RAQUEL TATIANE PEREIRA

NILE TILAPIA (*Oreochromis niloticus*): ENTEROENDOCRINE PEPTIDES AND FUNCTIONAL AMINO ACIDS

LAVRAS – MG

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Thesis submitted in fulfillment of the requirements for the degree of Doctor of Animal Science, Areas of concentration: Production and Nutrition of Non Ruminant Animals at Federal University of Lavras, Brazil.

Advisor and co-advisor

Prof Dr. Priscila Vieira Rosa

Prof Dr. Delbert M. Gatlin III

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Dedicated to the amazing adventure of a lifetime

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Phillip D. Adams, Class of 1970

“The mind that opens up to a new idea never returns to its original size”

Albert Einstein

“No llores porque se terminó, sonríe porque sucedió”

Gabriel García Márquez

ABSTRACT

The present thesis provides research findings in two different approaches. The first study analyzed by immunohistochemistry the distribution of ECs producing gastrin (GAS), cholecystokinin-8 (CCK-8), neuropeptide Y (NPY), and calcitonin gene-related peptide (CGRP) along the gut of Nile tilapia. In addition, we assessed the effects of fasting and feeding in the distribution of immunoreactive ECs. Depending on the peptide produced and anatomical location, ECs were seen more concentrated in some segments while in other intestinal portions ECs were sparse. Regarding nutrient status, we found that the anterior segments of the midgut seem to be the main site which respond to luminal changes in Nile tilapia. The second study investigated the effects of dietary supplementation of glutamine and arginine for Nile tilapia. The fish were fed the six experimental diets formulated to contain different levels of supplemental Gln and/or Arg (Control, GLN 1%, GLN 2%, ARG 1%, ARG 2% and GLN+ARG 1%) for a nine weeks period. Growth performance, innate immune responses, and amino acids profile in plasma and whole-body were examined. Dietary Gln and/or Arg supplementation resulted in significant effects on weight gain, feed intake, feed efficiency ratio, protein efficiency ratio and protein retention. Moreover, the concentration of free AA in plasma at 6 h and 18 h were significantly affected by experimental diets.

Keywords: Nutrition. Peptides. Intestine. Glutamine. Arginine. Fish.

RESUMO

A presente tese fornece resultados de pesquisa em duas abordagens diferentes. O primeiro estudo analisou, por imunohistoquímica, a distribuição de células endócrinas (CEs) produtoras de gastrina (GAS), colecistoquinina (CCK-8), neuropeptídeo Y (NPY) e peptídeo relacionado ao gene da calcitonina (CGRP) ao longo do intestino da tilápia do Nilo. Adicionalmente, os efeitos do jejum e alimentação na densidade de CEs foram analisados. Dependendo do peptídeo produzido e segmento intestinal amostrado, as CEs foram mais concentradas em certos segmentos e mais escassas em outros. Em relação ao status de nutriente, nossos resultados mostraram que o início do intestino médio parece ser o principal local que responde a mudanças luminais na tilápia do Nilo. O segundo estudo investigou os efeitos da suplementação de glutamina (Gln) e arginina (Arg) em dietas para tilápia do Nilo. Os peixes foram alimentados com seis dietas experimentais formuladas para conter diferentes níveis de Gln e/ou Arg (Control, GLN 1%, GLN 2%, ARG 2% e GLN + ARG 1%) por nove semanas. Desempenho, resposta imune inata e perfil de aminoácidos no plasma e no corpo inteiro foram examinados. A suplementação dietética de Gln e/ou Arg resultou em efeitos significativos no ganho de peso, consumo, eficiência alimentar, eficiência e retenção protéica. Além disso, a concentração de aminoácidos livres no plasma as 6 h e 18 h foram significativamente afetada pelas dietas experimentais.

Palavras-chave: Nutrição. Peptídeos. Intestino. Glutamina. Arginina. Peixes.

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LIST OF ABBREVIATIONS

DNES	Diffuse neuroendocrine system
GAS	Gastrin
CCK-8	Cholecystokinin-8
NPY	Neuropeptide Y
CGRP	Calcitonin gene-related peptide
ECs	Endocrine cells
+ECs mm^{-2}	Immunoreactive endocrine cells per mm^2 of mucosal epithelium
S1 – S9	First to ninth segment of intestine
AA	Amino acid
DAA	Dispensable amino acid
IAA	Indispensable amino acid
ARG	Arginine in experimental diet
Arg	Arginine
GLN	Glutamine in experimental diet
Gln	Glutamine
SEC	Superoxide anion extracellular
SIC	Superoxide anion intracellular
NBT	Nitro blue tetrazolium
LYZ–P	Plasma lysozyme activity
LYZ–S	Spleen lysozyme activity
HACS	Hemolytic activity of complement system

LIST OF ACRONYMS

FAO	Food and Agriculture Organization of the United Nations
NRC	National Research Council

LIST OF SYMBOLS

°C	Celsius degree
g	Gram
kg	Kilogram
L	Liter
mg	Milligram
cm	Centimeter
mm	Millimeter
mL	Milliliter
µL	Microliter
nmol	Nanomole
mm^{-2}	Square millimeter

SUMMARY

1. OVERVIEW

Nile tilapia was chosen in this research due to its prominent position as cultured fish in Brazil and in the global aquaculture. Nile Tilapia *Oreochromis niloticus* has favorable characteristics already well known to intensive production systems. Robustness, rapid growth, year-round production, and great market acceptance make Nile tilapia the second most important fish in global aquaculture and the most important cultured fish in Brazil accounting for 50% of the national aquaculture production (ASSOCIAÇÃO CULTURAL E EDUCACIONAL DO BRASIL - ACEB, 2014; FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS - FAO, 2016).

The present doctoral thesis provides research findings in two different approaches. The first manuscript discusses the endocrine cells distribution along the intestine of Nile Tilapia and its relation to nutrients status (fasting and fed). While the second manuscript is dedicated to the effects of dietary supplementation of the functional amino acids arginine and glutamine for Nile tilapia.

The aim of the present theoretical framework is not to exhaust the knowledge about these two research subjects, but to provide a basic background information in order to support the understanding about the manuscripts discussed above.

2. THEORETICAL FRAMEWORK

2.1 The diffuse neuroendocrine system: the largest endocrine system

Two major systems are held responsible for the regulation of homeostasis of the human and animal body: the classic endocrine and the neuroendocrine system. Both interact with their target organs or target tissues via secretion of

ubiquitous messenger molecules which can be peptides, amines or steroids. The effects of such messengers have been broadly considered as endocrine, paracrine, neurocrine and autocrine. The majority of the glands and tissues that comprise the endocrine system had been identified by the end of the 19th century. Although they were initially described and studied individually, they were subsequently recognized to comprise an interrelated group of 'ductless glands' which were regarded as the source of 'internal secretions' (METTLER, 1947). In most instances secretion is modulated by a variety of interconnected feedback loops which, in some areas (pituitary, the thyroid, adrenal cortex), are relatively well-defined. In other circumstances, particularly the diffuse neuroendocrine system (DNES) of the liver, heart, kidney and epithelium of the gastrointestinal tract, clarification is still necessary for characterization and definition of the functional elements and their effectors, revised by Modlin et al. (2006).

The DNES comprises several types of gastrointestinal cells that produce and secrete different peptides. These peptides have several effects on the digestive process and feeding behavior (JENSEN, 2001; TONI, 2004). The endocrine cells (ECs) of the gastrointestinal mucosa form the largest endocrine system in the body, not only in terms of cell numbers but also in terms of the different produced substances (AHLMAN; NILSSON, 2001). These cells types can be also called enterochromaffin, neuroendocrine and enteroendocrine along the historical evolution of the study of DNES (MODLIN et al., 2006).

The distribution of ECs in the gastrointestinal tract differs from that of the classical endocrine glands, e.g. islets are spread in the exocrine pancreas while ECs are interspersed between another gut mucosal cells (AHLMAN; NILSSON, 2001). The ECs comprise about 1% of the total epithelial cells in the digestive tract and more than 30 peptides have been identified as produced by these cells (GUTIERREZ-AGUILAR; WOODS, 2011; RINDI et al., 2004; SCHONHOFF; GIEL-MOLONEY; LEITER, 2004). A given ECs secretes one or more hormone

or hormone-like substance, which is released directly into the lamina propria and diffuses into the capillaries (RINDI et al., 2004). The intestinal mucosal cells can also be regarded as a large sensory organ with the complex interaction between neurons, ECs, and the immune system. The enteric neurons system consists of 100 million neurons, the DNES utilizes some 100 identified messengers (among hormones, peptides, and neurotransmitters) and the gut immune system harbour more than 70% of the immune cells in the body (FURNESS; KUNZE; CLERC, 1999). More than 15 different cell types have been identified in mammals (AHLMAN; NILSSON, 2001; HELANDER; FÄNDRIKS, 2012) while in fish, due the complexity and extensive diversity of species, many cells types remain to be described (GROSELL; FARRELL; BRAUNER, 2011).

Even though ECs are numerically scarce, the intestinal DNES form one of the most complex physiological system in the body by integrating endocrine, neural (myenteric and submucosal plexus) and immune system through which regulates gastrointestinal function relative to the secretion of bile and pancreatic enzymes, motility, epithelial renewal, immune response and food intake (GUTIERREZ-AGUILAR; WOODS, 2011; MAY; KAESTNER, 2010; RINDI et al., 2004). This complex system is responsible for nutrient tasting and elicits stimulus-adequate responses in terms of motility and secretion via both intrinsic and extrinsic nervous reflexes. On their mucosal surface gut, ECs have microvilli, which may serve to taste the intraluminal milieu (NEWSON et al., 1982). Figure 1 shows a schematic overview of sensory and secretory functions of ECs of the gut as proposed by Engelstoft et al. (2008).

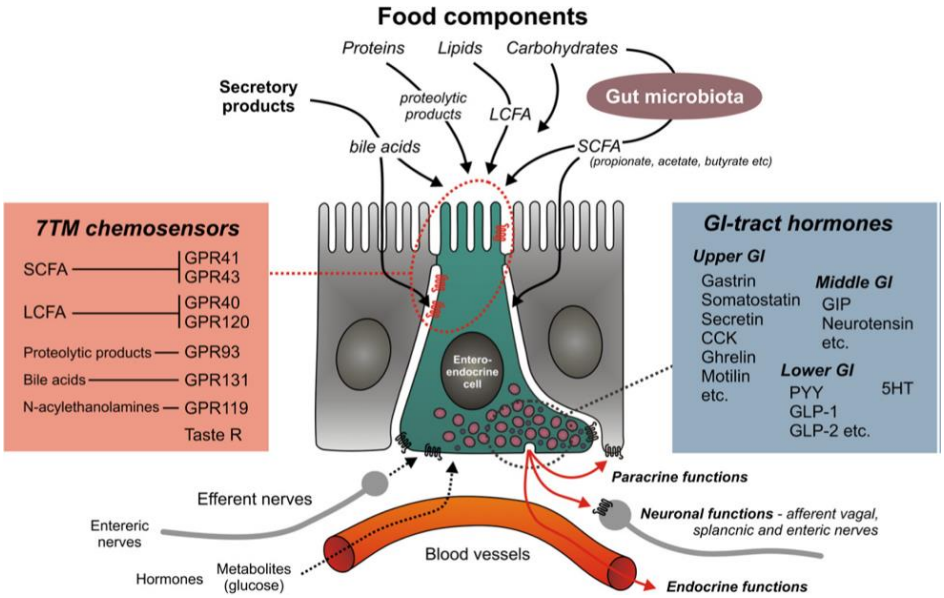

Figure 1 - A schematic overview of sensory and secretory functions of ECs of the gut. Between two enterocytes, a prototypical, conical endocrine cell is shown in green with its microvillus decorated apical pole reaching the gut lumen and with peptide hormone-filled secretory granules at the base. In the blue box to the right are listed a selection of classical gut hormones and with red arrows at the bottom are indicated the three main modes of action of such hormones: paracrine (and autocrine), neuronal, and endocrine functions. At the top are indicated the three main food components that are known to regulate gut hormone expression and secretion (proteins, lipids, and carbohydrates) and some of the main metabolites that are believed to be sensed by the endocrine cells (LCFA, long-chain fatty acids; SCFA, short-chain fatty acids). The gut microbiota is responsible for degrading complex polysaccharides to the main SCFAs: propionate, acetate, and butyrate. Secretory products such as bile acids—but conceivably also other components—from more proximal parts of the GI tract also affect enteroendocrine function. In the red box to the left are listed a number of 7TMG protein-coupled receptors that today are assumed to function as chemosensors in the endocrine cells (GPR131 is still often called TGR5). By small, red serpentine symbols are indicated the presumed location of these chemosensors on the basolateral membrane of the cell, and potentially also at the apical pole. Long black arrows—for simplicity only shown for SCFA and bile acids—indicate that a major site for chemosensing very likely could be the lateral space between the enteroendocrine cell and the enterocytes. Small, black serpentine symbols indicate 7TM receptors for hormones and neurotransmitters (neuropeptides and monoamines) and for the gut hormones themselves (ENGELSTOFT et al., 2008).

Among the peptides secreted by ECs gastrin (GAS), cholecystokinin (CCK-8), calcitonin gene-related peptide (CGRP) and neuropeptide Y were chosen in this study. While GAS and CCK-8 play a role in the stimulation of secretory products associated with digestion of nutrients, CGRP acts on the gastrointestinal motility and, NPY is most potent orexigenic factor known (GROSELL; FARRELL; BRAUNER, 2011).

The biological functions of the GAS and CCK were recognized for over 80 years (EDKINS, 1906; IVY; GOLDBERG, 1928). However, only in 1964, it was possible to isolate and determine the amino acid sequence (GREGORY et al., 1964; GREGORY; TRACY, 1961, 1964; TRACY; GREGORY, 1964). The GAS and CCK belong a family of peptides characterized by a tetrapeptide carboxy-terminal, common to mammals (CHANDRA; LIDDLE, 2007; REHFELD et al., 2007) and in fish (JOHNSEN, 1998; KUROKAWA; SUZUKI; ANDO, 2003).

Physiological functions attributed to GAS include stimulation of the secretion of HCl and pepsinogen in the stomach, contraction of the esophageal sphincter, pyloric sphincter relaxation and increases intestinal motility in mammals (HADLEY; LEVINE, 2006; MEZEY; PALKOVITS, 1992) and fish (GROSELL; FARRELL; BRAUNER, 2011; JENSEN, 2001). Gastrin-34 is the most common form produced by cells of the digestive tract (HADLEY; LEVINE, 2006).

The CCK functions involve gallbladder contraction, pancreatic enzyme secretion, gastrointestinal motility, inhibition of gastric emptying and satiety response in mammals (HADLEY; LEVINE, 2006) and fish (GROSELL; FARRELL; BRAUNER, 2011; JENSEN, 2001). Among biological forms of CCK including CCK-22, CCK-33, CCK-58 and CCK-8, the last one is the most abundant form (MORAN; KINZIG, 2004).

The NPY is a peptide consisting of 36 amino acid residues, which was isolated for the first time, from porcine brain (TATEMOTO, 1982). NPY is a

highly conserved peptide and is the most potent orexigenic factor known in mammals (CHEE; COLMERS, 2008; DUMONT et al., 1992). This peptide is mainly expressed in the hypothalamic neurons and in the ECs of the gastrointestinal tract (MACDONALD; VOLKOFF, 2009; YOKOBORI et al., 2012). NPY regulates feeding behavior and is a potent stimulator of food intake in mammals (CHEE; COLMERS, 2008; DUMONT et al., 1992) and fish (MACDONALD; VOLKOFF, 2009; SILVERSTEIN et al., 1999).

