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Special Issue Reprint

Amino Acid Supplementation in Fish Nutrition and Welfare

Edited by
Cláudia Aragão, Sofia Engrola and Benjamín Costas

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Guest Editors

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About the Editors

Cláudia Aragão

Cláudia Aragão received her PhD in Aquaculture, with a specialisation in Nutrition, from the University of Algarve in 2004. She is a researcher at the Centro de Ciências do Mar do Algarve (CCMAR/CIMAR-LA), Portugal. Cláudia has extensive experience in fish nutrition, particularly in amino acid metabolism. Her research focuses on the development of sustainable nutritional strategies that promote circularity and reduce reliance on marine-derived ingredients, aiming to enhance fish robustness and improve resilience to stress. With a strong emphasis on applied science, she works closely with industry partners to develop practical, research-based solutions that advance sustainability, welfare, and efficiency in aquaculture systems.

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Sofia Engrola holds a PhD in Aquaculture Nutrition and is the Principal Investigator at the Centro de Ciências do Mar do Algarve (CCMAR/CIMAR-LA, Portugal), where she leads the Aquaculture Research Group (AquaGroup). Her research focuses on sustainable strategies to improve fish performance and resilience through nutrition, with particular emphasis on protein metabolism and functional feeds. Over the past two decades, she has contributed to advancing knowledge on nutrient utilisation and early nutritional modulation in marine fish species. Sofia has led CCMAR's participation in several national and international research projects, often in close collaboration with industry. Her work has supported the development of innovative, practical solutions for aquaculture, including two patent applications related to improving fish robustness. By combining fundamental and applied research, she has helped to translate scientific advances into effective feeding protocols that support animal robustness and production efficiency, contributing to a more sustainable aquaculture sector.

Benjamín Costas

Benjamín Costas holds a PhD degree in Animal Science from the University of Porto, and is a Senior Researcher and Coordinator for the Aquatic Animal Health (A2S) group at CIIMAR. His most recent work and projects are dedicated to exploring innovative and sustainable solutions for enhancing the health and well-being of farmed animals. His team's efforts encompass both a fundamental understanding of underlying mechanisms and practical collaborations with industry partners. Their current research initiatives involve identifying robust biomarker signatures for animal health and welfare. These signatures will play a pivotal role in the development of cutting-edge predictive tools and non-invasive biosensors, marking a significant advancement in precision veterinary medicine. This progress extends to areas such as predicting animal disease susceptibility, enhancing diagnostic capabilities, and optimising treatment responses for smart farming practices.

Preface

As the aquaculture sector continues to expand to meet global food demands while addressing environmental sustainability challenges, the strategic use of amino acids in fish and shrimp nutrition is becoming increasingly relevant. Beyond their role as essential nutrients, amino acids play key roles in improving feed efficiency, nutrient retention, immune function, and stress resistance.

This Reprint compiles a series of studies that highlight the potential of amino acids, not only as essential nutrients, but also as functional ingredients capable of enhancing performance, robustness, and resilience in aquaculture species.

The Guest Editors would like to express their sincere gratitude to all the contributors to this Special Issue: to the authors for sharing the results of their cutting-edge research, to the reviewers for their time and their constructive evaluations, and to the editors for their precious input, ensuring the quality of the published work.

As Guest Editors, we expect that the knowledge compiled in this Reprint will support the development of more sustainable aquaculture practices, fostering the production of healthy, high-quality fish and shrimp, while optimising welfare and long-term productivity.

Cláudia Aragão, Sofia Engrola, and Benjamín Costas
Guest Editors

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Editorial

Amino Acid Supplementation in Fish Nutrition and Welfare

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Amino acids are not only the fundamental building blocks of proteins and, consequently, essential for animal growth, but they have also gained recognition in recent years for their critical roles in regulating key metabolic processes. In aquaculture, where the demand for more sustainable production systems continues to grow, nutrition remains a central pillar. Within this context, amino acids serve dual purposes: they are essential to balance the nutritional profiles of alternative protein sources, and they also act as functional ingredients that can enhance fish health, robustness, and resilience to stressors.

This Special Issue, "Amino Acid Supplementation in Fish Nutrition and Welfare", brings together a collection of two review articles and six original research papers that provide up-to-date and comprehensive insights into the multifaceted roles of amino acids in fish and shrimp nutrition.

Amino acids are vital for the proper functioning of aquatic organisms, as they regulate key metabolic processes including digestion, nutrient transport, and the synthesis of enzymes and hormones. Beyond their structural role in proteins, several amino acids also contribute significantly to immune competence and stress resilience [1]. In this Special Issue, Salamanca et al. (Contribution 1) present a comprehensive review of the current literature, highlighting the physiological roles of amino acids in supporting stress mitigation and immunological responses in fish. Amino acids such as tryptophan, isoleucine, valine, arginine, and gamma-aminobutyric acid (GABA) are involved in essential physiological functions, including energy metabolism, immune regulation, and neurotransmitter activity. Additionally, phenylalanine, tyrosine, tryptophan, methionine, and taurine have been shown to modulate stress responses, enhance immune function, and reduce oxidative stress. The authors conclude that the long-term effects of amino acid supplementation remain unclear and could potentially be negative due to the risks associated with unbalanced feeding. Nevertheless, they suggest that a strategic application of amino acids before stressful events could provide tangible benefits for fish welfare.

Nile tilapia (*Oreochromis niloticus*) is one of the most widely farmed species in global aquaculture [2]. Emerging research has highlighted the critical roles of amino acids beyond protein synthesis, including the regulation of growth, reproductive performance, health status, fillet yield, and flesh quality in this species (e.g., [3–5]). In this context, Furuya et al. (Contribution 2) reviewed and consolidated existing data on the amino acid requirements of Nile tilapia and proposed updated recommendations. They emphasised the importance of designing requirement assays that account for amino acid interactions. Their review revealed that amino acid needs in Nile tilapia can vary significantly depending on experimental conditions such as fish strain, size, rearing system, and basal diet composition.

Furthermore, amino acid recommendations may differ depending on the targeted production outcomes, such as weight gain, feed efficiency, or fillet yield. Therefore, selecting the most appropriate parameter—or combination of parameters—that aligns with the objectives of a given production system is essential. The authors also highlighted the need for more comprehensive data on amino acid requirements during the grow-out phase, as well as further validation of previously established values for key amino acids such as lysine, methionine, and threonine.

Tryptophan is an indispensable amino acid for fish, playing important roles not only in protein synthesis but also in immune tolerance mechanisms mediated by its metabolites. The main degradative pathway for tryptophan is the kynurenine pathway, which contributes to immune regulation. However, excessive activation of this pathway may lead to the accumulation of potentially cytotoxic metabolites, increasing physiological stress and suppressing immune function [6]. Vargas-Chacoff et al. (Contribution 3) evaluated the transcriptional effects of tryptophan and cortisol in primary cell cultures from the head and posterior kidney of coho salmon (*Oncorhynchus kisutch*). Their results revealed activation of the kynurenine pathway and enhanced serotonin activity following stimulation with tryptophan and cortisol, with approximately 95% of tryptophan being degraded through the kynurenine pathway. Their study emphasised the importance of understanding how this pathway is regulated and whether stressors commonly associated with aquaculture practices may trigger its activation, ultimately impacting fish health and welfare.

Considering the potential of tryptophan to modulate fish stress responses [7], Vasconcelos et al. (Contribution 4) evaluated the effects of different dietary tryptophan levels in juvenile meagre (*Argyrosomus regius*). At the end of the feeding trial, fish were exposed to a stress test consisting of 30 s of air exposure and to behaviour assessments, including anxiety-like behaviour, shoaling, and lateralisation tests. The study indicated that dietary supplementation with tryptophan, particularly at the higher inclusion level (0.8%), can reduce anxiety-like behaviour in response to acute stress (novel tank diving). Although the remaining results showed only mild effects, they provide promising evidence supporting the potential use of tryptophan as a functional dietary additive to mitigate stress in aquaculture.

The potential of dispensable (or non-essential) amino acids as functional dietary additives has received comparatively less attention in aquaculture, despite their diverse physiological roles [8]. For instance, glycine plays an important role in the synthesis of glutathione—together with glutamate and cysteine—thereby contributing to the antioxidant defence system in fish. Glycine is also known to stimulate the immune system [9]. In this context, Abbasi et al. (Contribution 5) analysed the effects of dietary glycine supplementation on the immunological and antioxidant capacities of common carp (*Cyprinus carpio*). Their findings showed that dietary glycine increased growth performance, enhanced glutathione-related antioxidant parameters and boosted humoral immune responses. Additionally, glycine supplementation improved several parameters mainly related to innate immunity in skin mucus, which may help prevent pathogen entry. Based on these results, the authors recommended a dietary supplementation level of 5 g/kg glycine for common carp feeding.

As the aquaculture industry increasingly uses alternative protein sources to replace fishmeal, methionine becomes the first limiting amino acid in many aquafeeds. This indispensable amino acid is essential for key physiological functions, including protein synthesis, detoxification, and methylation reactions [8]. Given that industrial shrimp feeds are among the largest global consumers of fishmeal, Nunes and Masagounder (Contribution 6) investigated the optimal levels of fishmeal and methionine required to maximise growth performance and economic efficiency in juvenile whiteleg shrimp (*Litopenaeus vannamei*).

DL-Methionyl-DL-methionine was used as the supplemental methionine source. Their results indicate that fishmeal levels in shrimp feeds can be minimised or even eliminated without impairing growth performance, provided that dietary methionine requirements are met through effective supplementation strategies. This approach supports both improved profitability and sustainability in shrimp aquaculture.

Taurine is a sulphur-containing amino acid with important physiological functions, such as the synthesis of bile salts, which are essential for the emulsification, digestion, and absorption of dietary lipids [10,11]. The shift from fishmeal to plant-based proteins in contemporary diets may lead to taurine deficiency, as it is abundant in marine sources but virtually absent in terrestrial plants. Therefore, Aragão et al. (Contribution 7) examined the effects of taurine supplementation in low-fishmeal diets for Senegalese sole (*Solea senegalensis*). Their study showed that high inclusion levels of plant proteins negatively affected lipid metabolism, likely due to reduced bile acid synthesis and/or limited taurine availability for bile salt formation. However, taurine supplementation in these plant-based diets helped mitigate some of these adverse effects, improving lipid utilisation and supporting enhanced metabolic performance. Moreover, while taurine supplementation had a positive impact on growth, achieving this benefit appeared to require supplementation levels that exceed those typically found in conventional fishmeal-based diets.

Dietary additives hold potential for enhancing immune responses, yet limited information is available regarding their application in diets for shrimp at larval and post-larval stages. Therefore, Barreto et al. (Contribution 8) evaluated the potential beneficial effects of vitamins C and E, β-glucans, taurine, and methionine in whiteleg shrimp post-larvae. Their results showed that post-larvae fed the taurine- and methionine-supplemented diet exhibited growth performance, survival, oxidative status, and immune condition comparable to those fed a positive control diet. The antioxidant capacity and robustness were further improved when vitamin C and E levels were similar to those of the positive control. However, since the positive control diet represents a premium option, it is expected that the benefits of these additives may be greater when incorporated into more cost-effective formulations. The inclusion of β-glucans in the diets showed the most promising effects, significantly reducing lipid peroxidation. These findings suggest that tailored diets with specific health-promoting additives may help overcome key challenges in shrimp larviculture, supporting the long-term success of whiteleg shrimp farming.

This Special Issue covers a broad spectrum of topics, ranging from amino acid metabolism and nutritional requirements to the use of non-proteinogenic amino acids—such as taurine—in aquafeeds. It also examines the impacts of amino acid supplementation on stress response, immune function, antioxidant capacity, and overall health status in several aquaculture species. Furthermore, the articles highlight how specific amino acids may act as functional ingredients to enhance performance, resilience, and robustness in fish and shrimp. Collectively, these studies showcase innovative strategies and scientific advancements that are essential for advancing the sustainable development of aquaculture systems worldwide.

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List of Contributions:

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Review

Amino Acids as Dietary Additives for Enhancing Fish Welfare in Aquaculture

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Simple Summary: In aquaculture, fish are often exposed to stressors that affect survival or limit growth. In recent years, extensive research has been performed to reduce stress in fish in order to improve the welfare of farmed fish. The inclusion of beneficial additives in the diet in order to mitigate the stress response to typical stressors has been an important research topic. In addition, nutritional studies have shown that dietary supplementation with several amino acids (e.g., arginine, glutamine, glutamate, leucine, and proline) modulates this stress response. Nevertheless, it must be taken into account the high diversity of physiological effects depending on species and stressor type. This review summarizes the different studies on amino acid supplementation and its various attributes in fish culture and welfare.

Abstract: The interest in fish welfare within aquaculture facilities has significantly increased over the past decade, recognizing the fundamental role of animal welfare in the quality of aquaculture products. It has been shown that stress in fish can affect their health, causing pathologies and immune failures, while stress-free fish grow faster and healthier. This has prompted aquaculture farmers to adopt strategies that reduce stress, improve water quality, and optimize stocking densities, thereby enhancing fish welfare. A key area is the role of amino acids in improving fish welfare. Amino acids, such as histidine, isoleucine, leucine, and tryptophan, are essential for various physiological processes, including neurotransmitter formation, energy metabolism, and immune function. Amino acids like tryptophan, arginine, and methionine play a crucial role in mitigating the effects of stress, improving immune function, and reducing oxidative stress. In the present review, the main roles of those amino acids related to fish stress have been shown, analyzing the physiological pathways involved in the link between amino acid ingestion and metabolization and stress responses.

