Doctoral Dissertation

Studies on effect of dietary arginine, ornithine, and citrulline supplementation on postprandial plasma amino acids, arginine catabolism and resistance to *Vibrio anguillarum* of rainbow trout *Oncorhynchus mykiss*

March 2018

Graduate school of Marine Science and Technology

Tokyo University of Marine Science and Technology

Doctoral Course of Applied Marine Biosciences

FAUZI ICHSAN ACHMAD

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[博士過程]

Doctoral Course

博士学位論文内容要旨

Abstract

専　攻 Major	APPLIED MARINE BIOSCIENCES	氏　名 Name	ICHSAN ACHMAD FAUZI
論文題目 Title	Studies on dietary arginine, ornithine, and citrulline supplementation on rainbow trout *Oncorhynchus mykiss* on postprandial amino acids, arginine catabolism, and resistance to *Vibrio anguillarum.*		

With its versatile nature and important function in the immune system, supplemental level of arginine should be explored as a solution for increasing the health status of cultured fish to mitigate the adverse effect of climate change in aquaculture industries. Although most of the beneficial and adverse effects of arginine supplementation in the human and terrestrial animal have been evaluated before, the responses of arginine supplementation on fishes are not fully comprehended due to limited research conducted on it. With that in mind, this first research is aimed to evaluate the effect of supplemental dietary arginine levels on growth performance, plasma amino acids, and genes expression of enzymes that catabolize arginine in rainbow trout. For feeding trial, basal diet was formulated with 50.3% protein and 17.8% lipid. to fulfill all essential amino acid requirements, crystalline amino acid was also used in this research. To simulate grade arginine level, 2% and 4% of l-arginine was used. Upon amino acid analysis, the diets were confirmed to contain 1.47 (CTRL), 3.89 (3.89A) and 5.64 % of arginine (5.64A). Feeding trial was conducted for 9 weeks using juvenile rainbow trout with average weight of 62.5 g. After 9 weeks, fish were then fasted for 3 days and subjected for postprandial plasma amino acid study. Positive correlation between dietary arginine level with

plasma arginine and ornithine were found while no increase of plasma arginine level found in all postprandial time of CTRL. Highest citrulline level was found in CTRL while high supplementation of arginine decreases plasma citrulline production. There is no difference was found in plasma glutamic acid among treatments. The peak of plasma glutamine in CTRL was observed at 6 hours and 18 hours postprandial while in 5.64A plasma glutamine was only observed at 12 hours postprandial. Increase of plasma proline in 5.64A occurred at 12 hours postprandial, earlier than CTRL at 18 hours postprandial. Increase of plasma urea was only observed in arginine supplemented groups and there is no plasma urea difference found between those treatments. There was also no significant difference found in growth performance and total amino acid content in muscle of all treatments. However, higher protein content was found in the muscle of 3.89A compared to CTRL.

Since Our first experiment suggests that rainbow trout fed diet supplemented with arginine shows higher plasma ornithine (a precursor of polyamine), and lower plasma citrulline after 18 hours postprandial. However, supplemental arginine is also reported to increase arginine degradation through urea cycle and consequently reduce arginine availability. Thus, to avoid excessive arginine degradation and to better understand the role of citrulline, two more researches were conducted to evaluate the effect of dietary supplementation of ornithine and citrulline on resistance of rainbow trout, Oncorhynchus mykiss against *Vibrio anguillarum*, while in the same time observe postprandial amino acid dynamics and growth performance of rainbow trout fed by those amino acids.

For the second experiment, 20 juvenile rainbow trout (average size 34.1 g) were reared in 60 L aquaria with a recirculating system at 15º C. Dietary treatment was consisted of control diet (CTRL) with 48 % protein and 16 % lipid level, while treatment diets were made by supplementing control diet with 1% l-ornithine (ORN), 1% l-citrulline (CIT), and combination of 1% of l-ornithine and 1% of l-citrulline (ORN-CIT). Fish were fed twice daily for six day a

week until apparent satiation. To evaluate the effect of short and long feeding period on immune system, feeding trial was conducted twice: 15 days and 30 days. However, growth performance was only evaluated in the fish that was fed for 30 days. After 15 days and 30 days feeding, fish were injected intraperitoneally with *Vibrio anguillarum* that was diluted with phosphate-buffered saline at 3.0 x 10^6 CFU per fish. At 1 day post injection, blood was collected for plasma amino acid analysis and RNA was also extracted from kidney for quantitative-real-time PCR analysis of inducible nitric oxide (iNOS), interleukin-1-betta (Il-1β), and arginase. There is no significant difference in growth performance and feed efficiency upon 30 days of feeding. Furthermore, pre-feeding with these supplemental amino acids did not affect survival upon challenge with *Vibrio anguillarum* in both feeding regime. However significant differences were found in the expression of iNOS and Il-1β in kidney in the case of 15 days feeding regime, and in iNOS, Il-1β, and arginase in kidney in the case of 30 days feeding regime. Moreover, it was also shown in the postprandial plasma amino acid analysis that CIT treatment produce higher plasma arginine compared other treatments.

Since the second experiment found that supplementation with citrulline can increase plasma arginine level, the third experiment was aimed at comparing dietary supplementation with citrulline, ornithine, and arginine. Juvenile rainbow trout with average size of 9.1 gram was reared with the same condition with second experiment. Dietary treatment was consisted of control diet (CTRL) which was formulated to have 47 % protein and 15 % lipid level, while treatment diets were made by supplementing control diet with 2% l-arginine (ARG), l-ornithine (ORN), and l-citrulline (CIT) at the expense of cellulose. Feeding was conducted in similar ways with the previous experiment for 30 days period. After 30 days, fish were also injected intraperitoneally with *Vibrio anguillarum* that was diluted with phosphate-buffered saline at 3.0 x 10^6 CFU per fish. After 24 hours post injection, blood was collected and RNA was extracted from kidney for plasma amino acid and quantitative-real-time PCR analysis

respectively. A better growth performance was found in ARG compared to CIT, while there is no significant difference found between CTRL with other treatments. Survival analysis after disease challenge showed an improve resistant in CIT treatment compared to CTRL and the gene analysis and significantly higher expression level of iNOS was observed in CIT then CTRL. Moreover, postprandial plasma analysis showed a similar level of plasma arginine between ARG and CIT treatment and both treatments were significantly higher than CTRL. Thus, based on the results of this study, it can be concluded that citrulline supplementation improves rainbow trout's resistance against *Vibrio anguillarum*.

As a general conclusion, dietary supplementation with 2% citrulline for at least 30 days can improve rainbow trout's resistance against *Vibrio anguillarum*. This is also the first-time l-ornithine and l-citrulline was used as dietary supplementation in rainbow trout and it is the first time citrulline supplementation was shown to increase plasma arginine level in rainbow trout.

CHAPTER 1

I. General introduction

1.1. Challenges in aquaculture

Aquaculture is among the fastest growing industries in the food production sector (Morgan, Terry, Rajaratnam, & Pant, 2017). In 2014, it was reported that around 44% (approximately 73.8 million tons) of the total fish produced (approximately 167.2 million tons) came from aquaculture, globally contributing a total first-sale value of US$ 160.2 billion (FAO Fisheries and Aquaculture Department, 2016). While greatly contributing to global seafood production, one of major obstacle in aquaculture dependency on fish meal in aquaculture feed. Recently, the increase of fish meal price due to increasing demand and stagnant fish meal production (Hardy, 2010) raise some concern regarding the use of fish meal as aquafeed ingredient in the long run (Gatlin et al., 2007). To overcome this, higher inclusion of plant based ingredient has already been used. However, due to amino acid profile differences in plant based ingredients, precision diet formulation and more elaborate knowledge on amino acid metabolism is beneficial to develop efficient fish feed that fulfilled all nutrition requirement for farmed fish.

Lately, there is a new development on amino acid study that highlight their tertiary role on modulating several physiological pathways that can enhance growth performance, immune system, or reproductive performance (Andersen, Waagbø, & Espe, 2015; Brosnan & Brosnan, 2013; Fernandes, Murakami, Martins, Sakamoto, & Garcia, 2009; Fligger, Gibson, Sordillo, & Baumrucker, 1997; Jobgen et al., 2009; S. W. Kim, McPherson, & Wu, 2004; Tan et al., 2009; Wu, 2010; Yao et al., 2008, 2011). One amino acid that is known to have functional role and considered as a potential candidate to be used at supplemental level in the aquafeed is arginine. Several studies have shown the beneficial effect of arginine on enhancing growth performance organ development of several terrestrial animals. In pigs, arginine enhances intestinal growth

and development, increase production of insulin in the plasma, enhances growth of milk-fed young pigs, and increase mammalian target of rapamycin (mTOR) signaling activity in skeletal muscle (S. W. Kim et al., 2004; Tan et al., 2009; Yao et al., 2008, 2011); in broilers, dietary arginine supplementation positively affect weight gain at starter phase (Fernandes et al., 2009), in calves, arginine supplementation increase average daily gain (Fligger et al., 1997); and in mice, arginine supplementation reduces white fat gain while in the same time increases skeletal muscle (Jobgen et al., 2009).

Although positive effect of arginine supplementation in growth performance has already demonstrated in terrestrial animal, confounding results are shown in fishes. While arginine supplementation shows a temporary stimulatory growth sign (Plisetskaya, Buchelli-Narvaez, Hardy, & Dickhoff, 1991) and improve growth performance when high percentage of plant based ingredients is used in diet (Tulli et al., 2007), a study conducted by Fournier et al. (2003) found that dietary arginine supplementation resulted in not only increase nitrogen excretion of rainbow trout and turbot, but also no differences in growth performance and feed efficiency compared to control treatment. Since the increase of nitrogen excretion due to dietary arginine supplementation can give undesirable effect into aquatic environment, further reevaluation on the effect of dietary arginine supplementation on growth performance is warranted.

Furthermore, aquaculture industries are also susceptible to economic losses caused by disease outbreak due to viruses, bacteria, and fungi (Defoirdt et al. 2011). It was reported that global loss from disease can reach up to US$ 9.58 billion annually (Shinn et al. 2015; Tavares-Dias and Martins 2017). The effect of disease outbreak in aquaculture is also magnified by the occurrence of changing climates. According to The Intergovernmental Panel on Climate Change (IPCC) working group in their 2007 assessment report (IPCC 2007), the earth surfaces temperature in the 21st century will increase into 2.4-6.4^{o} C in the high case scenario. This

irreversible climate changes (Solomon et al. 2010) may affect aquaculture activity in several ways such as (1) Sea level and temperature rise (Cochrane et al. 2009) (2) changes in ocean productivity (Schmittner 2005) and ocean circulation pattern (Shepherd and Jackson 2013). This changes in aquatic environment can affect both host and pathogen and alter disease occurrence through alteration of environmental variables (stressors), host, and pathogen itself. The details of impact of climate changes on infectious disease in aquaculture has already been provided in several publications (Harvell et al. 1999; Harvell et al. 2002; Marcogliese 2008; Burge et al. 2014).

In the past, disease prevention and control in farmed animals has been accomplished using drugs and antibiotics. However, the use of antibiotics has been found to not only promote drug-resistant strains, but also cause unintended consumption of antibiotics by humans, due to the presence of residual antibiotics (Cabello 2006). Thus, it is beneficial to evaluate the use of pharmaceutical effect of arginine to safely boost the immune function of farmed animals, without negative consequences.

From the list of functional nutrient that have a potential to be evaluated, arginine is considered as promising ingredients since it is known to have multiple physiological role in modulating growth and immune function.

1.2. Arginine metabolism

Arginine is considered as versatile amino acid, in most animal it usually comes from various source such as dietary protein, endogenous arginine synthesis, and protein turnover (Morris 2006). As stated by Ball et al. (2007) there are species variation on capability to produce arginine from endogenous arginine synthesis, thus dietary arginine requirement are varied ranging from 5% of crude protein intake in swine to more than 5% in pacific salmon.

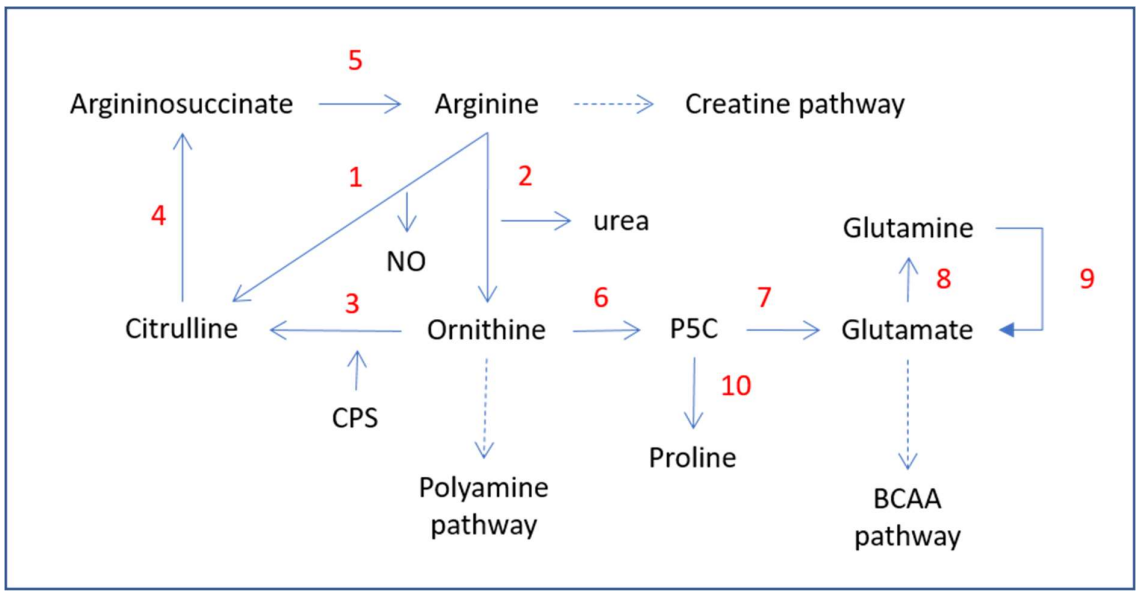

Figure 1.2.1 arginine pathway. Number in the figure represent enzymes: 1) nitric oxide synthase 2) arginase 3) ornithine carbamoyltransferase 4) argininosuccinate synthase 5) argininosuccinate lyase 6) ornithine amino transferase 7) pyrroline-5-carboxylate decarboxylase 8) glutamine synthetase 9) glutaminase 10) pyrroline-5-carboxylate reductase. Modified from Andersen et al. (2016).

Only one enzyme was known to be able to produce arginine: argininosuccinate lyase (ASL). Although only have one known synthesize enzyme, synthesis of arginine occurs on several steps; (1) ornithine and carbamoyl phosphate is converted into citrulline through ornithine carbamoyl transferase (2) citrulline is converted into argininosuccinate through argininosuccinate synthase (3) argininosuccinate is converted into arginine through argininosuccinate lyase. These steps can further complicate arginine pathway; ornithine can be converted into several compounds aside from citrulline thus connecting arginine pathway to proline, glutamine, and polyamine synthesis (figure 1.2.1.). Furthermore, synthesis of arginine can also involves several organ such as intestine, liver, and kidney (Morris 2004) which explained in figure 1.2.2.

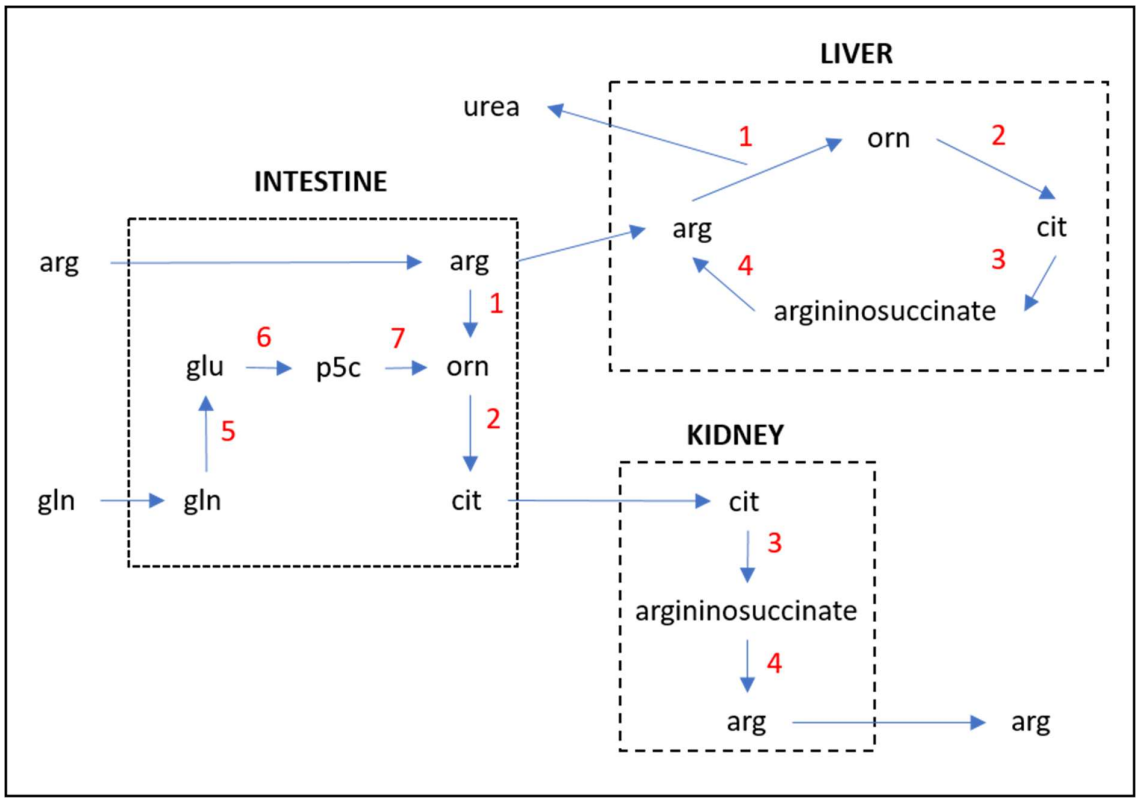

Figure 1.2.2. interorgan arginine. Adapted from Bahri et al. (2013). Arg, arginine; orn, ornithine; cit, citrulline; gln, glutamine; glu, glutamate; p5c, pyrroline-5-carboxylate. Number in the graph represent enzymes: 1) arginase 2) ornithine carbamoyltransferase 3) argininosuccinate synthetase 4) argininosuccinate lyase 5) glutaminase 6) P5C synthase 7) ornithine amino transferase.

The role of intestine on arginine synthesis pathway is first demonstrated by Windmueller and Spaeth (1974) who found conversion of glutamine into citrulline in intestine of mice (figure 1.2.2.). Conversion of glutamine into citrulline was done through l-Δ4-pyrroline-5-carboxylate (P5C), exclusively in intestine since P5C synthase is only in intestinal mucosa (Wakabayashi et al. 1983; Wakabayashi and Jones 1983; Wu 1998; Wu and Morris 1998). Furthermore, P5C are also connected with proline pathways whereas proline can be

used to produce P5C. This pathway connects proline and arginine synthesis in intestine. Citrulline that produce in intestine, is then released in blood circulation and converted into arginine in the kidney.

Kidney is known to be able to convert citrulline into arginine (Cohen and Hayano 1946; Wu and Morris 1998). Together with intestine, kidney plays an important for endogenous arginine synthesis. Conversion of citrulline into arginine in kidney is conducted with intermediary of argininosuccinate through argininosuccinate synthetase and argininosuccinate lyase. Arginine that was produced from this organ, if it is not used for nitric oxide synthesis, is then released into blood circulation (Levillain et al. 1990; Curis et al. 2005).

Liver has high activity of arginine synthesis enzyme and it also have complete enzyme to recycle arginine. However, urea cycle in the liver is regulated in a way so that the product of each enzymic reaction is channeled into next enzyme in pathways without net synthesis of arginine (Cheung et al. 1989; Watford 1991; Wu and Morris 1998). Thus, conversion of citrulline into arginine in liver is a strictly compartmentalized and isolated from other arginine pathways (Curis et al. 2005). Given that no net arginine synthesis in the liver, it is suggested by various studies that endogenous arginine synthesis is mainly comes from combination of intestine-kidney arginine synthesis (Wu and Morris 1998; Buentello and Gatlin III 2001b; Morris 2004; Morris 2006; Bahri et al. 2013; Breuillard et al. 2015).

Arginine can be catabolized by several enzymes: through arginase, arginine can be convert into ornithine while producing urea in the process; through nitric oxide synthase (NOS) arginine can be converted into nitric oxide and citrulline; along with methionine and glycine, arginine can be converted into creatine through l-arginine:glycine amidinotransferase (AGAT) and guanidinoacetate methyltransferase (GAMT). Ornithine is one of arginine catabolite through arginase.

Arginase itself has two isoforms: arginase I and arginase II. Arginase I is a cytosolic enzyme that commonly localize in liver and considered as main properties for urea cycle. Arginase II is a mitochondrial enzyme which can be found in various tissue except liver (Wu and Morris 1998). Aside from citrulline, ornithine can be converted into polyamine, proline, and glutamate. Conversion of ornithine into polyamine is catalyzed by ornithine decarboxylase while conversion into proline and glutamate is through ornithine aminotransferase.

Nitric oxide is one important compound that is known to have multiple role in signaling (Garthwaite and Boulton 1995), immune function (Bogdan et al. 2000), and cardiovascular function (Vallance 2001). Nitric oxide enzymes have several isoforms: NOS I which is constitutively expressed and originally found in neurons, NOS II also known as inducible nitric oxide (iNOS) which is induced by cytokine and commonly found in macrophage, NOS III which is constitutively expressed and originally found in endothelial cells (Förstermann and Kleinert 1995). From those isoform, a lot of interest has taken up into iNOS enzyme and due to its effect on innate immune system and interaction with arginase enzyme to regulate production of M1/M2 macrophage (Rath et al. 2015).

Creatine was considered to attract less attention compared to other arginine pathways, however recent study as a potent anticancer agent and in effect on athletic performance has initiated significant interest on this compound. (Wyss and Kaddurah-Daouk 2000). Synthesis of creatine from arginine is initiated by arginine:glycine amidinotransferase. In this reaction, guanidine group from arginine is transferred into glycine to form guanidinoacetate and ornithine. Guanidinoacetate is then methylated by guanidinoacetate N- methyltransferase to form creatine (Wu and Morris 1998).

1.3. Arginine metabolism in fish

Arginine is an essential amino acid in most fishes due to their inability to adequately acquire it through *de novo* synthesis (Mommsen et al. 2001; Li et al. 2009). However, Buentello

and Gatlin (2001a) and (Chiu et al. 1986) indicated the presence of endogenous synthesis of arginine in the form of conversion of ornithine into citrulline and arginine through plasma amino acid analysis and tracer study. In terrestrial animal, the interconversion between arginine, glutamine, and presence of endogenous arginine synthetic activity, was already demonstrated through observation of changes in plasma amino acid (Castillo et al. 1994; Castillo et al. 1995; Mateo et al. 2007; Deutz 2008). However in fish, due to limited study in fish arginine metabolism, association between glutamine and arginine pathways has only been found in the form of slightly reduce dietary arginine requirement of arginine in channel catfish when glutamine is supplemented in the feed (Buentello and Gatlin III 2000) which indicated by increased feed efficiency and higher plasma arginine production.

1.4. Arginine as substrate for nitric oxide synthase and arginase in macrophage

In the case of pharmacological benefit of arginine in fishes, it has been shown that arginine can: improved survival of channel catfish, *Ictalurus punctatus*, after exposure to *Edwardsiealla ictaluri* (Buentello and Gatlin III 2001a); improved phagocytosis and humoral defense response of Jian carp, *Cyprinus carpio* var. Jian (Chen et al. 2015); improved respiratory burst and nitric oxide production of Senegalese sole, *Solea senegalensis* (Costas et al. 2011); enhanced antioxidant capacity (Wang et al. 2015b) and increased tolerance to copper toxicity in grass carp, *Ctenopharyngodon idella* (Wang et al. 2015a); improved survival after *Aeromonas hydrophila* infection in yellow catfish, *Pelteobagrus fulvidraco* (Zhou et al. 2015); improvement of several immune parameters of hybrid stripe bass, *Morone chrysops* × *Morone saxatilis* (Cheng et al. 2012b); improved intestinal development of red drum, *Sciaenops ocellatus* (Cheng et al. 2011); and improved production of polyamines during inflammatory response of Atlantic salmon, *Salmo salar*, immune cells (Holen et al. 2014; Andersen et al.

2014). However, these aforementioned findings did not specifically explain the role of arginine supplementation in production of nitric oxide in macrophage.

The effect of arginine metabolism on macrophage has gain some interest recently (Rath et al. 2015). Since macrophage did not have required enzymes for endogenous arginine synthesis, transport of arginine from extracellular source to macrophage is conducted through amino acid transporter: cationic amino acid-acid transporter (CAT1 and CAT2) which is upregulated by lipopolysaccharide (Aktan 2004; Qualls et al. 2012).

Macrophage plays an important function in immune response again microbial pathogen or parasite through classically activated macrophage by production of reactive oxygen species and nitric oxide or through alternatively activated macrophage by increase production of phagocytic activity respectively (Joerink et al. 2006; Wiegertjes et al. 2016).

Nitric oxide that was produced in classically activated macrophage is considered as toxin that works again intracellular pathogen in fish. Production of nitric oxide is mainly through nitric oxide synthase pathway using arginine as it substrate thus arginine is considered as rate limiting in the activation of this macrophage (Gogoi et al. 2016).

Aside from used in NO production, arginine can be utilized by arginase in the macrophage. Utilization of arginine by arginase in the macrophage will produce ornithine and urea. Since arginase and nitric oxide synthase compete on the same substrate, arginase was used for regulate NO production and counterbalancing the classically activated macrophage (Wiegertjes et al. 2016).

CHAPTER 2

II. Effects of arginine supplementation on growth performance and plasma amino acid dynamics of rainbow trout, Oncorhynchus mykiss

2.1. Introduction

Arginine is an amino acid, which is known to have functional roles and is considered as a potential supplement candidate. It plays an important role in metabolism through production of multiple metabolites and is involved in wide variety of physiological phenomena in animals. Several studies have shown effects of arginine on growth performance, immunity, and nutrient metabolism of fish (Andersen, Waagbø, & Espe, 2015). Although the positive effect of arginine supplementation on growth performance has been already demonstrated in terrestrial animals, confounding results are seen in fishes. Arginine supplementation improved growth performance of coho salmon (Oncorhynchus kisutch), chinook salmon (Oncorhynchus tshawytscha), and rainbow trout (Cho, Kaushik, & Woodward, 1992; Fournier et al. 2003; Plisetskaya, Buchelli-Narvaez, Hardy, & Dickhoff, 1991). In addition, it was suggested that improvement of growth of European seabass (Dicentrarchus labrax) was more evident when a high percentage of plant-based ingredients were used in diet (Tulli, Vachot, Tibaldi, Fournier, & Kaushik, 2007). In contrast, there was no difference in growth in gilthead seabream (Sparus aurata) fed with a diet supplemented with graded levels of arginine (Coutinho et al. 2016; Oliva-Teles, Peres, & Kaushik 2017). Moreover, Fournier et al. (2003) found that dietary arginine supplementation did not affect nitrogen gain of rainbow trout and increased nitrogen excretion of rainbow trout and turbot.

In the terrestrial animal, arginine can be produced from various of non-essential amino acids, such as glutamine, glutamate, or proline, into arginine (G. Wu, 1997; G Wu et al., 2009). However, in the case of teleost fishes, limited endogenous arginine synthesis are shown due to low activity of carbamoyl phosphate synthase III, which consequently impede conversion of

ornithine into citrulline in ornithine-urea cycle. (Chiu, Austic, Rumsey, & Rumsey, 1986; Korte et al., 1997). Moreover, unlike in terrestrial animal, pathway that converts proline into arginine through pyrroline-5-carboxylic acid in fish has not been establish yet, thus the possibility of producing arginine from proline has not been evaluated yet. While most of studies in teleost fish agree that fish has limited CPS production, research conducted by Buentello & Gatlin III (2001) on channel catfish showed that administration of gabaculine, an effective ornithine aminotransferase inhibitor, reduced plasma citrulline and arginine level. They also suggested that endogenous citrulline synthesis occurred in channel catfish. Furthermore, given that previous research on rainbow trout show a positive correlation between citrulline supplementation and plasma arginine production (Chiu, Austic, Rumsey, & Rumsey, 1986) and most of studies in terrestrial animal suggest that the citrulline in the plasma circulation would be mainly taken up by kidney and converted into plasma arginine (Collins et al., 2007; Curis et al., 2005; Cynober, Moinard, & De Bandt, 2010; Deutz, 2008; Moinard & Cynober, 2007; Osowska, Moinard, Neveux, Loï, & Cynober, 2004; Windmueller & Spaeth, 1981). We feel that observing changes in plasma citrulline level can be used to observe endogenous arginine synthesis.

Along with excessive production of nitrogen waste, there is also concern that supplementation with dietary arginine can increase dietary arginine requirement due to lower arginine production from endogenous arginine synthesis. Previously, Ball et al. (2007) introduce the concept of partition in arginine metabolism and endogenous arginine synthesis (figure 2.1.1.). They stated that production of arginine through endogenous arginine synthesis can fluctuated and the gap between maximum endogenous arginine synthesis and arginine requirement for metabolism is fulfilled by arginine from dietary source. The fluctuation on endogenous arginine synthesis on fish has already been demonstrated by Buentello and Gatlin (2000) who found that at suboptimal level of arginine, plasma arginine and citrulline are

increase when glutamic acid is supplemented in the diets compared to treatments that are supplemented with glycine. While supplementation with glutamic acid can increase arginine that produce through endogenous arginine synthesis, it is not clear whether dietary supplementation with arginine can reduce arginine production from this arginine biosynthesis. Furthermore, it is not clear whether minimizing/maximizing arginine source from endogenous arginine synthesis will be beneficial in the terms of growth performance especially when CPS activity in fish is less active compared to human and other terrestrial mammals.