The CGRP is a 37 amino acid peptide originated by differential process tissue-specific (alternative splicing) which mRNAs are transcribed from the same gene of the calcitonin (AMARA et al., 1982; ROSENFELD et al., 1983). This peptide is encoded by different genes expressed in both cerebral and enteric neurons of mammals (AMARA et al., 1982; OGOSHI et al., 2006) and fish (HOLMGREN; OLSSON, 2009; KUROKAWA; SUZUKI; HASHIMOTO, 2003). CGRP mainly function involves inhibiting intestinal motility (SHAHBAZI et al., 1998).

As a consequence of the extraordinary diversity types of peptides and of the modest number of fish species that have actually been examined, our knowledge regarding the DNES of fish remains limited. Moreover, understanding the influence of nutrient status on ECs activity can contribute to clarifying the endocrine regulation in the gut.

2.2 Amino acids in animal nutrition: a paradigm shift

The research findings and approaches of this topic are focused on the newest knowledge obtained mainly from poultry and swine nutrition. Over the last 10 years, new approaches based on the review of biochemistry, metabolism, and nutrition of amino acids have been proposed and it will be considered above.

Amino acids (AA) are building blocks for proteins and must be present in cells for synthesis of polypeptides. AA had been classified traditionally as nutritionally essential or nonessential based on growth or nitrogen (N) balance of animals (WU, 2009). Nutritionally essential AA (EAA) are those AA whose carbon skeletons are not synthesized *de novo* or those AA that usually are not synthesized in adequate amounts to meet the animal's needs and, therefore, must be provided in diets to sustain life. By 1950, nine EAA had been identified for young and adult rats, including histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine, whereas one AA (arginine) was found to be a nutritionally semi-essential AA for young rats because its *de novo* synthesis only partially meets daily requirements for maximum growth (ROSE, 1968). Similar observations were made for swine, chickens, and pre-weaning ruminants between 1938 and 1952 (BAKER, 2005). According to the preceding definition, cysteine and tyrosine, whose carbon skeletons are not synthesized *de novo* in animals, should also be classified as EAA, although they can be formed from methionine and phenylalanine in the liver, respectively (WU, 2013). AA that are synthesized *de novo* in animal cells had been thought previously to be dispensable in diets and, therefore, were considered nutritionally nonessential AA (NEAA) (REEDS, 2000). This category of AA included alanine, arginine, asparagine, aspartate, glutamate, glutamine, glycine, proline, serine, and taurine for adult mammals other than carnivores.

Although N balance measurement is a simple and relatively inexpensive approach to estimate quantitative and qualitative requirements of individual AA by animals (ROSE, 1968), much evidence shows that this classical technique is not sufficiently sensitive to evaluate optimal dietary requirements of all AA and is beset with inherent limitations (REEDS, 2000; RHOADS; WU, 2009). Careful analysis of the literature reveals no compelling evidence for sufficient synthesis of all NEAA in animals to support their optimal growth, health, or production

performance (REEDS, 2000; WU, 2010a, 2010b). In recent years, some NEAA have been classified as conditionally essential because rates of their use are greater than rates of their synthesis under certain conditions (e.g., early weaning, lactation, pregnancy, burns, injury, infection, heat stress, and cold stress) (BAKER, 2005; WU, 2009). Examples of conditionally essential AA (CEAA) include (a) glutamate, glutamine, arginine, proline, glycine, and taurine for weanling piglets; (b) glutamate, glutamine, and taurine for rapidly growing chickens; and (c) glutamate, glutamine, glycine, and taurine for aquatic animals (WU, 2014; WU et al., 2014).

Among the factors affecting the AA synthesis, AA depends on substrate availability, species, developmental stage, physiological status, microbiota in the lumen of the gastrointestinal tract, environmental factors, and pathological states (WU et al., 2013). This illustrates the dynamic nature of dietary AA requirements and the complexity of classifying AA as nutritionally essential or nonessential among farm animals.

2.2.1 Functional amino acids: NEAA and beyond

Owing to technical limitations, analyses of some NEAA (e.g. free glutamine and proline, as well as glutamate, glutamine, and proline in protein) in animal tissues had been a daunting challenge until the 1970s when high-performance liquid chromatography became widely available for AA determination. In addition, research on AA biochemistry was limited primarily to EAA until 1971 when Marliss and co-workers discovered the release of glutamine from human skeletal muscle (MARLISS et al., 1971). Wu and colleagues proposed in 2000 that functional needs for AA beyond nitrogen balance and protein synthesis should be major criteria with which to classify AA as EAA or NEAA in nutrition (WU et al., 2000).

These findings, along with substantial amounts of experimental evidence, led to the new nutritional concept of functional AA, which are defined as those AA that participate in and regulate key metabolic pathways to improve the health, survival, growth, development, lactation, and reproduction of animals (WU, 2010a). This amino acid group encompasses arginine, cysteine, glutamine, glutamate, glycine, leucine, proline, and tryptophan. Metabolic pathways include: (1) intracellular protein turnover (synthesis and degradation) and associated events; (2) cell- and tissue-specific synthesis and catabolism of AA; (3) generation of small peptides, nitrogenous metabolites, and sulfur-containing substances (e.g. H_2S); (4) urea cycle and uric acid synthesis; (5) lipid and glucose metabolism; (6) one-carbon unit metabolism and DNA synthesis; and (7) cellular redox signaling (WU, 2010a, 2010b). Functional AA include both EAA and NEAA. Dietary NEAA requirements are affected by a plethora of nutritional, physiological, pathological, and environmental factors, revised by Hou, Yin and Wu (2015).

2.2.2 Amino acids metabolism in the gut

Intestinal metabolism of AA has profound impacts on nutrition and health (BERGEN; WU, 2009; STOLL; BURRIN, 2006). First, catabolism of glutamine, glutamate, and aspartate provides most of the ATP to maintain gut integrity and function. Second, because elevated levels of glutamine, glutamate, and aspartate in plasma exert a neurotoxic effect, their extensive catabolism by the small intestine is essential to the survival of organisms. Third, transformations of AA in the intestine play an important role in regulating the endogenous synthesis of AA (e.g., citrulline, arginine, proline, and alanine) and modulating the availability of dietary AA to extra-intestinal tissues. Thus, the ratios of most AA in diets relative to lysine differ markedly from those entering the portal vein from the small-intestinal lumen or appearing in plasma and body proteins. The discrepancies in

the patterns of AA between diets and body proteins are particularly large for arginine, cysteine, glutamate, glutamine, glycine, histidine, methionine, proline, and serine. Therefore, ratios of these AA to lysine in body proteins are not accurate estimates of their optimal dietary requirements by rapidly growing animals, revised by Wu et al. (2014).

2.2.3 Arginine and glutamine in fish nutrition

Glutamine and arginine have been classified as conditionally essential because the rates of their utilization are greater than rates of their synthesis under certain conditions such as under stressful conditions of high metabolic demand as experienced by young animals, or associated with injuries, infections, heat and cold stress (WU, 2010a, 2010b, 2013).

Glutamine is the most abundant free alpha-amino acid in the body and turns over rapidly in plasma (WATFORD, 2008; WATFORD; WU, 2005), which reflects a crucial role of this amino acid in whole-body nutrient metabolism and health (WU, 2010a, 2010b). Glutamine is a major fuel for the small-intestinal mucosal cells and is crucial for maintaining the integrity and function of the small intestine by regulating gene expression, protein turnover, immune function, cell proliferation and apoptosis. Because the small intestine is one of the most metabolically active tissues in the body, this tissue intestine plays important roles in the regulation of whole-body amino acid homeostasis, revised by Dai et al. (2013). Glutamine is utilized by the enterocytes of the small intestine as another major energy substrate. Glutamine may contribute more ATP to the animal's enterocytes than glucose and fatty acids. Additionally, glutamine is required for the functions of monocytes, macrophages, lymphocytes, and neutrophils (REZAEI et al., 2013).

Arginine is involved in numerous physiological pathways in the direct form or in the form of derivatives. This amino acid is the most abundant nitrogen carrier for tissue proteins and is used in multiple biosynthetic pathways, involving key regulatory enzymes, such as arginase, nitric oxide, nitric oxide synthase, arginyl-tRNA synthetase, among others (WU, 2010a, 2010b; WU et al., 2013). As such, arginine serves as a precursor for the synthesis of creatine, ornithine, proline, glutamate, polyamines and NO, displaying remarkable metabolic and modulatory versatility in animal cells (DAI et al., 2013; WU, 2013). In fish, arginine acts as a modulator of both the innate and adaptive immune system. Nitric oxide is an essential oxidative molecule used to combat a multitude of invading pathogens. Likewise, arginine may strongly regulate the expression of adhesion molecules, tissue factors, and cytokines and promote, among other important immune functions, the proliferation of lymphocytes and enhanced wound healing (POHLENZ et al., 2012a, 2012b).

For a number of fish species, dietary supplementation of glutamine and/or arginine has been shown to improve protein optimization and, hence growth performance. Optimization of somatic growth, feed efficiency and/or immune responses supported by glutamine and/or arginine dietary supplementation between 0.5% through 4% have been reported in tilapia (NEU et al., 2016; YUE et al., 2013), blunt snout bream (LIANG et al., 2016), jian carp (CHEN et al., 2015; HU et al., 2015), golden pompano (LIN et al., 2015), yellow catfish (ZHOU et al., 2015), channel catfish (POHLENZ et al., 2012a, 2012b; POHLENZ; GATLIN III, 2014), hybrid striped bass (CHENG; GATLIN III; BUENTELLO, 2012), and red drum (CHENG; GATLIN III; BUENTELLO, 2011). Among these studies, the best results were found with arginine supplementation from 1.4 to 3.6% and/or with the association arginine plus glutamine.

3. GENERAL CONCLUSIONS

Research about the physiology and anatomic distribution of the gut endocrine system are one of the most helpful tools for a better understanding of how digestion process and feed intake are regulated in animals. Although many studies carried out in the past decades have improved considerably the knowledge about the enteroendocrine peptides of fish, a lot remains to be elucidated.

Efforts have been focused on the expression of peptides using molecular methods for visualizing and isolating cells products based on messenger RNA or protein expression. Similarly, identifying ECs types and its gut anatomical distribution in response to stimuli is also important to acquire a better understanding about the DNES. For this reason, how feeding and fasting can influence ECs peptide-specific activity along the intestine is the the target of interest in this study.

Proper amino acids supply is critical not only to achieve optimal growth rates but also to maintain the health of cultured fish. Moreover, aquaculture must continue to move towards more eco-friendly and economically sustainable rearing systems. Matching up simultaneously all these features has been a challenge for commercial aquaculturists and the aquatic feed industry. Aiming to garner greater understanding, recent advances in metabolism and nutrition of amino acids has suggested new approaches that contribute to a deeper awareness of the roles of amino acids in animal nutrition and health.

Because of the great diversity of fish being cultured along with a lack of understanding regarding NEAA metabolism in fish and its relations to somatic growth and health, additional research is warranted in this field.

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Endocrine cells producing peptide hormones in the intestine of Nile tilapia: distribution and effects of feeding and fasting on the cell density

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Abstract

Endocrine cells (ECs) act as a luminal surveillance system responding to either the presence or absence of food in the gut through the secretion of peptide hormones. The aim of this study was to analyze the effects of feeding and fasting on the EC peptide-specific distribution along the intestine of Nile tilapia. We assessed the density of ECs producing gastrin (GAS), cholecystokinin-8 (CCK-8), neuropeptide Y (NPY), and calcitonin gene-related peptide (CGRP) in nine segments of the intestine using immunohistochemistry. Our results show that ECs immunoreactive to CCK-8, GAS, NPY, and CGRP can be found along all the intestinal segments sampled, from the midgut to hindgut, although differences in their distribution along the gut were observed. Regarding nutrient status, we found that the anterior segments of the midgut seem to be the main site responding to luminal changes in Nile tilapia. The NPY+ and CGRP+ EC densities increased in the fasted group, while the amount of CCK-8+ ECs were higher in the fed group. No effects of fasting or feeding were found in the GAS+ EC densities. Changes in ECs density were found only at the anterior segments of the intestine which may be due to the correlation between vagus nerve anatomy, EC location, and peptide turnover. Lastly, ECs may need to be considered an active cell subpopulation that may adapt and respond to different nutrient status as stimuli. Due to the complexity of the enteroendocrine system and its importance in fish nutrition, much remains to be elucidated and it deserves closer attention.

Keywords Cholecystokinin; Gastrin; Neuropeptide Y; Calcitonin gene-related peptide; Immunohistochemistry; Fish

Abbreviations

DNES	Diffuse neuroendocrine system
GAS	Gastrin
CCK-8	Cholecystokinin-8
NPY	Neuropeptide Y
CGRP	Calcitonin gene-related peptide
ECs	Endocrine cells
+ECs mm^{-2}	Immunoreactive endocrine cells per mm^2 of mucosal epithelium
S1 – S9	First to ninth segment of intestine

1. Introduction

Once food arrives in the gastrointestinal lumen, it promotes both physical and chemical stimulation to the diffuse neuroendocrine system (DNES), which is constituted by several types of endocrine cells (ECs). This complex endocrine system associated with the digestive tract is responsible for many functions, including digestion.

The ECs are a highly specialized mucosal cell subpopulation that comprises about 1% of the total epithelial cells, and more than 30 peptide hormones have been identified as produced by these cells (Rindi et al. 2004; Gutierrez-Aguilar and Woods 2011). These ECs in the gastrointestinal mucosa form the largest endocrine cell system in the body, comprising up to 15 different cell types in mammals (Ahlman and Nilsson 2001; Helander and Fändriks 2012). In fish, due to the complexity and extensive diversity of species, many cell types remain undescribed (Takei and Loretz 2011).

It is important to emphasize that ECs act as a luminal surveillance system responding to either the presence or absence of food in the gut lumen through their secretory peptide hormones (Dockray 2010; Dockray 2014). Collectively, peptide hormones regulate the course of digestion and determine the delivery of nutrients to the gut by controlling food intake. Afferent neurons of the vagus nerve are an important target of these peptide hormones, particularly for control of the pancreatic activity, gallbladder contraction, motility, and food intake (Volkoff et al. 2010; Dockray 2013).

Peptide hormones production by ECs are influenced by nutrient status; some of them play a role when food is present in the gut lumen, while other peptides increase their expression when food is absent (Rindi et al. 2004; Dockray 2010). Gastrin (GAS) and cholecystokinin-8 (CCK-8) stimulate gastric and pancreatic secretions, respectively, in response to the presence of digesta in the

gastrointestinal lumen (Jensen 2001; Takei and Loretz 2011). Neuropeptide Y (NPY) is an orexigenic factor mainly expressed in the hypothalamus and gut. Calcitonin gene-related peptide (CGRP) acts as an anorexigenic peptide, and its main action includes inhibiting intestinal motility, gastric secretion, and food intake (Takei and Loretz 2011; Volkoff et al. 2010). CCK-8, CGRP, and NPYare synthesized by both hypothalamic neurons and ECs of the gastrointestinal tract (Martínez-Álvarez et al. 2008; MacDonald and Volkoff 2009; Yokobori et al. 2012). Furthermore, CGRP is also expressed in neurons and nerve fibers of the myenteric plexus (Martínez-Álvarez et al. 2008).

Understanding the influence of nutrient status on EC activity can contribute to clarify the endocrine regulation pathways in the fish gut. Many studies carried out in the last decades have considerably improved our knowledge of the enteroendocrine system. Efforts have been mainly focused on the messenger RNA (mRNA) or protein expression of peptides (Takei and Loretz 2011). However, identifying EC types, their anatomical distribution in the gut and how they respond to stimuli are also important to acquire a better understanding on the DNES of fish. The distribution of ECs peptide-specific along the intestine and the influence of feeding and fasting status on the density of these ECs in Nile tilapia are the target of interest in this study.

