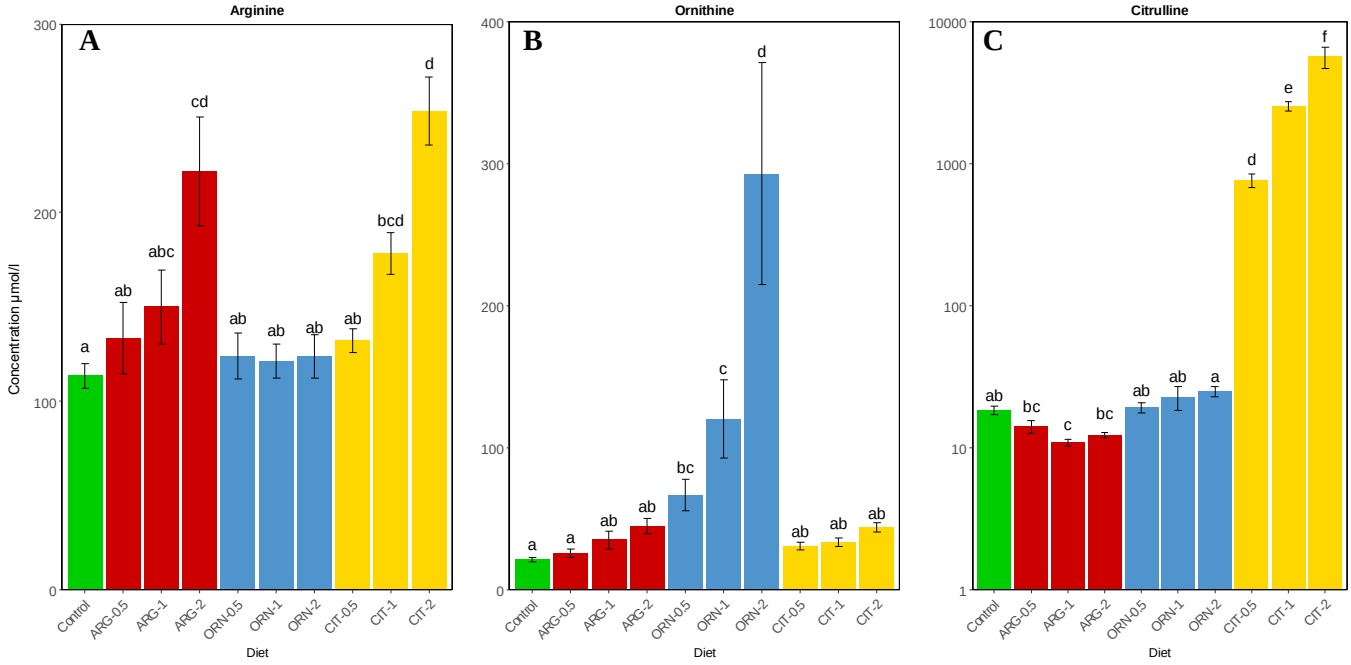

Arginine
A
Concentration µmol/l
300
200
100
0
a
ab
abc
cd
ab
ab
ab
ab
bcd
d
Control
ARG-0.5
ARG-1
ARG-2
ORN-0.5
ORN-1
ORN-2
CIT-0.5
CIT-1
CIT-2
Diet
Ornithine
B
400
300
200
100
0
a
a
ab
ab
bc
c
d
ab
ab
ab
Diet
Citrulline
C
10000
1000
100
10
1
ab
bc
c
bc
ab
ab
a
d
e
f
Diet

Figure 4.

