EVALUATION OF ARGININE AND GLUTAMINE AS DIETARY SUPPLEMENTS TO ENHANCE *Edwardsiella ictaluri* VACCINE EFFECTIVENESS IN CHANNEL CATFISH

A Dissertation

by

CAMILO POHLENZ CASTILLO

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2011

Major Subject: Nutrition

Evaluation of Arginine and Glutamine as Dietary

Supplements to Enhance *Edwardsiella ictaluri* Vaccine

Effectiveness in Channel Catfish

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ABSTRACT

Evaluation of Arginine and Glutamine as Dietary Supplements

to Enhance *Edwardsiella ictaluri* Vaccine Effectiveness

in Channel Catfish. (December 2011)

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Rapid expansion of the aquaculture industry in recent decades has resulted in infectious diseases emerging as a major constraint to fish production, causing large economical losses worldwide. Therefore, prevention practices are indispensable for maintaining the industry's profitability and sustainability. Vaccination is a proven effective strategy for disease control in aquaculture; however, improvements in vaccine efficacy are still needed. Because amino acid supplementation not only enhances fish growth but also immune responses, a series of experiments were conducted to test the hypothesis that dietary supplementation of arginine and glutamine, two amino acids with immunomodulatory roles, may promote growth and increase the efficacy of vaccination against *Edwardsiella ictaluri* in channel catfish.

An initial experiment demonstrated that dietary arginine supplementation at 2 and 4% of diet enhanced growth and feed efficiency of channel catfish. Dietary arginine deficiency diminished plasma levels of arginine, citrulline, ornithine, glutamine and

glutamate, and impaired innate performance of macrophages and neutrophils. In a separate experiment, dietary glutamine supplementation failed to enhance growth responses; however, supplementation at 2% of diet had strong positive effects on intestinal histology and enterocyte migration rate. In addition, serine, asparagine, glycine and threonine were increased in plasma of fish fed the diet with glutamine at 2%. A third experiment revealed that activated macrophages utilized large quantities of glutamine in media and to a lesser extent arginine. These two amino acids also were the most utilized by proliferating lymphocytes. Supplementing media with these amino acids positively modulated phagocytosis and bactericidal capacity of macrophages, as well as increased the proliferation rate of lymphocytes. A final experiment indicated that dietary supplementation of arginine (4%) and glutamine (2%) optimized the nutritional and immunological status of channel catfish, and enhanced responses to *E. ictaluri* vaccination. At the same time, this supplementation ameliorated some short-term adverse effects of vaccination on growth. Higher specific antibody titers, better lymphocyte responsiveness and survival to the bacterium were seen in vaccinated fish fed arginine- and glutamine-supplemented diets. These results support an expanded role of dietary arginine and glutamine manipulation as a tool to improve growth and vaccine efficacy of channel catfish.

DEDICATION

A mi amada esposa Nora Idania y mi recién nacido hijo Andrés Emiliano, los amo!

A mis padres Mario y Patricia

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CHAPTER I

INTRODUCTION

1. Fish nutrition

Aquaculture has become a major animal production activity worldwide and an important source of protein food for human kind, as well as a socioeconomically relevant industry. It continues to grow more rapidly than all other animal agriculture sectors. Freshwater fish culture towers over marine fish production, with an output of 27.8 million tons in 2006 (FAO, 2009). Among freshwater species, the channel catfish, *Ictalurus punctatus*, is the most important aquaculture species in the U.S. with approximately 150,000 surface acres in production accounting for $410 million in total sales for the year 2008 (USDA-NASS, 2009).

Despite the continuous expansion of aquaculture, there are several constraints that currently limit production, with infectious diseases being a major negative factor resulting in multimillion dollar loss per year worldwide. Diseases are natural events in all animal groups but these events get magnified by increased population densities, and fish are no different in this regard. Therefore, preventive measures must be taken in order to diminish the deleterious effects of diseases in aquaculture (Plumb, 2001; Klesius et al., 2004).

Adequate nutrition is critical to maintain fish's health and disease resistance (Sealey and Gatlin, 2001). This becomes apparent when nutrient imbalances lead to

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This dissertation follows the style of Aquaculture.

pathological changes (Chan, 2008). For that reason, fish nutrition focused for many years on determining nutrient requirements and preventing nutritional diseases (Tacon, 1985; NRC, 2011). Nevertheless the role of fish nutrition in health management, through modulation of immune responses and disease resistance, has become a priority research topic leading to the development of functional aquafeeds. These are defined as "feeds supplemented with specific ingredient to achieve desirable efficiency of metabolic transformation, growth performance, health, and/or compositional traits of aquacultured animals at various developmental stages" (Li et al., 2009).

2. Nutrition and immunology

Immunonutrition may be defined as the positive effect of providing specific nutrients, at levels above those required for normal growth, to improve immune function. This concept implies that besides having a direct effect on the defense system a particular dietary ingredient also may have a positive effect on other physiological processes that combine for an overall positive outcome on health and growth. Glutamine (GLN) and arginine (ARG) are two amino acids of enormous importance in this regard as they have been demonstrated to have an array of desirable biological properties.

2.1. Arginine supplementation

Arginine is an essential amino acid for maximal growth of young animals. It is the most abundant nitrogen carrier in tissue proteins and is used in multiple synthetic

pathways, involving enzymes such as arginase, nitric oxide synthase, arginine:glycine amidinotransferase, and arginyl-tRNA synthetase. As such, ARG serves as a precursor for the synthesis of creatine, ornithine, proline, glutamate, polyamines, and nitric oxide (NO) and thus displays remarkable metabolic and regulatory versatility in cells (Wu et al., 2004; Morris, 2006; Yao et al., 2008). Polyamines such as putrescine, spermine and spermidine are involved in cell growth and proliferation by stimulating DNA and protein synthesis. Polyamines are not only beneficial for growth but they also have wider effects such as affecting signal transduction pathways and modulating immune functions. For example, spermine and spermidine have been shown to inhibit the secretion of pro-inflammatory cytokines (Wu et al., 2005a; Morris, 2006).

Arginine deficiency is a major factor limiting maximal growth of young animals causing growth retardation, intestinal and reproductive dysfunction, impaired immune and neurological development, cardiovascular and pulmonary abnormalities, impaired wound healing, hyperammonemia, and even death(Wu et al., 2004). Dietary ARG supplementation markedly enhances protein accretion and the efficiency of nutrient utilization in milk-fed piglets. Additionally, an increase in circulating ARG through dietary supplementation or enhancing endogenous ARG synthesis was associated with enhanced protein synthesis in skeletal muscle of neonatal pigs (Yao et al., 2008).

Dietary supplementation with 0.4% ARG increased plasma concentrations of insulin and growth hormone by 24–27% in piglets, compared with controls. Between 7 and 21 days of age, the supplementation of 0.2 and 0.4% ARG to piglets enhanced average daily weight gain by 28 and 66%, and body weight by 15 and 32%, respectively,

compared with control piglets (Kim et al., 2004). Likewise, dietary ARG supplementation increased daily gain, plasma insulin concentration, and protein synthesis in skeletal muscle of piglets through the mTOR signaling activity (Yao et al., 2008).

As in mammals, ARG is an indispensable amino acid necessary for optimal growth of young fish including channel catfish (Robinson et al., 1981; NRC, 2011). It is noteworthy that the ARG requirement of channel catfish is one of the lowest when compared to other freshwater and saltwater fish species (Table 1.1). This amino acid also is a potent taste stimulant for channel catfish (Grosvenor et al., 2004). Arginine also has a role as a secretagogue, affecting pancreatic hormones plasma levels in rainbow trout, *Ochorynchus mikiss* (Mommsen et al., 2001) and barfin flounder, *Verasper moseri* (Andoh, 2007). However, no information on this respect is available for channel catfish to date. Supplementation of dietary ARG (4%) to channel catfish resulted in significantly higher growth, and hemoglobin and hematocrit levels (Buentello et al., 2007), but not in higher numbers of circulating leukocytes. Additionally, ARG is a unique source of nitric oxide, having an important role in the innate immune response of this species (Buentello and Gatlin, 1999). There is strong evidence for an ARG-sparing effect of dietary glutamate in channel catfish (Buentello and Gatlin, 2000, 2001a), but to date, this *de novo* pathway for endogenous synthesis of ARG from other amino acids (or vice versa) has not been fully elucidated.

Table 1.1
Interspecies ARG requirement for ammonotelic, uricotelic and ureotelic animals

Species	% of dry diet	% of crude protein	Physiological Status
Ammonotelic			
Channel catfish, [1] *Ictalurus punctatus*	1.0	4.3	Juvenile - growing
Rainbow trout, [1] *Oncorhynchus mykiss*	1.2	3.3	Juvenile - growing
Red drum, [2] *Sciaenops ocellatus*	1.4	4.2	Juvenile - growing
Yellow perch,[3] *Perca flavescens*	1.4	4.2	Juvenile - growing
Hybrid striped bass, [4] *Morone chrysops x M. saxatilis*	1.6	4.6	Juvenile - growing
Common carp, [1] *Cyprinus carpio*	1.6	4.3	Juvenile - growing
Tilapia, [1] *Oreochromis sp.*	1.6	4.0	Juvenile - growing
Gilthead sea bream, [1] *Sparus aurata*	1.7	5.0	Juvenile - growing
Japanese eel, [1] *Anguilla japonica*	1.7	4.5	Juvenile - growing
European seabass, [5] *Dicentrarchus labrax*	1.8	3.9	Juvenile - growing
Indian major carp, [6] *Cirrhinus mrigala*	1.8	4.6	Juvenile - growing
African catfish, [7] *Clarias gariepinus*	1.8	4.5	Juvenile - growing
Hybrid *Clarias,*[8] *C. gariepinus* x *C. macrocephalus*	2.0	5.0	Juvenile - growing
Japanese flounder, [9] *Paralichthys olivaceus*	2.1	4.1	Juvenile - growing
Atlantic salmon, [10] *Salmo salar*	2.2	5.1	Juvenile - growing
Coho salmon, [1] *Oncorhynchus kisutch*	2.3	5.8	Juvenile - growing
Chinook salmon, [1] *Oncorhynchus tshawytscha*	2.4	6.0	Juvenile - growing

Table 1.1 continued

Species	% of dry diet	% of crude protein	Physiological Status
Chum salmon, [1] *Oncorhynchus keta*	2.6	6.0	Juvenile - growing
Grouper, [11] *Epinephelus coioides*	2.7	5.5	Juvenile - growing
Silver perch, [12] *Bidyanus bidyanus*	2.7	6.8	Juvenile - growing
Black sea bream, [13] *Sparus macrocephalus*	2.8	7.3	Juvenile - growing
Uricotelic			
Chicken,[14] *Gallus domesticus*			
- Replacement pullets	0.7 - 1.0	4.5 - 5.5	Growing
- Laying hens	0.6 - 0.9	4.6 - 4.7	Producing
- Broilers	1.0 - 1.3	5.4 - 5.5	Growing
Turkey,[14] *Meleagris sp.*			
- Meat producing	0.6 - 1.6	4.3 - 5.7	Growing
- Breeders	0.5 - 0.6	4.2 - 4.3	Producing
Duck,[14] *Anas platyrhynchos domestica*	1.0 - 1.1	5.0 - 6.3	Growing
Ureotelic			
Swine,[15] *Sus scrofa*			
- Sows	0.0 - 0.04	0.0 - 0.3	Gestating
	0.4 - 0.5	2.0 - 2.6	Lactating
- Weanling pigs	0.4 - 0.5	2.1	Growing
- Finishing pigs	0.2 - 0.3	1.2 - 1.8	Growing
Rat,[16] *Rattus norvegicus*	0.4	2.9	Growing and Reproduction
Mouse,[16] *Mus musculus*	0.3	1.7	Maintenance
Guinea pig, [16] *Cavia porcellus*	1.2	6.7	Growing
Primates	N.D.	N.D.	

[1] NRC (2011); [2] Barziza et al. (2000); [3] Twibell & Brown (1997); [4] Griffin et al. (1994); [5] Tibaldi et al. (1994); [6] Ahmed and Khan (2004); [7] Fagbenro et al (1999); [8] Singh and Khan (2007); [9] Alam et al. (2002); [10] Berge et al.(1997); [11] Luo et al. (2007) (2007); [12] Ngamsnae et al. (1999); [13] Zhou et al. (2010); [14] NRC (1994); [15] NRC (1998); [16] NRC (1995)

2.2. Glutamine supplementation

Glutamine acts as an energy source for proliferating cells, and is essential for the synthesis of nucleic acids, pyrimidine and purine nucleotides, providing also the nitrogen necessary for the formation of glycosamines and other important cellular intermediates like NAD+ (Wu et al., 1996; Watford, 1999; Wilmore and Shabert, 1999). For humans, rats and pigs, GLN is the principal metabolic fuel for enterocytes, lymphocytes, macrophages (MØ), and fibroblasts (Wu and Flynn, 1995; Bartell and Batal, 2007). Furthermore, it has been shown that GLN improves whole-body protein synthesis. In addition, an enhanced hepatic uptake of GLN increases the synthesis of glucose, glutathione and acute phase proteins; improving also energy metabolism, supporting tissue growth, and assisting also in the maintenance of the gastrointestinal mucosa (Wilmore and Shabert, 1999).

Glutamine plays an important role as a regulator of cellular metabolism. It has been shown to induce protein synthesis and to inhibit intracellular protein degradation (Wu and Thompson, 1990; Mok et al., 2006). As mentioned above, GLN is a major fuel source for proliferating cells, so there are specific circumstances in which GLN supplementation aids cell function and metabolism, particularly during pathological or physiological challenges. Juvenile animals are not in a steady state regarding protein turnover, but in fact protein synthesis is greater than protein degradation. Thus, at this stage of development, GLN could be classified as a semi-essential or conditionally essential amino acid for rapidly growing animals (Lobley et al., 2001; Zou et al., 2006).

The small intestine is a major organ of GLN utilization in mammals in the post-absorptive state (Wu et al., 1995; Wu et al., 1996). Mucosal cells of the digestive tract have, along with other rapidly proliferating cells, an obligate requirement for GLN. The major challenges to the digestive tract, and its demands for GLN, arise during the early suckling period, the transition to weaning and in response to intestinal injury (Lobley et al., 2001). For example, the addition of GLN at 1% of diet improved the feed efficiency of weanling pigs (Kitt et al., 2002). Villi length in the duodenum and jejunum were significantly longer in birds and pigs fed diets supplemented with GLN (Yi et al., 2001; Kitt et al., 2002; Zou et al., 2006; Bartell and Batal, 2007)

Supplementation of GLN at 1% of diet also improved weight gain and feed efficiency of turkey poults (Yi et al., 2001) and chicks (Bartell and Batal, 2007). Moreover, at 1.2-2% of diet improved weight gain, feed efficiency, intestinal weight, fold height and digestive enzyme activities in juvenile jian carp, *Cyprinus carpio* var. Jian (Yan and Qiu-Zhou, 2006).

If the intestinal villi height can be increased early in the animal's life, then it may be able to utilize nutrients more efficiently due to increased surface area and thus have improved growth performance. Such response was reported by Wu et al. (1996) where dietary GLN supplementation (1.0%) prevented jejunal atrophy in weaned pigs during the first week post-weaning and improved pig's growth performance during the second week post-weaning.

Glutamine in total parenteral nutrition maintains gut integrity which is important in preventing bacterial infections (Karinch et al., 2001; Kessel et al., 2008), and also

enhances bowel mucosal proliferation, thereby repairing intestinal mucosal defects and maintaining the bowel barrier function (Wilmore and Shabert, 1999; Kessel et al., 2008). Challenges associated with diarrhea also are mitigated by GLN and the deleterious changes in digestive tract permeability associated with endotoxins also can be decreased (Lobley et al., 2001; Zou et al., 2006; Kessel et al., 2008).

Enterocytes from the new-born piglet can synthesize ARG from GLN and this may play an important early anabolic role. Later in development, GLN is a precursor for both ARG and proline synthesis within the digestive tract (Wu et al., 1994), and these synthetic processes may supplement dietary supplies of these amino acids and help prevent growth limitations (Lobley et al., 2001).

2.3. Role of GLN and ARG in immune system function

The amino acid GLN is usually included in the list of ‘‘immunonutrients’’ that possess various biological effects (Wilmore and Shabert, 1999). The rate of proliferation of lymphocytes in culture increases with external GLN concentration, as it is utilized at a high rate by cells of the immune system and is required to support optimal lymphocyte proliferation and cytokine production by lymphocytes and MØ (Calder and Yaqoob, 1999; Wilmore and Shabert, 1999; Zaloga and Siddiqui, 2004). Glutamine supports the potential for cytotoxic T cells to lyse target cells (Spittler et al., 1995). It is also important for the synthesis of Immunoglobulin (Ig) G antibodies and perhaps required for thymus derived T-cell helper function and response (Kew et al., 1999; Newsholme, 2001). For example, broiler chickens fed diets supplemented with 1% GLN

had significantly higher IgA and IgG concentrations in the serum, intestine and bile, as well as better gut barrier function due to higher IgA concentrations in the intestine thus, resulting in more resistance to infection (Bartell and Batal, 2007). In many cases, dietary GLN addition resulted in restoration of injured cell function to normal or supranormal levels (Wu et al., 1996; Karinch et al., 2001; Zaloga and Siddiqui, 2004; Kessel et al., 2008).

In regards to indirect actions, GLN stimulates growth hormone synthesis and this hormone can effectively up-regulate the immune system, through direct and indirect effects, with the latter occurring because of receptor homologies with interleukin-2 (Wilmore and Shabert, 1999). As a major source of glutamate, GLN regulates the synthesis of glutathione, a tripeptide crucial for defending cells from oxidative stress (Li et al., 2007). Glutamine supplementation has only been reported as an immunomodulatory strategy for hybrid sturgeon *Acipenser schrenckii* × *Huso dauricus*, where it increased the plasma levels of complement proteins C3 and C4 (Zhu et al., 2011). Finally, Glutamine is a precursor for the net synthesis of ARG, whose effects on the immune system will be mentioned next.

Similarly, ARG has been shown to exert a variety of roles on the animal's immune function (Efron and Barbul, 2000) including fish (Buentello and Gatlin, 1999, 2001b; Buentello et al., 2007). Arginine serves as the sole substrate for production of NO, which modulates expression of adhesion molecules, tissue factors, and cytokines (Zaloga and Siddiqui, 2004). Additionally, in MØ and neutrophils, NO is an essential defense mechanism against viruses, bacteria, fungi, malignant cells, intracellular

protozoa, and parasites in mammals, birds, terrestrial animals, lower vertebrates and invertebrates (Li et al., 2007). Arginine has been shown to increase thymus and spleen size in mice, increase cytokine production, and enhance lymphocyte proliferation (Bartell and Batal, 2007). It enhances T-lymphocyte mediated functions, stimulates replication of thymic lymphocytes, increases release of interleukin-2 from stimulated T-lymphocytes, and increases lymphocyte responses to mitogens. Arginine also improves nitrogen retention and enhances wound healing (Evoy et al., 1998; Zaloga and Siddiqui, 2004). Moreover, ARG supplementation before and during vaccination increased antibody titers in chicks vaccinated against infectious bursal disease virus, and diminished the adverse effects of the virus on immune cells (Tayade et al., 2006a; 2006b).

3. Enteric septicemia of catfish

Edwardsiella ictaluri is the causative agent of Enteric Septicemia of Catfish (ESC), the most prevalent and economically important disease in farmed-raised channel catfish (OIE, 2006). This disease accounts for about 30% of all disease-related losses in U.S. catfish aquaculture, with an annual economic impact of about $19 million related to both mortality losses and treatment costs.

This pathogen is a gram negative, rod-shaped, motile bacterium of the family *Enterobacteriacae*, which is capable of intra- and extra-cellular replication. It is β-hemolytic, positive for hemolysin activity, conferring it a high virulence (Williams and Lawrence, 2005; Booth et al., 2006). The channel catfish is the most susceptible

ictalurid species to ESC infection, while white, brown bullhead, and walking catfishes are rarely affected. In addition, blue catfish may be somewhat resistant to ESC infection (USDA-APHIS, 2003).

Enteric septicemia of catfish occurs in acute and chronic forms. The acute form is a bacterial septicemia characterized by multisystemic necrosis and hemorrhages that can rapidly progress in apparently healthy, fast-growing fish and can result in extensive mortality (Lobb et al., 1993; OIE, 2006). The chronic form is a meningoencephalitis with dorsal extension through the sutra fontanel of the skull (OIE, 2006). In the acute form, propagation of the bacterium throughout the body is rapidly achieved by crossing the intestinal mucosa. In a period of 15 min after infection the pathogen can be found in the head kidney, and as soon as 3 h initial necrosis of the intestine mucosa starts to appear, undergoing a rapid-onset septicemia (Baldwin and Newton, 1993; Booth et al., 2006). Affected fish will display hemorrhagic enteritis, systemic edema, ascites, and multiple necrotic foci in liver, spleen, head kidney, skeletal muscle, skin and other organs, generating granulomatous inflammation (OIE, 2006) within the following 48 h (Baldwin and Newton, 1993). Occurrence of ESC is primarily in the late spring - early summer and in the fall when water temperatures are between 22 and 28 C (Williams and Lawrence, 2005; OIE, 2006).

3.1. Immune response against E. ictaluri *infection*

Resistance to ESC is an extremely complex phenomenon. In general, fish are susceptible to the initial infection, but differ in their ability to limit the infection or

destroy the pathogen (Camp et al., 2000). Numerous observations suggest that variation in susceptibility to ESC is a function of differences in innate immune responses (Bilodeau-Bourgeois et al., 2008).

A multifaceted initial immune response to *E. ictaluri* infection has been observed, encompassing the complement cascade, iron regulation, inflammatory cell signaling, and antigen processing and presentation (Peatman et al., 2008). The acute phase response (APR) denotes a major role of innate responses, particularly up-regulation of genes involved in iron homeostasis (e.g., intelectin, hemopexin, haptoglobin, ferritin and transferrin). Up-regulation of the majority of the complement cascade is observed including the membrane attack complex components and complement inhibitors (Peatman et al., 2007). A number of pathogen recognition receptors (PRRs) and chemokines are also differentially expressed in the liver following infection (Bilodeau and Waldbieser, 2005; Baoprasertkul et al., 2006; Peatman et al., 2007).

The toll-like receptor (TLR)-5 is highly expressed in liver tissue of affected fish; its induction increases on days 5 and 8 post-exposure in liver and increases on day 5 in the head kidney, which may be due to MØ aggregation during ESC infection. In the same way, TLR3 expression in kidney is elevated and increases over time in spleen (Bilodeau and Waldbieser, 2005; Baoprasertkul et al., 2006). Toll-like receptors are an important component of the innate immune response of catfish, this family of PRRs is strongly associated with both innate and adaptive immune systems (Bilodeau-Bourgeois et al., 2008).

Also major histocompatibility complex (MHC)-I molecules play a major role in processing and presenting ESC antigens; MHC-I itself along with β_2M are highly expressed after day 3 post-infection, playing an important role in antigen presentation. In addition, some endoplasmic reticulum chaperons, such as calreticulin and endoplasmin, which have a role in peptide assembly to MHC-I, are highly up-regulated. In some ESC-sensitive catfish families, there is a slow expression of MHC-I related molecules along with the percentage of B lymphocytes increasing over time, with the greater responses on day 7 post-exposure; whereas, the resistant families have higher percentages of T-cells at each day post-exposure (Camp et al., 2000).

It is clear that during an ESC episode, the immune system is directed towards a cell-mediated response which is the classical response against intracellular pathogens, and it correlates with previous findings of survival and replication of this bacterium inside phagocytes (Booth et al., 2006). On the other hand, the kinetics of Ig production following infection shows that serum concentrations markedly increase at 13 days post-infection and the increase in mucus Ig concentrations occurs 14 days later (Zilberg and Klesius, 1997) or even 21 days post infection (Bader et al., 2004).

Fish from a population that has recovered from the disease are considered to be carriers. These fish will have protective immunity and may have high levels of *E. ictaluri* specific antibodies (OIE, 2006). This protective immunity in channel catfish is largely mediated by a cellular immune response with humoral antibodies having a secondary function (Russo et al., 2009). Thus, protection from ESC does not correlate with antibody production unless titers are very high, but antibodies do appear to play a

role in immunity when combined with phagocytic cells (Moore et al., 2002). Opsonization of *E. ictaluri* with serum from vaccinated fish enhanced phagocytosis by MØ from both vaccinated and non-vaccinated fish (Russo et al., 2009). Nevertheless, it is noteworthy that MØ from vaccinated fish were more efficient in phagocytosis and killing of *E. ictaluri* without opsonization compared to MØ from non-vaccinated fish, reflecting that other components of the immune system may enhance MØ killing efficiency. It has been noted that MØ from vaccinated catfish produce higher amounts of reactive oxygen species (ROS) and NO, as compared to MØ from non-vaccinated fish (Russo et al., 2009). Thus, MØ from vaccinated fish are activated and are responsible for rapid clearance of bacteria upon re-exposure to virulent *E. ictaluri*, along with a rapid and augmented traffic of activated MØ to the site of infection as shown in ESC-resistant catfish families (Camp et al., 2000).

3.2. Prevention of ESC

Prevention and control of ESC has proven difficult due to challenges present in intensive channel catfish husbandry and also because the widespread distribution of the bacterium throughout the catfish aquaculture industry. Although many efforts have been expended to develop a highly effective vaccine (Lawrence et al., 1997; Thune et al., 1997; Bader et al., 2004; Klesius et al., 2004), no cost-effective high-efficacy vaccine is on the market to date. Thus, management practices have been the best approach to reduce the incidence of *E. ictaluri*. Among these are proper feeding practices and nutrition, stress reduction, maintenance of adequate water quality and the correct use of

antibiotics and other chemicals (Hawke et al., 1998). However, governmental policies and consumer awareness, as well as pure economics have restrained the use of the latter. Hence, further development and use of appropriate vaccination strategies is vital for improving the efficiency and economics of catfish aquaculture and might have application to other cultured fish species (Adams and Thompson, 2006; Secombes, 2008).

Several studies have demonstrated as many as 15 immunogenic antigens present in *E. ictaluri* (Thune et al., 1997; Moore et al., 2002). Mainly two types of vaccines have been used against ESC, killed and live-modified bacterins. The latter is currently commercially available, and is the sole vaccine for fish currently licensed by the Animal and Plant Health Inspection Service – United States Department of Agriculture (USDA-APHIS, 2008). At an early stage of ESC vaccine development, efforts were primarily focused on the use of killed bacterins with equivocal results, and although antibodies were produced to a variety of preparations, a positive response did not correlate with protection unless very high titers were achieved (Thune et al., 1997; Hawke et al., 1998). In addition, a short-term humoral immunity would be expected from injection of subcomponents of the bacteria (Bader et al., 2004). For these reasons, the use of live-modified vaccines became the preferable strategy to improve efficacy (Klesius et al., 2004), due to long-term cell-mediated immunity likely requiring the entire live organism for stimulation and/or the addition of an adjuvant (Bader et al., 2004).

The efficacy of vaccines might be influenced by a variety of factors besides the immunogen *per se* (Klesius et al., 2004), and they need to be taken into account when

developing strategies for fish vaccination. One of the most important factors is nutrition; the role of nutrition on the immune system and health of fish is very complex and still not totally elucidated. On one hand, nutritional requirements need to be met in order to maintain proper growth, but also improved nutrition through supplementation of key nutrients may increase health and the disease resistance (Blazer et al., 1989; Hawke et al., 1998; Buentello and Gatlin, 2001b; Klesius et al., 2004; Buentello et al., 2007). Attempts to pursue the latter have been conducted by supplementing various vitamins, minerals, lipids, probiotics, prebiotics and amino acids among others, with some positive results (Wise et al., 1993; Sealey et al., 1997; Wang et al., 1997; Lim and Klesius, 2003; Welker et al., 2007; Peterson et al., 2009). Previous results in our laboratory demonstrated a positive effect of an ARG-enriched diet on the resistance of channel catfish to *E. ictaluri* infection (Buentello and Gatlin, 2001b).

Based on the preceding information, it was hypothesized that dietary supplementation of ARG and GLN at optimal levels would enhance various metabolic responses including vaccination efficacy of channel catfish against *E. ictaluri.* To test this hypothesis the following objectives were pursued:

1) To evaluate nutritional and metabolic responses of juvenile channel catfish fed graded amounts of ARG.
2) To evaluate nutritional and metabolic responses of juvenile channel catfish fed graded amounts of GLN.

3) To evaluate *in vitro* effects of ARG and GLN supplementation on channel catfish leukocytes performance.
4) To evaluate *in vivo* effects of ARG and GLN on *E. ictaluri*-specific immune responses in channel catfish.

CHAPTER II

EVALUATION OF NUTRITIONAL AND METABOLIC RESPONSES OF JUVENILE CHANNEL CATFISH FED GRADED AMOUNTS OF ARGININE

1. Introduction

Growth rates and efficiency of feed utilization are two of the most economically important factors in aquaculture. Hence, there is an increasing need to develop biotechnological applications that may help reduce production costs and grow fish more efficiently (Naylor et al., 2009). Pivotal roles of several amino acids on growth improvement and enhanced metabolic function have been documented for various fish species (Mommsen et al., 2001; Fournier et al., 2002; Gómez-Requeni et al., 2004; Andoh, 2007). More than just contributing to a balanced amino acid profile, recent evidence points to specific regulatory actions through which individual amino acids modulate key metabolic pathways which are indispensable for the optimization of somatic growth and immune function in cultured fish species (Li et al., 2009).

The formulation of aquafeeds with "functional nutrients" as a priority may be desirable as the supplementation of these nutrients often results in favorable effects including growth promotion and enhanced health of animals intended for human consumption. One such nutrient is arginine (ARG), an amino acid of enormous physiological importance due to its various beneficial actions, which have been demonstrated in different vertebrate models including fish (Morris, 2006; Buentello et

al., 2007; Wu et al., 2009). Of paramount importance is ARG's essentiality for maximization of growth potential in juvenile animals. This amino acid is the most abundant nitrogen carrier for tissue proteins and is used in multiple biosynthesis pathways, involving key regulatory enzymes such as arginase, nitric oxide (NO) synthase, arginyl-tRNA synthetase, among others (Morris, 2006; Wu et al., 2009). As such, ARG serves as a precursor for the synthesis of creatine, ornithine, proline, glutamate, polyamines, and NO, displaying remarkable metabolic and modulatory versatility in cells (Morris, 2006; Yao et al., 2008; Wu et al., 2009).

A growing number of reports on the enhancement of average daily weight gain, feed intake, protein efficiency and muscle protein synthesis emphasize ARG's unique ability to support rapid growth in different vertebrate species (Fligger et al., 1997; Kim et al., 2004; Fernandes et al., 2009; Ma and Li, 2009). Similarly, aquatic species also experience enhanced growth and improved protein metabolism upon supplementation of dietary ARG. For instance, acute stimulatory effects on growth have been observed in chinook salmon (*Oncorhynchus kisutch*) and rainbow trout (*O. mykiss*) fingerlings (Plisetskaya et al., 1991). In juvenile European seabass (*Dicentrarchus labrax*), supplementation of ARG to plant protein-based diets produced improvements of specific growth rate, feed efficiency and protein efficiency ratio (Tulli et al., 2007). Increased growth was also observed in Atlantic salmon (*Salmo salar*), but this effect was transient and observed in conjunction with glutamate supplementation (Oehme et al., 2010). However, in the South American pacu (*Piaractus mesopotamicus*), limited improvement was observed in protein efficiency ratio (Tesser et al., 2005).