Keywords: amino acid; fish; animal welfare; aquaculture; nutrition

1. Introduction

The interest in fish welfare in aquaculture facilities has grown significantly during the last decade. Important knowledge has been generated about fish stress, health, and pain; hence, currently, animal welfare is also a quality mark in aquaculture products, as in livestock species [1,2]. In fact, Wolke et al. [3] pointed out that fish farmers should recognize that the key to success is based on keeping fish stress-free. This statement is supported by fish farmers, who accept that healthy and well-being fish grow faster and better, resulting in

economic benefits, and poor welfare is related to pathologies and immune failures [4]. For instance, reducing handling stress in cultured Senegalese sole (*Solea senegalensis*) resulted in lower cortisol levels and improved growth [5]. Stocking density is also a typical stressor in sea farms, though the optimal range is very variable depending on species and culture conditions [6–8]. The right management of this variable can improve fish welfare and enhance growth significantly [9].

There are many procedures for improving animal welfare in sea farms. Innovative zootechnical procedures are based on the improvement of the water quality through new culture systems like IMTAs (Integrated Multi-Trophic Aquaculture), organic cultures, bioflocs, etc. [10]. Stocking density handling is also an efficient strategy for enhancing fish welfare, though it is very variable depending on species and culture conditions [7,11–13]. Lastly, the enrichment of tanks seems to be a useful tool but maybe not very practical for intensive or semi-intensive systems [14,15].

Nevertheless, it is widely known that fish feeding and nutrition are key for keeping fish healthy [16]. Besides nutritional factors, many other food ingredients can play a crucial role in the physiological state and responses to stressful conditions. They are usually additives that are added to the conventional feed formulation, representing less than 5% of meal weight [17]. Many proteins, carbohydrates, fatty acids, vitamins, amino acids, and minerals have been studied as welfare promoters since they can modulate many metabolic pathways involved in the stress response [18].

Amino acids are a heterogeneous group with diverse biochemical functions; hence, they are involved in very different physiological processes. For instance, amino acids can be involved in the formation of neurotransmitters, part of energy metabolism pathways, or play immune functions [9,19]. Therefore, amino acids are maybe the organic molecules that have been the most studied in order to improve fish welfare through their inclusion in fish feeds. Amino acids are usually included in crystalline form. This supposes some limitations regarding their utilization efficiency because of lower rates of intestinal absorption and alteration of acid–base and electrolyte balance during digestion, among other causes [9,20]. However, this matter can be improved through a suitable feeding strategy [21].

In this review, the literature on the effects on dietary amino acids of fish stress and welfare has been revised. The objective is to show the state of the art in this area, analyzing and classifying the main current findings and future prospects in this field.

2. General Amino Acid Physiological Roles in Animals

Amino acids (AAs) are the structural units of proteins. AAs play a critical role in the organism as they perform important metabolic functions, regulating processes such as digestion, nutrient transport, and the production of enzymes and hormones. AAs are also essential for maintaining healthy immune systems.

There are more than 700 AAs in nature, but only 20 of them are building blocks for proteins and present in cells for the synthesis of polypeptides. These include alanine, arginine, asparagine, aspartate, cysteine, glutamate, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, tyrosine, threonine, tryptophan, and valine. However, selenocysteine and pyrrolysine are also considered amino acids, although they are not part of the standard amino acid set related to fish protein synthesis [22,23]. Ten of them are considered "essential" (EAA), meaning their carbon skeletons are not synthesized de novo or are not synthesized in adequate amounts by the organism. These essential AAs are arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine. As a result, they must be provided through diets. On the other hand, the "nutritionally non-essential amino acids

(NEAA)" can be synthesized within the body from carbon skeletons provided by cells or intermediates of metabolic pathways [24].

Amino acids act as substrates in different biosynthetic reactions. Furthermore, nucleotide bases and numerous hormones, such as catecholamines, serotonin, melatonin, and the thyroid hormones thyroxine and triiodothyronine, as well as neurotransmitters, are derived from amino acids [25,26]. Additionally, AAs are precursors for the synthesis of other important molecules such as glutathione and polyamines, all of which have diverse physiological functions. They can be synthesized from glycolytic or Krebs cycle intermediates [22,25].

The crucial roles of AAs in metabolism, physiology, and immunity against infectious diseases are particularly notable. Amino acids serve primary roles as precursors for protein synthesis, and they modulate the protein synthetic machinery within target cells [27]. From a physiological point of view, several amino acids have been shown to support the intestinal barrier function and the intestinal endocrine function [28]. Furthermore, adequate dietary provision of all amino acids is necessary for sustaining normal immunocompetence and protecting the host from a variety of diseases in all species [29]. In recent years, investigations have focused on regulatory roles for dietary AA in nutrient metabolism, including protein turnover, as well as lipid synthesis and oxidation, to favor lean tissue growth and adipose tissue reduction [30,31].

The role of amino acids in the immune system can also be considered from two perspectives: the enhancement of the immune response, which protects individuals from infections and malignant neoplasms, and the reduction of over-responses such as inflammation and autoimmunity [32].

Animals, including fish, need the same ten EAAs in their diet for proper development [21]. Similarly, the metabolic pathways of amino acids in fish are analogous to those of mammals. However, there are some variations, such as differences in the distribution of enzymes that deaminate branched-chain amino acids or in the secondary pathways of amino acid metabolism, such as those involving serine [33].

Fish and mammals exhibit key differences in amino acid metabolism, such as the limited ability of fish to synthesize certain amino acids and their lower efficiency in storing and converting proteins. Due to these differences, fish have a higher protein requirement than mammals, as they need less non-protein energy compared to homeotherms. In addition to the ten essential amino acids (EAAs) mentioned above, each species has distinct EAA requirements, influenced by factors such as their position in the food chain and water temperature. Among these EAAs, arginine, isoleucine, lysine, and threonine are particularly important in the fish diet, and these nutritional needs should be carefully considered in aquaculture to optimize fish welfare and productivity [21].

Nutritional studies have demonstrated that dietary supplementation with certain amino acids (referred to as functional amino acids) regulates key metabolic pathways to improve survival, development, growth, health, welfare, and reproduction of organisms. In this context, the most studied amino acids include arginine, glutamine, glutamate, tryptophan, sulfur amino acids (methionine, cysteine, and taurine), and histidine [34–37]. However, the works focusing on the interactions between stress responses and dietary amino acids in fish are based on other amino acids, mainly the essential ones, like tryptophan, taurine, and phenylalanine (Figure 1).

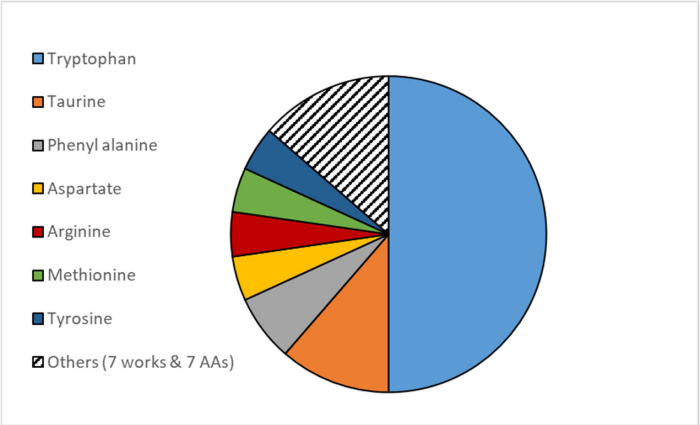

Figure 1. Distribution of papers dealing with the effects of amino acids on fish stress depending on amino acid type.

3. Stress Responses Depending on Amino Acids

Stress responses are diverse, including changes at different levels. Firstly, the endocrine and neuroendocrine changes are evident (primary response) and lead to alterations and failures in metabolism, immune system, reproduction, behavior, and growth [38]. Dietary amino acids can modulate those responses, though their effects are highly diverse depending on factors such as fish species, stressor type, and amino acid concentration. Table 1 summarizes the main physiological effects of amino acid additives on the stress response in fish, and that information has been developed below.

Table 1. Relevant stress responses related to every amino acid found in the literature.

Amino Acid	Relevant Physiological Stress Responses	Species	References
Tryptophan	Improves feeding intake	*Salmo trutta*	Höglund et al. [35]
	Reduces aggressiveness without affecting stress indicators	*Brycon amazonicus*	Wolkers et al. [39]
	Decreases growth Increases food consumption	*Oncorhynchus mykiss*	Papoutsoglou et al. [40]
	Increases growth parameters under stress conditions Decreases cortisol and glucose levels Decreases energy requirements	*Cirrhinus mrigala*	Tejpal et al. [41]
	Decrease cortisol, glucose levels and energy requirements	*Argyrosomus regius*	Teixeira et al. [42]
	Alters the prl and gh expressions after stress Increases Hsp70 expression	*Argyrosomus regius*	Herrera et al. [43]
	Decreases enzyme activities related to amino acid and carbohydrate metabolism Increases the liver kynurenine concentration	*Argyrosomus regius*	Herrera et al. [44]
	Decreases plasma lactate and mucus glucose	*Argyrosomus regius*	Fernández-Alacid et al. [45]
	Upregulates immune-related gene expressions	*Argyrosomus regius*	Asencio-Alcudia et al. [46]
	Keeps levels of protease, antiprotease, peroxidase and lysozyme unchanged	*Argyrosomus regius*	Gonzalez-Silvera et al. [47]
	Reduces SOD and CAT activities Increases serotonergic activity and plasma cortisol	*Totoaba macdonaldi*	Cabanillas-Gámez et al. [48]
	Decreased plasma cortisol levels Increased liver transaminase activity	*Gadus morhua*	Herrera et al. [49]
	Raises enzyme activity in glycolysis and gluconeogenesis Reduces plasma cortisol and lactate	*Solea senegalensis*	Salamanca et al. [50]
	Increases plasma cortisol Modulates plasma glucose and lactate	*Solea senegalensis*	Herrera et al. [18]
	Increases plasma cortisol Higher brain monoamine content	*Dicentrarchus labrax*	Azeredo et al. [51]
	Increases plasma cortisol Decreases plasma glucose Decreases lysozyme and ACH50 Decreases serum thyroid hormone Inhibits post-stress immunosuppression	*Acipenser persicus*	Hoseini et al. [52]
	Decrease serum thyroid hormones. Decreases aminotransferase and lactate dehydrogenase activities Reduces enzyme activities related to oxidative stress Higher growth, RGR and PER	*Labeo rohita*	Kumar et al. [53]

Table 1. *Cont.*

Amino Acid	Relevant Physiological Stress Responses	Species	References
Taurine	Improves growth performance, muscle composition and amino acid composition	*Takifugu rubripes*	Shi et al. [54]
	Enhances growth performance Improves intestine structure	*Mylopharyngodon piceus*	Tian et al. [55]
	Increases CAT activity Increases total serum immunoglobulin concentration	*Arapaima gigas*	Souto et al. [37]
	Increases growth Decreases ROS production and antioxidant enzyme gene expressions	*Dicentrarchus labrax*	Ceccotti et al. [56]
Phenylalanine	Lower plasma cortisol levels Increases liver transaminase activities Raises enzyme activity in glycolysis and gluconeogenesis	*Gadus morhua*	Herrera et al. [49]
	Reduces plasma stress markers	*Sparus aurata*	Salamanca et al. [57]
	Reduces plasma glucose and lactate	*Argyrosomus regius*	Salamanca et al. [57]
Aspartate	Enhances pomc-a expression Increases Hsp70 expression	*Argyrosomus regius*	Herrera et al. [43]
	Produces over-exudation of mucus metabolites and cortisol	*Argyrosomus regius*	Fernández-Alacid et al. [45]
	Keeps protease, antiprotease, peroxidase and lysozyme levels stable	*Argyrosomus regius*	Gonzalez-Silvera et al. [47]
Methionine	Decreases TG, TC, NEFA, LDL-C, and ALT Decreases lipid droplets in liver Increased ampkα and sirt1 expression Improves lipogenesis pathway gene expressions Up-regulates antioxidant enzyme activities and gene expression levels Decreases pro-inflammation and pro-apoptosis gene expressions Up-regulates anti-inflammatory cytokine and anti-apoptosis gene expressions	*Acanthopagrus schlegelii*	Yang et al. [36]
	Increases plasma cortisol Upregulates complement factor 3 Increases immune cells	*Dicentrarchus labrax*	Azeredo et al. [51]
Tyrosine	Reduces plasma stress markers	*Sparus aurata*	Salamanca et al. [57]
Arginine	Decreases plasma cortisol levels	*Scophthalmus maximus*	Costas et al. [34]
	Increases respiratory burst activity and nitric oxide production of head kidney leukocytes Enhances HIF-1, HAMP-1, MIP1-alpha and gLYS expressions	*Solea senegalensis*	Costas et al. [58]
Alanine + Glutamine	Increases body weight Increases fish survival during a bacterial challenge	*Cyprinus carpio*	Chen et al. [59]
Leucine	Promotes FW, WG, PWG, and SGR Decreases activities of serum parameters Decreases ROS, NO and $ONOO^-$ activities Increases mRNA levels of mitochondrial biogenesis genes and fusion-related genes Decreases mRNA levels of fission-related genes, mitophagy-related genes and autophagy-related genes	*Ctenopharyngodon idella*	Zhen et al. [60]
Valine + Isoleucine	Enhances growth Increases blood parameter levels	*Paralichthys olivaceus*	Shi et al. [61]
Gamma-aminobutyric acid	Improves macrophage maturation, autophagy activation, and antibacterial response to bacterial infection	*Paralichthys olivaceus*	Bae et al. [62]

Hsp70: mitochondrial heat shock protein 70; SOD: superoxide dismutase; CAT: catalase; ACH50: alternative complement pathway; RGR: relative growth rate; PER: Protein efficiency ratio; ROS: oxygen-containing reactive species; pomc-a: proopiomelanocortin-derived hormone; TG: triglyceride; TC: total cholesterol; NEFA: non-esterified fatty acid; LDL-C: low-density lipoprotein cholesterol; ALT: alanine transaminase; HIF-1: hypoxia-inducible factor 1; HAMP-1: hepcidin antimicrobial peptide 1; MIP1-alpha: macrophage inflammatory protein 1α; gLYS: g-type lysozyme; FW: final weight; WG: weight gain; PWG: percent weight gain; SGR: specific growth rate; NO: nitric oxide; $ONOO^-$: peroxynitrite.