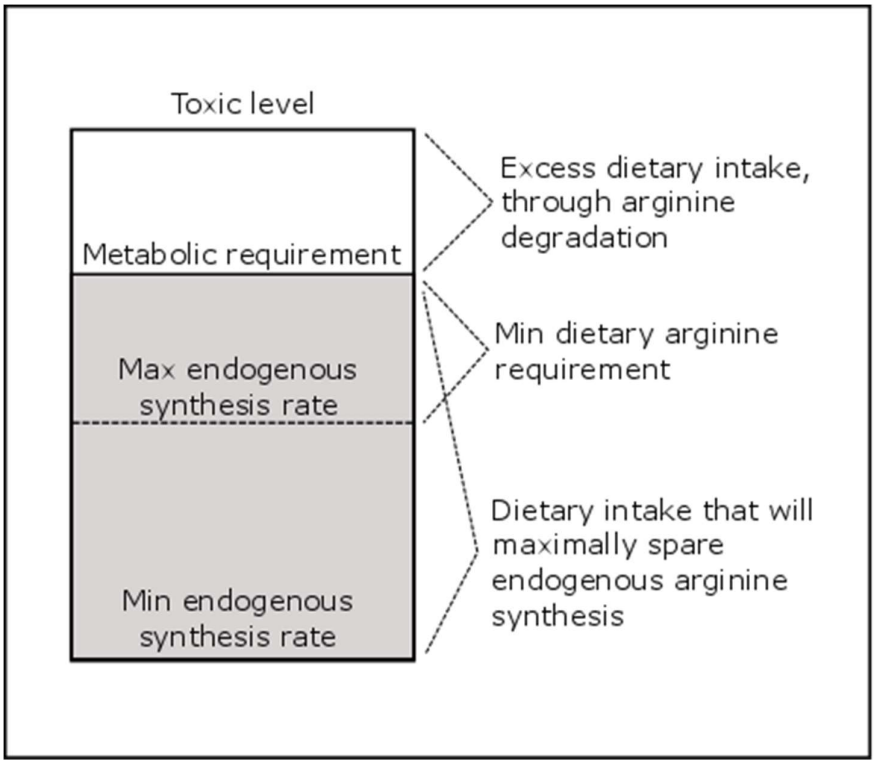

Figure 2.1.1 Partitioning on arginine metabolism and endogenous arginine synthesis. taken from ball et al. (2007)

Production of plasma ornithine from arginine through arginase is also known to have beneficial effect. Study in mice shows that increase of plasma ornithine through oral ornithine supplementation of wild and inducible nitric oxide knock out type mice increase their wound healing properties (Shi et al., 2002). Ornithine is also known as polyamine precursor.

Polyamine is known to have important role in cell growth and protein synthesis (Cynober, 1994). While in the terrestrial animal, liver is known as the organ with highest activity of arginase (Guoyao Wu & Morris, 1998), expression of non-hepatic arginase/ arginase II was also found in other organ such as intestine and kidney (Ozaki et al., 1999). In the intestine, it is suggested by Cynober (1994) that arginase II has function to remove excess arginine that comes from dietary source. However, in the case of fish, the protective properties of arginase in the intestine against excessive dietary arginine level has not been evaluated yet.

Previous studies have already reported arginine catabolites, such as ornithine and citrulline in plasma of fish fed with arginine-supplemented diets (Buentello & Gatlin III, 2001b; Chiu et al., 1986; Fournier et al., 2003; Gouillou-Coustans et al., 2002; Pohlenz, Buentello, Helland, & Gatlin, 2014; Riley, Higgs, Dosanjh, & Eales, 1996). However, these studies examined plasma amino acid with only single post prandial observation. The use of single observation to evaluate effect of dietary arginine provides very limited scope on temporal changes of plasma amino acid since peak level of amino acids is affected by type of amino acid (non-essential or essential) (Schuhmacher, Wax, & Gropp, 1997; Yamada, Simpson, Tanaka, & Katayama, 1981), as well as different type of protein sources (Ambardekar, Reigh, & Williams, 2009; Larsen, Dalsgaard, & Pedersen, 2012; Ogata, 1986; Xu et al., 2016; Yamamoto, Sugita, & Furuita, 2005; Yamamoto, Unuma, & Akiyama, 1998). Previous study that evaluate plasma amino acid on multiple time observation post-feeding (postprandial) can be found in Park et al. (2005) who evaluate the effect of plasma arginine in rainbow trout that fed with deficient arginine dietary level. However, their study mainly observes plasma amino acid on basal diet thus the effect of different dietary arginine supplementation on plasma arginine catabolites was not shown in their result. Careful control of arginine level in diet is necessary for maximizing the utilization of arginine supplementation on various physiological function while minimizing the negative impact on the environment. Thus, this study was aimed

to re-evaluate the effect of excessive arginine supplementation on growth performance, plasma urea production of rainbow trout; assess absorption of excessive dietary arginine supplementation on arginine level in blood circulation; and evaluate plasma ornithine production and citrulline availability in the plasma at 0, 6, 12, and 18 hour-postprandial. This study also aimed to observe expression of arginase Ⅱ (ARG 2), inducible nitric oxide synthase (iNOS), and heat shock protein 70 (HSP70) in the intestine after fed with excessive level of dietary arginine.

2.2. Materials & Methods

The basal diet was formulated using fishmeal, soybean meal, and corn gluten meal, with fish oil (Table 2.2.1). Mineral and vitamin mix was added to the basal diet to fulfill all mineral and vitamin requirements (Table 2.2.1). For the basal diet, arginine and other essential amino acids were formulated to meet requirements to support optimal growth (Kim, Kayes, & Amundson, 1992; Walton, Cowey, Coloso, & Adron, 1986). Essential amino acids in the basal diet were mostly provided from intact proteins by formulating the protein level at 50% dry weight based of the diet. Minimum amount of amino acid mixture was added to fulfill requirements for methionine, histidine, isoleucine, lysine, threonine, and valine (Table 2.2.1). To evaluate the effects of different arginine concentrations, crystalline L-arginine was supplemented into the basal diet at 0, 2.0, and 4.0% to provide a dry-weight base of 1.47 (which is close to arginine requirement for rainbow trout based on Kim et al., (1992)) 3.89; and 5.64 % of arginine, respectively (Table 2.2.2). The treatments were named CTRL, 3.89A, and 5.64A, respectively. Amino acid nitrogen was maintained equal in diets by replacing arginine with aspartic acid and glycine mixture (50:50) (Table 2.2.1). The experimental diet was then made by milling all the ingredients using Retsch Ultra Centrifugal Mill ZM 200 (Haan, Germany), mixing them homogeneously, shaping the dough into pellets using a pellet mill (AEZ12 M,

Hiraga Seishakusho, Kobe, Japan), and freeze-drying the pellets for 16 hours in a freeze dryer (RLE-II, Kyowa Vacuum, Saitama, Japan, Tables 2.2.1 and 2.2.2).

Table 2.2.1 Formulation of the experimental diets of rainbow trout fed with different supplementary level of l-arginine

Ingredients (crude protein (%) / ether extract (%))	**CTRL (g/100 g DW)**	**3.89A (g/100 g DW)**	**5.64A (g/100 g DW)**
Blue whiting fish meal (74.6/9.5)	20.0	20.0	20.0
Soybean meal (50.8/2.3)	14.1	14.1	14.1
Feather meal (90.8/4.8)	2.0	2.0	2.0
Blood meal (95.2/0.7)	2.0	2.0	2.0
Corn gluten meal (65.1/5.8)	20.7	20.7	20.7
Flour (17.1/4.5)	15.0	15.0	15.0
Fish Oil	12.6	12.6	12.6
Vitamin pre-mix[a]	3.0	3.0	3.0
Mineral pre-mix[b]	1.0	1.0	1.0
Monocalcium phosphate	0.5	0.5	0.5
Choline Cl (100%)	0.5	0.5	0.5
Vitamin E (50%)	0.1	0.1	0.1
Amino Acid pre-mix[c]	4.4	4.4	4.4
L-arginine[1]	0.0	2.0	4.0
Aspartic acid/Glycine pre-mix[d]	4.0	2.0	0.0
Cellulose	0.1	0.1	0.1

[a] Vitamin pre-mixture (amounts in kg−1): ascorbic acid, 368.902 g; biotin, 0.363 g; calcium pantothenate, 6.05 g; cyanocobalamin, 0.006 g; folic acid, 0.908 g; inositol, 121 g; niacin, 24.2 g; p-aminobenzoic acid, 3.025 g; pyridoxine hydrochloride, 2.42 g; riboflavin, 3.63 g; thiamin hydrochloride, 3.025 g; vitamin A acetate, 2,420,000 IU; vitamin D3, 2,420,000 IU; vitamin K3, 6.05 g

[b] based on Hernández et al. (2012)

[c] Amino acid pre-mixture (amount in g/100g): L-methionine[1], 3.6; L-histidine[1], 7.4; L-isoleucine[1], 23.9; L-lysine hydrochloride[1], 23.3; L-threonine[1], 13.2; L-valine[1], 28.7

[d] Aspartic acid:glycine; 50:50 used to make diet isonitrogenous

[1] Wako Pure Chemical Industry Inc., Osaka, Japan

Table 2.2.2 Proximate and total amino acid composition of the experimental diets of rainbow trout fed with different supplementary level of l-arginine *

	CTRL (g/100 g DW)	3.89A (g/100 g DW)	5.64A (g/100 g DW)
Proximate composition (% Dry matter)			
Crude protein	50.3	52.8	54.9
Crude lipid	17.8	17.3	16.7
Ash	5.9	5.8	6.1
Essential amino acid (% of diet)			
Threonine	1.81	1.69	1.50
Tryptophan	0.15	0.17	0.14
Valine	2.85	2.80	2.61
Methionine	0.69	0.66	0.60
Isoleucine	1.89	1.88	1.78
Leucine	3.25	2.85	2.78
Phenylalanine	1.68	1.50	1.54
Histidine	0.89	0.84	0.73
Lysine	2.49	2.43	2.10
Arginine	1.47	3.89	5.64

*Values are means of duplicate analysis

Rainbow trout fingerlings were acquired from Oizumi Station, Field Science Center, Tokyo University of Marine Science and Technology, Yamanashi, Japan. The care and use of fish in this research was in accordance with the handling regulation guideline of animal experiments in Tokyo University of Marine Science and Technology. Fish were stocked into nine conical glass tanks, three replications per treatment, with a volume of 60 L, at a stocking density of 20 fish per tank. Average initial weight of the fish was recorded around 60.57-65.04 g. The tanks were placed in a recirculating system and equipped with a thermostat to maintain water temperature. During nine weeks of the feeding period, average water temperature was 14.3 °C. Ammonia and nitrite were checked periodically using colorimetric test kit (Pack Test, Kyoritsu Chemical-check Lab Co., Tokyo, Japan) and confirmed to be within optimal range for rearing rainbow trout. To make sure oxygen was sufficient for the fish, aeration was provided into each tanks until saturation. Fish were fed six days per week, twice a day, in the

morning and afternoon. Feeding was conducted manually to apparent satiation, which was indicated by refusal or the presence of uneaten feed on the bottom of the tank.

After the nine weeks of the feeding trial, survival and final weight of the fish were recorded for growth and feed performance analyses. Before collecting tissue samples for biochemical analysis, fish were starved for two days in order to evacuate remaining diet in gastrointestinal tract. Three fish per tank were euthanized and dissected; dorsal muscle from each fish was collected for amino acid, protein, and lipid analysis. All remaining fish were then pooled per their treatment, and reared for another two weeks using similar feeding treatment and feeding regime for postprandial amino acid study.

In the postprandial amino acid study, fish was first fasted for two days to eliminate effect of previous feeding. After two days, three fish were taken from each treatment for blood collection at basal (0 hour) and the rest of the fish were fed according to their designated dietary treatment. After feeding, blood was taken from the caudal vein using a heparinized syringe at 6, 12, and 18 hour-postprandial while liver was only collected at 18 hour-postprandial for hepatic free amino acid analysis. Furthermore, intestine was also collected for gene expression analysis. Blood was taken from three fish per treatment per postprandial observation time. After blood collection, plasmas were separated by centrifuging at 1,000 × g at 4 °C for 15 minutes. Sulfosalicylic acid solution (3%) was added to the plasma and centrifuged at 1,000 × g for 15 minutes. The supernatant was then analyzed for amino acids and urea analysis using JLC-500/V AminoTacTM (JEOL Ltd, Tokyo, Japan).

Total amino acid analysis was conducted by first digesting the freeze-dried muscle sample with 4 M. methanesulfonic acid solution in a vacuum tube using digester at 110O C for 22 hours, neutralize the digested solution sample with 3.5 N NaOH, adjust the volume of sample of the sample until 10 ml by adding distilled water, filter the solution using a syringe filter with pore size of 0.45 μm and analyze using JLC-500/V AminoTacTM (JEOL Ltd, Tokyo,

Japan). For free amino acid analysis, liver samples were homogenized while adding 2% (w/v) sulfosalicylic acid; the homogenized mixture was then centrifuged at 3,000 × g and 4 °C, for 15 minutes. The supernatant was then filtered using a 0.45 μm syringe filter and analyzed using JLC-500/V AminoTacTM (JEOL Ltd, Tokyo, Japan).

RNA extraction was conducted using Trizol reagent (Invitrogen) according to manufacturer protocol. Before making cDNA, RNA quality was also measured using Nanodrop lite (Thermo Scientific) and treated with DNase using RQ1 RNase-Free DNase kit (Promega) to avoid false positive due to DNA contamination. Reverse transcription of total RNA to cDNA was conducted using High-capacity cDNA reverse transcription kits (Applied Biosystems). Primer design was conducted according to instruction manual for Thunderbird™ SYBR® qPCR mix (Toyobo, Tokyo, Japan). A list of primers that used for quantitative reverse-transcription polymerase chain reaction was shown in Table 2.2.3. qRT-PCR analysis was conducted using Thunderbird™ SYBR® qPCR mix on StepOne Plus real-time polymerase chain reaction system (Applied Biosystems) using β-actin gene as normalizer and ddCt method for data analysis.

Table 2.2.3 Primer of gene that used in this experiment

Gene name	**Forward**	**Reverse**	**NCBI refseq**
β-actin	ctcagtctcattggcatggc	gctgtttcaccgttccagtt	NM_001124235.1
Arginase 2	acacactaccgtcatgctgg	cgtcgacccagatcagacac	BK001400.1
iNOS	tggagctatcgtcagaccgg	tcacattgtctgccacctgtt	NM_001124359.1
HSP70	ccggggttgacatcagtacc	atggtgaaggtggtaaggcg	NM_001124347.2

Weight gain, feeding efficiency, protein and lipid content of muscle, and plasma urea content were subjected to analysis of variance (ANOVA) at $P \leq 0.05$ while all in the case of gene expression, Kruskal-wallis analysis was used at $P \leq 0.05$. To evaluate dynamics of plasma and hepatic-free arginine, ornithine, and citrulline levels, two-way ANOVA was employed for detecting statistical significance ($P \leq 0.05$). All statistical analyses were conducted using R

(version 3.3.0, The R Foundation for Statistical Computing, Vienna; Austria), and upon $P \leq 0.05$, a post-hoc test was conducted using Tukey's multiple range test.

2.3. Result

After nine weeks of the feeding trial, there were no significant differences found in growth, feeding efficiency, and survival among all groups. Significantly lower protein efficiency ratio was recorded in 5.64A than the control (Table 2.3.1). In the muscle, significantly higher protein content was found in 3.89A compared to CTRL, while there was no significant difference in lipid content among the groups (Table 2.3.1). There was no significant difference in essential and non-essential amino acid contents in muscle of rainbow trout among all groups (Tables 2.3.2 and 2.3.3). Regarding total essential amino acid in the muscle, the highest value was found in the 5.89A treatment, while the lowest was found in the CTRL treatment (Tables 2.3.2 and 2.3.3). On the other hand, the highest total non-essential amino acid was found in the 3.89A group, while the lowest was found in the CTRL treatment (Table 2.3.2 and 2.3.3). In the case of muscle protein, lipid, and amino acids on wet bases, there are no significant difference found among CTRL, 3.89A, and 5.64A.

Table 2.3.1 Growth performance, feed intake, feed efficiency, and protein and lipid contents of rainbow trout fed diets supplemented with arginine*

	CTRL	3.89A	5.64A	Pooled SEM[1]	Pr (> F)[2]
Initial weight (g)	63.8	64.4	63.4	0.5	0.788
Final weight (g)	149.9	160.3	152	1.0	0.428
Weight gain (% of initial weight)	134.9	149	140.2	5.0	0.609
Feed intake (g/fish)	78.01	83.9	80.0	2.1	0.571
Feeding Efficiency (% weight gain/feed intake)	92.2	88.9	93.4	1.65	0.541
Protein Efficiency Ratio (weight gain/protein intake)	2.16^{a}	2.12^{ab}	1.89^{b}	0.05	0.043
Survival (%)	98.3	96.7	91.6	1.2	0.068
Muscle's protein content (% dry weight/% wet basis)	81.6^{a}/18.7	89.2^{b}/20.2	88.1^{ab}/19.5	1.3/0.29	0.03/0.08
Muscle's lipid content (% dry weight/% wet basis)	9.1/2.2	10.1/2.3	10.8/2.4	0.83/0.19	0.805/0.762

*Values are means of three replicates, values with different superscript letters are statistically significantly different.
[1] Pooled standard error of the mean
[2] Probability associated with F statistic

For plasma arginine (Figure 2.3.1a), significant differences were found in dietary treatment and postprandial observation time ($P < 0.0001$ and $P = 0.0001$, respectively). By comparing the CTRL treatment with arginine-supplemented groups within postprandial observation time (Figure 2.3.1a), a significant difference was found in 3.89A and 5.64A at 6 and 12 hour-postprandial, while at 18 hour-postprandial, higher plasma arginine content was only found in 5.64A. Furthermore, there was no significant difference in arginine content in the CTRL group for all postprandial times examined. In the 3.89A group, a higher plasma arginine level was found at 6 and 12 hour-postprandial. While in the 5.64A group, plasma arginine levels at 6, 12, and 18 hour-postprandial were higher than the basal level (0 hour-postprandial).

Table 2.3.2. Essential amino acid content of muscle of rainbow trout fed diets supplemented with arginine*

Dietary treatment	**Arg dwb (wwb)** [1]	**His dwb (wwb)** [1]	**Ile dwb (wwb)** [1]	**Leu dwb (wwb)** [1]	**Lys dwb (wwb)** [1]	**Met dwb (wwb)** [1]	**Phe dwb (wwb)** [1]	**Thr dwb (wwb)** [1]	**Trp dwb (wwb)** [1]	**Val dwb (wwb)** [1]	**Total EAA dwb (wwb)** [1]
CTRL	3.1 (0.71)	1.3 (0.30)	1.7 (0.37)	4.6 (1.05)	5.1 (1.17)	1.8 (0.42)	2.9 (0.65)	2.8 (0.64)	0.54 (0.12)	2.1 (0.49)	26.1 (6.0)
3.89A	3.3 (0.81)	1.2 (0.29)	1.7 (0.39)	4.5 (1.09)	5.3 (1.26)	1.9 (0.44)	2.8 (0.68)	2.9 (0.70)	0.50 (0.12)	2.8 (0.68)	28.7 (6.5)
5.64A	3.7 (0.80)	1.4 (0.31)	1.8 (0.39)	5.0 (1.10)	5.8 (1.29)	2.0 (0.45)	3.1 (0.68)	3.0 (0.67)	0.46 (0.10)	2.7 (0.61)	29.0 (6.4)
Pooled SEM[2]	0.12 (0.02)	0.05 (0.03)	0.07 (0.13)	0.13 (0.03)	0.15 (0.03)	0.05 (0.01)	0.08 (0.02)	0.09 (0.02)	0.03 (0.01)	0.20 (0.04)	0.83 (0.15)
Pr (> F)[3]	0.145 (0.089)	0.701 (0.837)	0.855 (0.826)	0.668 (0.826)	0.187 (0.151)	0.302 (0.460)	0.583 (0.689)	0.458 (0.396)	0.575 (0.401)	0.259 (0.231)	0.367 (0.333)

*Means of three replicates
[1] dwb= dry weight base; wwb= wet weight base
[2] Pooled standard error of the mean
[3] Probability associated with F statistic
Amino acid abbreviation: Arg (arginine), His (histidine), Ile (isoleucine), Leu (leucine), Lys (lysine), Met (methionine), Phe (phenylalanine), Thr (threonine), Trp (tryptophan), Val (valine), EAA (essential amino acid)

Table 2.3.3. Non-essential amino acid content of muscle of rainbow trout fed diets supplemented arginine*

Dietary treatment	Ala dwb (wwb) [1]	Asp dwb (wwb) [1]	Cysta dwb (wwb) [1]	Cys dwb (wwb) [1]	Glu dwb (wwb) [1]	Gly dwb (wwb) [1]	Pro dwb (wwb) [1]	Ser dwb (wwb) [1]	Tau dwb (wwb) [1]	Tyr dwb (wwb) [1]	Total NEAA dwb (wwb)[1]
CTRL	4.5 (1.0)	6.9 (1.6)	0.09 (0.02)	0.008 (0.002)	11.5 (2.6)	3.6 (0.8)	2.0 (0.4)	2.9 (0.7)	0.11 (0.03)	1.9 (0.4)	26.6 (6.1)
3.89A	4.1 (1.0)	6.8 (1.6)	0.09 (0.02)	0.005 (0.002)	12.5 (3.1)	3.6 (0.8)	2.3 (0.6)	3.1 (0.8)	0.12 (0.04)	2.0 (0.5)	29.5 (6.7)
5.64A	4.5 (1.0)	7.5 (1.7)	0.06 (0.01)	0.009 (0.002)	13.2 (2.9)	3.3 (0.7)	2.4 (0.5)	3.2 (0.7)	0.31 (0.07)	2.2 (0.5)	28.9 (6.4)
Pooled SEM[2]	0.1 (0.02)	0.19 (0.04)	0.01 (0.002)	0.002 (0.0004)	0.47 (0.09)	0.09 (0.02)	0.09 (0.02)	0.11 (0.02)	0.05 (0.01)	0.07 (0.01)	0.78 (0.14)
Pr (> F)[3]	0.634 (0.503)	0.541 (0.687)	0.920 (0.591)	0.602 (0.946)	0.561 (0.138)	0.223 (0.156)	0.186 (0.168)	0.262 (0.153)	0.362 (0.379)	0.308 (0.242)	0.367 (0.228)

*Means of three replicates
[1] dwb= dry weight base; wwb= wet weight base
[2] Pooled standard error of the mean
[3] Probability associated with F statistic
Amino acid abbreviation: Ala (alanine), Asp (aspartate), Cysta (cystamine), Cys (cystine), Glu (glutamine), Gly (glycine), Pro (proline), Ser (serine), Tau (taurine), Tyr (tyrosine), NEAA (non-essential amino acid)

For plasma ornithine, significant differences were found in dietary treatment and postprandial observation time ($P < 0.0001$ and $P = 0.0274$, respectively). Higher plasma ornithine was only found in 5.64A at 12 and 18 hour-postprandial, while there was no increase in plasma ornithine between basal and 6 to 18 hour-postprandial in CTRL and 3.89A (Figure 2.3.1b).

For plasma citrulline, significant differences were found in postprandial observation time and in the interaction between dietary treatment and time ($P = 0.0004$ and $P = 0.0358$, respectively). By comparing the data within postprandial time, citrulline level in 5.64A was significantly lower than CTRL at 18 hour-postprandial. Moreover, by comparing group-based postprandial time within dietary treatment, higher plasma citrulline was found at 18 hour-postprandial in the CTRL group, and at 6 hour-postprandial in the 5.64A treatment (Figure 2.3.1c).

There is no significant difference found in terms of plasma lysine among all treatments in all hour-postprandial observation time. The highest level of plasma lysine was found in the 5.64A treatment at 18 hour-postprandial while the lowest level of plasma lysine was found in 3.89A at 12 hour-postprandial (Figure 2.3.1d).

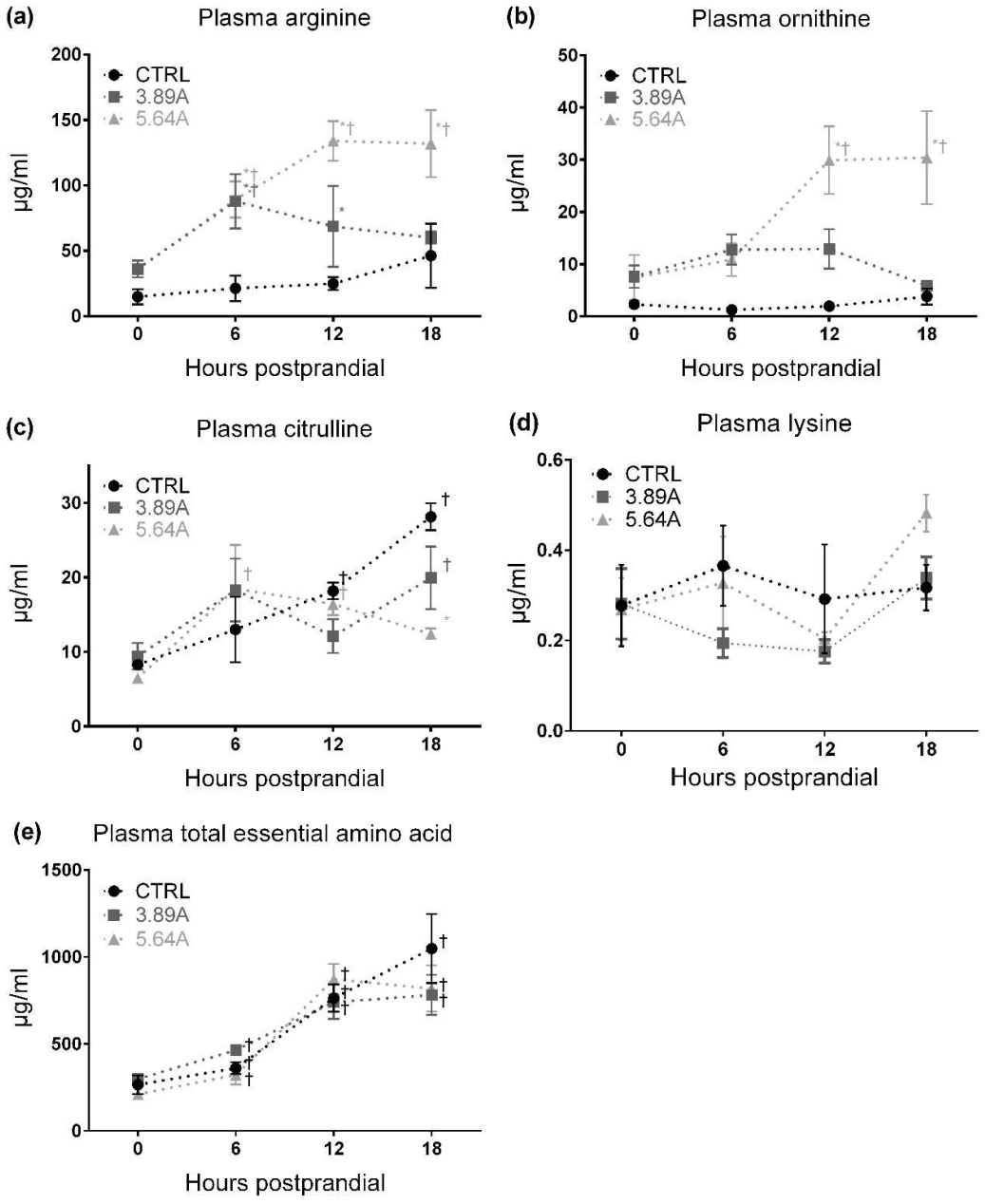

Figure 2.3.1. Plasma arginine (a), plasma ornithine (b), plasma citrulline (c), plasma lysine (d), and plasma of total essential amino acid (e) at 0, 6, 12, and 18 hours-postprandial.

Symbol "†" represents significant difference compare to basal state (0 hours) within dietary treatment at $P \leq 0.05$, while "*" represents significant difference compared to the CTRL treatment within postprandial observation time at $P \leq 0.05$.

Compared to the basal condition, all treatments showed an increase in plasma essential amino acids at 6 hour-postprandial. However, at each postprandial time, there was no significant difference between CTRL and arginine-supplemented groups (Figure 2.3.1e).

At 0 hour-postprandial, plasma urea in all treatments was not detected. In the CTRL, plasma urea was still not detected at 6, 12, and 18 hours. Moreover, while plasma urea was detected in arginine-supplemented groups, there was no significant difference these groups (Figure 2.3.2).

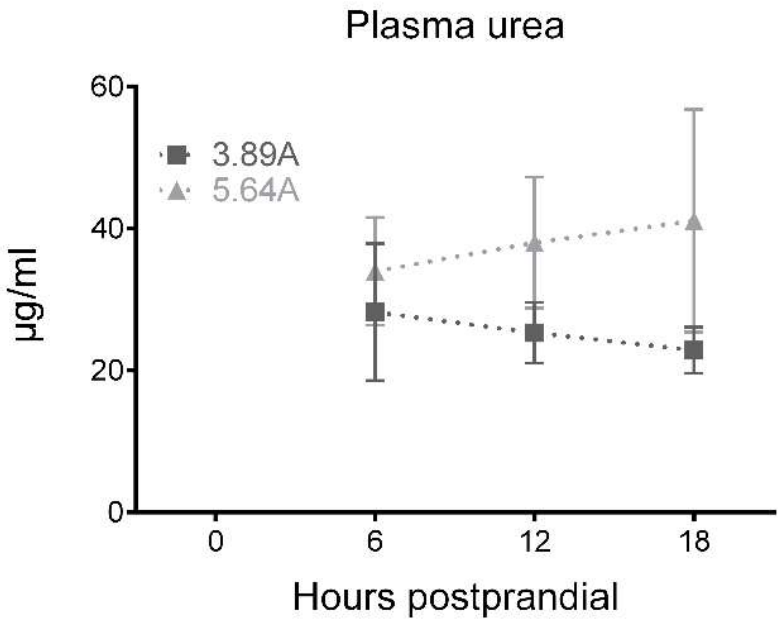

Figure 2.3.2 Plasma urea of rainbow trout fed with different levels of arginine, per postprandial time. Plasma urea in all treatments was not detected at 0 hours, while in CTRL, plasma urea was also not detected at 6, 12, and 18 hours-postprandial. There was no significant difference between 3.89A and 5.64A at 6, 12, and 18 hours-postprandial at a 95% confidence level.

In the 5.64A treatment, hepatic free amino acid was shown a significantly higher arginine concentration than that in the CTRL treatment at 0 hours. However, at 18 hour-postprandial, no significant difference was found among the treatments (Figure 2.3.3)**.** In the case of hepatic free ornithine, at 18 hour-postprandial a significant difference was found among treatments, with 5.64A showing a higher concentration than CTRL (Figure 2.3.3). There was no difference in hepatic free ornithine levels found at 0 hour-postprandial. In the case of citrulline, a significant difference among treatments was found only at 0 hour-postprandial, with the CTRL treatment showing a significantly higher hepatic free citrulline level than that in 3.89A and 5.64A (Figure2.3.3). However, at 18 hour-postprandial, no significant difference was found among the treatment.

For gene expression of intestinal iNOS, no significant difference was found between all treatment. While in the case of intestinal Arg 2 and HSP70, expression of these genes in arginine supplemented groups were significantly higher compared to CTRL (Figure 2.3.4).