In addition, several roles have been established for ARG as an immunomodulator of both the innate and adaptive immune systems of vertebrates. As previously indicated, ARG serves as the sole substrate for synthesis of NO – an essential oxidative molecule used to combat a multitude of invading pathogens. Likewise, ARG may strongly regulate the expression of adhesion molecules, tissue factors, cytokines and promote, among other important immune functions, the proliferation of lymphocytes and enhanced wound healing (Evoy et al., 1998; Li et al., 2007; Roth, 2007). In channel catfish, ARG supplementation (*in vivo* and *in vitro*) has been shown to influence NO production and phagocytosis in activated macrophages (MØ) of channel catfish (Buentello and Gatlin, 1999; Buentello et al., 2007) as well as enhance fish survival upon *Edwardsiella ictaluri* challenge (Buentello and Gatlin, 2001b). Because it is possible that other important components of the immune system may synergize with NO to exert deleterious reactions against bacteria and other pathogens (Secombes, 1996; Yano, 1996), further elucidation of the effects of ARG on the immune defense mechanisms becomes crucial to further understand its physiological role in fish. Therefore, the present study was conducted to evaluate the effects of dietary ARG supplementation on weight gain, protein optimization, circulating amino acid profiles and specific components of the innate immune system of juvenile channel catfish.

2. Materials and methods

2.1 Experimental diets

A basal diet was formulated to contain 26% crude protein from casein, gelatin and a crystalline L-amino acid premix; dextrin was provided at 25.4% and lipids from corn and menhaden oil at 8%, on a dry-matter basis, for an estimated available energy level of 12 kJ · g^{-1} (Table 2.1). The basal diet was analyzed to contain 0.5% ARG which is deficient based on the minimum dietary ARG requirement of 1% previously quantified for fingerling channel catfish (Robinson et al., 1981). Experimental diets were formulated and analyzed to provide ARG at 1, 2 and 4% of diet, by supplementing L-ARG·HCl (11500, Affymetrix, Santa Clara, CA) to the basal diet. Diets were maintained isonitrogenous by adjusting the levels of a 50:50 glycine-aspartate premix, as described by Buentello and Gatlin (2000).

2.2 Feeding trial

One hundred and eighty disease-free juvenile catfish, with an average weight of 22.9 ± 0.5 g were placed into 12, 110-L aquaria, at a density of 15 fish per aquarium. Each treatment was randomly assigned to triplicate aquaria which were arranged as a recirculating system equipped with a biofilter for ammonia removal and sand filter for mechanical filtration. A constant flow of 1L · min^{-1} was maintained in all tanks. Dissolved oxygen and water temperature were maintained at 90% of air saturation and 27 ± 1 °C, respectively. A 12:12 h light:dark cycle was provided through fluorescent lights regulated with a timer. Water quality was monitored every other day for pH,

Table 2.1
Formulation (% of dry weight) and proximate composition of experimental diets supplemented with graded levels of ARG

	Dietary ARG (% of diet)			
Ingredient	**0.5**	**1**	**2**	**4**
Casein, vitamin free[1]	10.4	10.4	10.4	10.4
Gelatin[1]	2.7	2.7	2.7	2.7
Amino acid premix[1,2]	7.1	7.1	7.1	7.1
Dextrin[1]	25.4	25.4	25.4	25.4
Celufil[1]	28.0	28.6	29.8	32.4
Corn oil[3]	4.0	4.0	4.0	4.0
Menhaden oil[3]	4.0	4.0	4.0	4.0
Vitamin premix[4]	3.0	3.0	3.0	3.0
Mineral premix[5]	4.0	4.0	4.0	4.0
$Ca(PO_4)$[6]	1.0	1.0	1.0	1.0
Carboxymethyl cellulose[1]	2.2	2.2	2.2	2.2
Aspartate:Glycine premix[1,2]	8.2	7.1	4.9	0.2
L-ARG·HCl[7]	0.0	0.5	1.5	3.6
Analyzed proximate composition (% dry weight)				
Dry matter	92.9	93.8	92.1	93.4
Crude protein	26.6	26.7	27.1	26.9
Crude lipid	8.3	8.3	8.5	8.4
Ash	3.7	3.7	3.7	3.6
ARG	0.5	1.0	1.9	4.0

[1] USB, Cleveland, OH, USA.

[2] Buentello and Gatlin (2000). Consisted of (% of diet) : L-histidine, 0.14; L-isoleucine, 0.19; L-leucine, 0.06; L-lysine, 0.64; LD-methionine, 0.32; L-phenylalanine, 0.42; L-serine, 1.57; L-threonine, 0.13; L- tryptophan, 0.02; L-valine, 0.11; L-proline, 1.57; L-alanine, 1.57.

[3] Omega Protein, Reedville, VA, USA.

[4] Moon and Gatlin (1991).

[5] MP Biomedicals, Solon, OH, USA.

[6] Fisher Scientific, Waltham, MA, USA.

[7] Affymetrix, Santa Clara, CA.

hardness, alkalinity, nitrite, ammonia, temperature and dissolved oxygen and remained within acceptable levels known to support optimal growth of channel catfish. Fish were acclimated for a period of 2 weeks to the experimental conditions during which fish were fed the basal diet. Thereafter, fish were fed the experimental diets for the duration of the feeding trial (6 weeks). Feeding rate was initially set at a level approaching satiation (4% of biomass) and provided in two daily feedings (a.m. and p.m.). Acceptance of diets, avidity of feeding and unconsumed feed were parameters considered for determining the feeding rate. Fish were weighed once a week and the feed ration was adjusted accordingly. Procedures used in this study were approved by the Texas A&M University System Animal Care and Use Committee.

2.3 Sample collection and analyses

At the end of the experimental period, three fish per tank were randomly selected for sample collection. Prior to all sampling fish were euthanized with tricaine methanesulphonate (MS-222, Western Chemical Inc., Ferndale, WA, USA, 300 mg/L). Blood samples (~1 mL) were obtained from the caudal vasculature with heparinized needles (1-mL syringe, 23-ga needle). Whole blood was used for neutrophil oxidative radical production (respiratory burst) and blood plasma was used for lysozyme activity and amino acid quantification after separation by centrifugation (3,800 x *g* for 12 min). Both head and trunk kidneys of each fish were excised and pooled per tank for phagocyte isolation. In addition, whole-body samples of three additional fish per tank

were separated for proximate composition analysis. Plasma and whole-body samples were quickly frozen and kept at –80 °C until analysis.

Performance indicators including relative weight gain (WG = final weight – initial weight x 100 / initial weight), feed efficiency ratio (FER = weight gain / dry feed intake) protein efficiency ratio (PER = weight gain / dry protein fed), protein retention (PR = [final body protein – initial body protein] x 100 / total protein fed), and survival rates were computed for fish fed each diet. Whole-body proximate composition was analyzed using established methodologies for crude protein (AOAC, 2005), lipids (Folch et al., 1957) and ash (AOAC, 1990).

Plasma amino acids, including ARG were analyzed via HPLC following a fluorometric technique (Buentello and Gatlin, 2000) using pre-column derivatization with o-phthaldialdehyde (P0657, Sigma, St. Louis, MO).

Neutrophil oxidative radical production was determined as described by Siwicki et al. (1994), absorbance was converted to nitro blue tetrazolium (NBT) units based on a standard curve of NBT diformazan · mL^{-1} blood. Serum lysozyme activity was determined by a turbidimetric assay described by Jørgensen et al. (1993), slightly modifying the pH (5.9) of the *Micrococcus lysodeikticus* suspension to maximize activity, as determined in preliminary assays with channel catfish plasma in our laboratory (data not shown). Phagocytes were isolated, enumerated and their viability assessed via trypan blue exclusion, as described in Buentello and Gatlin (2000). Viability was >95% in all cases. Their ability to produce both extracellular (EC) and

intracellular (IC) superoxide anion was analyzed following established methodology (Secombes, 1990; Sealey and Gatlin, 2002b).

2.4 Statistical analysis

Data was evaluated for normality using the Shapiro-Wilk test and for homogeneity of variance using the Levene's test. Results were analyzed via linear regression and analysis of variance. The post hoc Duncan's multiple-range test was used to identify means differences. The Statistical Analysis System (SAS, 9.2 v) software was used for all analyses. Statistical significance among treatments was considered at $P \leq 0.05$. Values are presented as means with a pooled standard error (P.S.E) per variable measured.

3. Results

Increasing levels of ARG in the diet resulted in significant effects on WG, FER, PER and PR (Table 2.2) after 6 weeks of feeding. Fish fed the 4% ARG diet had significantly higher values for all performance indicators, although these values were not different from those fed the 2% ARG diet, with the exception of PR. Weight gain, FER and PER values of fish fed the 2 and 4% ARG diets were 29-32 and 44-47% higher, respectively, than fish fed the 1% ARG. Also, PR was 65 and 180% higher in fish fed the 2 and 4% ARG diets, respectively, as compared with fish fed the 1% ARG diet. Accordingly, whole-body crude protein levels for the two highest supplementation levels were significantly greater than corresponding whole-body protein levels for the lower

levels of ARG inclusion. There were no significant differences in any other proximate composition parameter evaluated (Table 2.2).

Plasma ARG and related amino acid concentrations were significantly affected by dietary ARG levels (Table 2.3). Generally, fish fed the deficient diet (0.5%) had significantly lower values for all analyzed amino acids. Fish fed 2 and 4% ARG diets had significantly higher circulating levels of ARG as compared to the basal diet. These values were 2.2- and 2.3-fold higher than those observed for fish fed the basal diet, respectively. However, these values were not significantly different from levels observed in fish fed the 1% ARG diet. Circulating ornithine was 3.8-fold higher in fish fed the 2% ARG diet than in fish fed the basal diet; whereas, these values were only 2.4- and 2.6-fold for fish fed the 1 and 4% ARG, respectively, as compared to the level or ornithine found in fish fed the 0.5% ARG diet. Citrulline plasma levels were 3.7-, 3.7- and 5-fold higher in fish fed the 1, 2 and 4% ARG diets, respectively, compared to the citrulline concentration found in fish fed the basal diet. Glutamine concentrations exhibited a dose-dependent response as ARG increased from 0.5 to 4% of diet, with circulating glutamine in fish fed the 4% ARG diet being 3.6-fold higher than that of fish fed the basal diet. Similarly, fish fed the 2% ARG diet had 1.8-fold higher concentration of circulating glutamate than fish fed the basal diet. However, a further increase in dietary ARG, beyond 2% of diet, did not result in increased plasma glutamate.

All innate immune responses, except plasma lysozyme, were also significantly affected by dietary ARG supplementation (Table 2.4). Although fish fed the 2 and 4% ARG diets showed increased lysozyme values, they were not significantly different

Table 2.2
Growth performance responses of juvenile channel catfish fed incremental levels of ARG[1]

	ARG Level (% of diet)				*Pr* > F [2]	Pooled std. error	R^2
	0.5	**1**	**2**	**4**			
Initial biomass (g)	344	340	343	343	0.923	5.23	0.004
Final biomass (g)	520[3,c]	607[b]	740[a]	808[a]	0.0001	23.58	0.91[4]
Weight Gain (% initial weight)	51.1[c]	91.4[b]	120.8[a]	134.5[a]	<0.0001	6.17	0.72[4]
Feed Efficiency (g gain · g feed)	0.3[c]	0.4[b]	0.5[a]	0.6[a]	<0.0001	0.02	0.75[4]
Protein Efficiency Ratio (g gain · g protein fed)	1.0[c]	1.4[b]	1.9[a]	2.1[a]	<0.0001	0.08	0.76[4]
Protein Retention (%)	14.6[d]	27.7[c]	45.7[b]	77.6[a]	<0.0001	2.29	0.98[4]
Survival (%)	100	95	98	100	0.197	2.12	0.07
Proximate composition of whole-body							
Moisture (%)	68.2	68.2	69.3	68.9	0.5852	1.15	0.09
Crude Protein (%)	12.8[d]	15.3[c]	19.0[b]	27.1[a]	<0.0001	1.10	0.93[3]
Lipid (%)	9.0	11.9	10.1	9.6	0.1643	1.78	0.02
Ash (%)	4.3	3.7	3.1	3.9	0.1076	1.43	0.09

[1] Values represent means of three replicate tanks.

[2] Significance probability associated with the F-statistic.

[3] Different superscript letters indicate significant ($P < 0.05$) differences as evaluated by Duncan's multiple range test.

[4] Indicate significant ($P < 0.05$) differences as evaluated by linear regression.

Table 2.3
Circulating plasma levels (nmol · mL^{-1}) of selected amino acids in juvenile channel catfish fed incremental levels of ARG[1]

	ARG level (% of diet)				*Pr* > F [2]	Pooled std. error	R^2
	0.5	**1**	**2**	**4**			
Arginine	71.4[3,b]	124.6[ab]	156.1[a]	164.3[a]	0.015	10.21	0.44[4]
Ornithine	17.9[c]	43.1[b]	68.0[a]	46.1[b]	0.020	11.70	0.14
Citrulline	9.9[b]	36.2[a]	36.9[a]	49.9[a]	0.016	6.65	0.49[4]
Glutamine	38.4[c]	85.2[b]	135.7[a]	137.4[a]	0.048	7.36	0.40[4]
Glutamate	38.4[c]	57.8[b]	70.8[a]	63.7[ab]	0.001	3.59	0.34[4]

[1] Values represent means of two fish from each three replicate tanks.

[2] Significance probability associated with the F-statistic.

[3] Different superscript letters indicate significant ($P < 0.05$) differences as evaluated by Duncan's multiple range test.

[4] Indicate significant ($P < 0.05$) differences as evaluated by linear regression.

Table 2.4
Innate immune responses of juvenile channel catfish fed incremental levels of ARG[1]

	ARG level (% of diet)				*Pr* > F [2]	Pooled std. error	R^2
	0.5	**1**	**2**	**4**			
NBT[3]	2.9[4,b]	4.0[a]	3.7[a]	3.9[a]	< 0.0001	0.11	0.22[5]
Superoxide anion –EC[6]	5.2[b]	8.6[a]	5.4[b]	5.6[b]	0.0490	0.98	0.03
Superoxide anion –IC[7]	0.125[b]	0.547[a]	0.511[a]	0.484[a]	0.0030	0.08	0.12
Lysozyme[8]	166.7	179.7	206.2	218.8	0.572	28.87	0.05

[1] Values represent means of two fish from each three replicate tanks.

[2] Significance probability associated with the F-statistic.

[3] NBT, Nitroblue tetrazolium units (mg · mL^{-1}).

[4] Different superscript letters indicate significant ($P < 0.05$) differences as evaluated by Duncan's multiple range test.

[5] Indicate significant ($P < 0.05$) differences as evaluated by linear regression.

[6] EC, extracellular (nmol 0_2^-).

[7] IC, intracellular (ABS).

[8] units · mL^{-1}.

among treatments. Dietary ARG deficiency not only diminished the capacity of phagocytes to generate superoxide anion, but it also curtailed the capacity of blood neutrophils to generate oxidative radicals. All innate immunity indicators assessed in the present experiment were significantly decreased in fish fed the 0.5% ARG diet. However, no further enhancement was attained by supplementing ARG at levels beyond 1% of diet. In fact, EC superoxide anion production was significantly higher in fish fed the 1% ARG diet when compared to fish fed both ARG-supplemented and ARG-deficient diets.

4. Discussion

4.1 Effects of arginine on fish growth

The dietary arginine requirement of channel catfish was originally determined to be 1.0% of diet by Robinson et al. (1981). This requirement is one of the lowest when compared to other freshwater and saltwater fish species (NRC, 2011). In the present experiment, results indicate that dietary ARG supplementation beyond the previously determined minimum requirement increased PER, resulted in better PR, as well as improved WG and FE. These results are in agreement with previous reports with other fish species whose growth and metabolic rates are similar to that of channel catfish – e.g., *Clarias lazera* (Metwally and Fouad, 2009) – and other freshwater fish such as rainbow trout (Plisetskaya et al., 1991; Fournier et al., 2002), the South American pacu (Tesser et al., 2005), and marine fish including turbot, *Psetta maxima*, gilthead sea bream, *Sparus aurata* (Fournier et al., 2002), European seabass (Fournier et al., 2002;

Tulli et al., 2007), and Atlantic salmon (Oehme et al., 2010). The specific physiological mechanism by which ARG elicits an increased growth in fish species has not been fully elucidated. Investigations on mammalian species (Wideman et al., 2000) as well as fish (Mommsen et al., 2001), including our own ongoing research with channel catfish (unpublished data), points to a strong endocrine modulation, which may not have been previously considered in defining the nutritional requirement for this amino acid. Supplementation of ARG has been shown to increase plasma concentrations of insulin and growth hormone, and ultimately improving weight gain and protein synthesis in higher vertebrates (Kim et al., 2004; Collier et al., 2005; Wu et al., 2009). Although data in this area with fish is more sparse, similar effects have been reported for barfin flounder, *Verasper moseri* (Andoh, 2007), rainbow trout (Plisetskaya et al., 1991; Mommsen et al., 2001), chinook salmon (Plisetskaya et al., 1991), brown trout, *Salmo trutta*, common carp, *Cyprinus carpio* (Baños et al., 1997), and largemouth bass, *Micropterus salmoides* (Sink and Lochmann, 2007).

A plausible physiological alternative considers ARG's established roles as the most abundant nitrogen carrier in tissue proteins and its participation in multiple synthetic pathways (Morris, 2006; Wu et al., 2009) including protein (Kim et al., 2004; Yao et al., 2008) and proline synthesis (Wu et al., 2009). From these, ARG's role on polyamine biosynthesis deserves special consideration because numerous studies have shown that the polyamines putrescine, spermidine, and spermine have a significant effect on growth of the gastrointestinal mucosa of a variety of animals (McCormack and Johnson, 1991; Larque et al., 2007), including fish (Péres et al., 1997). As precursor of

ornithine, ARG is an essential and rate-controlling component for the biosynthesis of polyamines in animal tissues (Wu, 2010). Because several studies correlate high intracellular levels of polyamines with periods of increased growth in bacterial, plant and animal species (McCormack and Johnson, 1991; Li et al., 2009; Wu, 2010) it is possible that ARG also may influence growth through this hitherto unknown regulatory mechanism in fish. Interestingly, results from the present study (Table 2.3) indicate that ARG supplementation beyond 1% of diet did not result in significantly higher postprandial levels of plasma ARG but raised the concentrations of circulating ARG byproducts such as ornithine, glutamine and glutamate. As in the experiments listed above, it is quite possible that a fraction of the dietary ARG in excess of 1% could have been metabolized in the gastrointestinal tract, yielding higher concentrations of ARG-related products in both portal and post-hepatic circulation. This would up-regulate the usage of ornithine, glutamine and glutamate for the biosynthesis of polyamines in target tissues. Several reports on vertebrate species support this notion by describing a common metabolic pathway for ARG, ornithine, glutamine and glutamate. Evidence generated in our laboratory demonstrate that channel catfish is physiologically similar in this aspect to higher vertebrates (Buentello and Gatlin, 2001a).

The interorgan metabolism of ARG can provide citrulline either from ARG-derived ornithine or directly from ARG (Buentello and Gatlin, 2001a). The fact that in the present experiment plasma citrulline levels remained similar in fish fed 1-4% ARG and only those fed the ARG-deficient basal diet displayed a significantly lower citrulline concentration (Table 2.3) can be explained in two ways. First, if channel catfish has an

incomplete ornithine urea cycle, as indicated by some authors due to reduced activities of carbamoyl phosphate synthetase III (Felskie et al., 1998), this would result in reduced formation of citrulline from ornithine. On the other hand, if the activity of the enzyme arginase is of higher magnitude than NO synthase – as is the case for animals under no pathological challenge (Wong et al., 1998; Gouillou-Coustans et al., 2002), this would increase the formation of ornithine from ARG. Under either scenario ornithine appears to be more readily synthesized from ARG than citrulline and, this ARG-derived ornithine could be funneled to polyamine biosynthesis in support of rapid somatic (Li et al., 2009; Wu, 2010) and/or gastrointestinal (Péres et al., 1997; Larque et al., 2007) growth, as previously described.

4.2 Effects of arginine on fish immunity

It is well established that ARG is an important immunonutrient in vertebrate species such as humans, rodents, swine and poultry (Evoy et al., 1998; Li et al., 2007; Roth, 2007; Wu, 2010). Also, accumulating evidence demonstrates that, as in higher vertebrates, ARG potentiates several aspects of the immune responses in fish. For instance, improved survival of channel catfish was observed upon dietary ARG supplementation when fish were challenged with a virulent strain of *E. ictaluri* (Buentello and Gatlin, 2001b) and, the presence of ARG *in vivo* and *in vitro* up-regulated the synthesis of NO in lipopolysaccharide-activated catfish phagocytes (Buentello and Gatlin, 1999). In addition, ARG in the diet had positive effects on both hematological and innate immune responses such as hematocrit, hemoglobin,

phagocytosis, and circulating erythrocytes (Buentello et al., 2007). Data from the present experiment helps to highlight the crucial role that ARG has in maintaining proper phagocyte function. Specifically, the production of both superoxide anion by kidney phagocytes and oxidative radicals by blood neutrophils was severely compromised in fish fed the ARG-deficient basal diet (Table 2.4). That these indicators were not raised by levels of ARG above 1% of diet may be a reflection of the metabolic state of the cells. Neither neutrophils nor MØ were in an activated state. In contrast, an increase in oxidative radicals would be expected upon ARG enrichment in activated cells of the immune system, such as MØ (Buentello and Gatlin, 1999, 2001b; Li et al., 2007).

Although the levels of lysozyme activity increased as dietary ARG increased in the present experiment, a high degree of variability among treatments made these differences not significantly different ($P = 0.5$). An important consideration is that the activity of plasma lysozyme is not a direct measurement of cell performance but reflects the presence of this muramidase in plasma and can also be linked to the turnover rate of granulocytes and monocytes (Hansen, 1975; Yano, 1996). Because in the present experiment the sampled fish were presumably under a homeostatic state, a similar turnover rate of granulocytes and monocytes would be expected.

These results add to the growing body of literature concerning ARG and its effects on fish metabolism. The present results also confirm that dietary ARG is indispensable for optimal growth of juvenile fish as well as providing strong indications that this amino acid may have an important regulatory role in the biosynthesis of

polyamines and perhaps another one as an endocrine modulator in fish. Expanded ARG functions in maintaining proper phagocyte function were also demonstrated in the present study. However, more research is still necessary to further elucidate the specific mechanisms by which ARG promotes somatic growth in fish. In addition, the evaluation of ARG effects on other parameters of the immune system in health and disease is crucial to help optimize aquafeed formulations.

CHAPTER III

EVALUATION OF NUTRITIONAL AND METABOLIC RESPONSES OF JUVENILE CHANNEL CATFISH FED GRADED AMOUNTS OF GLUTAMINE

1. Introduction

Glutamine (GLN) plays an important role as a regulator of cellular metabolism, acting as an energy source for proliferating cells and being essential for the synthesis of nucleic acids, pyrimidine and purine nucleotides and other important cellular intermediates (Nakajo et al., 2005; Wu et al., 2011). Glutamine is the principal metabolic fuel for enterocytes, which makes the gastrointestinal tract (GIT) a major organ of GLN utilization in the post-absorptive state (Bartell and Batal, 2007; Rhoads and Wu, 2009; Wu et al., 2011).

Dietary GLN has been proven to elicit desirable effects for animal husbandry, and compelling evidence shows that GLN is a conditionally-essential amino acid under specific physiological conditions (Wu et al., 2011). The addition of GLN in diets improves the weight gain and feed efficiency in different mammalian and avian species. In addition, it not only prevents intestinal mucosal atrophy, but also promotes intestinal growth, and increases villi length in the duodenum and jejunum (Yi et al., 2005; Zou et al., 2006; Bartell and Batal, 2007; Murakami et al., 2007; Wang et al., 2008; Soltan, 2009; Wu et al., 2011). Additionally, challenges associated with diarrhea are mitigated by GLN and the deleterious changes in digestive tract permeability associated with

endotoxins also can be decreased (Lobley et al., 2001; Zou et al., 2006; Kessel et al., 2008). Moreover, GLN enhances recovery of the villous surface area in animals with ischemic-injured intestines (Blikslager et al., 1999). Noteworthy is the fact that GLN-enriched diets also enhance the transport of absorptive vesicles and amino acids across the enterocyte brush border (Salloum et al., 1990; Frankel et al., 1993; Curi et al., 2005).

Similar effects of dietary GLN supplementation have been reported to a limited extent in fish species. Improved weight gain, feed efficiency, intestinal weight, fold height and digestive enzyme activities were reported in juvenile Jian carp, *Cyprinus carpio* var. Jian (Yan and Qiu-Zhou, 2006) and juvenile hybrid sturgeon, *Acipenser schrenckii* × *Huso dauricus* (Qiyou et al., 2011). Additionally, it has been proven *in vitro* that GLN is essential for enterocyte proliferation (Jiang et al., 2009) as well as being an effective protector against H_2O_2-induced oxidative stress in jian carp (Chen et al., 2009).

Under normal conditions, the GIT epithelium has a high cell turnover and metabolic rate, both of which increase during physiological and/or pathological challenges. Thus, adequate nutrition is crucial to maintain proper function and integrity of the GIT, which in turn, has major repercussions on growth performance and health status of cultured animals. The GIT is not only the major site of digestion and absorption of nutrients, but it is also a critical player in intermediary metabolism, having a specific role in whole-body amino acid homeostasis and the availability of amino acids for the support of somatic growth (Reeds and Burrin, 2000; Wu et al., 2005b; Wang et al., 2009).

Therefore, the purpose of the present study was to evaluate the effects of graded amounts of GLN supplementation to semi-purified diets on intestinal structure, plasma amino acid profiles and growth performance of channel catfish. Also, based on results of the feeding trial, another experiment evaluated the effect of GLN supplementation on enterocyte migration rate using the incorporation of an exogenous marker bromodeoxyuridine (BrdU).

2. Materials and methods

2.1 Experimental diets

The basal diet was formulated to contain 28% crude protein from casein, gelatin and a crystalline L-amino acid premix; dextrin was provided at 24.5% and lipids from corn and menhaden oil at 8%, on a dry-matter basis, for an estimated available energy of 12 $kJ \cdot g^{-1}$ (Table 3.1). The basal diet was analyzed to contain 0% of free GLN. Five experimental diets were formulated, and analyzed, to provide 0.5, 1, 1.5, 2 and 3% free GLN by supplementing L-GLN (USB, 16285). Diets were maintained isonitrogenous by adjusting the levels of a 50:50 glycine-aspartate premix, as described by Buentello and Gatlin (2000). Diets were stored in sealed bags at -20 °C until used.

2.2 Experiment 1. Feeding trial

A first experiment was done to evaluate graded amounts of GLN supplementation on intestinal structure, plasma amino acid profiles and growth performance of channel catfish. Experimental fish were obtained from a local

Table 3.1

Formulation and proximate composition of the basal diet that was supplemented with graded levels of GLN

Ingredients	**% of dry weight**
Casein, vitamin free[1]	13.4
Gelatin[1]	3.7
Amino acid premix[1,2]	7.1
Dextrin[1]	24.7
Celufil[1]	27.5
Corn oil[1]	4.0
Menhaden oil[3]	4.0
Vitamin premix[4]	3.0
Mineral premix[5]	4.0
$CaHPO_4 \cdot 2H_2O$[6]	1.0
Carboxymethyl cellulose[1]	2.2
Aspartate/glycine premix[1,2]	5.0
L-Glutamine[1, 7]	0.0
L-Arginine[1]	0.4
Analyzed proximate composition (% dry weight)	
Dry matter	89.7
Crude protein	29.1
Lipid	8.3
Ash	3.4

[1] US Biochemical Corp., Cleveland, OH, USA.

[2] Buentello and Gatlin (2000). Consisted of (% of diet): L-histidine, 0.14; L-isoleucine, 0.19; L-leucine, 0.06; L-lysine, 0.64; LD-methionine, 0.32; L-phenylalanine, 0.42; L-serine, 1.57; L-threonine, 0.13; L- tryptophan, 0.02; L-valine, 0.11; L-proline, 1.57; L-alanine, 1.57.

[3] Omega Protein, Reedville, VA, USA.

[4] Moon and Gatlin (1991).

[5] MP Biomedicals, Solon, OH, USA.

[6] Fisher Scientific, Waltham, MA, USA.

[7] Analyzed GLN content (% - non-protein bound) in diets: 0, 0.44, 1.1, 1.4, 2.2, and 2.9

commercial hatchery and transported to the Texas A&M University Aquacultural Research and Teaching Facility. Fish were acclimated for 2 weeks to a recirculating system (flow rate: 1 L · min^{-1}) equipped with a biofilter for the removal of nitrogenous by-products. Fish were fed the basal diet during the conditioning period. Three hundred and sixty juvenile catfish, averaging 6.1 ± 0.2 g were placed at same density (20 fish / 0.11 m^3) into 18, 110-L aquaria. Oxygen saturation and water temperature were maintained at 90% and 27 ± 1 °C, respectively. A 12:12 h light:dark cycle was provided through fluorescent lights regulated with a timer. Water quality was monitored weekly for pH, hardness, alkalinity, nitrite, ammonia, temperature and dissolved oxygen and remained within optimal levels for channel catfish. Each aquarium was randomly assigned to a dietary treatment with a total of three replicate aquaria per diet. Fish were fed the experimental diets for a period of 10 weeks. Feeding rate was set at a level approaching satiation (4% of biomass) and was provided in two daily feedings (morning and evening). Fish were weighed once a week and the ration was adjusted accordingly.

2.2.1 Sample collection and analyses

Three representative fish per aquarium were randomly sampled at the end of week 10. Prior to all sample collection, fish were euthanized via tricainemethane sulphonate (MS-222, 300 mg/L) overdose. Whole body and GIT weight and length, along with liver, spleen, and intraperitoneal fat weight were recorded for morphometric calculations and tissue protein content analysis. Blood samples (approximately 0.5 mL) were drawn from the caudal vasculature with heparinized syringe (1 mL, 27 gauge

needle) at 15 h after the final feeding. Plasma was separated by centrifugation at 3,800 x *g* for 12 min, and stored frozen (-20 °C) until amino acid analysis. Whole-body samples from three additional fish per tank were taken for proximate composition analysis.

To evaluate changes in histological structures of the intestinal mucosa, GIT samples from three more fish were dissected from the gastro-pyloric region to the anal region, and tied at both ends with cotton mesh. Davidson's solution was injected into the intestinal lumen for preventing autolytic changes to the mucosa. Intestinal samples were kept in Davidson's fixative for 24 h and then transferred to a 70% ethanol solution for conservation until processing for histological slides.