3.1. Immunological Responses

The immune response is closely linked to fish stress and welfare since stress leads to an endocrine cascade through the HPI (hypothalamic–pituitary–interrenal) axis, which affects the immune system [63]. The stressors trigger a series of regulatory events, as the

hormonal activity increases, that interfere with the normal function of the immune system and facilitate a rapid pathogenic expansion [64]. For instance, post-stress blood cortisol increase can down-regulate the pro-inflammatory cytokine production [65] and depress phagocytosis of peripheral blood leukocytes [66].

It has been reported that some amino acids can modulate those stress immune effects. Tryptophan (Trp) is an essential amino acid and serotonin precursor, a neurotransmitter involved in stress response that can regulate the GALT (gut-associated lymphoid tissue) immune-related gene expression and anti-inflammatory signaling molecules [51,67,68]. It is hypothesized that stress modulation due to dietary Trp derives from the interaction between serotonergic activity and the HPI axis, though the physiological mechanisms regulating that interaction still remain unclear [44,69]. Additionally, Trp has also been shown to have significant impacts on immune functions controlled by specific pathways via its role as a structural component of specific transcription factors [46,70].

Herrera et al. [44] reported that Trp-enriched diets increased mucus antibacterial activity in the meager (*Argyrosomus regius*). Similarly, Gonzalez-Silvera et al. [47] analyzed several immunological parameters and concluded that dietary Trp improved the meager health by maintaining the levels of proteases and antiproteases under stress conditions. Lastly, Asencio-Alcudia et al. [46] concluded that Trp-supplemented diets can alleviate some of the most damaging aspects of the stress response on the mucosal immunity of the gut, such as elevated antimicrobial peptide expression that could change the microbiota and have long-term negative effects such as outbreaks of opportunistic bacterial pathogens.

Arginine (Arg) is a non-essential amino acid precursor for the synthesis of nitric oxide and polyamides [71], which is related to cellular defense mechanisms and can stimulate macrophages in fish [72,73]. Arg-enriched diets can influence the Senegal sole (*Solea senegalensis*) innate immune system [35]. Those authors have reported that stressed fish showed an increase in plasma lysozyme and alternative complement pathway activities as well as in the level of expression of g-type lysozyme (gLYS). In turbot (*Scophthalmus maximus*), dietary Arg could have enhanced some aspects of the stressed fish's immune system, such as an increase in the relative percentage of circulating lymphocytes and peripheral monocytes, probably due to a higher availability of polyamines (derived from Arg) for leukocyte growth and differentiation [34].

Taurine (Tau; 2-aminoethanesulfonic acid), the most abundant free amino acid in marine animals, is associated with many physiological functions, such as the innate immune response [74]. It has been reported that Tau dietary supplements can protect tiger puffer fish (*Takifugu rubripes*) against environmental stress by enhancing the plasma non-specific immunity (complement C4 concentration) [54].

Methionine (Met) is an essential amino acid involved in the lipid metabolism [75]. Yang et al. [36] have studied the effects of dietary methionine (Met) on growth and other physiological responses in black seabream (*Acanthopagrus schlegelii*) submitted to nutritional stress (high-fat diets), concluding that Met inclusion could alleviate hepatic inflammatory response and apoptosis.

Gamma-aminobutyric acid (GABA) is a natural amino acid present in bacteria, plants, and animals and is synthesized from glutamic acid inside presynaptic synapses of inhibitory nerve cells [76,77]. Although it is not an alpha amino acid, it has been used as a feed additive in some fish species [78–80]. In fact, Bae et al. [62] have reported that GABA supplements improved macrophage maturation, autophagy activation, and antibacterial response to bacterial infection and enhanced the host's innate immune response in olive flounder (*Paralichthys olivaceus*) under high stocking density stress.

3.2. Effects Involving Energy Metabolism

The secondary stress response leads to diverse metabolic changes, mainly those related to intermediate metabolism, in order to cope with a stressful condition and recover homeostasis, since stress is an energy-demanding process [49]. Several amino acids can act as energy substrates in those metabolic pathways; hence, their roles during the stress response can be crucial, affecting plasma stress markers related to energy mobilization as lactate and glucose [49]. For instance, amino acid metabolism involves aminotransferase reactions, which are stress markers in many animals, including humans [81,82]. Additionally, some amino acids can be converted into hormones and other substances that act directly on the energy metabolism [9]. Although it seems that amino acid supplementations usually provide beneficial metabolic effects, the addition of amino acids to the diet in an unbalanced manner can negatively affect growth parameters, as the requirements for other amino acids may be displaced by the supplemented one [75].

Tryptophan (Trp) is a neurotransmitter precursor (see previous sections), though 95% of ingested Trp is catabolized through the kynurenine pathway, producing niacin, pyruvate, and acetyl-CoA as the final products [67]. Acetyl-CoA and pyruvate can then enter into the Krebs cycle for making energy. In this sense, stress conditions induce a hypermetabolic status, which may induce the mobilization of amino acids as a coping mechanism for the increased energy demand, and Teixeira et al. [42] have reported that hepatic amino acid catabolism enzyme activity due to stress was affected by dietary Trp, decreasing with the increased level of Trp. Nevertheless, Herrera et al. [49] reported high gluconeogenic and glycolytic activities in stressed cods (*Gadus morhua*) fed Trp supplements. Those metabolic alterations can affect circulating plasma metabolites, and some works have reported that dietary Trp supplementations reduce plasma glucose and lactate levels in several fish species after stress submission [41,43,44,49,52]. However, Salamanca et al. [50] did not describe variations in glucose and lactate throughout the experimental period, which could be attributed to the long feeding duration with that amino acid. This aligns with the findings of Herrera et al. [18], who reported a decrease in plasma glucose and lactate concentrations when Trp levels were increased, but not when the feeding duration was prolonged. The metabolic changes under stress lead to growth alterations, and Trp supplements can reduce some growth parameters since they alter the food intake [42,44,48,50]. However, Tejpal et al. [41] described an improvement in growth parameters in *Cirrhinus mirgala* fed Trp supplements and subjected to high stocking density stress.

Phe is an essential amino acid that is metabolized through two metabolic pathways, namely oxidation to tyrosine (Tyr) and transamination to phenylpyruvate [83], and Tyr could be catabolized to hydroxyphenyl pyruvate and become a part of the energy metabolism [84]. It has been reported that both amino acids are mobilized to the brain as a stress response, resulting in a higher accumulation in fish fed Phe or Tyr supplements [57]. Additionally, Phe supplements kept or reduced enzyme activities related to the energy metabolism in the cod (*Gadus morhua*) [49]. Regarding plasma metabolites, it has been reported that Phe reduces glucose and lactate levels in specimens subjected to stress [49]. This is consistent with Salamanca et al. [57], who found a reduction in glucose and lactate levels in specimens fed diets supplemented with Phe and Tyr, except after 90 days of feeding. These results indicate that the effects of amino acids vary depending on the feeding duration and species. In previous studies, lactate levels were reduced in gilthead seabream and meager fed Phe and Tyr, but the reduction was more pronounced in meager [57]. The effects of those amino acids on growth have been studied in the gilthead seabream (*Sparus aurata*), describing that those supplements could decrease the growth parameters due to the unbalanced formulation (amino acid excess) provided on a long-term basis [57].

As methionine (Met) is involved in lipid metabolism (see previous section), its role in the intermediate metabolism can be crucial, mainly in stress conditions due to unbalanced feeding [85,86]. In this sense, Yang et al. [36] have reported that Met-supplemented diets can promote lipogenesis and suppress lipolysis, consequently reducing excess lipid accumulation and alleviating liver damage in stressed black sea breams (*Acanthopagrus schlegelii*) due to high-fat diets.

Valine (Val) and isoleucine (Ile) are branched-chain amino acids involved in several biochemical reactions, such as glycolysis and ketogenesis [87,88], key pathways in energy metabolism. Regarding stress response effects, a high level of Ile combined with a high level of Val in the diet improved the tolerance against low salinity stress in the Japanese flounder [61].

Taurine (Tau; 2-aminoethanesulfonic acid) regulates heart function and metabolism [89,90]. Therefore, Dixon et al. [91] have demonstrated that reducing cardiac taurine levels (through feeding) significantly affects whole animal sensitivity to acute thermal and hypoxic stress, impairs heart function, and influences energy metabolism in the brook char (*Salvelinus fontinalis*). However, dietary Tau did not reduce the plasma glucose concentration in *Mylopharyngodon piceus* and *Arapaima gigas*, though improved growth parameters [37,55].

GABA (gamma-aminobutyric acid) induces stable metabolic processes and immune and antioxidant responses by inhibiting overactive neurons in stressful situations [62]. In fish, it has been reported that dietary GABA had a beneficial effect on growth performance and feed utilization [80,92,93]. Bae et al. [62] did not find differences in GOT (aspartate aminotransferase) and GPT (alanine aminotransferase) activities in the olive flounder (*Paralichthys olivaceus*) between control fish and fish submitted to stress density and fed GABA additives; hence, they stated that was a good indicator of the beneficial effects of GABA as a feed additive.

Alanine–glutamine (Ala-Gln) is a dipeptide composed of the amino acids alanine and glutamine, both involved in various metabolic functions, such as protein synthesis and acid–base balance regulation [55,59]. Despite its role in cellular homeostasis and energy metabolism, it has been reported that Ala-Gln does not reduce glucose levels in the organism, regardless of its concentration in the diet [37,59]. This suggests that, although its supplementation may have other physiological benefits, it does not exert a modulatory effect on glucose under stress conditions or in basal situations.

Aspartate (Asp) has been identified as a regulator of glucose metabolism, acting as an intermediary in the Krebs cycle and gluconeogenesis [43]. Its impact on glucose levels varies depending on the type of stress the organism is exposed to, indicating that its effect is dependent on the physiological and environmental context. Studies have shown that Asp can play a key role in metabolic adaptation to adverse conditions, adjusting energy availability according to the organism's demands [43].

3.3. Endocrine and Neuroendocrine Processes

Stress in fish induces significant hormonal changes, primarily affecting thyroid hormones, cortisol, and catecholamines. The hypothalamic–pituitary–interrenal (HPI) axis activation results in cortisol release, impacting immune functions and metabolic processes. Thyroid hormones regulate growth and metabolism, while catecholamines mediate acute stress responses. Several amino acids have been studied for their role in modulating these hormonal changes [44,69].

Tryptophan is a serotonin precursor, a neurotransmitter involved in stress response and regulation of the HPI axis. It is hypothesized that dietary Trp modulates stress through serotonergic activity, though exact mechanisms remain unclear. Studies indicate that Trp

does not significantly reduce cortisol levels [43,52,68]. However, its supplementation may influence thyroid hormones and catecholamine levels.

Phenylalanine and tyrosine are precursors for catecholamines, impacting stress responses and metabolic functions. In gilthead seabream, Phe and Tyr supplementation led to decreased catecholamine levels, suggesting an influence on stress regulation [84]. However, in meager, these effects were not observed. Regarding cortisol, no significant differences were noted between stressed specimens supplemented with Phe and Tyr compared to controls [57,84]. Nonetheless, Herrera et al. [18] found that Phe supplementation influenced cortisol levels under air exposure stress but not thermal stress. Additionally, Tyr supplementation was associated with increased T4 (thyroxine) levels in meager [84].

Ala-Gln supplementation has been associated with increased T3 (triiodothyronine) and T4 levels, suggesting a stimulatory effect on thyroid function. This effect can be explained by the influence of glutamine on energy metabolism and hormone synthesis. Glutamine, as a key substrate for gluconeogenesis and the Krebs cycle, supports ATP production, providing the necessary energy for thyroid gland activity. Additionally, alanine participates in the glucose–alanine cycle, contributing to metabolic homeostasis and enhancing the availability of precursors for hormone synthesis [59].

Aspartate has not been shown to significantly reduce cortisol levels in stressed fish [43]. However, its role in energy metabolism suggests potential indirect effects on stress adaptation. By participating in the Krebs cycle and gluconeogenesis, aspartate contributes to ATP production, providing the necessary energy to maintain metabolic homeostasis and support the organism's adaptive response to stressful conditions [94].

3.4. Responses Related to Oxidative Stress

Reactive oxygen species (ROS) are produced mainly in mitochondria under physiological conditions. When the level of ROS cannot be controlled and its content rises, oxidative stress develops [56]. Antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione (GSH), are produced by cells to counteract oxidative stress by reducing ROS levels.

In aquaculture, numerous situations cause physiological stresses, including oxidative stress. The inclusion of certain amino acids in the diet can mitigate oxidative stress, significantly reducing ROS levels and decreasing the activity of antioxidant enzymes [36, 56].

Several studies have shown that tryptophan, an essential amino acid precursor for serotonin and implicated in several behavioral patterns such as fear, stress [45], aggression [39], appetite regulation [35], immune response [47], social dominance, and sex behavior, could be a useful tool for reducing stress associated with farming practices [18,44,50]. An experiment investigating stress mitigation effects of dietary tryptophan (L-TRP) under thermal stress in rohu, *Labeo rohita* [53], showed that CAT and SOD activities were significantly higher in the control groups (0% L-TRP), whereas decreasing activities of these enzymes were observed with the increasing level of dietary L-TRP. It could be concluded that L-TRP might have antioxidative properties in mitigating oxidative stress caused by increased temperature.