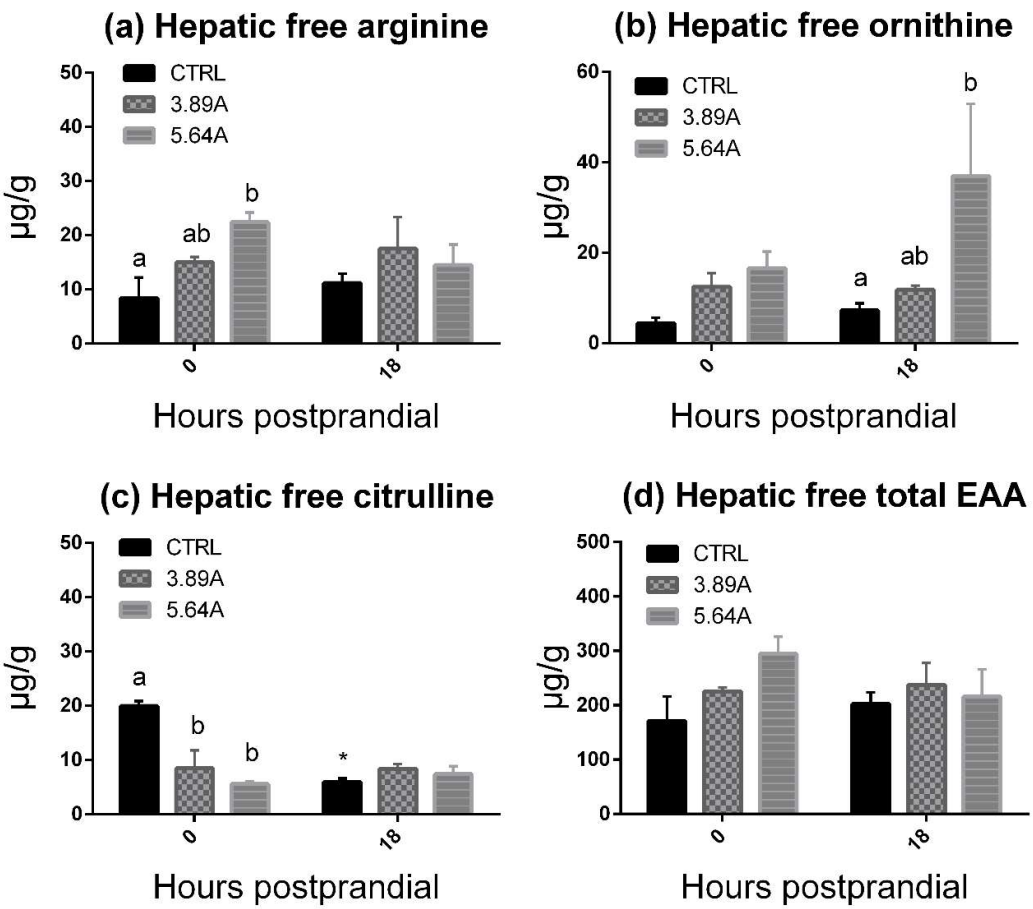

Figure 2.3.3. Hepatic free arginine (a), ornithine (b), citrulline (c), and total essential amino acid (EAA) (d) of rainbow trout fed with different levels of arginine supplementation. Letter above bar represent significant difference within same postprandial hours while asterisk represent significant difference compared to 0 hours postprandial.

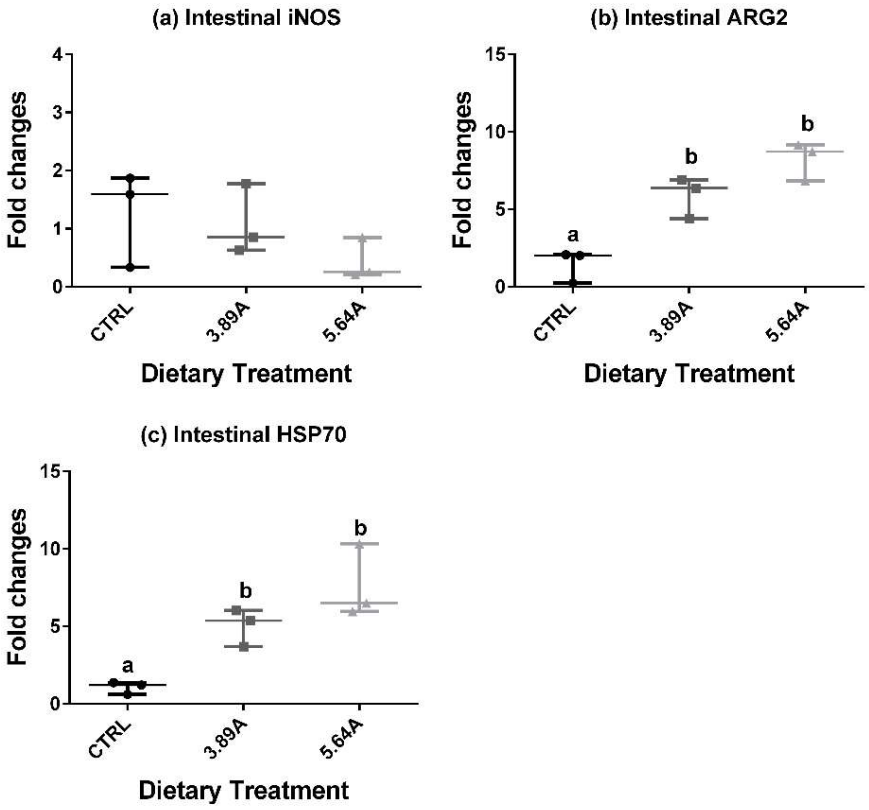

Figure 2.3.4. Quantitative PCR analysis of intestinal inducible nitric oxide synthase (a) intestinal arginase II (b) and intestinal heat shock protein 70 (c) after 6 hours postprandial. Long Horizontal bar represents median while vertical bar represents interquartile range. Letter above the upper horizontal bar represent significant difference among treatments.

2.4. Discussion

Dietary arginine supplementation and growth performance and amino acid composition in muscle

Although 3.89A had the highest growth performance and final body weight, it was not statistically different compared to CTRL at a 95% confidence level. In a previous study (Fournier et al., 2003), final body weight of rainbow trout was improved when dietary arginine level increased from 1.72% to 3.09%, but retarded when it increased from 2.51% to 3.61% and from 3.09% to 4.01%. In contrast, there was no negative impact on weight gain and final body weight of rainbow trout when dietary arginine level increased from 1.47% to 5.64% in the present study. It is possible that this the negative impact of excessive arginine could be

enhanced when dietary protein is limited and the contrasting result from both of studies come from different level of crude protein. Our study used diets that met the protein recommendation for rainbow trout (NRC, 2011), therefore the negative effect of excessive arginine appeared to be masked in part by sufficient amounts of dietary protein. Since all essential amino acid value except for arginine in this study were set similar to essential amino acid requirement based on (K. Il Kim, Kayes, & Amundson, 1992; M. J. Walton et al., 1986) it is likely that the high protein content in all the experimental diets was come from non-essential amino acid. Study conducted by Encarnação, de Lange, & Bureau (2006) on rainbow trout demonstrate that adding non-essential amino acid and setting the protein level as high as 65-68% did not affect growth performance if the essential amino acids, especially the limiting amino acid is set at adequate level. However, it is still unknown whether high protein content in the diet can ameliorate the negative effect of excessive dietary arginine since there are limited studies has been conducted on relation between different protein level and dietary arginine supplementation. Thus the notion that high level of protein can ameliorate negative effect of excessive dietary arginine level should be further evaluate. Additionally, similar no difference of growth was also reported in Atlantic salmon and gilthead sea bream fed with a dietary surplus of arginine (Andersen et al., 2015; Coutinho et al., 2016; Oliva-Teles, Peres, & Kaushik, 2017).

In the case of muscle composition, higher protein content was found in 3.89A than CTRL treatment. Several studies in terrestrial animals have shown beneficial effects of arginine supplementation on growth and muscle gain (Jobgen et al., 2009; Yao et al., 2008). In a previous study, elevation of crude protein content in muscle was observed in rainbow trout (Cho, Kaushik, & Woodward, 1992). However, our study failed to observe growth promotion by dietary arginine supplementation. In addition, an increase of total essential amino acid content was not observed in the muscles. Because crude protein content includes non-protein

nitrogen compounds, such as urea, ammonia, and nucleotide, it is more likely that the increase in muscle protein in this study was associated with the increase of non-protein nitrogen compounds. This notion was supported by the presence of plasma urea, possibly come from arginine catabolism, that only appear in arginine-supplemented groups; numerically higher plasma ornithine; and no significant difference in total amino acid content in muscle among treatments (Tables 4 and 5). Similarly, an increase in protein content accompanied with no changes in total essential amino acid profile was observed in cobia fed with diet supplemented with graded levels of arginine (Ren, Ai & Mai, 2014).

Although there are significant differences found in plasma arginine, ornithine, and citrulline of treatments and control, there are no significant difference found in arginine, ornithine, and citrulline content in the muscle. This difference between muscle and plasma might be caused by utilization and conversion of those amino acids so that it will not be incorporated into muscle.

Furthermore, in the terms of effect of excessive arginine supplementation on growth performance, there are phenomenon that called as arginine-lysine antagonism whereas increasing level of dietary lysine in the diet will also increase lysine requirement. This arginine lysine antagonism is first observed in the chicken (Jones, 1964) and also found in other animal such as dog and rat (Ball et al., 2007). In the case of fish, arginine-lysine antagonism is reported to be exist in rainbow trout (Kaushik & Fauconneau, 1984) although only in the of ureagenesis while other study conducted in the channel catfish (Robinson, Wilson, & Poe, 1981) demonstrated that increasing level of dietary arginine did not affect weight gain, feed efficiency, and serum lysine.

The result of this study was similar to study conducted by Robinson et al., (1981). There excessive dietary arginine did not affect growth performance and there is no difference in plasma lysine even when it was observed using multiple hour-postprandial observation. Thus,

there are no visible indication that arginine-lysine antagonism was exist in this study.

Dietary arginine supplementation on postprandial plasma and hepatic free amino acid

The significantly higher plasma arginine in the arginine-supplemented groups was observed in this study. This is consistent with finding made in another study on juvenile rainbow trout (Cho, Kaushik, & Woodward, 1992). In their study, rainbow trout fed dietary arginine content above their minimum requirement level showed higher plasma arginine level at 6 hour-postprandial. Moreover, similar results are also shown in studies conducted on different fish species, such as Atlantic salmon (Berge, Sveier, & Lied, 2002), and Japanese flounder (Paralichthys olivaceus) (Alam, Teshima, Koshio, & Ishikawa, 2002). Interestingly, plasma arginine level of CTRL treatment also shown slower increasing trend compared to arginine supplemented group where noticeable increase was only found between 12-18 hour-postprandial. These different plasma arginine dynamics between the CTRL and the arginine-supplemented groups could be explained by the source of arginine. Arginine was absorbed from dietary arginine in arginine-supplemented groups but synthesized by endogenous synthesis in the CTRL group. Because a remarkable increase in plasma citrulline level was found in the CTRL group at around 12 hour-postprandial, it was possible that an increase in arginine level at 12–18 hour-postprandial in CTRL group occurred by the conversion of citrulline to arginine in. The present study demonstrated that plasma arginine level kept increasing until 12 hour-postprandial when the fishes were fed a high level of dietary arginine (5.64%). Although previous studies measured plasma arginine level at 6 hour-postprandial, it was suggested that measuring plasma arginine levels after 6-12 hour-postprandial is recommended.

There was no significant increase in plasma ornithine in the CTRL and 3.89A treatments, while there was an increase in the 5.64A group at 12 and 18 hour-postprandial. Therefore, it is suggested that more than 5.64% dietary arginine is required to elevate plasma

ornithine in rainbow trout. Plasma ornithine was produced from dietary arginine through arginase enzyme. The presence of this enzyme in rainbow trout has been reported in intestine, kidney, gill, and red muscle (Portugal & Aksnes 1983; Walton, Cowey, Coloso, & Adron 1986; Wright, Campbell,Morgan, 2004). The observation of increased plasma ornithine by arginine supplementation was consistent with the previous studies on salmon (Berge, Lied, & Sveier, 1997).

Although dynamics of plasma arginine and ornithine were positively associated with dietary arginine in this study, plasma citrulline level was not well correlated. In the CTRL treatment, plasma citrulline levels kept increasing until 18 hour-postprandial and reached maximum measured values at 18 hour-postprandial, which was the highest among all the treatments at all postprandial times examined. The increase in plasma citrulline level was observed at 6 and 18 hour-postprandial in the 3.89A group, whereas it reached maximum measured values at 6 hour-postprandial and deceased until 18 hour-postprandial in the 5.64A group. This difference seems to reflect different sources of arginine, i.e., arginine from endogenous production in CTR vs. dietary arginine in 3.89A and 5.64A. Because previous studies showed that plasma citrulline was associated with arginine production (Bahri et al., 2013; Breuillard, Cynober, & Moinard, 2015; Curis et al., 2005; Deutz, 2008; Moinard & Cynober, 2007; Schwedhelm et al., 2008; Windmueller & Spaeth, 1981), it is likely that low plasma citrulline level in the highest dietary arginine-supplemented group suggests little endogenous arginine synthesis in this group. A previous study conducted by Buentello & Gatlin (2000) shows that different dietary levels of arginine did not significantly affect plasma-free citrulline levels in channel catfish. This contrasting result may be explained in several ways; e.g., wider range of dosage in the present study than their study (1.5–5.6% vs. 0–5-2%, respectively) and different feeding preferences and nutritional requirements between species.

The result from postprandial plasma citrulline and plasma arginine also suggested that

monitoring plasma citrulline level can be one of the good indicator of endogenous arginine synthesis of rainbow trout. By maximizing endogenous arginine synthesis, it is possible to minimize dietary arginine requirement and further reduce nitrogen excretion in trout farming sites.

The liver is an organ with high arginase activity. Therefore, the present study examined hepatic free arginine, ornithine, and citrulline levels at 0 and 18 hours after feeding. Arginine uptake by liver and stimulation of arginase activity by arginine intake was demonstrated in humans (van de Poll et al. 2007). It is possible that significant differences in hepatic arginine and citrulline levels observed at 0 hour-postprandial could reflect dietary arginine intake for nine weeks thus 48 hours fasting was not enough to eliminate the effect of previous feeding. In contrast, there was no significant difference in hepatic free arginine at 18 hour-postprandial, suggesting that the effect of dietary arginine on the levels of hepatic free arginine and its metabolites can be observed within 18 hour-postprandial. Accordingly, Walton & Wilson (1986) demonstrated that the highest concentration of hepatic free arginine was found at 48 hour-postprandial in rainbow trout. Difference in the timing of the maximum measured values of hepatic free arginine between the present study and their study could reflect different dietary arginine sources (crystalline vs. protein bound). Significant differences in hepatic free citrulline levels among the treatments were found at 0 hour-postprandial, whereas those were significantly higher in CTRL than in arginine-supplemented groups. It is possible that higher levels of hepatic free citrulline in the CTRL reflect endogenous arginine synthesis.

No difference was observed in hepatic arginine and citrulline levels among groups 18 hour-postprandial. In contrast, a significantly higher ornithine level was found in 5.64A at 18 hour-postprandial than in CTRL. The higher ornithine level in 5.64A appeared to reflect endogenous ornithine synthesis from dietary arginine. However, a significantly higher ornithine level was observed in plasma at 18 hour-postprandial. Ornithine is the precursor of

citrulline in the urea cycle. However, the elevation of ornithine levels in 5.64A was not accompanied with increase in hepatic citrulline levels. The reason why hepatic citrulline level failed to increase with increasing hepatic ornithine level was unclear. However, rainbow trout is known to have a limited capability to produce carbamoyl phosphate synthase (CPS; Chiu et al., 1986; Fournier et al., 2003; Todgham, Anderson, & Wright, 2001), which produces carbamoyl phosphate for citrulline production via ornithine, with the aid by ornithine transcarbamoylase. Relatively lower CPS activity may limit citrulline synthesis from ornithine, eventually resulted in high hepatic ornithine levels without corresponding increases in citrulline levels.

Urea production

Plasma urea is normally generated by amino acid consumption for energy synthesis, gluconeogenesis, and/or liponeogenesis. Plasma urea was not detected in CTRL at all times examined. Total plasma essential amino acid was highest in CTRL at 18 hour-postprandial, implying that the potential for protein synthesis was higher in the CTRL treatment than the arginine-supplemented groups. However, there was no increase in muscle protein content in the CTRL treatment, consistent with an earlier report (Fournier et al. 2003). A significantly higher plasma urea level was reported in rainbow trout fed with a diet supplemented with graded levels of arginine (Fournier et al., 2003). Although we failed to detect plasma urea in CTRL and could not test whether dietary arginine elevation significantly elevated plasma urea level, we successfully detected plasma urea in the arginine-supplemented groups. In contrast, Cho, Kaushik, & Woodward (1992) did not observe any significant increase in plasma urea level in rainbow trout when dietary arginine was increased from 1.75% to 2.75%. However, Fournier et al. (2003) observed increase in plasma urea level at 6 hour-postprandial when rainbow trout was fed 1.72–4.01% dietary arginine. The present study could not detect significant differences in plasma urea levels when the fish were fed with 3.89% and 5.64%

dietary arginine probably because 3.89% is sufficiently excessive for elevating plasma urea from basal levels.

No report was made on plasma urea after 6 hour-postprandial in rainbow trout fed dietary arginine supplementation (Cho, Kaushik, & Woodward, 1992; Fournier et al., 2003). The present study demonstrated that plasma urea level decreased when the fish were fed 3.89% dietary arginine, whereas it tended to increase when fed 5.89% dietary arginine, suggesting that plasma urea level should be monitored even after 6 hour-postprandial.

Gene expressions

Result from expression of ARG 2 in intestine support statement from Cynober (1994) that arginase has protective properties against excessive level of dietary arginine and also confirm presence of arginase in intestine of fish. It is not clear how much contribution of intestinal arginase on production of plasma ornithine in this study since arginase in liver was not measured. Supplementation with dietary arginine also did not increase expression of iNOS. Thus, it is possible that iNOS was tightly regulated and the abundance of substrate was not enough to increase its expression in intestine during observation at 6 hours postprandial.

Additionally, it is interesting that excessive arginine supplementation increased expression of HSP70 gene in this study. HSP70 gene is known to have various role in intestine such as folding and refolding of protein and transport through sub-cellular organelle membranes (Liu, Dicksved, Lundh, & Lindberg, 2014). Study from Wu et al. (2010) in terrestrial animal also found increased HSP70 expression due to arginine supplementation and it was suggested that this gene has an important function in protecting the intestinal mucosa (David, Grongnet, & Lalles, 2002).

In conclusion, there was no significant difference in growth performance and total amino acid of muscle in all treatments. The present study demonstrated that plasma ornithine and

availability of plasma citrulline was affected by dietary arginine intake in rainbow trout and this effect was observed even after 12-18 hour-postprandial in fish fed high dietary arginine intake. Hepatic citrulline level decreased with increasing dietary arginine level after nine weeks of feeding arginine supplemented diet. After 18 hour-postprandial, although no difference was observed in hepatic arginine and citrulline levels, significantly higher hepatic ornithine level was observed with increasing dietary arginine intake in rainbow trout. Excessive supplementation of dietary arginine also increases expression of non-hepatic arginase and HSP70 gene in the intestine.

CHAPTER 3

III. Effect of dietary arginine, ornithine, and citrulline on postprandial plasma amino acids, arginine catabolism and resistance to *Vibrio anguillarium* of rainbow trout *Oncorhynchus mykiss*

3.1. Introduction

Previous the first research had shown that dietary supplementation of arginine did not enhance growth performance of rainbow trout. Furthermore, association between arginine supplementation with plasma ornithine and plasma citrulline was found in the treatments. Ornithine and citrulline is considered as part of endogenous arginine pathways, in interorgan ornithine-urea cycle, ornithine that was synthesize in the intestine can be converted into citrulline and released into blood circulation to be used for arginine synthesis by kidney. Previously, most of effort to increase arginine concentration in plasma pool in fish is mainly conducted by dietary arginine supplementation. Study conducted on ornithine and citrulline supplementation to increase plasma arginine pool through endogenous arginine synthesis are still limited in fish.

First study that conduct ornithine and citrulline supplementation on fish is found on (Chiu et al. 1986) who tried to evaluate urea cycle activity in the rainbow trout and found that production of plasma arginine is possible through supplementation of citrulline. Although this study was already shown the effect of ornithine and citrulline supplementation on growth performance and arginine production, casein based diet was used in dietary formulation which did not give similar characteristic with common commercial feed that was used in aquaculture industry. Furthermore, evaluating effect of ornithine and citrulline supplementation with multiple postprandial observation will give a more complete description on amino acid dynamics of rainbow trout.

Aside from research conducted by (Chiu et al. 1986), there are no other study of ornithine and arginine supplementation on fish. Since several research has already been

conducted on arginine and ornithine supplementation and it is found that this amino acids can be beneficial for increasing growth performance indicator, wound healing, and substrate for nitric oxide production (Cynober 1994; Norris et al. 1995; Shi et al. 2002a; Schwedhelm et al. 2008; Breuillard et al. 2017) it is beneficial to also evaluate supplementation of arginine and ornithine on growth performance while also observe the effect of supplementation on arginine production and survival upon challenge with bacterial pathogen.

3.2. Materials and methods

This study was divided into two experiments. First experiment was conducted to evaluate postprandial plasma amino acid dynamic after fed with fed supplemented with arginine, ornithine, and citrulline, while in the second experiment feeding trial and disease challenge was conducted to evaluate the effect of dietary supplementation on growth and immune performance of rainbow trout. Basal diet was first formulated using intact protein such as fish meal, soybean meal, blood meal, feather meal, corn gluten meal. To fulfill essential amino acid requirements, mixture of crystalline amino acid was added into diet. Wheat flour was used in this basal diet as a source of carbohydrate. To fulfill lipid and fatty acid requirement, fish oil was added into the diet. To emulate supplemental level of ornithine, citrulline, and mixture of ornithine and citrulline, 1 % of l-ornithine and l-citrulline was used. The complete dietary formulation that was used in this experiment was presented in table 3.2.1. Supplemental level and nutrient composition was then confirmed using amino acid and proximate analysis (table 3.2.2 and 3.2.3).

Table 3.2.1. Formulation of experimental diets for rainbow trout fed with dietary supplementation of l-ornithine, l-citrulline, and mixture or l-ornithine and l-citrulline

Ingredients	CTRL (g/100 g DW)	ORN (g/100 g DW)	CIT (g/100 g DW)	ORN-CIT (g/100 g DW)
Fish meal	20.00	20.00	20.00	20.00
Soybean meal	11.98	11.98	11.98	11.98
Feather meal	2.00	2.00	2.00	2.00
Blood meal	2.00	2.00	2.00	2.00
Corn gluten meal	25.00	25.00	25.00	25.00
Flour	15.00	15.00	15.00	15.00
Fish oil	10.41	10.41	10.41	10.41
Vitamin premix	3.00	3.00	3.00	3.00
Mineral premix	1.00	1.00	1.00	1.00
Choline Cl 100%	0.50	0.50	0.50	0.50
Vit E 50%	0.10	0.10	0.10	0.10
Monophosphate	2.00	2.00	2.00	2.00
Celullose	3.76	2.76	2.76	1.76
Amino acid premix	4.25	4.25	4.25	4.25
L-ornithine	0.00	1.00	0.0	1.00
L-citrulline	0.00	0.00	1.00	1.00

[a] Vitamin pre-mixture (amounts in g/kg): ascorbic acid, 368.902 g; biotin, 0.363 g; calcium pantothenate, 6.05 g; cyanocobalamin, 0.006 g; folic acid, 0.908 g; inositol, 121 g; niacin, 24.2 g; p-aminobenzoic acid, 3.025 g; pyridoxine hydrochloride, 2.42 g; riboflavin, 3.63 g; thiamin hydrochloride, 3.025 g; vitamin A acetate, 2,420,000 IU; vitamin D3, 2,420,000 IU; vitamin K3, 6.05 g

[b] based on Hernández et al., (2012)

[c] Amino acid pre-mixture (amount in g/100g): L-methionine[1], 3.6; L-histidine[1], 7.4; L-isoleucine[1], 23.9; L-lysine hydrochloride[1], 23.3; L-threonine[1], 13.2; L-valine[1], 28.7

[1] Wako Pure Chemical Industry Inc., Osaka, Japan

Table 3.2.2. Proximate analysis, ornithine and citrulline content of the treatment diets rainbow trout fed with dietary supplementation of l-ornithine, l-citrulline, and mixture or l-ornithine and l-citrulline

Nutrient (% d.w base)	CTRL	ORN	CIT	ORN-CIT
Protein	48.1	50.4	50.6	51.1
Lipid	16.4	16.7	17.3	16.8
Ash	13.8	15.2	14.1	15.4
Ornithine	0.02	0.66	0.06	0.75
Citrulline	0.05	0.05	0.68	0.70

*Values are means of duplicate analysis

Table 3.2.3. Essential amino acid of the treatment diets rainbow trout fed with dietary supplementation of l-ornithine, l-citrulline, and mixture or l-ornithine and l-citrulline

Essential amino acid (% d.w diet)	CTRL	ORN	CIT	ORN-CIT	**Amino acid requirement
Threonine	1.81	1.66	1.73	1.82	1.20
Valine	2.36	2.25	2.28	2.12	1.12
Methionine	0.83	0.77	0.72	0.75	0.80
Isoleucine	1.81	1.76	1.78	1.70	0.84
Leucine	3.64	3.40	3.23	3.32	1.56
Phenylalanine	1.77	1.68	1.58	1.29	1.12
Histidine	0.96	1.03	0.89	0.86	0.64
Lysine	2.47	2.39	2.37	2.20	1.90
Arginine	1.83	1.76	1.71	1.62	1.41

*Values are means of duplicate analysis
**Based on Kim *et al*. (1992) and Walton *et al*. (1986

This study was divided into several experiments: (1) evaluation of postprandial plasma amino acid upon feeding with ornithine, citrulline and combination of ornithine citrulline. (2) short term growth analysis (3) disease challenge and evaluation of quantitative-real time PCR and plasma amino acid post intraperitoneal injection with *Vibrio anguilarum.*

For the first study, rainbow trout with average body weight of 34.1 gram was fasted for 14 days to eliminate the effect of previous feeding and reared in 60 liter aquaria that was equipped with recirculating system. Before feeding, blood was harvested from 3 fish for plasma amino acid analysis at basal condition. While after feeding until apparent satiation, blood was then collected from 3 fish for each treatment at 7, 15, and 30 hours postprandial. Blood collection was conducted using heparinized syringe at caudal vein.

For the second study, fish were reared in 60 liter aquaria in the same recirculating system. Fish was fed twice daily, six day a week until apparent satiation which indicated by the presence of uneaten feed. Rearing was conducted for 30 days period. After 30 days, weight

of fish and amount of eaten feed per treatment was measured to calculate growth performance and feeding efficiency.

For the third study, 15 fish per treatments that comes from feeing trial in the 2nd experiment was used for disease challenge. Disease challenge was conducted through intraperitoneal injection with *Vibrio anguilarum* diluted with phosphate-buffered saline at 3.0 x 10^6 CFU per fish. Fish was reared in aquaria with small isolated circulating system and temperature control. At 24 hours post-injection, blood was collected from 5 fish per treatment for plasma amino acid while kidney and spleen was collected for RNA extraction. To compare the effect of short-term and long-term feeding, 15 fish per treatment was reared for 15 days. Rearing condition was set to be similar with previous and after 15 days fish was then injected peritoneally with *Vibrio anguilarum* at the same concentration and reared in aquaria with small and isolated circulating system that was equipped with temperature control. At 24 hours post-injection, blood was collected for plasma amino acid analysis, kidney and spleen was also harvested for RNA extraction.

For plasma-amino-acid analysis, plasma was first mixed with 3% sulfosalicylic acid solution (w/v) and then centrifuged at 1000 *g* at 4ºC for 15 minutes after. The supernatant was then filtered using 0.45 µm pore size syringe filter and then diluted with HPLC grade water at 1:5 dilution before analyzed using amino acid analyzer, JLC-500/V AminoTac™ (JEOL Ltd, Tokyo, Japan).

Ribonucleic acid (RNA) was extracted using Trizol reagent (Invitrogen) according to manufacturer protocol. RNA quality was measured spectrophotometrically at 260/280 optical density using Nanodrop lite (Thermo Scientific). To avoid false positive due to DNA contamination, RNA samples were treated with DNase using RQ1 RNase-Free DNase kit (Promega). Reverse transcription of total RNA to cDNA was conducted using High-capacity cDNA reverse transcription kits (Applied Biosystems). Quantitative-real-time polymerase

chain reaction (qRT-PCR) analysis was conducted using Thunderbird™ SYBR® qPCR mix on StepOne Plus real-time polymerase chain reaction system (Applied Biosystems). Primer that was used in this research was presented in table 3.2.3 and designed according to protocols from Thunderbird™ SYBR® qPCR.

Table 3.2.3. Sequence of primer used for chapter 3.

Gene name	Forward	Reverse	NCBI refseq
B-actin	ctcagtctcattggcatggc	gctgtttcaccgttccagtt	NM_001124235.1
Arginase 2	acacactaccgtcatgctgg	cgtcgacccagatcagacac	BK001400.1
iNOS	tggagctatcgtcagaccgg	tcacattgtctgccacctgtt	NM_001124359.1
Il-1β	ccggggttgacatcagtacc	atggtgaaggtggtaaggcg	NM_001124347.2

After feeding trial, Initial weight, final weight, growth performance, and feed efficiency were analyzed using one-way anova. At $P \leq 0.05$. Postprandial plasma arginine, ornithine and citrulline were analyzed using two-way anova without interaction $P \leq 0.05$. Upon significant difference, all data in feeding trial and postprandial plasma experiment was analyzed using Tukey's range test. After disease challenge, survival was analyzed using Kaplan-Meier survival plot combined with log-rank test upon significant result at $P \leq 0.05$. Post-injection plasma arginine, ornithine, and citrulline was analyzed using one-way anova At $P \leq 0.05$ and upon significant difference, Tukey range test was used for post-hoc analysis. Gene expression was analyzed using Kruskal-Wallis at $P \leq 0.05$ and upon significant result, Dunn's multiple comparison test to compare between treatments. Graphpad Prism® 6 (version 6.01, Graphpad software, La Jolla, USA)

3.3. Result

After two-way anova analysis of postprandial plasma arginine, ornithine, and citrulline, in the case of plasma arginine, compared to basal state, the increase of plasma arginine was found in CTRL, CIT, and ORN-CIT at 15 hours postprandial and in ORN, CIT, and ORN-CIT at 30 hours postprandial (table 3.3.1). At 6-hours postprandial, by comparing dietary effect between different treatment, no significant difference was found in this study. at 15 hours postprandial, significant difference was found between ORN and CIT groups. Furthermore, at 30 hours postprandial plasma arginine of CIT treatment was significantly higher compared to other treatments.

Table 3.3.1. Postprandial plasma arginine, ornithine, and citrulline of rainbow trout after fed with dietary supplementation of ornithine, citrulline, and mixture of ornithine and citrulline.