Nile tilapia (*Oreochromis niloticus*) feed on a wide variety of dietary sources, including phytoplankton, zooplankton, insects, larval fish, and benthic detritus. Adult Nile tilapia are omnivorous and readily adapt to complete commercial diets based on plant and animal protein sources (El-Sayed 2006; Mjoun Kamal et al. 2010). Dietary protein requirements for this species vary from 40% for larvae to 28% of the diet for adult fish (NRC 2011). Robustness, rapid growth, feed efficiency, year-round production, and great market acceptance make tilapia the second most important fish in global aquaculture, and the most

important cultured fish in Brazil, accounting for 41% of the national aquaculture production (ACEB 2014; FAO 2016).

Histologically, the Nile tilapia intestine is long and coiled, microscopically divided into two sections. The first three quarters of the total length of the intestine correspond to midgut, and the final quarter refers to the posterior portion extending up to the anus, as shown in Fig. 1 (Morrison and Wright 1999).

This study was designed to determine the distribution of ECs immunoreactive to CCK-8, GAS, NPY, and CGRP throughout intestine of Nile tilapia. Moreover, we aimed to determine if EC density is influenced by feeding or fasting status.

2. Material and methods

2.1 Fish and sampling procedures

Juvenile Nile tilapia were obtained from Aquaminas Fish Farm (Minas Gerais, Brazil) and acclimated during a 3-month period to the experimental conditions. Fish were fed a commercial diet (Pirá 32% crude protein; www.guabi.com.br) twice a day at 9:00 am and 4:00 pm ad libitum. All 30 fish were maintained in the same 500-L tank in an indoor recirculating system at the Fish Laboratory Facilities, Department of Animal Science, Federal University of Lavras, UFLA, Brazil. Water quality parameters such as temperature, dissolved oxygen, and toxic ammonia were monitored daily at the beginning and ending of the day and remained appropriate for Nile tilapia. Average water temperature was 28 ± 1 °C and dissolved oxygen was 6.5 ± 2 mg L^{-1} throughout the experimental period.

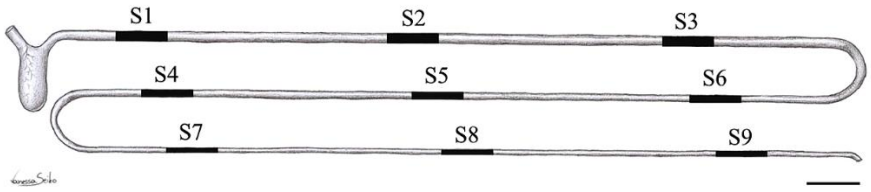

Fig. 1 Schematic drawing of the Nile tilapia (*O. niloticus*) digestive tract in which the intestinal segments sampled for immunohistochemistry are indicated. The nine intestinal segments were sampled every ~5 cm along the intestine. Segments 1 to 7 (S1–S7) corresponded to midgut, while S8 and S9 were collected from hindgut. Bar = 1 cm. Drawn by Vanessa Seiko Sugihara

Following the acclimation period, fish were divided into two groups, fasted and fed. Fasted fish (n = 11, average weight 24.2 ± 6.2 g, average length 11.23 ± 0.89 cm) were food deprived for 24 h, while fed fish (n = 10, average weight 29.5 ± 6.1 g, average length 11.63 ± 0.66 cm) were fed normally. After 24 h of fasting, fish of the fasted group were euthanized as described below and samples were collected. Fed fish were also euthanized and sampled 3 h after the last feeding. This experimental design was applied to give opposite nutrient status. In Nile tilapia, the return of the appetite, following a satiation meal, occurs approximately 4 h at 28 °C when the fish have evacuated 64% of their meal. Inversely, fasted Nile tilapia was archived at 18 h at 28 °C following the first feeding when the gastric residuum was zero and gut have evacuated 95% (Riche et al. 2004).

Fish were euthanized with an overdose of Benzocaine 250 mg L−1, followed by severance of the spinal cord. After dissecting the coelomic cavity, the entire intestine was removed. In both groups, seven sections from the midgut (S1–S7) and two sections from hindgut (S8–S9) were sampled. Average intestinal length for the Nile tilapia was 45.32 ± 5 cm, i.e., ~4 times longer than its total body length. The S1 was dissected ~1 cm behind the stomach. Cross sections (1-cm length) were removed and fixed in Bouin's solution for 12 h and then stored in 70% ethanol until processing. The immunohistochemistry assays were carried

out at the Histology and Immunohistochemistry Laboratory, Department of Animal Science, Federal University of Lavras, Brazil. All procedures applied to fish were properly analyzed and authorized by the Animal Ethics Committee of the Federal University of Lavras, under protocol number 013/2012.

2.2 Immunohistochemistry and light microcopy

Samples were dehydrated in ethanol, diaphanized in xylene, and embedded in paraffin wax. Histological serial sections (3–4 μm in thickness) were obtained, placed on silanized slides, and dried in an oven overnight at 37 °C. The histological sections were dewaxed and stained with hematoxylin and eosin for morphological analysis of the structures and measurement of the area of the mucosal epithelium, according to routine histological methods for intestine (Kumar and Rudbeck 2009). The slides were made as serial sections; the immune staining procedure was carried out in the same morphological region for each fish from each group. For this reason, it was possible to analyze all the four peptide hormones simultaneously in each gut segment of each fish.

For immunohistochemistry, all incubations were performed in a humid chamber and all washing procedures consisted of three successive immersions in 0.1 M phosphate buffered saline (PBS), pH 7.20, for 5 min. The endogenous peroxidase activity was blocked for 30 min by Peroxidase Block reagent (S200389, DakoCytomation, USA). Non-specific antibody binding was blocked in two ways: incubation of the sections in 5% non-fat dry milk in PBS for 5 min and incubation of the sections in Block Serum reagent (X0909, DakoCytomation, USA) for 15 min. Following the blocking step, the histological sections were incubated with rabbit polyclonal primary antibodies against CCK-8, GAS, NPY, and CGRP, as presented in Table 1. After washes in PBS, the anti-rabbit IgG secondary antibody (K401111 EnVision + System/HRP, DakoCytomation, USA)

was applied over the samples for 30 min. The reactions were detected with an enzymatic method, using 3,3′-diaminobenzidine tetrahydrochloride (DAB; K346811 DakoCytomation, USA) for 25 s and then counterstaining with Carazzi's hematoxylin. Intestine histological sections were included as positive controls, and the negative controls were the same samples in which the primary antibodies were replaced by PBS.

The polyclonal antibodies used in this study were selected based on their accuracy to recognize the peptide hormones CCK-8, GAS, CGRP, and NPY produced by ECs of the DNES in several fish species including dorado (*Salminus brasiliensis*) (Pereira et al. 2015), South American catfish (*Rhamdia quelen*) (Hernández et al. 2012), pejerrey (*Odontesthes bonariensis*) (Vigliano et al. 2011), and turbot (*Scophthalmus maximus*) (Bermúdez et al. 2007).

Table 1 List of antibodies used to identify gut peptides in Nile tilapia, *O. niloticus*

Polyclonal antibody against	Antibody working dilution	Incubation variables	Source (code)
CCK-8 (synthetic)	1:1000	3h, RT	Bachem* T-4254
GAS (human)	1:600	3h, RT	Bachem*T-4347
NPY (swine)	1:1500	ON, 4 °C	Bachem* T-4454
CGRP (rat)	1:800	ON, 4 °C	Bachem* T-4032

GAS: gastrin; CCK-8: cholecystokinin-8; NPY: neuropeptide Y; CGRP: calcitonin gene-related peptide; ON: overnight; RT: room temperature (22 to 25 °C).
*www.bachem.com

In addition, the protein sequences for Nile tilapia CCK (NP_001266659.1), GAS (XP_003453823.1), CGRP (XP_005460012.1), and NPY (AJD19684.1) were retrieved from the protein database of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/protein) and

compared using BLASTP to the sequences of the peptide hormones employed as immunogens to confirm if cross-reactivity observed in Nile tilapia may be due to the binding of each antibody to homologous fish proteins.

2.3 Endocrine cell counting and statistical analysis

The whole histological transverse section of the intestine was used for both cell counting and mucosal epithelium area measurement. The average number of ECs per square millimeter of mucosal epithelium (ECs mm^{-2}) was determined for CCK-8, GAS, CGRP, and NPY, in the nine segments (S1–S9) sampled from fasted and fed fish groups. The total number of ECs was counted manually using a microscope, and mucosal epithelium area was photographed with a CX31 light microscope (Olympus, Japan) coupled to an Altra SC30 digital camera (Olympus, Japan). The mucosal epithelium area was measured using the morphometric analysis software ImageJ, version 1.46a (National Institute of Mental Health, USA).

The density values of ECs producing each peptide were analyzed using repeated measures analysis of variance conducted with the MIXED SAS procedure (version 9.3). This approach was chosen because several measurements were taken on the same fish; that is, the measurements of each peptide hormone were taken for each fish over the nine intestinal segments. We considered the linear mixed model (LMM):

$$y_{ijk} = \mu + d_i + \alpha_j + \beta_k + \alpha\beta_{jk} + \varepsilon_{ijk}$$

which evaluated the fixed effects of nutrient status (α_j) ($j = 1,2$), segment (β_k) ($k = 1,2,\ldots,9$), and their interaction ($\alpha\beta_{jk}$) on the observed density of ECs peptide-specific (y_{ijk}). The term d_i is the random effect related to the ith fish, where $d_i \sim N(0, \sigma_d^2)$, and $\varepsilon_{ijk} \sim N(0, \sigma^2)$.

Statistical analyses were applied to the original data from each fish, and the significance level was set at $p < 0.05$. When significant effects were detected, the means were compared using Tukey-Kramer tests (Gonçalves et al. 2016). Results were reported as least square means with standard error and percentage of ECs mm^{-2} mucosal epithelium.

3. Results

3.1 Diffuse neuroendocrine system: EC distribution along the intestinal segments

The 3-h fed group fish were satiated and their digestive tracts were full. On the other hand, after 24 h of food deprivation, fasted fish had empty stomachs and very little *digesta* in the gut, and then only in the distal segments. The BLASTP alignment resulted in at least 78% of identities between sequences for all the gut peptide hormones employed in this study.

Nile tilapia ECs had a triangular shape with their base situated over the basement membrane. The nucleus of these cells was euchromatic, rounded or oval, and was located in a medial or basal position. Some ECs presented one or two cytoplasmic processes toward the gut lumen and in the opposite direction toward the base of the cell (Fig. 2).

ECs immunoreactive to CCK-8, GAS, NPY, and CGRP were found throughout all intestinal segments sampled from midgut to hindgut (Table 2). Differences in the distribution of ECs depended on the type of peptide hormone produced, which resulted in peptide-specific ECs being more concentrated in some intestinal segments than others of Nile tilapia. The average number of ECs mm^{-2} for each peptide along the gut are shown in Table 2. In absolute numbers,

the amount of immunoreactive ECs was higher for CCK-8 followed by GAS, NPY, and lastly CGRP (Table 2).

The percentages refer to the proportion of ECs immunoreactive to each antibody in each intestinal segment with respect to the total number of cells recorded in the whole histological cross section. As we expected, the density of ECs mm^{-2} mucosal epithelium changed significantly along the gut segments sampled for all peptide hormones assessed ($p < 0.0001$), regardless of whether the fish were fasted or fed (Table 2).

CCK-8+ ECs mm^{-2} were distributed in the mucosal epithelium from S1 to S9 with a similar trend in both fasted and fed fish. The largest number of CCK-8+ ECs mm^{-2} was found in S1, with 39.4 and 45.1% found in fasted and fed fish, respectively (Table 2 and Fig. 4). Overall, the number of CCK-8+ ECs mm^{-2} was higher in S1 and then decreased throughout the following segments of the intestine.

GAS+ ECs mm^{-2} showed a similar distribution pattern to that described for CCK-8, in which the ECs density was higher in S1 and then decreased gradually from S2 to S9. The highest GAS+ ECs mm^{-2} was observed in S1 with 42.9 and 41.7% in fasted and fed fish, respectively (Table 2 and Fig. 4).

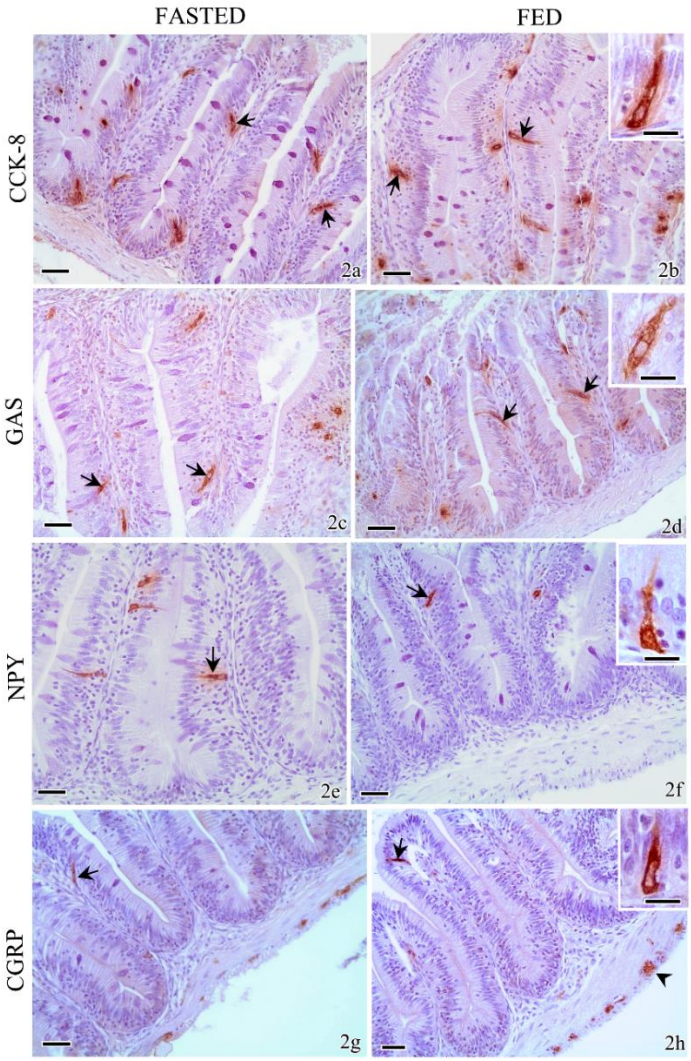

Fig. 2 Photomicrograph of ECs of the DNES and myenteric plexus immunoreactive to CCK-8, GAS, NPY, and CGRP in the midgut of fed (3 h) and fasted (24 h) Nile tilapia, *O. niloticus*. *Arrows* indicate immunopositive ECs in the mucosal epithelium of the intestine. (a, b CCK-8) immunoreactivity of EC mucosal epithelium. (c, d GAS) immunoreactivity of EC mucosal epithelium. (e, f NPY) immunoreactivity of EC mucosal epithelium. (g, h CGRP) immunoreactivity of EC mucosal epithelium (a–h, bar = 50 μm). *Inserts* higher magnification showing the morphology of immunoreactive cells. Note the ECs showing a triangular shape with their base situated over the basement membrane and their rounded and euchromatic nucleus. Inserts (a–h, bar = 20 μm). Photomicrographs (a–g) correspond to immunoreactivity of intestinal segments sampled from S1 and (h) from S5.