Specific amino acids can scavenge ROS, and their dietary inclusion can alleviate oxidative stress. This is the case for methionine (Met) and taurine (Tau). Appropriate dietary levels of methionine in black seabream (*Acanthopagrus schlegelii*) alleviated physiological stress produced by a high-fat diet, reducing ROS levels while notably up-regulating the activity of antioxidant enzymes [36]. A similar situation was observed in European seabass *(Dicentrarchus labrax*) under sustained swimming conditions. A diet supplemented with

1.5% Tau resulted in significantly lower ROS levels than in a control diet, along with a significant decrease in the gene expression of antioxidant enzymes [56].

Leucine (Leu) is a functional amino acid that can be oxidized to supply energy. A recent study where Leu was added to the diet of sub-adult grass carp [60] showed that this AA could mitigate the negative effects of nitrite-induced stress response in this species. This may be attributed to leucine alleviating mitochondrial dysfunction by reducing ROS production.

4. Conclusions

Amino acid supplements have a significant influence on the stress response in fish. However, their long-term effects remain unclear and could potentially be negative due to the risks of unbalanced feeding. Despite this, their application prior to stressful conditions may offer benefits for fish welfare. Most studies have focused on essential amino acids such as tryptophan, phenylalanine, and tyrosine, although others, such as aspartate and taurine, have also been investigated. Since many amino acids are involved in both endocrine and neuroendocrine responses, as well as energy supply, understanding the primary pathways through which specific amino acids affect the stress system should be a key focus of future research.

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Abbreviations

The following abbreviations are used in this manuscript:

AA	amino acid
ACH50	alternative complement pathway
Ala-Gln	alanine–glutamine
ALT	alanine transaminase
Arg	arginine
Asp	aspartate
ATP	adenosine triphosphate
CAT	catalase
EAA	essential amino acid
FW	final weight
GALT	gut-associated lymphoid tissue
gLYS	g-type lysozyme
GPT	alanine aminotransferase
Gpx	glutathione peroxidase
GOT	aspartate aminotransferase
GSH	glutathione
Ile	isoleucine
IMTA	integrated multitrophic aquaculture

HAMP-1	hepcidin antimicrobial peptide 1
HIF-1	hypoxia-inducible factor 1
HPI	hypothalamic–pituitary–interrenal
Hsp70	mitochondrial heat shock protein 70
Leu	leucine
LDL-C	low-density lipoprotein cholesterol
Met	methionine
MIP1-alpha	macrophage inflammatory protein 1α
NEAA	nutritionally non-essential amino acids
NEFA	non-esterified fatty acid
NO	nitric oxide
$ONOO^-$	peroxynitrite
PER	protein efficiency ratio
Phe	phenylalanine
pomc-a	proopiomelanocortin-derived hormone
PWG	percent weight gain
RGR	relative growth rate
ROS	oxygen-containing reactive species
SGR	specific growth rate
SOD	superoxide dismutase
Tau	taurine
TC	total cholesterol
TG	triglyceride
Trp	tryptophan
Tyr	tyrosine
Val	valine
WG	weight gain

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animals

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Review

Amino Acid Requirements for Nile Tilapia: An Update

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Simple Summary: The concept of optimizing growth performance and supporting fish health through precise amino acid (AA) nutrition is well-accepted in current Nile tilapia, *Oreochromis niloticus*, farming. Emerging studies have evidenced the crucial role of essential amino acids (EAAs) and non-essential amino acids (NEAAs) beyond protein synthesis, regulating growth and reproductive performance, health status, fillet yield, and flesh quality responses of Nile tilapia. Balanced AAs can contribute to accurately implementing the "Precision Nutrition" concept and may help assess the economic dimension of the aquaculture system. Additionally, information on the precise dietary AA requirements may help produce environmentally sustainable diets for Nile tilapia farming in compliance with sustainability principles.

Abstract: This review aims to consolidate the relevant published data exploring the amino acid (AA) requirements of Nile tilapia, *Oreochromis niloticus*, and to reach a new set of recommendations based on those data. There are still inconsistencies in lysine, sulfur-containing AA, threonine, tryptophan, branched-chain AA, and total aromatic AA recommendations in data that have appeared since 1988. This review finds that strain, size, basal diet composition, and assessment method may have contributed to the inconsistencies in AA recommendations. Currently, the expansion of precision AA nutrition diets for Nile tilapia is receiving more attention because of the demand for flexibility in widespread ingredient substitutions which will allow compliance with environmentally sustainable principles. Such approaches involve changes in diet ingredient composition with possible inclusions of non-bound essential and non-essential AAs. Increasing the inclusion of non-bound AAs into Nile tilapia diets may modify protein dynamics and influence AA requirements. Emerging evidence indicates that not only essential but also some non-essential amino acids regulate growth performance, fillet yield, and flesh quality, as well as reproductive performance, gut morphology, intestinal microbiota, and immune responses. Thus, this review considers current AA recommendations for Nile tilapia and proposes refinements that may better serve the needs of the tilapia industry.

Keywords: amino acid requirement; ideal amino acid ratio; growth performance; health status; Nile tilapia

1. Introduction

Global tilapia production is projected to continue growing until 2031 through the sustainable management and utilization of natural resource principles based on improved nutrition practices [1]. In this way, the growth performance, reproduction, and health of tilapias have been improved by genetic selection breeding programs coupled with precision nutritional strategies to meet this increasing demand. However, this need has generated challenges for the tilapia industry, including concerns of food security, food safety, feed ingredient shortages, diseases, and environmental issues.

Emerging evidence suggests a variety of economically and environmentally sustainable feedstuffs for use in aquafeeds [2–5]. One of these groups is the plant-protein feedstuffs;

however, vegetable feedstuffs may contain antinutrients and limiting amounts of certain amino acids (AAs) that may impair protein synthesis [3,6–9]. Consistently, the deficiency of a single essential AA may impair several physiological functions and, subsequently, growth performance [10–12]. Emerging evidence suggests that AAs also act as signaling molecules regulating protein synthesis [13,14] and energy metabolism in the Nile tilapia, *Oreochromis niloticus* [15]. Therefore, the provision of tilapia feeds closely matching optimum AA requirements is a crucial strategy for overcoming various challenges, including optimizing sustainable raw materials, lowering feeding costs, and attenuating nitrogen loss into the environment.

Fish, like other animals, synthesize body proteins from AAs that are provided in the diet as well as some AAs that can be synthesized in the body from precursors. Those which must be provided in the diet due to lack of endogenous synthesis capabilities have traditionally been referred to as dietary indispensable or essential amino acids (EAAs). Classical fish nutrition textbooks consider arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine as nutritionally essential AAs to maintain normal physiological functions of cells and tissues [16,17]. These EAAs are most critical to provide in the diet because a deficiency of any one can limit protein synthesis, which is often manifested as reduced weight gain as well as other specific deficiency signs.

Another group of AAs commonly referred to as dispensable or non-essential amino acids (NEAAs) includes alanine, asparagine, aspartate, cysteine, glutamate, glutamine, glycine, proline, serine, and tyrosine. Those NEAAs traditionally have been classified as such because they can be synthesized in the body from precursor biochemicals. They also may be found in dietary protein and used for synthesizing tissue proteins as well as participating in various metabolic pathways. Emerging evidence has indicated that dietary supplementation of NEAAs also may have beneficial effects on the growth performance [18,19], reproduction [20], health [21,22], and flesh quality [23] of Nile tilapia that are fed plant-based diets. Some AAs may be classified as conditionally essential amino acids (CEAAs) because their rates of use may exceed their rates of synthesis under certain physiological conditions. AAs in this category include glutamate, glutamine, glycine, proline, and hydroxyproline, as well as the sulfonic acid taurine. The CEAA term also has been applied where the reduction or elimination of certain protein feedstuffs in the diet which are rich in certain AAs requires the supplementation of such AAs to prevent growth reduction or other metabolic impairment. The participation of AAs in various metabolic processes beyond protein synthesis such as cell signaling, gene expression, and metabolic regulation has led to the term "functional" AAs, which can include EAAs, CEAAs, and NEAAs [24,25]. The relevance of the concept of functional AAs to aquatic animal nutrition has been established [26] and is beginning to receive heightened attention.

Notably, many relevant outcomes on AA requirements of tilapia have been published since the last National Research Council (NRC) edition in 2011 [27]. However, the summary of AA requirements of tilapia has not been updated for more than a decade. Variables such as fish strain and size, feed composition and processing technology, as well as feed management and statistical methods, may influence experimental estimates of AA requirements of Nile tilapia and may explain some of the inconsistencies in AA recommendations throughout the literature. Importantly, some of these new data provide insights for optimal production efficiency, welfare, health, and flesh quality responses. Thus, this review comprehensively consolidates the relevant data on dietary AA requirements, identifies the shortfalls, and recommends directions for future research in tilapia nutrition.

2. Methodology

The studies selected in this review were completed since 1988, and the impacts of single AA additions on the growth performance of Nile tilapia were determined in most of them, although statistical models using the deletion method were applied to estimate AA requirements in the remaining studies. Where data permit, the impacts of dietary

AA recommendations based on growth performance, reproduction, and health responses are tabulated. Also, all data are expressed on a dry-matter basis. Generally, growth rate, feed conversion ratio, and protein retention efficiency were identified as the most appropriate parameters for deriving AA recommendations, and their simple mean (±standard deviation) recommendations were used to summarize the AA recommendations reported during the past 34 years. While it is well-established that feedstuffs contain more total than digestible amino acids, few studies have reported dietary amino acid requirements that consider amino acid digestibility. Therefore, in this review, we focused on data based on diets' total amino acid content. Furthermore, relevant Nile tilapia production stage ranges for the recommendations are included as footnotes. Standard deviations of the mean AA values are recorded to illustrate the variations caused by units in expressing requirements (g/kg diet or % crude protein). These mean values are somewhat problematic because AA requirements are expressed on a total basis, and variations in respective fish size ranges, feed ingredients and diet composition, response variable, and statistical methods for estimating AA requirements are the main influential factors on these outcomes. Recent studies have used various estimation methods to arrive at AA recommendations for Nile tilapia, including linear, quadratic, and polynomial broken-line regressions. An alternative method proposed to determine the optimal AA ratio for pigs [28] and applied for other species such as Atlantic salmon, *Salmo salar* [29], and Nile tilapia [12] is the deletion method. This method involves monitoring the nitrogen balance as AAs are reduced from the diet and assuming that reducing a non-limiting AA does not affect nitrogen retention [30]. This approach differs from the conventional procedure by allowing for the determination of the requirements for all essential AAs in one set of experiments [30] and is well-accepted as an efficient and rapid tool to estimate the ideal AA profile in Nile tilapia [10,11]. Additionally, there are some studies in which more than one assessment method was applied to compare the impacts of different methods on estimated AA requirements. Broken-line models generated 27% of tabulated recommendations, while both linear and broken-line assessments generated 9%. Additionally, polynomial regressions generated 50%, while the deletion method generated 14% of the recommendations. Finally, we established recommendations for the dietary essential AA profile (plus cystine and tyrosine) for each Nile tilapia production stage relative to dietary protein, as these values have not yet been established for all AAs and production stage ranges. For this, we considered previously established dietary protein requirement values in the literature for each production stage.

3. Results

3.1. Amino Acid Composition of Tilapia Tissues

The AA composition of eggs and whole bodies of Nile tilapia at different production stages are shown in Table 1. Lysine was the dominant essential AA, while glutamic acid was the major non-essential AA. However, there is little information in the literature about the impact of dietary AA supplementation on egg and tissue AA profiles.

Table 1. Amino acid profile (g/100 crude protein) of eggs and whole bodies of Nile tilapia.

AA	Egg [1]	Fry [2]	Nursery [3]	Pre-growout [4]	Growout [5]	Mean ± SD
EAA						
Arg	4.7	3.7	4.2	5.4	6.3	4.9 ± 1.0
His	1.9	5.2	2.3	2.1	1.5	2.6 ± 1.5
Ile	2.8	3.2	3.9	3.9	4.3	3.6 ± 0.6
Leu	5.9	6.8	6.0	6.5	7.1	6.5 ± 0.5
Lys	5.8	6.0	7.2	6.7	7.4	6.6 ± 0.7
Met	2.9	5.3	2.0	2.3	2.5	3.0 ± 1.3
Phe	2.2	3.5	3.2	3.7	3.5	3.2 ± 0.6
Thr	4.5	3.7	3.3	3.9	4.1	3.9 ± 0.4
Trp	n.d.	n.d.	0.9	0.9	0.8	0.9 ± 0.1
Val	4.4	4.4	4.4	4.6	4.7	4.5 ± 0.1

Table 1. *Cont.*

AA	Egg [1]	Fry [2]	Nursery [3]	Pre-growout [4]	Growout [5]	Mean ± SD
NEAA						
Ala	8.4	7.1	5.6	6.1	7.0	6.8± 1.1
Asp	8.1	7.0.	8.2	8.2	8.1	8.2 ± 0.1
Cys	n.d.	n.d.	0.9	0.7	0.7	0.8 ± 0.1
Glu	9.4	11.8	12.1	12.0	14.5	12.0 ± 1.8
Gly	4.3	n.d.	6.8	7.3	9.5	7.0 ± 2.1
Pro	7.2	7.2	n.d	n.d	n.d	7.2 ±0.0
Ser	8.8	5.2	3.2	3.6	3.6	4.9 ± 2.3
Tyr	2.7	3.4	2.9	3.0	2.8	3.0 ± 0.3

Abbreviations: EAA, essential amino acid; NEAA, non-essential amino acid; n.d., non-determined; [1] Egg composition of Nile tilapia females fed a 350 g/kg crude protein diet [31]; [2] Yolk-sac resorbed Nile tilapia fry of 12 mg body weight [32]; [3] Body weight of ~1 g [33]; [4] Body weight of ~44 g [13]; [5] Body weight of ~829 g [34].