		Basal	6-hours	15 hours	30 hours
Plasma arginine (µg/ml plasma)	CTRL	6.88 ± 0.45	6.42 ± 0.33	18.59 ± 1.21$^{\dagger ab}$	12.79 ± 1.35^{a}
	ORN		6.89 ± 0.02	8.43 ± 0.18^{a}	23.12 ± 6.04$^{\dagger a}$
	CIT		11.75 ± 1.77	25.5 ± 2.92$^{\dagger b}$	36.96 ± 7.56$^{\dagger b}$
	ORN-CIT		11.39 ± 0.92	19.43 ± 0.6$^{\dagger ab}$	20.29 ± 2.36$^{\dagger a}$
Plasma ornithine (µg/ml plasma)	CTRL	1.32 ± 0.13	0.89 ± 0.15^{a}	2.29 ± 0.59^{a}	1.39 ± 0.37^{a}
	ORN		14.41 ± 1.05$^{\dagger b}$	7.4 ± 0.52^{ab}	8.64 ± 0.68$^{\dagger b}$
	CIT		2.03 ± 0.25^{a}	3.7 ± 0.14^{a}	6.86 ± 1.16^{ab}
	ORN-CIT		17.89 ± 0.43$^{\dagger b}$	18.73 ± 2.23$^{\dagger b}$	13.68 ± 5.05$^{\dagger b}$
Plasma Citrulline (µg/ml plasma)	CTRL	0.64 ± 0.05	1.07 ± 0.28^{a}	1.27 ± 0.08^{a}	1.28 ± 0.15^{a}
	ORN		0.98 ± 0.11^{a}	1.67 ± 0.06^{a}	2.02 ± 0.35^{a}
	CIT		124.21 ± 8.54$^{\dagger b}$	198.35 ± 15.93$^{\dagger b}$	316.12 ± 28.25$^{\dagger b}$
	ORN-CIT		156.8 ± 7.11$^{\dagger b}$	278.17 ± 17.1$^{\dagger b}$	324.46 ± 30.9$^{\dagger b}$

Values are mean of triplicate analysis.
Superscript letter indicates significant difference between dietary treatment in the same post-prandial observation time.
† indicates significant difference compared to basal condition

The increase of plasma ornithine compared to its basal state were found in ORN and ORN-CIT treatment at 7 hours postprandial, in ORN-CIT at 15 hours postprandial, and in ORN and ORN-CIT treatment at 30 hours postprandial. Meanwhile, by comparing dietary effect in each postprandial observation time, at 7 hour postprandial there are no significant difference

found between all the diets, at 15 hours postprandial ORN-CIT was significantly higher compared to CTRL and CIT, at 30 hours postprandial ORN and ORN-CIT was significantly higher compared to CTRL treatment.

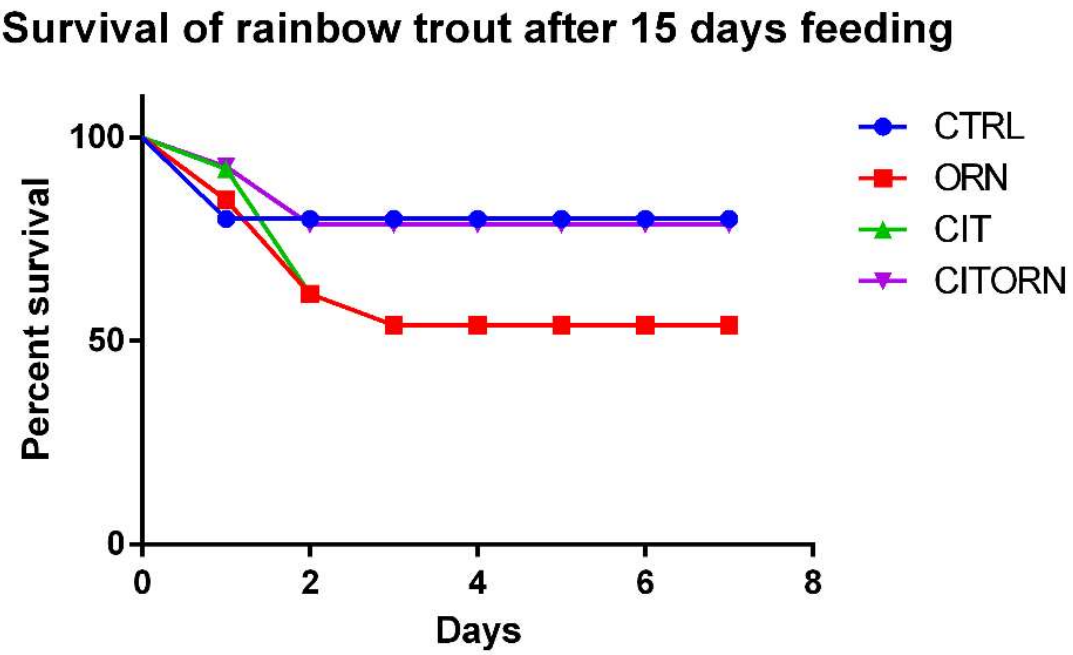

Figure 3.3.1 Survival of rainbow trout pre-fed for 15 days with supplemental ornithine, citrulline, and mixture of ornithine and citrulline and injected intraperitoneally with *Vibrio anguillarum*. Disease challenge was conducted for 7 days.

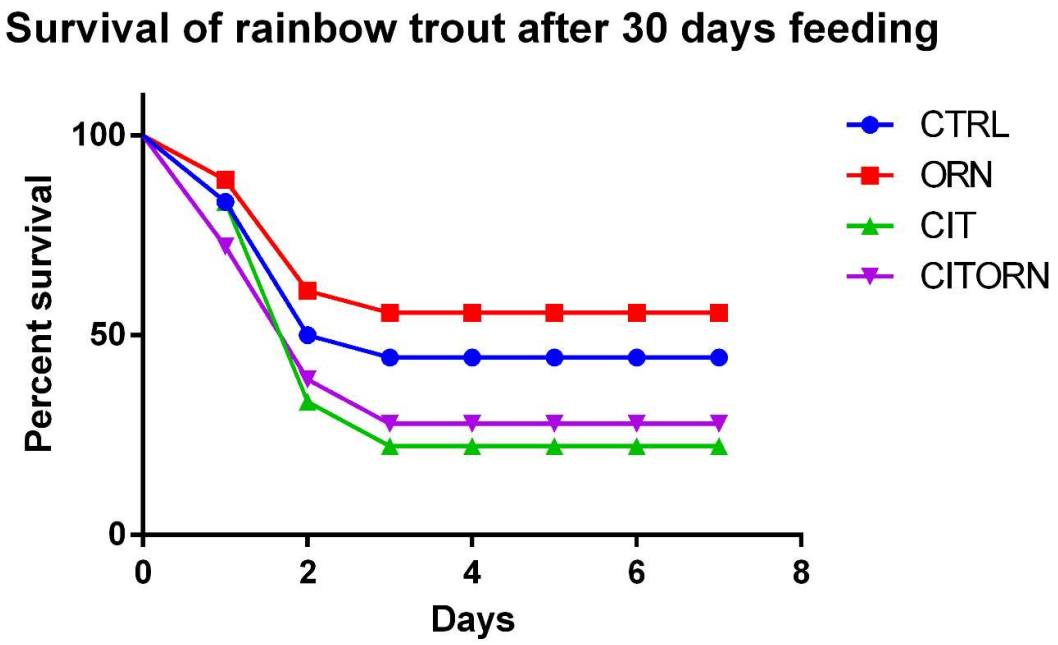

Figure 3.3.2 Survival of rainbow trout pre-fed for 30 days with supplemental ornithine, citrulline, and mixture of ornithine and citrulline and injected intraperitoneally with *Vibrio anguillarum*. Disease challenge was conducted for 7 days.

For disease challenge, upon log-rank test was conducted in both feeding regime, there is no significant difference found among diets (figure 3.3.1 and figure 3.3.2). Highest numerical percent survival in 30 days feeding was found in ORN treatment while lowest numerical percent survival was found in CIT treatment. In the case of 15 days feeding before injected with *Vibrio anguillarum*, highest numerical percent survival was found in CTRL treatment while lowest numerical percent survival was found in CIT and CIT-ORN treatments.

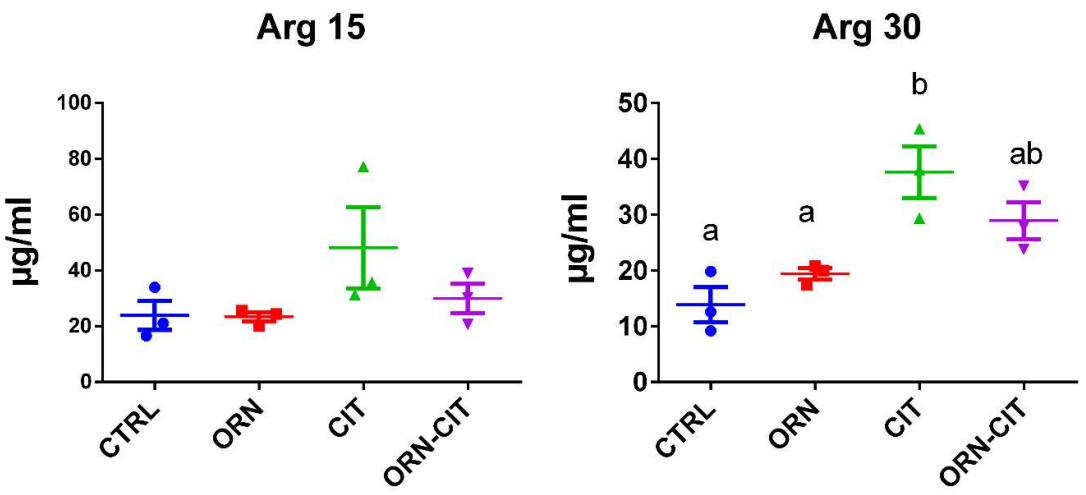

Figure 3.3.3. Plasma arginine of rainbow trout pre-fed for 15 days (left) and 30 days (right) respectively with supplementary level of ornithine, citrulline, and mixture of ornithine and citrulline upon challenge with *Vibrio anguillarum*.

After peritoneal injection with *Vibrio anguillarum*, in treatment that was fed for 15 days there are no significant difference found among all treatment. However, in the case of rainbow trout that was fed for 30 days before exposed to *Vibrio anguillarum*, plasma arginine of CIT treatment was significantly higher compared to CTRL and ORN but not with ORN-CIT treatment (figure 3.3.3.).

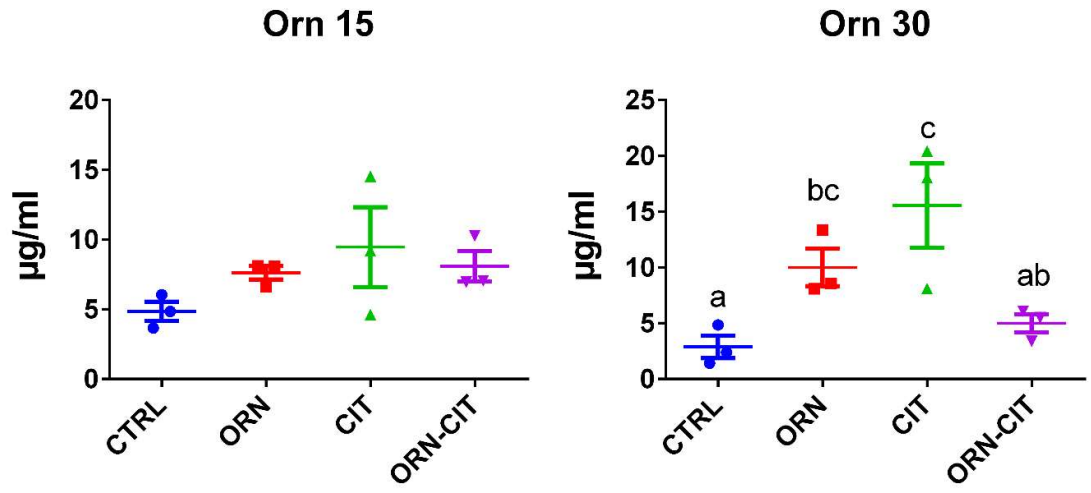

Figure 3.3.4. Plasma ornithine of rainbow trout pre-fed for 15 days (left) and 30 days (right) respectively with supplementary level of ornithine, citrulline, and mixture of ornithine and citrulline upon challenge with *Vibrio anguillarum*.

In the case of plasma ornithine, in rainbow trout that was fed for 15 days before exposed to *Vibrio anguillarum*, there are also no significant difference found among all treatment. However, in rainbow trout that was fed for 30 days, plasma ornithine of CIT treatment was significantly higher compared to other treatment (figure 3.3.4). Furthermore, plasma ornithine of ORN treatment was also higher compared to CTRL treatment.

In the case of plasma citrulline, in rainbow trout that was fed for 15 days before exposed to *Vibrio anguillarum*, significantly higher plasma citrulline was found in ORN-CIT compared to ORN and CTRL (figure 3.3.5). Furthermore, in rainbow trout that was fed for 30 days, significantly higher plasma citrulline was found in ORN-CIT compared to ORN and CTRL while CIT treatment was also significantly higher compared to CTRL treatment.

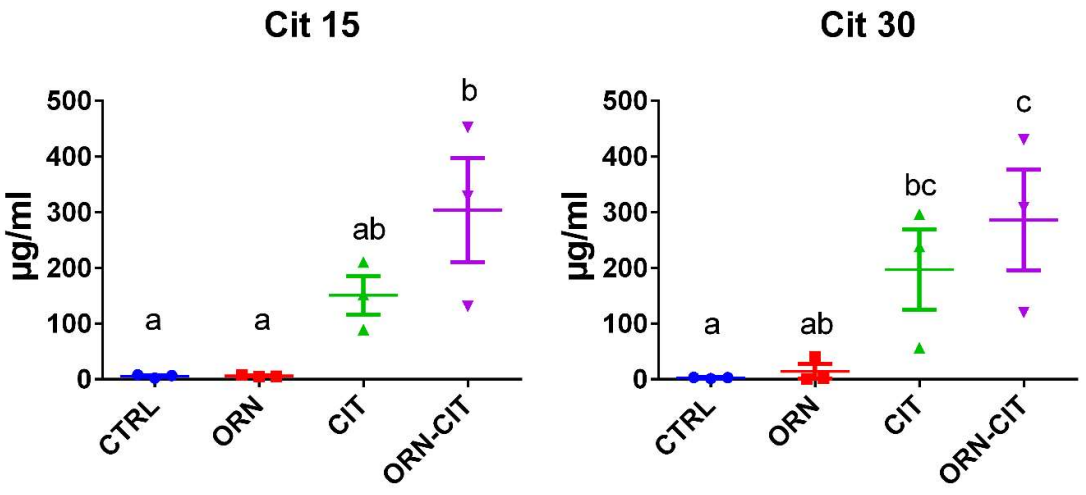

Figure 3.3.5. Plasma citrulline of rainbow trout pre-fed for 15 days (left) and 30 days (right) respectively with supplementary level of ornithine, citrulline, and mixture of ornithine and citrulline upon challenge with *Vibrio anguillarum*.

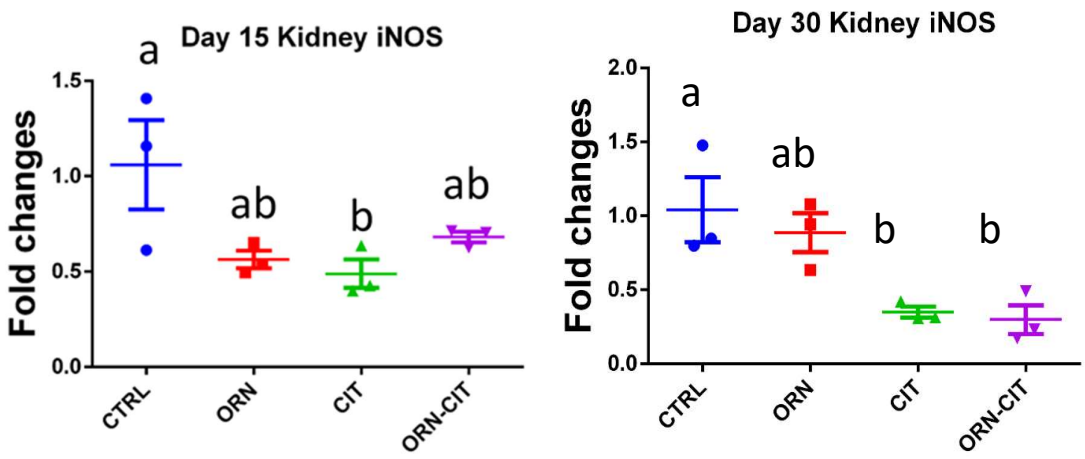

Figure 3.3.6. Expression of renal iNOS of rainbow trout pre-fed for 15 days (left) and 30 days (right) respectively with supplementary level of ornithine, citrulline, and mixture of ornithine and citrulline upon challenge with *Vibrio anguillarum*.

In gene expression of renal iNOS, in rainbow trout that was pre-fed for 15 days, significant difference was found between CTRL and CIT treatment (figure 3.3.6). Meanwhile, in the case of rainbow trout that was pre-fed for 30 days, statistically higher expression of iNOS was found in CTRL compared to CIT and ORN-CIT (figure 3.3.7). Furthermore, there is no significant difference found between ORN with CTRL, CIT, and ORN-CIT.

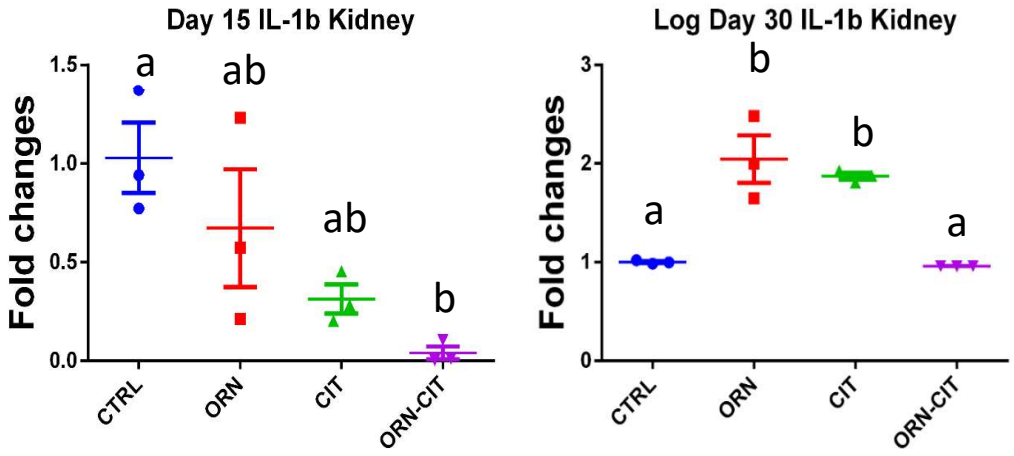

Figure 3.3.7. Expression of renal 1L-1β of rainbow trout pre-fed for 15 days (left) and 30 days (right) respectively with supplementary level of ornithine, citrulline, and mixture of ornithine and citrulline upon challenge with *Vibrio anguillarum*.

In renal 1L-1β, after 15 days feeding and disease challenge, it was found that statistically higher expression of IL-1β was found in CTRL compared to ORN-CIT treatment. While in the case of 30 days feeding regime, statistically higher expression of IL-1β was found in ORN and CIT treatment compared to CTRL while there was no difference found between CTRL and ORN-CIT treatment (figure 3.3.7).

In renal arginase, after 15 days feeding regime and disease challenge, there are no significant differences found among all the treatment. However, in the case of rainbow trout that was fed with 30 days feeding regime before exposed to bacterial injection, significant difference was found between CTRL and ORN treatment (figure 3.3.8).

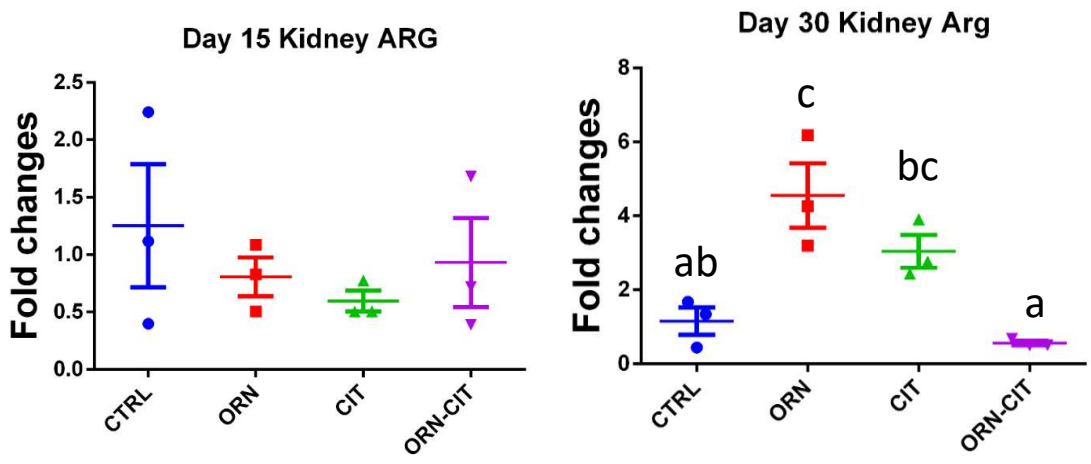

Figure 3.3.8. Expression of renal arginase II of rainbow trout pre-fed for 15 days (left) and 30 days (right) respectively with supplementary level of ornithine, citrulline, and mixture of ornithine and citrulline upon challenge with *Vibrio anguillarum*.

Table 3.3.2. Initial weigh, final weight, growth, and feed efficiency of rainbow trout fed with dietary supplementation of l-ornithine, l-citrulline, and combination of l-ornithine and l-citrulline after 30 days feeding period.

	CTRL	ORN	CIT	ORN-CIT
Initial weight (g)	9.08 ± 0.89	9.08 ± 0.69	9.03 ± 0.75	9.11 ± 0.9
Final weight (g)	30.38 ± 1.72	30.73 ± 2.16	31.02 ± 1.84	30.71 ± 1.67
Growth (% of initial weight)	242.3 ± 16.96	251.7 ± 42.97	258 ± 41.38	257.1 ± 50.85
Feeding efficiency (%)	87.31 ± 7.68	101.6 ± 10.82	88.29 ± 9.45	89.65 ± 9.25

Values are mean of triplicate analysis.

After 30 days feeding, there was no significant difference found in growth and feeding efficiency among all treatment. in the case of average growth, the highest numerical value was found in CIT treatment while the lowest was found in CTRL treatment (table 3.3.2). Furthermore, in the case of feeding efficiency, the highest numerical value was found in ORN treatment while the lowest numerical value was found in CTRL treatment.

3.4.Discussion

Postprandial plasma amino acid

Plasma arginine increase of CTRL treatment was found in 15 hours postprandial compared to its basal condition. The increase of plasma arginine at 12 hours postprandial is in line with postprandial arginine increase which is found in other study on fish (Schuhmacher et al. 1997). At 30 hours postprandial plasma arginine of CTRL already shown a reduced number. It is previously stated plasma arginine that diminished from blood circulation is mainly used for protein synthesis (Deutz 2008). In ORN treatment, increase of plasma arginine was found only at 30 hours postprandial, the increase of plasma arginine in ORN was also occurred at later time compared to other treatments. In CIT treatment, similar to CTRL, the increase of plasma arginine was occurred at 15 hours postprandial. At that observation time, although plasma arginine of CIT treatment was numerical higher compared to other treatment, significant difference was only found between CIT and ORN. At 30 hours postprandial, plasma arginine of CIT treatment was significantly higher compared to other treatment. in ORN-CIT, the increase of plasma arginine was occurred at 15 hours postprandial. plasma arginine of ORN-CIT treatment was still showing increasing trend although at slower pace compared to CIT treatment.

Plasma ornithine of CTRL and CIT did not shown increase compared to its basal condition. The increase of plasma ornithine can only be observed in treatments with ornithine supplementation (ORN and ORN-CIT) as early as 6 hours postprandial. In ORN treatment, higher than basal plasma ornithine was found at 6 hours and 30 hours postprandial; at 15 hours postprandial concentration of plasma ornithine was fall into almost half of previous hour and was not significantly different compared to basal state. Meanwhile. plasma ornithine of ORN-CIT shown numerical highest concentration at 6, 15, and 30 hours postprandial and was shown a significantly higher than CTRL at those postprandial observation. Interestingly, plasma arginine of ORN treatment in this study was shown a significant increase at slower time among

other treatments. Thus, the possibility that plasma ornithine concentration can regulate plasma arginine production should be evaluated.

Increase of plasma citrulline was only found in treatments with citrulline supplementation (CIT and ORN-CIT) and shown an increasing trend from 6 until 30 hours postprandial. at 30 hours postprandial, plasma citrulline of CIT and ORN-CIT treatment was immensely higher compared to the CTRL treatment.

In table 4.3.1., the increase of plasma ornithine and plasma citrulline in the treatments that were supplemented by those amino acids can be observe at 6 hours postprandial while the increase of plasma arginine can be observe as early as 15 hours postprandial. It is possible that plasma ornithine and citrulline were appeared early since it was come directly from dietary source while plasma arginine come from either protein turnover or endogenous arginine synthesis.

Survival; plasma amino acids; and expression of renal inducible nitric oxide synthase, interleukin-1-beta, and arginase.

It was stated previously that plasma arginine may partially regulate intracellular availability of arginine and ensure sustain production of nitric oxide (Buentello & Gatlin, 1999). However, based on survival analysis, it seems that higher concentration of plasma arginine that was found in CIT treatment was not enough to increase immune performance of rainbow trout after peritoneal injection with *Vibrio anguillarum*. Interestingly, renal iNOS expression of CIT treatment shown lower relative fold change compared to CTRL at 24 hours post-injection with *Vibrio anguillarum*. Furthermore, the lower fold change of CIT compared to CTRL was also consistent in 15 and 30 days feeding regime. Since iNOS is reliable marker for M1 macrophage (Wiegertjes et al. 2016), it can be concluded that at 24 hours post-injection, M1 macrophage was not activated in this study. Activation of M1 macrophage is linked with protection against acute infectious disease by stimulating intracellular killing of bacteria such as *Vibrio*

anguillarum (Boesen et al. 2001; Benoit et al. 2008; Wiegertjes et al. 2016). In this research, it is not clear why iNOS expression level was lower compared those in CTRL treatment. However, from previous publication in terrestrial animal, it is found that expression of iNOS can be inhibited by several factors such as blockage of I-kappa beta, overproduction of nitric oxide, exposure to tumor growth factor beta, and extracellular arginine availability (Perrella et al. 1994; Lee et al. 2003; Aktan 2004).

In ORN treatment, higher than CTRL expression of arginase II was observed at 24 hours post-injection. Since arginase II is considered as indicator for M2/ alternatively activated macrophage (Wiegertjes et al. 2016), it is possible that M2 was activated in this study. While activation of M2 in fish was mainly linked into anti-inflammatory and healing process, M2 macrophage is divided into several types in terrestrial animal: M2a which is induced by Il-4 or Il-14, M2b which is induced by Il-1β, and M2c which is induced by Il-10 and glucocorticoid hormone (Benoit et al. 2008; Wiegertjes et al. 2016). In the case of fish, although differentiation of M2 macrophage was not been proved yet, higher Il-1β and arginase II might imply the similarity between those in M2b of mammals. However, further in deep study is still required to reach that conclusion especially since stimuli for M1/M2 activation was not fully evaluated in this study.

In conclusion, this study confirms that supplementation with l-citrulline increase plasma arginine production. Furthermore, l-citrulline supplementation also shown higher plasma arginine level compared to other treatment at 24 hours post-injection with *Vibrio anguillarum*. The higher plasma arginine level in CIT treatment however is not enough to enhance immune performance of rainbow trout after disease challenge. qPCR analysis in the renal also shown that supplementation with citrulline, ornithine, and combination of ornithine and citrulline at 1% of diet did not enough to increase mRNA level of iNOS.

CHAPTER 4

IV. Effect of dietary arginine, ornithine, and citrulline supplementation on postprandial plasma amino acids, arginine catabolism and resistance to *Vibrio anguillarium* of rainbow trout *Oncorhynchus mykiss*"

4.1. Introduction

Arginine is a versatile amino acid that plays important roles in various physiological functions. It regulates the production of aliphatic polyamine, which controls cellular metabolism and is related to cell growth and differentiation through ornithine production. Arginine can also enhance wound healing (Shi et al. 2002b; Wu et al. 2009; Andersen et al. 2013) and nitric oxide production through nitric oxide synthase (NOS) enzymes. Nitric oxide is beneficial to the immune system, maintains cardiovascular health, and plays a role in neural signaling (Garthwaite and Boulton 1995; Wu and Morris 1998; Förstermann and Münzel 2006).

Recent studies have shown that arginine plays an important role in the activation of macrophages: increased NOS activity occurs in classically activated macrophages, while increased arginase activity is an indicator of alternatively activated macrophages (Forlenza et al. 2011). Nitric oxide in classically activated macrophages, known as M1 macrophages, is a potent anti-microbial compound, and with superoxide, has anti-parasitic properties (Nathan and Hibbs 1991). Alternatively activated macrophages, known as M2 macrophages, promote cell proliferation during wound healing (Mills et al. 2015). Classically activated and alternatively activated macrophages compete for L-arginine as a substrate (Forlenza et al. 2011), so L-arginine availability also plays an important role in the activation of classically and alternatively activated macrophages.

Because arginine is important for macrophage functioning, several studies have aimed to supplement animals with various compounds that increase the availability of arginine. Dietary supplementation with L-arginine increases plasma arginine concentrations in pigs (Yao

et al. 2008; Yao et al. 2011), chickens (Ruiz-Feria et al. 2001), and fish (Fournier et al. 2003; Cheng et al. 2011; Cheng et al. 2012a; Pohlenz et al. 2013; Andersen et al. 2014). Plasma arginine concentrations can be increased by L-arginine supplementation, while arginine is involved in the production of various compounds and is regulated by various pathways; therefore, not all supplemental arginine can be used to improve the immune system. Chen et al. (2015) reported an increase in inducible nitric oxide expression in the head kidney of Jian carp, *Cyprinus carpio* var. Jian, when fed 2.19% arginine and challenged with *Aeromonas hydrophila.* However, in the channel catfish *Ictalurus punctatus*, there was no significant increase in innate immunity when the fish were fed excessive amounts of arginine, above their optimum level for growth (Pohlenz et al. 2014). Arginine supplementation also increases urea excretion due to the conversion of arginine by arginase (Fournier et al. 2003; Tulli et al. 2007; Oliva-Teles et al. 2017). Increased urea excretion in aquaculture is problematic, because it can pollute the surrounding water column and cause eutrophication (Talbot and Hole 1994).

In addition to arginine, citrulline and ornithine are compounds that can also be supplemented into feed, and potentially used to increase arginine availability or improve fish immunity. Citrulline is an arginine metabolite that is obtained from the ornithine-urea cycle or nitric oxide synthesis. It can increase arginine synthesis through an argininosuccinate intermediary, which involves argininosuccinate lyase and argininosuccinate synthase. Ornithine is another arginine metabolite, and is important for polyamine and proline synthesis. In terrestrial animals, supplementation with this amino acid can accelerate wound repair and healing [23,24]. In mammals, citrulline can increase plasma arginine levels more than arginine itself (Bahri et al. 2013). In fish, it is unclear whether ornithine or citrulline are able to increase the availability of arginine, so dietary arginine is used. In addition, it is unclear whether these compounds improve fish immunity. Therefore, this study aimed to evaluate the effects of dietary supplementations of ornithine and citrulline on the resistance of rainbow trout

Oncorhynchus mykiss against *Vibrio anguillarum*. In addition, we evaluated the expression levels of arginase, interleukin-1-beta, and NOS during disease challenge, and evaluated the postprandial behavior of plasma arginine and its metabolites after L-ornithine and L-citrulline supplementation.