Performance indicators were analyzed as follows, relative weight gain (WG = final weight – initial weight x 100 / initial weight), feed efficiency ratio (FER = weight gain / dry feed intake), protein efficiency ratio (PER = weight gain / dry protein fed) and protein retention (PR = [final body protein – initial body protein] x 100 / total protein fed). In addition, body indexes were computed as follows, condition factor (CF= fish weight x 100 / fish length ^ 3), hepatosomatic index (HSI= liver weight x 100 / whole-body weight) and intraperitoneal fat ratio (IPF= intraperitoneal fat weight x 100 / whole-body weight), relative gut weight (RGW= gut weight x 100 / whole-body weight), relative gut length (RGL= gut length x 100 / whole-body length), and relative spleen weight (RSW= spleen weight x 100 / whole-body weight). Finally, proximate composition of whole-body samples were analyzed using established methodology: Dumas protocol (AOAC, 2005) for crude protein (N factor = 6.25), Folch et al. (1957) for lipids, AOAC (1990) for ash. Crude protein content of liver, intestine and spleen

were analyzed as cited above. Plasma amino acid levels were analyzed via HPLC following the methodology of Buentello and Gatlin (2000).

For histological analysis, two cross-sectional rings of approximately 0.5 cm were cut from each of the anterior, mid and posterior regions of the intestine. Intestinal regions were processed for paraffin embedding, and 5-μm sectioning was made for glass slide mounting and hematoxylin-eosin staining. All slides were evaluated in an Olympus BC-2 series light microscope linked to a digital camera. Three fields at 4x and five fields at 40x objectives were captured for each region. Images were then analyzed with the ImageJ (v.1.4g) Software (NIH, freeware). Variables measured included fold length (distance between the base and tip of the fold), and enterocyte and microvilli height for all intestinal regions.

2.3 Experiment 2. Enterocyte migration rate

A second experiment was done to further evaluate the enterocyte migration rates. A second batch of 120 fish, averaging 33 ± 0.9 g, were placed in six 110-L glass aquaria and kept under the same conditions as described earlier. Based on the results from the first experiment (feeding trial), fish were only fed the basal and 2% GLN diets (three replicates each). After 2 weeks of feeding, all fish were intraperitoneally injected with an aqueous solution of BrdU (Sigma B5002) at a dose of 0.1 mg · g^{-1} of body weight (Alfei et al., 1993). Three randomly selected fish per treatment were sampled every day until 11 d post injection (dpi), then every other day until 19 dpi. Before sampling, fish were euthanized as described earlier. Fish intestines were carefully removed and placed

in 4% formalin for 24 h and moved to 70% ethanol for conservation until processing for histological slides.

2.3.1 Immunohistochemistry detection of BrdU

Intestinal samples were processed for paraffin embedding and unstained slides were obtained for immunohistochemistry (IHC) detection of BrdU in cross-sections of the anterior, mid and posterior intestinal regions. Tissue detection of BrdU was done using a commercial kit (Millipore BrdU IHC kit, 2760). Briefly, tissue sections were deparaffinized with xylene, and rehydrated with several dilutions of ethanol (100-70%) and phosphate buffer solution (PBS). Endogenous peroxidase was quenched submerging the slides in 30% hydrogen peroxide:methanol (1:10, v/v). Tissue DNA was denatured and the sections were blocked to prevent non-specific binding. Sections were incubated with primary antibody (mouse anti-BrdU) and then secondary antibody (Streptavidin-Horse radish peroxidase conjugated goat anti-mouse IgG). Slides were developed with the addition of the substrate solution (3,3' diaminobenzidine). Then, tissues were counterstained with hematoxylin. Slides were dehydrated and mounted with coverslip for further analysis.

2.3.2 Quantification and orientation of BrdU

Quantification and orientation of BrdU positive cells were done using the equipment and software described earlier for histometric analysis. For enterocyte migration rate, BrdU positive cells were analyzed. At least 10 measurements per section

per fish were taken, where only appropriately oriented folds were used. Enterocyte migration per day was calculated by measuring the distance (μm) between the base of the fold and the farthest $BrdU^+$ cell (Fan et al., 2001). This distance was then correlated to the fold height (μm) and a migration percentage per day was obtained (MP = distance from base to $BrdU^+$ cells x 100/ fold height). All animal procedures used in this study were approved by the Texas A&M University Animal Care and Use Committee.

2.4 Statistical analysis

Data was evaluated for normality using the Shapiro-Wilk test and for homogeneity of variance using the Levene's test. Results from feeding trial were subjected to linear regression analysis, and analysis of variance, using Duncan's multiple-range test to compare treatment means when appropriate. Data from the enterocyte migration trial were subjected to analysis of covariance to detect differences in slopes and intercepts. All analyses were conducted using the Statistical Analysis System (SAS v9.2) software. Statistical significance was set at a P-value of ≤ 0.05.

3. Results

3.1 Performance parameters and condition indices

Although fish fed the diet supplemented with 2% GLN tended to have higher values for WG, FER, PER, PR, no statistical differences were observed (Table 3.2). Similarly, whole-body proximate composition values were equal among treatments in dry matter, crude protein, and ash, but a significant difference was obtained in lipid

content where fish fed the diet supplemented with 2% GLN had higher values than those fed 0, 0.5 and 1% GLN but not compared to those fed diets supplemented with 1.5 and 3% GLN (Table 3.2).

In agreement with productive parameters, 2% free GLN in the diet tended to improve the majority of body indexes and the protein content of intestine, but again, no significant differences were found among treatments (Table 3.3).

3.2 Plasma amino acids

Plasma levels of asparagine, serine, glycine and threonine were significantly higher in fish fed the diet supplemented with 2% GLN (Table 3.4). Glutamine and related amino acids did not exhibit any significant differences, although they had similar patterns as the parameters reported above. Namely, fish fed 2% GLN tended to have higher values than other supplementation levels.

3.3 Intestinal structure

Intestinal histometrics are presented in Table 3.5. The mucosal intestinal histology showed a high degree of responsiveness to free dietary GLN supplementation. Increased fold length as well as enhanced enterocyte and microvilli height were observed in a dose-dependent manner. The anterior, mid and posterior intestinal sections had significantly higher values in all parameters measured for fish fed the diets supplemented with 2-3% GLN.

Table 3.2
Growth performance responses and proximate composition of juvenile channel catfish fed graded levels of GLN for 10 weeks[1]

Variables	L-GLN inclusion level (%)						*Pr* > F[2]	Pooled std. error	R^2
	0	0.5	1	1.5	2	3			
Initial average fish weight (g)	6.1	6.2	6.1	6.0	6.0	6.1	0.821	0.11	0.01
Final average fish weight (g)	15.9	16.3	16.4	15.8	17.6	16.3	0.704	0.99	0.039
Weight gain (% initial weight)	161	162	167	162	191	165	0.686	11.42	0.045
Feed efficiency (g gain · g feed)	0.36	0.37	0.38	0.37	0.40	0.36	0.794	0.02	-0.043
Protein efficiency ratio (g gain · g protein fed)	1.11	1.17	1.21	1.15	1.26	1.17	0.619	0.06	0.068
Protein retention (%)	19.6	20.7	20.9	19.6	20.8	20.9	0.979	1.68	-0.047
Proximate composition of Whole-body									
Moisture (%)	70.1	71.1	69.8	71.5	71.0	70.0	0.503	0.74	0.385
Crude protein (%)	16.7	16.7	16.5	16.4	16	16.9	0.976	0.75	-0.059
Lipid (%)	8.2[3,bc]	7.9[c]	8.0[c]	8.9[ac]	10.0[a]	9.7[ab]	0.049	0.79	-0.062
Ash (%)	3.7	3.4	3.8	3.6	3.3	3.8	0.211	0.4	-0.059

[1] Values represent means of three replicate tanks.

[2] Significance probability associated with the F-statistic.

[3] Different superscript letters indicate significant ($P < 0.05$) differences as evaluated by Duncan's multiple range test.

Table 3.3
Tissue indices and composition of juvenile channel catfish fed graded levels of GLN for 10 weeks[1]

Variables	L-GLN inclusion level (%)						*Pr* > F [2]	Pooled std. error	R^2
	0	0.5	1	1.5	2	3			
Hepatosomatic index	1.44	1.57	1.59	1.49	1.48	1.38	0.336	0.07	0.077
Intraperitoneal fat index	3.08	3.16	3.17	2.79	3.18	3.27	0.787	0.24	0.008
Condition factor	0.72	0.71	0.74	0.71	0.74	0.73	0.74	0.02	0.053
Relative GIT length	1.11	1.19	1.27	1.42	1.38	1.2	0.251	0.1	0.048
Relative GIT weight	2.29	2.35	2.66	2.79	2.81	2.37	0.194	0.18	0.024
Relative spleen weight	0.08	0.07	0.08	0.09	0.07	0.10	0.083	0.01	0.015^{ns}
GIT protein content (%)	13.0	14.7	15.0	14.5	15.1	15.4	0.283	0.99	0.153^{ns}
Liver protein content (%)	15.6	14.8	13.8	13.4	14.7	14.9	0.179	0.39	0.035^{ns}
Spleen protein content (%)	19.0	18.1	19.6	19.5	19.6	20.9	0.275	0.98	0.104^{ns}

[1] Values represent means of three fish from each three replicate tanks.

[2] Significance probability associated with the F-statistic.

Table 3.4
Plasma amino acid profile (nmol · mL^{-1}) of juvenile channel catfish fed graded levels of GLN for 10 weeks[1]

	L-GLN inclusion level (%)						*Pr* > F [2]	Pooled std. error	R^2
Amino acid	**0**	**0.5**	**1**	**1.5**	**2**	**3**			
Aspartate	68.7	60.1	59.0	66.5	71.1	66.0	0.876	8.17	0.012
Glutamate	102.9	110.5	90.3	99.8	122.7	112.0	0.589	12.44	0.028
Asparagine	9.1[3,b]	13.1[b]	13.7[b]	14.7[b]	37.6[a]	10.2[b]	0.02	5.2	0.031
Serine	118.6[b]	114.7[b]	119.0[b]	138.2[b]	197.6[a]	103.3[b]	0.04	18.71	0.025
Glutamine	200.4	157.7	143	146.7	201.4	133.3	0.36	27.16	0.043
Histidine	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	--	--	--
Glycine	113.4[b]	110.4[b]	123.3[b]	113.4[b]	163.8[a]	103.9[b]	0.048	12.24	0.037
Threonine	67.3[b]	55.4[b]	70.0[b]	55.4[b]	104.0[a]	68.0[b]	0.008	7.72	0.011
Citrulline	16.17	33.9	36.9	22.0	31.24	15.6	0.679	11.66	0.005
Arginine	143.4	138.9	119.7	130.5	172.0	142.1	0.767	23.11	0.010
Taurine	383.6	359.8	391.7	380.6	428.7	422.7	0.851	42.78	0.086
Alanine	351.5	307.3	307.1	319.4	400	345.9	0.381	32.97	0.051
Tyrosine	109.1	100.3	123.7	103.7	134.8	132.7	0.412	14.3	0.097
Tryptophan	20.4	16.1	18.4	14.4	35.9	21.0	0.418	7.42	0.051
Methionine	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	--	--	--
Valine	139.1	114	113.1	116.2	152.9	152.4	0.491	19.76	0.076
Phenylalanine	73.7	60.9	72.6	62.7	82.9	68.8	0.655	9.87	0.009
Isoleucine	115.2	103.4	92.2	110.6	123.5	119.2	0.875	19.41	0.027
Leucine	228.3	216.1	196.4	204.0	251.1	242.9	0.653	26.4	0.047
Ornithine	81.0	71.2	71.7	83.5	103.3	86.6	0.815	17.84	0.059
Lysine	369.4	300.8	282.7	295	429.4	341.1	0.617	65.58	0.014

[1] Values represent means of three fish from each three replicate tanks.

[2] Significance probability associated with the F-statistic.

[3] Different superscript letters indicate significant ($P < 0.05$) differences as evaluated by Duncan's multiple range test.

3.4 Enterocyte migration rates

Patterns of enterocyte migration rates, obtained from experiment 2, were influenced by dietary glutamine supplementation. In fish fed the basal diet, enterocytes reached the tip of the fold on 11dpi for the anterior intestine and on 10 dpi for the mid and posterior intestinal sections. Fish fed the diet supplemented with 2% GLN had a significant increase in enterocyte migration rate, reaching the tip of the fold on 9, 6 and 8 dpi for the anterior, mid and posterior regions, respectively. Linear regression indicated a migration rate for fish fed the basal and GLN supplemented diets of 5.4 ± 0.3 vs. 6.5 ± 0.3 % · d^{-1} for the anterior intestine, 5.5 ± 0.4 vs. 8.9 ± 0.7 % · d^{-1} for the mid intestine, and 6.4 ± 0.4 vs. 7.8 ± 0.5 % · d^{-1} for the posterior intestine. All slopes were significantly different among treatments in all three intestinal sections; whereas, the intercept was only different in the anterior and posterior sections (Fig. 3.1).

4. Discussion

Glutamine is a versatile amino acid which plays important roles in a variety of biochemical functions. This versatility would seem to give it great potential in normal animal production as well as during situations involving pathological challenges. It is well known that GLN serves as the main energy source for proliferating cells and its supply may improve the outcome of catabolic states and/or disease challenges (Wu et al., 2005b; Wang et al., 2009). Therefore, dietary supplementation of GLN could result in enhanced growth and survival of cultured fish. In practice, however, responses of

Table 3.5
Intestinal histometrics (μm) of juvenile channel catfish fed graded levels of GLN for 10 weeks[1]

	L-Glutamine Inclusion Level (%)						*Pr* > F [2]	Pooled std. error	R^2
	0	0.5	1	1.5	2	3			
Anterior Section									
Fold length	741.83[3,b]	732.57[b]	814.29[b]	767.9[b]	910.88[a]	770.25[b]	<0.0001	27.25	0.076[4]
Enterocyte height	29.31[c]	28.61[c]	31.98[b]	31.49[b]	36.40[a]	35.44[a]	<0.0001	0.76	0.195[4]
Microvilli height	1.29[cd]	1.22[d]	1.52[a]	1.44[ab]	1.55[a]	1.38[bc]	<0.0001	0.04	0.110[4]
Medium Section									
Fold length	169.29[cd]	150.98[d]	213.44[b]	187.1[c]	214.38[b]	255.88[a]	<0.0001	8.29	0.226[4]
Enterocyte height	28.43[d]	27.06[d]	30.64[c]	28.64[d]	36.22[a]	32.47[b]	<0.0001	0.66	0.268[4]
Microvilli height	1.32[b]	1.28[b]	1.39[ab]	1.47[a]	1.49[a]	1.49[a]	0.0002	0.04	0.065[4]
Posterior Section									
Fold length	246.81[b]	234.84[b]	239.98[b]	214.23[b]	283.47[a]	285.46[a]	<0.0001	11.13	0.083[4]
Enterocyte height	27.27[d]	29.06[c]	30.48[bc]	31.05[ab]	32.83[a]	32.32[ab]	<0.0001	0.63	0.132[4]
Microvilli height	1.16[d]	1.26[cd]	1.36[bc]	1.49[a]	1.43[ab]	1.51[a]	<0.0001	0.04	0.154[4]

[1] Values represent means of three fish from each three replicate tanks.

[2] Significance probability associated with the F-statistic.

[3] Different superscript letters indicate significant ($P < 0.05$) differences as evaluated by Duncan's multiple range test.

[4] Indicate significant ($P < 0.05$) differences as evaluated by linear regression.

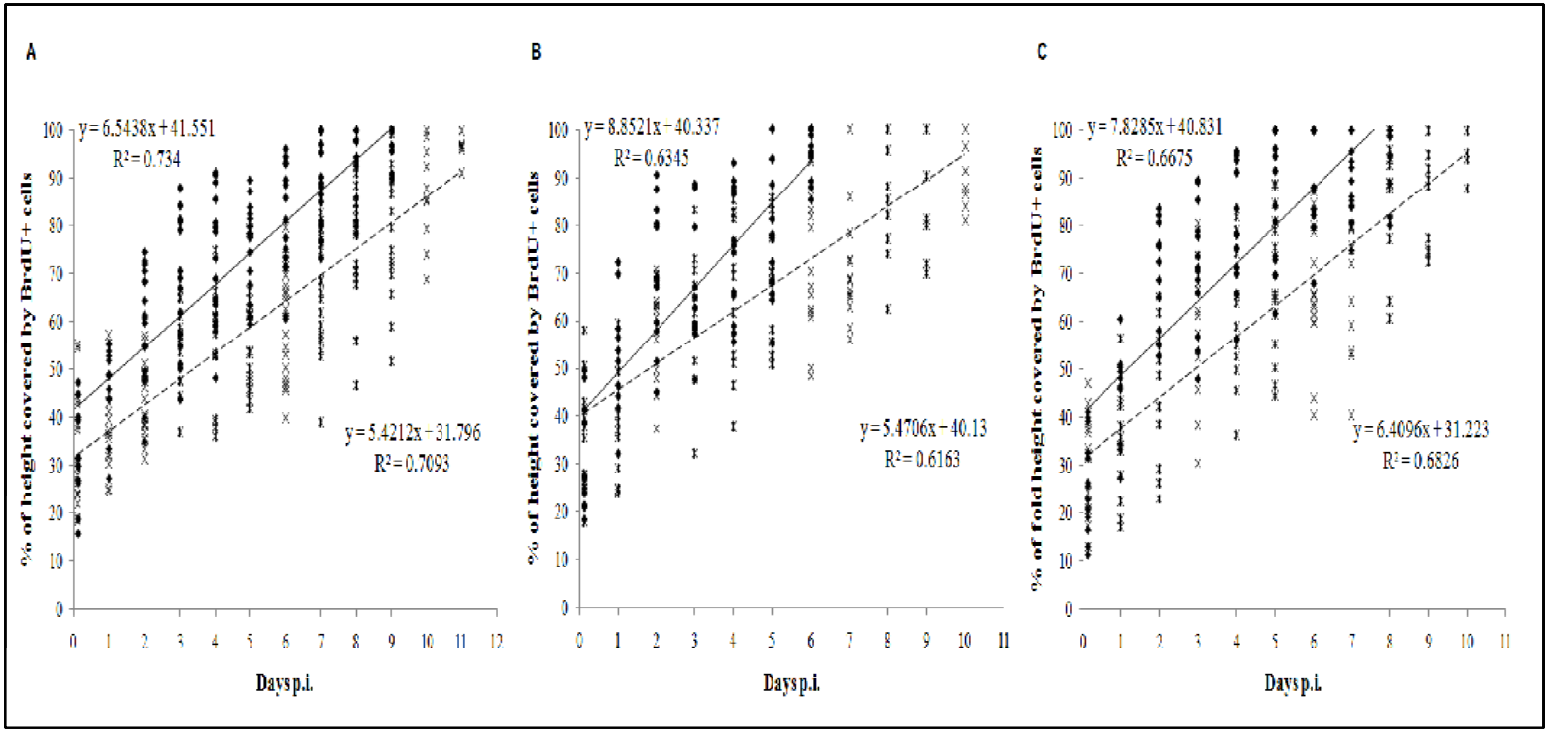

Fig. 3.1. Enterocyte migration rates for the anterior, mid and posterior intestine of fish fed the basal or GLN-supplemented diet (20 g · kg^{-1}) from experiment 2. Regression analysis indicated a linear relationship ($P < 0.05$) between percentage of enterocyte migration and days after BrdU injection (p.i.) in all intestinal sections. The linear regression equations indicated a migration rate for fish fed the basal (∗) vs. GLN (♦) supplemented diets of 5.42 ± 0.25 vs. 6.54 ± 0.31 %·d^{-1} for the anterior intestine (A), 5.47 ± 0.37 vs. 8.85 ± 0.73 %·d^{-1} for the mid intestine (B), and 6.41 ± 0.39 vs. 7.83 ± 0.54 %·d^{-1} for the posterior intestine (C). Slopes were significantly ($P < 0.05$) different among treatments in all intestinal sections. The intercept was significantly ($P < 0.05$) different in the anterior and posterior intestine.

various animal species to dietary GLN enrichment have been thus far inconsistent (Lobley et al., 2001).

The intestine is an important organ for its utilization in the post-absorptive state. The latter is well established in mammals (Curi et al., 2005; Rhoads and Wu, 2009; Wu et al., 2011), and it is thought to be similar in fish (Buentello and Gatlin, 2000; Li et al., 2009). However, information on the effects of GLN as a dietary supplement in fish feeds is rather limited to date.

In seawater eel, GLN was reported to be metabolized to produce ATP, increase oxygen consumption and net water flux, and the effects of GLN depended on its concentration (Ando, 1988). Likewise, GLN effects were reported to be more prominent on the mucosal side, where its absorption limited the rate of end-product formation (Ando, 1988). In addition, *in vitro* studies have indicated that GLN significantly enhanced proliferation, growth and differentiation of fish enterocytes, increasing protein retention and alkaline phosphatase activity (Jiang et al., 2009). Glutamine supplementation in cultured enterocytes protected against peroxide-induced cell damage, inhibiting enterocyte lipid oxidation, facilitating recovery of Na^+-K^+ ATPase, superoxide dismutase, catalase and glutathione peroxidase activities, and maintaining glutathione content and its redox ratio. This information appears to indicate that GLN is able to restore enteric absorption and restitute enterocyte integrity after oxidative damage (Chen et al., 2009).

Because previously listed GLN actions point to improvements of GIT integrity, results from the present experiment are in line with the current understanding of GLN

metabolism in monogastric animals. The GIT of channel catfish experienced significant increases in fold length and both enterocyte and microvilli height (Table 3.5). More importantly, these dimensional modifications were not associated with any microscopic signs of enteritis, but in fact resulted in a somewhat improved fish performance (Table 3.2). Our results are also in agreement with other studies which consistently report beneficial effects upon GLN supplementation in fish (Yan and Qiu-Zhou, 2006; Qiyou et al., 2011), poultry (Yi et al., 2005; Bartell and Batal, 2007; Murakami et al., 2007; Soltan, 2009) and swine (Wu et al., 1996).

In addition, in fish fed GLN-supplemented diet, the enterocyte turnover rate was increased by 19, 46 and 20% in the anterior, mid and posterior intestine, respectively, deducted from an increased enterocyte migration rate. The latter corroborates the role of GLN as a growth factor and not just as a required nutrient, with mitogenic and anti-apoptotic effects (Curi et al., 2005; Rhoads and Wu, 2009). Although Jiang et al. (2009) reported an *in vitro* GLN promotion of enterocyte proliferation in fish as well as cellular structural integrity, the present study reports for the first time an enhanced enterocyte migration rates upon dietary GLN supplementation in fish.

The GIT is not only the chief organ of nutrient digestion and absorption, but performs a number of physiological functions different from nutrient assimilation, being also important for whole-body amino acid homeostasis (Reeds and Burrin, 2000). About 65-75% of dietary GLN is oxidized by the intestine (Wu, 1998), which makes it almost unavailable to extra-intestinal tissues illustrating its crucial role to intestinal metabolism. Besides GLN, the intestine metabolizes several other dietary amino acids such as

arginine, ornithine, proline, valine, leucine, isoleucine, lysine, methionine, phenylalanine, threonine, glycine, and serine (Wu et al., 2005b; Wang et al., 2009), some of which have concurrent fates with GLN. In the present experiment, dietary supplementation of 2% GLN significantly increased values of plasma threonine, glycine, serine and asparagine (Table 3.4). These results are in agreement with plasma profiles found in humans fed free dietary GLN (Boza et al., 2001). The enteric metabolism of these amino acids is not directly connected to that of GLN but rather, it appears to exert a sparing effect on them. The fact is that the metabolic fate of glycine, serine and threonine is interconnected and these amino acids may be used by the intestine for synthesis of purines and pyrimidines, which are important compounds for cell proliferation and protein synthesis (Wu, 1998; Wu et al., 2005b). In the current trial, it appeared as if having an increased GLN source primed catfish enterocytes to utilize this amino acid for the production of proliferative compounds instead of other dietary amino acids as reported for rats (Salloum et al., 1990). Also, asparagine is a potent stimulator of ornithine decarboxylase – a key enzyme in polyamine metabolism – and, although it is not metabolized by the enterocytes, it may assist, together with GLN, in maintaining adequate proliferation rates for the turnover of these enteric cells (Kandil et al., 1995).

Despite the positive effects mentioned earlier no statistical differences were established among treatments in the evaluated productive parameters. This disagrees with findings in Jian carp where GLN supplementation (1.2 to 2%) improved weight gain, feed intake, feed efficiency and intestinal weight (Yan and Qiu-Zhou, 2006), as well as with findings in young hybrid sturgeon were supplementation (0.6 to 1.5%)

improved weight gain and feed efficiency (Qiyou et al., 2011). Glutamine has been proven to beneficially affect several animal species with supplementation in the range of 1% in the diet, improving performance parameters such as weight gain and/or feed efficiency of swine (Wu et al., 1996; Kitt et al., 2002; Zou et al., 2006) and poultry (Yi et al., 2001; 2005; Bartell and Batal, 2007; Soltan, 2009). In contrast, House et al. (1994), Plazier et al. (2001), Murakami et al. (2007), and Sakamoto et al. (2006) found no effects of GLN supplementation on growth or other productive parameters of different animal species.

The results of the present study indicate an efficient utilization of GLN by intestinal cells of channel catfish, perhaps due to the reported roles of this amino acid as metabolic fuel, without a significant positive outcome on growth parameters when supplemented in purified diets. Longer-term trials and/or the use of practical diets may result in improved fish growth due to increased absorptive areas in the gastrointestinal tract, but more research is needed to evaluate this notion.

CHAPTER IV

EVALUATION OF IN VITRO PERFORMANCE OF CHANNEL CATFISH LEUKOCYTES IN ARGININE AND/OR GLUTAMINE SUPPLEMENTED MEDIA

1. Introduction

Infectious diseases worldwide are a major constraint for the continuous expansion and production of aquaculture, resulting in multimillion dollar losses each year (Plumb, 2001). Significant research efforts have been implemented to prevent, control and treat diseases in aquaculture in order to maintain it as a sustainable industry. Consequently, as the primary line of defense against invading pathogens, it is crucial to study the immune system of fish, including specific components (e.g., leukocytes) of both the innate and adaptive immune responses.

In vitro models are important for functional studies involving fish leukocytes, and although under this approach the roles of particular nutrients on immune competence have been evaluated (Villena, 2003), there is still a lack of understanding regarding how specific amino acids may modulate the immune function of fish.

Glutamine (GLN) and arginine (ARG) are two amino acids with proven immunomodulatory effects in higher vertebrates (Li et al., 2007), including the enhancement of phagocytosis and bacterial killing (Wallace and Keast, 1992; Newsholme, 2001; Muhling et al., 2002), lymphocyte proliferation (Newsholme et al., 1999; Choi et al., 2009), cytokine production (Newsholme, 2001; Mori and Gotoh, 2004;

Li et al., 2007), T-cells responses (Roth, 2007; Abdukalykova et al., 2008), and immunoglobulin synthesis (Newsholme, 2001; Tayade et al., 2006a). As unique precursor for nitric oxide (NO), ARG donates nitrogen; whereas, GLN provides metabolic fuel to support this reaction's kinetics. Most of this research, however, has been conducted with mammalian species, whose immune system has important differences from that of teleost fish. Therefore, further research is needed to assess the effects of these two amino acids on the immune system of fish, with the targeted goal of using them as dietary tools for health preservation and disease management in aquaculture.

In fish, GLN's role on leukocyte metabolism is a complex one and appears to be species specific. This is in contrast with GLN's role on mammalian leukocytes, which is consistent across species (Crawford and Cohen, 1985). Conflicting reports document GLN-dependent (Rosenberg-Wiser and Avtalion, 1982) and GLN-independent (McBride and Keast, 1997; Ganassin et al., 1998) responses of proliferating cultured lymphocytes of fish. Interestingly, GLN plasma levels fall sharply in disease-affected fish (Walker et al., 1996), which reflects increased GLN utilization; and is analogous to the endogenous GLN production via muscle breakdown, a well-documented catabolic state in humans undergoing trauma and sepsis (Griffiths, 2001), such condition may be reverted by provision of parenteral GLN. The essentiality of GLN as metabolic fuel for active cells of the immune system in fish is further supported by the increased NO production documented for activated channel catfish macrophages (MØ) when the culture media was supplemented with GLN (Buentello and Gatlin, 1999). Moreover,

dietary GLN supplementation has been shown to increase plasma levels of complement proteins C3 and C4 in juvenile hybrid sturgeon, *Acipenser schrenckii* × *Huso dauricus* (Zhu et al., 2011).

Similarly, ARG also enhanced NO production in catfish MØ but, through a different mechanism (Buentello and Gatlin, 1999). In addition, increased phagocytosis in channel catfish (Buentello et al., 2007) together with increased lysozyme activity and an enhanced respiratory burst in the Japanese flounder, *Paralichthys olivaceus* (Galindo-Villegas et al., 2006) have been reported upon ARG supplementation. More importantly, an experimental challenge with pathogenic bacteria is all-encompassing in that it tests all components of the immune system at once and, ARG has also been reported to increase survival of fish after exposure to *Edwardsiella ictaluri* (Buentello and Gatlin, 2001b). On the other hand, it also has been proven that supplementation of ARG to culture media enhances NO production by activated MØ of channel catfish (Buentello and Gatlin, 1999).

Based on the preceding information, the present study was conducted to further elucidate the immunomodulatory roles of ARG and GLN via *in vitro* studies with various cells of the channel catfish immune system.

2. Materials and methods

2.1 Fish

Healthy channel catfish with an average weight of 600 g were held in a 1100-L round fiberglass tank arranged as a recirculation system (flow 1.1 L/min). This system

included a common settling chamber, biological filter and sand filter. Fish were fed a commercial catfish diet (32% crude protein, 8% crude lipid, 90% dry matter, Land O' Lakes Purina Feed LLC, Shoreview, MN), and sampled as needed. Before drawing blood, fish were anesthetized with tricaine methanesulfonate (MS-222, Western Chemical Inc, Ferndale, WA, 100 mg · L^{-1}). Blood was drawn from the caudal vasculature of three fish with heparinized syringes (5 mL, 22-ga needle), pooled into a composite sample and used immediately for peripheral blood lymphocyte proliferation assays. Three more fish were euthanized with a more concentrated dose of MS-222 (300 mg · L^{-1}) and head kidneys were aseptically excised and placed in cold incomplete catfish media (sterile, described below) and pooled into a composite sample. All immunological assays were repeated twice using two separate composite samples.

2.2 Culture media

Incomplete catfish medium (ICM, pH 7.0) consisted of equal portions of AIM-V (31035, Invitrogen™, Carlsbad, CA) and L-15 (L5520, Sigma, St. Louis, MO) media, 8% cell culture grade water (SH30529, HyClone®, Logan UT), 50 units · mL^{-1} of penicillin – 0.05 mg · mL^{-1} of streptomycin (P0781, Sigma), 0.02 mg · mL^{-1} gentamicin (G1397, Sigma), 0.05 mM of 2-mercaptoethanol (M3148, Sigma), and 0.09% Na_2HCO_3 (S-233, Fisher Scientific, Waltham, MA). Complete catfish medium (CCM) consisted of ICM plus 5% of heat-inactivated and pooled channel catfish serum (Miller et al., 1994). The control medium consisted of plain CCM which contained 0.83 mM of each ARG and GLN. Based on previous studies with catfish MØ (Buentello and Gatlin, 1999;

Buentello et al., 2007), CCM was supplemented with ARG (A3784, Sigma) and GLN (G5763, Sigma) at 0.5 and 1 mM, respectively. Media enrichment with ARG and GLN was applied individually and in combination, resulting in seven different media treatments.