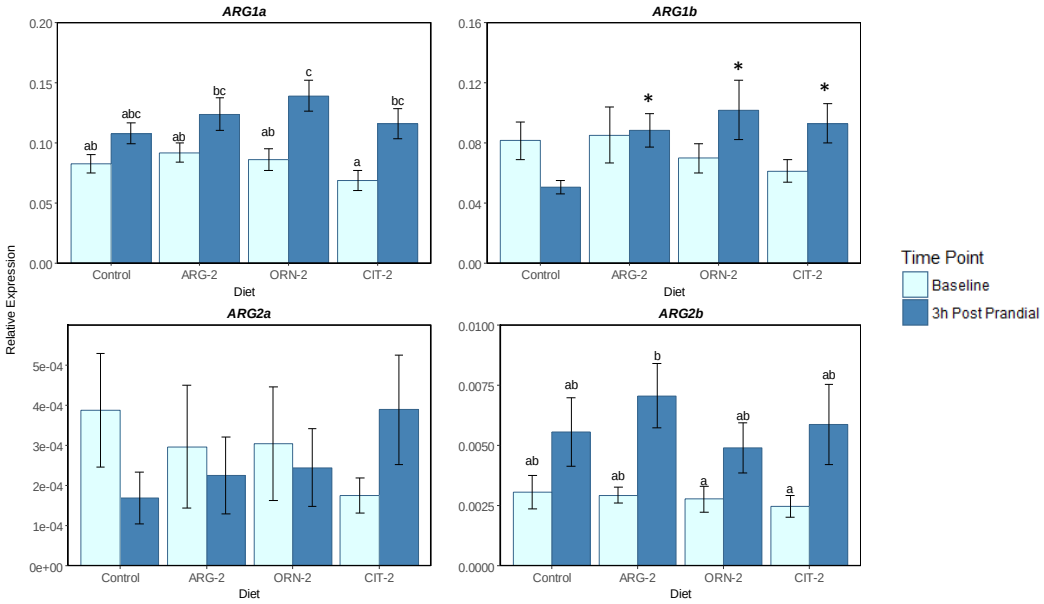

ARG1a
ARG1b
ARG2a
ARG2b
Relative Expression
Diet
Control
ARG-2
ORN-2
CIT-2
Time Point