Table 2 Distribution of immunoreactive ECs to CCK-8, GAS, CGRP, and NPY along the intestine of fed (3 h) and fasted (24 h) Nile tilapia, *O. niloticus*

		Intestinal segment									Effects and p-values		
		Midgut							Hindgut				
Peptide	Nutrient status	S1	S2	S3	S4	S5	S6	S7	S8	S9	Nutrient status	Segment	Nutrient status × segment
CCK-8	Fasted	12.31^{a} 39.5%	3.37^{b} 10.8%	3.28^{b} 10.5%	3.01^{b} 9.7%	3.46^{b} 11.1%	2.28^{b} 7.3%	2.00^{b} 6.4%	1.48^{bc} 4.7%	0.01^{c} 0.0%	0.3100	<.0001	0.0047
	Fed	14.99^{a} 45.1%	3.15^{b} 9.5%	2.34^{b} 7.0%	3.25^{b} 9.8%	3.20^{b} 9.6%	3.40^{b} 10.2%	1.84^{bc} 5.5%	1.07^{c} 3.2%	0.03^{c} 0.1%			
GAS	Fasted	10.52^{a} 42.9%	2.95^{b} 12.0%	2.01^{b} 8.2%	1.90^{bc} 7.8%	2.14^{bc} 8.7%	2.00^{bc} 8.2%	1.83^{bcd} 7.5%	0.93^{cd} 3.8%	0.23^{d} 0.9%	0.5335	<.0001	0.5772
	Fed	10.85^{a} 41.7%	3.18^{b} 12.2%	3.09^{b} 11.9%	2.97^{bc} 11.4%	1.89^{bc} 7.3%	2.16^{bc} 8.3%	1.12^{bcd} 4.3%	0.65^{cd} 2.5%	0.10^{d} 0.4%			
NPY	Fasted	6.21^{a} 40.1%	4.36^{a} 28.2%	3.05^{a} 19.7%	1.39^{a} 9.0%	0.10^{b} 0.6%	0.00^{b} 0.0%	0.17^{b} 1.1%	0.04^{b} 0.3%	0.17^{b} 1.1%	0.0881	<.0001	0.0022
	Fed	3.12^{a} 26.6%	3.59^{a} 30.6%	2.64^{a} 22.5%	1.67^{a} 14.2%	0.40^{b} 3.4%	0.25^{b} 2.1%	0.03^{b} 0.3%	0.04^{b} 0.3%	0.01^{b} 0.1%			
CGRP	Fasted	2.66^{a} 23.3%	1.62^{a} 14.2%	1.93^{a} 16.9%	2.26^{a} 19.8%	1.62^{abc} 14.2%	0.67^{bc} 5.9%	0.29^{c} 2.5%	0.29^{c} 2.5%	0.10^{c} 0.9%	0.0296	<.0001	0.1592
	Fed	1.18^{a} 21.4%	0.90^{a} 16.3%	0.89^{a} 16.1%	0.85^{a} 15.4%	0.70^{abc} 12.7%	0.67^{bc} 12.1%	0.18^{c} 3.3%	0.13^{c} 2.4%	0.02^{c} 0.3%			

The results are expressed as average number of peptide-specific ECs mm−2 mucosal epithelium and its percentage in each intestinal segment. The percentage of ECs immunoreactive to each antibody in each segment was calculated in relation to the total of ECs recorded in the whole histological cross section for each peptide. The effects of the segment on the average number of ECs per mm−2 epithelial mucosa are expressed as least squares means and was obtained from MIXED SAS procedure and Tukey-Kramer’ s tests performed at $p < 0.05$. Different letters within a row indicate significant differences among segments of the intestine for each peptide CCK-8 cholecystokinin-8, GAS gastrin, NPY neuropeptide Y, CGRP calcitonin gene-related peptide, S1–S7 intestinal segments sampled in the midgut, S8–S9 intestinal segments sampled in the hindgut

Although NPY+ ECs mm^{-2} were also observed along the whole gut, it was mainly concentrated in the anterior segments of the midgut S1–S4, which correspond to 74.0 and 69.2% of the ECs in fasted and fed fish, respectively (Table 2 and Fig. 4).

CGRP+ ECs mm^{-2} showed a similar pattern to that reported for NPY (Table 2 and Fig. 4). The CGRP+ ECs mm^{-2} were concentrated in the first four segments, accounting for 97.5 and 97.2% of the total ECs in fasted and fed fish, respectively.

3.2 Enteric nervous system immunoreactivity

Structures and cells of the enteric nervous system associated with the muscle layer were observed using immunohistochemistry. Neurons and nerve fibers in the myenteric plexus showed a strong positive immunoreactivity to CGRP in all nine segments sampled (Fig. 3a, b). In addition, nerve fibers (but not neurons) in the myenteric plexus of both fed and fasted fish also exhibited immunoreactivity for NPY mainly in S8 and S9, where very few or no ECs was recorded in the mucosal epithelium (Fig. 3c, d).

3.3 Effects of nutrient status on ECs density along the intestine

Effects of fasting and feeding on density of CCK-8+, GAS+, NPY+, and CGRP+ ECs along the intestine as well the statistical interaction nutrient status × segment were examined (Table 2).

Interaction effects between nutrient status and segments of the intestine were statistically significant for CCK-8+ ($p = 0.0047$) and NPY+ ($p = 0.0022$) ECs mm^{-2} as shown in Table 2 and Fig. 4. In S1, the density of NPY+ ECs mm^{-2} was greater in the fasted fish group than in the fed fish group ($p < 0.0001$) (Fig.

4). The opposite was found for CCK-8+ ECs mm−2, in which the fed group had the highest density in S1 ($p = 0.0212$) (Fig. 4).

In the case of CGRP+ ECs mm^{-2}, both nutrient status and gut segments, as isolated effects, were statistically significant on the cells density, as shown in Table 2 ($p = 0.0296$ and $p < 0.0001$, respectively), but not the interaction nutrient status × segment ($p = 0.1592$), as shown in Table 2 and Fig. 4. Notably, the CGRP+ ECs mm^{-2} in S1–S4 were higher in the fasted fish group compared to the fed fish group (Fig. 4).

Unlike, GAS+ ECs mm^{-2} were not statistically affected by neither interaction nutrient status × segment ($p = 0.5772$) or nutrient status ($p = 0.5335$) (Table 2 and Fig. 4). Hence, fasted and fed fish groups had the same GAS+ EC density along the segments of the intestine sampled.

Overall, the intestinal segments sampled from the anterior portion (S1–S4) of the midgut were the main anatomical regions influenced by feeding and fasting status. No differences were found in the distal segments of midgut or hindgut (S6–S9).

4. Discussion

Morphological description for EC types in Nile tilapia matches with the expected features when compared to those from other species of fish (Bosi et al. 2004; Vigliano et al. 2011; Pereira et al. 2015) and mammals (Rindi et al. 2004).

Most studies on EC distribution along the digestive tract sample few intestinal segments from midgut and hindgut (Dezfuli et al. 2000; Pan et al. 2000; Bosi et al. 2004; Çınar et al. 2006; Bermúdez et al. 2007; Micale et al. 2012). Likewise, molecular studies characterizing endocrine peptides from the intestine usually have similar sampling patterns (Bosi et al. 2004; Martínez-Álvarez et al. 2008; MacDonald and Volkoff 2009; Webb et al. 2010; Yuan et al. 2014).

However, due to the extensive complexity of DNES and its relation to the different feeding habits of fish, this might generate a misconception about EC distribution along the intestine and, consequently, their function. Therefore, the results obtained in this study emphasize the need for extensive samplings and provide a detailed description of the distribution pattern of ECs producing CCK-8, GAS, CGRP, and NPY along the intestine of the Nile tilapia.

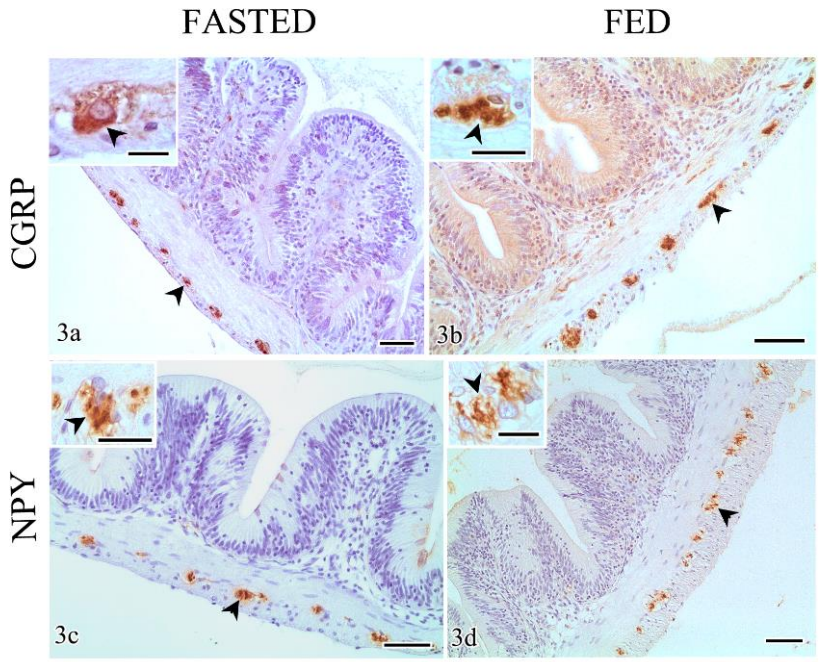

Fig. 3 Photomicrographs of the NPY and CGRP immunoreactivity in nerve fibers and cells in the intestine of fed (3 h) and fasted (24 h) Nile tilapia, *O. niloticus*. (a, b) Photomicrographs of CGRP immunoreactive neurons and nerve fibers in the myenteric plexus (a S7, b S5). *Arrowheads* indicate CGRP immunoreactivity in neurons (a) and nerve fibers (b) in the myenteric plexus of fed and fasted fish (a, b, bar = 50 μm). Note the neuron and the nerve fibers in a higher magnification in (c, d), respectively. Inserts (a, b), bar = 20 μm. (c, d) Photomicrographs of NPY immunoreactivity of nerve fibers in the plexus myenteric (c S9, d S9). *Arrowheads* in all photomicrographs indicate the immunoreactivity of nerve fibers in the myenteric plexus of fed and fasted fish (c, d, bar = 50 μm). In the *inserts* in b, the lack of immunoreactivity for NPY can be observed in a neuron located in the myenteric plexus. *Inserts* (c, d, bar = 20 μm).

4.1 CCK-8+ and GAS+ EC distribution

The EC distribution pattern results obtained in this study can be explained in terms of anatomical and morphological features of the digestive tract. An expected distribution model for CCK+ ECs was proposed based on coiled intestine versus straight intestine (Kamisaka et al. 2005; Rønnestad et al. 2007). The straight intestine is common in carnivorous species, while coiled intestines are typical in omnivorous and herbivorous fish (Wilson and Castro 2011). Thus, in the fish with coiled intestines, CCK+ cells are concentrated primarily in the anterior segment of the midgut (Kurokawa et al. 2003; Webb et al. 2010), while in fish with straight intestines, such as Atlantic herring (*Clupea harengus*), have cells expressing CCK mRNA scattered throughout the entire intestine (Kamisaka et al. 2005).

Our results in Nile tilapia for CCK-8+ and GAS+ ECs match with the distribution model proposed for coiled intestines. Although this model is not associated with GAS, previous studies have reported a similar distribution pattern for CCK-8 and GAS. Higher density of ECs producing CCK and/or GAS in the anterior segments of the midgut was described for dorado (Pereira et al. 2015), blacktip grouper (*Epinephelus fasciatus*) (Hur et al. 2013), white sea bream (*Diplodus sargus*) (Micale et al. 2012), red drum (*Sciaenops ocellatus*) (Webb et al. 2010), turbot (*S. maximus*) (Bermúdez et al. 2007), brown trout (*Salmo trutta*) (Bosi et al. 2004), Korean aucha perch (*Coreoperca herzi*) (Lee et al. 2004), and Atlantic cod (*Gadus morhua*) (Jönsson et al. 1987).

CCK-8 and GAS comprise a small, yet pivotal, hormone family in the digestive process (Takei and Loretz 2011). CCK-8 plays a key role in digestive physiology by stimulating the release of pancreatic enzymes, gut motility, gallbladder contraction (Aldman et al. 1992), and by delaying gastric emptying (Olsson et al. 1999). Moreover, it has been hypothesized that hindgut CCK-8+

ECs might participate in the feedback control of digestive processes, by receiving chemical signals from incompletely digested food reaching the hindgut (Olsson et al. 1999; Hartviksen et al. 2009; Micale et al. 2012; Micale et al. 2014). GAS stimulates sphincter motility, HCl, and pepsin secretion in the stomach (Takei and Loretz 2011).

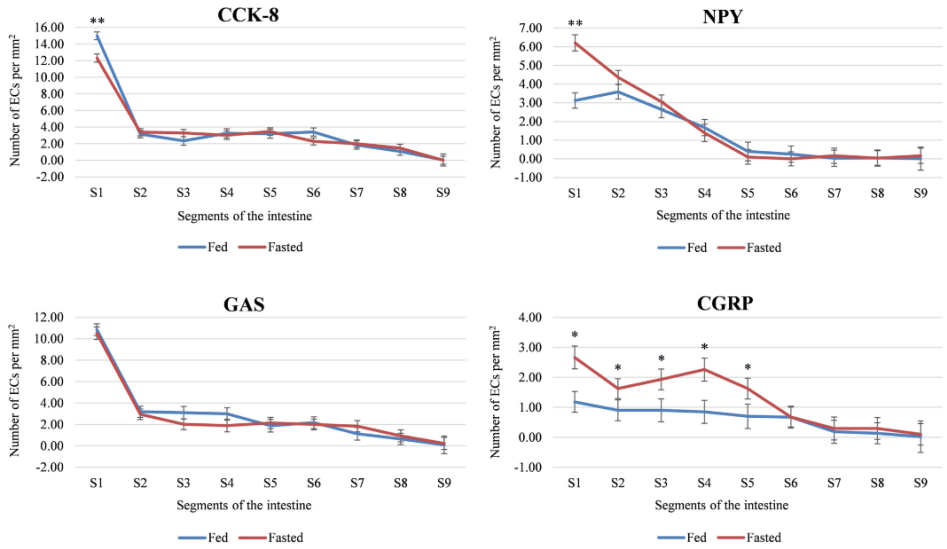

Fig. 4 Effect of nutritional status on the density of immunoreactive ECs to CCK-8, GAS, CGRP, and NPY in the different segments of the digestive tract of Nile tilapia, *O. niloticus*. The fed fish group corresponds to fish sampled 3 h from the last feeding, and the fasted fish group was starved for 24 h. Average number of ECs per mm−2 epithelial mucosa are expressed as least squares means with standard error and was obtained from MIXED SAS procedure and Tukey-Kramer's tests performed at $p < 0.05$. *Double asterisks* indicate the significant differences on the interaction nutrient status × segment effect between fasted and fed fish groups at the same intestinal segment for CCK-8 ($p = 0.0047$) and NPY ($p = 0.0022$). *Single asterisk* indicates the significant differences between fasted and fed fish groups among the intestinal segment for CGRP ($p = 0.0296$).

Pancreatic and hepatic ducts empty into the intestinal lumen in an anatomically strategic region characterized by the pyloric sphincter on the cranial side and by the duodenum on the caudal side (Olsson et al. 1999). This

region is a crucial transit site because it regulates the flow of digesta from the stomach to the intestine (Olsson and Holmgren 2001). In the present study, the higher density of CCK-8+ and GAS+ ECs mm^{-2} in S1 is thought to be because it is a key location for both peptides. In S1, ECs are able to monitor the chyme as soon as it arrives in the intestinal lumen and then activate the pancreas, liver, and myenteric plexus.

Similarly, the anterior segments of the intestine can also be understood as the expanded domain of GAS. Even though GAS is a peptide mainly present in the stomach, and CCK is found mainly in the gut, both peptides can be found in ECs of the stomach as well as the intestine of fish (Bosi et al. 2004; Takei and Loretz 2011). Results from the current study show that GAS+ and CCK-8+ EC densities and distribution patterns of both peptides are quite similar in Nile tilapia gut (Table 2). Similar findings were reported in dorado (Pereira et al. 2015), a carnivorous fish.