Previous research identified that the AA composition of eggs varies with dietary protein [31]. Conversely, early studies have reported that dietary AA supplementation does not alter the whole-body AA composition of nursery, pre-growout, and growout Nile tilapia [35–37].

3.2. Dietary Amino Acid Recommendations for Nile Tilapia

The AA requirements recommended for some Nile tilapia production stages are presented in Table 2. Of note, there are tangible differences across the literature, and the genesis of these inconsistencies could include genetic differences between strains, diet composition, fish management, rearing conditions, and the statistical method by which AA requirements were assessed. The AA requirements of Nile tilapia were published by the NRC (2011) and are considered one of the main references for AA recommendations. However, genetic improvements and higher performance objectives set by the modern tilapia industry have motivated researchers to review the NRC (2011) recommendations over the past decade. Notably, a number of recent studies have been published, although different experimental methodologies have been used. Several experiments were conducted in which the requirement of one single or multiple AAs was determined, as shown in Table 2.

Table 2. Amino acid recommendations for Nile tilapia.

Amino Acid	Fish Stage	Dietary Requirement		Response	P:E	Reference
		g/kg Diet (DM)	% Protein			
Arg	1	11.8	4.2	WG	26.8	[38]
	1	18.2	6.2	WG	n.p.	[39]
	1	13.6	4.9	WG	21.4	[40]
	2	16.7	5.2	WG	18.5	[41]
	2	13.7	4.9	NR	16.1	[11]
	Mean ± SD	14.8 ± 2.6	5.1 ± 0.7		18.7 ± 2.7	
His	1	4.8	1.7	WG	26.8	[38]
	2	4.8	1.8	NR	16.1	[11]
	2	8.2	3.1	WG	15.4	[42]
	3	8.8	2.8	WG	19.4	[43]
	Mean ± SD	6.7 ± 2.2	2.4 ± 0.7		19.4 ± 5.2	
Ile	1	8.7	3.1	WG	26.8	[38]
	2	9.1	3.3	NR	16.1	[11]
	1	13.7	5.0	WG	19.6	[13]
	Mean ± SD	10.5 ± 2.8	3.8 ± 1.0		20.8 ± 5.5	

Table 2. *Cont.*

Amino Acid	Fish Stage	Dietary Requirement		Response	P:E	Reference
		g/kg Diet (DM)	% Protein			
Leu	1	9.5	0.34	WG	26.8	[38]
	2	13.5	0.48	NR	16.1	[11]
	1	12.5	0.43	WG	n.p.	[44]
	Mean ± SD	11.8 ± 2.1	4.2 ± 007		21.5 ± 7.6	
Lys	1	14.3	5.1	WG	26.8	[38]
	2	16.5	5.9	NR	16.1	[11]
	3	15.1	6.0	WG	19.2	[35]
	2	18.0	5.6	WG	18.5	[45]
	Mean ± SD	16.0 ± 1.6	5.7 ± 0.4		20.2 ± 4.6	
Met	1	7.5	2.7	WG	26.8	[38]
	3	6.8	2.3	WG	20.6	[46]
	1	9.1	3.2	WG	n.p.	[47]
	1	8.1	2.9	WG	19.1	[48]
	Mean ± SD	7.9 ± 1.0	2.8 ± 0.4		22.2 ± 4.1	
Met +Cys	1	9.0	3.2	WG	26.8	[38]
	3	11.2	3.8	WG, FY	20.6	[46]
	1	10.0	3.5	WG	n.p.	[47]
	1	8.5	3.0	WG	19.1	[48]
	Mean ± SD	9.7 ± 1.2	3.4 ± 0.4		22.2 ± 4.1	
Phe	1	10.5	3.8	WG	26.8	[38]
	1 [1]	8.8	3.0	WG	21.4	[49]
	1	12.1	3.5	WG	20.9	[50]
	Mean ± SD	10.5 ± 1.2	3.4 ± 0.4		23.0 ± 3.3	
Phe + Tyr	1	15.5	5.6	WG	26.8	[38]
	1 [1]	18.6	6.4	WG		[49]
	1	20.6	5.9	WG		[50]
	Mean ± SD	18.2 ± 2.6	6.0 ± 0.4		23.0 ± 3.3	
Thr	1	10.5	3.8	WG	26.8	[38]
	1	13.3	4.7	WG		[51]
	2	13.5	4.8	NR	16.1	[11]
	3	11.5	4.0	WG, FY	22.7	[34]
	Mean ± SD	12.2 ± 1.4	4.3 ± 0.5		21.9 ± 5.4	
Trp	1	2.8	1.0	WG	26.8	[38]
	2	2.4	0.9	NR	16.1	[11]
	1	3.4	1.1	WG	19.3	[52]
	1	3.8	1.2	WG	18.5	[53]
	1	3.1	1.0	WG	n.p.	[54]
	Mean ± SD	3.1 ± 0.5	1.0 ± 0.1		20.2 ± 4.6	
Val	1	7.8	2.8	WG	26.8	[38]
	2	9.7	3.5	NR	16.1	[11]
	1	12.7	4.5	WG	17.4	[55]
	Mean ± SD	10.1 ± 2.5	3.6 ± 0.9		20.1 ± 5.8	

Abbreviations: SD, standard deviation; DM, dry matter; WG, weight gain; NR, nitrogen retention; FY, fillet yield; P:E, protein-to-energy ratio (g/MJ gross or digestible energy); n.p., non-presented; [1] Hybrid tilapia, *Oreochromis niloticus* × *Oreochromis aureus*.

Based on data displayed in Table 1, the dietary recommendation of each essential AA (plus cystine and tyrosine) was computed relative to the dietary crude protein content and shown in Table 3. For this, we considered the dietary protein content for each fish production stage based on previous values established for Nile tilapia.

Table 3. Dietary amino acid recommendation (g/kg diet) for Nile tilapia [1].

Amino Acid	Production Stage [2]		
	Fry	Nursery/Pre-Growout	Growout
	Crude Protein (g/kg Diet) [3]		
	460	350	320
Arg	23.5	17.9	16.3
His	11.0	8.4	7.7
Ile	17.5	13.3	12.2
Leu	19.3	14.7	13.4
Lys	26.2	20.0	18.2
Met	12.9	9.8	9.0
Met + Cys	15.6	11.9	10.9
Phe	15.6	11.9	10.9
Phe + Tyr	27.6	21.0	19.2
Thr	19.8	15.1	13.8
Trp	0.5	0.4	0.3
Val	16.1	12.3	11.2

[1] Each amino acid is estimated relative to crude protein (mean value) displayed in Table 2; [2] Previously established for Nile tilapia as nursery (1.6 to 30 g of body weight), pre-growout (31 to ≤220 g of body weight), and growout (>220 g of body weight) [56]; [3] Mean values previously established for Nile tilapia fry [57,58], nursery and pre-growout [57], and growout Nile tilapia [59].

Previous evidence shows that the culture system influences the dietary protein requirement of Nile tilapia broodstock. Thus, based on the percentage of each AA relative to dietary protein, the dietary AA recommendations for Nile tilapia broodstock are displayed in Table 4.

Table 4. Dietary amino acid recommendations (g/kg diet) for broodstock Nile tilapia [1].

Amino Acid	Crude Protein (g/kg Diet; Dry Matter)		
	350 [2]	380 [3]	400 [4]
Arg	17.9	19.4	20.4
His	8.4	9.1	9.6
Ile	13.3	14.4	15.2
Leu	14.7	16.0	16.8
Lys	20.0	21.7	22.8
Met	9.8	10.6	11.2
Met + Cys	11.9	12.9	13.6
Phe	11.9	12.9	13.6
Phe + Tyr	21.0	22.8	24.0
Thr	15.1	16.3	17.2
Trp	0.4	0.4	0.4
Val	12.3	13.3	14.0

[1] Each amino acid is estimated relative to crude protein (mean value) displayed in Table 2; [2] Broodstock raised in earthen pond [31,60]; [3] Broodstock raised in recycling system [61]; [4] Broodstock raised in water salinity up to 14‰ [62].

The concept of ideal protein for domestic animals was first proposed by Mitchell over 60 years ago [63] and remains relevant in poultry and pig nutrition [64]. The ideal protein concept refers to dietary protein with an AA profile that exactly meets an animal's requirements [65]. According to this concept, dietary protein should have an AA profile that exactly meets the animal's requirements, as shown in Table 5. Lysine is used as a reference AA to express the requirements for other AAs, which simplifies diet formulation, as solid requirement data for most AAs are not readily available [66]. Additionally, the concept of ideal AA ratios was introduced for Nile tilapia in 1994 to aid in the development of cost-effective feed formulations [67,68].

Table 5. Dietary amino acid profile (% of lysine) based on the ideal protein concept for Nile tilapia.

Amino Acid	Fish Production Stage [1]			Mean ± SD
	Nursery [2]	Pre-Growout [2]	Growout [2]	
Lysine	100	100	100	10 0 ± 0
Arginine	86	125	81	97 ± 24
Histidine	30	34	34	33 ± 2
Isoleucine	56	57	51	55 ± 3
Leucine	84	96	66	82 ± 15
Methionine	41	64 [3]	41 [3]	49 ± 13
Phenylalanine	64	101 [4]	70 [4]	78 ± 20
Threonine	103	93	89	95 ± 7
Tryptophan	16	24	23	21 ± 4
Valine	60	76	73	70 ± 9

Abbreviation: SD, standard deviation; [1] Previously established for Nile tilapia as nursery (1.6 to 30 g of body weight), pre-growout (31 to ≤220 g of body weight), and growout (>220 g of body weight) [56]; [2] Data established for nursery [11], pre-growout [12], and growout [10] Nile tilapia by deletion method; [3] Methionine plus cystine; [4] Phenylanaline plus tyrosine.

Of note, the ideal protein concept also has been applied to reduce dietary protein [69] and optimize fishmeal-free diets [70] for Nile tilapia. Importantly, further studies are required to continuously update ideal AA profiles considering the ideal protein concept in Nile tilapia diets.

3.3. Importance of Amino Acids in Nile Tilapia

3.3.1. Lysine

Lysine is the first limiting essential AA in typical corn, wheat, and cereal coproduct–based diets for Nile, and supplementation of feed-grade lysine has been largely adopted in practical and experimental diets for Nile tilapia. Lysine's primary metabolic role in protein synthesis also is the basis for it to be the reference AA in computing ideal AA ratios. Interestingly, an early study demonstrated that the effectiveness of using intact lysine from high-lysine corn protein concentrate was not significantly different from that of crystalline lysine in Nile tilapia [71]. It is noteworthy that accurate estimations of dietary lysine requirements are critical because recommendations for the balance of AAs based on the ideal protein concept are expressed as ratios to lysine (Table 5). In this sense, dietary lysine is generally considered the first limiting essential AA. As shown in Table 3, there is a relatively low variation in dietary lysine recommendations for body weight gain of fish. On the basis of body weight gain, the simple means of total [45] and digestible lysine [72] intakes are 14 and 12 mg/g body weight gain, respectively. Previous work indicated that lysine utilization efficiency remained relatively high (+63%) in nursery and pre-growout Nile tilapia and decreased (48%) in growout Nile tilapia [73]. In this sense, the dietary lysine requirements for the maintenance of nursery, pre-growout, and growout Nile tilapia have been established to be 2.7, 45.1, and 56.3 mg lysine/$kg^{0.8}$ body, respectively [74]. Previous work determined that growout Nile tilapia required an intake of 23 mg of lysine to deposit 1 g of body weight gain [35]. Moreover, lysine has been shown to improve body weight, feed efficiency, and fillet yield in Nile tilapia [35,75]. Additionally, high quantitative lysine requirements have been described for Nile tilapia reared in saline water [76], with Nile tilapia reared in brackish water (8‰) showing higher lysine requirements than those raised in 0‰ water (23 vs. 21 g/kg diet, respectively) [77].

3.3.2. Sulfur-Containing Amino Acids

Methionine and cysteine (cystine forms from two cysteine residues) are the total sulfur-containing AAs considered in tilapia feeds. Of note, methionine is considered the first limiting AA in Nile tilapia fed cereal-based diets, particularly soybean-meal-rich diets [3,4]. Methionine plays an essential role in cellular metabolism as a methyl donor and acts as the precursor to cysteine [17]. Notably, methionine is one of the most supplemented

feed-grade AAs in fish feeds, including those for Nile tilapia [48]. In addition, the total sulfur-containing AA requirement of Nile tilapia is often met by supplementing methionine and considering the replacement values of cysteine and methionine in tandem. Previous research identified that cystine could spare up to 49% of the methionine requirement on a molar sulfur basis in diets for Nile tilapia [48]. Moreover, reports on quantitative methionine requirements for body weight gain are very similar when expressed as individual methionine contents (CV of 13%) compared to methionine-plus-cystine contents (CV of 12%). These suggest that total sulfur-containing AAs may be used to express the dietary requirement, along with considering the minimum level of dietary methionine. The optimal methionine-plus-cystine requirement is well established in the literature, averaging 9.7 g/kg diet (3.5% of crude protein), as displayed in Table 2. A previous study established methionine utilization efficiencies of 0.76 and 0.55 and determined the methionine maintenance requirements of 3.12 and 16.5 mg methionine/$kg^{0.7}$ body for pre-growout and growout Nile tilapia, respectively [78]. Importantly, a previous work determined that growout Nile tilapia required an intake of ~15.2 mg of methionine to deposit 1 g of body weight gain [34].