2.3 Macrophage primary culture

Macrophages were isolated from head kidney as previously reported (Secombes, 1990) with slight modifications. Briefly, head kidney tissue was filtered through a 100-μm nylon mesh. The resulting cell suspension was layered on a Percoll (77237, Sigma) gradient (34%/51% v/v) and centrifuged at 400 x *g* for 30 min. The cell layer at the interface was collected and washed two times with ice-cold (PBS) at 200 x *g* for 10 min. A final wash was conducted with antibiotic free (af)-ICM, then the cell pellet was resuspended in 1 mL of af-CCM. Macrophages were enumerated using a hemocytometer and viability assessed by Trypan blue (T8154, Sigma) staining. Viability was > 95% in all cases. Cell suspension was adjusted to 1 x 10^7 cells · mL^{-1} in af-CCM and 100 μL of the MØ suspension were added per well in a sterile flat bottom 96-well microplate (351172, Falcon, Le Pont De Claix, France).

2.4 Phagocytosis assay

Phagocytosis of *E. ictaluri* by catfish MØ cultured with different ARG, GLN, and ARG + GLN concentrations, was evaluated using the method described by Ainsworth and Chen (1990), with some modifications. Namely, *E. ictaluri* isolated from

a natural outbreak at the Aquacultural Research and Teaching Facility, Texas A&M University was cultured in brain heart infusion (BHI) broth (211059, BBL^{TM}, Sparks, MD) for 18 h at 27 °C. The bacterial broth was then centrifuged at 2000 x *g* for 10 min after which the pellet was washed once in phenol-free Hank's balanced salt solution (HBSS, pH 7.3, H4891, Sigma) for 10 min and resuspended in 1 mL af-ICM. Bacteria were enumerated using a bacterial counter chamber and the suspension was adjusted to 6 x 10^7 cells · mL^{-1}. Before the addition of 100 µL of bacterial suspension per well (6 x 10^6 cells), amino acids were added (in af-CCM) to triplicate sets of MØ primary culture plates. The control medium consisted of plain af-CCM also added to triplicate wells. After the addition of bacterial cells, MØs were cultured at 27 °C in a humidified 5% CO_2 atmosphere for 1 h with occasional shaking. At the end of the incubation period, 150 µL of MØ-bacteria suspension from each of three replicate wells were smeared onto a glass slide, fixed and stained with Wright's stain (WS16, Sigma). A light microscope (Olympus BH-2) was used to enumerate phagocytes (300) with at least one internalized bacterium. The total number of engulfed bacteria per MØ was recorded as well. Phagocytic index (PI = total engulfed bacteria / total phagocytes) and phagocytic activity (PA= number of phagocytes with engulfed bacteria x100/ total MØ) were computed for each slide.

2.5 Bactericidal assay

The MØ ability to kill *E. ictaluri* was evaluated at different ARG, GLN, and ARG + GLN media concentrations using the method described by Secombes (1990), as

modified by Shoemaker et al. (1997). Bacterial suspension was prepared as described earlier, but with a final concentration of 1 x 10^8 cells · mL^{-1}. The MØ primary culture was incubated for 2 h, then washed twice with 200 µL of af-CCM, and supplemented with corresponding treatments into sets of six wells (*section 2.2*). The bacterial suspension was added (20 µL) to each well and the plate was centrifuged at 150 x *g* for 5 min. The combined cell cultures (MØ + bacteria) were incubated for 0 h (control, three wells) or 2.5 h (three wells), under the same conditions as in section *2.4*. After each incubation period, supernatants were removed and MØ lysed with 50 µL of 0.2% Tween 20 (H285, Mallinckrodt, St. Louis, MO) solution. Fresh BHI was added (100 µL) to each well and the plate was further incubated for 18 h at 27 °C in an orbital incubator. After the incubation period, 20 µL of thiazolyl blue tetrazolium bromide (MTT, 10 mg · mL^{-1}, M5655, Sigma) were added per well and the plate incubated for an additional 15 min. The plate was then read at 620 nm. Bacterial concentrations were calculated by comparing the absorbance (ABS) obtained for each well to a standard curve previously constructed (data not shown). Bactericidal capacity (% killing = bacteria 0 h – bacteria 2.5 h x 100/ bacteria 0 h) was computed for each well and are presented as mean % killing per treatment.

2.6 Lymphocyte primary culture

Lymphocytes were isolated from peripheral blood as previously described by Miller and Clem (1988) and Miller et al. (1994). Briefly, channel catfish blood (~ 4 mL) was diluted 1:2 with ICM, then 4 mL were layered over 3 mL of Lymphoprep™

(1114544, Axis-Shield, Oslo, Norway) and centrifuged at 350 x *g* for 20 min. Lymphocytes were recovered from the interface. Cells were washed with ICM at 600 x *g* for 10 min. The resulting cell pellet was resuspended in 1 mL of CCM, enumerated and checked for viability as described in section *2.3* (viability was > 95%). Lymphocyte concentration was adjusted to 2.5 x 10^6 cells · mL^{-1} and 200 μL were added per well to a sterile round bottom 96-well microplate (163320, Nunc, Roskilde, Denmark).

2.7 Proliferation assay

Lymphocyte proliferation stimulated by non-specific mitogens was evaluated using ARG, GLN and ARG + GLN media enrichment and assessed using the method described by Miller and Clem (1988), with modifications. Briefly, supplemented media was added to appropriate wells (sets of six for each treatment) of lymphocyte primary culture plate as indicated earlier (section *2.2*). Lipopolysaccharide (LPS, from *Salmonella thyphimuryum*, L6511, Sigma), concavalin A (ConA, 150710, MP Biologicals, Solon, OH) and a mixture of phorbol myristate acetate (PMA, P8139, Sigma) and Ca^{++} ionophore, A24187 (Ca^{2+}, C4403, Sigma) were added to sets of 21 wells each to achieve a final concentration of 500 mg · mL^{-1}, 50 mg · mL^{-1} and 0.00001:0.0001 mg · mL^{-1}, respectively. One set of 21 wells did not receive any mitogen and served as control. Cells were incubated at 27 °C in a 5% CO_2 - 95% air incubator; media with PMA + Ca^{2+} was removed after 18 h and replaced with fresh CCM. Cell proliferation was quantified using bromodeoxyuridine (BrdU) incorporation to cell DNA following manufacturer's instructions (BrdU cell proliferation kit, 2752,

Millipore™, Billerica, MA). After incubating cells for 18 h, lymphocytes were pulsed with BrdU and further incubated for 24 h. Detection of BrdU was conducted through an enzyme-linked immunosorbent assay, using mice anti-BrdU monoclonal antibodies and peroxidase conjugated goat anti-mouse IgG antibodies. Absorbance was read at 450 nm using a plate reader (Biorad, iMark™). Lymphocyte proliferation capacity was computed and presented as stimulation index (SI = ABS stimulated cells / ABS non-stimulated [control] cells).

2.8 Amino acid profiles

Amino acid levels in CCM, before and after cell addition, were evaluated as indirect assessment of amino acid utilization by MØ and lymphocytes under activated conditions such as bacterial killing and proliferation. Supernatants of cell culture media were sampled during the bactericidal (0 h and 2.5 h) and proliferation (0 h and 18 h) assays, and kept at -80 °C until further analysis. Amino acid levels in culture media were determined using ultraperformance liquid chromatography (UPLC-Acquity system®, Waters™) and the commercial kit MassTrak™ (186004094, Waters™, Milford, MA). Samples were deproteinized with 1.5 M $HClO_4$ (9552-05, J.T. Baker, Phillipsburg, NJ) and neutralized with 2 M K_2CO_3 (P5833, Sigma) before derivatization following manufacturer's instructions with associated reagents.

2.9 Statistical analysis

Because each phagocytosis, bactericidal, and proliferation assay was replicated twice, values from the replicate assays were first compared, via Student's T-test, and found to be non-significantly different; therefore, data from the replicate assays were pooled and analyzed via one-way analysis of variance, after which Duncan's multiple-range test was used to detect potential differences in treatment means. Data from amino acid profiles were compared before and after each assay using Student's T-test. All analyses were conducted using Statistical Analysis System (SAS, 9.2v) software. Statistical differences among treatments were considered significant at $P \leq 0.05$.

3. Results

3.1 Amino acid utilization

Changes in amino acid levels in CCM after bactericidal assays are summarized in Table 4.1. The concentrations of most amino acids decreased after 2.5 h of co-incubation MØ-*E. ictaluri*. The sum total of amino acids in the culture media also decreased by 23% and this reduced level was significantly different from the initial overall amino acid level. Figure 4.1 illustrates changes (%Δ) in amino acid levels sharing a common biochemical pathway with ARG and GLN. Both ARG and GLN significantly decreased from the original levels – 19 and 39% reduction, respectively. Also, the culture media was completely devoid of citrulline after 2.5 h of incubation (from 2.8 to 0 nmol $\cdot mL^{-1}$). Together with citrulline, serine experienced one of the most remarkable drops in concentration (100 and 75% reduction, respectively). On the other

Table 4.1
Medium amino acid profile (nmol · mL^{-1}) before and after bactericidal assay with *E. ictaluri*[1]

Amino acid	0 h	18 h	Change	Δ	%Δ	*P*-Value	Pooled std. error
Hydroxyproline	5.6	2.1	-	3.6	63.4	0.015	0.9
Histidine	376.8	277.2	-	99.7	26.4	0.025	28.5
Phosphoethanolamine	2.5	1.4	-	1.1	43.2	0.0001	0.1
Asparagine	520.6	309.3	-	211.3	40.6	0.006	39.2
3-Methylhistidine	1.1	1.0	-	0.1	9.3	0.102	0.05
Taurine	13.3	8.2	-	5.2	38.7	0.025	1.5
1-Methylhistidine	2.2	4.3	+	2.1	94.7	0.163	1.2
Serine	436.4	108.7	-	327.7	75.1	0.001	35.2
Glutamine	834.7	513.3	-	321.4	38.5	0.010	70.8
Carnosine	4.9	4.6	-	0.3	7.0	0.197	0.2
Arginine	829.7	673.9	-	155.8	18.8	0.004	25.3
Glycine	650.0	528.7	-	121.3	18.7	0.048	43.2
Anserine	12.6	1.6	-	11.1	87.7	0.019	1.5
Ethanolamine	6.8	3.0	-	3.7	55.3	0.002	0.5
Aspartate	4.0	4.0	+	0.0	0.5	0.927	0.2
Sarcosine	24.0	19.8	-	4.2	17.4	0.001	0.5
Glutamate	68.8	91.1	+	22.2	32.3	0.010	4.9
Citrulline	2.8	0.0	-	2.8	100	0.0001	0.01
Threonine	634.0	467.9	-	166.1	26.2	0.021	44.9
Alanine	563.7	497.0	-	66.7	11.8	0.114	33.0
γ-Aminobutyric acid	2.6	2.8	+	0.2	8.2	0.411	1.3
Proline	41.0	45.1	+	4.1	10.0	0.145	2.3
β-Aminoisobutyric acid	2.5	2.3	-	0.2	9.8	0.428	0.3
Hydroxy-lysine 1	0.9	0.8	-	0.1	8.2	0.100	0.03
Hydroxy-lysine 1	5.4	5.8	+	0.4	7.1	0.274	0.3
α-Aminobutyric acid	3.3	4.0	+	0.7	20.5	0.078	0.3
Cysteine	8.9	7.2	-	1.6	18.2	0.028	0.5
Ornithine	9.6	12.6	+	3.0	31.3	0.080	1.3
Cystine	21.5	20.1	-	1.4	6.4	0.337	1.3
Lysine	281.5	289.7	+	8.2	2.9	0.629	15.8
Tyrosine	399.4	285.8	-	113.7	28.5	0.024	32.2
Methionine	132.2	124.1	-	8.0	6.1	0.308	6.9
Valine	292.6	259.8	-	32.8	11.2	0.107	15.8
Isoleucine	294.2	243.2	-	51.0	17.3	0.049	18.2
Leucine	322.5	337.7	+	15.2	4.7	0.461	16.9
Phenylalanine	221.0	212.6	-	8.4	3.8	0.492	11.1
Tryptophan	31.4	30.1	-	1.3	4.1	0.507	1.7
SUM	7067	5403		1664	23.5	0.013	391.7

[1] Values represent means of three replicate wells.

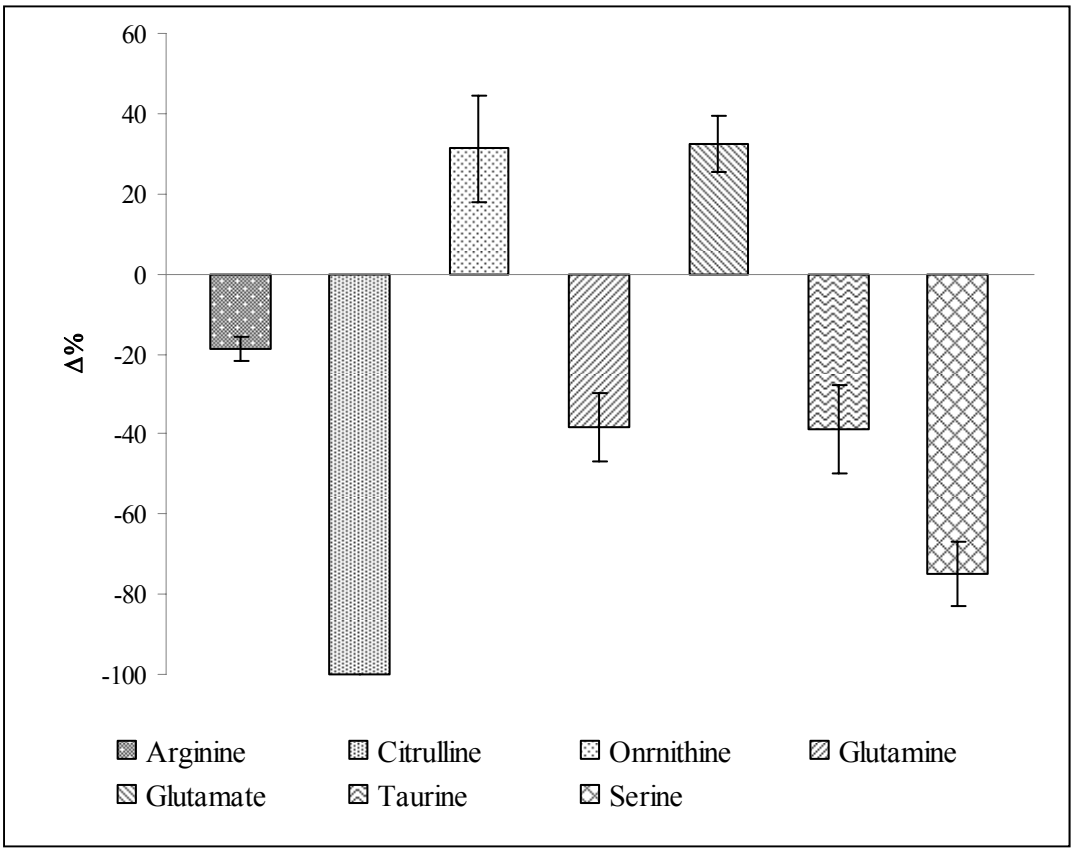

Fig. 4.1. Amino acid changes in medium after a bactericidal assay with head-kidney-derived MØ. Peripheral blood lymphocyte proliferation assay. Bars represent means (± S.E.) of the % of the difference from the initial value (Δ%). All presented values, but ornithine's, were significantly ($P < 0.05$) different from the initial value.

hand, ornithine increased 31%, although this level was not significantly different (P = 0.08) from the original value. Glutamate concentration significantly increased after the bactericidal assays; whereas, taurine significantly decreased. Interestingly, proline levels remained unchanged.

Changes in amino acid levels in the culture media after proliferation assays are summarized in Table 4.2. In general, these changes were more pronounced than those observed for the bactericidal assay. The sum of total amino acids in the culture media significantly decreased by 45% from the original level after lymphocyte proliferation. Figure 4.2 illustrates changes in amino acid levels sharing a common biochemical pathway with ARG and GLN, after proliferation. As in the bactericidal assays, both ARG and GLN significantly decreased from the original levels – 46 and 52% reduction, respectively. Citrulline followed the same pattern as in the bactericidal assay, being completely cleared from the culture media. Also, ornithine, glutamate and proline levels were significantly diminished (68, 40, and 44%, respectively) from before-assay levels. There was no change in the levels of taurine before or after proliferation but, aspartate evidently increased 3.8-fold after lymphocyte proliferation in a significant manner.

3.2 Head kidney-derived MØ phagocytic and bactericidal capacity

Phagocytosis of head kidney MØ, expressed as PI and PA, is presented in Table 4.3. The PI significantly increased with ARG supplementation to the culture media, irrespective of GLN addition. Phagocytic index increased 2.3-fold upon ARG

Table 4.2
Medium amino acid profile (nmol · mL^{-1}) before and after lymphocyte proliferation assay[1]

Amino acid	0 h	18 h	Change	Δ	%Δ	*P*-Value	Pooled std. error
Hydroxyproline	5.6	3.8	-	1.8	32.2	0.166	0.9
Histidine	376.8	218.7	-	158.1	42.0	0.005	28.2
Phosphoethanolamine	2.5	1.6	-	0.9	36.6	0.622	1.6
Asparagine	520.5	285.0	-	235.5	45.2	0.005	40.9
3-Methylhistidine	1.1	26.4	+	25.3	2271	0.001	2.6
Taurine	13.3	11.0	-	2.3	17.2	0.357	2.2
1-Methylhistidine	2.2	2.5	+	0.3	15.4	0.910	2.8
Serine	436.4	252.1	-	184.3	42.2	0.009	39.1
Glutamine	834.7	400.5	-	434.2	52.0	0.004	72.3
Carnosine	4.9	0.9	-	4.0	81.9	0.044	0.9
Arginine	829.7	446.7	-	383.0	46.2	0.0001	31.9
Glycine	650.0	379.1	-	271.0	41.7	0.003	41.7
Anserine	12.6	1.5	-	11.1	88.5	0.016	1.5
Ethanolamine	6.8	5.1	-	1.7	25.1	0.099	0.8
Aspartate	4.0	19.0	+	15.0	373.1	0.016	2.0
Sarcosine	24.0	0.0	-	24.0	100	0.0001	0.1
Glutamate	68.8	41.6	-	27.2	39.6	0.003	4.3
Citrulline	2.8	0.0	-	2.8	100	0.0001	0.01
Threonine	634.0	356.6	-	277.4	43.8	0.004	45.6
Alanine	563.7	315.5	-	248.3	44.0	0.002	32.8
γ-Aminobutyric acid	2.6	1.8	-	0.8	31.2	0.374	2.2
Proline	41.0	22.7	-	18.4	44.8	0.001	2.0
β-Aminoisobutyric acid	2.5	0.0	-	2.5	100	0.0001	0.1
Hydroxy-lysine 1	0.9	0.0	-	0.9	100	0.0001	0.1
Hydroxy-lysine 1	5.4	0.7	-	4.7	86.6	0.0001	0.4
α-Aminobutyric acid	3.3	0.5	-	2.8	83.5	0.035	0.5
Cysteine	8.8	5.7	-	3.1	35.8	0.012	0.4
Ornithine	9.6	3.0	-	6.6	68.8	0.005	1.2
Cystine	21.5	12.1	-	9.4	43.9	0.0001	0.7
Lysine	281.5	129.2	-	152.3	54.1	0.0001	11.6
Tyrosine	399.4	230.6	-	168.8	42.3	0.006	32.3
Methionine	132.2	71.4	-	60.7	46.0	0.0001	5.8
Valine	292.6	160.6	-	132.1	45.1	0.001	14.0
Isoleucine	294.2	162.2	-	132.0	44.9	0.001	17.0
Leucine	322.5	175.0	-	147.5	45.7	0.001	14.9
Phenylalanine	221.0	131.4	-	89.6	40.5	0.002	12.3
Tryptophan	31.4	18.2	-	13.2	42.2	0.003	2.0
SUM	7067	3893		3174	44.9	0.002	431.7

[1] Values represent means of three replicate wells.

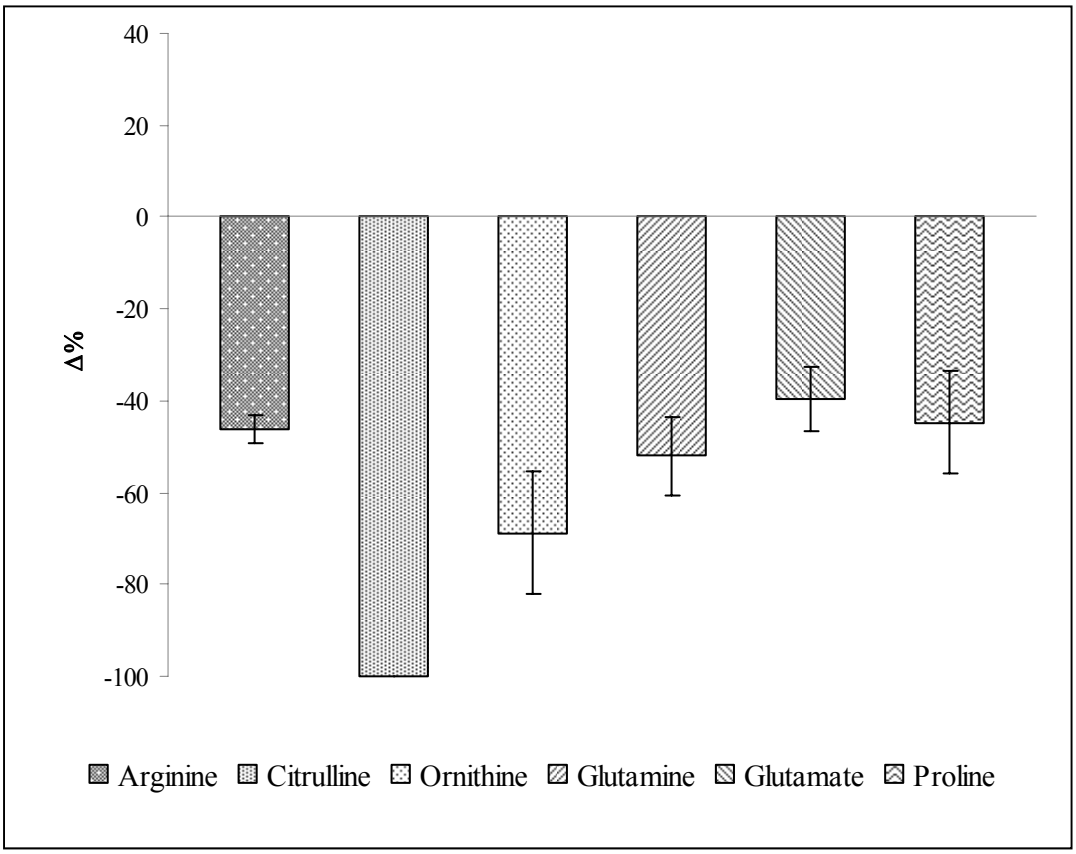

Fig. 4.2. Amino acid changes in medium after a proliferation assay with peripheral blood lymphocytes. Bars represent means (± S.E.) of the % of the difference from the initial value (Δ%). All presented values were significantly ($P < 0.05$) different from the initial value.

fortification at 0.5 mM but, further supplementation (1 mM) did not elicit additional improvements. Glutamine supplementation alone made no difference on PI values. Although PA levels were noticeably higher for all MØ cultured in amino acid-supplemented media as compared to control media, only those cultured with 1 mM ARG, 0.5 mM ARG + GLN and 1 mM ARG + GLN displayed significantly higher PA values than those of MØ cultured in control media. The highest PA index was observed for MØ cultured in 0.5 mM ARG + GLN, a 3-fold improvement from the control media. Table 4.3 also illustrates MØ bactericidal ability, presented as mean % killing. Arginine supplementation at 0.5 mM elicited a significant 45% increase over that attained by MØ in control medium. Further supplementation of ARG and/or GLN did not change the ability of MØ to kill *E. ictaluri*.

3.3 Proliferation of peripheral blood lymphocytes

Proliferation of peripheral blood lymphocytes upon non-specific mitogenic stimulation is summarized in Table 4.4 and presented as SI. The response of channel catfish lymphocytes to non-specific mitogens was positively modulated by supplementation of either ARG or GLN to the culture media. Arginine + GLN at 0.5 mM consistently exhibited a significantly higher SI value. Concavalin A-treated cells significantly increased proliferation rates when ARG or GLN (either alone or in combination) were present in the culture media at a concentration of 1 mM. Lymphocytes cultured in the presence of 0.5 mM ARG + GLN and exposed to PMA + Ca^{2+} had the highest proliferation rates but, no further enhancement was attained above

Table 4.3
Phagocytic and killing capacity against *E. ictaluri* of head-kidney-derived MØ incubated with supplemental levels of ARG and/or GLN[1]

	Phagocytosis Assay		Bactericidal Assay
Cell culture media	**Phagocytosis index**	**Phagocytosis activity**	**Mean % killing**
CCM	1.9[2,c]	13.7[c]	34.6[b]
+ GLN 0.5 mM	2.1[c]	23.6[bc]	37.5[b]
+ GLN 1 mM	2.4[bc]	25.6[bc]	47.6[ab]
+ ARG 0.5 mM	4.3[a]	24.7[bc]	50.4[a]
+ ARG 1mM	4.4[a]	39.7[ab]	45.6[ab]
+ ARG + GLN 0.5 mM	3.7[ab]	42.7[a]	42.4[ab]
+ ARG + GLN 1 mM	4.8[a]	38.6[ab]	42.3[ab]
*P*r > F[3]	0.003	0.01	0.044
Pooled std. error	0.439	7.11	7.98

[1] Values represent means of 6 replicate wells.
[2] Different superscript letters indicate significant ($P < 0.05$) differences as evaluated by Duncan's multiple range test.
[3] Significance probability associated with the F-statistic.

Table 4.4
Proliferation capacity upon non-specific stimulation of peripheral blood lymphocytes incubated with supplemented levels of ARG and/or GLN[1]

	Stimulation index		
Cell culture media	**ConA**	**LPS**	**PMA + Ca^{2+}**
CCM	2.0[2,d]	2.5[c]	4.0[c]
+ GLN 0.5 mM	4.2[c]	3.3[b]	6.5[ab]
+ GLN 1 mM	6.2[ab]	5.0[a]	6.1[ab]
+ ARG 0.5 mM	5.1[bc]	3.2[b]	5.4[bc]
+ ARG 1 mM	6.1[ab]	4.9[a]	5.7[ab]
+ ARG + GLN 0.5 mM	6.6[a]	5.4[a]	7.4[a]
+ ARG + GLN 1 mM	6.0[ab]	3.6[b]	6.3[ab]
*P*r > F[3]	< 0.0001	< 0.0001	0.014
Pooled std. error	0.430	0.206	0.530

[1] Values represent means of 6 replicate wells.
[2] Different superscript letters indicate significant ($P < 0.05$) differences as evaluated by Duncan's multiple range test.
[3] Significance probability associated with the F-statistic.

this concentration. There was a step-wise increase in SI for LPS-treated lymphocytes in that, as the concentration of each amino acid increased in the culture media so did SI. Also, a limited synergistic effect was accomplished by supplementing both amino acids at 0.5 mM (a 2-fold increase over control values) but not at 1 mM. Finally, GLN supplementation appeared to have a greater impact on lymphocyte proliferation rates than ARG.

4. Discussion

In quiescent immune cells, found in homeostatic conditions, nutrient utilization should remain at a minimum level for maintenance purposes. However, during an immune challenge, utilization of key nutrients (including amino acids) by these cells should sharply increase (Newsholme et al., 1999). In the present experiment, the amino acid analysis performed on culture media proved to be ideally suited to detect differences in uptake and utilization of ARG, GLN and other amino acids by channel catfish leukocytes, MØ and lymphocytes, experimentally induced to proliferate, phagocytize or kill.

Because CCM adequately satisfies the metabolic requirements of channel catfish immune cells it is widely used for *in vitro* studies with this species (Miller et al., 1994; Stuge et al., 1997; Khayat et al., 2001; Edholm et al., 2010) and thus constitutes a suitable control media. The significant decline in amino acid concentration after cell induction observed in the present experiment (tables 4.1 and 4.2), is consistent with earlier studies on mammalian cells (Straus et al., 1977; Segel, 1992; Muhling et al.,

2002). As in leukocytes from higher vertebrates, it is likely that fish cells actively transport amino acids against a concentration gradient, such that the intracellular concentration became many times higher than the concentration in the medium. In the present study it is important to point out that cell proliferation demanded nearly twice as much amino acids from the culture medium as did the bactericidal assay. This may be attributed to the increased amino acid demand, both as energy substrates and synthetic precursors, exerted by proliferating immune cells (Newsholme et al., 1999; Hotamisligil and Erbay, 2008; Choi et al., 2009). It is also possible that increased amino acid usage may be directed to the production of immune mediators, such as cytokines (Mori and Gotoh, 2004; Hotamisligil and Erbay, 2008).

It also should be noted that 21 amino acids accounted for most of media amino acid loss during lymphocyte proliferation, compared to only 12 amino acids during the bacterial killing process. This partially agrees with previous mammalian reports (Segel, 1992), highlighting ARG, GLN, isoleucine, leucine, lysine, methionine, threonine, tryptophan, valine, tyrosine, histidine, cysteine, phenylalanine, serine and alanine (15 amino acids) as necessary for optimal *in vitro* proliferation, DNA synthesis and survival of lymphocytes. In addition, channel catfish lymphocytes also consumed citrulline, ornithine, proline, glutamate, asparagine and glycine.

The sharp decline in ARG and GLN concentrations after cell activation is also in line with previous studies (Newsholme et al., 1999) indicating that from the many amino acids available in cell culture medium, mammalian MØ utilize only GLN and ARG in significant quantities. Moreover, Nishiyama et al. (2010) reported that activated MØ

produce greater amounts of glycine, glutamate, alanine and histidine, and consume serine and GLN at the highest rates. Data from the present MØ trials is similar in that GLN and ARG accounted for 30% of the absolute amino acid losses; and GLN and serine were consumed at the highest rates. However, only glutamate was produced in greater amounts.

Because commercially available culture media is designed to emulate the nutrient composition of specific body fluids (e.g., catfish plasma), the data reported herein corroborates the essentiality of adequate amino acid supplies to support a physiologically-efficient immune cell function in fish. The possibility of using nutritional interventions to enhance the immune system in fish is further confirmed by the fact that the diet strongly affects the concentration of most amino acids in the body (Li et al., 2007).