4.2 NPY+ EC distribution

Unlike other peptides discussed, NPY has not been associated with any enzymatic or peristaltic process in the digestive tract (Takei and Loretz 2011). Most of the effects exerted by NPY have been reported in the centra nervous system on feeding behavior, mainly in the hypothalamus (Hoskins and Volkoff 2012; Volkoff 2016). Similar to our results in Nile tilapia, the larger amount of NPY+ ECs was concentrated in the anterior segments of the midgut of dorado (Pereira et al. 2015), South American catfish (Hernández et al. 2012), channel catfish (*Ictalurus punctatus*) (He et al. 2009), flower fish (*Pseudophoxinus antalyae*) (Çınar et al. 2006), and European eel (*Anguilla anguilla*) (Domeneghini et al. 2000). Interestingly, in all above-cited studies, NPY producing ECs have always been described at the anterior segments of the midgut, regardless of

feeding habit (omnivorous or carnivorous). Considering the restricted distribution of NPY+ ECs to the four anterior segments of the midgut and the orexigenic effects of this peptide, it can be suggested that the anterior segments of the midgut are the main signaling area to the brain center that modulates food intake.

Likewise, the strongest NPY mRNA expression has been reported in the anterior segments of the intestine in Atlantic cod (*Gadus morhua*) (Kehoe and Volkoff 2007), winter skate (*Raja ocellata*) (MacDonald and Volkoff 2009), grass carp (*Ctenopharyngodon idella*) (Zhou et al. 2013), and blunt snout bream (*Megalobrama amblycephala*) (Ji et al. 2015), among others.

4.3 CGRP+ EC distribution

CGRP exerts a wide range of biological actions, including reduction of food intake and gastrointestinal motility (Nag et al. 2006; Martínez-Alvarez et al. 2009). Immunoreactivity for CGRP in ECs in the digestive tract was previously reported in dorado (Pereira et al. 2015), South American catfish (Hernández et al. 2012), pejerrey (Vigliano et al. 2011), and brown trout (Dezfuli et al. 2000).

In Nile tilapia, CGRP+ EC distribution and myenteric plexus immunoreactivity swept the whole gut. Higher CGRP+ ECs densities were found in the five first segments and decreased after. CGRP acts as an inhibitory agent of intestinal motility in fish (Shahbazi et al. 1998; Olsson and Holmgren 2001) and mammals (Martinez et al. 2006). Peristalsis is a result of contraction and relaxation of the circular muscle of the intestine to conduce the ingested food from mouth to anus (Olsson and Holmgren 2001). Intrinsic sensory neurons in the plexus are activated by products released from ECs in the mucosal epithelium (Olsson 2009). Therefore, it is reasonable that CGRP+ ECs have a broad distribution along the intestine to be able to gather luminal stimuli to control motility. However, the reason for decreasing CGRP+ ECs in the posterior

segments of midgut and hindgut is still unclear. It has been suggested that greater CGRP+ EC density in midgut than hindgut could be associated with food intake inhibition through satiety signals (Olsson 2009; Dockray 2014).

4.4 Effects of nutrient status on ECs density along the intestine

We found that the anterior segments of the midgut seem to be the main site responding to luminal changes in Nile tilapia. Therefore, our results show that ECs behave as luminal surveillance in both the presence and the absence of food in the intestinal lumen. Any change in the intestinal environment can be a stimulus to EC action; hence, the presence and absence of food in the lumen could be understood as an elementary reference to the EC activity (Dockray 2009).

Reports describing the influence of nutrient status on the EC distribution are still limited in Nile tilapia and other fish due to the great diversity of fish species and the complexity of the DNES (Volkoff et al. 2010; Hoskins and Volkoff 2012). When available, most studies analyzing the effects of nutrient status on the intestinal endocrine system focus on EC products based on mRNA or protein expression. No studies about the effects of fasting and feeding on GAS+ and CGRP+ ECs were found in fish. Moreover, studies have examined CCK using mRNA quantification, and/or EC identification methods are greater in number (Micale et al. 2014; Volkoff 2016). In addition, endocrine regulation of food intake in fish has been more thoroughly studied in the brain than in the digestive tract (revised by Volkoff 2016).

Two research studies have applied a similar sampling strategy to the current study and reported results which are consistent with our findings in Nile tilapia. The first study found that CCK+ EC distribution in White Sea bream was affected by fasting (24 h) and feeding (3 h). Fasting appeared to induce a pronounced decrease in CCK expression and also the density of immunoreactive

cells in the pyloric ceca, as well as the anterior and posterior midgut, but not in the hindgut (Micale et al. 2012). In the second study, the NPY gene expression after 24 h of fasting and 1 h after feeding was quantified
in the anterior and posterior segments of the intestine of grass carp. The authors reported the strongest NPY expression in the anterior segment of the intestine of fasted grass carp. In contrast, a weak or no expression of NPY was observed in the intestine of grass carp (Zhou et al. 2013).

Changes in EC density were found only in the anterior segments of the intestine. This could be based on the correlation between vagus nerve anatomy, endocrine cell location, and peptide turnover (Dockray 2013; Dockray 2014). Nonetheless, further studies need to be conducted in order to determine the functional meaning of ECs in the distal segments of the intestine.

Anatomically, the vagal nerves mainly innervate the anterior (cranial) part of the digestive tract, including the esophagus, stomach, and proximal intestine of teleost and elasmobranch species (Olsson 2009). Gastrointestinal vagal afferents are more prevalent in the proximal gut, and their nerve terminals are distributed within the gut wall, including mucosa and submucosa as well as muscle and enteric ganglia. Importantly, 80 to 90% of axons in the vagus nerve are afferent nerve fibers and this innervation is both mechano- and chemo-sensitive in animals (Steinert and Beglinger 2011).

Due to gut peptide hormones being subject to rapid breakdown by proteolytic enzymes, the largest concentrations are found very near the site of secretion, via neurocrine and paracrine systems rather than systemic circulation in fish (Olsson 2009) and mammals (Steinert and Beglinger 2011). Therefore, peptides released from ECs may act locally on vagal afferent fibers running close to the basolateral membrane (Engelstoft et al. 2008). In turn, vagal afferent neurons activate mechanisms that control nutrient delivery to the small intestine by regulating food intake and gastric emptying (Dockray 2010). This mechanism

may be either stimulated or inhibited by neurohumoral factors whose releasing depends on the presence or absence of nutrients (Dockray 2013; Dockray 2014).

Additionally, it is generally accepted assertion that gastric satiation signals arise primarily from mechanical distention, whereas those from the intestine derive largely from the chemical effects of food on the enteroendocrine system (Powley and Phillips 2004; Cummings and Overduin 2007). GAS is secreted in response to mechanical and chemical stimuli such as the presence of proteins in the lumen of the stomach/intestine after food intake in mammals (Schubert and Makhlouf 1992) and fish (Aldman et al. 1989; Takei and Loretz 2011).

Unlike the other gut peptide hormones analyzed in this study, GAS+ EC density was similar in fasted and fed fish. It could be possible that changes in GAS+ EC density may occur at earlier or later periods of feeding, and therefore, our results may be a consequence of the sampling strategy adopted and/or the method applied to analyze GAS. Moreover, GAS functionality have been focused on its effects on the digestive process, including acid secretion and motility stimuli (Jensen 2001; Olsson and Holmgren 2001; May and Kaestner 2010; Takei and Loretz 2011), while GAS effects on feeding intake
regulation are scarce (reviewed by Volkoff et al. 2010; Volkoff 2016).

Although many particularities exist among species, it is possible to establish an association between EC distribution pattern along the intestine and feeding habits (Domeneghini et al. 2000; Bosi et al. 2004; Vigliano et al. 2011). Our current results for the omnivorous Nile tilapia and the previous results for the carnivorous dorado (Pereira et al. 2015) support this correlation between EC peptide-specific distribution and feeding habits/anatomical features. Therefore, because these specific anatomical features are conserved among fish species, it could feasibly provide an expected distribution pattern of ECs in the fish gut. These research findings provide a tool for enteroendocrine sampling sites along

the digestive tract in fish; it also provides unique physiological insight into the DNES of Nile tilapia.

5. Conclusions

In conclusion, ECs producing CCK-8, GAS, CGRP, and NPY were observed throughout the whole intestine of juvenile Nile tilapia. However, differences in the concentration and distribution patterns of ECs were particular for each peptide hormone produced depending on the intestinal segment and/or the presence or absence of food. Higher density of ECs mm^{-2} mucosal epithelium was described in the first segments of the midgut for all peptides studied regardless of whether the fish were fasted or fed. This EC distribution pattern in Nile tilapia is similar to that observed in other omnivorous species. Feeding and fasting status significantly affected CCK-8+, CGRP+, and NPY+ EC density, but not GAS+, in the intestine of Nile tilapia in the present study. The number of CGRP+ and NPY+ ECs mm^{-2} were higher in fasted fish, while for ECs producing CCK-8 the fed fish showed the higher amount.

Despite the slight differences in our results, overall, ECs may need to be considered an active cell subpopulation that may adapt and respond to different nutrient status in Nile tilapia such as these cells act in mammals (Rindi et al. 2004).

Although particularities exist among species, it seems that there is a distribution pattern that can partially be justified by the feeding habits of the species and the anatomical features of the digestive system. So far, our current results in tilapia and previous study in dorado (Pereira et al. 2015) indicate that ECs producing GAS, CCK-8, CGRP, and NPY had a distribution pattern consistent with the intestinal features of omnivorous and carnivorous fish, respectively. This can extend the application and understanding of the expected distribution model proposed for CCK+ ECs (Kamisaka et al. 2005; Rønnestad et

al. 2007) based on coiled intestine versus straight intestine to other peptide hormones. Due to the complexity of the enteroendocrine system and its importance in fish nutrition and physiology, much remains to be elucidated and it deserves closer attention.

Conflict of interest The authors declare that they have no conflict of interest.

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Arginine and glutamine in diets for Nile tilapia: Effects on growth, innate immune responses, plasma amino acid profiles and whole-body composition

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Abstract

Glutamine (Gln) and arginine (Arg) are functional amino acids (AA) known to improve growth, immunity and nutrient utilization of animals. Juvenile Nile tilapia were fed six experimental diets formulated to contain different levels of supplemental Gln and/or Arg (Control, GLN 1%, GLN 2%, ARG 1%, ARG 2% and GLN+ARG 1%) for a 9-week period. Growth performance, innate immune responses, AA profiles in plasma and whole-body were examined. Dietary Gln and/or Arg supplementation resulted in significant effects on weight gain, feed intake, feed efficiency, protein efficiency and protein retention. Moreover, the concentration of free AAs in plasma at 6 h and 18 h were significantly affected by experimental diets. The AA concentrations significantly affected at the 6 h sampling were Cys, Asp, Ser, Gly and Hyp while at 18 h, differences were observed for Arg, Val, Cys, Ser, Gly and Pro. In contrast, only differences in Gln, Gly and Ser concentrations were observed regarding AA composition of the whole-body tissues. Therefore, the AA profiles of plasma were more affected by the dietary GLN and/or ARG supplementation than whole-body. Most of the immunity indicators were not raised by dietary levels of Gln and/or Arg probably as a reflection of the non-activated state of the immune cells. Although Gly was included to the experimental diets to adjust nitrogen content, this inclusion resulted in effects on the growth performance and physiological parameters. Finally, Nile tilapia fed the combined supplement of GLN + ARG at 1% had more improved growth performance than those fed the diets supplemented individually with Gln or Arg.

Keywords Functional amino acids; Protein optimization; Amino acids metabolism; Innate immunity; Nutrition; Fish.

Abbreviations

AA	Amino acid
DAA	Dispensable amino acid
IAA	Indispensable amino acid
ARG	Arginine in experimental diet
Arg	Arginine
GLN	Glutamine in experimental diet
Gln	Glutamine
SEC	Superoxide anion extracellular
SIC	Superoxide anion intracellular
NBT	Nitro blue tetrazolium
LYZ–P	Plasma lysozyme activity
LYZ–S	Spleen lysozyme activity
HACS	Hemolytic activity of complement system

1. Introduction

In pursuing a greater understanding of the roles of amino acids (AAs) in animal nutrition and health, numerous studies in recent years have focused on the potential effects of indispensable and dispensable AAs on various physiological and immunological functions. Similarly in fish nutrition, research on both categories of AAs has expanded in the last decade and have reported the importance of various AAs on nitrogen balance, protein and energy utilization, as well as health of species such as channel catfish (Pohlenz et al., 2013), tilapia (Gaye-Siessegger et al., 2007; Mambrini and Kaushik, 1995; Wu et al., 2015) and Atlantic salmon (Larsson et al., 2014).

The gap between traditional AA classification and physiological importance has led to the emergence of the "Functional amino acids" concept proposed by Wu (2010). Functional AAs are defined as those which participate and regulate key metabolic pathways to improve growth and health in mammals and fish. This AA group encompasses arginine, cysteine, glutamine, glutamate, glycine, leucine, proline, and tryptophan regardless of their designation as dispensable or indispensable. Due to many of their metabolic roles, functional AAs are known to improve the efficiency of nutrient utilization by animals (Wu, 2013a, 2010; Wu et al., 2014). The functional AA concept also has led to a paradigm shift regarding the classification of AA as nutritionally dispensable or indispensable. It has been proposed that animals (focused on poultry and swine) have dietary requirements for not only indispensable, but also dispensable AAs to achieve maximum growth and production performance (Wu, 2014; Wu et al., 2014). Indeed, this assumption encourages discussion regarding whether AA requirements in animals could be underestimated under certain conditions and/or if ratios between dispensable or indispensable AAs have been taken into account as they should. Because of the great diversity of fish being cultured along with a

lack of understanding regarding AA metabolism in fish and its relations to somatic growth and health, additional research is warranted in this field (Kiron, 2012; Pohlenz and Gatlin, 2014;Wu et al., 2014).

As functional AAs, glutamine (Gln) and arginine (Arg) occupy a prominent position. Gln is the major source of nitrogen and carbon in the interorgan metabolism of AAs (Watford, 2008), which reflects a crucial role of this AA in whole-body nutrient metabolism and health of mammals (Wu, 2010, 2009) and fish (Cheng et al., 2012; Pohlenz et al., 2012a, 2012b, 2012c).Moreover, Gln is a major fuel for the mucosal cells of the intestine (Burrin and Stoll, 2009) and it is an AA required for the functions of several cell populations of the immune systemin mammals (Dai et al., 2013) and fish (Cheng et al., 2011; Pohlenz et al., 2012a, 2012b, 2012c).

Arg is involved in numerous physiological pathways directly or in the form of derivatives. This AA is the most abundant nitrogen carrier for tissue proteins and it is used in multiple biosynthetic pathways, involving key regulatory enzymes, such as arginase, nitric oxide synthase, arginyl-tRNA synthetase, among others (Wu, 2013a, 2010). As such, Arg serves as a precursor for the synthesis of creatine, ornithine, proline, glutamate, polyamines and nitric oxide, displaying remarkable metabolic and modulatory versatility in animal cells (Dai et al., 2012) and fish (Bogdan, 2015; Buentello and Gatlin, 1999; Pohlenz et al., 2014, 2013, 2012c)

The minimum dietary requirement for Arg, based on weight gain, in different fish species may vary between 1.0 and 3.1% of diet while for Nile tilapia the requirement has been reported at 1.2% of the diet (or 4.2% of crude protein) according to the NRC (2011). However, the fish minimum requirement for Gln is not currently available.

For a number of fish species, dietary supplementation of Gln and Arg has been shown to improve protein optimization and, hence growth performance.

Optimization of somatic growth, feed efficiency and/or immune responses supported by dietary supplementation of Gln and/or Arg between 0.5% through 4% of diet have been reported in tilapia (Neu et al., 2016; Yue et al., 2013), blunt snout bream (Liang et al., 2016), Jian carp (Chen et al., 2015; Hu et al., 2015), golden pompano (Lin et al., 2015), yellow catfish (Zhou et al., 2015), channel catfish (Pohlenz et al., 2014, 2012a, 2012b, 2012c), hybrid striped bass (Cheng et al., 2012), and red drum (Cheng et al., 2011). Among these studies, the best results were found with Arg supplementation from 1.4 to 3.6% of the diet and/or when included along with supplemental Gln.