4.2. Materials and Methods

The study was divided into postprandial plasma amino acid level observations and a feeding trial with a disease challenge using *V. anguillarum*. Juvenile rainbow trout were acquired from Oizumi Research Station, Tokyo University of Marine Science and Technology, Yamanashi, Japan, and acclimated for two weeks. During this period, fish were fed the CTRL diet (see below). In both experiments, fish were reared in eight rectangular aquaria with a total water volume of ~50 L per treatment. The aquaria were equipped with recirculating systems that had sedimentation tanks, biofiltration, and automatic thermostats. The average temperature during the trial period was 14.4 °C. In the recirculating system of each aquarium, aeration was provided to maintain the oxygen level at saturation. During the trials, NO_2 and NO_3 levels were periodically checked using Packtest (Kyoritsu, Tokyo, Japan), and were under 0.05 and 2 ppm, respectively. The water flow rate was maintained at 0.75 L min^{-1}. Animal experimentation was conducted according to the guidelines of the Animal Experiment Treaty of the Tokyo University of Marine Science and Technology.

Dietary treatments

The basal diet (CTRL) for both experiments was formulated using a combination of intact protein sources, such as blue whiting fish meal, soybean meal, feather meal, blood meal, corn gluten meal, and a crystalline amino acid mixture (Table 4.2.1). Wheat flour was used as a source of carbohydrate, while the lipid requirement was mainly provided by fish oil. For the treatment diets, 2% L-arginine (+ARG), 2% L-ornithine (+ORN), and 2% L-citrulline (+CIT) supplements were used (Table 4.2.1). A proximate analysis revealed that the protein and lipid

percentages of the diets were 48.6 and 18.9% in CTRL, 53.1 and 18.7% in +ARG, 51.7 and 18.1% in +ORN, and 52.9 and 18.9% in +CIT (Table 4.2.1). An amino acid analysis revealed that the arginine percentage in +ARG was 3.91% (compared to 1.80% in CTRL), the ornithine percentage in +ORN was 1.61% (compared to 0.01% in CTRL), and the citrulline percentage in +CIT was 1.76% (compared to 0.04% in CTRL) (Table 4.2.2). For preparing the experimental diet, all the ingredients were ground using a Retsch Ultra Centrifugal Mill ZM 200 (Haan, Germany) to a particle size of ~0.5 μm; thereafter, these were mixed thoroughly, pelleted using a pellet mill (AEZ12 M, Hiraga Seishakusho, Kobe, Japan), and the pellets were freeze-dried for 16 hours in a freezer-dryer (RLE-II, Kyowa Vacuum, Saitama, Japan; Tables 4.2.1 and 4.2.2). The diets were stored at 5 °C until use.

Table 4.2.1. Formulation and proximate analysis of experimental diets for rainbow trout fed with supplemental level of l-arginine, l-ornithine, and l-citrulline

Ingredients (crude protein (%) / ether extract (%))	**CTRL (g/100 g DW)**	**ARG (g/100 g DW)**	**ORN (g/100 g DW)**	**CIT (g/100 g DW)**
Formulation of experimental diets				
Blue whiting fish meal (71.8/9.6)	20.0	20.0	20.0	20.0
Soybean meal (48.5/2.3)	11.9	11.9	11.9	11.9
Feather meal (90.7/4.8)	2.0	2.0	2.0	2.0
Blood meal (95.2/0.7)	2.0	2.0	2.0	2.0
Corn gluten meal (63.9/1.6)	25.0	25.0	25.0	25.0
Flour (21.5/2.5)	15.0	15.0	15.0	15.0
Fish Oil	10.4	10.4	10.4	10.4
Vitamin premix[b]	3.0	3.0	3.0	3.0
Mineral premix[c]	1.0	1.0	1.0	1.0
Monocalcium phosphate	0.5	0.5	0.5	0.5
Choline Cl (100%)	0.5	0.5	0.5	0.5
Vitamin E (50%)	0.1	0.1	0.1	0.1
Amino acid premix[1]	4.4	4.4	4.4	4.4
Celullose	4.1	4.1	4.1	4.1
L-arginine	0	2.0	0	0
L-ornithine	0	0	2.0	0
L-citrulline	0	0	0	2.0
Proximate analysis of experimental diet				
Crude protein	48.6	53.1	51.7	52.9
Crude lipid	18.9	18.7	18.1	18.9

Ash	8.2	8.6	8.6	8.6

[a] Vitamin pre-mixture (amounts in g/kg): ascorbic acid, 368.902 g; biotin, 0.363 g; calcium pantothenate, 6.05 g; cyanocobalamin, 0.006 g; folic acid, 0.908 g; inositol, 121 g; niacin, 24.2 g; p-aminobenzoic acid, 3.025 g; pyridoxine hydrochloride, 2.42 g; riboflavin, 3.63 g; thiamin hydrochloride, 3.025 g; vitamin A acetate, 2,420,000 IU; vitamin D3, 2,420,000 IU; vitamin K3, 6.05 g

[b] based on Hernández et al., (2012)

[c] Amino acid pre-mixture (amount in g/100g): L-methionine[1], 3.6; L-histidine[1], 7.4; L-isoleucine[1], 23.9; L-lysine hydrochloride[1], 23.3; L-threonine[1], 13.2; L-valine[1], 28.7

[1] Wako Pure Chemical Industry Inc., Osaka, Japan

Table 4.2.2. Total amino acid content of experimental diets for rainbow trout fed with supplemental level of l-arginine, l-ornithine, and l-citrulline [*]

	CTRL (g/100 g DW)	**ARG (g/100 g DW)**	**ORN (g/100 g DW)**	**CIT (g/100 g DW)**
Taurine	0.12	0.14	0.14	0.14
Aspartic acid	3.18	3.20	3.21	3.23
Threonine	1.80	2.11	2.13	2.42
Serine	2.17	2.26	2.23	2.26
Glutamic acid	7.32	7.48	7.47	7.49
Glycine	1.87	1.94	1.91	1.88
Alanine	2.78	2.86	2.87	2.81
Citrulline	0.04	0.04	0.05	1.76
Valine	2.22	2.36	2.26	2.40
Cysteine	0.32	0.32	0.31	0.33
Methionine	0.77	0.69	0.71	0.71
Cystathionine	0.05	0.08	0.05	0.04
Isoleucine	1.94	2.07	1.89	1.95
Leucine	3.99	4.07	3.93	3.87
Tyrosine	1.53	1.64	1.52	1.54
Phenylalanine	1.85	1.87	1.86	1.92
Ornithine	0.01	0.02	1.61	0.10
Histidine	0.94	0.95	1.07	1.00
Lysine	2.42	2.51	2.51	2.55
Tryptophan	0.06	0.24	0.16	0.28
Arginine	1.80	3.91	1.88	1.79
Proline	3.07	3.16	2.97	2.46

[*]Values are means of duplicate analysis

Postprandial plasma arginine levels

For the postprandial plasma experiment, to avoid any confounding effects of previous feeding, rainbow trout (average mass, 82.9 g) were first fasted for 14 days before being fed

once with one of the designated basal and treatment diets until apparent satiation, which was indicated by a refusal to feed and the presence of uneaten food at the bottom of the aquarium. After 6, 15, and 30 postprandial hours, three fish per treatment were anaesthetized using ethylene glycol monophenyl ether at 400 ppm before being blood sampled. Blood was taken from the caudal vein using a heparinized syringe, and the plasma was separated by centrifugation at 1000 × *g* at 4 °C for 15 minutes, while the liver and kidney were harvested and preserved at -40 °C for free amino acid analysis.

Feeding and disease challenge

For the feeding and disease challenge, a short feeding trial was conducted for 30 days using 15 fish per aquarium (average mass, 9.1 g). The fish were fed twice a day, six days a week until apparent satiation. After 30 days, the final weight gain was recorded, and the fish were then injected with 3.0×10^6 colony forming units of *V. anguillarum* diluted with phosphate-buffered saline (1 mL per fish) and kept in isolation tanks. These tanks were equipped with recirculating systems and automatic thermostats that were set at 18 °C. At 24 hours post-injection, five fish per treatment were anaesthetized using 2-phenoxyethanol at 400 ppm, and blood was collected from the caudal vein using a heparinized syringe. The liver, intestine, muscle, and kidney were preserved for quantitative polymerase chain reaction (qPCR) and amino acid analysis. Blood was separated using the same blood preparation method, while the liver and kidney were collected and preserved in a sealed plastic bag at -40 °C for free amino acid analysis. A small part of the head kidney was preserved in RNAlater® for real-time qPCR analysis.

For plasma amino acid analysis, plasma was first mixed with 3% sulfosalicylic acid solution (w/v) and then centrifuged at 1000 × *g* at 4 °C for 15 minutes. The supernatant was then filtered using a 0.45-μm syringe filter, and the filtered supernatant was diluted with HPLC grade water at 1:4 dilution before being analyzed with the amino acid analyzer JLC-500/V

AminoTac™ (JEOL Ltd., Tokyo, Japan), according to the method described by Boonyoung et al. (2013).

Renal samples were first defrosted in running water. The defrosted renal samples (0.2–0.5 g) were then homogenized in 2% sulfosalicylic acid and centrifuged at 3000 × *g* and 4 °C for 15 minutes. The supernatants were then collected and the pellets re-homogenized using the same method, in order to ensure that all of the free amino acids were extracted from the organs. The pooled supernatants were then made up to 50 mL and aliquots were filtered using a 0.45-μm-pore-size syringe filter, which were then analyzed using the JLC-500/V AminoTac™ (JEOL Ltd.) according to the method described by Boonyoung et al. (2013).

RNA extraction was conducted using TRIzol reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's protocol. Before preparing cDNA, the RNA quality was spectrophotometrically measured using a Nanodrop™ Lite Spectrophotometer (Thermo Scientific, Waltham, USA), and the RNA was treated with DNase using a RQ1 RNase-Free DNase Kit (Promega, Madison, USA) to avoid false positives caused by DNA contamination. The reverse transcription of total RNA to cDNA was conducted using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, USA). Primer design was conducted using Primer3web version 4.1.0 (http://primer3.ut.ee/) following the guidelines of the Thunderbird® SYBR® qPCR Mix (Toyobo, Tokyo, Japan): a primer length of ~20–30 mer, a primer guanine-cytosine content of ~40–60%, and a target length of ≤150 bp. A list of primers that were used in the qPCR is presented in Table 4.2.3. qPCR analysis was conducted using Thunderbird™ SYBR® qPCR Mix in a StepOnePlus™ Real-Time PCR System (Applied Biosystems) using beta-actin as a housekeeping gene (as a normalizer) and the ddCt method of data analysis.

Table 4.2.3. Sequence of primer used for chapter 4.

Gene name	Forward	Reverse	NCBI refseq
B-actin	ctcagtctcattggcatggc	gctgtttcaccgttccagtt	NM_001124235.1
Arginase 2	acacactaccgtcatgctgg	cgtcgacccagatcagacac	BK001400.1
iNOS	tggagctatcgtcagaccgg	tcacattgtctgccacctgtt	NM_001124359.1
Il-1β	ccggggttgacatcagtacc	atggtgaaggtggtaaggcg	NM_001124347.2

Statistical analyses

Fish growth and amino acid contents were analyzed using an analysis of variance (ANOVA) at $P \leq 0.05$. Postprandial plasma arginine, ornithine, citrulline, and ammonia levels were analyzed using a two-way ANOVA without interactions ($P \leq 0.05$), and gene expression was analyzed using a Kruskal-Wallis test at $P \leq 0.05$. Upon detecting a significant difference, a post-hoc analysis was conducted using several tests: Tukey's range test for weight gain, postprandial and post-injection plasma amino acid levels, and free renal amino acid levels, and Dunn's multiple comparison test for gene expression analysis. For the disease challenge, survival was analyzed using a log rank test for trend combined with a Mantel-Cox test with significance set at $P \leq 0.05$, and post-hoc analysis was conducted using Fisher's least significant difference test. All statistical analyses were conducted using Graphpad Prism® 6 version 6.01 (Graphpad software, La Jolla, USA).

4.3.Result

Significant differences were found in postprandial plasma amino acid levels among the dietary treatments and among the postprandial amino acid observation timepoints (Table 4.3.1). The plasma arginine level was higher than the basal level in +ARG and +CIT. In +ARG, high plasma arginine levels were observed at 6, 15, and 30 hours postprandial, while in +CIT, they were observed at 15 and 30 hours postprandial. Regarding each timepoint, at 6 hours

postprandial, plasma arginine levels were significantly higher in +ARG than in CTRL; at 15 hours postprandial, plasma arginine levels in +ARG were significantly higher than in the other diets, and they were higher in +CIT than in CTRL; and at 30 hours postprandial, they were significantly higher in +ARG and +CIT than in CTRL and +ORN.

Significant differences were found in plasma ornithine levels among the treatments and timepoints (Table 4.3.1). An increase in plasma ornithine levels was only observed in +ORN, and at each timepoint, the plasma ornithine level in +ORN was significantly higher than in the other treatments.

Table 4.3.1. Post-prandial plasma amino acids of rainbow trout fed with supplemental level of arginine, ornithine, and citrulline

		Basal	6-hours	15 hours	30 hours
Plasma arginine (μ g/ml plasma)	CTRL	12.0 ± 0.6	16.9 ± 1.6^{a}	9.0 ± 3.0^{a}	18.8 ± 2.7^{a}
	ARG		40.0 ± 7.0$^{\dagger b}$	45.6 ± 3.7$^{\dagger c}$	44.1 ± 1.2$^{\dagger b}$
	ORN		19.3 ± 0.8^{ab}	22.1 ± 0.5^{ab}	24.2 ± 3.8^{a}
	CIT		24.9 ± 3.6^{ab}	30.0 ± 5.4$^{\dagger b}$	40.4 ± 3.3$^{\dagger b}$
Plasma ornithine (μ g/ml plasma)	CTRL	3.1 ± 0.1	1.9 ± 0.1^{a}	1.8 ± 0.2^{a}	1.8 ± 0.2^{a}
	ARG		5.9 ± 1.0^{a}	6.6 ± 0.3^{a}	7.2 ± 1.6^{a}
	ORN		34.1 ± 1.9$^{\dagger b}$	67.4 ± 6.9$^{\dagger b}$	24.8 ± 6.5$^{\dagger b}$
	CIT		2.8 ± 0.5^{a}	3.0 ± 0.6^{a}	6.0 ± 1.3^{a}
Plasma Citrulline (μ g/ml plasma)	CTRL	0.47 ± 0.08	1.48 ± 0.23^{a}	0.74 ± 0.16^{a}	1.25 ± 0.18^{a}
	ARG		1.25 ± 0.31^{a}	1.19 ± 0.33^{a}	1.47 ± 0.46^{a}
	ORN		1.92 ± 0.28^{a}	2.18 ± 0.26^{a}	2.21 ± 0.04^{a}
	CIT		351.89 ± 112.41$^{\dagger b}$	240.54 ± 38.53$^{\dagger b}$	396.81 ± 194.81$^{\dagger b}$
Plasma NH^3 (μ g/ml plasma)	CTRL	6.7 ± 0.1	13.7 ± 1.0†	13.7 ± 1.4†	9.6 ± 1.3^{ab}
	ARG		13.4 ± 1.2†	11.2 ± 1.2	14.3 ± 2.1$^{\dagger b}$
	ORN		11.9 ± 0.6†	14.1 ± 0.7†	10.7 ± 0.6^{ab}
	CIT		12.5 ± 1.9†	9.6 ± 0.6	8.2 ± 0.8^{a}

Values are mean of triplicate analysis.
Superscript letter indicates significant difference between dietary treatment in the same post-prandial observation time.
† indicates significant difference compared to basal condition

Significant differences in plasma citrulline levels were only found among the dietary treatments, and there were no significant differences among the postprandial timepoints (Table

4.3.1). A Tukey's multiple comparison test revealed that higher than basal plasma citrulline levels were found in +CIT at 6, 15, and 30 hours postprandial. Furthermore, at each postprandial timepoint, the plasma citrulline level in +CIT was significantly higher than that in the other treatments.

Significant differences were found in plasma ammonia levels among the postprandial timepoints, but not among the dietary treatments (Table 4.3.1). However, a Tukey's multiple comparison test revealed a significant difference between +ARG and +CIT at 30 hours postprandial.

Feeding and disease challenge

Results of ANOVA showed no significant difference in growth performance, based on the final weight of the fish (Table 4.3.2), but a post-hoc analysis revealed that growth in +ARG was significantly higher than that in +CIT. However, there was no significant difference between CTRL and +CIT. There was no significant difference in the food conversion ratio among the treatments.

Table 4.3.2. Initial weight, final weight, growth, and food conversion ratio (FCR) of rainbow trout fed with dietary arginine, ornithine, and citrulline supplementation.

	Dietary treatments			
	CTRL	**ARG**	**ORN**	**CIT**
Initial weight (g)	34.0 ± 2.3	34.2 ± 1.7	34.2 ± 1.7	34.0 ± 2.0
Final weight (g)	82.8 ± 4.5	85.5 ± 3.8	84.0 ± 4.5	78.8 ± 3.7
Growth (% of initial weight)	146.5 ± 4.2^{ab}	151.4 ± 3.8^{a}	145.1 ± 2.2^{ab}	133.6 ± 4.2^{b}
FCR	1.17 ± 0.05	1.21 ± 0.05	1.21 ± 0.07	1.16 ± 0.05

Values are mean of triplicate analysis.
Superscript letter indicates significant difference between dietary treatment in the same post-prandial observation time.

After seven days of disease challenge, a Mantel-Cox post-hoc analysis revealed that the only significant difference in survival was between CTRL and +CIT: survival in +CIT (69.2%) was significantly higher than that in CTRL (25.0%) (Figure 4.3.1).

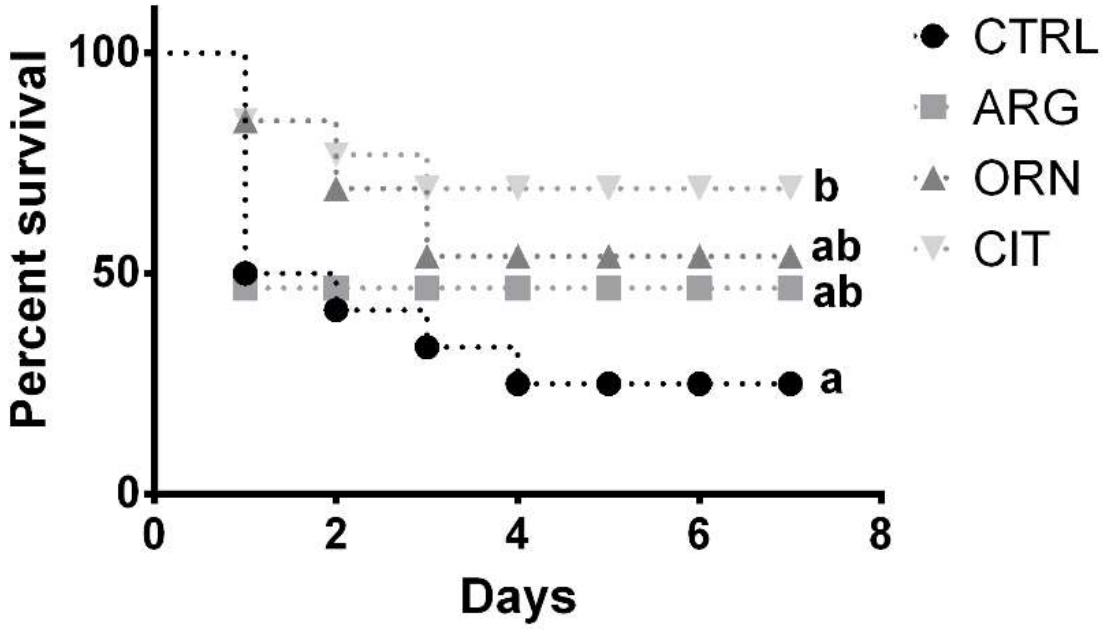

Figure 4.3.1. Survival analysis of rainbow trout pre-fed with supplemental level of arginine, ornithine, and citrulline upon *Vibrio anguilarum* challenge through peritoneal injection. Disease challenge was performed for 7 days. Analysis was conducted using log-rank test.

Twenty-four hours after injecting *V. anguillarum*, plasma samples from each treatment were collected and analyzed (Table 4.3.3). Significantly higher plasma arginine levels were found in +CIT than in +ARG or +ORN, while no significant difference was found between CTRL and +CIT. Moreover, +CIT fish had significantly higher plasma citrulline levels than those in the other treatments.

Regarding free renal amino acids (Table 4.3.4), significantly higher plasma arginine levels were found in +CIT than in +ORN or +ARG. There was no significant difference between CTRL and +CIT, but CTRL had significantly higher plasma arginine levels than +ORN. Significant differences were found in plasma citrulline levels between +CIT and the other treatments. There were no significant differences in plasma ornithine levels.

Table 4.3.3. Plasma amino acid of rainbow trout pre-fed supplemental level of arginine, ornithine, and citrulline after 24 hours post-injection with *Vibrio anguilarum*

	Dietary treatments			
	CTRL	**+ARG**	**+ORN**	**+CIT**
Plasma arginine (μ g/ml plasma)	25.8 ± 4.6^{a}	29.8 ± 5.2^{a}	25.9 ± 5.1^{a}	63.7 ± 5.4^{b}
Plasma ornithine (μ g/ml plasma)	5.1 ± 0.8^{a}	10.0 ± 2.4^{a}	19.6 ± 5.5^{ab}	29.2 ± 5.8^{b}
Plasma citrulline (μ g/ml plasma)	3.8 ± 0.9^{a}	3.5 ± 1.1^{a}	3.9 ± 0.4^{a}	148.8 ± 58.4^{b}

Values are mean of triplicate analysis.
Superscript letter indicates significant difference between dietary treatment

Table 4.3.4. Renal free amino acid of rainbow trout pre-fed supplemental level of arginine, ornithine, and citrulline after 24 hours post-injection with *Vibrio anguilarum*

	Dietary treatments			
	CTRL	**ARG**	**ORN**	**CIT**
arginine (10^{-3} μ g/ml plasma)	47.0 ± 9.0^{ab}	26.0 ± 2.0^{a}	20.0 ± 8.0^{a}	52.0 ± 18.0^{b}
ornithine (10^{-3} μ g/ml plasma)	2.2 ± 2.4	1.9 ± 0.6	3.6 ± 0.9	3.9 ± 0.9
citrulline (10^{-3} μ g/ml plasma)	1.85 ± 0.59^{a}	1.04 ± 0.13^{a}	1.29 ± 0.22^{a}	38.72 ± 12.89^{b}
Plasma glutamic acid (10^{-3} μ g/ml plasma)	80.2 ± 12.3^{a}	18.7 ± 3.7^{b}	16.1 ± 1.7^{b}	26.56 ± 2.0^{a}

Values are mean of triplicate analysis.
Superscript letter indicates significant difference between dietary treatment

A real-time qPCR analysis of the kidney revealed significant differences in inducible nitric oxide synthase (iNOS) and interleukin-1-beta Il-1β levels (Figure 2). Significantly higher iNOS fold-changes were only found between +CIT and CTRL, while significantly higher Il-1β fold-changes were found between +ORN and CTRL. There was no significant difference in arginase 2 expression in the kidney (figure 4.3.2).

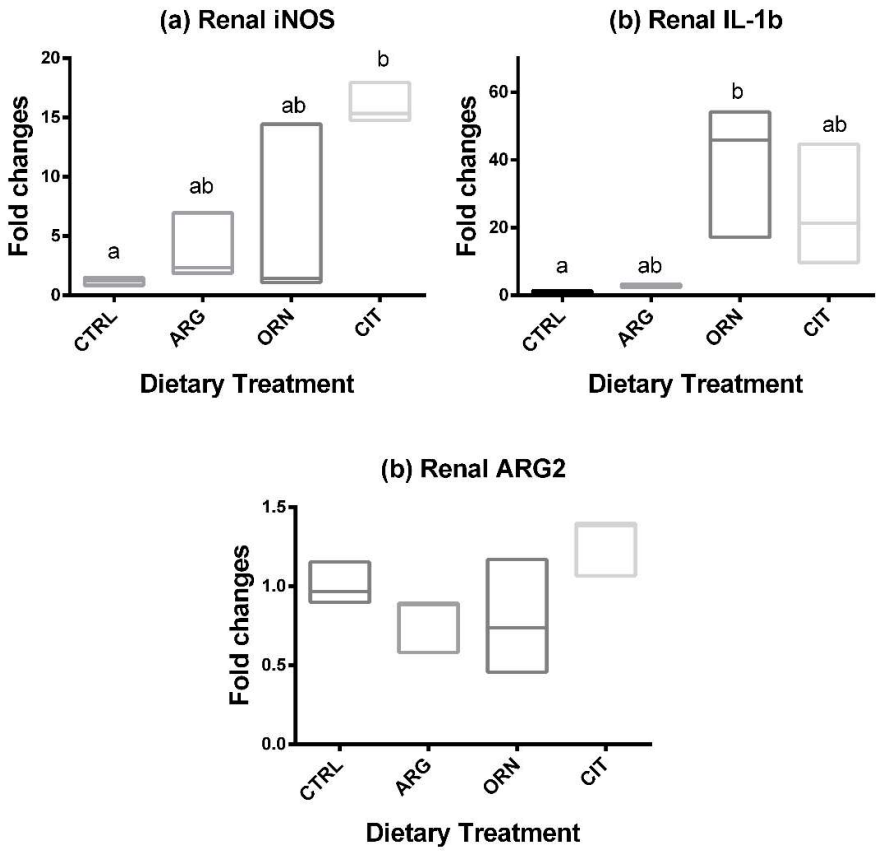

Figure 4.3.2. relative expression of iNOS **(a)**, interleukin-1-beta **(b)**, and arginase 2 **(c)**, in the kidney of rainbow trout at 24 hours after disease challenge with *Vibrio anguillarum* after fed 30 days fed with dietary supplementation of arginine, ornithine, and citrulline.

4.4. Discussion

Postprandial amino acid levels

Several studies on fish have shown that arginine supplementation increases postprandial plasma arginine levels (Barziza et al. 2000; Buentello and Gatlin III 2000; Alam et al. 2002; Tesser et al. 2005), and we found that plasma arginine levels in +ARG were still high even at 30 hours postprandial. To the best of our knowledge, this is the first time that citrulline supplementation in fish has resulted in similar observable plasma arginine levels as arginine supplementation, even 30 hours after feeding. Interestingly, the fish in +CIT had

higher plasma arginine levels at 30 hours postprandial than those in CTRL. Moreover, at the same postprandial timepoint, the plasma arginine level in +CIT was as high as that in +ARG. It is possible that the increased plasma arginine level in +CIT resulted from the conversion of citrulline to arginine through the ornithine-urea cycle. Overall, we found that only arginine or citrulline supplementation could increase plasma arginine levels.

Plasma arginine originates from several sources: protein that is consumed during feeding, protein catabolism and turnover, and endogenous arginine synthesis (Morris 2006; Deutz 2008). It is probable that the increased plasma arginine level in +ARG was caused by dietary arginine supplementation, which supports the results of studies on humans (Castillo et al. 1993), pigs (Bruins et al. 2002), *I. punctatus* (Pohlenz et al. 2013), and flounder *Paralichthys olivaceus* (Alam et al. 2002). It is probable that the plasma arginine increase in +CIT was caused by the conversion of citrulline into arginine through endogenous arginine pathways, which are interorgan pathways that involve the synthesis of citrulline from ornithine in the intestine, the release of citrulline into the blood, and the conversion of citrulline into arginine in the kidney (Van De Poll et al. 2007; Deutz 2008). This pathway in *O. mykiss* was discussed by Chiu et al. (1986), who found that citrulline can be used as a precursor in arginine synthesis. Interestingly, the plasma arginine increase in +CIT occurred at a later timepoint than in +ARG, possibly because the plasma arginine levels in +CIT were not affected by feeding activity, as they were in +ARG. This difference highlights the importance of using multiple postprandial observation timepoints when measuring postprandial plasma amino acid levels, particularly when the amino acid targeted is involved in multiple pathways and can be endogenously produced.

Regarding postprandial plasma ornithine levels, an increase in plasma ornithine levels was only found in +ORN at 6, 15, and 30 hours postprandial. The maximum plasma ornithine level was observed at 15 hours postprandial, and at 30 hours postprandial, some of the plasma

ornithine had already been utilized by the body. Furthermore, a two-way ANOVA found no significant difference between CTRL, +ARG, and +CIT. Ornithine is an arginine catabolite that is obtained through the actions of the enzyme arginase. In the intestines of terrestrial animals, ornithine is a reversible, intermediate compound between proline and arginine through P5C (Wu 1997; Wu et al. 2011), and links the glutamine pathway with arginine through glutamate and P5C (Wu and Morris 1998). While ornithine can be synthesized from glutamine and proline, increased plasma ornithine concentrations are only observed after ornithine supplementation (Ewtushik et al. 2000; Sugino et al. 2008), with the increased plasma arginine levels caused by arginine supplementation (Daly et al. 1988; Castillo et al. 1995; Wilson et al. 2007), or after supplementation with compounds that elevate plasma arginine concentrations, such as citrulline and N-carbamylglutamate (Osowska 2004; Collins et al. 2007; Wu et al. 2010). However, we found that plasma ornithine levels in +ARG and +CIT only tended to be higher than those in CTRL at all postprandial timepoints, and there was no significant increase in plasma ornithine due to arginine or citrulline supplementation.

An increase in postprandial plasma citrulline levels was only found in +CIT. Citrulline obtained from food is taken up by a transporter (Vadgama and Evered 1992). In terrestrial animals, citrulline has an exceptionally high bioavailability, a high intestinal absorption velocity, and an ability to be taken up by various amino acid transporters (Bahri et al. 2008; Moinard et al. 2008; Cynober et al. 2010). These facts could explain the high loading capacity of plasma citrulline in +CIT (800-times its basal state) compared to that of plasma arginine in +ARG (3.8-times its basal state) and that of plasma ornithine in +ORN (21.7-times its basal state).