Interestingly, after the bactericidal challenge, concentrations in media of ARG and GLN decreased 18 and 38%, respectively. Serine and GLN were the most used amino acids by MØ in absolute terms during the bactericidal assay; whereas, ARG was the fifth. Similarly, it is widely accepted that MØ activation *in vitro,* leads to a significant increase in GLN utilization for important cell metabolites needed for proper phagocytosis and bacterial killing (Wallace and Keast, 1992; Newsholme, 2001). Additionally, it would be expected that ARG is more readily used by MØ to produce NO during the bacterial killing process (Mori and Gotoh, 2004). However, even though ARG transport is increased in MØ generating NO (Bogle et al., 1992), activated MØ possess a high rate of arginase activity, using the extracellular ARG for ornithine

production (Mills, 2001), which concurs with the increased ornithine values (38%, not significantly different) observed in this experiment. Newsholme et al. (1999) reported that activated mammalian MØ use GLN at a higher rate to meet the intracellular ARG needs and then use this ARG for NO production. The current finding of a more distinct decrease in extracellular GLN than ARG partially correlates with the previous statement and is in agreement with previous results in our laboratory (Buentello and Gatlin, 1999). In addition, Wu and Brosan (1992) reported that mammalian MØ can convert citrulline into ARG, and this is also thought to be the case for channel catfish (Buentello and Gatlin, 1999). Accordingly, in the present experiment, medium citrulline levels surprisingly decreased below detection levels after the incubation period, suggesting a substantial usage of this amino acid by activated MØ. While the fate of GLN and citrulline for ARG synthesis cannot be proven through the approach used in this experiment, it could be a plausible explanation for the current findings.

In regard to the amino acid changes observed during the proliferation assay, the ARG value decreased 46%, while the GLN concentration decreased 52%, which indicates a 2.5- and 1.4-fold increase over that observed in the bactericidal assay. In this case, both GLN and ARG were the most used amino acids by lymphocytes during proliferation, accounting for 26% of the absolute loss of amino acids. These results are in agreement with previous findings for both amino acids in mammals (Li et al., 2007; Hotamisligil and Erbay, 2008; Choi et al., 2009), but differ from the scarce reports with fish lymphocytes from other species, which appear to minimize GLN essentiality for cell proliferation (Bols et al., 1994; McBride and Keast, 1997; Ganassin et al., 1998). To our

knowledge, no reports exist for ARG metabolism during lymphocyte proliferation in fish. Arginine and GLN have been proven to influence several lymphocyte metabolic functions in mammals including protein synthesis (Segel, 1992), regulation of cell-cycle progression (Rodriguez et al., 2007), and energy utilization (Newsholme et al., 1999), hence their preferential use by these cells during immune challenges (Hotamisligil and Erbay, 2008).

Interactions between *E. ictaluri* and channel catfish immune cells have been extensively studied in the past (Ainsworth and Chen, 1990; Shoemaker et al., 1997; Booth et al., 2009). Amino acid management to enhance health status also has been studied in this species (Buentello and Gatlin, 1999; Buentello et al., 2007). However, to our knowledge, the present study is pioneer in assessing these two factors in combination.

In this experiment, phagocytic and bactericidal capacities of head-kidney MØ against *E. ictaluri* were modulated by amino acid supplementation to the culture media. This is in accordance with similar studies in mammalian (Wallace and Keast, 1992; Wang et al., 2003; Blanc et al., 2005; Roth, 2007) and fish species (Galindo-Villegas et al., 2006; Buentello et al., 2007). It has been proven that ARG modulates MØ phagocytosis (Nii et al., 1992; Moffat et al., 1996; Choi et al., 2009), where NO seems to play a crucial role affecting the MØ cytoskeleton (Moffat et al., 1996). Both, supplemental ARG (Buentello and Gatlin, 1999) and *E. ictaluri* (Schoor and Plumb, 1994), has been proven to support an up-regulated synthesis of NO in channel catfish MØ. Furthermore, it is interesting that fish MØ utilize serine in similar amounts as

GLN. Serine is needed for *de novo* synthesis of ceramide, which may act as a secondary molecule and appears to play a critical role in many important MØ inflammatory signaling, mainly in response to LPS, such as stimulation of inducible isoform of NO synthase (Knapp and English, 2000). Therefore, it is feasible that the increased phagocytosis observed in the present experiment, may have been driven by NO modulation, where amino acids are being coordinately utilized to promote a sustained response against *E. ictaluri*. However, despite the high GLN utilization by activated MØ, limited modulation of phagocytosis and killing capacity was achieved by supplementing GLN alone to the culture media. These findings emphasize the need for extracellular ARG to enhance channel catfish MØ function against this pathogen.

Higher mean % killing of *E. ictaluri* was expected with higher levels of either amino acid. Nevertheless, it is noteworthy that ARG supplementation at 0.5 mM had the highest bactericidal effect and further supplementation with or without GLN did not increase this parameter, despite having similar phagocytic performance. One possible reason for the observed effect is the high arginase activity in activated MØ as previously discussed. Nevertheless, if that is the case, GLN supplementation should have solved this paradox, but results showed that GLN supplementation did not have a strong effect on bacterial killing. Furthermore, previous results in our laboratory indicated that medium supplementation with ARG + GLN significantly increased NO production in channel catfish activated MØ (Buentello and Gatlin, 1999), but this does not appear to correlate with *E. ictaluri* killing in the current experiment. This discrepancy may be explained by findings from a recent study indicating that *E. ictaluri* encodes an acid-

activated urease that is required for intracellular replication in channel catfish MØ (Booth et al., 2009), suggesting the utilization of urea produced by MØ (Mills, 2001; Munder, 2009) to increase intracellular pH to evade killing. Furthermore, it seems feasible that *E. ictaluri* possesses the genetic makeup to either up-regulate host arginase or to enzymatically use microenvironmental ARG for urea synthesis (Thune et al., 2007). Therefore, it might be possible that supplementing higher levels of ARG or GLN may raise levels of urea in MØ, providing higher levels of substrate for the bacteria's urease, hence its increased survival in the present experiment. Yet further research is needed to evaluate this notion.

Phagocytic efficiency and killing capacity of MØ are crucial in channel catfish for *E. ictaluri* immunity and resistance (Chen et al., 2002). Here, ARG had an increase on both parameters, but only with mid level of supplementation. Results in the present study corroborates with the findings reported by Buentello and Gatlin (2001b) where increased levels of ARG pool elevated by dietary ARG supplementation, increased resistance of channel catfish to *E. ictaluri* infection. Interestingly, in that experiment, the highest level of ARG had lower survival than a moderate level of supplementation, what might be related to the findings reported here for the highest levels of ARG supplementation to the media. Thus, although our findings appears promising for the utilization of ARG and/or GLN as dietary tools for enhancing disease resistance, attention needs to be put on a possible synergy among high levels of these two amino acids and the pathogenesis of *E. ictaluri*. However, the finding of increased

phagocytosis with supplemented levels of ARG warrants further research with other pathogen models.

In this study, naïve lymphocyte proliferation capacity upon non-specific mitogenic stimulation with ARG and/or GLN supplemented culture media was evaluated. Results herein showed a clear effect on stimulation index by supplementing media with either of these two amino acids, which agrees with previous finding in other animal models (Chang et al., 1999; Newsholme et al., 1999; Abdukalykova et al., 2008; Choi et al., 2009). It is known that GLN is obligatory in mammalian lymphocytes cultures (Newsholme, 2001; Roth, 2007); however, as previously discussed, differences seem to exist in fish species, and channel catfish lymphocyte responded differently than those from rainbow trout, *Onchorynchus mykiss* (Ganassin et al., 1998), or snapper, *Pargus auratus* (McBride and Keast, 1997). On the other hand, ARG has also proliferative promoting effects in mammalian lymphocytes (Newsholme et al., 1999; Ochoa et al., 2001; Suarez Butler et al., 2005), but no data have been published in fish cell cultures to date. In the present study, T lymphocytes (ConA sensitive), B lymphocytes (LPS sensitive) and both (PMA + Ca^{2+} sensitive) proliferation was achieved as in previous studies with channel catfish lymphocytes (Miller and Clem, 1988; Lin et al., 1992; Miller et al., 1994). This proliferation rate was enhanced by supplementing with either ARG or GLN, and generally was higher in media supplemented with either amino acid at 1 mM. Although a strong synergistic effect was not evident, supplementation of a combination of both amino acids at 0.5 mM consistently produced the best performance in the proliferation assays. The latter might

be explained by different fates of these two amino acid in lymphocytes when both are present in high levels in the surrounding environment instead of a possible common usage when one is low or even lacking (Buentello and Gatlin, 1999; Newsholme et al., 1999; Moinard et al., 2000; Blanc et al., 2005). As expected, SI in the CCM and PMA+Ca^{2+} treated cells was higher when compared to the other mitogens. Interestingly, SI changes by supplementing either or both amino acids in the PMA+Ca^{2+} treated cells was of a lesser magnitude to those obtained in the cells treated with ConA or LPS, when all are compared to their appropriate control. In this sense, PMA+Ca^{2+} stimulation of channel catfish cells involves more than one type of leukocytes, namely, B and T lymphocyte, and monocytes (Miller and Clem, 1988; Lin et al., 1992), which seems to result in a higher demand for these two amino acids.

The adaptive immune response is mainly driven by different lymphocyte subsets, which have the capability to create memory. This unique characteristic increases the chances of rapid clearance and survival on subsequent infection by the same pathogen. However the capacity to generate memory relies on an initial activation and expansion of naïve lymphocytes after encounter with the appropriate antigen and cytokine stimulation (Boyman et al., 2009). Therefore, the findings of an increased proliferation of naïve lymphocytes driven by ARG or GLN supplementation gain importance for fish immunology because it raises the possibility for *in vivo* supplementation with these two amino acids to promote creation of memory during vaccination.

In conclusion, the current findings appear promising for fish immunonutrition. This study has demonstrated that these two amino acids play a pivotal role in both

branches of the immune system, being important for the very first response against an invading pathogen, increasing phagocytosis and killing capacity of MØ against *E. ictaluri*, and for expanding the response upon activation, of either naïve T or B lymphocyte subsets and thus increase the creation of memory.

CHAPTER V

EVALUATION OF DIETARY ARGININE AND/OR GLUTAMINE SUPPLEMENTATION ON THE EFFICACY OF *Edwardsiella ictaluri* VACCINATION OF JUVENILE CHANNEL CATFISH

1. Introduction

Growth and disease resistance are two traits of pivotal importance to the aquaculture industry. Impaired disease resistance will have a strong negative impact on growth and survival of fish potentially resulting in large economical losses (Plumb, 2001). Therefore, disease prevention is extremely relevant to all aquaculture ventures aiming for profitability and sustainability (Klesius et al., 2004).

Different prophylactic measures may be taken to increase disease resistance including vaccination, proper nutrition and feeding practices, stress reduction, maintenance of adequate water quality and correct use of antibiotics and other chemicals (Klesius et al., 2004). Vaccination has been proven to be somewhat effective against disease occurrence in aquaculture. However, there is a considerable need to develop high-efficacy vaccines because the lack of effectiveness is a factor that constrains the widespread use of this method of disease control (Secombes, 2008; Shoemaker et al., 2009).

The efficacy of vaccines might be influenced by a variety of factors besides the immunogen *per se*. Although nutritional factors are recognized as having major

consequences on the immune responses of fish (Klesius et al., 2004), and great efforts have been expended in this area of research, the role of nutrition on the immune system and health of fish is very complex and still not totally elucidated. Nutritional requirements need to be met in order to support proper growth, but also, nutritional intervention through supplementation of key nutrients may improve health and increase disease resistance. Generally, such research has been conducted by feeding the fish for a specific time period and then evaluating immune parameters or survival after a disease challenge (Waagbø, 2006). However, few studies have combined immunization with nutrient supplementation (Sealey and Gatlin, 2002a; Li et al., 2004). Under this scenario, the amino acids glutamine (GLN) and arginine (ARG) may prove fundamental as they have been demonstrated not only to promote growth (Wu, 2010) but also to have an array of desirable immunological attributes (Li et al., 2007).

Immunity is a complex process involving multiple humoral and cellular components from both the innate and adaptive immune system, such as cytokines, immunoglobulins, mononuclear cells (macrophages [MØ] and lymphocytes), as well as polymorphonuclear cells including neutrophils (Secombes, 2008). Adequate amino acid availability plays a key role in the performance of the immune system while combating invading pathogens. For example, GLN improves the secretion of important cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-2, IL-4, IL-6 and IFN-γ (Newsholme, 2001; Yeh et al., 2003; Yi et al., 2005; Li et al., 2007). In addition, GLN improves phagocytosis and killing capacity of neutrophils and MØ (Calder and Yaqoob, 1999), enhancing the expression of major histocompatibility complex-II molecules in

MØ (Newsholme, 2001). Also, lymphocyte proliferation is improved in the presence of this amino acid, and it plays an important role for plasma cell differentiation and immunoglobulin synthesis (Newsholme, 2001; Li et al., 2007). There is only a single report of dietary GLN supplementation in fish increasing plasma levels of complement proteins C3 and C4 (Zhu et al., 2011)

Similarly, ARG possesses several immunological functions. As the sole precursor for nitric oxide (NO), ARG has a key role for the innate and adaptive immune systems. High concentrations of ARG increases cytotoxicity of monocytes and natural killer cells, as well as synthesis of IL-2 and CD3 expression in T-cells (Li et al., 2007; Choi et al., 2009). In addition, ARG increases phagocytosis and killing capacity of MØ and neutrophils (Muhling et al., 2002; Choi et al., 2009), also modulating lymphocyte subsets and positively affecting their proliferation, adhesion molecules and chemotaxis (Zaloga and Siddiqui, 2004; Abdukalykova et al., 2008; Choi et al., 2009). Dietary ARG supplementation to poultry has proven to increase cell-mediated immunity and antibody titers after vaccination (Abdukalykova and Ruiz-Feria, 2006; Tayade et al., 2006a; Ruiz-Feria and Abdukalykova, 2009). In channel catfish, ARG-supplemented diets increased production of NO by MØ (Buentello and Gatlin, 1999) and their phagocytic capacity (Buentello et al., 2007), as well as resistance to experimental *E. ictaluri* infection (Buentello and Gatlin, 2001b).

Enteric septicemia of catfish (ESC) is caused by *E. ictaluri*, a gram negative, rod-shaped, motile bacterium of the family *Enterobacteriacae* (Hawke et al., 1981), which is capable of intra- and extra-cellular replication (Thune et al., 1993; Booth et al., 2006).

This disease is the most prevalent and economically devastating disease in farmed-raised channel catfish, costing the US catfish industry $40–60 million annually (Shoemaker et al., 2009). A lived-attenuated vaccine was developed against ESC (Klesius and Shoemaker, 1999) and made into a commercially available option for ESC control (Intervet/Schering plough Animal Health, Summit, NJ AQUAVAC-ESC®), which is presently the only licensed vaccine against this disease (APHIS-USDA, 2008). Although this vaccine has proven to build protection against *E. ictaluri*, this protection seems to be not permanent and not entirely effective as it does not encompass all bacterial strains causing ESC (Klesius and Shoemaker, 1999; Shoemaker et al., 2009). Apparently, fish survival upon vaccination is dependent on specific application protocols (Carrias et al., 2008) and the time lapsed after immunization. In addition, vaccine administration often triggers response mechanisms that may negatively affect fish growth and metabolism (Midtlyng and Lillehaug, 1998; Ronsholdt and McLean, 1999; Lönnström et al., 2001).

Here, it is hypothesize that dietary ARG and GLN intervention in channel catfish may enhance vaccine effectiveness against ESC, while preventing detrimental changes in fish growth. Therefore, the objective of the present experiment was to evaluate immune and metabolic performance of juvenile channel catfish fed supplemental levels of ARG, GLN and their combination, before and after vaccination against ESC, and after infection with *E. ictaluri*.

2. Materials and methods

2.1 Experimental diets

A basal diet was formulated to contain 26% crude protein from casein, gelatin and a crystalline L-amino acid premix. Dextrin was provided at 15.9% and lipids from corn and menhaden oil at 8%, on a dry-matter basis, for an estimated available energy level of 12 kJ · g^{-1} (Table 5.1). This diet met minimum dietary requirements of channel catfish (NRC, 2011). Three experimental diets were formulated to provide either ARG at 4%, GLN at 2% or a combination of both (same levels) by supplementing L-ARG (11490 USB, Cleveland, OH) and/or GLN (16285-USB) to the basal diet while maintaining them isonitrogenous by adjusting the levels of a 50:50 glycine-aspartate premix (Buentello and Gatlin, 2000). All diets were prepared as previously described (Bai and Gatlin, 1994) and maintained at -20 °C until used.

2.2 Feeding trial

Four hundred and eighty disease-free and *E. ictaluri* naïve juvenile catfish, with an average weight of 18.8 ± 0.6 g were placed into 24, 110-L aquaria, at a density of 20 fish per aquarium. The aquaria were arranged as a recirculating system equipped with a sand filter for mechanical filtration and biofilter for ammonia removal. A constant flow of 1L · min^{-1} was maintained in all aquaria. Dissolved oxygen and water temperature were maintained at 90% of air saturation and 27 ± 1 °C, respectively. A 12:12 h light:dark cycle was provided through fluorescent lights regulated with a timer. Water

Table 5.1
Formulation and proximate composition of the basal diet

Ingredient	% of dry weight
Casein[1]	16.2
Gelatin[1]	3.7
Amino acid premix[1,2]	7.1
Dextrin[1]	15.9
Celufil[1]	29.3
Corn oil[3]	4.0
Menhaden oil[3]	4.0
Vitamin premix[4]	3.0
Mineral premix[5]	4.0
$Ca(PO_4)$[6]	1.0
Carboxymethyl cellulose[1]	2.2
Aspartate:Glycine premix[1,2]	8.2
L-ARG[1]	0.2
L-GLN[1]	0.0
Analyzed proximate composition (% dry weight)	
Dry matter	90.2
Crude protein	33.5
Crude lipid	7.9
Ash	3.8

[1] USB, Cleveland, OH, USA.

[2] Buentello and Gatlin (2000). Consisted of (% of diet) : L-histidine, 0.14; L-isoleucine, 0.19; L-leucine, 0.06; L-lysine, 0.64; LD-methionine, 0.32; L-phenylalanine, 0.42; L-serine, 1.57; L-threonine, 0.13; L- tryptophan, 0.02; L-valine, 0.11; L-proline, 1.57; L-alanine, 1.57.

[3] Omega Protein, Reedville,VA, USA.

[4] Moon and Gatlin (1991).

[5] MP Biomedicals, Solon, OH, USA.

[6] Fisher Scientific, Waltham, MA, USA

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quality was monitored every other day for pH, hardness, alkalinity, nitrite, ammonia, temperature and dissolved oxygen and remained within acceptable levels known to support optimal growth of channel catfish. Fish were acclimated to the experimental conditions for a period of 2 weeks and fed the basal diet. Thereafter, each dietary treatment was randomly assigned to six aquaria, and fish were fed the experimental or basal diets for a period of 4 weeks. Feeding rate was set at a level approaching satiation (4% of biomass) and provided in two daily feedings (a.m. and p.m.). Fish were weighed once a week and the feed ration was adjusted accordingly. Procedures used in this study were approved by the Texas A&M University System Animal Care and Use Committee.

2.3 Vaccination

After 2 weeks of the feeding trial, fish were vaccinated against *E. ictaluri* using the commercial vaccine Aquavac-ESC. Vaccination protocol followed the manufacturer's instructions. Briefly, one vaccine vial was dissolved with water to reach a dosage sufficient to immunize 1 kg of fish biomass in 1.15 L of tank water. Fish from three aquaria per treatment were removed and placed in previously cleaned plastic containers with an appropriate amount of water and oxygen supply, and exposed to the vaccine solution for 30 min. After vaccination, fish were returned to their appropriate aquarium. Fish in the remaining three aquaria were sham-treated as the vaccinated fish, by placing them in a container with tank water and no vaccine. The feeding trial continued for 2 more weeks.

2.4 Disease challenge

After 14 d post-vaccination (dpv, 4 weeks into the feeding trial) 20 fish per treatment were challenged with a pathogenic strain of *E. ictaluri* via intraperitoneal (i.p.) injection of 5 x 10^8 colony forming units (CFU)/fish. This dose was previously determined in a median lethal dose (LD_{50}) trial (described below). Bacteria-exposed fish were monitored for morbidity and mortality every 12 h until the vaccinated control fish reached 100% mortality (day 8), and every 24 h until day 21 post-infection (pi).

For the LD_{50} trial, inoculums were prepared with a pathogenic *E. ictaluri* isolated and molecularly identified (Williams and Lawrence, 2010) from a natural outbreak as described by Buentello and Gatlin (2001b). Dilutions were made with brain heart infusion (BHI) broth to obtain four different concentrations. The dosages were i.p. injected to four different groups of fish (n = 6), weighing 15 g ± 1, at a dose of 0.5 mL/fish. A fifth group received sterile BHI to serve as a control. Fish were checked for mortality for a 4-d period.

2.5 Sample collection

Samples from nine randomly selected fish per treatment (three per aquarium) were taken at 2 weeks of the feeding trial (pre-vaccine), 7 dpv and 14 dpv. Prior to all sampling, fish were euthanized with tricaine methanesulphonate (MS-222, Western Chemical Inc., Ferndale, WA, USA, 300 mg $\cdot$ L^{-1}). Blood samples (~1 mL) were obtained from the caudal vasculature with heparinized needles (1-mL syringe, 23-ga needle). Heparinized blood (n=3) was used for peripheral blood lymphocyte isolation

(described below). Also, plasma was obtained (n=6) by centrifuging whole blood at 3800 *x* g for 10 min. Intestinal mucus samples were obtained by placing both sides of a 1-cm^2 filter paper (Wathman No.2, 1002-042) in contact with the enteric mucosa for 1 min each side; protein was eluted by shaking filter papers in 1 mL of PBS (Phosphate buffer solution, pH 7.2, Sigma P4417) for 2 h (Zilberg and Klesius, 1997). Bile was collected using a 1-mL syringe, 23-ga needle, and centrifuged at 2000 x *g* for 10 min (Coscia and Oreste, 2000). Fish whole-body and intestinal length and weight were recorded along with liver, spleen, head and trunk kidney, and left side fillet muscle weight. Spleen and both head and trunk kidneys, from three fish, were placed in sterile Hank's buffered salt solution (HBSS, Sigma H4891) and used for lymphocyte isolation. All remaining tissue samples and plasma (from six fish) were quickly frozen in liquid nitrogen and kept at -80 °C for further analysis. Intestinal mucus and bile samples were kept at -20 °C. In addition, available fish were sampled at 3 dpi for plasma and 14 dpi for plasma, intestinal mucus and bile.

2.6 Growth and performance parameters

To corroborate previous effects of ARG and GLN supplementation in channel catfish and to determine if these amino acids affected growth and other responses after vaccination, the following indicators were analyzed. Relative weight gain (WG = final weight – initial weight x 100 / initial weight), feed efficiency ratio (FER = weight gain / dry feed intake), protein efficiency ratio (PER = weight gain / dry protein fed), condition factor (CF= fish weight x 100 / length ^ 3), fillet yield (FY= muscle weight x 2 x 100 /

whole-body weight), hepatosomatic index (HSI= liver weight x 100 / whole-body weight), relative intestinal length (RIL= intestine length x 100 / whole-body length), relative intestinal weight (RIW = intestine weight x 100 / whole-body weight), relative spleen weight (RSW = spleen weight x 100 / whole-body weight), relative kidney weight (RKW = kidney weight x 100 / whole-body weight) and percent survival (final no. of living fish x 100 / initial no. of fish) were computed. Also crude protein (AOAC, 2005) was estimated for muscle, intestine, liver, spleen and kidney to obtain protein retention (PR = [final tissue protein – initial tissue protein] x 100 / total protein fed) for each tissue.

2.7 Amino acid analysis

To evaluate changes and modulation of amino acids, free-pool (perchloric acid extracted) amino acid profiles were analyzed in plasma and muscle at each sampling point during the feeding trial. Sample preparation before derivatization was as described by Buentello and Gatlin (2002) with modifications. Briefly, muscle (~500 mg) was homogenized in 3 mL of 1.5 M $HClO_4$ (9552-05, J.T. Baker, Phillipsburg, NJ), centrifuged at 3000 x *g* for 15 min, then supernatant was neutralized with 2M K_2CO_3 (P5833, Sigma). Plasma was deproteinized ($HClO_4$) and neutralized (K_2CO_3) as muscle samples. Amino acid were analyzed using an ultraperformance liquid chromatograph (UPLC-Acquity system®, Waters™, Milford, MA) and the commercial kit MassTrak™ (186004094, Waters™).

2.8 Anti-E. ictaluri *antibody detection*

To evaluate specific humoral responses against *E. ictaluri*, specific antibody titers in plasma, intestinal mucus and bile were measured through enzyme linked immunosorbent assay (ELISA). Plates were prepared as described by Waterstrat et al. (1989), and stored at room temperature until used.

Based on previous titration assays of positive and negative samples (data not shown), PBS was used to dilute (v/v) plasma 1:160, and bile 1:20. Intestinal mucus was not diluted. In order to detect specific antibodies against *E .ictaluri*, 50 μL of sample were dispensed to duplicate wells of the ELISA plate. After 1 h incubation, the plate was washed three times with PBS. Mouse anti-channel catfish IgM (9E1, University of Mississippi) was used as a primary antibody, adding 50 μL (1:10 dilution) to each well; repeating incubation and washing steps. The secondary antibody consisted of peroxidase-conjugated sheep anti-mouse IgG (Sigma, A5906), adding 50 μL (1:1000 dilution) to each well; repeating both incubation and washing steps. Substrate solution (1 tetramethylbenzidine tablet [Sigma, T5525] + 1 mL dimethyl sulfoxide [Sigma, 154838] + 9 mL citric acid buffer [Sigma, P4809]+ 2 mL H_2O_2 [Sigma, H3410]) was added (100 μL) to each well. Reaction was stopped after 5 min with 50 μL of 1M H_2SO_4 (VWR, 7662-93-9). An ELISA plate reader (Biorad, iMark^{TM}) was used to read the absorbance (ABS) at 450 nm. Inter-plate differences were prevented by standardizing raw values to a positive control value.

2.9 Lymphocyte isolation from blood and tissues

To study immune cellular responses, lymphocytes were isolated from peripheral blood as described by Miller et al. (1994). These cells were isolated from spleen and both head and trunk kidney following the procedures of Secombes (1990), with modifications. Briefly, heparinized blood was diluted 1:2 with PBS (pH 7.2). Each tissue was mechanically disaggregated using a glass tissue homogenizer and filtered through a 100-μm nylon mesh. Then cell suspensions were layered over Lymphoprep™ (Axis-Shield PoC AS, 1114545) and centrifuged at 350 x *g* for 20 min. Lymphocytes were recovered from the interface. Cells were washed three times with PBS at 600 x *g* for 10 min, red blood lysing buffer (0.15 M NH_4Cl + 0.01 M $KHCO_3$ + 0.0003 M ethylenediaminetetraacetic acid) was used to lyse remanent red blood cells. Cells were resuspended in 1 mL ice-cold PBS and enumerated using a hemocytometer and viability assessed by trypan blue (Sigma, T8154) staining. Viability was > 95% in all cases. Lymphocytes for flow cytometry were kept in cold PBS; whereas, those cells intended for responsiveness assays were transferred to a complete channel catfish medium (CCM, L-15:AIM-V:de-ionized water, 45:45:10; 50 units · mL^{-1} of penicillin – 0.05 mg · mL^{-1} of streptomycin, 0.02 mg · mL^{-1} gentamicin, 0.05 mM of 2-mercaptoethanol, 0.09% Na_2HCO_3, and 5% pooled heat-inactivated channel catfish serum).

2.10 Flow cytometry for B-lymphocytes

To analyze lymphocyte population modulation, the proportion of B-lymphocyte (IgM^+ cells) was analyzed. Isolated lymphocytes were transferred (5 x 10^5) to a flow

cytometry tube (VWR, 60818-408) and washed once with 1 mL of PBS. Supernatant was decanted and the tube blotted on paper towel. Cells were resuspended in the remaining PBS. Primary antibody (9E1, 1:10 dilution) was added (10 μL) incubating in ice for 1 h. Cells were then washed two times with 2 mL of PBS. After the second wash, supernatant was decanted and tube blotted on paper towel, resuspending cells in remaining PBS. Secondary antibody (FITC conjugated Goat anti-mouse IgG, Sigma F8521, 1:100 dilution) was added (5 μL) and incubated for 1 hr in ice. After washing the cells twice they were resuspended in 300 μL of PBS. Stained cells were counted in a cell-coulter (FACSCalibur, Becton-Dickinson). Results are presented as the mean percentage amount of IgM^+ cells per 10,000 events.

2.11 Lymphocyte responsiveness against E. ictaluri

In order to evaluate formation of memory cells, proliferation of lymphocytes upon exposure to a known antigen (*E. ictaluri*) was analyzed. Isolated lymphocyte concentration was adjusted to 2.77×10^6 cell · mL^{-1} with CCM. Lymphocytes were seeded by adding 180 μL to sextuplet wells of a sterile round bottom 96-well microplate (Nunc, 163320). Formalin-killed *E. ictaluri* (bacteria suspension cultured with 1% formalin for 24 h) was added (20 μL) to three wells to achieve 1×10^5 bacteria per well. The remaining three wells received CCM to serve as control (non-stimulated) cells. A separate set of triplicate wells with cells from a control fish received Concavalin-A to serve as a positive control for the assay. Cells were cultured at 27 °C in a humidified 5% CO_2 atmosphere for 72 h. Cells were pulsed with 0.5 μCi of ^{3}H-thymidine (MP

biologicals, 124066) 18 h before harvesting. Incorporation of the radionucleotide was measured with a liquid scintillation β–counter (Perkin Elmer, Wallac MicroBeta TriLux). Results are presented as stimulation index (SI = counts per minute stimulated cells / counts per minute control cells). The positive control cells validated the assays as proliferation was detected (data not-shown).

2.12 Statistical analysis

Pre-vaccine data were subjected to one-way analysis of variance (ANOVA). Post-vaccine data were subjected to two-way ANOVA with diet and vaccine as main effects and the interaction of both. The Statistical Analysis System (SAS, 9.2 v) software was used for all analyses. A $P \leq 0.05$ was taken to indicate statistical significance among treatment means, which were separated using Duncan's multiple range test. Values are presented as means with a pooled standard error (P.S.E) per variable measured.