Tilapia *Oreochromis* sp. holds a notable position as it has favorable characteristics already well known to intensive production systems. Robustness, rapid growth, year-round production and great market acceptance make Nile tilapia the second most important fish in global aquaculture, and the most important cultured fish in Brazil accounting for 41% of the national aquaculture production (ACEB, 2014; FAO, 2016).

We designed this study aiming to investigate the effects of dietary glutamine and/or arginine supplementation on growth performance, innate immune responses, circulating AA profiles and whole-body AA composition of Nile tilapia *Oreochromis niloticus*. To the best of our knowledge, this is the first experiment to investigate the effects of Gln and Arg combined in diets for Nile tilapia.

2. Materials and methods

2.1 Experimental diets and feeding trial

A control diet was formulated from menhaden fishmeal and dehulled soybean meal to contain 36% crude protein and meet the tilapia's nutritional

requirements based on the most recent publication of the National Research Council (NRC, 2011) (Table 1). That Control diet was analyzed to contain 0.37% glutamine and 2.01% arginine. Six experimental diets composed of the same ingredients were formulated to contain different levels of supplemental Gln and/or Arg (GLN 1%, GLN 2%, ARG 1%, ARG 2% and GLN+ARG 1%) as shown in Table 1. Diets were maintained iso-nitrogenous by adjusting the glycine (Gly) level as previously reported for fish (Buentello and Gatlin, 2000; Cheng et al., 2012). The Gly was chosen because it does not act as a precursor of arginine or glutamine in their metabolic turnover (Buentello and Gatlin, 2001, 2000), and it is structurally the simplest AA. Experimental diets provided arginine at a level above that previously established to meet the minimum requirement of Nile tilapia (NRC, 2011).

All ingredients were weighed individually and mixed in a commercial V-mixer for 30 min for each experimental diet prior to the addition of oil and water and mixing for another 30 min. Pellets were manufactured by passing the moistened mixture through a 3-mm die using a Hobart meat grinder as described by (Webb and Gatlin, 2003). Lastly, pellets were broken and sieved to 2–3-mm in length and stored at −18 °C during the feeding trial.

The feeding trial was conducted in an indoor recirculation system at the Aquacultural Research and Teaching Facility, Texas A&M University, Texas, USA. The culture system was equipped with a biofilter for ammonia removal and sand filter for mechanical filtration. Water flow rate remained of 1 L min^{-1} throughout the experiment. Juvenile Nile tilapia, genetically modified to be all males, were obtained from a commercial producer (Louisiana Specialty Aquafarms. Robert, LA). A period of 2 weeks was applied to acclimate the fish to the experimental conditions during which all fish were fed the control diet. After the conditioning period, fish initially averaging 7.14 ± 0.15 g each were placed into 24, 38-L glass aquaria at a density of 15 fish.

Table 1

Formulation and analyzed chemical composition of six experimental diets supplemented with arginine and/or glutamine for Nile tilapia.

Ingredient (g/100 g)	**Control**	**GLN 1%**	**GLN 2%**	**ARG 1%**	**ARG 2%**	**GLN+ARG 1%**
Menhaden fish meal[a]	11.00	11.00	11.00	11.00	11.00	11.00
Soybean meal[b]	43.26	43.26	43.26	43.26	43.26	43.26
Dextrinized starch[c]	23.25	23.25	23.25	23.25	23.25	23.25
Soy oil[d]	4.58	4.58	4.58	4.58	4.58	4.58
Vitamin premix[e]	3.00	3.00	3.00	3.00	3.00	3.00
Mineral premix[e]	4.00	4.00	4.00	4.00	4.00	4.00
Carboxymethyl cellulose[c]	2.00	2.00	2.00	2.00	2.00	2.00
Calcium phosphate, dibasic[f]	1.00	1.00	1.00	1.00	1.00	1.00
DL-Methionine[g]	0.25	0.25	0.25	0.25	0.25	0.25
Glycine[g]	3.95	2.92	1.89	2.22	0.50	1.20
Cellulose[c]	3.71	3.74	3.77	4.44	5.16	4.46
Glutamine[g]	0.00	1.00	2.00	0.00	0.00	1.00
Arginine[g]	0.00	0.00	0.00	1.00	2.00	1.00

Analyzed composition (g/100 g dry sample)						
Dry matter	89.3	89.5	89.3	89.3	89.3	89.6
Crude Protein	36.0	36.3	36.0	36.3	35.7	35.7
Lipids	8.0	7.6	7.9	7.7	8.3	8.0
Ash	8.7	8.6	9.9	8.6	8.7	8.6
Glutamine[h]	0.37	1.17	1.59	0.27	0.28	0.81
Arginine[i]	2.01	2.37	2.17	3.56	4.38	3.34
Glycine[i]	1.49	1.28	1.01	0.94	0.72	0.85

[a] Crude protein 67.0%, crude lipid 15%, dry matter 92%.
[b] Crude protein 54.0%, crude lipid 4%, dry matter 94%.
[c] MP Biomedicals, Santa Ana, CA, USA.
[d] Commercial refined soybean oil, TX, USA.
[e] Moon & Gatlin III (1991).
[f] Fisher Scientific, Waltham, MA, USA.
[g] USB Corporation, Cleveland, OH, USA.
[h] Analyzed as non-protein bound amino acid.
[i] Analyzed as protein-bound amino acid

The six dietary treatments were each randomly assigned to four replicate aquaria, for a total of 60 fish per treatment. Fish were fed twice a day (at 8:00 am and 4:00 pm) for 9 weeks. The feeding rate was set at 5% of fish body weight during weeks 1 through 6 and 4% during weeks 7 through 9. Fish in each tank were weighted as a group each week and feed quantities adjusted accordingly.

Water quality parameters were monitored weekly during the feeding trial and measured according to APHA (1992) procedures. Throughout the trial, the average water temperature was 27.9 ± 2.1 °C, dissolved oxygen was 5.52 ± 1.97 mg L^{-1}, pH was 8.3 ± 0.24, ammonia was 0.15 ± 0.10 mg L^{-1}, nitrite was 0.03 ± 0.03 mg L^{-1}, and salinity was 0.69 ± 0.06 g L^{-1} A 12-h light/dark photoperiod was provided by fluorescent lights controlled by timers and temperature was controlled by conditioning ambient air. Procedures used in this study were approved by the Texas A&M University System Animal Care and Use Committee.

2.2 Sampling procedures

At the end of the feeding trial, all fish were counted and weighed to determine survival, weight gain, feed intake, feed efficiency, protein efficiency, and protein retention. The sampling collection was divided into two time periods. The first period was 6 h after the last feeding while the second was 18 h sampled after feeding. Three fish per aquarium (n = 12/treatment) were randomly sampled after being euthanized with tricaine methanesulphonate (Western Chemical, Ferndale, WA, USA, 300 mg L^{-1}). Fish from the 6 h sampling period were sampled for whole-body proximate composition and AAs profile analysis. Fish from the 18 h sampling period were collected for condition index including hepatosomatic index (HSI), intraperitoneal fat (IPF) ratio and fillet yield, as well as innate immune responses from spleen and kidney samples. Blood samples were collected at both time periods, 6 h and 18 h for AA profile of plasma. All blood

samples (~1 mL) were obtained from the caudal vasculature with heparinized needles (1-mL syringe, 23-ga needle) and, in turn, plasma was separated by centrifugation at 3800 ×g for 12 min. Whole-body and plasma samples were stored at −80 °C until analysis.

Growth performance and body condition indexes data were computed using the following calculations a) weight gain % = [(*final weight − initial weight/initial weight) × 100*]; b) feeding intake rate % = (*100 × [dry feed intake*/*square root of initial body weight × final body weight* (*g*))/*days on feed*]; c) feed efficiency ratio = (*weight gain*/*dry feed intake*); d) protein efficiency ratio = (*weight gain*/*dry protein fed*) and e) protein retention={[(*final body weight (g) × final body protein* (*%*)) − (*initial body weight* (*g*) × *initial body protein* (*%*))]/(*protein intake* (*g*)} × *100*; f) HSI (%) = [(*liver weight*/*fish weight*) × *100*] and g) IPF ratio (%) = [(*IPF weight*/*fish weight*) × *100*].

2.3 Proximate composition: diets and whole-body

The composition of whole-body samples was determined on pooled samples of three fish per aquarium (n = 4/treatment). Dry matter, crude protein, crude lipid, and ash were determined in diets and whole-body using standard procedures as previously described (Webb and Gatlin, 2003; Wu et al., 2015) and approved (AOAC, 1993).

2.4 Innate immune responses

The ability of phagocytes to produce extracellular (SEC) and intracellular (SIC) superoxide anion was analyzed in ex vivo samples from fish fed each experimental diet following established methodology (Sealey and Gatlin, 2002; Secombes, 1990). Immediately after dissection, both head and trunk kidney

samples from three fish per aquarium were pooled (n = 4/treatment) and placed into Leibowitz 2% cell culture media (L-15 enriched with 2% fetal calf serum and 100 U/mL penicillin). Phagocytes were isolated, enumerated and their viability assessed via trypan blue exclusion, as described in Buentello and Gatlin (1999). Viability was > 95% in all cases. Kidney phagocyte samples (100 μL of 2×10^6 cells/mL suspension) were incubated in the dark for 2 h at 25 °C, after which the supernatant was removed and the attached cells washed twice with warm (18–20 °C) L-15 media, in order to form a cell monolayer in the 96-well microplates.

For SEC, the phagocyte monolayer was covered with 100 μL of 2 mg/mL ferricytochrome C from equine heart (C2506, Sigma Aldrich) with 1 μg/mL phorbol 12-myristate 13-acetate (PMA, P8139, Sigma Aldrich) in phenol red-free Hank's balanced salt solution (HBSS). Following, the absorbance was read on a spectrophotometer every 5 min for 30 min at 550 nm. The amount of SEC/well was calculated as: $[(\Delta_{absorbance} \times 100)/6.3]$.

For SIC, the phagocyte monolayer was covered with 100 μL of 1 mg/mL of nitrotetrazolium blue (N6876, Sigma Aldrich) with 1 μg/mL PMA in phenol red-free HBSS and incubated in the dark for 45 min at 25 °C. Following, the supernatant was gently removed, the phagocyte monolayer was incubated with 200 μL of 70% MeOH in the dark for 10 min at 25 °C and then washed and dried in the dark for 45min at 25 °C and then, 120 μL of 2M KOH was added. Lastly, the phagocyte monolayer was covered with 140 μL of dimethyl sulfoxide (DMSO D8418, Sigma Aldrich) and the absorbance was immediately read in a spectrophotometer at 620 nm. The amount of SIC/well was calculated as follows: $(A_{absorbance\ sample} - A_{absorbance\ control\ (SOD)})$. Superoxide dismutase (SOD) from bovine erythrocyte (S7571-75KU, Sigma Aldrich) was included in both SEC and SIC assays as a negative control (600 U/mL) to block the reaction and verify specificity.

Neutrophil oxidative radical production (NBT) was determined as described by Siwicki et al. (1994); in fresh whole blood samples from three fish per aquarium (n=12/treatment). Blood samples (100 µL) were incubated with 100 µL of nitrotetrazolium blue chloride for 30 min at 25 °C, then 50 µL of this mixture was transferred into a microplate with 1 mL of N, N-dimethyl formamide (D4551, Sigma Aldrich) and afterward centrifuged at 3000 ×g for 5 min. The supernatant was transferred to cuvettes and absorbance read in a spectrophotometer at 545 nm. Absorbance was converted to nitrotetrazolium blue chloride units/mL of blood based on a standard curve of nitrotetrazolium blue diformazan solution: $40 \times (A_{absorbance545} - 0.0245)/5.8564$.

Lysozyme activity was determined in pooled plasma (LYZ–P) and spleen (LYZ–S) from three fish per aquarium (n = 4/treatment) using a turbidimetric assay described by (Jørgensen et al., 1993). One slight modification was to adjust the pH of the *Micrococcus lysodeikticus* (M0508, ATCC No 4698, Sigma Aldrich) to 6.0 to maximize the activity, as determined in preliminary assays with tilapia plasma in this laboratory (data not shown). The LYZ–S was determined in extracts of spleen tissue homogenized in four volumes (*w/v*) of 0.1 M Tris/HCl Buffer (pH 7.8) and centrifuged at 13,000 ×g for 30 min at 4 °C. Following the centrifugation, the supernatant was collected and used as the crude enzyme solution. In both assays, plasma and spleen, 10 µL of sample was mixed with 200 µL *Micrococcus lysodeikticus* suspension in PBS at pH 6.0 and absorbance read in a spectrophotometer at 450 nm. The lysozyme activity (unit/mL) was defined as the amount of enzyme producing a decrease in absorbance using the following formula: $[(A_{absorbance\ (4\ -\ 1\ min)}/3)/0.001] \times 100$. Lysozyme activity assays were similar as described for red drum and channel catfish by Sutili et al. (Sutili et al., 2016a, 2016b), Pohlenz et al. (2014) and Cheng et al. (2012).

Hemolytic activity of the complement system (HACS) was determined as described for red drum by (Sutili et al., 2016a, 2016b) and adapted from Morales-

delaNuez et al. (2009) using sheep red blood cells as targets. Total hemolysis control (distilled water + sheep blood) and spontaneous lysis (PBS 0.1 M + sheep blood) were performed individually. Tilapia plasma samples (75 μL) (n=12/treatment) were incubated at room temperature for 15 min with sheep blood (25 μL) and then, cold-PBS (100 μL) was added and the microplate was centrifuged at 3000 ×g for 5 min at 4 °C. The supernatant was transferred to 96-well microplates and the absorbance of the samples was read in a spectrophotometer at 405 nm. Percentage of hemolysis (%) of the saline solution for each tilapia sample was calculated relative to the respective total hemolysis as follows: $[(A_{405\ sample} - A_{405\ no\text{-}hemolysis})/(A_{405\ total\ hemolysis} - A_{405\ no\text{-}hemolysis})] \times 100$.

2.5 Amino acids profiles in plasma, whole-body and diets

Plasma circulating AA profiles were determined at both 6 h and 18 h after feeding. These sampling times were chosen to provide comparisons between post-absorptive dynamics at those two time periods and compare with other fish species fed diets with intact protein and free AAs (Ambardekar et al., 2009; Schuhmacher et al., 1997).

Amino acid profiles in plasma (free AAs), whole-body (protein-bound) and diets (protein-bound) were quantified using ultra-performance liquid chromatography (UPLC-Acquity system®, Waters™ Corporation) with an integrated tunable ultraviolet (UV) detector and MassTrak AAA Solutions Kit (186,004,094, Waters) following established methodology (Ambardekar et al., 2009; Castillo et al., 2015; Pohlenz et al., 2012a; Schuhmacher et al., 1997). The MassTrak kit uses pre-column derivatization of AAs with a 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate tag (AccQTag), followed by reversed phase UPLC on a C18 column (1.7 μm; 2.1 × 150 mm) and UV detection at 260 nm. Analyzed AAs concentrations in all samples were confirmed the targeted values

with SDs no N0.05 g/100 g. Samples were deproteinized with 1.5 M HClO4 (9552-05, J.T. Baker, Phillipsburg, NJ) and neutralized with 2MK2CO3 (P5833, Sigma Chemical) before derivatization. Total glutamine content in whole-body and diet samples were achieved as non-protein bound after fast hydrolysis. Plasma AAs concentration was expressed in nmol mL^{-1} while whole-body AAs composition was expressed as % of sum according to Helland and Grisdale-Helland (2011, 2006).

2.6 Statistical analysis

All data were analyzed through the GLM Procedure of SAS version 9.4. One-way ANOVA was performed and, the model included fixed effects of diets on performance, proximate composition, immune responses and AA profiles in plasma and whole-body. Normality and homogeneity of variance were evaluated by Shapiro-Wilk' and Levene's test, respectively. The significance level was set at p b 0.05 and differences among means were compared by Tukey's test. Results were reported as least square means with standard error mean (S.E.M) and coefficient of variation (C.V.).