While it is probable that citrulline in the postprandial plasma of the fish in +CIT was obtained from dietary supplementation, it can also be produced by two enzymes: ornithine carbamoyl transferase (OCT) and NOS. OCT is commonly found in the intestinal mucosa and

livers of terrestrial animals (Ryall et al. 1985; Curis et al. 2005), converts ornithine into citrulline, and is part of the ornithine-urea cycle. In the intestine, citrulline can be produced *de novo* from glutamine, proline, or arginine, and plays a role in regulating nitrogen homeostasis (Wu 1997; Wu and Morris 1998; Moinard and Cynober 2007; Cynober et al. 2010; Wu et al. 2011). Citrulline, and nitric oxide, can also be synthesized from arginine by NOS. This enzyme is found in neural cells, macrophages, and endothelial cells, and is classified as nNOS, iNOS, and eNOS, respectively (Curis et al. 2005). iNOS is a type of nitric oxide that can be induced by stimulatory cytokines and infections, and plays an important role in innate immunity (Bogdan 2001; Aktan 2004). However, it is unlikely that plasma citrulline originates in the nitric oxide pathway, because the production of nitric oxide is strictly regulated as it can induce apoptosis (Mori 2007). In humans and other terrestrial animals, circulating citrulline can be taken up by cells that metabolize arginine into nitric oxide, or it can be converted into arginine in the kidney, enter the blood, and increase circulating arginine levels. The potential use of citrulline to increase plasma arginine production has been suggested previously (Bahri et al. 2013), including in rainbow trout (Chiu et al. 1986). In the present study, it was not only shown that citrulline can produce similar plasma arginine levels as +ARG at 30 hours postprandial, but also that it can be more effective in increasing circulating arginine levels than +ARG, because plasma citrulline levels in +CIT were very high.

Feeding and disease challenge

Survival was higher in +CIT than in CTRL after an intraperitoneal injection of *V. anguillarum*, and kidney iNOS expression was also higher in +CIT than in CTRL. This could indicate the activation of a M1 macrophage, in which iNOS is specifically expressed (Wiegertjes et al. 2016). A review of macrophage polarization in fish suggested that the activation of M1/classically activated macrophages is characterized by cell-mediated immunity, killing intracellular pathogens such as *V. anguillarum* (Boesen et al. 2001; Wiegertjes et al.

2016). Therefore, citrulline increased iNOS expression in the kidney and killed *V. anguillarum*, which eventually increased fish survival.

Extracellular arginine is important for maintaining nitric oxide production (Wu and Morris 1998), and we found that arginine levels in the plasma and kidneys of +CIT fish were significantly higher than those in the plasma and kidneys of +ORN and CTRL fish, respectively, and +ARG and +ORN fish, respectively. Furthermore, citrulline levels in the plasma and kidneys of +CIT fish were significantly higher than those in the plasma and kidneys of fish in the other treatments. In terrestrial animals, supplementation with 80 or 120 μM of citrulline can produce more nitric oxide than supplementation with arginine at a similar concentration. Rapovy et al. (2015) demonstrated that supplementation with L-citrulline alone or with both L-arginine and L-citrulline results in higher nitric oxide production than with supplementation with only L-arginine. Therefore, it is possible that both arginine and citrulline in the kidney play important roles in the sustainable production of nitric oxide.

The renal expression of Il-1β was significantly higher in +ORN than in CTRL. Il-1β is a pro-inflammatory cytokine that plays an important role in the innate immune system (Dinarello 1996). It is possible that high IL-1β expression is related to innate immune system activation (Gioacchini et al. 2008; Awad et al. 2011); however, further studies are required to determine an association between high Il-1β expression and dietary ornithine supplementation, because arg2 expression did not significantly differ between CTRL and +ORN.

In summary, dietary supplementation of rainbow trout with 2% citrulline increased plasma arginine production to a similar level as arginine supplementation. Furthermore, when challenged with *V. anguillarum*, 2% citrulline supplementation increased survival compared to CTRL, increased post-injection plasma and renal arginine and citrulline levels, and significantly increased iNOS expression. Therefore, citrulline supplementation is better than

arginine supplementation in enhancing the immune performance of rainbow trout when challenged with *V. anguillarum*.

CHAPTER 5

V. General Summary

For general conclusion, arginine supplementation did not enhance growth performance and feed efficiency based on 9 weeks feeding trial on rainbow trout. Furthermore, using postprandial study, it seems that supplementation with different level of dietary arginine increase production of plasma urea and imply negative association with endogenous arginine synthesis through lower production of plasma citrulline especially at 18 hours postprandial compared to control treatment that has sufficient level of dietary arginine. In the case of plasma ornithine of this postprandial study, positive association between dietary arginine supplementation and plasma ornithine was found.

Since, it was found that arginine supplementation did not produce significant difference found in growth, no higher arginine composition found in muscle of rainbow trout feed with arginine supplemented group and increase plasma urea production. These findings raise possibility to use arginine catabolite to emulate the beneficial properties of arginine. One of potential compounds are ornithine and citrulline. In the first chapter, it is stated that in the endogenous arginine synthesis, ornithine can be converted into citrulline in intestine and citrulline will then converted into arginine in kidney. Thus, supplementation with ornithine and citrulline was aimed to utilize endogenous arginine pathways to increase production of plasma arginine.

In second experiment, result in postprandial study shows that supplementation with citrulline can increase arginine production compared to control treatment. However, supplementation with ornithine did not seems to increase plasma arginine production up until 30 hours postprandial observation. Furthermore, upon survival challenge, with *Vibrio anguillarum*, although higher post-injection plasma arginine was found in group that was supplemented with only 1% of citrulline in rainbow trout that was fed for 30 days, survival analysis did not show significant difference among all treatment. This result was also

corroborated with gene expression analysis in kidney who did not found any increase of iNOS in the group that was supplemented with 1% citrulline.

Upon result from the second experiment, the third experiment was aimed to evaluate whether citrulline or ornithine can be used to replace arginine as functional ingredients. Since previous research with only 1 % supplementation did not produce better result on bacterial challenge, the supplementation dosage in the third experiment was increase into 2% of diet.

On the third experiment, postprandial study shows that citrulline supplementation can produce plasma arginine at similar level with arginine supplementation. However, short feeding trial shows that treatment with 2% citrulline supplementation produce lower growth compared to treatment with 2% of arginine supplementation. While produce lower growth performance, treatment with 2% citrulline supplementation shows better survival upon challenge with *Vibrio anguillarum*. The effect of citrulline supplementation is also supported with higher plasma arginine, high free arginine and citrulline availability in kidney, and higher expression of inducible nitric oxide in kidney which indicate activation of M1 macrophage for intracellular immune activity.

References

Aktan F (2004) iNOS-mediated nitric oxide production and its regulation. Life Sci 75:639–653 . doi: 10.1016/j.lfs.2003.10.042

Alam MSS, Teshima S, Koshio S, Ishikawa M (2002) Arginine requirement of juvenile Japanese flounder *Paralichthys olivaceus* estimated by growth and biochemical parameters. Aquaculture 205:127–140 . doi: 10.1016/S0044-8486(01)00670-6

Andersen SM, Holen E, Aksnes A, Rønnestad I, Zerrahn J-E, Espe M (2013) Dietary arginine affects energy metabolism through polyamine turnover in juvenile Atlantic salmon (Salmo salar). Br J Nutr 110:1968–1977 . doi: 10.1017/S0007114513001402

Andersen SM, Taylor R, Holen E, Aksnes A, Espe M (2014) Arginine supplementation and exposure time affects polyamine and glucose metabolism in primary liver cells isolated from Atlantic salmon. Amino Acids 46:1225–1233 . doi: 10.1007/s00726-014-1684-4

Andersen SM, Waagbø R, Espe M (2015) Functional amino acids in fish nutrition, health and welfare. Front Biosci (Elite Ed) 8:143–169 . doi: 10.2741/757

Awad E, Mitchell WJ, Austin B (2011) Effect of dietary supplements on cytokine gene expression in rainbow trout, *Oncorhynchus mykiss* (Walbaum). J Fish Dis 34:629–634 . doi: 10.1111/j.1365-2761.2011.01271.x

Bahri S, Curis E, El Wafi F-Z, Aussel C, Chaumeil J-C, Cynober L, Zerrouk N (2008) Mechanisms and kinetics of citrulline uptake in a model of human intestinal epithelial cells. Clin Nutr 27:872–880 . doi: 10.1016/j.clnu.2008.08.003

Bahri S, Zerrouk N, Aussel C, Moinard C, Crenn P, Curis E, Chaumeil JC, Cynober L, Sfar S (2013) Citrulline: From metabolism to therapeutic use. Nutrition 29:479–484

Ball RO, Urschel KL, Pencharz PB (2007) Nutritional consequences of interspecies differences in arginine and lysine metabolism. J Nutr 137:1626S–1641S . doi: 10.1093/jn/137.6.1626S

Barziza DE, Buentello JA, Gatlin III DM (2000) Dietary arginine requirement of juvenile red drum (*Sciaenops ocellatus*) based on weight gain and feed efficiency. J Nutr 130:1796–1799

Benoit M, Desnues B, Mege J-L (2008) Macrophage polarization in bacterial infections. J Immunol 181:3733–3739 . doi: 10.4049/jimmunol.181.6.3733

Boesen HT, Larsen MH, Larsen JL, Ellis AE (2001) In vitro interactions between rainbow trout (*Oncorhynchus mykiss*) macrophages and *Vibrio anguillarum* serogroup O2a. Fish Shellfish Immunol 11:415–431 . doi: 10.1006/fsim.2000.0328

Bogdan C (2001) Nitric oxide and the immune response. Nat Immunol 2:907–16 . doi: 10.1038/ni1001-907

Bogdan C, Röllinghoff M, Diefenbach a (2000) The role of nitric oxide in innate immunity. Immunol Rev 173:17–26 . doi: 10.1034/j.1600-065X.2000.917307.x

Boonyoung S, Haga Y, Satoh S (2013) Preliminary study on effects of methionine hydroxy analog and taurine supplementation in a soy protein concentrate-based diet on the biological performance and amino acid composition of rainbow trout [<i>Oncorhynchus mykiss <i/> (Walbaum)]. Aquac Res. doi: 10.1111/j.1365-2109.2012.03138.x

Breuillard C, Curis E, Le Plénier S, Cynober L, Moinard C (2017) Nitric oxide production by peritoneal macrophages from aged rats: A short term and direct modulation by citrulline. Biochimie 133:66–73 . doi: 10.1016/j.biochi.2016.10.020

Breuillard C, Cynober L, Moinard C (2015) Citrulline and nitrogen homeostasis: An overview. Amino Acids 47:685–691 . doi: 10.1007/s00726-015-1932-2

Bruins MJ, Soeters PB, Lamers WH, Deutz NEP (2002) L-arginine supplementation in pigs decreases liver protein turnover and increases hindquarter protein turnover both during and after endotoxemia. Am J Clin Nutr 75:1031–44

Buentello J, Gatlin III DM (1999) Nitric oxide production in activated macrophages from

channel catfish (*Ictalurus punctatus*): influence of dietary arginine and culture media. Aquaculture 179:513–521 . doi: 10.1016/S0044-8486(99)00184-2

Buentello JA, Gatlin III DM (2000) The dietary arginine requirement of channel catfish (Ictalurus punctatus) is influenced by endogenous synthesis of arginine from glutamic acid. Aquaculture 188:311–321 . doi: 10.1016/S0044-8486(00)00344-6

Buentello JA, Gatlin III DM (2001a) Effects of Elevated Dietary Arginine on Resistance of Channel Catfish to Exposure to *Edwardsiella ictaluri*. J Aquat Anim Health 13:194–201 . doi: 10.1577/1548-8667(2001)013<0194:eoedao>2.0.co;2

Buentello JA, Gatlin III DM (2001b) Plasma citrulline and arginine kinetics in juvenile channel catfish, *Ictalurus punctatus*, given oral gabaculine. Fish Physiol Biochem 24:105–112 . doi: 10.1023/A:1011991312908

Burge CA, Mark Eakin C, Friedman CS, Froelich B, Hershberger PK, Hofmann EE, Petes LE, Prager KC, Weil E, Willis BL, Ford SE, Harvell CD (2014) Climate change influences on marine infectious diseases: implications for management and society. Ann Rev Mar Sci 6:249–277 . doi: 10.1146/annurev-marine-010213-135029

Cabello FC (2006) Heavy use of prophylactic antibiotics in aquaculture: A growing problem for human and animal health and for the environment. Environ. Microbiol. 8:1137–1144

Castillo L, Chapman TE, Sanchez M, Yu YM, Burke JF, Ajami AM, Vogt J, Young VR (1993) Plasma arginine and citrulline kinetics in adults given adequate and arginine-free diets. Proc Natl Acad Sci U S A 90:7749–53 . doi: 10.1073/pnas.90.16.7749

Castillo L, Sanchez M, Chapman TE, Ajami A, Burke JF, Young VR (1994) The plasma flux and oxidation rate of ornithine adaptively decline with restricted arginine intake. Proc Natl Acad Sci 91:6393–6397 . doi: 10.1073/pnas.91.14.6393

Castillo L, Sánchez M, Tannenbaum SR, Young VR (1995) Plasma arginine, citrulline and ornithine kinetics in adults, with Observations on nitric oxide synthesis. Crit Care Med

23:A252 . doi: 10.1097/00003246-199501001-00445

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

Cheng Z, Buentello JA, Gatlin III DM (2011) Effects of dietary arginine and glutamine on growth performance, immune responses and intestinal structure of red drum, <i>Sciaenops ocellatus<i/>. Aquaculture 319:247–252 . doi: 10.1016/j.aquaculture.2011.06.025

Cheng Z, Gatlin DM, Buentello A (2012a) Dietary supplementation of arginine and/or glutamine influence growth performance, immune responses and intestinal morphology of hybrid striped bass (*Morone chrysops* × *Morone saxatilis*). Aquaculture

Cheng Z, Gatlin Iii DM, Buentello A (2012b) Dietary supplementation of arginine and/or glutamine influences growth performance, immune responses and intestinal morphology of hybrid striped bass (Morone chrysops × Morone saxatilis). Aquaculture 362–363:39–43 . doi: http://dx.doi.org/10.1016/j.aquaculture.2012.07.015

Cheung CW, Cohen NS, Raijman L (1989) Channeling of urea cycle intermediates in situ in permeabilized hepatocytes. J Biol Chem 264:4038–4044

Chiu YN, Austic RE, Rumsey AL, Rumsey GL (1986) Urea cycle activity and arginine formation in rainbow trout (*Salmo gairdneri*). J Nutr 116:1640–1650

Cochrane K, de Young C, Soto D, Bahri T (2009) Climate change implications for fisheries and aquaculture: overview of current scientific knowledge. FAO Fish Aquac Tech Pap 1–212 . doi: ISSN 2070-7010

Cohen P., Hayano M (1946) The conversion of citrulline to arginine (transimination) by tissue slices and homogenates. J Biol Chem 166:239–250

Collins JK, Wu G, Perkins-Veazie P, Spears K, Claypool PL, Baker RA, Clevidence BA (2007) Watermelon consumption increases plasma arginine concentrations in adults. Nutrition 23:261–266 . doi: 10.1016/j.nut.2007.01.005

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

Curis E, Nicolis I, Moinard C, Osowska S, Zerrouk N, Bénazeth S, Cynober L (2005) Almost all about citrulline in mammals. Amino Acids 29:177–205 . doi: 10.1007/s00726-005-0235-4

Cynober L (1994) Can arginine and ornithine support gut functions? Gut 35:S42–S45 . doi: 10.1136/gut.35.1_Suppl.S42

Cynober L, Moinard C, De Bandt JP (2010) The 2009 ESPEN Sir David Cuthbertson. Citrulline: A new major signaling molecule or just another player in the pharmaconutrition game? Clin Nutr 29:545–551 . doi: 10.1016/j.clnu.2010.07.006

Daly JM, Reynolds J, Thom A, Kinsley L, Dietrick-Gallagher M, Shou J, Ruggieri B (1988) Immune and metabolic effects of arginine in the surgical patient. Ann Surg 208:512–23 . doi: 10.1097/00000658-198810000-00013

Defoirdt T, Sorgeloos P, Bossier P (2011) Alternatives to antibiotics for the control of bacterial disease in aquaculture. Curr Opin Microbiol 14:251–258 . doi: 10.1016/j.mib.2011.03.004

Deutz NEP (2008) The 2007 ESPEN Sir David Cuthbertson Lecture: Amino acids between and within organs. The glutamate-glutamine-citrulline-arginine pathway. Clin Nutr 27:321–327 . doi: 10.1016/j.clnu.2008.03.010

Dinarello CA (1996) Biologic basis for interleukin-1 in disease. Blood 87:2095–147 . doi:

10.2302/kjm.43.131

Ewtushik AL, Bertolo RFP, Ball RO (2000) Intestinal development of early-weaned piglets receiving diets supplemented with selected amino acids or polyamines

Forlenza M, Fink IR, Raes G, Wiegertjes GF (2011) Heterogeneity of macrophage activation in fish. Dev Comp Immunol 35:1246–1255 . doi: 10.1016/j.dci.2011.03.008

Förstermann U, Kleinert H (1995) Nitric oxide synthase: expression and expressional control of the three isoforms. Naunyn Schmiedebergs Arch Pharmacol 352:351–364 . doi: 10.1007/BF00172772

Förstermann U, Münzel T (2006) Basic Science for Clinicians Endothelial Nitric Oxide Synthase in Vascular Disease. 1708–1715 . doi: 10.1161/CIRCULATIONAHA.105.602532

Fournier V, Gouillou-Coustans M. F, Métailler R, Vachot C, Moriceau J, Le Delliou H, Huelvan C, Desbruyeres E, Kaushik S. J (2003) Excess dietary arginine affects urea excretion but does not improve N utilisation in rainbow trout *Oncorhynchus mykiss* and turbot *Psetta maxima*. Aquaculture 217:559–576 . doi: 10.1016/S0044-8486(02)00420-9

Garthwaite J, Boulton CL (1995) Nitric oxide signaling in the central nervous system. Annu Rev Physiol 57:683–706 . doi: 10.1146/annurev.ph.57.030195.003343

Gioacchini G, Smith P, Carnevali O (2008) Effects of Ergosan on the expression of cytokine genes in the liver of juvenile rainbow trout (*Oncorhynchus mykiss*) exposed to enteric red mouth vaccine. Vet Immunol Immunopathol 123:215–222 . doi: 10.1016/j.vetimm.2008.01.037

Gogoi M, Datey A, Wilson KT, Chakravortty D (2016) Dual role of arginine metabolism in establishing pathogenesis. Curr Opin Microbiol 29:43–48 . doi: 10.1016/J.MIB.2015.10.005

Harvell CD, Kim K, Burkholder JM, Colwell RR, Epstein PR, Grimes DJ, Hofmann EE,

Lipp EK, Osterhaus ADME, Overstreet RM, Porter JW, Smith GW, Vasta GR (1999) Emerging Marine Disease--Climate Links and Anthropogenic Factors. Science (80-) 285:1505–1510 . doi: 10.1126/science.285.5433.1505

Harvell CD, Mitchell CE, Ward JR, Altizer S, Dobson AP, Ostfeld RS, Samuel MD (2002) Climate warming and disease risks for terrestrial and marine biota. Science 296:2158–2162 . doi: 10.1126/science.1063699

Hernández AJ, Satoh S, Kiron V (2012) Supplementation of citric acid and amino acid chelated trace elements in low-fish meal diet for rainbow trout affect growth and phosphorus utilization. J World Aquac Soc 43:688–696 . doi: 10.1111/j.1749-7345.2012.00589.x

Holen E, Espe M, Andersen SM, Taylor R, Aksnes A, Mengesha Z, Araujo P (2014) A co culture approach show that polyamine turnover is affected during inflammation in Atlantic salmon immune and liver cells and that arginine and LPS exerts opposite effects on p38MAPK signaling. Fish Shellfish Immunol 37:286–298 . doi: 10.1016/j.fsi.2014.02.004

IPCC (2007) Climate Change 2007: impacts, adaptation and vulnerability: contribution of Working Group II to the fourth assessment report of the Intergovernmental Panel

Joerink M, Forlenza M, Ribeiro CMS, de Vries BJ, Savelkoul HFJ, Wiegertjes GF (2006) Differential macrophage polarisation during parasitic infections in common carp (Cyprinus carpio L.). Fish Shellfish Immunol 21:561–571 . doi: 10.1016/j.fsi.2006.03.006

Lee J, Ryu H, Ferrante RJ, Morris SM, Ratan RR (2003) Translational control of inducible nitric oxide synthase expression by arginine can explain the arginine paradox. Proc Natl Acad Sci 100:4843–4848 . doi: 10.1073/pnas.0735876100

Levillain O, Hus-Citharel A, Morel F, Bankir L (1990) Localization of arginine synthesis

along rat nephron. Am J Physiol 259:F916-23

Li P, Mai K, Trushenski J, Wu G (2009) New developments in fish amino acid nutrition: Towards functional and environmentally oriented aquafeeds. Amino Acids 37:43–53 . doi: 10.1007/s00726-008-0171-1

Marcogliese DJ (2008) The impact of climate change on the parasites and infectious diseases of aquatic animals. Rev Sci Tech 27:467–84

Mateo RD, Wu G, Bazer FW, Park JC, Shinzato I, Kim SW (2007) Dietary L-arginine supplementation enhances the reproductive performance of gilts. J Nutr 137:652–656 . doi: 137/3/652 [pii]

Mills CD, Lenz LL, Ley K (2015) Macrophages at the fork in the road to health or disease. Front Immunol 6:59 . doi: 10.3389/fimmu.2015.00059

Moinard C, Cynober L (2007) Citrulline: a new player in the control of nitrogen homeostasis. J Nutr 137:1621S–1625S

Moinard C, Nicolis I, Neveux N, Darquy S, Bénazeth S, Cynober L (2008) Dose-ranging effects of citrulline administration on plasma amino acids and hormonal patterns in healthy subjects: the Citrudose pharmacokinetic study. Br J Nutr 99:855–862 . doi: 10.1017/S0007114507841110

Mommsen TP, Moon TW, Plisetskaya EM (2001) Effects of arginine on pancreatic hormones and hepatic metabolism in rainbow trout. Physiol Biochem Zool 74:668–678 . doi: PBZ000127 [pii]\r10.1086/322924

Mori M (2007) Regulation of Nitric Oxide Synthesis and Apoptosis by Arginase and Arginine Recycling. J Nutr. doi: 137/6/1616S [pii]

Morris SM (2006) Arginine: beyond protein. Am J Clin Nutr 83:508S--512S

Morris SM (2004) Recent advances in arginine metabolism. Curr. Opin. Clin. Nutr. Metab. Care 7:45–51

Nathan CF, Hibbs JB (1991) Role of nitric oxide synthesis in macrophage antimicrobial activity. Curr Opin Immunol 3:65–70 . doi: 10.1016/0952-7915(91)90079-G

Norris KA, Schrimpf JE, Flynn JL, Morris SM (1995) Enhancement of macrophage microbicidal activity: Supplemental arginine and citrulline augment nitric oxide production in murine peritoneal macrophages and promote intracellular killing of Trypanosoma cruzi. Infect Immun 63:2793–2796

Oliva-Teles A, Peres H, Kaushik S (2017) Dietary arginine supplementation does not improve nutrient utilisation in gilthead seabream. Aquaculture 479:690–695 . doi: 10.1016/j.aquaculture.2017.07.016

Osowska S (2004) Citrulline increases arginine pools and restores nitrogen balance after massive intestinal resection. Gut 53:1781–1786 . doi: 10.1136/gut.2004.042317

Perrella M a, Yoshizumi M, Fen Z, Tsai JC, Hsieh CM, Kourembanas S, Lee ME (1994) Transforming growth factor-beta 1, but not dexamethasone, down-regulates nitric-oxide synthase mRNA after its induction by interleukin-1 beta in rat smooth muscle cells. J Biol Chem 269:14595–14600

Pohlenz C, Buentello JA, Helland SJ, Gatlin III DM (2014) Effects of dietary arginine supplementation on growth, protein optimization and innate immune response of channel catfish Ictalurus punctatus (Rafinesque 1818). Aquac Res 45:491–500 . doi: 10.1111/j.1365-2109.2012.03252.x

Pohlenz C, Buentello JA, Miller T, Small BC, Mackenzie DS, Gatlin III DM (2013) Effects of dietary arginine on endocrine growth factors of channel catfish, *Ictalurus punctatus*. Comp Biochem Physiol A Mol Integr Physiol 166:215–21 . doi: 10.1016/j.cbpa.2013.06.016

Qualls JE, Subramanian C, Rafi W, Smith AM, Balouzian L, Defreitas AA, Shirey KA, Reutterer B, Kernbauer E, Stockinger S, Decker T, Miyairi I, Vogel SN, Salgame P,

Rock CO, Murray PJ (2012) Sustained generation of nitric oxide and control of mycobacterial infection requires argininosuccinate synthase 1. Cell Host Microbe 12:313–323 . doi: 10.1016/j.chom.2012.07.012

Rapovy SM, Zhao J, Bricker RL, Schmidt SM, Setchell KDR, Qualls JE (2015) Differential requirements for l-citrulline and l-arginine during antimycobacterial macrophage activity. J Immunol 195:3293–3300 . doi: 10.4049/jimmunol.1500800

Rath M, Müller I, Kropf P, Closs EI, Munder M (2015) Metabolism via arginase or nitric oxide synthase: two competing arginine pathways in macrophages. In: Mills CD, Lenz LL, Ley K (eds) M1/M2 Macrophages: The Arginine Fork in the Road to Health and Disease. Frontiers Media SA, p 13

Ruiz-Feria CA, Kidd MT, Wideman RF (2001) Plasma Levels of Arginine, Ornithine, and Urea and Growth Performance of Broilers Fed Supplemental L-Arginine During Cool Temperature Exposure. Poult Sci 80:358–369 . doi: 10.1093/ps/80.3.358

Ryall J, Nguyen M, Bendayan M, Shore GC (1985) Expression of nuclear genes encoding the urea cycle enzymes, carbamoyl-phosphate synthetase I and ornithine carbamoyl transferase, in rat liver and intestinal mucosa. Eur J Biochem 152:287–292 . doi: 10.1111/j.1432-1033.1985.tb09196.x

Schmittner A (2005) Decline of the marine ecosystem caused by a reduction in the Atlantic overturning circulation. Nature 434:628–33 . doi: 10.1038/nature03476

Schuhmacher A, Wax C, Gropp JM (1997) Plasma amino acids in rainbow trout (*Oncorhynchus mykiss*) fed intact protein or a crystalline amino acid diet. Aquaculture 151:15–28 . doi: 10.1016/S0044-8486(96)01502-5

Schwedhelm E, Maas R, Freese R, Jung D, Lukacs Z, Jambrecina A, Spickler W, Schulze F, Böger RH (2008) Pharmacokinetic and pharmacodynamic properties of oral L-citrulline and L-arginine: Impact on nitric oxide metabolism. Br J Clin Pharmacol 65:51–59 . doi:

10.1111/j.1365-2125.2007.02990.x

Shepherd CJ, Jackson AJ (2013) Global fishmeal and fish-oil supply: inputs, outputs and markets. J Fish Biol 83:1046–66 . doi: 10.1111/jfb.12224

Shi HP, Fishel RS, Efron DT, Williams JZ, Fishel MH, Barbul A (2002a) Effect of supplemental ornithine on wound healing. J Surg Res 106:299–302 . doi: 10.1006/jsre.2002.6471

Shi HP, Fishel RS, Efron DT, Williams JZ, Fishel MH, Barbul A (2002b) Effect of supplemental ornithine on wound healing. J Surg Res 106:299–302 . doi: 10.1006/jsre.2002.6471

Shinn A, Pratoomyot J, Bron JE, Paladini G, Brooker E, Brooker AJ (2015) Economic impacts of aquatic parasites on global finfish production. Glob Aquac Advocate 82–84

Solomon S, Daniel JS, Sanford TJ, Murphy DM, Plattner G-K, Knutti R, Friedlingstein P (2010) Persistence of climate changes due to a range of greenhouse gases. Proc Natl Acad Sci U S A 107:18354–9 . doi: 10.1073/pnas.1006282107

Sugino T, Shirai T, Kajimoto Y, Kajimoto O (2008) l-Ornithine supplementation attenuates physical fatigue in healthy volunteers by modulating lipid and amino acid metabolism. Nutr Res 28:738–743 . doi: 10.1016/j.nutres.2008.08.008

Talbot C, Hole R (1994) Fish diets and the control of eutrophication resulting from aquaculture. J Appl Ichthyol 10:258–270 . doi: 10.1111/j.1439-0426.1994.tb00165.x

Tavares-Dias M, Martins ML (2017) An overall estimation of losses caused by diseases in the Brazilian fish farms. J Parasit Dis 1–6 . doi: 10.1007/s12639-017-0938-y

Tesser MB, Terjesen BF, Zhang Y, Portella MC, Dabrowski K (2005) Free- and peptide-based dietary arginine supplementation for the South American fish pacu (*Piaractus mesopotamicus*). Aquac Nutr 11:443–453 . doi: 10.1111/j.1365-2095.2005.00373.x

Tulli F, Vachot C, Tibaldi E, Fournier V, Kaushik SJ (2007) Contribution of dietary arginine

to nitrogen utilisation and excretion in juvenile sea bass (*Dicentrarchus labrax*) fed diets differing in protein source. Comp Biochem Physiol - A Mol Integr Physiol 147:179–188 . doi: 10.1016/j.cbpa.2006.12.036

Vadgama J V, Evered DF (1992) Characteristics of L-citrulline transport across rat small intestine in vitro. 32:472–478

Vallance P (2001) GENERAL CARDIOLOGY: Endothelial function and nitric oxide: clinical relevance. Heart 85:342–350 . doi: 10.1136/heart.85.3.342

Van De Poll MCG, Siroen MPC, Van Leeuwen PAM, Soeters PB, Melis GC, Boelens PG, Deutz NEP, Dejong CHC (2007) Interorgan amino acid exchange in humans: Consequences for arginine and citrulline metabolism. Am J Clin Nutr 85:167–172

Wakabayashi Y, Henslee JG, Jones ME (1983) Pyrroline-5-carboxylate synthesis from glutamate by rat intestinal mucosa. Subcellular localization and temperature stability. J Biol Chem 258:3873–3882

Wakabayashi Y, Jones ME (1983) Pyrroline-5-carboxylate synthesis from glutamate by rat intestinal mucosa. J Biol Chem 258:3865–3872

Wang B, Feng L, Jiang W-D, Wu P, Kuang S-Y, Jiang J, Tang L, Tang W-N, Zhang Y-A, Liu Y, Zhou X-Q (2015a) Copper-induced tight junction mRNA expression changes, apoptosis and antioxidant responses via NF-κB, TOR and Nrf2 signaling molecules in the gills of fish: Preventive role of arginine. Aquat Toxicol 158:125–137 . doi: 10.1016/j.aquatox.2014.10.025

Wang B, Liu Y, Feng L, Jiang W-D, Kuang S-Y, Jiang J, Li S-H, Tang L, Zhou X-Q (2015b) Effects of dietary arginine supplementation on growth performance, flesh quality, muscle antioxidant capacity and antioxidant-related signalling molecule expression in young grass carp (*Ctenopharyngodon idella*). Food Chem 167:91–99 . doi: 10.1016/j.foodchem.2014.06.091

Watford M (1991) The urea cycle: a two-compartment system. Essays Biochem 26:49–58

Wiegertjes GF, Wentzel AS, Spaink HP, Elks PM, Fink IR (2016) Polarization of immune responses in fish: The 'macrophages first' point of view. Mol Immunol 69:146–156 . doi: 10.1016/J.MOLIMM.2015.09.026