3.3.3. Threonine

Threonine is a potential limiting AA in conventional corn-, wheat-, soybean-, and coproducts-based diets fed to Nile tilapia [6,7]. Feed-grade threonine is commercially available and may be included in diets for Nile tilapia. Threonine is an important AA because it is prominent in intestinal mucin secretion and in the production of antibodies [79], as well as influencing digestive and absorptive capacities and antioxidant status in the intestine [80]. However, the underlying mechanisms of action of threonine on mucin secretion and intestine health status in Nile tilapia are not fully understood, although many studies have reported the positive impacts of adequate and excess provision of dietary threonine on Nile tilapia performance. Noteworthily, the optimal threonine requirement is well established in the literature and averages 12 g/kg diet (4.3% crude protein), as shown in Table 2. An early study identified the dietary threonine requirement as lower for body weight gain (10.5 g/kg diet; 3.6% crude protein) compared to fillet yield (11.5 g/kg diet; 4% crude protein) in growout Nile tilapia [34]. Additionally, these authors also established that growout Nile tilapia require an intake of 15.9 mg of threonine to deposit 1 g of body weight.

3.3.4. Tryptophan

Tryptophan is considered a potential limiting AA in conventional plant-based diets, particularly in Nile tilapia fed corn and its coproducts [6]. Feed-grade tryptophan is also commercially available for inclusion in Nile tilapia aquafeeds. In addition to protein synthesis, tryptophan plays an important role in producing several metabolites, mainly the neurotransmitter/neuromodulator serotonin and the hormone melatonin in teleost fish [81]. Thus, as a precursor of serotonin, dietary tryptophan has been linked to various behavioral patterns [82]. Consistently, adequate tryptophan supplementation leads to reduced aggressive behavior and stress in Nile tilapia [83], resulting in positive effects on growth, feed efficiency [54], and survival [84]. In this review, the tryptophan requirement averages 3.1 g/kg diet (1% crude protein), as shown in Table 2. Previous work established that pre-growout Nile tilapia require an intake of ~3 mg of tryptophan to deposit 1 g of body weight [52].

3.3.5. Branched-Chain Amino Acids

Isoleucine, leucine, and valine are branched-chain AAs that attract less attention, as their requirements are usually met by typical protein feedstuffs in conventional Nile tilapia diets. In addition, it is important to elaborate diets with a balanced profile of branched-chain AAs because interactions between leucine and valine exist, and imbalances have been reported to depress performance [85]. In this sense, it is possible that branched-chain AA interactions may have influenced the tabulated requirement values. Variations in

branched-chain AA recommendations are summarized in Table 2. Growing evidence has suggested the optimal branched-chain AAs ratio (Ile:Leu:Val) to be 1:1.3:0.9 in diets for pre-growout Nile tilapia [13]. However, very few studies have been designed to investigate the interactive effects of branched-chain AAs in Nile tilapia. Feed-grade isoleucine, leucine, and isoleucine have yet to become economically feasible. In addition to protein synthesis, an adequate supply of leucine and valine is important for maintaining immune responses [44, 55]. Recently, a study reported that leucine and valine improved intestinal function, enhanced digestive and absorptive capacities, and positively regulated glucose and fatty acid metabolism in the liver, thereby improving the growth performance of Nile tilapia [85]. Dietary isoleucine, leucine and valine requirements average 10.5 g/kg diet (3.8% crude protein), 11.8 g/kg diet (4.2% crude protein), and 10.1 g/kg diet (3.6% crude protein), respectively, as described in Table 2. Previous studies reported that Nile tilapia require intakes of ~15 [13], ~18 [44], and 19 mg [55] of isoleucine, leucine, and valine, respectively, to deposit 1 g of body weight.

3.3.6. Arginine

Arginine has received little consideration in Nile tilapia because its requirement is usually met by typical protein feedstuffs in conventional Nile tilapia aquafeeds. It is noteworthy that the optimal arginine requirement is well established in the literature and averages 14.8 g/kg diet (5.1% crude protein), as shown in Table 2. Early works showed that Nile tilapia required an intake of 20–27 mg of arginine to deposit 1 g of body weight [39,41]. Additionally, a previous study found that arginine at 16.7 g/kg diet stimulated the mRNA expression of myogenic regulatory factors (MRFs), growth hormone (GH), insulin-like growth factors (IGFs) [41], and consequently hypertrophic muscle processes, supporting enhanced growth of Nile tilapia [40]. Also of note, a previous study found that Nile tilapia fed 23.9 g arginine/kg diet exhibited higher immune responses and survival when challenged by Streptococcus agalactiae [86]. Furthermore, emerging evidence suggests that arginine positively changes intestinal microbiota, activates intestinal fatty acid oxidation, and alleviates triglyceride accumulation in intestinal tissue and intracellular cells of Nile tilapia [87]. Additionally, the beneficial effect of 29 g arginine/kg diet on liver health was reported in Nile tilapia reared under high-density (500 fish/m^3; 63 $\pm$ 20 g body weight) conditions in floating net cages [88]. On the contrary, the above authors also reported that a higher level of arginine (41 g/kg diet) promoted the incidence of liver necrosis, further supporting the observation that excess arginine may lead to increased plasma ammonia concentration, decreasing the excretion efficiency of this metabolite, as described in Jian carp, *Cyprinus carpio* var. Jian [89]. However, more extensive research is necessary to investigate the effects of excess arginine on the nitrogen excretion of Nile tilapia.

3.3.7. Histidine

Histidine is considered a marginally limiting AA in typical plant-based protein feedstuffs used in Nile tilapia diets [6,7,90]. Feed-grade histidine is generally not commercially available for use in Nile tilapia aquafeeds. As shown in Table 2, dietary histidine requirements average 7.3 g/kg diet (3.4% crude protein), and previous studies reported that Nile tilapia require an intake of ~9 mg of histidine to deposit 1 g of body weight. An early work reported the positive effects of adequate histidine supplementation on muscle growth by hypertrophy and hyperplasia in pre-growout Nile tilapia [43]. Another study established that histidine also increased mRNA levels of muscle growth-related genes, myoblast determination protein (MyoD), and myogenin, as well as protein synthesis of growout Nile tilapia [42]. Furthermore, emerging evidence has identified the antioxidant capacity of histidine to improve flesh quality attributes in grass carp [91], while another study reported influences on fillet quality of growout Nile tilapia [75]. However, few studies have been conducted to evaluate the health status of Nile tilapia in response to dietary histidine supplementation. This approach is of major importance in applying the precision nutrition concept in Nile tilapia operations.

3.3.8. Total Aromatic Amino Acids

The academic community has not extensively explored the dietary total aromatic AA (phenylalanine plus tyrosine) requirements of Nile tilapia, possibly because both AAs are not considered marginally limiting in feed ingredients typically used for tilapia. Therefore, feed-grade phenylalanine and tyrosine are not commercially available for diet supplementation. However, it is possible that in low-protein diets, phenylalanine may be a limiting AA for Nile tilapia. Importantly, the total aromatic AA requirements of Nile tilapia can be met by supplementing phenylalanine and also considering phenylalanine and tyrosine in tandem because tyrosine can spare some of the dietary phenylalanine otherwise required for tyrosine synthesis [92]. In this regard, the total aromatic AA requirement is influenced by dietary tyrosine levels. Recent work determined the tyrosine replacement value for phenylalanine on a molar basis to be 37% in Nile tilapia [50]. Another recent study confirmed that adequate phenylalanine supplementation influenced growth rate in nursery Nile tilapia [38]. As displayed in Table 2, the optimal phenylalanine-plus-tyrosine requirement averaged 18.3 g/kg diet (5.9% crude protein). A recent study reported that Nile tilapia require an intake of ~35 mg of phenylalanine plus tyrosine to deposit 1 g of body weight [50].

3.3.9. Non-Essential Amino Acids

Growing evidence suggests that non-essential AAs are closely related to the growth performance, health, and flesh quality of Nile tilapia. Non-essential AAs assume more important roles in fish fed plant-rich diets because of their more limited presence [3,4,6,7,90,93]. Additionally, anti-nutritional factors in vegetable ingredients may have adverse effects on AA digestion and absorption and also impair fish health [94]. Emerging evidence has identified that glutamine plays crucial roles in growth and intestinal function [95] and enhances leucocyte function in Nile tilapia [22]. In addition, early studies identified that supplementation of glycine could enhance the antioxidant ability of Nile tilapia [96] and has beneficial effects on growth [19,21]. Previous research reported a positive association between dietary taurine intake and lipid digestion/absorption with carbohydrate and AA metabolism that promoted enhanced growth performance of Nile tilapia [97]. Along with these beneficial effects, taurine plays an antioxidant role [98] which may have positive effects on flesh quality attributes [23] as well as on reproductive performance [20]. Of note, it has been well established that carnitine plays a central role in regulating the lipid β-oxidation of long-chain fatty acids to produce energy in fish species such as zebrafish, *Danio rerio* [99], and this may explain the decreased mesenteric and fillet fat accumulation in growout hybrid tilapia [100]. In addition, carnitine supplementation was found to improve antioxidant functionality in flesh quality attributes [100] and ameliorate or prevent induced liver, intestine, and gill histopathological lesions in Nile tilapia [101].

4. Conclusions and Implications

The environmental impact of fish farming is becoming a major challenge that could warrant restrictions on tilapia production. Higher nitrogen excretion levels into the environment are an increasing issue, and well-balanced aquafeeds have been identified as a potential solution. Of note, most studies have estimated the requirement of one individual AA at a time without considering the interactive effects of other dietary AAs. Therefore, it is important to design AA requirement assays for Nile tilapia that take AA interactions into consideration. A lack of attention to protein synthesis dynamics in non-protein-bound AA diets is important because they are supplemented at relatively higher levels in plant-based diets. Therefore, it is important to investigate the digestive dynamics of proteins as well as protein synthesis while maintaining good health status and flesh quality. Moreover, it is evident that AA nutrition of broodstock needs more attention in terms of the impact of AAs on reproductive performance responses. Interestingly, some new studies have reported that non-essential AAs improve growth and reproductive performance as well as the health of fish. Worthy of note is the fact that emerging evidence shows that genomic approaches

constitute an important tool for better understanding the underlying mechanisms of fish growth, behavior, and health in order to estimate the AA requirements of Nile tilapia. However, it is clear that the AA requirements of Nile tilapia might vary as a consequence of numerous experimental conditions, including fish strain, size, culture system, and the basal diet used. Notably, genetic selection in Nile tilapia has improved growth performance, fillet yield, and feed efficiency. Therefore, dietary formulations should consider increased amino acid requirements. Interestingly, the current survey highlighted low variations in lysine recommendations relative to protein content, considering that lysine is used as the reference AA in applying the ideal protein concept. This review also identified that emerging studies considered this concept to determine multiple AA requirements using the deletion method. More comprehensive data in the literature are needed for AA requirements through growout in tilapia production in addition to confirming previously established values for lysine, methionine, and threonine. This review observed variations in AA recommendations for different commercial production parameters such as weight gain, feed efficiency, and fillet yield. Therefore, it is important to choose the most appropriate parameter or combination of parameters that represents the business model of the tilapia industry. Finally, these considerations indicated that well-balanced AA diets might be useful for improving the economic and ecological sustainability of tilapia farming into the future.

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animals

MDPI

Article

Tryptophan and Cortisol Modulate the Kynurenine and Serotonin Transcriptional Pathway in the Kidney of *Oncorhynchus kisutch*

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Simple Summary: Our results indicate activation of the kynurenine pathway and serotonin activity when stimulated with tryptophan and cortisol supplementation. An amount of 95% of tryptophan is degraded by the kynurenine pathway, indicating the relevance of knowing how this pathway is activated and if stress levels associated with fish culture trigger its activation. Additionally, it is essential to know the consequence of increasing KYNA levels in different species in the short and long term, and even during the fish ontogeny.

Abstract: Aquaculture fish are kept for long periods in sea cages or tanks. Consequently, accumulated stress causes the fish to present serious problems with critical economic losses. Fish food has been supplemented to reduce this stress, using many components as amino acids such as tryptophan. This study aims to determine the transcriptional effect of tryptophan and cortisol on primary cell cultures of salmon head and posterior kidney. Our results indicate activation of the kynurenine pathway and serotonin activity when stimulated with tryptophan and cortisol. An amount of 95% of tryptophan is degraded by the kynurenine pathway, indicating the relevance of knowing how this pathway is activated and if stress levels associated with fish culture trigger its activation. Additionally, it is essential to know the consequence of increasing kynurenic acid "KYNA" levels in the short and long term, and even during the fish ontogeny.

Keywords: stress; neurotransmitter; cell culture; fish; aquaculture

1. Introduction

Fish in aquaculture suffer high stress levels due to abiotic variables such as temperature, hypoxia, salinity, pH, or eutrophication and biotic variables such as viruses, bacterial, sea lice, or harmful micro-algae blooms [1–3]. As a reaction to this event, Wendelaar-Bonga (1997) [3] mentioned that the fish have two axis stress responses, having different actions at different times: the first is for acute stress, "brain-sympathetic-chromaffin" (BSC), and the

second axis is for chronic stress, "hypothalamus-pituitary-interrenal" (HPI) [3,4]. Farmed fish are typically kept for a long period in sea cages or tanks. Consequently, accumulated stress causes the fish to present serious problems with critical economic losses. Chronic stress modulates the endocrine response, with cortisol being the final product and used as a stress indicator. This hormone release is controlled by the adrenocorticotropic hormone (ACTH), and its secretion is controlled by corticotropin-releasing factor (CRF) [2]. Cortisol binds with glucocorticoid receptors (GRs) and generates several physiological effects in peripheral tissues in order to overcome stress and recover the pre-stress homeostatic state [3,5,6]. Øverli et al. [7,8] described that neurotransmitters of brain origin, such as noradrenaline (NAd), dopamine (DA), or serotonin (5-HT), are involved in the control and integration of responses to physiological stress in teleost fish [7,9–11].