Baseline
3h Post Prandial

Figure 5.

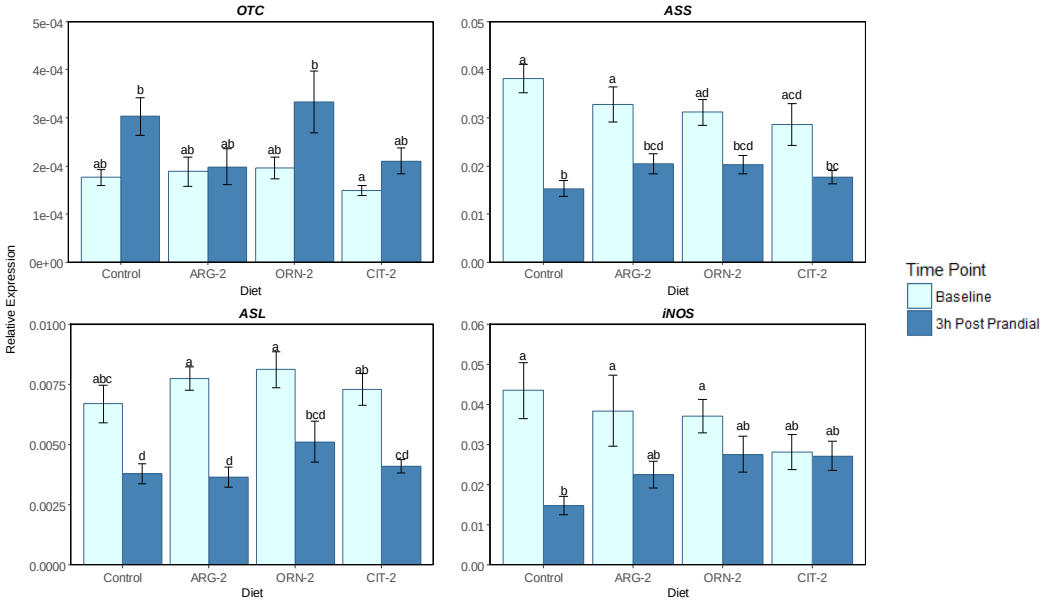
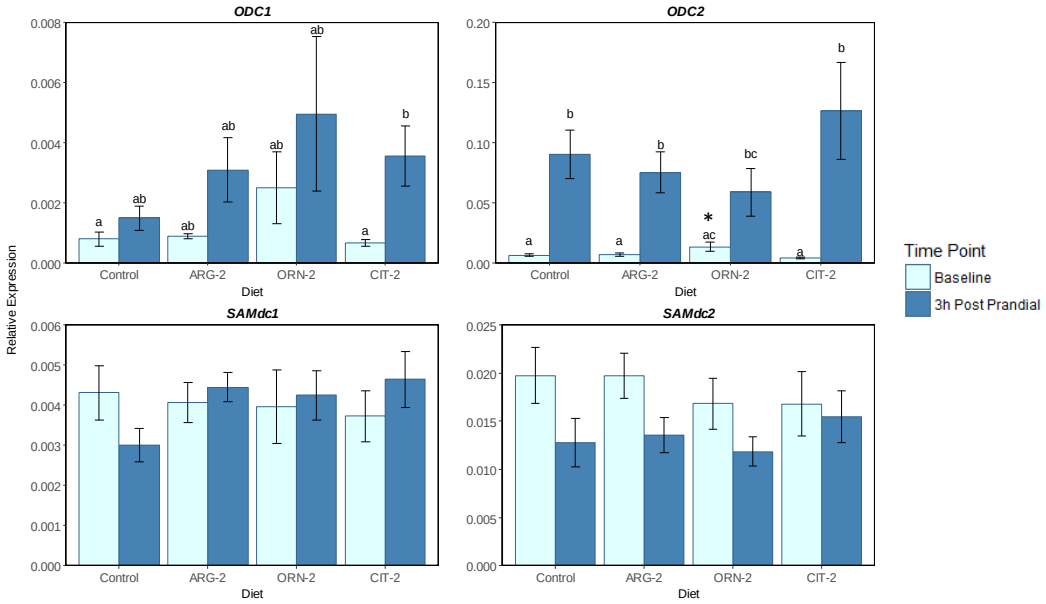