3. Results

3.1 Growth performance

Dietary glutamine and/or arginine supplementation resulted in significant effects on weight gain, feed efficiency ratio, protein efficiency ratio and protein retention, but not in survival after 9 weeks of feeding (Table 2). Overall, fish fed with GLN + ARG 1% diet had the greatest values for all performance indicators, although these values were not different from those fish fed the Control, GLN and

ARG supplemented diets in a few cases. The weight gain was higher in fish fed with GLN+ARG 1% or GLN 2% followed by ARG 1%, Control, ARG 2% and lastly GLN 1% whichwas significantly lower than all others. The feed intake was lower in fish fed with GLN+ARG 1%, Control, and GLN 1–2% while both ARG 1–2% supplementation resulted in higher feed intake.

The feed efficiency ratio was greater for GLN + ARG 1%, despite not being significantly different from the Control and GLN 1%. Similar findings were observed regarding both protein efficiency and protein retention where fish fed with GLN+ARG 1% had greater values, although not significantly different from those fed the Control diet. Furthermore, in general, fish fed with ARG-supplemented diets had the lowest growth performance values.

3.2 Whole-body composition and condition indexes

Moisture, crude protein and ash concentrations in the whole body of Nile tilapia were not significantly affected by dietary GLN and/or ARG supplementation of the diet (Table 3). Similarly, none of the conditions indexes, HSI, IPF and fillet yield, were affected in the fish fed the GLN and/or ARG supplemented diets (Table 3).

3.3 Innate immune responses

Among the immunity parameters analyzed, only spleen lysozyme activity (LYZ–S) was significantly increased in fish fed the GLN 2% and GLN+ARG 1% diets as shown in Table 4. However, no effects on respiratory burst (SEC, SIC, NBT), plasma lysozyme activity (LYZ–P) or hemolytic activity of the complement system (HACS) were observed with dietary supplementation of Gln and/or Arg.

Table 2

Growth performance of juvenile Nile tilapia fed diets supplemented with glutamine and/or arginine at two different levels for 9 weeks.

	Control	**GLN 1%**	**GLN 2%**	**ARG 1%**	**ARG 2%**	**GLN+ARG 1%**			
Glutamine Arginine	0.37 2.01	1.17 2.37	1.59 2.17	0.27 3.56	0.28 4.38	0.81 3.34	**S.E.M**	**C.V.**	**Pr > F***
Survival, %	98.3	98.3	98.3	93.3	100	98.33	1.85	4.36	0.3817
Weight gain[1]	589^{ab}	538^{c}	607^{a}	590^{ab}	571^{ab}	635^{a}	12.69	4.38	0.0088
Feed intake[2]	3.77^{b}	3.70^{b}	3.80^{b}	3.89^{ab}	4.13^{a}	3.81^{b}	0.03	4.44	0.0010
Feed efficiency[3]	0.93^{ab}	0.93^{ab}	0.91^{bc}	0.84^{d}	0.86^{cd}	0.97^{a}	0.01	2.06	<.0001
Protein efficiency[4]	2.59^{ab}	2.52^{b}	2.47^{b}	2.31^{c}	2.42^{bc}	2.71^{a}	0.02	2.26	<.0001
Protein retention[5]	40.53^{a}	38.27^{bc}	39.77^{b}	35.09^{c}	39.62^{b}	40.08^{a}	0.61	2.94	0.0012

Initial fish average weight was 7.22; 7.12; 7.18; 7.17; 7.02 and 7.13, (S.E.M ± 0.03) respectively.
Final fish average weight was 47.04; 46.13; 49.49; 48.52; 47.10 and 51.64, (S.E.M ± 0.82) respectively.
Results obtained from One-way ANOVA and Tukey's test. Different superscript letters within a row indicate significant differences ($p < 0.05$).
1 Weight gain, % = [(final weight − initial weigh/initial weight) × 100].
2 Feed intake, % = (100 × [dry feed intake/square root of initial body weight − final body weight (g))/days on feed].
3 Feed efficiency ratio, %= (weight gain/dry feed intake).
4 Protein efficiency ratio, % = (weight gain/dry protein fed).
5 Protein retention, % = [(protein finalWB ∗ weight final − n (protein initialWB ∗ weight initial)/total protein fed) × 100].

Table 3

Body condition indexes and whole-body composition of juvenile Nile tilapia fed diets supplemented with glutamine and/or arginine at two different levels for 9 weeks.

	Control	**GLN 1%**	**GLN 2%**	**ARG 1%**	**ARG 2%**	**GLN+ARG 1%**			
Glutamine Arginine	0.37 2.01	1.17 2.37	1.59 2.17	0.27 3.56	0.28 4.38	0.81 3.34	**S.E.M**	**C.V.**	**Pr > F***
HSI[a]	2.49	2.63	2.8	2.31	2.37	2.52	0.33	12.69	0.3341
IPF[b]	0.71	0.58	0.65	0.6	0.56	0.63	0.16	27.62	0.8708
Fillet yield, %	23.61	24.04	23.89	23.24	24.33	24.17	1.29	5.86	0.8910
Moisture, %	74.87	74.46	75.00	74.98	74.01	75.72	1.18	1.62	0.5765
Crude Protein, %	15.69	14.31	16.17	15.36	16.07	14.89	1.07	6.14	0.0881
Lipids, %	4.73	4.82	4.25	5.27	4.97	5.07	0.54	9.80	0.1035
Ash, %	4.37	3.82	4.61	4.07	4.31	4.34	0.71	17.72	0.8042

Results obtained from One-way ANOVA and Tukey's test. Different superscript letters within a row indicate significant differences ($p < 0.05$).

[a] HSI: Hepatosomatic index, % = [(liver weight / fish weight) × 100]

[b] IPF: Intraperitoneal fatty, % = [(IPF weight / fish weight) × 100]

3.4 Plasma amino acids profiles

Plasma free AA concentrations at 6 h and 18 h sampling time periods are shown in Table 5. Overall, higher AA concentration with the exception of Gly, were observed at 6 h regardless of diet. Numerically, the highest concentration of indispensable AAs was observed for Arg followed by Val, Phe, Leu, Lys, Ile, Thr, Met, His and Cys. Regarding dispensable AA, the highest values were observed for Asp, Ser, and Gln and then, followed Ala, Tau, Glu, Gly, Pro, Tyr and Hyp.

The dietary supplementation of GLN and/or ARG significantly affected the concentration of free AAs in plasma at 6 h and 18 h. The AAs concentrations significantly affected at 6 h sampling were Cys, Asp, Ser, Gly and Hyp while at 18 h, differences were observed for Arg, Val, Cys, Ser, Gly and Pro (Table 5).

The concentration of Gly at 18 h was higher in fish fed the Control diet, with an intermediate concentration found in the fish fed the GLN 1% diet, followed by the other diets (not different among each other). Fish fed the Control and GLN 1% diets had a higher concentration of Ser at 6 h and, for Asp at 6 h.

The lowest amounts for Asp and Ser at 6 h were observed in the fish fed the ARG 1%, ARG 2% and GLN + ARG 1% diets. Additionally, fish fed the Control diets had a higher concentration of Hyp while those fish fed the ARG 2% diet showed the lowest amount.

Fish fed the ARG 2% diet showed the larger concentration of Arg, Val and Pro at 18 h, and for Cys at 6 h and 18 h. Additionally, fish fed the GLN + ARG 1% diet did not differ from those fed the Control and GLN 1% diets for Gly (6 h), Arg, Val and Pro at 18 h.

Table 4

Innate immune responses of juvenile Nile tilapia fed with diets supplemented with glutamine and/or arginine at two different levels for 9 weeks.

	Control	**GLN 1%**	**GLN 2%**	**ARG 1%**	**ARG 2%**	**GLN+ARG 1%**			
Glutamine Arginine	0.37 2.01	1.17 2.37	1.59 2.17	0.27 3.56	0.28 4.38	0.81 3.34	**S.E.M**	**C.V.**	**Pr > F***
SEC[1]	8.69	8.62	9.29	9.51	9.14	9.16	0.15	8.51	0.5672
SIC[2]	0.611	0.670	0.872	0.847	0.960	0.878	0.039	23.24	0.1383
NBT[3]	4.46	4.01	4.16	3.94	4.08	4.08	0.07	6.41	0.4476
LYZ–P[4]	487.96	481.48	498.15	456.17	476.39	466.67	22.20	21.58	0.9952
LYZ–S[5]	377.78^{b}	574.07^{ab}	897.22^{a}	555.56^{ab}	616.67^{ab}	1077.78^{a}	44.13	28.45	0.0074
HACS[6]	56.18	61.39	55.80	58.71	58.58	59.07	1.84	15.71	0.9640

Results obtained from One-way ANOVA and Tukey's test. Different superscript letters within a row indicate significant differences ($p < 0.05$).

[1] Superoxide anion extracellular $^{-}$EC, nmol O_2^{-} from kidney macrophage.

[2] Superoxide anion intracellular $^{-}$IC, absorbance at 620 nm from kidney macrophage.

[3] Neutrophil oxidative radical production, nitroblue tetrazolium units expressed in mg/mL from blood.

[4] Plasma lysozyme activity, units/mL.

[5] Spleen lysozyme activity, units/mL.

[6] Plasma hemolytic activity of complement system, % of hemolysis.

3.5 Whole-body amino acids composition

The AAs concentrations in the Nile tilapia whole-body, as a percentage of the sum of useful amino acids (those that can be synthesized into protein), are shown in Table 6. Few differences were observed regarding the AA composition of whole-body tissues. Experimental diets most significantly affected the concentrations of Gln and Gly as well as Ser. The concentration of Gln in whole-body was highest in fish fed the ARG 2% diet and differed significantly from that of the Control diet. In contrast, the Ser concentration was higher in fish fed the Control diet and lowest in fish fed the ARG 2% diet. Whole-body Gly was highest in fish fed the GLN 1% diet and lowest in fish fed the ARG 1% diet.

4. Discussion

4.1 Growth performance

The described feeding trial with Nile tilapia emphasizes the importance of dietary GLN and ARG on performance indicators and AAs metabolism. Based on growth performance, the combined supplementation of Gln plus Arg at 1% to an otherwise nutritionally complete diet showed the best results due mainly to greater feed efficiency and weight gain. These results are in agreement with previous studies in which the dietary supplementation of Gln and Arg to hybrid striped bass (Cheng et al., 2012) and red drum (Cheng et al., 2011) also resulted in improved growth by the combination supplementation of Gln and Arg at 1%. However, the specific mechanism by which Arg and/or Gln may affect fish somatic growth and nutrient efficiency needs to be further elucidated.

Although the current feeding trial was designed to investigate the effects of Gln and Arg on Nile tilapia nutrition, the observations regarding Gly are also

interesting. The Gly was included in the experimental diets to adjust the nitrogen content, but this inclusion resulted in effects on the growth performance and physiological parameters. The higher Gly inclusion was in Control (3.95%) and GLN 1% (2.92%) diets while the lowest was in ARG 2% (0.50%) and GLN + ARG 1% (1.20%) diets as shown in Table 1. Although GLN + ARG 1% diet had the highest values for growth performance, these results were, in some cases, similar to the results obtained by fish fed the Control and GLN 1% diets. Hence, it is possible that Gly conferred some beneficial effect on the growth of tilapia. The performance improvement by dietary Gly have been reported in tilapia (Gaye-Siessegger et al., 2007; Mambrini and Kaushik, 1994; Xie et al., 2016), grass carp (Jin et al., 2016) and rainbow trout (Schuhmacher et al., 1995). Despite the goal and the methodological approaches in those experiments were slightly different, the results are generally in agreement with the findings in the current study with tilapia.

The Gly functions include synthesis and structure of collagen proteins and it is a major AA for the conjugation of bile acids (Bender, 2012). Gly is also a precursor for many antioxidants, creatine, and uric acid (Senthilkumar et al., 2004), and has been regarded to have an important role in anti-oxidative ability in fish (Xie et al., 2016). Moreover, Gly is involved with modulation of intracellular Ca^{2+} and it is a neurotransmitter in the central nervous system (Wu, 2013b). Despite the physiological importance of Gly, its dietary requirements are not currently available for fish (NRC, 2011).

Table 5 Plasma free-amino acid profiles at 6h and 18h post feeding from juvenile Nile tilapia fed diets with glutamine and/or arginine at two different levels for 9 weeks. Amino acid concentrations in plasma are expressed as nmol mL^{-1}.

		Control	GLN 1%	GLN 2%	ARG 1%	ARG 2%	GLN+ARG 1%	S.E.M	C.V.	Pr > F*
Glutamine		0.37	1.17	1.59	0.27	0.28	0.81			
Arginine		2.01	2.37	2.17	3.56	4.38	3.34			
Indispensable AA										
Arg	6h	122.3	122.6	116.8	108.0	124.1	104.6	17.5	33.87	0.9683
	18h	29.5^{ab}	29.2^{ab}	26.8^{b}	26.9^{b}	43.1^{a}	31.7^{ab}	2.47	18.28	0.0070
Val	6h	76.7	68.2	62.7	56.3	73.6	60.3	12.2	38.84	0.8571
	18h	36.5^{ab}	35.7^{ab}	35.9^{ab}	28.1^{c}	42.8^{a}	37.3^{ab}	2.50	16.75	0.0096
Phe	6h	63.1	52.3	43.1	45.3	36.5	40.8	10.4	50.03	0.6562
	18h	41.5	36.7	29.1	30.1	30.5	36.4	4.55	30.17	0.4958
Leu	6h	48.4	44.1	42.0	41.1	50.1	38.8	8.5	43.43	0.9536
	18h	23.9	22.8	23.8	30.4	27.8	23.6	2.61	25.93	0.5411
Lys	6h	43.8	38.5	33.3	40.2	47.7	32.4	5.7	33.14	0.5431
	18h	39.2	37.1	34.0	29.4	41.3	32.9	2.95	17.27	0.1255
Ile	6h	34.4	40.4	43.0	45.6	42.8	35.1	8.2	43.17	0.9200
	18h	26.1	31.6	35.6	31.0	34.4	33.1	7.37	49.36	0.9662
Thr	6h	28.5	25.6	20.5	26.6	26.9	21.9	3.3	26.96	0.5256
	18h	16.6	14.7	13.9	15.6	24.8	17.0	2.12	28.92	0.0656
Met	6h	26.0	28.7	23.7	20.4	25.2	24.5	3.8	35.25	0.8482
	18h	15.2	11.2	12.4	14.1	15.4	12.8	1.47	23.85	0.4125
His	6h	21.7	20.6	19.7	19.5	22.0	19.2	2.6	27.03	0.9662
	18h	13.5	14.4	14.3	14.9	16.1	13.0	1.38	21.12	0.7657
Cys	6h	13.3^{b}	11.4^{b}	8.5^{b}	15.6^{b}	29.9^{a}	15.1^{b}	2.5	34.99	0.0006
	18h	10.2^{b}	8.9^{b}	8.7^{b}	10.5^{b}	19.9^{a}	12.1^{b}	0.80	15.99	<.0001

Dispensable AA										
Asp	6h	127.8^{a}	99.3^{a}	58.9^{bc}	48.3^{bc}	41.44^{c}	43.3^{c}	10.18	32.01	<.0001
	18h	22.3	21.0	21.9	21.4	23.7	22.5	0.83	8.58	0.4319
Ser	6h	121.2^{a}	105.3^{ab}	54.4^{bc}	43.4^{c}	41.0^{c}	39.6^{c}	11.57	38.22	0.0004
	18h	66.2^{a}	41.0^{ab}	33.0^{b}	30.8^{b}	21.1^{b}	23.3^{b}	4.59	34.59	0.0010
Gln	6h	127.8	115.8	106.0	113.7	143.1	104.1	15.55	29.55	0.6263
	18h	86.3	84.1	89.3	86.8	109.8	70.8	9.07	22.31	0.1981
Ala	6h	81.4	77.8	55.8	47.3	66.3	57.6	10.88	36.67	0.3177
	18h	36.6	32.1	38.1	31.3	38.8	34.4	2.21	14.93	0.2599
Tau	6h	51.6	47.2	55.6	52.5	42.7	41.3	5.05	21.79	0.3608
	18h	54.8	38.9	50.9	42.9	41.2	44.3	5.12	24.09	0.3332
Glu	6h	46.8	67.4	56.6	56.6	54.6	47.9	7.90	29.52	0.5373
	18h	28.9	41.6	34.9	34.9	33.7	29.5	4.87	29.52	0.5370
Gly	6h	34.7^{ab}	30.6^{ab}	27.5^{b}	51.6^{ab}	66.3^{a}	37.1^{ab}	7.35	40.05	0.0306
	18h	68.7^{a}	55.0^{ab}	44.4^{b}	43.6^{b}	35.5^{b}	40.5^{b}	3.87	21.19	0.0028
Pro	6h	29.9	24.7	20.7	25.2	25.8	18.7	3.71	34.73	0.5044
	18h	20.4^{b}	16.6^{b}	17.6^{b}	21.9^{ab}	33.53^{a}	21.6^{ab}	1.87	21.87	0.0014
Hyp	6h	25.85^{a}	23.23^{ab}	18.75^{ab}	19.84^{ab}	16.49^{b}	19.36^{ab}	1.83	18.94	0.0385
	18h	17.52	17.18	14.70	15.17	14.25	16.96	1.83	25.72	0.7887
Tyr	6h	18.6	27.8	17.2	18.4	22.9	14.9	3.93	52.12	0.5683
	18h	15.4	11.9	11.7	14.0	18.6	15.5	2.88	43.93	0.6602

Results obtained from One-way ANOVA and Tukey's test. Different superscript letters within a row indicate significant differences ($p < 0.05$).