This activity can be traduced into high levels of brain serotoninergic activity after exposure to different stressors. In aquaculture, the fish are exposed to stressors such as handling, pollutants, crowding, diseases, and the presence of ectoparasites (sea lice). These stressors induce high levels of serotoninergic activity, as was observed in several fish species including the coho salmon *Onchorynchus kisutch* [11–13]. Several authors indicated that fishes presented high levels of serotonin (5-HT) due to tryptophan supplementation, which reduced cortisol levels and underwent changes, for example, a decrease in aggressive behavior, reduction in cortisol at high temperature, exposure to air, chasing, and high stocking density [14–22]. However, all these studies are short-term experiments, and therefore long-term experiments are required [23].

Tryptophan is an essential amino acid for mammals and fish. It is involved in immune tolerance mechanisms mediated by its metabolites, following the enzymatic activity of indoleamine 2,3-dioxygenase in leucocytes [24,25]. Results in European seabass HKL primary cell cultures suggest altered pro-inflammatory signals that counteract the inflammatory response caused by increased tryptophan availability [26]. This amino acid is converted to 5HT through the activity of tryptophan hydroxylase and aromatic L-amino acid decarboxylase in the presence of vitamin B6 [27]. Another pathway is the kynurenine pathway (KP). It is mediated by two rate-limiting enzymes, namely tryptophan 2,3-dioxygenase (TDO) and indoleamine 2,3-dioxygenase (IDO) [28], which metabolizes to kynurenine (KYN) [29], and KYN is metabolized in kynurenic acid by kynurenine aminotransferase 2 (KIAT 2) [30–32] (Figure 1). Due to its toxic potential, this pathway may cause stress, which can suppress the immune system, as mentioned by [3]. Kaczorek et al. (2017) [33] indicated that feed supplementation with kynurenic acid in rainbow trout aggravated damage signs in the liver, kidneys, and gills, and the effect was dose-dependent in fish infected with *Yersinia ruckeri*.

In Chile, salmon farming is an important economic activity, with Atlantic salmon (*Salmo salar*) and coho salmon (*Oncorhynchus kisutch*) being the main aquaculture species in Chile, and placed first in coho salmon production worldwide. Generally, coho salmon are less susceptible to sea lice than Atlantic salmon, but they are still affected [11]. The sector is also suffering the effects of climate change due to rising temperatures, low oxygen levels, and harmful micro-algae blooms [2].

Therefore, this study aimed to describe how supplementation with tryptophan and cortisol modulates the neuroendocrine and kynurenic pathway. We used the mRNA transcription of *Tryptophan Hydroxylase* (*TPH2*), *5HT1α receptor*, *INFγ*, and *KIAT 2* as markers in the primary cell culture of the head and posterior kidney of coho salmon (*Oncorhynchus kisutch*) following the pathway (Figure 1).

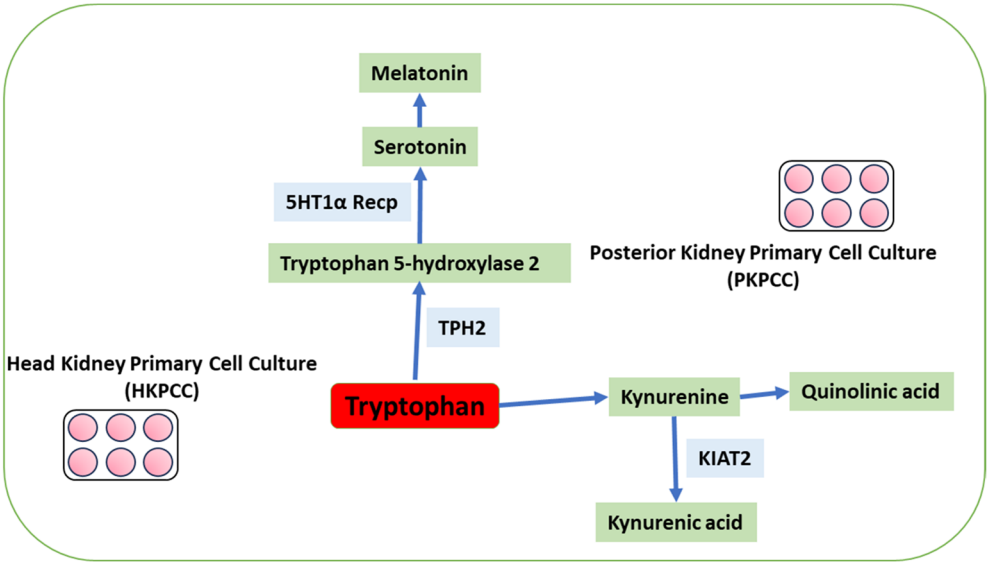


Figure 1. Diagram of the kynurenine pathway from tryptophan. Blue arrows show the product in each box. Light blue boxes are enzymes or receptors of this pathway, previous to the final product.

2. Materials and Methods

2.1. Animals

We used immature healthy specimens of coho salmon (*Oncorhynchus kisutch*). A total of 12 fish weighing approximately 300 ± 12 g with a length of 39 ± 5 cm, post-smolt stage, were obtained from Metri Station (Universidad de Los Lagos, Osorno, Chile). They were transported to laboratories at the Faculty of Science (Universidad Austral de Chile, Valdivia, Chile) and distributed into seawater (35 psu) tanks (500 L) with a continuous flow-through system, 12:12 h light/dark photoperiod cycle, and a water temperature of 13 ± 2 °C. The fish were acclimated for two weeks to avoid the stress from handling and transportation. During these acclimation and maintenance stages, fish were fed ad libitum using EWOS Transfer 100 pellet feed, without boost. Three fish were sampled, and each fish head kidney and posterior kidney were removed; each tissue had three replicates. All fish were captured, anesthetized with a lethal dose of 2-phenoxyethanol (1 mL/L, Fluka-77699-500ML SIGMA-ALDRICH, SLS Ireland, Dublin, Ireland), and euthanized through spinal sectioning before tissue removal [1–34].

All experimental protocols complied with guidelines for using laboratory animals, as established by the Chilean National Commission for Scientific and Technological Research (ANID), the Universidad Austral de Chile with the approved protocol number 261/2016, and the Universidad de los Lagos with the approved protocol number 001/2023.

2.2. Primary Culture Cells

For the primary culture, we obtained small pieces of tissue (approximately 10 mg) or explant of the head and posterior kidney from *O. kisutch* under aseptic or sterile conditions, following Nualart et al. (2023)'s [34] protocol, which was then seeded and kept in a six-well plate and maintained at 18 °C for 24 h under air atmosphere. The cell and tissue growth medium was Leibovitz's 15 (L-15) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) and 1% penicillin-streptomycin (P/S) (Gibco, Thermo Fisher, Waltham, MA, USA).

2.3. In Vitro Treatment

Twenty-four hours after seeding the explant of the head and posterior kidney, primary cell cultures were treated with cortisol 200 ng/mL, tryptophan 5 µg/mL, tryptophan 50 µg/mL, and a combination of cortisol 200 ng/mL + tryptophan 5 µg/mL and cortisol 200 ng/mL + tryptophan 50 µg/mL, using the same concentrations previously described

for Castro et al. (2011) [35] and Mardones et al. (2018) [36]. Primary cell cultures in six-well plates were exposed for 1, 3, 6, 12, 24, and 48 h at 18 °C. Control plates had the same volume of medium without the treatment. All experiments were run in triplicate (biological replicates) and were repeated twice independently (as a technical replicate).

2.4. Total RNA Extraction

Total RNA from the head and posterior kidney portions, from both stimulated and control tissues, were isolated using TRIzol reagent (Sigma, St. Louis, MO, USA) following the manufacturer's instructions and stored at −80 °C. Subsequently, RNA was quantified at 260 nm on a NanoDrop spectrophotometer (NanoDrop Technologies®, Wilmington, DE, USA), and the quality was determined through electrophoresis on a 1% agarose gel. Finally, total RNA (2 µg) was used as a reverse transcription template to synthesize cDNA, applying MMLV-RT reverse transcriptase (Promega, Madison, WI) and the oligo-dT primer (Invitrogen, Waltham, MA, USA) according to standard procedures.

2.5. qRT-PCR Analysis of Gene Expression

Reactions were carried out on an AriaMx Real-time PCR System (Agilent, Santa Clara, CA, USA). CDNA was diluted to 100 ng and used as a qRT-PCR template with reactive Brilliant SYBRGreen qPCR (Stratagene, San Diego, CA, USA). Reactions were performed in triplicate, in a total volume of 14 µL, which contained 6 µL SYBRGreen, 2 µL cDNA, 1.08 µL of primers mix, and 4.92 µL of PCR-grade water. The applied PCR program was as follows: 95 °C for 10 min, followed by 40 cycles at 90 °C for 10 s, 60 °C for 15 s, and 72 °C for 15 s. Melting curve analysis of the amplified products was performed after each PCR to confirm that only one PCR product was amplified and detected. Expression levels were analyzed using the comparative Ct method ($2^{-\Delta\Delta CT}$) [37]. The data are presented as the fold change in gene expression normalized to an endogenous reference gene (*18S*) and relative to unstimulated cells (control). The primers used for *TPH2*, *5-HT 1α receptor*, *INFγ receptor*, *KIAT 2* are listed in Table 1. PCR efficiencies were determined through linear regression analysis of sample data using LinRegPCR [38] from the serial dilutions when Log dilution was plotted against DCT (threshold cycle number).

Table 1. Primer sequences.

Primer	Nucleotide Sequences (5′→3′)	Efficiency Head Kidney (%)	Efficiency Posterior Kidney (%)	
18S Fw	GTCCGGGAAACCGTC	101.9	100.5	XR_006760234.1
18S Rv	TTGAGTCAAATTAAGCCGCA			
TPH 2 Fw	AGTGTAGCTCAGTGGAGGA	101.4	103.2	XM_014125607.2
TPH 2 Rv	AATGCACTGGAGAGGATGTT			
INF γ receptor Fw	ATCGCTCCCTATTTCTCTGTG	99.1	100.2	NM_001360942.1
INF γ receptor Rv	CCAAGACACCCAACAGGAT			
KIAT 2 Fw	TGCACAGCGGAGAAGGTACAGTGG	101.6	104.8	XM_045693024.1
KIAT 2 Rv	GGCTCCGACAGTGACCAGGATGT			
5-HT 1α receptor Fw	TGGAGTGCTCAGTGACTGGT	97.4	100.2	XM_014173861.2
5-HT 1α receptor Rv	AGCCCTTTAGTCCAGCCTCTAC			

2.6. Statistical Analysis

All statistical analyses and graphs were performed using the software Sigma Plot 11 and Minitab 19. The assumptions of normality, independence, and homogeneity of the residuals for the between-group variances were also tested using a Shapiro–Wilk test and a Levene test, respectively. Significant differences in gene expression between different treatments were determined through three-way analysis of variance (three-way ANOVA),

while the factors of variance were the conditions cortisol, tryptophan, and a combination of cortisol + tryptophan in high and low concentrations and times (1–48 h). Tukey tests were used to evaluate the a posteriori difference between groups in relative gene expression ($p < 0.01$), and all data are shown as the mean ± standard error (SE) and represent the relative expression ($2^{-\Delta\Delta Ct}$) normalized to a reference gene (*18S*) and compared to the control group at each time point.

3. Results

3.1. Tryptophan Hydroxylase (TPH2) mRNA Gene Expression Changes

The mRNA gene expression of TPH2 in head kidney cells stimulated with cortisol significantly increased at 3 and 12 h in O. kisutch (Figure 2A). Meanwhile, the gene expression of tryptophan significantly increased at 3 and 48 h in both concentrations. Nevertheless, the combined effect of cortisol and tryptophan (at 5 and 50 µg/mL) presented high levels at 1, 3, and 48 h. However, cortisol and tryptophan at 50 µg/mL also increased their levels at 24 h. Coincidentally, at 6 h, all experimental groups presented a downregulation (Figure 2A and Table 2).

Table 2. *p*-values from three-way ANOVA, with the interaction of parameters (condition*time) in the head kidney primary cell culture (HKPCC) of *O. kisutch* treated with cortisol, tryptophan, and a combination of cortisol and tryptophan in high and low concentrations. Statistical differences between different treatments (control, cortisol, and tryptophan) at the same time point, condition, and times are the main factors. NS, not significant; significant ($p < 0.01$).