3. Results

3.1 Growth and performance parameters

Growth parameters, pre- and post- vaccination, are summarized in Table 5.2. Before vaccination, there was a significant positive effect on WG, FE and PER of fish fed ARG-supplemented diets, with or without GLN addition. No synergistic effect was observed by supplementing both amino acids to the diet. Similarly, consistent trends were observed at 7 and 14 dpv in all these parameters when diet was considered a

Table 5.2
Growth parameters pre- and post-vaccination with Aquavac-ESC®[1]

Pre-vaccine					Factor		WG		FE		PER		Survival	
Diet	**WG[2]**	**FER[3]**	**PER[4]**	**Survival**	**Diet**	**Vaccine**	**7dpv**	**14dpv**	**7dpv**	**14dpv**	**7dpv**	**14dpv**	**7dpv**	**14dpv**
Basal	11.6[5,b]	0.27[b]	0.64[b]	97	**Basal**	**Yes**	6.1	8.6	0.1	0.1	0.3	0.3	98	87
						No	15.1	17	0.2	0.2	0.6	0.5	96	77
GLN	13.3[b]	0.31[b]	0.73[b]	98	**GLN**	**Yes**	12.3	11.9	0.2	0.2	0.5	0.4	98	84
						No	11.1	15	0.2	0.2	0.5	0.5	100	96
ARG	19.8[a]	0.45[a]	1.01[a]	96	**ARG**	**Yes**	25.0	24.9	0.4	0.3	1.0	0.7	100	88
						No	21.0	24.4	0.3	0.3	0.8	0.7	94	77
ARG + GLN	18.3[a]	0.42[a]	1.07[a]	98	**ARG + GLN**	**Yes**	16.7	14.4	0.3	0.2	0.7	0.5	98	98
						No	17.9	19.5	0.3	0.2	0.7	0.6	100	85
Pooled std. error	1.9	0.04	0.07	2.81	**Pooled std. error**		3.1	3.78	0.05	0.05	0.12	0.11	1.8	6.6
					***Pr* > F[6]**									
***Pr* > F**	0.001	0.001	0.001	0.83	**Diet**		0.0002	0.002	0.001	0.047	0.01	0.059	0.598	0.502
					Vaccination		0.328	0.04	0.44	0.108	0.76	0.179	0.482	0.323
					Diet*Vaccination		0.046	0.515	0.09	0.695	0.23	0.7	0.242	0.339

[1] Values represent the mean of three replicate tanks.
[2] WG, weight gain = final weight – initial weight x 100 / initial weight.
[3] FE, feed efficiency ratio = weight gain / dry feed intake.
[4] PER, protein efficiency ratio = weight gain / dry protein fed.
Survival = final no. of living fish x 100 / initial living fish.
[5] Different superscript letters indicate significant ($P < 0.05$) differences as evaluated by Duncan's multiple range test.
[6] Significance probability associated with the F-statistic.

factor. On the other hand, vaccination only had a significant effect at 14 dpv, where WG was lower in the vaccinated than unvaccinated groups. Significant interaction of these two factors was only observed at 7 dpv, as WG was reduced in vaccinated fish fed the basal diet by 60% in comparison to unvaccinated animals. Importantly, supplementing the diet with either GLN or ARG ameliorated this effect. In addition, ARG tended (P = 0.09) to increase WG in vaccinated fish.

Organosomatic indices for the pre-vaccine period are showed in Table 5.3. Only CF, FY and RIL were affected by diet in the pre-vaccine period. Fish fed diets supplemented with GLN or ARG had significantly higher CF compared to fish fed the basal diet. However, in those fed the diet supplemented with ARG + GLN, CF was lower compared to those fed the diet in which GLN was supplemented alone. Fillet yield was significantly higher in fish fed the two diets supplemented with ARG. However, FY from fish fed the diet supplemented with GLN was not different from the ARG-fed fish or those fed the basal diet. Concerning the RIL, all supplemented diets had a significant increase of up to 30%; however, no synergistic effect was observed by providing both amino acids in the diet. After vaccination (Table 5.4), diet continued to have an effect on CF and RIL at 7 dpv, with the same pattern as described earlier. Vaccination affected FY at 7 dpv, as well as RIW, RSW and RKW at 14 dpv. In all cases but IRW, vaccinated groups had higher indexes than the non-vaccinated. An interaction between vaccine and diet was only observed in FY and RKW at 7 dpv, and in RIW at 14 dpv.

Table 5.3
Organosomatic indices pre-vaccination with Aquavac-ESC®[1]

Diet	CF[2]	FY[3]	RIL[4]	RIW[5]	HSI[6]	RSW[7]	RKW[8]
Basal	0.71[9,c]	20.2[b]	0.62[b]	1.03	1.37	0.084	0.55
GLN	0.75[a]	23.8[ab]	0.90[a]	1.15	1.45	0.061	0.65
ARG	0.74[ab]	27.1[a]	0.90[a]	1.11	1.53	0.057	0.65
ARG + GLN	0.72[b]	27.0[a]	0.89[a]	1.25	1.38	0.065	0.66
Pooled std. error	0.012	0.84	0.081	0.20	0.11	0.016	0.08
***Pr* > F[10]**	0.003	0.024	0.003	0.586	0.390	0.991	0.309

[1] Values represent the mean of six randomly sampled fish.
[2] CF, condition factor = fish weight * 100 / length ^ 3
[3] FY, fillet yield = muscle weight * 100 / whole-body weight.
[4] HSI, hepatosomatic index = liver weight * 100 / whole-body weight.
[5] RIL, relative intestinal length = intestine length * 100 / whole-body length
[6] RIW, relative intestinal weight = intestine weight * 100 / whole-body weight.
[7] RSW, relative spleen weight = spleen weight * 100 / whole-body weight.
[8] RKW, relative kidney weight = kidney weight * 100 / whole-body weight.
[9] Different superscript letters indicate significant ($P < 0.05$) differences as evaluated by Duncan's multiple range test.
[10] Significance probability associated with the F-statistic.

Table 5.4
Organosomatic indices post-vaccination with Aquavac-ESC®[1]

Factor		CF[2]		FY[3]		RIL[4]		RIW[5]		HSI[6]		RSW[7]		RKW[8]	
Diet	**Vaccine**	**7dpv**	**14dpv**	**7dpv**	**14dpv**	**7dpv**	**14dpv**	**7dpv**	**14dpv**	**7dpv**	**14dpv**	**7dpv**	**14dpv**	**7dpv**	**14dpv**
Basal	**Yes**	0.65	0.68	33.0	33.2	0.71	0.81	0.83	0.73	1.28	1.20	0.061	0.082	0.65	0.84
	No	0.69	0.68	33.0	33.2	0.76	0.95	0.89	1.05	1.42	1.28	0.060	0.077	0.76	0.58
GLN	**Yes**	0.71	0.66	35.8	36.3	0.97	0.95	0.85	0.95	1.33	1.21	0.078	0.131	1.01	0.98
	No	0.72	0.68	28.4	35.1	0.92	0.94	0.90	0.87	1.24	1.29	0.059	0.078	0.71	0.78
ARG	**Yes**	0.71	0.70	36.5	35.8	0.91	0.91	0.95	0.85	1.34	1.20	0.052	0.109	0.66	0.90
	No	0.73	0.68	30.7	36.7	0.90	0.91	0.92	1.06	1.16	1.38	0.057	0.085	0.65	0.69
ARG + GLN	**Yes**	0.75	0.65	34.9	33.0	0.94	0.92	0.90	0.90	1.31	1.26	0.070	0.074	0.56	0.79
	No	0.71	0.69	28.5	33.9	0.90	0.87	0.96	0.95	1.36	1.19	0.074	0.077	0.90	0.88
Pooled std. error		0.03	0.03	1.53	1.73	0.11	0.08	0.11	0.10	0.14	0.12	0.009	0.022	0.13	0.14
***Pr* > F[9]**															
Diet		0.004	0.764	0.230	0.074	0.043	0.762	0.73	0.832	0.716	0.358	0.064	0.135	0.135	0.284
Vaccination		0.696	0.433	0.0001	0.859	0.839	0.606	0.509	0.027	0.764	0.259	0.580	0.017	0.715	0.029
Diet*Vaccination		0.362	0.536	0.01	0.850	0.917	0.372	0.930	0.050	0.356	0.500	0.290	0.332	0.022	0.256

[1] Values represent the mean of six randomly sampled fish.
[2] CF, condition factor = fish weight x 100 / length ^ 3.
[3] FY, fillet yield = muscle weight x 2 x 100 / whole-body weight.
[4] RIL, relative intestinal length = intestine length x 100 / whole-body length.
[5] RIW, relative intestinal weight = intestine weight x 100 / whole-body weight.
[6] HSI, hepatosomatic index = liver weight x 100 / whole-body weight.
[7] RSW, relative spleen weight = spleen weight x 100 / whole-body weight.
[8] RKW, relative kidney weight = kidney weight x 100 / whole-body weight.
[9] Significance probability associated with the F-statistic.

Protein retention by channel catfish organs, pre- and post-vaccination, is presented in Table 5.5. After 2 weeks of the feeding trial, although fish fed the diet supplemented with ARG had higher numerical values, there were no significant differences among treatments in any of the tissues analyzed. In contrast, 7 d after vaccination, PR in all tissues was affected by dietary treatment. Fish fed supplemental levels of ARG or GLN (but not combined) had significantly higher PR. Interestingly, fish fed the diet supplemented with ARG had the highest muscle PR; whereas, those fed the diet supplemented with GLN had the highest kidney PR. At this point (7 dpv), only PR in muscle was significantly affected by vaccination, with vaccinated fish having higher PR than non-vaccinated fish. Additionally, an interaction between diet and vaccination was observed in liver. Vaccinated fish fed diets supplemented with GLN and/or ARG and non-vaccinated fish fed the diet supplemented with ARG alone had significantly higher PR than vaccinated and non-vaccinated fish fed the basal diet. Protein retention at 14 dpv was only affected in kidney. Fish fed the basal diet had significantly lower PR than the other treatments; whereas, vaccinated fish had significantly higher PR than the non-vaccinated group.

3.2 Amino acid profiles

Plasma amino acid profile for the pre-vaccine period is presented in Table 5.6. Only a few changes in amino acids were noticed. Plasma ARG levels were significant higher (2-fold) in fish fed diets supplemented with ARG. Glutamine supplementation did not affect levels of this amino acid. Ornithine levels were higher only in fish fed the

Table 5.5
Protein retention[1] by individual tissues pre- and post-vaccination with Aquavac-ESC®[2]

Pre-vaccine						Factor		Gut		Liver		Muscle		Spleen		Kidney	
Diet	**Gut**	**Liver**	**Muscle**	**Spleen**	**Kidney**	**Diet**	**Vaccine**	**7dpv**	**14dpv**	**7dpv**	**14dpv**	**7dpv**	**14dpv**	**7dpv**	**14dpv**	**7dpv**	**14dpv**
Basal	0.26	0.52	3.36	0.03	0.01	**Basal**	Yes	0.05	0.03	0.11	0.21	3.3	5.80	0.008	0.032	0.05	0.17
							No	0.01	0.21	0.21	0.22	2.6	5.00	0.010	0.035	0.11	0.01
GLN	0.33	0.45	3.28	0.02	0.20	**GLN**	Yes	0.21	0.21	0.62	0.27	10.8	8.5	0.051	0.059	0.58	0.33
							No	0.33	0.31	0.20	0.45	5.7	8.5	0.027	0.036	0.25	0.24
ARG	0.41	0.60	7.42	0.02	0.24	**ARG**	Yes	0.40	0.48	0.44	0.43	10.7	11.0	0.030	0.041	0.23	0.34
							No	0.44	0.25	0.47	0.30	10.4	7.4	0.025	0.043	0.33	0.14
ARG + GLN	0.67	0.75	4.59	0.06	0.31	**ARG + GLN**	Yes	0.34	0.32	0.43	0.38	8.6	9.6	0.041	0.039	0.15	0.23
							No	0.14	0.33	0.32	0.30	4.4	7.6	0.032	0.028	0.19	0.26
Pooled std. error	0.196	0.184	1.86	0.017	0.126	**Pooled std. error**		0.13	0.12	0.09	0.10	1.53	1.70	0.010	0.010	0.11	0.07
						***Pr* > F[3]**											
***Pr* > F**	0.574	0.713	0.469	0.465	0.403	**Diet**		0.022	0.163	0.014	0.366	0.001	0.143	0.003	0.635	0.036	0.043
						Vaccination		0.881	0.746	0.109	0.925	0.030	0.201	0.093	0.421	0.669	0.035
						Diet*Vaccination		0.636	0.293	0.034	0.401	0.324	0.751	0.360	0.701	0.200	0.340

[1] Protein retention = (initial tissue protein – initial tissue protein) x 100 / total protein fed.
[2] Values represent the mean of tissues from six randomly sampled fish.
[3] Significance probability associated with the F-statistic.

Table 5.6
Plasma amino acid profiles (nmol · mL^{-1}) pre-vaccination with Aquavac-ESC®[1]

	Diet				Pooled std. error	*Pr* > F[2]
Amino acid	**Basal**	**GLN**	**ARG**	**ARG + GLN**		
Hydroxyproline	352.9	438.8	574.4	805.4	163.1	0.296
Histidine	86.2	88.9	78.5	126.9	19.3	0.354
Phosphoethanolamine	117.8[3,a]	44.9[b]	28.2[b]	31.2[b]	9.6	0.001
Asparagine	151.5	104.4	136.9	149.7	24.3	0.527
3-Methylhistidine	55.4	24.0	48.7	71.0	10.5	0.070
Taurine	678.4	505.1	380.6	585.3	524.7	0.300
1-Methylhistidine	17.4[b]	13.5[b]	20.9[b]	36.8[a]	4.4	0.024
Serine	370.5[a]	198.8[b]	171.1[b]	188.8[b]	27.0	0.003
Glutamine	277.9	221.3	254.8	303.0	28.3	0.735
Carnosine	n.d	n.d	n.d	n.d	--	--
Arginine	103.9[b]	110.1[b]	229.8[a]	282.7[a]	26.2	0.049
Glycine	484.0	323.5	268.3	353.8	72.1	0.262
Anserine	2.6	10.2	19.1	27.6	9.1	0.305
Ethanolamine	64.7	46.6	40.3	63.3	0.9	0.224
Aspartate	45.9	43.7	33.1	23.1	6.5	0.121
Sarcosine	18.1	11.9	8.3	15.3	2.4	0.090
Glutamate	181.4	133.4	105.7	732.8	299.8	0.446
Citrulline	2.7	4.4	4.4	7.2	2.0	0.504
β-Alanine	10.7	10.5	9.0	11.7	1.3	0.549
Threonine	246.9	196.5	207.8	169.1	20.5	0.135
Alanine	706.5	597.8	517.5	728.3	93.0	0.398
γ-Aminobutyric acid	31.2[a]	17.9[ab]	12.4[b]	31.8[a]	4.7	0.046
Proline	344.5	467.5	594.3	829.9	310.8	0.727
Hydroxylysine	18.6	14.5	16.9	21.0	4.5	0.776
α-Aminobutyric acid	78.3[a]	36.8[b]	38.5[b]	37.7[b]	7.1	0.008
Cysteine	16.4	7.5	4.7	4.2	4.0	0.196
Ornithine	42.1[b]	39.3[b]	68.2[b]	151.1[a]	24.0	0.035
Cystine	17.1	28.0	19.7	23.3	5.7	0.585
Lysine	341.5	230.0	270.8	285.3	55.9	0.588
Tyrosine	102.3	57.5	84.1	103.4	17.5	0.286
Methionine	121.3	57.6	32.9	101.9	24.2	0.109
Valine	551.9	455.8	397.0	513.9	50.9	0.230
Isoleucine	355.9	267.6	219.5	304.6	30.0	0.062
Leucine	638.6	475.3	451.5	543.0	50.6	0.113
Homocysteine	3.4	3.4	5.9	4.3	1.6	0.685
Phenylalanine	207.0	167.2	157.9	251.3	32.1	0.231

Table 5.6 continued

Amino acid	Diet				Pooled std. error	*Pr* > F[2]
	Basal	GLN	ARG	ARG + GLN		
Tryptophan	76.3	59.8	68.4	123.5	19.9	0.185

[1] Values represent the mean of six randomly sampled fish.
[2] Significance probability associated with the F-statistic.
[3] Different superscript letters indicate significant ($P < 0.05$) differences as evaluated by Duncan's multiple range test.

diet supplemented with both amino acids, with values of 3.6, 3.8 and 2.2-fold those of fish fed the basal, GLN and ARG diets, respectively. Other amino acids affected were γ-aminobutyric acid (GABA), which was lower in fish fed ARG-supplemented diets. Serine, α-aminobutyric acid (AABA) and phosphoethanolamine (PEA) were higher in fish fed basal diet; whereas, methylhistidine (MH) was higher in fish fed the ARG + GLN diet.

At 7 dpv (Table 5.7), plasma amino acid profile changes were more accentuated than during the pre-vaccine period. Diet had an effect on circulating ARG but not in circulating GLN. Fish fed diets supplemented with either ARG or GLN had significantly higher ($P < 0.05$) plasma ARG than those fed the basal and ARG + GLN diets. Citrulline levels were higher in fish fed the GLN diet than the rest of the treatments. Diet had also an important effect on hydroxyproline, MH, citrulline, threonine, AABA, cysteine, leucine and tryptophan. On the other hand, asparagine, glycine and AABA were significantly higher in non-vaccinated fish, while ethanolamine, GABA and methionine remained lower. Vaccine and diet interaction had an effect on hydroxyproline, asparagine, serine, glutamine and AABA. Non-vaccinated fish fed the diet supplemented with GLN had significantly higher levels of circulating GLN than vaccinated animals. Also, the former group of fish had higher levels of hydroxyproline, asparagine and AABA. A similar pattern was seen in fish fed ARG + GLN but without significant differences ($P > 0.05$) in the GLN values. Fish fed either ARG-supplemented diet had a noticeable tendency to lower plasma ARG levels when vaccinated ($P = 0.09$). In contrast, fish fed the basal diet or the GLN-

Table 5.7
Plasma amino acid profiles (nmol · mL^{-1}) 7 dpv with Aquavac-ESC®[1]

Amino acid	Diet / Vaccine								Pooled std. error	*Pr* > F[2]		
	Basal		GLN		ARG		ARG + GLN			Diet	Vaccine	Diet *Vaccine
	No	Yes	No	Yes	No	Yes	No	Yes				
Hydroxyproline	80.7	75.1	256.6	58.3	174.8	212.3	66.9	78.5	34.1	0.006	0.128	0.012
Histidine	43.2	49.5	59.7	49.3	49.0	46.8	42.8	33.3	7.0	0.173	0.437	0.617
Phosphoethanolamine	11.8	12.8	9.1	18.8	13.4	13.2	14.7	20.9	4.2	0.585	0.175	0.608
Asparagine	65.1	85.1	83.0	45.4	55.7	40.8	97.1	40.6	11.9	0.176	0.018	0.030
3-Methylhistidine	13.3	18.3	46.1	46.0	53.6	19.2	18.0	14.5	16.5	0.215	0.491	0.637
Taurine	1105.7	958.3	558.2	900.2	655.0	780.7	554.0	1282.4	202.7	0.365	0.086	0.215
1-Methylhistidine	6.8	6.0	10.4	5.6	11.9	14.5	10.3	9.4	2.2	0.043	0.541	0.451
Serine	125.8	176.4	160.6	130.6	109.6	84.4	164.1	88.3	20.4	0.069	0.182	0.048
Glutamine	377.4	464.1	682.7	235.1	507.9	593.2	500.9	397.7	90.8	0.53	0.16	0.030
Carnosine	3.1	1.6	1.3	15.5	1.4	0.6	1.4	0.6	5.0	0.411	0.441	0.352
Arginine	88.7	112.3	167.8	191.5	205.2	165.9	162.8	113.1	21.4	0.021	0.283	0.096
Glycine	154.7	179.7	194.1	142.9	164.8	116.1	234.8	99.5	32.8	0.794	0.038	0.156
Anserine	4.2	7.4	10.9	11.3	9.0	12.3	2.7	5.3	3.0	0.075	0.283	0.960
Ethanolamine	26.9	34.9	29.3	42.1	29.3	31.0	25.1	37.6	4.7	0.652	0.019	0.627
Aspartate	12.2	18.0	21.5	16.4	16.5	23.0	10.7	10.0	3.8	0.094	0.553	0.398
Sarcosine	12.7	18.6	7.1	7.1	6.0	7.1	14.2	4.7	5.1	0.288	0.868	0.503

Table 5.7 continued

Amino acid	Diet / Vaccine								Pooled std. error	*Pr* > F[2]		
	Basal		GLN		ARG		ARG + GLN			Diet	Vaccine	Diet *Vaccine
	No	Yes	No	Yes	No	Yes	No	Yes				
Glutamate	51.2	76.0	69.8	64.3	55.6	52.0	41.9	63.2	8.7	0.291	0.151	0.207
Citrulline	1.5	2.0	6.6	9.6	1.8	3.4	1.3	3.6	2.4	0.05	0.295	0.963
β-Alanine	6.7	6.0	6.8	6.0	6.1	6.0	7.2	5.0	0.9	0.969	0.137	0.688
Threonine	124.2	131.2	154.6	119.5	96.8	68.3	91.2	71.8	19.9	0.02	0.196	0.732
Alanine	210.7	295.7	286.2	220.6	265.9	303.8	309.2	232.6	47.8	0.891	0.888	0.288
γ-Aminobutyric acid	17.8	24.3	10.8	31.0	11.5	18.9	12.1	20.7	6.3	0.708	0.029	0.673
Proline	406.7	119.3	356.6	77.8	179.6	368.9	79.0	86.6	145.9	0.552	0.384	0.319
Hydroxylysine	7.7	6.0	6.8	10.1	4.7	9.7	2.7	8.5	2.4	0.722	0.091	0.432
α-Aminobutyric acid	18.9	32.6	35.0	16.7	16.8	10.6	30.6	14.5	3.7	0.014	0.02	0.002
Cysteine	5.2	9.0	6.4	4.8	1.8	1.2	2.6	2.3	1.3	0.001	0.708	0.192
Ornithine	39.1	34.2	20.2	66.3	37.4	35.7	47.3	45.4	17.8	0.927	0.463	0.441
Cystine	0.7	2.5	4.2	6.5	5.8	4.8	2.2	0.8	2.0	0.113	0.777	0.712
Lysine	166.6	230.6	288.2	270.2	169.6	146.3	204.4	136.8	57.8	0.193	0.787	0.720
Tyrosine	45.6	57.3	55.3	79.1	45.6	42.3	57.6	46.4	9.3	0.128	0.435	0.280
Methionine	15.8	10.3	7.1	24.6	12.8	18.7	14.8	42.6	6.3	0.102	0.021	0.090
Valine	219.4	264.8	284.5	256.5	175.8	212.6	202.5	169.5	28.8	0.029	0.794	0.401

Table 5.7 continued

Amino acid	Diet / Vaccine								Pooled std. error	*Pr* > F[2]		
	Basal		GLN		ARG		ARG + GLN			Diet	Vaccine	Diet *Vaccine
	No	Yes	No	Yes	No	Yes	No	Yes				
Isoleucine	171.0	181.7	182.0	203.9	103.5	131.3	136.9	107.9	32.0	0.072	0.732	0.811
Leucine	251.7	304.7	337.7	315.2	192.7	247.8	243.0	207.2	36.7	0.033	0.638	0.477
Homocysteine	3.8	6.8	12.3	6.5	5.4	3.3	4.3	1.4	2.5	0.099	0.285	0.386
Phenylalanine	101.8	128.8	118.5	157.7	120.6	116.2	104.9	94.7	12.6	0.056	0.167	0.188
Tryptophan	34.1	44.0	44.8	53.8	43.2	42.6	34.5	33.0	4.9	0.037	0.236	0.522

[1] Values represent the mean of six randomly sampled fish.
[2] Significance probability associated with the F-statistic.

supplemented diet tended to have higher values of this amino acid when vaccinated. Finally, serine decreased in vaccinated fish fed the diet supplemented with ARG + GLN.

At 14 dpv (Table 5.8) only ARG, GLN, MH, taurine, β-alanine, alanine and methionine were significantly affected by diet. Arginine levels remained high in fish fed either ARG-supplemented diet and, for the first time, GLN was higher in fish fed ARG-enriched diets. Also, these fish had higher values of MH. Fish fed the GLN diet had significantly higher values of circulating taurine and methionine than fish fed the basal diet or the ARG + GLN-supplemented diet. Fish fed the basal diet had the lowest levels of β-alanine. Conversely, vaccinated fish had higher values of carnosine and methionine, and lower values of β-alanine and alanine. Interactions between factors were only seen for GLN and MH. Contrary to observations at 7 dpv, plasma GLN was significantly higher in vaccinated fish fed GLN-supplemented diets. However, only fish fed the ARG + GLN-supplemented diet yielded significant differences between vaccinated and non-vaccinated fish. Fish vaccinated and fed the basal diet had significantly higher values of MH than non-vaccinated animals under the remaining dietary treatments.

Pre-vaccine muscle total free pool amino acids profile is presented in Table 5.9. The sum of total amino acids among treatments remained unchanged after 2 weeks of feeding. Muscle total ARG, ornithine and glutamate pools were significantly higher in both groups of fish fed ARG-supplemented diets, with or without GLN supplementation. A 15- and 8-fold increase was seen in muscle total ARG pool in fish fed the ARG and ARG + GLN diets, respectively, when compared to fish fed the control diet. In addition

Table 5.8
Plasma amino acid profiles (nmol · mL^{-1}) 14 dpv with Aquavac-ESC®[1]

Amino acid	Diet / Vaccine								Pooled std. error	*Pr* > F[2]		
	Basal		GLN		ARG		ARG + GLN			Diet	Vaccine	Diet *Vaccine
	No	Yes	No	Yes	No	Yes	No	Yes				
Hydroxyproline	244.6	106.1	128.6	144.9	254.2	261.0	330.8	252.4	95.0	0.365	0.482	0.825
Histidine	48.5	52.7	43.0	55.6	60.6	57.0	89.1	52.4	13.9	0.415	0.558	0.339
Phosphoethanolamine	16.3	15.2	13.7	19.1	12.9	19.5	13.5	11.1	4.2	0.746	0.489	0.641
Asparagine	78.4	77.6	55.2	56.9	99.2	81.5	56.4	83.0	12.0	0.069	0.775	0.358
3-Methylhistidine	28.8	56.9	27.3	14.7	27.9	23.1	31.5	34.8	6.7	0.025	0.468	0.041
Taurine	642.0	817.6	905.4	987.2	538.1	936.3	426.5	559.6	146.0	0.050	0.074	0.717
1-Methylhistidine	10.5	7.9	9.5	10.0	18.6	13.1	16.7	19.8	2.5	0.005	0.522	0.366
Serine	170.2	179.1	97.1	122.4	197.1	122.5	398.7	114.1	81.6	0.373	0.178	0.247
Glutamine	625.5	441.0	294.4	414.1	688.8	594.3	367.5	692.3	88.3	0.036	0.517	0.046
Carnosine	0.5	0.9	0.5	1.2	0.5	2.4	0.7	1.9	0.3	0.113	0.000	0.131
Arginine	135.0	128.0	117.4	168.1	189.5	196.5	167.8	201.8	29.8	0.018	0.759	0.606
Glycine	242.3	205.2	138.9	172.6	272.4	176.3	339.9	198.4	47.6	0.166	0.093	0.319
Anserine	12.7	13.0	6.1	9.7	7.3	11.1	7.0	8.5	4.0	0.573	0.426	0.964
Ethanolamine	27.1	36.3	24.0	33.0	26.6	30.4	32.5	26.5	4.8	0.897	0.253	0.374
Aspartate	18.4	11.8	17.5	22.5	22.8	18.3	57.6	13.6	14.1	0.518	0.227	0.353
Sarcosine	8.6	11.0	7.1	9.2	9.9	7.8	13.7	10.4	2.9	0.582	0.908	0.691

Table 5.8 continued

Amino acid	Diet / Vaccine								Pooled std. error	*Pr* > F[2]		
	Basal		GLN		ARG		ARG + GLN			Diet	Vaccine	Diet *Vaccine
	No	Yes	No	Yes	No	Yes	No	Yes				
Glutamate	61.9	50.2	46.4	65.9	64.3	65.1	52.9	63.6	12.5	0.886	0.595	0.636
Citrulline	2.3	2.6	3.7	4.4	2.6	2.2	6.6	1.7	1.3	0.378	0.270	0.169
β-Alanine	7.0	6.1	11.3	8.2	12.8	9.4	12.4	8.5	1.0	0.002	0.001	0.483
Threonine	164.6	149.8	149.0	166.0	145.0	143.0	155.6	95.3	28.9	0.658	0.474	0.595
Alanine	331.7	308.2	256.8	259.3	401.8	261.5	369.0	244.5	36.3	0.232	0.013	0.165
γ-Aminobutyric acid	15.8	12.4	10.8	24.0	12.9	13.7	32.6	7.2	9.3	0.887	0.583	0.255
Proline	1003.8	557.1	423.7	292.7	426.2	256.2	157.5	271.1	277.9	0.235	0.432	0.796
Hydroxylysine	7.8	5.6	9.8	5.4	5.8	6.1	8.0	6.8	1.6	0.722	0.105	0.538
α-Aminobutyric acid	30.4	23.6	19.9	23.3	29.2	33.9	22.1	20.8	4.3	0.096	0.994	0.553
Cysteine	2.7	6.6	3.6	4.6	4.1	2.7	5.2	1.7	1.3	0.724	0.980	0.050
Ornithine	18.2	33.7	25.0	29.2	65.6	48.7	112.3	57.4	31.8	0.238	0.570	0.704
Cystine	4.4	4.2	3.3	5.5	6.3	5.4	6.6	10.5	2.1	0.183	0.399	0.654
Lysine	195.2	253.6	175.1	228.1	209.2	234.5	204.1	206.5	37.8	0.905	0.211	0.870
Tyrosine	39.7	62.6	41.5	51.0	45.6	50.2	61.9	49.5	10.9	0.832	0.437	0.468
Methionine	11.6	15.5	17.5	33.1	14.5	23.3	10.1	11.2	5.1	0.050	0.050	0.527
Valine	263.1	276.0	184.4	259.8	217.6	273.3	221.1	221.0	29.9	0.347	0.108	0.563

Table 5.8 continued

Amino acid	Diet / Vaccine								Pooled std. error	*Pr* > F[2]		
	Basal		GLN		ARG		ARG + GLN			Diet	Vaccine	Diet *Vaccine
	No	Yes	No	Yes	No	Yes	No	Yes				
Isoleucine	170.1	196.8	106.1	169.7	133.3	178.1	135.3	133.6	30.7	0.392	0.144	0.747
Leucine	298.7	320.6	209.4	313.0	237.9	327.8	253.2	251.8	40.0	0.506	0.077	0.503
Homocysteine	11.9	7.3	6.2	10.2	8.0	9.1	10.8	11.9	3.5	0.807	0.879	0.671
Phenylalanine	113.1	116.2	101.9	115.4	115.7	106.8	127.0	106.9	15.1	0.948	0.775	0.711
Tryptophan	37.7	43.7	32.1	41.6	41.6	44.7	43.4	40.9	6.0	0.734	0.359	0.785

[1] Values represent the mean of six randomly sampled fish.
[2] Significance probability associated with the F-statistic.

ARG-supplementation (with/without GLN) increased the free ornithine pool by 39- and 37-fold, respectively; in turn, the glutamate pool was 2.2 and 1.9 higher. The total free serine pool was 5.8-fold and 4.3-fold higher in fish fed the basal diet as compared to fish fed the ARG or the ARG + GLN diets, respectively. Lastly, MH was significantly higher in fish fed the ARG + GLN diet.