Wilson AM, Harada R, Nair N, Balasubramanian N, Cooke JP (2007) L-arginine supplementation in peripheral arterial disease: No benefit and possible harm. Circulation 116:188–195 . doi: 10.1161/CIRCULATIONAHA.106.683656

Windmueller HG, Spaeth AE (1974) Uptake and metabolism of plasma glutamine by the small intestine. J Biol Chem 249:5070–5079 . doi: 10.1074/jbc.M601576200

Wu G (1997) Synthesis of citrulline and arginine from proline in enterocytes of postnatal pigs. Am J Physiol 272:G1382–G1390

Wu G (1998) Intestinal mucosal amino acid catabolism. J Nutr 128:1249–52

Wu G, Bazer F, Davis T, Kim S, Li P, Marc Rhoads J, Carey Satterfield M, Smith S, Spencer T, Yin Y, Guoyao W (2009) Arginine metabolism and nutrition in growth, health and disease. Aminoacids 37:153–168 . doi: 10.1007/s00726-008-0210-y.Arginine

Wu G, Bazer FW, Burghardt RC, Johnson GA, Kim SW, Knabe DA, Li P, Li X, McKnight JR, Satterfield MC, Spencer TE (2011) Proline and hydroxyproline metabolism: Implications for animal and human nutrition. Amino Acids 40:1053–1063 . doi: 10.1007/s00726-010-0715-z

Wu G, Morris SM (1998) Arginine metabolism: nitric oxide and beyond. Biochem J 336:1–17 . doi: 10.1042/bj3360001

Wu X, Ruan Z, Gao Y, Yin Y, Zhou X, Wang L, Geng M, Hou Y, Wu G (2010) Dietary supplementation with l-arginine or N-carbamylglutamate enhances intestinal growth and heat shock protein-70 expression in weanling pigs fed a corn- and soybean meal-based diet. Amino Acids 39:831–839 . doi: 10.1007/s00726-010-0538-y

Wyss M, Kaddurah-Daouk R (2000) Creatine and Creatinine Metabolism. Physiol Rev 80:1107–1213 . doi: 10.1016/S1286-0115(06)74505-2

Yao K, Guan S, Li T, Huang R, Wu G, Ruan Z, Yin Y (2011) Dietary l-arginine supplementation enhances intestinal development and expression of vascular endothelial growth factor in weanling piglets. Br J Nutr 105:703–709 . doi: 10.1017/S000711451000365X

Yao K, Yin Y, Chu W, Liu Z, Deng D, Li T, Huang R, Zhang J, Tan B, Wang W, Wu G (2008) Dietary Arginine Supplementation Increases mTOR Signaling Activity in Skeletal Muscle of Neonatal Pigs. J Nutr 138:867–872 . doi: 10.1093/jn/138.5.867

Zhou Q, Jin M, Elmada ZC, Liang X, Mai K (2015) Growth, immune response and resistance to Aeromonas hydrophila of juvenile yellow catfish, *Pelteobagrus fulvidraco* , fed diets with different arginine levels. Aquaculture 437:84–91 . doi: 10.1016/j.aquaculture.2014.11.030

APPENDIXES

Chapter II:

Initial weight

Anova

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1.489238889	2	0.744619	0.248682	0.787485	5.143253
Within Groups	17.96556667	6	2.994261			
Total	19.45480556	8				

Final Weight

Anova

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*
Between Groups	182.1977	2	91.09885	0.980838	0.427997
Within Groups	557.2716	6	92.8786		
Total	739.4693	8			

Weight gain

Anova

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*
Between Groups	306.9	2	153.4	0.5390	0.6092
Within Groups	1708	6	284.7		
Total	2015	8			

Feed intake

Anova

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	35968.99	2	17984.5	1.288209	0.342402	5.143253
Within Groups	83765.09	6	13960.85			
Total	119734.1	8				

Feeding efficiency

Anova

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*
Treatment (between columns)	33.07	2	16.54	F (2, 6) = 0.6825	P = 0.5407
Residual (within columns)	145.4	6	24.23		
Total	178.4	8			

Protein efficiency ratio

Anova

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*
Treatment (between columns)	0.1313	2	0.06567	5.522	0.0436
Residual (within columns)	0.07135	6	0.01189		
Total	0.2027	8			

Tukey's multiple comparison

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary
CTRL vs. 3.89A	0.04601	-0.2272 to 0.3192	No	ns
CTRL vs. 5.64A	0.2761	0.002943 to 0.5493	Yes	*
3.89A vs. 5.64A	0.2301	-0.04306 to 0.5033	No	ns

Survival

Raw data

CTRL	**3.89A**	**5.64A**
95	100	90
100	95	95
100	95	90

Anova

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*
Between Groups	72.22222	2	36.11111	4.333333	0.068464
Within Groups	50	6	8.333333		
Total	122.2222	8			

Muscle protein content

Anova

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*
Treatment (between columns)	100.9	2	50.46	6.724	0.0294
Residual (within columns)	45.02	6	7.504		
Total	145.9	8			

Tukey's multiple comparison

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary
CTRL vs. 3.89A	-7.566	-14.43 to -0.7032	Yes	*
CTRL vs. 5.64A	-6.526	-13.39 to 0.3369	No	ns
3.89A vs. 5.64A	1.040	-5.823 to 7.903	No	ns

Muscle lipid content

Anova

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*
Treatment (between columns)	4.195	2	2.097	0.2433	0.7914
Residual (within columns)	51.72	6	8.619		
Total	55.91	8			

Amino acid of muscle

Threonine

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
				F (2, 6) =	P =
Treatment (between columns)	0.1409	2	0.07044	0.8929	0.4577
Residual (within columns)	0.4733	6	0.07888		
Total	0.6142	8			

Valine

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
				F (2, 6) =	P =
Treatment (between columns)	1.171	2	0.5854	1.707	0.2588
Residual (within columns)	2.057	6	0.3428		
Total	3.228	8			

Arginine

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
				F (2, 6) =	P =
Treatment (between columns)	0.5567	2	0.2783	2.707	0.1452
Residual (within columns)	0.6169	6	0.1028		
Total	1.174	8			

Isoleucine

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
			0.00943	F (2, 6) =	P =
Treatment (between columns)	0.01886	2	0	0.1605	0.8552
Residual (within columns)	0.3524	6	0.05874		
Total	0.3713	8			

Leucine

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
				F (2, 6) =	P =
Treatment (between columns)	0.1729	2	0.08644	0.4317	0.6681
Residual (within columns)	1.202	6	0.2003		
Total	1.374	8			

Phenylalanine

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
				F (2, 6) =	P =
Treatment (between columns)	0.08779	2	0.04389	0.5912	0.5830
Residual (within columns)	0.4455	6	0.07425		
Total	0.5333	8			

Histidine

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
				F (2, 6) =	P =
Treatment (between columns)	0.02238	2	0.01119	0.3776	0.7007
Residual (within columns)	0.1778	6	0.02964		
Total	0.2002	8			

Lysine

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
				F (2, 6) =	P =
Treatment (between columns)	0.7805	2	0.3903	2.240	0.1877
Residual (within columns)	1.045	6	0.1742		
Total	1.826	8			

Tryptophan

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	0.01151	2	0.005755	F (2, 6) = 0.6087	P = 0.5745
Residual (within columns)	0.05672	6	0.009454		
Total	0.06823	8			

Methionine

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*
Between Groups	0.001544	2	0.000772127	0.88696095	0.459762
Within Groups	0.005223	6	0.000870531		
Total	0.006767	8			

Serine

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*
Between Groups	0.013603533	2	0.006802	2.610194	0.152908
Within Groups	0.015635081	6	0.002606		
Total	0.029238614	8			

Glutamate

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*
Between Groups	0.301081782	2	0.150541	2.806993	0.137883
Within Groups	0.321784001	6	0.053631		
Total	0.622865783	8			

Glycine

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*
Between Groups	0.01142072	2	0.00571	2.574522	0.155862
Within Groups	0.013308164	6	0.002218		
Total	0.024728884	8			

Alanine

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*
Between Groups	0.006122747	2	0.003061	0.770353	0.503753
Within Groups	0.023843934	6	0.003974		

Total	0.029966681	8			

Cysteine

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*
Between Groups	1.70218E-07	2	8.51E-08	0.05594	0.946083
Within Groups	9.12866E-06	6	1.52E-06		
Total	9.29888E-06	8			

Cystathionine

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*
Between Groups	8.86096E-05	2	4.43E-05	0.575701	0.590582
Within Groups	0.000461748	6	7.7E-05		
Total	0.000550357	8			

Tryrosine

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*
Between Groups	0.005495672	2	0.002748	1.817289	0.241521
Within Groups	0.009072315	6	0.001512		
Total	0.014567987	8			

Proline

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*
Between Groups	0.012444055	2	0.006222	2.433092	0.168354
Within Groups	0.015343507	6	0.002557		
Total	0.027787562	8			

Taurine

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*
Between Groups	0.003120888	2	0.00156	1.146507	0.378718
Within Groups	0.008166249	6	0.001361		
Total	0.011287137	8			

Aspartate

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*
Between Groups	0.010436418	2	0.005218	0.399643	0.68717
Within Groups	0.078343136	6	0.013057		
Total	0.088779554	8			

Plasma amino acid

Plasma arginine

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	10490	6	1748	F (6, 18) = 4.303	P = 0.0073
Time	14404	3	4801	F (3, 18) = 11.82	P = 0.0002
Column Factor	30230	2	15115	F (2, 6) = 25.14	P = 0.0012
Subjects (matching)	3607	6	601.1	F (6, 18) = 1.479	P = 0.2408
Residual	7315	18	406.4		

Dunnett's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary
0				
CTRL vs. 3.89A	-21.16	-62.08 to 19.76	No	ns
CTRL vs. 5.64A	-21.21	-62.13 to 19.72	No	ns
6				
CTRL vs. 3.89A	-66.53	-107.5 to -25.61	Yes	**
CTRL vs. 5.64A	-67.90	-108.8 to -26.98	Yes	**
12				
CTRL vs. 3.89A	-43.77	-84.69 to -2.847	Yes	*
CTRL vs. 5.64A	-109.1	-150.0 to -68.18	Yes	****
18				
CTRL vs. 3.89A	-13.84	-54.76 to 27.09	No	ns
CTRL vs. 5.64A	-85.68	-126.6 to -44.76	Yes	***

Dunnett's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary
CTRL				
0 vs. 6	-6.431	-48.62 to 35.76	No	ns
0 vs. 12	-10.01	-52.21 to 32.18	No	ns
0 vs. 18	-31.39	-73.58 to 10.80	No	ns
3.89A				
0 vs. 6	-51.80	-93.99 to -9.608	Yes	*
0 vs. 12	-32.62	-74.81 to 9.572	No	ns
0 vs. 18	-24.06	-66.26 to 18.13	No	ns
5.64A				
0 vs. 6	-53.13	-95.32 to -10.94	Yes	*

0 vs. 12	-97.91	-140.1 to -55.72	Yes	****
0 vs. 18	-95.87	-138.1 to -53.67	Yes	****

Plasma ornithine

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	975.6	6	162.6	F (6, 18) = 3.410	P = 0.0200
Time	492.9	3	164.3	F (3, 18) = 3.446	P = 0.0388
Column Factor	1813	2	906.4	F (2, 6) = 23.68	P = 0.0014
Subjects (matching)	229.6	6	38.27	F (6, 18) = 0.8026	P = 0.5807
Residual	858.3	18	47.69		

Dunnett's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary
0				
CTRL vs. 3.89A	-5.330	-18.25 to 7.586	No	ns
CTRL vs. 5.64A	-5.133	-18.05 to 7.783	No	ns
6				
CTRL vs. 3.89A	-11.53	-24.45 to 1.383	No	ns
CTRL vs. 5.64A	-9.613	-22.53 to 3.303	No	ns
12				
CTRL vs. 3.89A	-10.95	-23.86 to 1.970	No	ns
CTRL vs. 5.64A	-27.95	-40.87 to -15.03	Yes	****
18				
CTRL vs. 3.89A	-2.083	-15.00 to 10.83	No	ns
CTRL vs. 5.64A	-26.61	-39.53 to -13.70	Yes	***

Dunnett's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary
CTRL				
0 vs. 6	1.047	-13.41 to 15.50	No	ns
0 vs. 12	0.3367	-14.12 to 14.79	No	ns
0 vs. 18	-1.490	-15.94 to 12.96	No	ns
3.89A				
0 vs. 6	-5.157	-19.61 to 9.297	No	ns
0 vs. 12	-5.280	-19.73 to 9.174	No	ns
0 vs. 18	1.757	-12.70 to 16.21	No	ns
5.64A				
0 vs. 6	-3.433	-17.89 to 11.02	No	ns
0 vs. 12	-22.48	-36.93 to -8.026	Yes	**
0 vs. 18	-22.97	-37.42 to -8.516	Yes	**

Plasma citrulline

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	428.7	6	71.45	F (6, 18) = 3.414	P = 0.0199
Time	697.6	3	232.5	F (3, 18) = 11.11	P = 0.0002
Column Factor	71.48	2	35.74	F (2, 6) = 0.8610	P = 0.4691
Subjects (matching)	249.1	6	41.51	F (6, 18) = 1.984	P = 0.1216
Residual	376.7	18	20.93		

Dunnett's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary
0				
CTRL vs. 3.89A	-1.127	-10.92 to 8.669	No	ns
CTRL vs. 5.64A	1.777	-8.019 to 11.57	No	ns
6				
CTRL vs. 3.89A	-5.287	-15.08 to 4.509	No	ns
CTRL vs. 5.64A	-5.483	-15.28 to 4.312	No	ns
12				
CTRL vs. 3.89A	6.043	-3.752 to 15.84	No	ns
CTRL vs. 5.64A	1.743	-8.052 to 11.54	No	ns
18				
CTRL vs. 3.89A	8.197	-1.599 to 17.99	No	ns
CTRL vs. 5.64A	15.73	5.931 to 25.52	Yes	**
Dunnett's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary
CTRL				
0 vs. 6	-4.730	-14.30 to 4.845	No	ns
0 vs. 12	-9.883	-19.46 to -0.3084	Yes	*
0 vs. 18	-19.85	-29.42 to -10.27	Yes	***
3.89A				
0 vs. 6	-8.890	-18.46 to 0.6849	No	ns
0 vs. 12	-2.713	-12.29 to 6.862	No	ns
0 vs. 18	-10.52	-20.10 to -0.9484	Yes	*
5.64A				
0 vs. 6	-11.99	-21.56 to -2.415	Yes	*
0 vs. 12	-9.917	-19.49 to -0.3417	Yes	*
0 vs. 18	-5.897	-15.47 to 3.678	No	ns

Plasma lysine

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	1.662	6	0.2770	F (6, 18) = 0.6509	P = 0.6892
Time	1.933	3	0.6445	F (3, 18) = 1.515	P = 0.2448
Column Factor	0.06533	2	0.03267	F (2, 6) = 0.04417	P = 0.9571
Subjects (matching)	4.437	6	0.7395	F (6, 18) = 1.738	P = 0.1694
Residual	7.659	18	0.4255		

Plasma essential amino acid

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	180228	6	30038	F (6, 18) = 1.783	P = 0.1594

Time	2.514e+006	3	838035	F (3, 18) = 49.75	P < 0.0001
Column Factor	18361	2	9180	F (2, 6) = 0.1774	P = 0.8417
Subjects (matching)	310501	6	51750	F (6, 18) = 3.072	P = 0.0300
Residual	303233	18	16846		

Dunnett's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary
0				
CTRL vs. 3.89A	-32.81	-339.6 to 274.0	No	ns
CTRL vs. 5.64A	54.03	-252.7 to 360.8	No	ns
6				
CTRL vs. 3.89A	-103.6	-410.4 to 203.1	No	ns
CTRL vs. 5.64A	38.97	-267.8 to 345.7	No	ns
12				
CTRL vs. 3.89A	23.39	-283.4 to 330.2	No	ns
CTRL vs. 5.64A	-107.7	-414.4 to 199.1	No	ns
18				
CTRL vs. 3.89A	266.7	-40.10 to 573.4	No	ns
CTRL vs. 5.64A	229.4	-77.37 to 536.2	No	ns

CTRL				
0 vs. 6	-95.30	-367.0 to 176.4	No	ns
0 vs. 12	-498.9	-770.5 to -227.2	Yes	***
0 vs. 18	-782.7	-1054 to -511.1	Yes	****
3.89A				
0 vs. 6	-166.1	-437.8 to 105.5	No	ns
0 vs. 12	-442.7	-714.3 to -171.0	Yes	**
0 vs. 18	-483.3	-754.9 to -211.6	Yes	***
5.64A				
0 vs. 6	-110.4	-382.0 to 161.3	No	ns
0 vs. 12	-660.6	-932.2 to -388.9	Yes	****
0 vs. 18	-607.4	-879.0 to -335.7	Yes	****

Plasma urea

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	117.8	2	58.90	F (2, 12) = 0.2300	P = 0.7979
Row Factor	2.347	2	1.174	F (2, 12) = 0.004583	P = 0.9954
Column Factor	674.7	1	674.7	F (1, 12) = 2.635	P = 0.1305
Residual	3073	12	256.1		

Hepatic free AA

Arginine

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value

Interaction	111.9	2	55.95	F (2, 12) = 1.582	P = 0.2455
Row Factor	3.339	1	3.339	F (1, 12) = 0.09442	P = 0.7639
Column Factor	244.3	2	122.1	F (2, 12) = 3.454	P = 0.0654
Residual	424.3	12	35.36		

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary
0				
CTRL vs. 3.89A	-6.600	-19.55 to 6.353	No	ns
CTRL vs. 5.64A	-14.03	-26.98 to -1.076	Yes	*
3.89A vs. 5.64A	-7.429	-20.38 to 5.525	No	ns
18				
CTRL vs. 3.89A	-6.354	-19.31 to 6.599	No	ns
CTRL vs. 5.64A	-3.329	-16.28 to 9.624	No	ns
3.89A vs. 5.64A	3.024	-9.929 to 15.98	No	ns

Sidak's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary
0 - 18				
CTRL	-2.787	-16.24 to 10.66	No	ns
3.89A	-2.541	-15.99 to 10.91	No	ns
5.64A	7.912	-5.538 to 21.36	No	ns

Ornithine

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	379.3	2	189.7	F (2, 12) = 1.340	P = 0.2984
Row Factor	253.2	1	253.2	F (1, 12) = 1.789	P = 0.2059
Column Factor	1390	2	695.1	F (2, 12) = 4.910	P = 0.0277
Residual	1699	12	141.6		

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary
0				
CTRL vs. 3.89A	-8.083	-34.00 to 17.83	No	ns
CTRL vs. 5.64A	-12.24	-38.16 to 13.68	No	ns
3.89A vs. 5.64A	-4.157	-30.07 to 21.76	No	ns
18				
CTRL vs. 3.89A	-4.580	-30.50 to 21.34	No	ns
CTRL vs. 5.64A	-29.73	-55.64 to -3.810	Yes	*
3.89A vs. 5.64A	-25.15	-51.06 to 0.7697	No	ns

Sidak's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary
0 - 18				
CTRL	-2.840	-29.75 to 24.07	No	ns

3.89A	0.6627	-26.25 to 27.57	No	ns
5.64A	-20.33	-47.24 to 6.584	No	ns

Citrulline

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	224.3	2	112.1	F (2, 12) = 14.66	P = 0.0006
Row Factor	74.97	1	74.97	F (1, 12) = 9.800	P = 0.0087
Column Factor	131.7	2	65.84	F (2, 12) = 8.606	P = 0.0048
Residual	91.80	12	7.650		

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary
0				
CTRL vs. 3.89A	11.45	5.428 to 17.48	Yes	***
CTRL vs. 5.64A	14.38	8.359 to 20.41	Yes	****
3.89A vs. 5.64A	2.930	-3.095 to 8.955	No	ns
18				
CTRL vs. 3.89A	-2.475	-8.500 to 3.550	No	ns
CTRL vs. 5.64A	-1.456	-7.481 to 4.570	No	ns
3.89A vs. 5.64A	1.020	-5.005 to 7.045	No	ns

Sidak's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary
0 - 18				
CTRL	14.00	7.748 to 20.26	Yes	***
3.89A	0.07554	-6.181 to 6.332	No	ns
5.64A	-1.835	-8.091 to 4.422	No	ns

Total EAA

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	10293	2	5147	F (2, 12) = 1.357	P = 0.2943
Row Factor	641.7	1	641.7	F (1, 12) = 0.1692	P = 0.6881
Column Factor	14686	2	7343	F (2, 12) = 1.936	P = 0.1868
Residual	45522	12	3794		

Intestinal qPCR

ARG2

Kruskal-Wallis test	
P value	0.0107
Exact or approximate P value?	Exact
P value summary	*

Do the medians vary signif. (P < 0.05)	Yes
Number of groups	3
Kruskal-Wallis statistic	6.489

Dunn's multiple comparisons test	Mean rank diff.	Significant?	Summary
CTRL vs. 3.89A	-3.333	No	ns
CTRL vs. 5.64A	-5.667	Yes	*
3.89A vs. 5.64A	-2.333	No	ns

iNOS

Kruskal-Wallis test	
P value	0.2321
Exact or approximate P value?	Exact
P value summary	ns
Do the medians vary signif. (P < 0.05)	No
Number of groups	3
Kruskal-Wallis statistic	3.289

HSP70

Kruskal-Wallis test	
P value	0.0107
Exact or approximate P value?	Exact
P value summary	*
Do the medians vary signif. (P < 0.05)	Yes
Number of groups	3
Kruskal-Wallis statistic	6.489

Dunn's multiple comparisons test	Mean rank diff.	Significant?	Summary
CTRL vs. 3.89A	-3.333	No	ns
CTRL vs. 5.64A	-5.667	Yes	*
3.89A vs. 5.64A	-2.333	No	ns

Chapter III

Postprandial plasma amino acid

Plasma arginine

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	788.4	9	87.60	F (9, 32) = 2.691	P = 0.0188
Row Factor	2148	3	716.1	F (3, 32) = 22.00	P < 0.0001
Column Factor	651.1	3	217.0	F (3, 32) = 6.667	P = 0.0013
Residual	1042	32	32.55		

Dunnett's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary
CTRL				
0 vs. 7	0.3067	-11.18 to 11.79	No	ns
0 vs. 15	-11.89	-23.38 to -0.4062	Yes	*
0 vs. 30	-6.093	-17.58 to 5.394	No	ns
ORN				
0 vs. 7	-0.1600	-11.65 to 11.33	No	ns
0 vs. 15	-1.727	-13.21 to 9.761	No	ns
0 vs. 30	-16.39	-27.88 to -4.906	Yes	**
CIT				
0 vs. 7	-5.027	-16.51 to 6.461	No	ns
0 vs. 15	-18.79	-30.28 to -7.306	Yes	***
0 vs. 30	-30.23	-41.71 to -18.74	Yes	****
ORN-CIT				
0 vs. 7	-4.693	-16.18 to 6.794	No	ns
0 vs. 15	-12.73	-24.21 to -1.239	Yes	*
0 vs. 30	-13.59	-25.08 to -2.106	Yes	*

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary
0				
CTRL vs. ORN	0.0	-12.62 to 12.62	No	ns
CTRL vs. CIT	0.0	-12.62 to 12.62	No	ns
CTRL vs. ORN-CIT	0.0	-12.62 to 12.62	No	ns
ORN vs. CIT	0.0	-12.62 to 12.62	No	ns
ORN vs. ORN-CIT	0.0	-12.62 to 12.62	No	ns
CIT vs. ORN-CIT	0.0	-12.62 to 12.62	No	ns
7				
CTRL vs. ORN	-0.4667	-13.09 to 12.16	No	ns
CTRL vs. CIT	-5.333	-17.96 to 7.289	No	ns
CTRL vs. ORN-CIT	-5.000	-17.62 to 7.622	No	ns
ORN vs. CIT	-4.867	-17.49 to 7.755	No	ns

ORN vs. ORN-CIT	-4.533	-17.16 to 8.089	No	ns
CIT vs. ORN-CIT	0.3333	-12.29 to 12.96	No	ns
15				
CTRL vs. ORN	10.17	-2.455 to 22.79	No	ns
CTRL vs. CIT	-6.900	-19.52 to 5.722	No	ns
CTRL vs. ORN-CIT	-0.8333	-13.46 to 11.79	No	ns
ORN vs. CIT	-17.07	-29.69 to -4.445	Yes	**
ORN vs. ORN-CIT	-11.00	-23.62 to 1.622	No	ns
CIT vs. ORN-CIT	6.067	-6.555 to 18.69	No	ns

Plasma ornithine

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	468.1	9	52.01	F (9, 32) = 5.356	P = 0.0002
Row Factor	428.8	3	142.9	F (3, 32) = 14.72	P < 0.0001
Column Factor	931.0	3	310.3	F (3, 32) = 31.96	P < 0.0001
Residual	310.7	32	9.710		

Dunnett's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary
CTRL				
0 vs. 7	0.4333	-5.840 to 6.707	No	ns
0 vs. 15	-0.9633	-7.237 to 5.310	No	ns
0 vs. 30	-0.07667	-6.350 to 6.197	No	ns
ORN				
0 vs. 7	-13.08	-19.36 to -6.810	Yes	****
0 vs. 15	-6.073	-12.35 to 0.2002	No	ns
0 vs. 30	-7.310	-13.58 to -1.036	Yes	*
CIT				
0 vs. 7	-0.7100	-6.984 to 5.564	No	ns
0 vs. 15	-2.372	-8.645 to 3.902	No	ns
0 vs. 30	-5.510	-11.78 to 0.7635	No	ns
ORN-CIT				
0 vs. 7	-16.57	-22.84 to -10.29	Yes	****
0 vs. 15	-17.40	-23.68 to -11.13	Yes	****
0 vs. 30	-12.38	-18.65 to -6.103	Yes	****

0				
CTRL vs. ORN	0.0	-6.893 to 6.893	No	ns
CTRL vs. CIT	0.0	-6.893 to 6.893	No	ns
CTRL vs. ORN-CIT	0.0	-6.893 to 6.893	No	ns
ORN vs. CIT	0.0	-6.893 to 6.893	No	ns
ORN vs. ORN-CIT	0.0	-6.893 to 6.893	No	ns
CIT vs. ORN-CIT	0.0	-6.893 to 6.893	No	ns
7				
CTRL vs. ORN	-13.52	-20.41 to -6.623	Yes	****

CTRL vs. CIT	-1.143	-8.037 to 5.750	No	ns
CTRL vs. ORN-CIT	-17.00	-23.89 to -10.11	Yes	****
ORN vs. CIT	12.37	5.480 to 19.27	Yes	***
ORN vs. ORN-CIT	-3.485	-10.38 to 3.408	No	ns
CIT vs. ORN-CIT	-15.86	-22.75 to -8.965	Yes	****
15				
CTRL vs. ORN	-5.110	-12.00 to 1.783	No	ns
CTRL vs. CIT	-1.408	-8.302 to 5.485	No	ns
CTRL vs. ORN-CIT	-16.44	-23.33 to -9.547	Yes	****
ORN vs. CIT	3.702	-3.192 to 10.59	No	ns
ORN vs. ORN-CIT	-11.33	-18.22 to -4.437	Yes	***
CIT vs. ORN-CIT	-15.03	-21.92 to -8.138	Yes	****
30				
CTRL vs. ORN	-7.233	-14.13 to -0.3400	Yes	*
CTRL vs. CIT	-5.433	-12.33 to 1.460	No	ns
CTRL vs. ORN-CIT	-12.30	-19.19 to -5.407	Yes	***
ORN vs. CIT	1.800	-5.093 to 8.693	No	ns
ORN vs. ORN-CIT	-5.067	-11.96 to 1.827	No	ns
CIT vs. ORN-CIT	-6.867	-13.76 to 0.02662	No	ns

Plasma citrulline

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	174760	9	19418	F (9, 32) = 28.50	P < 0.0001
Row Factor	171197	3	57066	F (3, 32) = 83.75	P < 0.0001
Column Factor	367619	3	122540	F (3, 32) = 179.8	P < 0.0001
Residual	21804	32	681.4		

Dunnett's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary
CTRL				
0 vs. 7	-0.4237	-52.98 to 52.13	No	ns
0 vs. 15	-0.6220	-53.17 to 51.93	No	ns
0 vs. 30	-0.6237	-53.18 to 51.93	No	ns
ORN				
0 vs. 7	-0.3403	-52.89 to 52.21	No	ns
0 vs. 15	-1.025	-53.58 to 51.53	No	ns
0 vs. 30	-1.390	-53.94 to 51.16	No	ns
CIT				
0 vs. 7	-123.6	-176.1 to -71.01	Yes	****
0 vs. 15	-197.7	-250.3 to -145.2	Yes	****
0 vs. 30	-315.5	-368.0 to -262.9	Yes	****
ORN-CIT				
0 vs. 7	-156.2	-208.7 to -103.6	Yes	****
0 vs. 15	-277.5	-330.1 to -225.0	Yes	****

0 vs. 30	-323.8	-376.3 to -271.2	Yes	****

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary
0				
CTRL vs. ORN	0.0	-14.58 to 14.58	No	ns
CTRL vs. CIT	0.0	-14.58 to 14.58	No	ns
CTRL vs. ORN-CIT	0.0	-14.58 to 14.58	No	ns
ORN vs. CIT	0.0	-14.58 to 14.58	No	ns
ORN vs. ORN-CIT	0.0	-14.58 to 14.58	No	ns
CIT vs. ORN-CIT	0.0	-14.58 to 14.58	No	ns
7				
CTRL vs. ORN	0.002055	-14.58 to 14.59	No	ns
CTRL vs. CIT	-24.00	-38.58 to -9.412	Yes	***
CTRL vs. ORN-CIT	-31.48	-46.06 to -16.89	Yes	****
ORN vs. CIT	-24.00	-38.58 to -9.414	Yes	***
ORN vs. ORN-CIT	-31.48	-46.06 to -16.89	Yes	****
CIT vs. ORN-CIT	-7.481	-22.06 to 7.103	No	ns
15				
CTRL vs. ORN	-0.1059	-14.69 to 14.48	No	ns
CTRL vs. CIT	-31.57	-46.15 to -16.99	Yes	****
CTRL vs. ORN-CIT	-76.34	-90.92 to -61.75	Yes	****
ORN vs. CIT	-31.47	-46.05 to -16.88	Yes	****
ORN vs. ORN-CIT	-76.23	-90.81 to -61.65	Yes	****
CIT vs. ORN-CIT	-44.77	-59.35 to -30.18	Yes	****
30				
CTRL vs. ORN	-0.02976	-14.61 to 14.55	No	ns
CTRL vs. CIT	-42.37	-56.95 to -27.78	Yes	****
CTRL vs. ORN-CIT	-54.77	-69.35 to -40.18	Yes	****
ORN vs. CIT	-42.34	-56.92 to -27.75	Yes	****
ORN vs. ORN-CIT	-54.74	-69.32 to -40.15	Yes	****
CIT vs. ORN-CIT	-12.40	-26.98 to 2.185	No	ns