4.2 Whole-body composition and condition indexes

In the present study dietary GLN and/or ARG did not affect the proximate composition of whole-body tissues or body condition indexes which is contradictory to some previous studies in tilapia. Similar to the findings in the present study, Yue et al. (2013) studying different supplementation levels of Arg, and Graciano et al. (2014) and Da Silva et al. (2010) studying Gln supplementation in tilapia also reported no differences in whole-body composition. In contrast, Neu et al. (2016) reported changes in the moisture and ash content in the body of tilapia fed increasing levels of Arg.

4.3 Innate immune responses

Most of the innate immunity indicators measured in the present study, except for LYZ–S, were not raised by dietary levels of Gln and/or Arg, probably a reflection of the non-activated state of the immune cells because neither neutrophils nor phagocytes were exposed to living or non-living pathogen during the feeding trial.

In the present study, fish fed the GLN 2% and GLN + ARG 1% diets had increased LYZ–S. Lysozyme is a widely expressed enzyme and one of the fundamental components of the immune system in fish. As a potent antimicrobial element, the lysozymes (EC 3.2.1.17) catalyze the hydrolysis of β-(1, 4)-glycosidic linkages between N-acetylmuramic acid and N-acetyl-d-glucosamine residues in a peptidoglycan that is found in the cell walls of Gram-positive bacteria (Saurabh and Sahoo, 2008). In fish, lysozyme is highly expressed in the hematopoietic organs and mucosal tissues such as spleen, kidney, intestine, and gills (Kim and Nam, 2015; Saurabh and Sahoo, 2008), but is known to be synthesized in the liver of blue tilapia as well (Gao et al., 2012). The lysozyme

activity is dependent on stressor factors (degree, intensity and duration), infection conditions and/or nutrition (Saurabh and Sahoo, 2008). However, to date, no study reporting the effects of dietary Gln and/or Arg supplementation on the spleen lysozyme activity was available in fish.

Although the laboratory assays employed immunostimulatory substances, such as PMA, it was not sufficient to trigger an immune response in Nile tilapia. Also, LYZ– P and SIC results had a high degree of variability among treatments; therefore, the numerical differences were not statistically significant. An important consideration is that the activity of plasma LYZ– P is not a direct measurement of cell performance, but reflects the presence of this muramidase in plasma and it can also be linked to the turnover rate of granulocytes and monocytes (Hansen, 1975; Yano, 1997). Even though SIC methodology is well established for a number of fish, including channel catfish (Cheng et al., 2012; Pohlenz et al., 2014), red drum(Cheng et al., 2011) and hybrid striped bass (Sealey and Gatlin, 2002), this was the first time that SIC methodology was performed on Nile tilapia. Therefore, the SIC discrepancies among the results could possibly indicate either an insufficient internalization of PMA or inadequate amount of SOD addition to the media (Sealey and Gatlin, 2002).

Overall, because no challenge was carried out in the current study, the immune system was under homeostatic state and consequently, no difference among treatments would be expected. In contrast, although differences in the immunity parameters have been reported in species unchallenged such as jian carp (Hu et al., 2015), channel catfish (Pohlenz et al., 2014), hybrid striped bass (Cheng et al., 2012) and red drum (Cheng et al., 2012), it is important to emphasize that immune system is affected by many factors. It may be explained in terms of differences in fish species physiology and variable experimental procedures employed (Grayfer et al., 2014; Kiron, 2012; Wiegertjes et al., 2016).

Table 6

Whole-body amino acid (AA) composition of juvenile Nile tilapia fed diets with glutamine and/or arginine at two different levels for 9 weeks. Amino acid concentrations are expressed as % of sum of AA.

	Control	**GLN 1%**	**GLN 2%**	**ARG 1%**	**ARG 2%**	**GLN+ARG 1%**			
Glutamine	0.37	1.17	1.59	0.27	0.28	0.81	**S.E.M**	**C.V.**	**Pr > F***
Arginine	2.01	2.37	2.17	3.56	4.38	3.34			
Indispensable AA									
Arg	6.14	6.08	6.05	6.08	6.06	6.10	0.67	2.01	0.9213
Val	4.78	4.70	4.77	4.70	4.61	4.76	0.05	2.93	0.5287
Phe	4.14	4.10	4.15	4.17	3.96	4.09	0.08	3.61	0.4411
Leu	7.59	7.44	7.57	7.49	7.26	7.57	0.03	3.16	0.3882
Lys	8.32	8.04	8.09	7.95	7.92	8.36	0.05	3.92	0.2793
Ile	4.25	4.16	4.27	4.18	4.07	4.30	0.39	3.61	0.3421
Thr	13.66	13.43	13.53	13.41	13.36	13.79	0.06	1.81	0.2260
Met	2.46	2.44	2.48	2.51	2.43	2.48	0.14	3.30	0.7911
His	2.45	2.42	2.47	2.43	2.39	2.22	0.04	8.60	0.5703
Cys	0.24	0.24	0.25	0.25	0.25	0.25	0.10	6.40	0.5560

Dispensable AA									
Asp	7.85	7.70	7.75	7.67	7.60	7.95	0.19	2.56	0.1826
Ser	4.37^{a}	4.28^{ab}	4.25^{ab}	4.29^{ab}	4.11^{b}	4.20^{ab}	0.01	2.47	0.0496
Gln	4.65^{b}	6.85^{ab}	6.28^{ab}	6.60^{ab}	7.83^{a}	6.49^{ab}	0.29	16.05	0.0140
Ala	6.43	6.37	6.27	6.25	6.28	6.44	0.06	3.42	0.6951
Tau	0.89	0.92	0.91	0.90	0.89	0.89	0.04	7.69	0.9909
Glu	4.39	4.35	4.38	4.35	4.26	4.39	0.06	2.15	0.3599
Gly	8.39^{ab}	8.85^{a}	7.85^{ab}	7.53^{b}	8.16^{ab}	8.16^{ab}	0.32	5.53	0.0130
Pro	4.51	4.53	4.28	4.35	4.46	4.46	0.11	9.45	0.9507
Hyp	1.28	1.34	1.09	1.14	1.37	0.96	0.08	13.21	0.7780
Tyr	3.24	3.24	3.34	3.38	3.19	3.26	0.06	4.43	0.4465

Results obtained from One-way ANOVA and Tukey's test. Different superscript letters within a row indicate significant differences ($p < 0.05$).

Because Nile tilapia is known to be a robust freshwater fish specie, it is important to submit the fish to a challenge in order to investigate immune modulatory properties of nutrients (Pohlenz and Gatlin, 2014; Yue et al., 2013). However, such a disease challenge was not possible in the present study.

4.4 Plasma amino acids profiles

The experimental diets varied in terms of Gln, Arg and Gly contents. Changes in the plasma free concentrations of Arg, Val, Cys, Asp, Ser, Gly and Pro and Hyp were observed in this study (Table 5). The absorption dynamics of AA in plasma can be influenced by a variety of factors including the form of the AA in diets (free and protein-bound) and the types of ingredients contributing AAs. Free AAs appear in circulation more quickly than those from intact protein (e.g., Ambardekar et al., 2009; Schuhmacher et al., 1997). The supplementation of crystalline Arg, Gln and Gly in the present study likely influenced the dynamics of those amino acids in circulation.

The findings of this study in Nile tilapia regarding AAs biochemistry dynamics and its inter-relations are in agreement with previously reports in mammals (Bender, 2012; Wu, 2013b) and fish (Mambrini and Kaushik, 1994; NRC, 2011; Wu et al., 2015.

The Arg catabolism occurs via multiple pathways, generating nitric oxide, ornithine, urea, polyamines, proline, glutamate, creatine, agmatine, CO_2, and water (Wu, 2013b). In the present study, the highest Arg inclusion (ARG 2% diet) reflected the highest plasmatic concentration of this AA (Table 5). Arg as the most abundant nitrogen carrier in tissue protein participates in multiple synthetic pathways, including protein and Pro synthesis (Wu, 2009). Thus, it is quite possible that dietary supplementation of Arg in Nile tilapia fed the ARG 2% diet

contributed to the higher plasma concentration of Pro through intestine derived ornithine pathway both via synthesis or sparing effect of Pro (Wu, 2013b).

The higher Cys plasma concentration in fish fed the ARG 2% diet could possibly be explained through an interorgan-transsulfuration pathway between Arg and Cys via creatine (Bender, 2012; Wu, 2013b). In mammals, Arg is converted into creatine (and ornithine) through guanidinoacetate, and in turn, is converted into homocysteine which, lastly, is converted into Cys (Stead et al., 2001). This could possibly up-regulate the usage of Arg for the biosynthesis of Cys in target tissues (kidney and liver) of Nile tilapia in the present study. This Cys synthesis pathway has been described previously in Atlantic salmon (Nordrum et al., 2000) and rainbow trout (Yokoyama, 1998). Additionally, creatine consumes more methyl groups than all other methylation reactions in the body combined, therefore, creatine synthesis from Arg regulates the availability of methyl group donors for other methylation reactions, such as the synthesis of methionine from homocysteine. Thus, Arg can indirectly affect one-carbon-unit metabolism in the whole body (Wu, 2013b).

Fish fed the higher levels of dietary Gly (notably the Control and GLN 1% diets) showed effects on plasma concentrations of Gly and Ser (Table 5). Both Gly and Ser are readily interconvertible AAs and Gly can provide Ser as a precursor for gluconeogenesis (Wu, 2013b). Moreover, Gly and Ser are the main sources of one-carbon units used for biosynthetic reactions, forming methylene tetrahydrofolate through the actions of serine hydroxymethyltransferase and the Gly cleavage system in animal cells (Bender, 2012). These processes could potentially explain the increased concentrations of Gly and Ser in plasma. Besides, Gly supply would be in excess because both AAs levels were increased in plasma. The use of these AAs for energy also may be of considerable importance, due to the enhanced ability of fish to use dietary protein for energy (Mambrini and Kaushik, 1994).

Tilapia fed the ARG 2% diet resulted in a higher plasma concentration of Val although it was not statistically different fromthose of fish fed the Control, GLN 1–2%, and GLN+ARG 1% diets. Because Val is known as a purely glucogenic AA (Bender, 2012), it can be speculated that the increased plasma levels may have been trigged through energy supply pathways due the greater carbon skeletons being supplied by other AA, hence avoiding Val oxidation (Wu, 2013b). However, the potential regulatory mechanisms through which Arg, Gln and/or Gly play a role in Val turnover in Nile tilapia is hitherto unknown.

Asp can be formed from NH_3 and oxaloacetate in mammals and fish (Wu, 2013b). Results from the present study show the Asp was more concentrated in the plasma of fish fed the Control and GLN 1% diets. It is reasonable to assume that higher levels of dietary Gly could lead to ammonia excess due the increased AAs supplying energy in Nile tilapia. As such, higher levels of Asp may have been a mechanism for ammonia detoxification from AA catabolism. Similarly, the high concentrations of Hyp in those fish fed the Control and GLN 1% diets would be trigged by the Gly supply. Because Hyp is the major substrate for renal synthesis of Gly in animals (Bender, 2012), this supply could have resulted in a sparing effect of Hyp for Gly synthesis.

4.5 Whole-body amino acids composition

There are several ways to express AA concentrations in fish whole-body tissues, none of which are perfect due to the complex relationships with various components of the fish body. The sum of AA (Helland and Grisdale-Helland, 2006) was chosen to express AA composition of the fish protein. In the present study, among the AAs in whole-body tissues, only Gln, Gly and Ser were altered by the experimental diets.

Because intestinal metabolism may have a profound impact on AA composition, the discrepancies in patterns of AAs between diets and body proteins have been reported to be particularly large for arginine, cysteine, glutamate, glutamine, glycine, histidine, methionine, proline, and serine (Wu, 2014;Wu et al., 2014).

Neu et al. (2016) investigated increasing levels of dietary Arg for Nile tilapia and reported that the maximum whole-body retention for all indispensable AAs was observed in fish fed diets containing Arg at 1.31– 1.37% of the diet. In contrast, Zhou et al. (2015) did not find any change in the muscle of yellow catfish fed diets with increasing levels of dietary Arg. Regarding Gln, Graciano et al. (2014) examined the effects of increasing levels of AminoGut® in diets for tilapia and, also, did not report any differences in the whole-body indispensable AAs composition. Results from the present study differ somewhat from the above-mentioned studies. Taking into account that Arg can be synthesized from Gln, through ornithine/citrulline, by enterocytes (Wu, 2013b) it can be hypothesized that this conversion could have saved the usage of Gln in this pathway and, consequently, increased its deposition into tissues/protein in the whole-body of fish fed the ARG 2% diet. In regards to Gly, it is potentially possible that because Gly was in excess in most diets (Table 1), it also would be reflected in higher concentrations of this AA in whole-body tissues of Nile tilapia. Additionally, because Gly and Ser are readily interconvertible AAs, the mentioned Gly supply would also have triggered the deposition of Ser in whole-body tissues.

In summary, these divergences in tissue AA patterns in this and previous studies may be due to differences in experimental design, fish size, dietary protein sources, the reference AA pattern, feeding regime, feed allowance, presence of adequate levels of other nutrients and fish culture conditions.

So far, the mechanisms by which Gln and/or Arg (and also Gly) play a role in regulating protein synthesis in fish remain unclear and further study I

needed to elucidate them. Moreover, the limited attention received by dispensable AAs to date presents an additional difficulty in comparing results of the current study with others.

5. Conclusions

The supplementation of GLN and ARG in the diet beyond the previously determined minimum requirements improved weight gain and feed efficiency as well as resulted in better protein retention and deposition in Nile tilapia. Fish fed the combined supplement of GLN+ARG at 1% had more improved growth performance than fish fed the AAs individually. Plasma AA profiles reasonably reflected the AA profile of the experimental diets. Whole-body AAs composition was less affected by dietary GLN and/or ARG supplementation. Most of the innate immune responses measured in this study, except for LYZ–S, were not raised by the experimental diets supplemented with GLN and ARG which could be partially explained by the non-activated state of the immune cells.

The dietary Gly inclusion to adjust nitrogen content contributed for the Nile tilapia AAs metabolism and performance which require close attention. Metabolism of Gln and Arg and their utilization for protein synthesis and as energy sources are complex subjects, and require further investigation.

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