At 7 dpv (Table 5.10), the total muscle-free amino acid pool was affected by diet or vaccination but with no significant interaction of factors. Fish fed the ARG or GLN diets had significantly higher total free pool than fish fed the basal diet while fish fed ARG + GLN did not differ from either treatment. Vaccinated fish had a higher muscle-free amino acid pool than non-vaccinated. Arginine total free pool remained unaffected by any of the factors evaluated, although higher numerical values were observed in fish fed the ARG diets. In contrast, free citrulline pool was affected by dietary treatment with fish fed the basal diet having significantly lower citrulline levels than fish from any other treatment. Muscle-free ornithine was higher in fish fed ARG and ARG + GLN diets, but not in fish fed the GLN diet. Other diet-affected amino acids were hydroxyproline, which was higher in fish fed the ARG-supplemented diet; taurine, glycine and alanine which were higher in fish fed ARG- and GLN-supplemented diets; β-alanine, GABA and AABA which were lower in fish fed the basal diet; and cysteine, which was higher in fish fed the GLN-supplemented and basal diets. Vaccinated fish had significantly higher taurine, MH, carnosine, EA, GABA, and ornithine. Interaction diet * vaccination was found in fish fed the ARG diet, for which vaccinated and non-

Table 5.9
Muscle free-pool amino acid profiles (nmol · wet muscle weight^{-1}) pre-vaccination with Aquavac-ESC®[1]

	Diet**				Pooled std. error	*Pr* > F[2]
Amino acid	**Basal**	**GLN**	**ARG**	**ARG + GLN**		
Hydroxyproline	33167	39738	57821	45062	13768	0.646
Histidine	5972	8804	8357	6026	1871	0.607
Phosphoethanolamine	747	478	770	992	326	0.745
Asparagine	376	1309	4389	3056	1014	0.088
3-Methylhistidine	2017	1067	3285	1035	1060	0.441
Taurine	428365	389296	497889	423688	52086	0.545
1-Methylhistidine	340[3,b]	223[b]	297[b]	685[a]	89	0.026
Serine	30790[a]	22538[ab]	5297[b]	7230[b]	5690	0.036
Glutamine	30946	27447	60094	51001	11304	0.202
Carnosine	1509	1408	2329	1708	416	0.445
Arginine	2904[b]	1149[b]	45095[a]	23676[a]	9571	0.036
Glycine	132159	98115	70545	68871	34488	0.556
Anserine	6499	5529	5921	6449	1653	0.970
Ethanolamine	1593	1427	2956	1316	497	0.147
Aspartate	685	1459	1108	1530	271	0.186
Sarcosine	894	605	617	754	148	0.509
Glutamate	12739[c]	14684[bc]	28205[a]	23775[ab]	2900	0.015
Citrulline	235	280	443	333	179	0.858
β-Alanine	1819	1938	2189	2458	507	0.815
Threonine	9892	12022	12712	5858	2273	0.217
Alanine	63134	69888	77911	79206	14553	0.848
γ-Aminobutyric acid	1716	2530	6558	5043	1683	0.231
Proline	95181	118299	239299	162615	68655	0.502
Hydroxylysine	445	230	394	220	116	0.450
α-Aminobutyric acid	2917	2468	3942	3319	707	0.535
Cysteine	2064	1693	1314	862	419	0.285
Ornithine	787[b]	1355[b]	31036[a]	29382[a]	5750	0.007
Cystine	133	94	156	155	56	0.843
Lysine	12562	10662	30271	13860	6860	0.235
Tyrosine	0	0	0	0	--	--
Methionine	147	108	226	190	75	0.718
Valine	3611	3107	3794	3472	510	0.807
Isoleucine	2439	1873	2231	2284	338	0.692
Leucine	4094	3190	4230	3891	525	0.539
Homocysteine	0	0	0	0	--	--
Phenylalanine	1152	966	1097	1331	92	0.114

Table 5.9 continued

Amino acid	Diet**				Pooled std. error	*Pr* > F[2]
	Basal	GLN	ARG	ARG + GLN		
Tryptophan	426	343	454	485	76	0.6066
SUM	895087	847112	1214062	982658	195491	0.5811

[1] Values represent the mean of six randomly sampled fish.

[2] Significance probability associated with the F-statistic.

[3] Different superscript letters indicate significant ($P < 0.05$) differences as evaluated by Duncan's multiple range test.

Table 5.10
Muscle free-pool amino acid profiles (nmol · wet muscle weight^{-1}) 7 dpv with Aquavac-ESC®[1]

Amino acid	Diet / Vaccine								Pooled std. error	*Pr* > F[2]		
	Basal		GLN		ARG		ARG + GLN			Diet	Vaccine	Diet *Vaccine
	No	Yes	No	Yes	No	Yes	No	Yes				
Hydroxyproline	9426	12397	40593	7855	50669	63352	9515	32463	7468	<0.0001	0.785	0.010
Histidine	6521	8028	13667	11209	12089	11616	6468	9167	2273	0.075	0.845	0.689
Phosphoethanolamine	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	--	--	--	--
Asparagine	2423	3011	10246	986	2615	1886	780	5515	2061	0.393	0.435	0.025
3-Methylhistidine	845	1438	2528	1552	992	2135	1281	1236	500	0.313	0.620	0.217
Taurine	531011	572731	477800	914333	751675	773281	502006	642801	75132	0.037	0.008	0.050
1-Methylhistidine	0	261	0	512	0	257	0	242	82	0.329	<0.0001	0.329
Serine	23244	40375	35133	35523	25183	20176	15945	18084	8251	0.146	0.539	0.589
Glutamine	29247	48232	91262	36308	83930	76868	32562	103408	17472	0.156	0.581	0.018
Carnosine	1756	1952	1354	2866	2651	3347	1827	2970	433	0.089	0.011	0.481
Arginine	3197	2905	5139	8027	6953	41433	16885	25188	9782	0.118	0.121	0.306
Glycine	87816	56336	135925	84907	129066	129681	47878	73475	24285	0.040	0.425	0.428
Anserine	1193	4279	114	6733	6027	5537	7097	5514	1926	0.218	0.180	0.176
Ethanolamine	361	1831	1101	4040	1203	1882	678	1313	579	0.057	0.003	0.201
Aspartate	2543	1784	1294	2670	1529	2324	1298	1531	666	0.711	0.395	0.448
Sarcosine	2223	2092	994	4182	1246	1489	1598	1308	793	0.385	0.199	0.136

Table 5.10 continued

Amino acid	Diet / Vaccine								Pooled std. error	*Pr* > F[2]		
	Basal		GLN		ARG		ARG + GLN			Diet	Vaccine	Diet *Vaccine
	No	Yes	No	Yes	No	Yes	No	Yes				
Glutamate	10727	18263	26015	20636	20774	19703	17862	17184	4157	0.218	0.973	0.491
Citrulline	311	284	1095	1255	1582	1410	1071	1186	159	<0.0001	0.869	0.725
β-Alanine	1782	1898	3343	3863	4017	3592	2460	2868	634	0.025	0.735	0.880
Threonine	8275	13040	18507	14999	13780	12962	8367	10135	2906	0.089	0.792	0.543
Alanine	37788	64104	92583	85242	110718	133435	46132	108850	19661	0.018	0.079	0.392
γ-Aminobutyric acid	2799	2322	2051	7016	4177	5123	1934	4348	817	0.042	0.004	0.026
Proline	47734	31341	91268	21451	65186	125016	38128	93934	26570	0.243	0.701	0.077
Hydroxylysine	898	645	916	1758	777	700	685	516	287	0.091	0.679	0.236
α-Aminobutyric acid	3125	3199	6745	5603	7164	5223	3927	4530	944	0.012	0.381	0.540
Cysteine	4875	2346	4720	4689	1738	2399	2446	694	921	0.014	0.180	0.311
Ornithine	3027	1779	4603	8776	15018	28019	11823	20751	3844	0.001	0.036	0.316
Cystine	126	69	215	259	206	290	177	154	57	0.061	0.769	0.610
Lysine	11537	15356	32294	28281	12696	25331	14495	18793	5981	0.062	0.337	0.596
Tyrosine	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	--	--	--	--
Methionine	539	107	291	530	163	213	177	160	142	0.309	0.696	0.158
Valine	5425	5390	5482	9929	4695	5827	3886	4177	1405	0.108	0.161	0.391

Table 5.10 continued

Amino acid	Diet / Vaccine								Pooled std. error.	*Pr* > F[2]		
	Basal		GLN		ARG		ARG + GLN			Diet	Vaccine	Diet *Vaccine
	No	Yes	No	Yes	No	Yes	No	Yes				
Isoleucine	4138	3504	3592	7619	2563	3565	2575	2301	1431	0.181	0.324	0.380
Leucine	6033	5703	6229	10725	5083	6348	4417	4770	1708	0.179	0.249	0.522
Homocysteine	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	--	--	--	--
Phenylalanine	2512	1946	1750	3775	2366	2025	1904	1738	629	0.533	0.600	0.182
Tryptophan	676	577	547	863	840	790	707	618	163	0.687	0.867	0.540
SUM	855781	931724	1121268	1367302	1350408	1519407	811850	1224876	133281	0.004	0.029	0.641

[1] Values represent the mean of six randomly sampled fish.
[2] Significance probability associated with the F-statistic.

vaccinated groups had higher hydroxyproline free pool than those fed the basal diet, regardless of this group's vaccination status, and than vaccinated GLN-fed fish and non-vaccinated ARG + GLN-fed fish. In addition, non-vaccinated fish fed GLN diet had higher asparagine free pool than all other treatments. Taurine free pool was affected by vaccination status only in fish fed the GLN diet, where vaccinated fish had a 2-fold increase in its muscle-free pool than the non-vaccinated group. Glutamine free pool was changed by vaccination in fish fed the GLN and ARG + GLN diets. In the first group, vaccinated fish had a fourth of the non-vaccinated animals muscle GLN free pool; whereas, ARG + GLN fed fish had a 3-fold increase in the vaccinated group. Also, non-vaccinated fish fed GLN diet had higher GLN free pool than fish fed basal diet with the same vaccination status. Also, the GABA free pool had a similar pattern, with vaccinated fish fed the GLN and ARG + GLN diets having a 3.4- and 2.5-fold increase, respectively, compared to non-vaccinated fish. Proline tended to be higher ($P = 0.0773$) in vaccinated fish as well as in those fed ARG diets.

At 14 dpv (Table 5.11), the sum total of muscle-free amino acid pool was not significantly affected by either factor evaluated. Diet did affect the muscle-free amino acid pool of nine amino acids and had a strong tendency to affecting three more amino acids. In contrast, vaccination did not have noticeable effects on muscle-free amino acid pools, and a significant interaction of both factors was only observed for ornithine. The total muscle-free pool of GLN was significantly higher in fish fed the ARG diet. Histidine values were 2-fold higher in fish fed the GLN diet as compared to the fish fed the basal diet. Fish fed ARG and ARG + GLN diets had twice as much carnosine in the

muscle-free amino acid pool as compared to those fed the basal diet. Furthermore, the same pool for ornithine was significantly higher in fish fed ARG and ARG + GLN diets. Cysteine and tryptophan were higher only in fish fed the ARG + GLN compared to fish fed the basal diet. Aspartate and threonine free pools were higher in fish fed GLN diet. In contrast, citrulline free pool was remarkably lower in fish fed the control diet. Arginine, anserine and GABA free pools tended ($P = 0.06$) to be higher in fish fed the ARG and ARG + GLN-supplemented diet. On the other hand, ornithine was the only amino acid where an interaction between factors was observed. Vaccinated animals fed ARG + GLN had an ornithine muscle-free amino acid pool 4-fold that of non-vaccinated fish. The former treatment had also higher ornithine free pool values than fish fed the basal and GLN diets, despite of their vaccination status. Arginine tended ($P = 0.07$) to be higher in non-vaccinated fish fed the ARG diet. Finally, taurine also tended ($P = 0.09$) to be higher in vaccinated fish fed ARG and ARG + GLN diets.

3.3 Immune parameters

Specific antibodies against *E. ictaluri* were analyzed and presented as ABS (OD at 450 nm). Low ABSs were seen in plasma, bile and intestinal mucus before vaccination with no significant differences among treatments (Table 5.12). In addition, all titres in all non-vaccinated groups remained similar throughout the post-vaccination period and were significantly different from the vaccinated groups. Plasma from fish vaccinated and fish fed the ARG + GLN diet had, at 7 dpv, significantly higher titres

Table 5.11
Muscle free-pool amino acid profiles (nmol · wet muscle weight^{-1}) 14 dpv with Aquavac-ESC®[1]

Amino acid	Diet / Vaccine								Pooled std. error	*Pr* > F[2]		
	Basal		GLN		ARG		ARG + GLN			Diet	Vaccine	Diet *Vaccine
	No	Yes	No	Yes	No	Yes	No	Yes				
Hydroxyproline	21706	28137	43882	55633	57001	82164	101904	65488	30472	0.279	0.937	0.768
Histidine	10655	14435	22969	22976	14766	16076	13716	13598	3505	0.037	0.622	0.939
Phosphoethanolamine	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	--	--	--	--
Asparagine	9711	20362	20835	26626	17294	13563	7313	10196	7884	0.337	0.494	0.831
3-Methylhistidine	1119	1531	2747	2351	2614	1708	1687	2572	549	0.196	0.998	0.390
Taurine	616380	605003	895359	745115	727754	771570	595962	871571	79095	0.103	0.491	0.095
1-Methylhistidine	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	--	--	--	--
Serine	48231	70025	36131	55615	56028	40012	38434	20243	16218	0.360	0.879	0.460
Glutamine	78257	67397	99248	116885	153350	138601	56235	142901	22280	0.034	0.230	0.126
Carnosine	1094	1012	1754	1378	1620	2523	2568	2227	284	0.001	0.897	0.124
Arginine	2951	3792	4777	5558	30072	9171	5802	20535	6214	0.066	0.799	0.073
Glycine	109546	109460	127199	198807	136973	143982	167873	135007	51127	0.752	0.756	0.779
Anserine	3831	2047	5465	6100	7899	8839	4218	7887	1819	0.062	0.511	0.537
Ethanolamine	1475	1781	1019	3153	1198	1859	1934	1475	598	0.803	0.138	0.217
Aspartate	2505	2416	5363	4070	1388	1483	2617	2079	972	0.024	0.516	0.893
Sarcosine	1264	1600	625	1062	694	1543	4378	1441	947	0.172	0.630	0.205

Table 5.11 continued

Amino acid	Diet / Vaccine								Pooled std. error	*Pr* > F[2]		
	Basal		GLN		ARG		ARG + GLN			Diet	Vaccine	Diet *Vaccine
	No	Yes	No	Yes	No	Yes	No	Yes				
Glutamate	26259	19988	27101	24315	20185	25565	21737	21435	4681	0.845	0.768	0.656
Citrulline	816	648	1206	1232	1232	1767	1363	1467	154	0.001	0.270	0.177
β-Alanine	3364	2766	3543	4355	3196	4469	4419	3806	809	0.589	0.708	0.555
Threonine	19149	25885	44361	37239	18522	16333	14860	11579	8252	0.020	0.805	0.859
Alanine	100625	85230	114762	139204	107462	128646	106304	104899	22133	0.463	0.651	0.778
γ-Aminobutyric acid	2280	2779	2521	2948	8339	4167	3303	8019	1597	0.065	0.749	0.089
Proline	56527	156456	208836	182571	113031	141103	68876	126549	64932	0.447	0.398	0.801
Hydroxylysine	1043	1144	1063	1306	725	864	1540	615	282	0.557	0.588	0.175
α-Aminobutyric acid	5557	5248	6519	6478	6752	4936	5070	6897	1268	0.858	0.926	0.569
Cysteine	4204	7750	4515	7150	3215	3164	8036	2436	2627	0.701	0.944	0.332
Ornithine	3199	4825	8422	3475	19202	13731	3892	22968	3762	0.013	0.348	0.016
Cystine	160	240	291	225	256	191	484	410	87	0.047	0.623	0.784
Lysine	9425	18165	22029	17469	14063	12534	10482	11863	6009	0.539	0.816	0.721
Tyrosine	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	--	--	--	--
Methionine	82	208	163	372	332	291	880	308	254	0.370	0.706	0.438
Valine	6299	5790	4463	6651	5028	6774	8612	5769	1584	0.755	0.898	0.390

Table 5.11 continued

Amino acid	Diet / Vaccine								Pooled std. error	*Pr* > F[2]		
	Basal		GLN		ARG		ARG + GLN			Diet	Vaccine	Diet *Vaccine
	No	Yes	No	Yes	No	Yes	No	Yes				
Isoleucine	4215	3873	2298	4486	2707	3754	7355	3188	1865	0.691	0.812	0.378
Leucine	6990	6174	4938	7490	5022	7188	10174	6182	1912	0.685	0.987	0.319
Homocysteine	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	--	--	--	--
Phenylalanine	2069	1609	1813	2172	2040	2215	4013	2196	741	0.345	0.418	0.470
Tryptophan	569	407	549	675	633	943	996	862	155	0.046	0.760	0.399
SUM	1165258	1282892	1731439	1699539	1543723	1614294	1291675	1641312	235248	0.237	0.458	0.871

[1] Values represent the mean of six randomly sampled fish.
[2] Significance probability associated with the F-statistic.

than plasma from vaccinated fish fed the basal, ARG or GLN diets. In contrast, at 14 dpv, titres from fish fed the GLN, ARG and ARG + GLN were significantly different from those fed the basal diet. After exposure to *E. ictaluri*, no interaction of factors was observed at 3 or 14 dpi. However, titers in the vaccinated groups were significantly higher than in naïve fish being exposed for the first time to the bacterium. Also, fish fed the basal diet had lower antibody titers than fish fed all other dietary treatments. Due to the progressing mortality, at 14 dpi only antibody titers from fish fed the ARG and GLN diets were secured, but, antibody levels at this time remained similar to those collected from fish at 3 dpi. No interactions between diet and vaccination were obtained among treatments, and vaccinated fish had higher titers than non-vaccinated fish. In addition, fish fed the ARG diet had higher titers than the GLN group.

Intestinal mucus titers, pv and pi, were different between vaccinated and non-vaccinated groups, always being significantly higher in vaccinated fish (Table 5.12). Titers from 7 dpv to 14 dpv decreased in most cases; however, they had ~3-fold increase after 14 dpi. A diet-realted tendency ($P = 0.07$) was observed in mucus antibody titers at 7 dpv and 14 dpi, with fish fed ARG + GLN or GLN diets displaying numerically higher values, although these were not significantly different. At day 7, vaccinated fish that were fed the ARG diet had the lowest mucus antibody titer. In contrast, at 14 dpi, vaccinated fish fed the GLN diet had the highest titer concentration.

Titers from bile and intestinal mucosa shared a common pattern (Table 5.12). At 7 dpv, fish fed GLN-supplemented diets had numerically higher bile titers. However,

Table 5.12
Plasma, gut mucus and bile antibody titers (ABS at 450 nm) pre- and post-vaccination with Aquavac-ESC®[1]

	Pre-vaccine			Factor		Plasma				Gut mucus			Bile		
Diet	**Plasma**	**Gut mucus**	**Bile**	**Diet**	**Vaccination**	**7dpv**	**14dpv**	**3dpi**	**14dpi**	**7dpv**	**14dpv**	**14dpi**	**7dpv**	**14dpv**	**14dpi**
Basal	0.215	0.085	0.153	**Basal**	**Yes**	0.515	0.539	0.639	n.d.	0.123	0.116	n.d.	0.337	0.591	n.d.
					No	0.162	0.216	0.262	n.d.	0.079	0.076	n.d.	0.183	0.231	n.d.
GLN	0.265	0.077	0.233	**GLN**	**Yes**	0.556	0.627	0.688	1.369	0.117	0.109	0.659	0.422	0.768	0.861
					No	0.235	0.212	0.404	0.662	0.08	0.075	0.409	0.260	0.240	0.638
ARG	0.298	0.083	0.264	**ARG**	**Yes**	0.512	0.652	0.633	1.597	0.106	0.109	0.487	0.303	0.643	0.972
					No	0.216	0.202	0.484	0.748	0.089	0.078	0.436	0.234	0.228	0.817
ARG + GLN	0.29	0.078	0.264	**ARG + GLN**	**Yes**	0.647	0.687	0.714	n.d.	0.127	0.100	n.d.	0.472	0.597	n.d.
					No	0.183	0.175	0.349	n.d.	0.083	0.078	n.d.	0.217	0.211	n.d.
Pooled std. error	0.061	0.004	0.06	**Pooled std. error**		0.042	0.041	0.054	0.186	0.005	0.006	0.045	0.059	0.167	0.082
				***Pr* > F**[2]											
***Pr* > F**	0.533	0.411	0.32	**Diet**		<0.0001	0.044	0.0363	0.013	0.072	0.193	0.067	0.076	0.738	0.015
				Vaccination		<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.008	<0.0001	0.0002	0.002
				Diet*Vaccination		0.0003	0.0102	0.2366	0.877	0.001	0.209	0.031	0.188	0.722	0.513

[1] Values represent the mean of six randomly sampled fish.
[2] Significance probability associated with the F-statistic.

only fish fed the ARG diet had significantly higher bile titers than fish fed the GLN diet at 14 dpi. No other differences were observed.

Flow cytometry results (Table 5.13) showed that the proportion of IgM^+ cells in peripheral blood lymphocytes (PBL) tended to be higher at 7 dpv and 14 dpv in vaccinated fish but no significant differences were found. In contrast, at 7 dpv dietary GLN increased the IgM^+ spleen cell population significantly augmenting their relative number by 63 and 38% (for fish fed GLN and ARG + GLN diets, respectively) as compared to cells from fish fed the basal diet. On the contrary, at 14 dpv, fish fed the basal diet had the highest number of IgM^+ cells in spleen. Also, vaccinated fish had a higher percent of these cells. Further analysis indicated that, with the exception of basal group, cell populations were significantly different ($P < 0.05$) between vaccinated and non-vaccinated fish, within the same dietary treatment. Individually, both GLN and ARG groups had higher percent of B-lymphocytes in vaccinated fish; whereas, ARG + GLN had the opposite effect. As for B-lymphocytes from the head kidney, fish fed the GLN diet had higher relative numbers than the rest of the treatments. Also, vaccination raised the proportion of these cells by 29%. In this case, all groups were different between vaccinated and non-vaccinated within the same dietary treatment. In all cases except for fish fed ARG + GLN, vaccination increased the population of B-cells. A vaccine-GLN synergy was displayed by an increased proportion of B-cell, followed by a similar interaction between vaccination and ARG.

When the responsiveness of leukocytes to *E. ictaluri* was evaluated (Table 5.13), vaccinated fish had significantly higher SI than non-vaccinated fish in all cases. In

PBLs, this was the only difference found at both, 7 and 14 dpv, although vaccinated fish fed the ARG and ARG + GLN diets had a tendency for higher numerical values. In contrast, at 14 dpv, spleen and head kidney derived leukocyte responsiveness was affected by both diet and vaccination. Vaccinated fish fed the ARG or GLN diets had significantly higher SI than fish fed the basal or the ARG + GLN diets.

3.4 Disease challenge

The cumulative mortality after *E. ictaluri* challenge is showed in Fig. 5.1. At 84 h pi (3.5 days), significant differences were found based on diet, vaccine status and interactions between both. Vaccinated fish had lower mortality than non-vaccinated fish. Within the vaccinated fish, those fed ARG or GLN diets had lower mortality (65% for both) than fish fed the ARG + GLN diet (85%). The highest mortality was observed in non-vaccinated fish fed the control diet (90%). Moreover, at 96 h pi (4 days), non-vaccinated fish fed the control diet and both groups of fish fed the ARG + GLN diet reached 100% mortality. It was not until 192 h pi (8 days) that the vaccinated fish fed the basal diet reached 100% mortality. At this point, vaccinated fish fed the ARG or GLN diet had 85 and 90% mortality, respectively; whereas, non-vaccinated fish fed these two diets had 95% mortality.

4. Discussion

Growth parameters before vaccination were as expected, namely, ARG supplemented diets had the best performance. The inclusion of GLN to the basal diet

Table 5.13
Cell immune response post-vaccination with Aquavac-ESC®[1]

Factor		IgM$^+$ cells population (%)[2]					Leukocyte responsiveness (SI)[3]			
		7 dpv		14 dpv			7 dpv	14 dpv		
Diet	**Vaccination**	**PBLs[4]**	**Spleen**	**PBLs**	**Spleen**	**HK[5]**	**PBLs**	**PBLs**	**Spleen**	**HK**
Basal	**Yes**	20.0	17.8	46.6	35.0	28.1	1.59	1.84	1.92	1.30
	No	18.1	17.1	24.0	32.3	22.9	0.94	0.98	1.04	1.02
GLN	**Yes**	28.6	29.3	51.7	33.7	40.6	1.78	1.81	3.15	2.34
	No	10.5	25.8	37.7	26.9	24.9	0.91	1.01	0.95	0.89
ARG	**Yes**	21.7	15.5	45.4	33.1	29.4	1.76	2.49	4.43	2.37
	No	9.1	18.2	51.0	23.7	18.8	0.91	0.99	0.91	1.04
ARG + GLN	**Yes**	20.8	17.4	45.1	26.7	24.3	2.28	2.52	2.25	1.62
	No	18.1	29.2	41.5	33.3	27.8	0.97	0.98	1.05	0.91
Pooled std. error		11.3	3.6	17.8	2.3	0.8	0.52	0.26	2.95	0.21
***Pr* > F[6]**										
Diet		0.936	0.002	0.773	0.029	<0.0001	0.194	0.809	0.637	0.0067
Vaccination		0.138	0.167	0.346	0.015	<0.0001	<0.0001	<0.0001	0.025	<0.0001
Diet*Vaccination		0.725	0.04	0.703	0.001	<0.0001	0.486	0.256	0.004	0.0003

[1] Values represent the mean of three randomly sampled fish.
[2] %, relative numbers of cells in 10,000 events.
[3] SI, stimulation index = counts per minute stimulated cells / counts per minute control cells.
[4] PBLs, peripheral blood lymphocytes.
[5] HK, head kidney.
[6] Significance probability associated with the F-statistic.

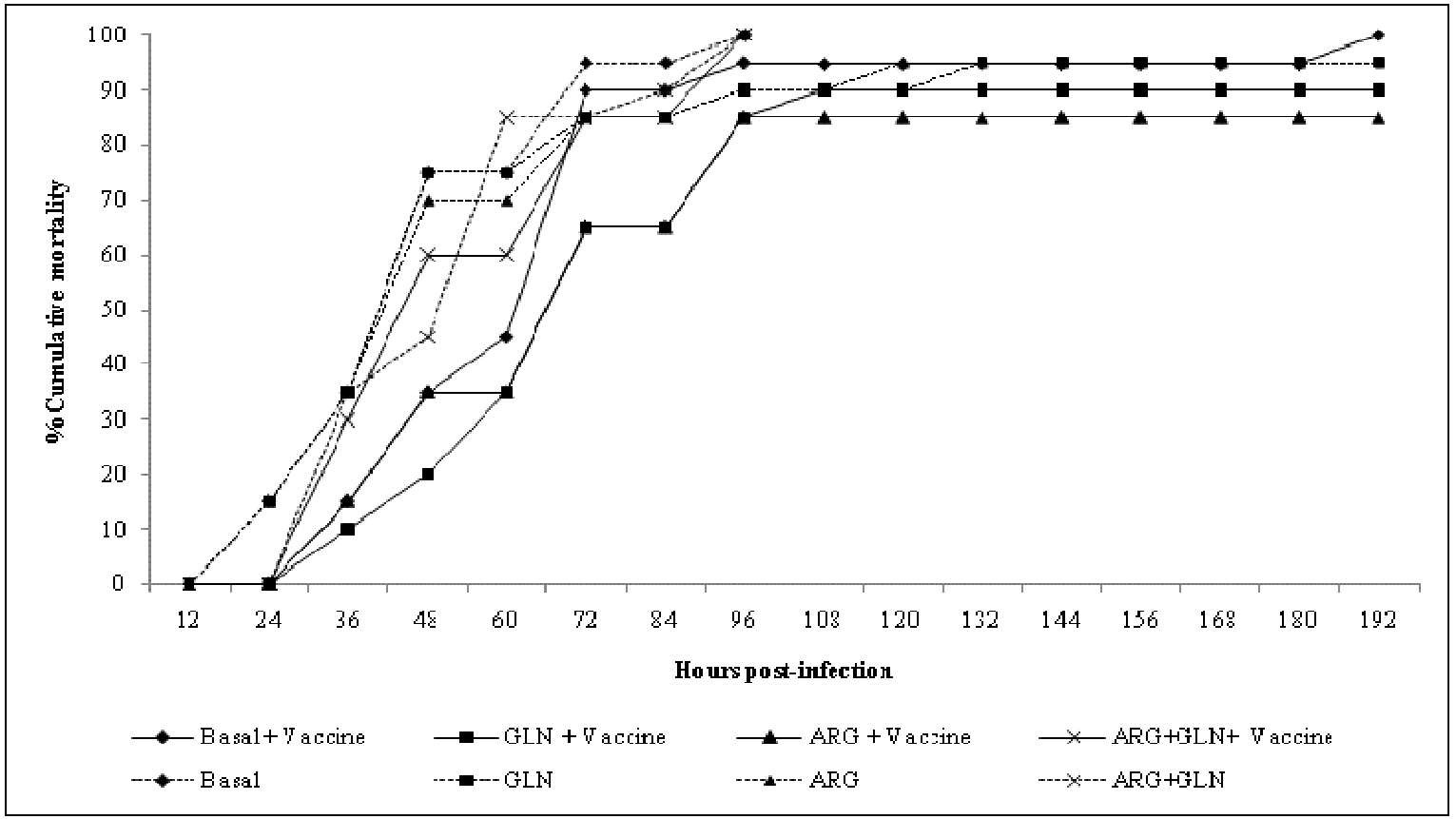

Fig. 5.1. Cumulative mortality of channel catfish by treatment after *E. ictaluri* challenge at 14 dpv. Fish were i.p. injected with 5 x 10^8 bacteria · mL^{-1}. Markers represent the mean percentage mortality of two replicate tanks (n=20). At 84 h pi, vaccinated fish had significantly ($P < 0.05$) lower mortality than non-vaccinated fish; within the vaccinated fish, those fed the ARG and GLN diets had significantly ($P < 0.05$) lower mortality than those fed the ARG + GLN and the basal diets. Non-vaccinated fish fed the basal diet reached 100% at 96 h pi. Vaccinated fish fed the basal diet reached 100% at 194 h pi.

did not have any positive effects on growth, nor did its co-inclusion with ARG. These results are in agreement with previous reports with fish (Plisetskaya et al., 1991; Fournier et al., 2002; Oehme et al., 2010). All these parameters had the same trend after vaccination, where fish fed ARG and ARG + GLN diets performed better than fish fed the control and GLN diets. However, there was a notable decrease in WG, FE and PER for fish fed the basal diet after vaccination. All parameter levels were ~50% lower than those from non-vaccinated fish. This is in accord with previous reports of side effects of vaccination on other species. For instance, Ronsholdt and McLean (1999) and Midtlyng and Lillehaug (1998) reported decreases of 8% and 23% in weight gain for rainbow trout (*Ochorynchus mykiss*) and Atlantic salmon (*Salmo salar*), respectively, vaccinated against furunculosis. These results, however, contrast with field reports for Aquavac-ESC® (Carrias et al., 2008; Shoemaker et al., 2009), which indicate no adverse effects on larger fingerlings and improved FE. Differences may be explained by the latter reports being based on long-term period, longer than the 7 dpv reported herein, which was the interval at which significant differences in growth occurred in the present experiment. Because parameters tended to revert to normal at 14 dpv, it would be logical to expect a similar outcome had our experiment continue for a longer term.