Growth performance

Initial weight

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	0.02298	3	0.007661	F (3, 20) = 0.001939	P = 0.9999
Residual (within columns)	79.01	20	3.951		
Total	79.04	23			

Final weight

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value

Treatment (between columns)	1.259	3	0.4196	F (3, 20) = 0.02035	P = 0.9959
Residual (within columns)	412.4	20	20.62		
Total	413.6	23			

Weight gain

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	936.3	3	312.1	F (3, 20) = 0.03234	P = 0.9919
Residual (within columns)	192985	20	9649		
Total	193921	23			

Feeding efficiency

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	661.1	3	220.4	F (3, 16) = 0.5024	P = 0.6860
Residual (within columns)	7018	16	438.6		
Total	7679	19			

Survival

Survival of 15 days feeding

Comparison of Survival Curves	
Log-rank (Mantel-Cox) test (recommended)	
Chi square	3.225
df	3
P value	0.3582
P value summary	ns
Are the survival curves sig different?	No

Survival of 30 days feeding

Comparison of Survival Curves	
Log-rank (Mantel-Cox) test (recommended)	
Chi square	4.849
df	3
P value	0.1832
P value summary	ns
Are the survival curves sig different?	No

Plasma arginine 15 days feeding upon challenge with *Vibrio anguillarum*

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	1204	3	401.4	F (3, 8) = 1.975	P = 0.1964
Residual (within columns)	1626	8	203.3		
Total	2831	11			

Plasma arginine 30 days feeding upon challenge with *Vibrio anguillarum*

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
				F (3, 8) =	P =
Treatment (between columns)	989.0	3	329.7	10.15	0.0042
Residual (within columns)	259.8	8	32.47		
Total	1249	11			

Uncorrected Fisher's LSD	Mean Diff.	Significant?	Summary	Individual P Value
CTRL vs. ORN	-5.540	No	ns	0.2679
CTRL vs. CIT	-23.75	Yes	***	0.0009
CTRL vs. ORN-CIT	-15.05	Yes	*	0.0120
ORN vs. CIT	-18.21	Yes	**	0.0045
ORN vs. ORN-CIT	-9.505	No	ns	0.0753
CIT vs. ORN-CIT	8.703	No	ns	0.0983

Plasma ornithine 15 days feeding upon challenge with *Vibrio anguillarum*

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	33.60	3	11.20	F (3, 8) = 1.479	P = 0.2919
Residual (within columns)	60.58	8	7.572		
Total	94.18	11			

Plasma ornithine 30 days feeding upon challenge with *Vibrio anguillarum*

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
				F (3, 8) =	P =
Treatment (between columns)	286.0	3	95.35	6.777	0.0138
Residual (within columns)	112.6	8	14.07		
Total	398.6	11			

Uncorrected Fisher's LSD	Mean Diff.	Significant?	Summary	Individual P Value
CTRL vs. ORN	-7.108	Yes	*	0.0488
CTRL vs. CIT	-12.64	Yes	**	0.0033
CTRL vs. ORN-CIT	-2.107	No	ns	0.5110
ORN vs. CIT	-5.533	No	ns	0.1084
ORN vs. ORN-CIT	5.002	No	ns	0.1411
CIT vs. ORN-CIT	10.54	Yes	**	0.0088

Plasma citrulline 15 days feeding upon challenge with *Vibrio anguillarum*

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	182214	3	60738	F (3, 8) = 8.132	P = 0.0082
Residual (within columns)	59751	8	7469		
Total	241965	11			

Uncorrected Fisher's LSD	Mean Diff.	Significant?	Summary	Individual P Value
CTRL vs. ORN	-0.2400	No	ns	0.9974
CTRL vs. CIT	-145.0	No	ns	0.0740
CTRL vs. ORN-CIT	-298.1	Yes	**	0.0029
ORN vs. CIT	-144.7	No	ns	0.0744
ORN vs. ORN-CIT	-297.8	Yes	**	0.0029
CIT vs. ORN-CIT	-153.1	No	ns	0.0618

Plasma citrulline 30 days feeding upon challenge with *Vibrio anguillarum*

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	175169	3	58390	F (3, 8) = 5.755	P = 0.0214
Residual (within columns)	81165	8	10146		
Total	256334	11			

Uncorrected Fisher's LSD	Mean Diff.	Significant?	Summary	Individual P Value
CTRL vs. ORN	-0.3320	No	ns	0.6131
CTRL vs. CIT	-3.709	Yes	***	0.0004
CTRL vs. ORN-CIT	-2.701	Yes	**	0.0027
ORN vs. CIT	-3.377	Yes	***	0.0007
ORN vs. ORN-CIT	-2.369	Yes	**	0.0056
CIT vs. ORN-CIT	1.008	No	ns	0.1489

iNOS expression after 15 days feeding upon challenge with *Vibrio anguillarum*

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	0.5800	3	0.1933	F (3, 8) = 4.052	P = 0.0504
Residual (within columns)	0.3817	8	0.04771		
Total	0.9617	11			

iNOS expression after 30 days feeding upon challenge with *Vibrio anguillarum*

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	1.267	3	0.4223	F (3, 8) = 7.393	P = 0.0108
Residual (within columns)	0.4570	8	0.05712		
Total	1.724	11			

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary
CTRL vs. ORN	0.1560	-0.4689 to 0.7809	No	ns
CTRL vs. CIT	0.6924	0.06745 to 1.317	Yes	*
CTRL vs. ORN-CIT	0.7425	0.1176 to 1.367	Yes	*
ORN vs. CIT	0.5364	-0.08857 to 1.161	No	ns

ORN vs. ORN-CIT	0.5865	-0.03846 to 1.211	No	ns
CIT vs. ORN-CIT	0.05010	-0.5748 to 0.6750	No	ns

IL-1b expression after 15 days feeding upon challenge with *Vibrio anguillarum*

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	1.666	3	0.5555	F (3, 8) = 5.799	P = 0.0209
Residual (within columns)	0.7664	8	0.09580		
Total	2.433	11			

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary
CTRL vs. ORN	0.3563	-0.4529 to 1.166	No	ns
CTRL vs. CIT	0.7158	-0.09346 to 1.525	No	ns
CTRL vs. ORN-CIT	0.9891	0.1798 to 1.798	Yes	*
ORN vs. CIT	0.3595	-0.4498 to 1.169	No	ns
ORN vs. ORN-CIT	0.6327	-0.1765 to 1.442	No	ns
CIT vs. ORN-CIT	0.2733	-0.5360 to 1.083	No	ns

IL-1b expression after 30 days feeding upon challenge with *Vibrio anguillarum*

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	2.916	3	0.9719	F (3, 8) = 21.68	P = 0.0003
Residual (within columns)	0.3586	8	0.04482		
Total	3.274	11			

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary
CTRL vs. ORN	-1.043	-1.597 to -0.4899	Yes	**
CTRL vs. CIT	-0.8699	-1.423 to -0.3163	Yes	**
CTRL vs. ORN-CIT	0.04206	-0.5115 to 0.5956	No	ns
ORN vs. CIT	0.1736	-0.3800 to 0.7271	No	ns
ORN vs. ORN-CIT	1.086	0.5320 to 1.639	Yes	**
CIT vs. ORN-CIT	0.9120	0.3584 to 1.466	Yes	**

Arginase II expression after 15 days feeding upon challenge with *Vibrio anguillarum*

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
				F (3, 8) =	P =
Treatment (between columns)	0.6788	3	0.2263	0.6358	0.6126
Residual (within columns)	2.847	8	0.3558		
Total	3.526	11			

Arginase II expression after 30 days feeding upon challenge with *Vibrio anguillarum*

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value

				F (3, 8) =	P =
Treatment (between columns)	29.78	3	9.927	12.04	0.0025
Residual (within columns)	6.595	8	0.8244		
Total	36.38	11			

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary
CTRL vs. ORN	-3.393	-5.767 to -1.019	Yes	**
CTRL vs. CIT	-1.885	-4.259 to 0.4887	No	ns
CTRL vs. ORN-CIT	0.5917	-1.782 to 2.966	No	ns
ORN vs. CIT	1.508	-0.8662 to 3.882	No	ns
ORN vs. ORN-CIT	3.985	1.611 to 6.359	Yes	**
CIT vs. ORN-CIT	2.477	0.1030 to 4.851	Yes	*

Chapter IV:

Postprandial plasma amino acid

Plasma arginine

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	1380	9	153.3	F (9, 32) = 3.559	P = 0.0037
Row Factor	2568	3	856.0	F (3, 32) = 19.87	P < 0.0001
Column Factor	2706	3	902.0	F (3, 32) = 20.93	P < 0.0001
Residual	1379	32	43.09		

Dunnett's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary
CTRL				
0 vs. 6	-4.900	-18.12 to 8.315	No	ns
0 vs. 15	3.060	-10.16 to 16.28	No	ns
0 vs. 30	-6.745	-19.96 to 6.470	No	ns
Arginine				
0 vs. 6	-20.92	-34.14 to -7.705	Yes	**
0 vs. 15	-34.93	-48.14 to -21.71	Yes	****
0 vs. 30	-32.12	-45.33 to -18.90	Yes	****
Ornithine				
0 vs. 6	-7.220	-20.44 to 5.995	No	ns
0 vs. 15	-10.06	-23.28 to 3.155	No	ns
0 vs. 30	-12.19	-25.40 to 1.030	No	ns
Citrulline				
0 vs. 6	-12.86	-26.08 to 0.3551	No	ns
0 vs. 15	-18.01	-31.22 to -4.790	Yes	**
0 vs. 30	-28.34	-41.55 to -15.12	Yes	****

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary
0				
CTRL vs. Arginine	0.0	-14.52 to 14.52	No	ns
CTRL vs. Ornithine	0.0	-14.52 to 14.52	No	ns
CTRL vs. Citrulline	0.0	-14.52 to 14.52	No	ns
Arginine vs. Ornithine	0.0	-14.52 to 14.52	No	ns
Arginine vs. Citrulline	0.0	-14.52 to 14.52	No	ns
Ornithine vs. Citrulline	0.0	-14.52 to 14.52	No	ns
6				
CTRL vs. Arginine	-16.02	-30.54 to -1.499	Yes	*
CTRL vs. Ornithine	-2.320	-16.84 to 12.20	No	ns
CTRL vs. Citrulline	-7.960	-22.48 to 6.561	No	ns
Arginine vs. Ornithine	13.70	-0.8207 to 28.22	No	ns
Arginine vs. Citrulline	8.060	-6.461 to 22.58	No	ns
Ornithine vs. Citrulline	-5.640	-20.16 to 8.881	No	ns

15				
CTRL vs. Arginine	-37.99	-52.51 to -23.46	Yes	****
CTRL vs. Ornithine	-13.12	-27.64 to 1.401	No	ns
CTRL vs. Citrulline	-21.07	-35.59 to -6.544	Yes	**
Arginine vs. Ornithine	24.87	10.34 to 39.39	Yes	***
Arginine vs. Citrulline	16.92	2.399 to 31.44	Yes	*
Ornithine vs. Citrulline	-7.945	-22.47 to 6.576	No	ns
30				
CTRL vs. Arginine	-25.37	-39.89 to -10.85	Yes	***
CTRL vs. Ornithine	-5.440	-19.96 to 9.081	No	ns
CTRL vs. Citrulline	-21.59	-36.11 to -7.069	Yes	**
Arginine vs. Ornithine	19.93	5.409 to 34.45	Yes	**
Arginine vs. Citrulline	3.780	-10.74 to 18.30	No	ns
Ornithine vs. Citrulline	-16.15	-30.67 to -1.629	Yes	*

Plasma ornithine

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	4818	9	535.3	F (9, 32) = 19.31	P < 0.0001
Row Factor	1669	3	556.4	F (3, 32) = 20.08	P < 0.0001
Column Factor	7405	3	2468	F (3, 32) = 89.06	P < 0.0001
Residual	886.9	32	27.71		

Dunnett's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary
CTRL				
0 vs. 6	1.245	-9.354 to 11.84	No	ns
0 vs. 15	1.310	-9.289 to 11.91	No	ns
0 vs. 30	1.305	-9.294 to 11.90	No	ns
Arginine				
0 vs. 6	-1.990	-12.59 to 8.609	No	ns
0 vs. 15	-3.515	-14.11 to 7.084	No	ns
0 vs. 30	-4.085	-14.68 to 6.514	No	ns
Ornithine				
0 vs. 6	-30.95	-41.55 to -20.35	Yes	****
0 vs. 15	-64.30	-74.89 to -53.70	Yes	****
0 vs. 30	-21.69	-32.29 to -11.09	Yes	****
Citrulline				
0 vs. 6	0.3550	-10.24 to 10.95	No	ns
0 vs. 15	0.1200	-10.48 to 10.72	No	ns
0 vs. 30	-2.930	-13.53 to 7.669	No	ns

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary
0				
CTRL vs. Arginine	0.0	-11.65 to 11.65	No	ns
CTRL vs. Ornithine	0.0	-11.65 to 11.65	No	ns

CTRL vs. Citrulline	0.0	-11.65 to 11.65	No	ns
Arginine vs. Ornithine	0.0	-11.65 to 11.65	No	ns
Arginine vs. Citrulline	0.0	-11.65 to 11.65	No	ns
Ornithine vs. Citrulline	0.0	-11.65 to 11.65	No	ns
6				
CTRL vs. Arginine	-3.235	-14.88 to 8.411	No	ns
CTRL vs. Ornithine	-32.20	-43.84 to -20.55	Yes	****
CTRL vs. Citrulline	-0.8900	-12.54 to 10.76	No	ns
Arginine vs. Ornithine	-28.96	-40.61 to -17.31	Yes	****
Arginine vs. Citrulline	2.345	-9.301 to 13.99	No	ns
Ornithine vs. Citrulline	31.31	19.66 to 42.95	Yes	****
15				
CTRL vs. Arginine	-4.825	-16.47 to 6.821	No	ns
CTRL vs. Ornithine	-65.61	-77.25 to -53.96	Yes	****
CTRL vs. Citrulline	-1.190	-12.84 to 10.46	No	ns
Arginine vs. Ornithine	-60.78	-72.43 to -49.13	Yes	****
Arginine vs. Citrulline	3.635	-8.011 to 15.28	No	ns
Ornithine vs. Citrulline	64.42	52.77 to 76.06	Yes	****
30				
CTRL vs. Arginine	-5.390	-17.04 to 6.256	No	ns
CTRL vs. Ornithine	-23.00	-34.64 to -11.35	Yes	****
CTRL vs. Citrulline	-4.235	-15.88 to 7.411	No	ns
Arginine vs. Ornithine	-17.61	-29.25 to -5.959	Yes	**
Arginine vs. Citrulline	1.155	-10.49 to 12.80	No	ns
Ornithine vs. Citrulline	18.76	7.114 to 30.41	Yes	***

Plasma citrulline

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	213447	9	23716	F (9, 32) = 1.663	P = 0.1395
Row Factor	72917	3	24306	F (3, 32) = 1.704	P = 0.1858
Column Factor	602209	3	200736	F (3, 32) = 14.07	P < 0.0001
Residual	456396	32	14262		

Dunnett's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary
CTRL				
0 vs. 6	-1.010	-241.4 to 239.4	No	ns
0 vs. 15	-0.2700	-240.7 to 240.2	No	ns
0 vs. 30	-0.7750	-241.2 to 239.7	No	ns
Arginine				
0 vs. 6	-1.110	-241.5 to 239.3	No	ns
0 vs. 15	-0.4435	-240.9 to 240.0	No	ns
0 vs. 30	-0.9950	-241.4 to 239.4	No	ns
Ornithine				
0 vs. 6	-1.445	-241.9 to 239.0	No	ns
0 vs. 15	-1.710	-242.1 to 238.7	No	ns
0 vs. 30	-1.735	-242.2 to 238.7	No	ns

Citrulline				
0 vs. 6	-351.4	-591.9 to -111.0	Yes	**
0 vs. 15	-290.1	-530.5 to -49.66	Yes	*
0 vs. 30	-396.3	-636.8 to -155.9	Yes	***

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary
0				
CTRL vs. Arginine	0.0	-264.2 to 264.2	No	ns
CTRL vs. Ornithine	0.0	-264.2 to 264.2	No	ns
CTRL vs. Citrulline	0.0	-264.2 to 264.2	No	ns
Arginine vs. Ornithine	0.0	-264.2 to 264.2	No	ns
Arginine vs. Citrulline	0.0	-264.2 to 264.2	No	ns
Ornithine vs. Citrulline	0.0	-264.2 to 264.2	No	ns
6				
CTRL vs. Arginine	-0.1000	-264.3 to 264.1	No	ns
CTRL vs. Ornithine	-0.4350	-264.6 to 263.8	No	ns
CTRL vs. Citrulline	-350.4	-614.6 to -86.21	Yes	**
Arginine vs. Ornithine	-0.3350	-264.5 to 263.9	No	ns
Arginine vs. Citrulline	-350.3	-614.5 to -86.11	Yes	**
Ornithine vs. Citrulline	-350.0	-614.2 to -85.78	Yes	**
15				
CTRL vs. Arginine	-0.1735	-264.4 to 264.0	No	ns
CTRL vs. Ornithine	-1.440	-265.6 to 262.8	No	ns
CTRL vs. Citrulline	-289.8	-554.0 to -25.64	Yes	*
Arginine vs. Ornithine	-1.267	-265.5 to 262.9	No	ns
Arginine vs. Citrulline	-289.7	-553.8 to -25.46	Yes	*
Ornithine vs. Citrulline	-288.4	-552.6 to -24.20	Yes	*
30				
CTRL vs. Arginine	-0.2200	-264.4 to 264.0	No	ns
CTRL vs. Ornithine	-0.9600	-265.2 to 263.2	No	ns
CTRL vs. Citrulline	-395.6	-659.8 to -131.4	Yes	**
Arginine vs. Ornithine	-0.7400	-264.9 to 263.5	No	ns
Arginine vs. Citrulline	-395.4	-659.5 to -131.2	Yes	**
Ornithine vs. Citrulline	-394.6	-658.8 to -130.4	Yes	**

Plasma NH_3

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	77.34	9	8.594	F (9, 32) = 1.697	P = 0.1307
Row Factor	274.6	3	91.53	F (3, 32) = 18.07	P < 0.0001
Column Factor	31.58	3	10.53	F (3, 32) = 2.079	P = 0.1226
Residual	162.1	32	5.064		

Dunnett's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary
CTRL				
0 vs. 6	-7.033	-11.56 to -2.503	Yes	**
0 vs. 15	-7.033	-11.56 to -2.503	Yes	**
0 vs. 30	-2.867	-7.397 to 1.664	No	ns
Arginine				
0 vs. 6	-6.700	-11.23 to -2.169	Yes	**
0 vs. 15	-4.500	-9.031 to 0.03073	No	ns
0 vs. 30	-7.600	-12.13 to -3.069	Yes	***
Ornithine				
0 vs. 6	-5.200	-9.731 to -0.6693	Yes	*
0 vs. 15	-7.400	-11.93 to -2.869	Yes	***
0 vs. 30	-3.967	-8.497 to 0.5641	No	ns
Citrulline				
0 vs. 6	-5.800	-10.33 to -1.269	Yes	**
0 vs. 15	-2.933	-7.464 to 1.597	No	ns
0 vs. 30	-1.467	-5.997 to 3.064	No	ns

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary
0				
CTRL vs. Arginine	0.0	-4.978 to 4.978	No	ns
CTRL vs. Ornithine	0.0	-4.978 to 4.978	No	ns
CTRL vs. Citrulline	0.0	-4.978 to 4.978	No	ns
Arginine vs. Ornithine	0.0	-4.978 to 4.978	No	ns
Arginine vs. Citrulline	0.0	-4.978 to 4.978	No	ns
Ornithine vs. Citrulline	0.0	-4.978 to 4.978	No	ns
6				
CTRL vs. Arginine	0.3333	-4.645 to 5.312	No	ns
CTRL vs. Ornithine	1.833	-3.145 to 6.812	No	ns
CTRL vs. Citrulline	1.233	-3.745 to 6.212	No	ns
Arginine vs. Ornithine	1.500	-3.478 to 6.478	No	ns
Arginine vs. Citrulline	0.9000	-4.078 to 5.878	No	ns
Ornithine vs. Citrulline	-0.6000	-5.578 to 4.378	No	ns
15				
CTRL vs. Arginine	2.533	-2.445 to 7.512	No	ns
CTRL vs. Ornithine	-0.3667	-5.345 to 4.612	No	ns
CTRL vs. Citrulline	4.100	-0.8783 to 9.078	No	ns
Arginine vs. Ornithine	-2.900	-7.878 to 2.078	No	ns
Arginine vs. Citrulline	1.567	-3.412 to 6.545	No	ns
Ornithine vs. Citrulline	4.467	-0.5117 to 9.445	No	ns
30				
CTRL vs. Arginine	-4.733	-9.712 to 0.2450	No	ns
CTRL vs. Ornithine	-1.100	-6.078 to 3.878	No	ns
CTRL vs. Citrulline	1.400	-3.578 to 6.378	No	ns

Arginine vs. Ornithine	3.633	-1.345 to 8.612	No	ns
Arginine vs. Citrulline	6.133	1.155 to 11.11	Yes	*
Ornithine vs. Citrulline	2.500	-2.478 to 7.478	No	ns

Growth performance

Initial weight

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	0.2045	3	0.06817	F (3, 56) = 0.001249	P > 0.9999
Residual (within columns)	3057	56	54.58		
Total	3057	59			

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary
CTRL vs. ARG	-0.1200	-7.263 to 7.023	No	ns
CTRL vs. ORN	-0.1200	-7.263 to 7.023	No	ns
CTRL vs. CIT	-0.006667	-7.150 to 7.137	No	ns
ARG vs. ORN	5.086e-007	-7.143 to 7.143	No	ns
ARG vs. CIT	0.1133	-7.030 to 7.257	No	ns
ORN vs. CIT	0.1133	-7.030 to 7.257	No	ns

Final weight

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	374.8	3	124.9	F (3, 56) = 0.4867	P = 0.6929
Residual (within columns)	14375	56	256.7		
Total	14750	59			

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary
CTRL vs. ARG	-2.780	-18.27 to 12.71	No	ns
CTRL vs. ORN	-1.213	-16.70 to 14.28	No	ns
CTRL vs. CIT	3.973	-11.52 to 19.46	No	ns
ARG vs. ORN	1.567	-13.92 to 17.06	No	ns
ARG vs. CIT	6.753	-8.738 to 22.24	No	ns
ORN vs. CIT	5.187	-10.30 to 20.68	No	ns

Weight gain

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value

				F (3, 56) =	P =
Treatment (between columns)	2550	3	850.0	4.491	0.0068
Residual (within columns)	10600	56	189.3		
Total	13150	59			

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary
CTRL vs. ARG	-4.938	-18.24 to 8.364	No	ns
CTRL vs. ORN	1.433	-11.87 to 14.73	No	ns
CTRL vs. CIT	12.86	-0.4387 to 26.17	No	ns
ARG vs. ORN	6.370	-6.932 to 19.67	No	ns
ARG vs. CIT	17.80	4.499 to 31.10	Yes	**
ORN vs. CIT	11.43	-1.871 to 24.73	No	ns

FCR

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	0.03341	3	0.01114	F (3, 56) = 0.2269	P = 0.8773
Residual (within columns)	2.749	56	0.04908		
Total	2.782	59			

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary
CTRL vs. ARG	-0.03649	-0.2507 to 0.1777	No	ns
CTRL vs. ORN	-0.04286	-0.2571 to 0.1713	No	ns
CTRL vs. CIT	0.01283	-0.2014 to 0.2270	No	ns
ARG vs. ORN	-0.006370	-0.2206 to 0.2078	No	ns
ARG vs. CIT	0.04932	-0.1649 to 0.2635	No	ns
ORN vs. CIT	0.05569	-0.1585 to 0.2699	No	ns

Survival, plasma amino acid, renal amino acid, and renal qPCR analysis upon challenge with *Vibrio anguillarum*

Survival

Logrank test for trend (recommended)	
Chi square	5.321
df	1
P value	0.0211
P value summary	*
Sig. trend?	Yes

CTRL vs +ARG

Log-rank (Mantel-Cox) test

Chi square	0.8126
df	1
P value	0.3673
P value summary	ns
Are the survival curves sig different?	No

CTRL vs +ORN

Log-rank (Mantel-Cox) test	
Chi square	2.513
df	1
P value	0.1129
P value summary	ns
Are the survival curves sig different?	No

CTRL vs +CIT

Log-rank (Mantel-Cox) test	
Chi square	4.906
df	1
P value	0.0268
P value summary	*
Are the survival curves sig different?	Yes

+ARG vs +ORN

Log-rank (Mantel-Cox) test	
Chi square	0.4446
df	1
P value	0.5049
P value summary	ns
Are the survival curves sig different?	No

+ARG vs +CIT

Log-rank (Mantel-Cox) test	
Chi square	1.657
df	1
P value	0.1980
P value summary	ns
Are the survival curves sig different?	No

+ORN vs +CIT

Log-rank (Mantel-Cox) test	
Chi square	0.5236
df	1
P value	0.4693

P value summary	ns
Are the survival curves sig different?	No

Plasma arginine

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
				F (3, 8) =	P =
Treatment (between columns)	3968	3	1323	23.61	0.0003
Residual (within columns)	448.1	8	56.01		
Total	4416	11			

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary
CTRL vs. Arginine	-4.033	-27.05 to 18.99	No	ns
CTRL vs. Ornithine	-0.06667	-23.09 to 22.95	No	ns
CTRL vs. Citrulline	-37.93	-60.95 to -14.91	Yes	**
Arginine vs. Ornithine	3.967	-19.05 to 26.99	No	ns
Arginine vs. Citrulline	-33.90	-56.92 to -10.88	Yes	**
Ornithine vs. Citrulline	-37.87	-60.89 to -14.85	Yes	**

Plasma ornithine

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
				F (3, 8) =	P =
Treatment (between columns)	1025	3	341.6	6.506	0.0154
Residual (within columns)	420.1	8	52.51		
Total	1445	11			

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary
CTRL vs. Arginine	-4.933	-23.88 to 14.01	No	ns
CTRL vs. Ornithine	-14.50	-33.45 to 4.447	No	ns
CTRL vs. Citrulline	-24.10	-43.05 to -5.153	Yes	*
Arginine vs. Ornithine	-9.567	-28.51 to 9.380	No	ns
Arginine vs. Citrulline	-19.17	-38.11 to -0.2199	Yes	*
Ornithine vs. Citrulline	-9.600	-28.55 to 9.347	No	ns

Plasma citrulline

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	47336	3	15779	F (3, 8) = 6.139	P = 0.0180
Residual (within columns)	20562	8	2570		
Total	67898	11			

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary
CTRL vs. Arginine	0.3000	-132.3 to 132.9	No	ns
CTRL vs. Ornithine	-0.1667	-132.7 to 132.4	No	ns

CTRL vs. Citrulline	-145.0	-277.6 to -12.44	Yes	*
Arginine vs. Ornithine	-0.4667	-133.0 to 132.1	No	ns
Arginine vs. Citrulline	-145.3	-277.9 to -12.74	Yes	*
Ornithine vs. Citrulline	-144.8	-277.4 to -12.27	Yes	*

Renal arginine

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	0.002270	3	0.000756	F (3, 8) = 4.801	P = 0.0338
Residual (within columns)	0.001261	8	0.000157		
Total	0.003531	11			

Uncorrected Fisher's LSD	Mean Diff.	Significant?	Summary	Individual P Value
CTRL vs. ARG	0.0215	No	ns	0.0692
CTRL vs. ORN	0.02723	Yes	*	0.0290
CTRL vs. CIT	-0.005187	No	ns	0.6265
ARG vs. ORN	0.005730	No	ns	0.5914
ARG vs. CIT	-0.02669	Yes	*	0.0314
ORN vs. CIT	-0.03242	Yes	*	0.0133

Renal ornithine

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	9.076e-006	3	3.025e-006	F (3, 8) = 1.017	P = 0.4345
Residual (within columns)	2.380e-005	8	2.975e-006		
Total	3.288e-005	11			

Uncorrected Fisher's LSD	Mean Diff.	Significant?	Summary	Individual P Value
CTRL vs. ARG	0.00028	No	ns	0.8474
CTRL vs. ORN	-0.001407	No	ns	0.3471
CTRL vs. CIT	-0.001738	No	ns	0.2523
ARG vs. ORN	-0.001687	No	ns	0.2653
ARG vs. CIT	-0.002018	No	ns	0.1898
ORN vs. CIT	-0.000331	No	ns	0.8201

Renal citrulline

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	0.003136	3	0.001045	F (3, 8) = 8.373	P = 0.0075

Residual (within columns)	0.0009989	8	0.000124 9
Total	0.004135	11	

Uncorrected Fisher's LSD	Mean Diff.	Significant?	Summary	Individual P Value
CTRL vs. ARG	0.0008133	No	ns	0.9312
CTRL vs. ORN	0.0005633	No	ns	0.9523
CTRL vs. CIT	-0.03687	Yes	**	0.0037
ARG vs. ORN	-0.00025	No	ns	0.9788
ARG vs. CIT	-0.03768	Yes	**	0.0033
ORN vs. CIT	-0.03743	Yes	**	0.0034

Renal iNOS

Kruskal-Wallis test	
P value	0.0165
Exact or approximate P value?	Exact
P value summary	*
Do the medians vary signif. (P < 0.05)	Yes
Number of groups	4
Kruskal-Wallis statistic	8.077

Dunn's multiple comparisons test	Mean rank diff.	Significant?	Summary
CTRL vs. +ARG	-4.000	No	ns
CTRL vs. +ORN	-2.000	No	ns
CTRL vs. +CIT	-8.000	Yes	*
+ARG vs. +ORN	2.000	No	ns
+ARG vs. +CIT	-4.000	No	ns
+ORN vs. +CIT	-6.000	No	ns

Renal IL-1B

Kruskal-Wallis test	
P value	0.0006
Exact or approximate P value?	Exact
P value summary	***
Do the medians vary signif. (P < 0.05)	Yes
Number of groups	4
Kruskal-Wallis statistic	9.667

Dunn's multiple comparisons test	Mean rank diff.	Significant?	Summary
CTRL vs. +ARG	-3.000	No	ns
CTRL vs. +ORN	-8.333	Yes	*

CTRL vs. +CIT	-6.667	No	ns
+ARG vs. +ORN	-5.333	No	ns
+ARG vs. +CIT	-3.667	No	ns
+ORN vs. +CIT	1.667	No	ns

Renal ARG2

Kruskal-Wallis test	
P value	0.0918
Exact or approximate P value?	Exact
P value summary	ns
Do the medians vary signif. (P < 0.05)	No
Number of groups	4
Kruskal-Wallis statistic	6.179

Dunn's multiple comparisons test	Mean rank diff.	Significant?	Summary
CTRL vs. +ARG	3.667	No	ns
CTRL vs. +ORN	2.667	No	ns
CTRL vs. +CIT	-3.000	No	ns
+ARG vs. +ORN	-1.000	No	ns
+ARG vs. +CIT	-6.667	No	ns
+ORN vs. +CIT	-5.667	No	ns