OTC
ASS
ASL
iNOS
Relative Expression
Diet
Control
ARG-2
ORN-2
CIT-2
Time Point
Baseline
3h Post Prandial

Figure 6.


ODC1
ODC2
SAMdc1
SAMdc2
Relative Expression
Diet
Control
ARG-2
ORN-2
CIT-2
Time Point
Baseline
3h Post Prandial

Supplementary material

Supplementary Table 1. Relative gene expression of urea cycle enzymes, polyamine synthesis enzymes and *iNOS* at the baseline time point of rainbow trout fed either a control diet or a diet supplemented with either arginine, ornithine or citrulline at one of 3 levels (0.5%, 1% or 2%) (mean ± SEM, n=9)

		Arginine			Ornithine			Citrulline			
Gene	**Control**	**0.5%**	**1%**	**2%**	**0.5%**	**1%**	**2%**	**0.5%**	**1%**	**2%**	**ANOVA**
ARG1a	0.08± 0.008	0.07±0.006	0.09±0.007	0.09± 0.008	0.07± 0.007	0.10± 0.012	0.09± 0.009	0.07± 0.007	0.08± 0.010	0.07± 0.009	0.33
ARG1b	0.08± 0.01	0.06±0.01	0.07±0.01	0.08± 0.02	0.07± 0.01	0.10± 0.02	0.07± 0.01	0.08± 0.01	0.06± 0.01	0.06± 0.008	0.26
ARG2a	0.0004± 0.0001	0.0001±0.0001	0.0004±0.0001	0.0003± 0.0002	0.0003± 0.0001	0.0002± 0.0001	0.0003± 0.0001	0.0005± 0.0003	0.0002± 0.0001	0.0002± 0.0001	0.62
ARG2b	0.003± 0.0007	0.002±0.0004	0.003±0.0003	0.003± 0.0003	0.002± 0.0002	0.003± 0.0005	0.003± 0.0006	0.004± 0.0007	0.002± 0.0004	0.002± 0.0005	0.35
OTC	0.0002± 2E-05	0.0002±2E-05	0.0002±2E-05	0.0002± 3E-05	0.0002± 2E-05	0.0001± 3E-05	0.0002± 2E-05	0.0002± 1E-05	0.0002± 3E-05	0.0001± 1E-05	0.18
ASS	0.04± 0.003	0.03±0.004	0.04±0.003	0.03± 0.004	0.03± 0.003	0.04± 0.006	0.03± 0.003	0.03± 0.002	0.04± 0.003	0.03± 0.004	0.59
ASL	0.007± 0.0008	0.007±0.0008	0.007±0.0012	0.008± 0.0005	0.008± 0.0004	0.007± 0.0009	0.008± 0.0007	0.008± 0.0006	0.008± 0.0008	0.007± 0.0007	0.61
ODC1	0.001± 0.0002	0.001±0.0002	0.001±0.0003	0.001± 0.0001	0.001± 0.0005	0.002± 0.0009	0.003± 0.0012	0.002± 0.0006	0.001± 0.0003	0.001± 0.0001	0.28
ODC2	0.006± 0.001^{ab}	0.007±0.002^{ab}	0.006±0.001^{ab}	0.007± 0.002^{ab}	0.006± 0.001^{ab}	0.009± 0.002^{ab}	0.013± 0.004^{a*}	0.011± 0.002^{ab}	0.005± 0.002^{ab}	0.004± 0.001^{b}	0.023
SAMdc1	0.004± 0.0007	0.004±0.0008	0.004±0.0004	0.004± 0.0005	0.004± 0.0005	0.004± 0.0006	0.004± 0.0009	0.004± 0.0005	0.003± 0.0004	0.004± 0.001	0.93
SAMdc2	0.02± 0.003	0.02±0.003	0.01±0.002	0.02± 0.002	0.02± 0.002	0.02± 0.004	0.02± 0.003	0.02± 0.002	0.01± 0.001	0.02±0.003	0.82
iNOS	0.04± 0.007	0.03±0.004	0.03±0.004	0.04± 0.009	0.03± 0.003	0.03± 0.003	0.04± 0.004	0.05± 0.010	0.03± 0.002	0.03± 0.004	0.29

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

[2] Concentration values in the same row with a "*" represent a significant difference from the control diet (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$)

Supplementary Table 2. Relative gene expression of urea cycle enzymes, polyamine synthesis enzymes and *iNOS* at the 3-h post-prandial time point of rainbow trout fed either a control diet or a diet supplemented with the maximal levels of either arginine, ornithine or citrulline (mean ± SEM, n=9).

Gene	Control	ARG-2	ORN-2	CIT-2	ANOVA
ARG1a	0.11± 0.01	0.12± 0.01	0.14± 0.01	0.12± 0.01	0.33
ARG1b	0.05± 0.004	0.09± 0.01*	0.10± 0.02*	0.09± 0.01*	0.037
ARG2a	0.0002 ± 0.0001	0.0002± 0.0001	0.0003± 0.0001	0.0004± 0.0001	0.53
ARG2b	0.006± 0.001	0.007± 0.001	0.005± 0.001	0.006± 0.002	0.63
OTC	0.0003± 0.00004	0.0002± 0.00004	0.0003± 0.00006	0.0002± 0.00003	0.059
ASS	0.02± 0.002	0.02± 0.002	0.02± 0.002	0.02± 0.001	0.16
ASL	0.004± 0.0004	0.004± 0.0004	0.005± 0.0008	0.004± 0.0003	0.21
ODC1	0.001± 0.0004	0.003± 0.001	0.005± 0.003	0.004± 0.001	0.41
ODC2	0.09± 0.02	0.07± 0.02	0.06± 0.02	0.13± 0.04	0.22
SAMdc1	0.003± 0.0003	0.004± 0.0004	0.004± 0.001	0.005± 0.001	0.14
SAMdc2	0.013± 0.003	0.014± 0.002	0.012± 0.002	0.015± 0.003	0.7
iNOS	0.01± 0.002	0.02± 0.003	0.03± 0.004	0.03± 0.004	0.069

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

[2] Concentration values in the same row with a "*" represent a significant difference from the control diet (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$)