In the present work, significant differences in growth were found only at 7 dpv, compared to 14 or 21 dpv. The finding that ARG and/or GLN supplementation ameliorates adverse growth effects supporting similar performance for vaccinated and non-vaccinated fish may be of importance to the producer. More so, as this supplementation also enhanced WG when comparing fish that were both vaccinated and

fed the supplemented diets to those vaccinated and fed the basal diet. The present results constitute the first report on the interaction between ARG and/or GLN supplementation and vaccination in fish and are very much in line with previous studies using other animal models which document similar responses in vaccinated bovine calves (Fligger et al., 1997) and broiler chicken (Yi et al., 2005) upon ARG and GLN supplementation, respectively.

Organosomatic indexes at the pre-vaccine stage had variable responses. Condition factor, FY and RIL were significantly higher in fish fed ARG, GLN or ARG + GLN diet. Accordingly, Yan and Qiu-Zhou (2006) found that RIL increased in common carp (*Cyprinus carpio*) fed graded levels of GLN after 80 days. In contrast, previous experiments in our laboratory with channel catfish failed to prove this effect after 70 days (Chapter III). In that experiment, a diet supplemented with the same GLN level used in the current trial had a tendency to produce higher RIL values than in fish fed the basal diet, but no significant differences were found. Interestingly, in the present experiment, feeding the fish for only 14 days (instead of 70) brought about significant differences ($P < 0.05$) in RIL between fish fed diets supplemented with GLN and those fed the basal diet. In contrast, Oehme et al. (2010) found that supplementing ARG and glutamate to the diet of Atlantic salmon increased the RIW, although these authors did not measure RIL.

During the post-vaccination period, similar dietary effects were observed for CF and RIL at 7 dpv but not at 14 dpv, where vaccination did not have an effect on these parameters. Results on fillet yield after vaccination diverged from those obtained during

the pre-vaccine period. Immunized fish which were also fed the supplemented diets had higher FY than the non-vaccinated groups. Interestingly, vaccinated fish fed the ARG diet had the highest FY. These findings may explain in part differences in growth performance described above. However, it is still puzzling that for fish fed the basal diet there were no differences in FY between vaccinated and non-vaccinated animals. These results appear to indicate that in vaccinated fish an ARG and GLN-supported mechanism exists which prevents the loss of muscle tissue in fish fed supplemented diets. That this mechanism is absent, or rather, that essential materials are missing (e.g., ARG and GLN) in fish fed the basal diet is evidenced by the observed reduced growth in this latter group upon vaccination. It is well established in metabolic studies with channel catfish that injection with lypopolysaccharide (LPS) transiently increases the abundance of the mRNA of MyoD, a muscle regulating factor, and decreases the abundance of the mRNA of myostatin, a negative regulator of skeletal muscle mass (Weber et al., 2005). Because the cell wall of gram-negative bacteria, including *E. ictaluri,* is composed in part of LPS, its presence may help explain the anabolic response observed in vaccinated fish. Weber et al. (2005) also suggest the involvement of nuclear factor kappa B (NF-kB) in the regulation of these two muscle factors in channel catfish. This mechanism, in combination with ARG action down-regulating NF-kB activity in muscle fibers (Hnia et al., 2008), may also help explain the increased FY in vaccinated fish fed supplemented diets.

In the present experiment muscle relative weight associated well with RKW at 7 dpv. Hence, the participation of skeletal muscle in the vaccination-triggered immune

response may be also possible as reported for muscle tissue from mammals (Marino et al., 2011) and fish (Purcell et al., 2006). The head and trunk kidneys are known to be key immune organs for the response to *E. ictaluri* infection (Russo et al., 2009). In the present study, RKW at 7 dpv were affected by supplementing GLN to the diet. Also, vaccinated fish had significantly heavier kidneys than non-vaccinated fish fed this diet. Due to the reported roles of GLN on cells of the immune system as both metabolic fuel and proliferative compound (Newsholme, 2001) the significant ($P < 0.05$) increase in renal weight may reflect an increased cellularity of these organs. Similarly, the spleen also displayed increased weight upon GLN supplementation, although this increase was not significantly different from that of fish fed the basal diet. Vaccination significantly ($P < 0.05$) increased RSW and RKW at 14 dpv. However, diet did not significantly affect either parameter. Consistent with these findings, Ronsholdt and McLean (1999) found an increased RSW at 7 weeks after inoculating Atlantic salmon with *Aeromonas salmonicida*; however, they did not find any increase in RKW. More recently, Harun et al. (2011) found an increased RSW in rainbow trout exposed to *Yersinia ruckeri*. In addition, Tayade et al. (2006a) and Ruiz-Feria and Abdukalykova (2009) found augmentation in spleen and other immune organ weights between vaccinated and non-vaccinated chicks, but they did not find differences among vaccinated chicks fed a control or ARG-supplemented diet.

On the other hand, protein retention by specific tissues further supports the preceding ideas. Muscle had higher protein retention at 7 dpv in fish fed the supplemented diets, compared to the basal group. Also, vaccinated fish had higher PR

than non-vaccinated fish, which would be expected if MyoD is up-regulated (Weber et al., 2005). Similarly, kidney had higher PR at 7 and 14 dpv, and at the latter time point, vaccinated fish had higher PR than non-vaccinated fish. Spleen PR was only significant at 7 dpv and was affected only by diet.

Metabolism in all these organs is presumably changed during immune responses or disease conditions, such as with *E. ictaluri* infection (Booth and Bilodeau-Bourgeois, 2009). In this sense, Pridgeon et al. (2010) recently showed that, from numerous important up-regulated genes in head kidney of channel catfish vaccinated with Aquavac-ESC®, 21% were related to organ metabolism (including protein); whereas, 28% to the immune response. Consistently, 16% and 19% of up-regulated genes in muscle were related to metabolism and immune response, respectively, in rainbow trout vaccinated against hematopoietic necrosis virus (Purcell et al., 2006). Although gene expression does not necessarily reflect actual protein levels, it could be considered an appropriate correlate. Altogether, it is apparent that the supplementation of amino acids played an important role in catfish immune organs, and possibly in muscle, in the response to *E. ictaluri* vaccination; however, it seemed that muscle participation was limited to 7 dpv, whereas spleen and kidney responses continued up to 14 dpv, time frame consistent with previous experiments using this vaccine (Shoemaker et al., 2009).

It is well known that during immune responses or pathological conditions there is significant mobilization of GLN and ARG from the skeletal muscle and plasma of mammals (Wu, 2010) and fish (Walker et al., 1996; Buentello and Gatlin, 2001b). Thus these two compounds might be considered as immuno-essential. Plasma amino acid

pools depend in great part on other tissue free pool. Because of its mass, skeletal muscle has the highest amino acid pool in a living organism, playing a crucial role in their homeostasis (Ballantyne, 2001). In channel catfish, muscle accounts for 70 and 30-60% of the whole-body total pool for ARG and GLN, respectively (Wilson and Poe, 1974), thus it becomes a key organ to maintain a proper supply of these amino acids during adverse conditions (Newsholme, 2001; Salem et al., 2010). In line with these previous reports, in the present study amino acid profiles were distinct among treatments throughout the trial. The proposed rationale for supplementing ARG and GLN was to create and maintain an ideal nutrient microenvironment, at least for these two amino acids, to enable an enhanced immune response to new antigens. Evidence showed here that feeding fish with supplemented levels of ARG did accomplish the proposed goal before vaccination and after vaccination; whereas, supplemented levels of GLN accomplish it only after vaccination.

At the pre-vaccine period, from the seven and five amino acids affected by diet in plasma and muscle, respectively, only four were affected in both tissues. In addition, results demonstrate that feeding channel catfish the diet supplemented with ARG, but not with GLN, increased ARG and ornithine pools in both tissues. The latter correlates with the understanding of circulating levels of plasma amino acids strongly depend on both diet and free pool in tissues (Ballantyne, 2001). In contrast, neither plasma GLN nor glutamate were affected by diet, although the later tended to be higher in fish fed the diet supplemented with ARG + GLN. Interestingly, glutamate was higher in muscle total pool in fish fed diets supplemented with ARG, but not with GLN. The finding of

significantly higher plasma ARG levels in fish fed diets supplemented with ARG concurs with previous experiments in our laboratory (Buentello and Gatlin, 2000, 2001b).

Total free pool amino acid was higher in vaccinated fish than non-vaccinated only at 7 dpv, however, not finding a factor interaction in this variable shows that pools were not different between vaccinated and non-vaccinated fish of the same group of diet fed. Free pools of amino acids depend on diet or proteolysis. The levels of proteolysis in fish muscle vary with metabolic state and are higher under demanding physiological or pathological conditions (Ballantyne, 2001). Methylhistidines are appropriate markers for proteolysis (Nakashima et al., 2008). Besides, 1-MH and anserine may activate the proteolytic protein calpain; whereas, carnosine may increase its inhibition by the activation of calpastatin (Johnson and Hammer, 1989). In the current results, although 3-MH or anserine pools were not affected by either factor at 7 dpv, 1-MH was higher in muscle pool of vaccinated fish. However, along with a raised 1-MH in muscle, an increase of carnosine levels were observed in these groups, and tended to be higher in fish fed supplemented diets. This evidence may indicate an increase in protein turnover in muscle after 7 dpv, what matches with an increased intramuscular free amino acid pool at this time. Nevertheless, net protein lost seems not to be happening, as FY and muscle PR, discussed previously, were higher in vaccinated fish, suggesting a stronger effect of diet on free pool amino acids than proteolysis. This statement is supported by the fact that no distinct pattern of increased levels of 3-MH pools were noticeable as reported for poultry, where plasma and muscle 3-MH increased as a sign of net protein

lost when infected with *Eimeria spp. (Fetterer and Allen, 2000; Fetterer and Augustine, 2001)*. Accordingly, the increase in muscle carnosine pool concurs with the previously discussed idea of vaccination triggering a metabolic response to prevent muscle loss. Consistently, Johansen et al. (2006) found an up-regulation of calpastatin in LPS-injected rainbow trout, while no muscle protein content changes were observed. In addition, Salem et al. (2010) found no substantial increase in any major proteolytic pathways, only relative abundance of calpain in atrophying rainbow trout muscle. Moreover, Bohe et al. (2003) reported that the rates of synthesis of all classes of human muscle proteins are regulated by the blood essential amino acids, where the stimulation of protein synthesis depends on the sensing of the concentration of their extracellular, rather than intramuscular levels. In this sense, the current data showed that the essential amino acids, ARG, threonine, valine, leucine and tryptophan had higher plasma levels at 7 dpv in either or both groups of fish fed the diets supplemented with ARG and GLN; whereas, methionine was higher in vaccinated fish.

As discussed so far, adequate supplies of ARG and GLN gain relevance during changes in metabolic states, but not only to maintain metabolic homeostasis but also to promote an adequate immune response against invading pathogens (Li et al., 2007). For channel catfish, Buentello and Gatlin (2001b) reported a correlation between higher plasma ARG levels before exposure to *E. ictaluri* and survival afterwards. These authors found a significant decrease in plasma ARG and citrulline levels after 24 h of the exposure. In addition, in fish fed supplemental ARG, even when the decrease was of greater magnitude than in fish fed the basal diet, its plasma levels were still supranormal.

Accordingly, in the present experiment, plasma ARG levels tended to decrease in vaccinated fish fed the ARG-supplemented diets at 7 dpv. In contrast citrulline tended to be higher in vaccinated fish at this time period, and within these fish, those fed the diet supplemented with GLN had the highest levels. Interestingly, plasma citrulline levels correlate with its pool in muscle, as well as with the muscle pool of its metabolic predecessor ornithine, which was also higher in vaccinated fish. Regardless of the high levels of plasma ARG in fish fed supplemented diets, its muscle pool remained unchanged, contrasting with findings at the pre-vaccine period. These results suggest that plasma ARG continued to be higher when fish were fed supplemented diets, although it seems the muscle was using this ARG to synthesize ornithine and, supported by the fact that plasma ornithine did not change, tunneled it to synthesize citrulline that served as a supply for plasma. Channel catfish has particularly high levels of muscle arginase and ornithine carbamoyl transferase (OTC), and although levels of carbamoyl phosphate synthetase (CPS)-III are low, it is possible that these enzymes are induced at much higher levels for specific functions (Felskie et al., 1998), such as muscle growth and development (Korte et al., 1997), or in this case the immune response against a vaccine

Interestingly, plasma and muscle glutamine were significantly decreased after vaccination in fish fed the diet supplemented with this amino acid. This suggests that supplementing GLN into the diet may prime the fish to utilize plasma GLN for the synthesis of key molecules. Under a challenging situation, as is the immune response against a vaccine, and in the absence of a surplus of ARG, GLN may be used for

arginine, ornithine and citrulline synthesis (Wu, 2010), which correlates with findings of these amino acids in this fish group. The decrease of GLN under pathological conditions is well documented in mammals (Wu, 2010) and fish (Walker et al., 1996).

Evidence of the amino acid profiles support the idea that supplementing diets with ARG and GLN help to maintain an adequate homeostasis of these amino acids, having a surplus of plasma ARG and citrulline. Because amino acids are delivered to most cells in free form by the blood (Ballantyne, 2001) it would be appropriate to deduce that supra-normal levels of these amino acids would be readily utilize by immune cells, and that should be reflected in an enhanced immune performance against vaccination, because of their immuno-modulating properties (Li et al., 2007; Wu, 2010).

One classic way to assess vaccine effectiveness is by measuring antibody titers or humoral immune responses (Secombes, 2008). The current results showed that vaccination had a positive effect on fish antibody titers in all body fluids where they were measured. In addition, results showed that diet affected antibody titers among vaccinated fish, although the effects depended on body fluid.

Because of their difference in sample processing and analysis, titers values among fluids may not be compared, however, tendencies in each of those fluids are quite representative of diet or vaccine effects, and are appropriate for discussing such effects. In line with the current results are those reports for common carp (Irie et al., 2003), where plasma antibody titers showed more responsiveness to diet and vaccination than intestinal mucus and bile. Fish lack the specific mucosal isotype IgA; to date, in channel catfish IgM seems to be the main functional immunoglobulin against invading pathogens

(Bengten et al., 2006; Edholm et al., 2010). Hence, it is feasible that structural variation within the IgM may offer a means to achieve diverse effector functionality with this single isotype (Ye et al., 2011), making possible its appearance in other tissue fluids besides the blood.

A short synergistic effect was observed at 7 dpv in plasma titers when fish were fed the diet supplemented with ARG + GLN; however, titers among fish fed supplemented diets, although higher than those of fish fed the basal diet, were equal among them at 14dpv. In addition, at 3 dpi, titers of naïve and vaccinated fish fed these supplemented diets were higher than those of fish fed the basal diet. These results suggest that high levels of ARG and GLN in the diet accelerate antibody production, but this acceleration will take longer when feeding ARG or GLN alone. Reports in higher vertebrates demonstrated similar results. Broiler chickens fed supplemental levels up to three times its ARG dietary requirement had highest sera specific antibody titers when vaccinated and challenged against hydropericardium syndrome virus (Munir et al., 2009) and infectious bursal disease virus (Abdukalykova and Ruiz-Feria, 2006; Tayade et al., 2006a; Ruiz-Feria and Abdukalykova, 2009), or inoculated with sheep red blood cells (Ruiz-Feria and Abdukalykova, 2009). Likewise, the production of specific antibodies against *Pseudomonas aeruginosa* significantly increased in mice fed supplemental levels of ARG (Shang et al., 2003). On the other hand, in a separate experiment, mice fed a GLN-supplemented diet and vaccinated against *P. aeruginosa* had a similar response to those fed the ARG-supplemented diet (Yeh et al., 2003). Accordingly, Bartell and Batal

(Bartell and Batal, 2007) found an increase in non-specific IgG and IgA in the serum, intestine and bile of broiler chickens fed a diet supplemented with GLN.

Interestingly, diet had limited effects on specific antibodies in gut mucus and bile during the post-vaccine period. Opposite to findings in plasma titers, fish fed the diet supplemented with ARG had a lower titer in gut mucus than the remaining treatments at 7 dpv. The tendency of higher titers in both fluids in fish fed the diet supplemented with GLN agrees with the above mentioned reference (Bartell and Batal, 2007). The lower titers in gut mucus at 14 dpi than 7 dpi reflects a transient increase of specific antibodies against *E. ictaluri* in this fluid. This effect is somehow expected when considering *E. ictaluri* has been reported to translocate from the intestine to the channel catfish circulation in a short period of time (Thune et al., 1993). However, contrasting are the results found with bile titers suggesting that antibody secretion into bile seems to be more lasting. In higher vertebrate, immunoglobulins are secreted into intestinal mucus by plasma cells residing in liver (through bile) or in gut associated lymphoid tissue-GALT (Jones et al., 1989). However, because channel catfish have low numbers of B-cells in intestinal mucosa (Hebert et al., 2002) this function may be assigned to plasma cells residing in liver, a fact reported for other teleosts (Abelli et al., 2005). The results reported herein for the post-vaccine period, seems to indicate that although there was not an increase in IgM in intestinal mucus after 7 dpv, plasma cells were still active and secreting IgM into the bile at 14 dpv. Also, IgM secretion was readily boosted after re-exposure with the appropriate antigen, as shown in the intestinal mucus and bile titers at

14 dpi (Table 5.12). Nevertheless, the paradoxical effects of ARG or GLN on bile or intestinal titers need further investigation.

To further elucidate the effects of ARG and GLN on immune responses of channel catfish upon vaccination, cellular responses were analyzed. Lymphocytes positive to membrane IgM (IgM^+-cells) correspond to B-cells, which under appropriate signals, evolve to antibody secreting cells (Secombes, 2008). The biological implications of the results presented herein are not fully elucidated and are limited by the fact that we failed to assess the proportion of T-cells. However, insight of modulation of IgM^+-cells are worthy of discussion. The proportion present of IgM^+-cells in peripheral blood tended to be higher in vaccinated fish than non-vaccinated fish at 7 and 14 dpv, but no effects of the diet were observed. In contrast are the findings of Abdukalykova et al. (2008), where feeding high levels of ARG increased the proportion of circulating B-cells and T-cells in vaccinated fish.

It was not until 14 dpv in the present study that the proportion of IgM^+-cells was affected by vaccination in both spleen and head kidney. Noteworthy is that values of these cells in spleen of fish fed the basal diet did not differ between vaccinated and non-vaccinated groups, which was not so in all three groups of fish fed the supplemented diets. A strong relationship was found among immune cell proportions in head kidney and spleen but only in fish fed diets supplemented with GLN or ARG. These observations are in agreement with the common knowledge of the kidney serving as a hematopoietic tissue and spleen as an immunoreactive tissue in channel catfish (Petrie-Hanson and Ainsworth, 2001). Also, in line with current results, Manhart et al. (2001)

reported a high yielding of B and T cell Peyer's patches in mice treated with LPS. Due to the lack of the latter immune tissue in fish, the spleen's function gains increased relevance in pathogen processing and clearance. These results suggest that dietary GLN and ARG supplementation in vaccinated channel catfish modulate B-cell production in the head kidney and further homing to the spleen. Such effects have been reported at least for ARG supplementation in mice (de Jonge et al., 2002).

Also, the current results partially correlate with findings in plasma antibody titres and both SRW and RKW discussed earlier. Higher number of B-cells may result in higher plasma cells, hence higher antibody titres (Petrie-Hanson and Ainsworth, 2001). However, this interpretation is limited in our model because of the fact that vaccinated fish fed the diet supplemented with ARG + GLN also had higher antibody titers, but the IgM^{+}-cell population was lower in these fish than non-vaccinated fish fed the same diet. Further utilization of different lymphocyte surface antigens for cell identification and sorting should give a clearer panorama of this situation. However, to date, the lack of specific antibodies against these cell markers is a limiting factor for the study of lymphocyte subsets in most teleosts.

Memory cells are the most unique and advantageous characteristic of the adaptive immune system. Confronting lymphocytes to a known antigen will trigger proliferation of those cells with specific receptors to that antigen (memory cells). Lymphocyte responsiveness against formalin-killed whole *E. ictaluri* was modulated by dietary ARG and GLN supplementation, but only in lymphocytes residing in spleen and head kidney. Although, PBL proliferation was higher in vaccinated fish, only tendencies

of higher response were observed in fish fed the ARG + GLN diet at 7 dpv and the same group plus those fed the diet supplemented with ARG at 14 dpv. In contrast are the findings of Munir et al. (2009), where vaccinated chicks fed supplemental ARG in the diet had higher PBL proliferation than those fed a control diet. Nonetheless, it is important to state that these authors used the non-specific mitogen concavalin-A to stimulate proliferation, failing to test memory-cell proliferation. However, Tayade et al. (2006a, 2006b), did use a specific antigen to test PBL proliferation finding a positive effect on vaccinated chicks fed supplemental levels of ARG.

In the present experiment, responsiveness of lymphocytes residing in spleen and head kidney was higher in vaccinated fish fed supplemental levels of ARG or GLN but not by feeding both in the same diet. Consistent to these results, Tayade et al. (2006a) found higher proliferation of intestinal lymphocytes when chicks were fed a diet supplemented with ARG. Interestingly, results herein showed that spleen lymphocyte proliferation was higher than that of the head kidney, which supports the previously mentioned immune functions of these two tissues (Petrie-Hanson and Ainsworth, 2001). In regard to dietary GLN supplementation, no reports exist concerning vaccination and lymphocytes responsiveness. However, supplementation of dietary GLN did enhance non-specific mitogen proliferation of spleen and PBLs lymphocytes in mice (Kew et al., 1999) and swine (Yoo et al., 1997; Lee et al., 2003).

The present experiment did not have the aim to test the efficacy of the commercial vaccine used, which has been tested elsewhere (Klesius and Shoemaker, 1999; Shoemaker et al., 2009). Yet the main objective was to evaluate if dietary ARG

and/or GLN supplementation increased the efficacy of the immune response of channel catfish to *E. ictaluri* vaccination, which was found to be accurate with the current data. Still, an enhanced immune response against a given pathogen should result in better protection upon re-exposure. Mortality upon *E. ictaluri* challenge was correlated with metabolic and immune findings. The highest survival was observed in vaccinated fish fed diets supplemented with ARG and GLN.

The finding of an increased survival in non-vaccinated fish fed the ARG and GLN supplemented diets compared to those vaccinated and fed the basal diet is intriguing. These results may suggest a lack of protection of the vaccine, since it is not effective against all *E. ictaluri* strains (Shoemaker et al., 2009), but it seems to be more related to the aggressiveness of the infection imposed by i.p. injection, as seen in the overall mortality rate of all treatments (Fig. 4). However, the infection model used appeared sound in the sense that it permitted resolution of differences among treatments. Nonetheless, this evidence suggests that supplementing these two amino acids, by themselves, is sufficient enough to slightly increase the survival against ESC without previous exposure to the etiological agent. These results are in agreement with previous reports from our laboratory (Buentello and Gatlin, 2001b) where channel catfish fed diets supplemented with ARG at 2 and 4% of diet had significantly higher survival than fish fed an ARG-deficient and sufficient diet.

Contrasting results, however, were observed for fish fed the diet supplemented with ARG + GLN which reached 100% mortality, regardless of their vaccination status. Reasons of these effects may not be determined with the current data, because immune

responses in this group had similar trends as the other two groups fed diets supplemented with individual amino acids. Perhaps the pathogenesis of *E. ictaluri* is enhanced by high levels of ARG and GLN or their related compounds. Evidence of the latter was given by Booth et al. (2009) who suggest the utilization of urea to increase intracellular pH to evade killing by phagocytic cells. Interesting, the above-cited research (Buentello and Gatlin, 2001b) also reports slightly less survival in fish fed higher amounts of ARG in diet. Likewise, in the pacific flounder, *Paralichthys olivaceus*, although ARG supplementation benefited some innate immune parameters, when fish were exposed the closely related bacterium *E. tarda*, they had higher mortality rate than the control (Galindo-Villegas et al., 2006).

In conclusion, dietary supplementation with ARG or GLN had beneficial metabolic and immunological effects on channel catfish vaccinated against ESC. Both amino acids while enhancing the immune responses and further protection against *E. ictaluri*, also prevented, and in some cases improved, the early adverse effects of vaccination seen in fish fed the basal diet. Although the evidence from this study is promising as related to vaccinology and immunonutrition fields for aquatic species, more research is needed before recommending supplementing aqua-feeds with both ARG and GLN. This is mainly because of what appeared to be an antagonic effect of feeding high levels of these amino acids at the same time.

CHAPTER VI

CONCLUSIONS

Four separate experiments were conducted with channel catfish to evaluate arginine (ARG) and glutamine (GLN) supplementation in terms of their effects on growth, metabolic and immune performance after vaccination against *Edwardsiella ictaluri*.

In the first experiment, incremental dietary ARG resulted in significant improvements in weight gain (WG), feed efficiency ratio, protein efficiency ratio and protein retention (PR). Fish fed the diet supplemented with ARG at 4% had significantly ($P < 0.0001$) higher values for all performance indicators, although these values were not different from those of fish fed the diet supplemented with ARG at 2% with the exception of PR. Similarly, plasma amino acid concentrations were significantly affected by dietary ARG levels. Fish fed the deficient diet (0.5% ARG) had significantly ($P < 0.05$) lower values for all analyzed amino acids. With the exception of plasma lysozyme, innate immune responses were also significantly affected by dietary ARG. Both superoxide anion production and respiratory burst were significantly ($P < 0.05$) decreased in fish fed the 0.5% ARG diet compared to those fed the ARG-supplemented diets. These results demonstrate that the indispensability of ARG is not only related to optimized protein utilization and growth, but also, the supplementation of this amino acid in fish diets may significantly impact several aspects of the immune system, including phagocyte function, thus improving overall fish health.

In a second experiment, intestinal microstructures were positively affected by dietary GLN with the highest levels of supplementation (2 and 3% of diet) significantly ($P < 0.05$) increasing enterocyte and microvilli height in anterior, mid and posterior intestinal sections. Moreover, a subsequent experiment revealed that dietary GLN supplementation significantly ($P < 0.05$) increased the enterocyte migration rate in all three intestinal segments. Plasma amino acid levels 15 h postprandial showed significantly ($P < 0.05$) higher levels of asparagine, serine, glycine and threonine in fish fed the diet supplemented with GLN at 2%. Despite a consistent trend of higher values seen in fish fed the diet supplemented with GLN at 2%, there were no significant ($P > 0.05$) differences in any growth-related parameters among treatments. Although the present results indicate an efficient utilization of free GLN by intestinal mucosal cells of channel catfish, which resulted in enhancement of the enteric microstructure along with increased migration rates of enterocytes and modified plasma amino acid profiles, improved weight gain at the end of a 10-week feeding trial was not observed.

In a third experiment, an *in vitro* bactericidal assay showed that total free amino acid pool in media decreased 23%, while GLN levels in media decreased by 38% and ARG by 18%. Similarly, in a lymphocyte proliferation assay, a decrease of 45, 52 and 46% was observed for total free amino acids, GLN and ARG pools, respectively. Phagocytosis was significantly ($P < 0.05$) modulated by 0.5 and 1 mM ARG supplementation to the media regardless of GLN supplementation. However, a significant ($P < 0.05$) increase in *E. ictaluri* killing ability was achieved only with 0.5 mM supplementation of ARG. Proliferation of T- and B-lymphocytes was positively (*P*

< 0.05) modulated by supplementing the medium with either or both amino acids; however, a limited synergistic effect was observed. These findings demonstrate that both ARG and GLN are important substrates for fish leukocytes from both branches of the immune system, as illustrated by the utilization rate of these two amino acids and further corroborated by leukocyte performance in supplemented media. This suggests that these two amino acids play an important role in the first response against an invading pathogen by increasing phagocytosis and killing capacity of MØ. In addition, the increased peripheral blood lymphocyte proliferation upon non-specific mitogenic stimulation suggests that high levels of ARG and GLN in fish would be beneficial for the expansion of either T or B lymphocyte subsets, and this would impact the generation of immune memory.

In a fourth and final experiment, feeding channel catfish with diets supplemented with ARG (4%) and GLN (2%) created an appropriate nutritional environment for proper metabolic and immune performance enhancing vaccination efficacy against *E. ictaluri*. Plasma and muscle pools of ARG and related amino acids were significantly ($P < 0.05$) increased in fish fed these diets before and after vaccination. Results suggested that increased pools of key amino acids significantly ($P < 0.05$) ameliorated the decrease of weight gain observed in vaccinated fish fed the basal diet, as well as significantly ($P < 0.05$) increased the fillet yield and kidney relative weight at 7 d post-vaccination (pv). Also at this time point, protein retention in all evaluated tissues was higher ($P < 0.05$) in fish fed the supplemented diets. Both humoral and cellular immune responses were modulated by the addition of ARG and GLN to the diet. Plasma-specific antibodies

titers were elevated ($P < 0.05$) in fish fed supplemented diets as soon as 7 dpv and remained higher throughout the experiment. Similarly, bile-specific antibodies titers were higher in fish fed the diet supplemented with GLN at 7 dpv. B-cell population in spleen was significantly ($P < 0.05$) higher in vaccinated fish fed the GLN diet at 7 dpv. When comparing groups by their vaccination status, fish fed the GLN and ARG diet had higher number of B-cells in spleen and head kidney in vaccinated fish than non-vaccinated fish at 14 dpv. In addition, at 14 dpv, lymphocyte responsiveness against *E. ictaluri* showed a modulation by dietary supplementation of ARG and GLN in cells isolated from spleen and head kidney. Finally, fish survival upon ESC challenge revealed an increased protection in fish vaccinated and fed ARG or GLN in diet, but a lower protection was observed when the diet was supplemented with both amino acids at the same time. In conclusion, diet supplementation with ARG or GLN had beneficial metabolic and immunological effects in channel catfish vaccinated against ESC. Both amino acids were shown to enhance the immune responses of channel catfish and increase vaccine-related protection against *E. ictaluri*. In addition, ARG and GLN supplementation also prevented some adverse effects caused by vaccination (e.g., decreased WG) which were evident in fish fed the basal diet. Results from the present experiments are quite promising from the vaccinology and immunonutrition perspectives. However, more research is needed before recommending dietary supplementation of aquafeeds because of the potentially antagonic effects observed while feeding supplemental ARG and GLN at the same time.

Altogether, these results indicate that ARG is a strong alternative to be used to promote growth on both homeostatic and non-homeostatic states such as vaccination or disease. Arginine may also promote improved immunity upon vaccination, increasing its efficacy and hence fish survival. Likewise, GLN supplementation may have similar effects; however, such effects seem to be accentuated when fish are in a non-homeostatic state, as seen in fish responses after vaccination, rather than in an interior milieu, as seen during the GLN feeding trial. Further research is needed for the elucidation of other roles of these two amino acids on specific physiological effects in fish, such as activation of synthetic pathways, endocrine system modulation or even a possible synergism in the pathogenesis of *E. ictaluri*.

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