Tissue	Genes	Times	Control	Cortisol 200 ng/mL	Tryptophan 5 µg/mL	Tryptophan 50 µg/mL	Cortisol 200 ng/mL + Tryptophan 5 µg/mL	Cortisol 200 ng/mL + Tryptophan 50 µg/mL
Head Kidney	**5HTP1a**	**1**	NS	<0.01	<0.01	<0.01	<0.01	<0.01
		3	NS	<0.01	<0.01	<0.01	<0.01	<0.01
		6	<0.01	NS	<0.01	<0.01	NS	NS
		12	NS	<0.01	<0.01	<0.01	<0.01	<0.01
		24	<0.01	<0.01	<0.01	<0.01	NS	<0.01
		48	<0.01	NS	<0.01	NS	<0.01	<0.01
Head Kidney	**INFg**	**1**	NS	<0.01	NS	<0.01	<0.01	NS
		3	<0.01	<0.01	<0.01	<0.01	NS	<0.01
		6	NS	NS	<0.01	NS	NS	NS
		12	<0.01	<0.01	<0.01	<0.01	<0.01	NS
		24	<0.01	NS	NS	<0.01	<0.01	<0.01
		48	<0.01	NS	NS	NS	<0.01	<0.01
Head Kidney	**KIAT**	**1**	NS	NS	<0.01	<0.01	<0.01	NS
		3	NS	NS	NS			NS
		6	NS	NS	NS	NS	NS	NS
		12	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
		24	<0.01	NS	<0.01	<0.01	<0.01	<0.01
		48	<0.01	NS	NS	NS	<0.01	<0.01
Head Kidney	**TPH2**	**1**	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
		3	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
		6	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
		12	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
		24	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
		48	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01

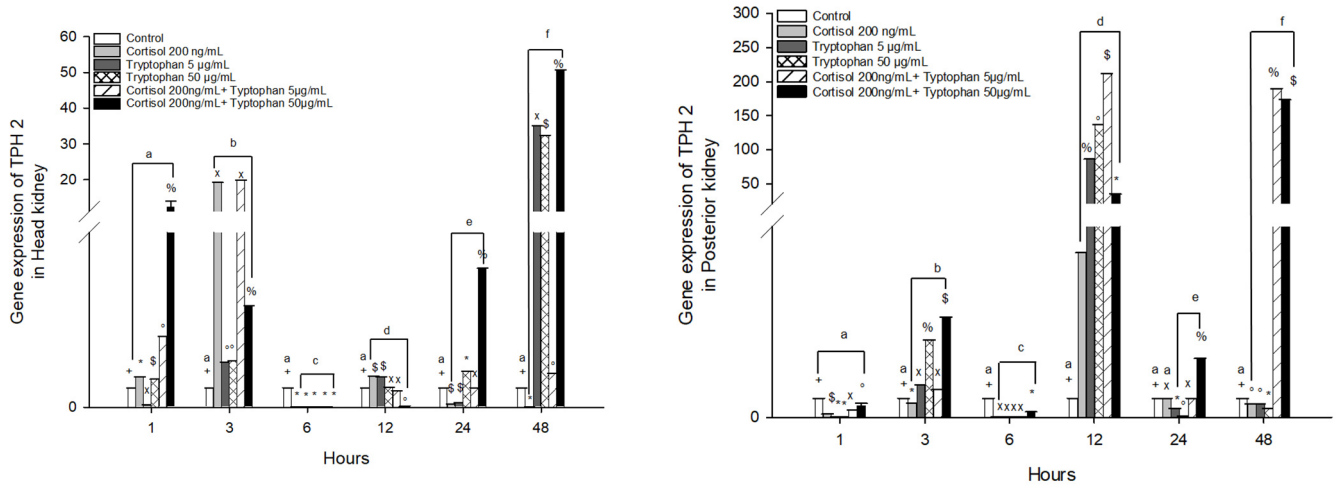


Figure 2. Gene expression of *TPH2* in the (**A**) head kidney primary cell culture (HKPCC) and (**B**) posterior kidney primary cell culture (PKPCC) of *O. kisutch* treated with cortisol, tryptophan, and a combination of cortisol and tryptophan at high and low concentrations. Each value represents the mean ± S.E.M. (n = 3). Different letters indicate statistical differences within the same treatment between time points. Symbols (*, °, %, x, $, and +) indicate statistical differences between different treatments (control, cortisol, and tryptophan) at the same time point (three-way ANOVA, $p < 0.01$).

In posterior kidney cells stimulated with cortisol and tryptophan, 5 µg/mL significantly increased at 12 h in O. kisutch (Figure 1B), while tryptophan 50 µg/mL significantly upregulated at 3 and 12 h. The combined effect of cortisol and tryptophan (at 5 and 50 µg/mL) presented high levels at 3, 12, and 48 h (Figure 2B). Likewise, at 1 and 6 h, all experimental groups presented downregulation (Figure 2B and Table 3).

Table 3. *p*-values from three-way ANOVA, with the interaction of parameters (condition*time) in the posterior kidney primary cell culture (PKPCC) of *O. kisutch* treated with cortisol, tryptophan, and a combination of cortisol and tryptophan in high and low concentrations. Statistical differences between different treatments (control, cortisol, and tryptophan) at the same time point, Condition, and Times are the main factors. NS, not significant; significant ($p < 0.01$).

Tissue	Genes	Times	Control	Cortisol 200 ng/mL	Tryptophan 5 µg/mL	Tryptophan 50 µg/mL	Cortisol 200 ng/mL + Tryptophan 5 µg/mL	Cortisol 200 ng/mL + Tryptophan 50 µg/mL
Posterior Kidney	**5HTP1a**	**1**	NS	<0.01	<0.01	<0.01	<0.01	NS
		3	NS	NS	<0.01	<0.01	<0.01	NS
		6	NS	<0.01	NS	NS	NS	NS
		12	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
		24	NS	<0.01	<0.01	NS	<0.01	NS
		48	NS	NS	NS	NS	<0.01	<0.01
Posterior Kidney	**INFg**	**1**	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
		3	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
		6	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
		12	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
		24	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
		48	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01

Table 3. *Cont.*

Tissue	Genes	Times	Control	Cortisol 200 ng/mL	Tryptophan 5 µg/mL	Tryptophan 50 µg/mL	Cortisol 200 ng/mL + Tryptophan 5 µg/mL	Cortisol 200 ng/mL + Tryptophan 50 µg/mL
Posterior Kidney	**KIAT**	**1**	NS	<0.01	NS	<0.01	<0.01	<0.01
		3	NS	<0.01	<0.01	<0.01	<0.01	<0.01
		6	NS	<0.01	<0.01	NS	<0.01	NS
		12	<0.01	<0.01	NS	<0.01	NS	<0.01
		24	<0.01	NS	<0.01	<0.01	<0.01	<0.01
		48	<0.01	NS	<0.01	<0.01	<0.01	<0.01
Posterior Kidney	**TPH2**	**1**	NS	NS	NS	NS	<0.01	<0.01
		3	NS	<0.01	<0.01	<0.01	<0.01	<0.01
		6	NS	NS	NS	NS	NS	NS
		12	NS	<0.01	<0.01	<0.01	<0.01	<0.01
		24	NS	<0.01	<0.01	NS	<0.01	<0.01
		48	NS	<0.01	<0.01	<0.01	<0.01	<0.01

3.2. 5HT1α Receptor mRNA Gene Expression Changes

The mRNA gene expression of *5-HT 1α* in head kidney cells stimulated with cortisol significantly increased at 3, 12, and 24 h in *O. kisutch* (Figure 3A and Table 2), while tryptophan stimulation with 5 µg/mL presented an upregulation at 12 h, instead of stimulation with 50 µg/mL which presented an upregulation at 1, 12, and 24 h. The combined effect of cortisol and tryptophan at 5 µg/mL presented an upregulation at 1, 3, and 48 h, while cortisol and tryptophan at 50 µg/mL were highest at 3, 24, and 48 h.

Posterior kidney cells stimulated with cortisol presented an upregulation at 12 h; instead, tryptophan 5 µg/mL presented an upregulation at 3 and 12 h, while tryptophan 50 µg/mL was highest at 12 h. The combined effect of cortisol and tryptophan 5 µg/mL presented an upregulation at 1, 3, 12, and 48 h, and cortisol and tryptophan 50 µg/mL presented the highest levels at 12 and 24 h (Figure 3B and Table 3).

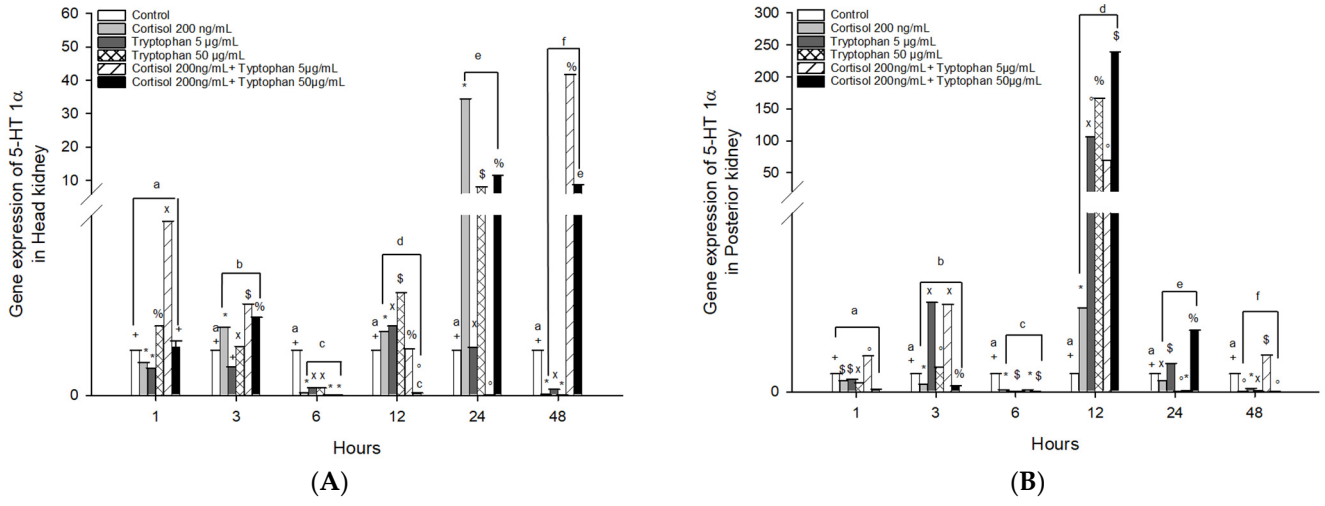
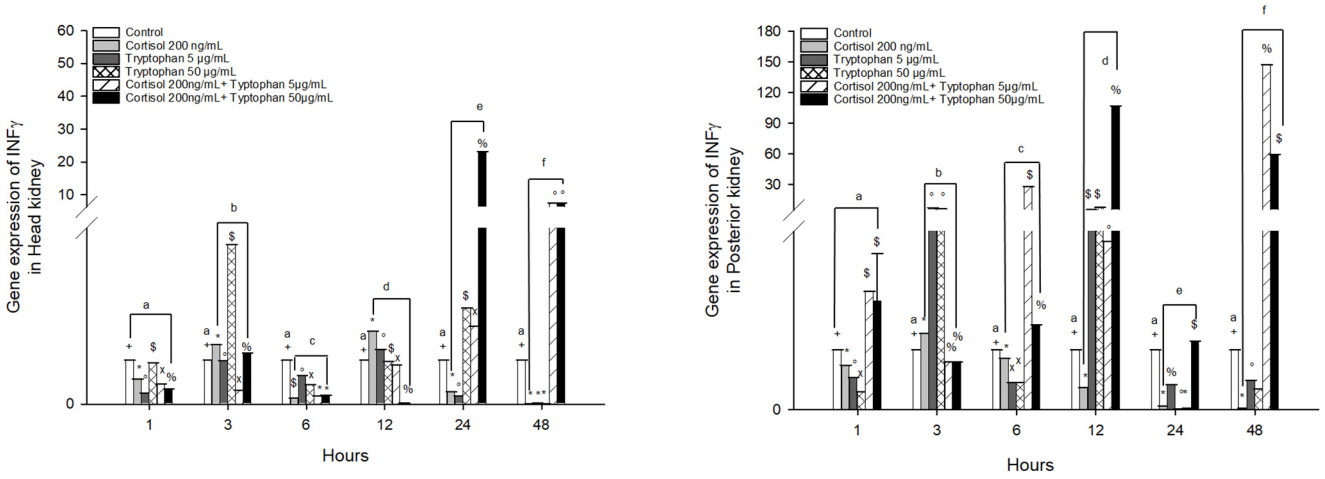


Figure 3. Gene expression of *5HT 1α* in the (**A**) head kidney primary cell culture (HKPCC) and (**B**) posterior kidney primary cell culture (PKPCC) of *O. kisutch* treated with cortisol, tryptophan, and a combination of cortisol and tryptophan in high and low concentrations. Each value represents the mean ± S.E.M. (n = 3). Different letters indicate statistical differences within the same treatment between time points. Symbols (*, °, %, x, $, and +) indicate statistical differences among different treatments (control, cortisol, and tryptophan) at the same time point (three-way ANOVA, $p < 0.01$).

3.3. INFγ Receptor mRNA Gene Expression Changes

The mRNA gene expression of *INFγ* in head kidney cells stimulated with cortisol significantly increased at 3 and 12 h in *O. kisutch* (Figure 4A and Table 2), while tryptophan stimulation with 5 µg/mL did not present changes compared to the control group; instead, stimulation with 50 µg/mL presented upregulation at 3 and 24 h. The combined effect of cortisol and tryptophan at 5 and 50 µg/mL had the highest expression levels at 24 and 48 h.

Posterior kidney cells stimulated with cortisol presented an upregulation at 12 h; instead, tryptophan 5 and 50 µg/mL presented an upregulation at 3 and 12 h. The combined effect of cortisol and tryptophan 5 and 50 µg/mL presented an upregulation at 1, 6, 12, and 48 h (Figure 4B and Table 3).



(**A**) (**B**)

Figure 4. Gene expression of *IFNγ* in the (**A**) head kidney primary cell culture (HKPCC) and (**B**) posterior kidney primary cell culture (PKPCC) of *O. kisutch* treated with cortisol, tryptophan, and a combination of cortisol and tryptophan at high and low concentrations. Each value represents the mean ± S.E.M. (n = 3). Different letters indicate statistical differences within the same treatment between time points. Symbols (*, °, %, x, $, and +) indicate statistical differences between different treatments (control, cortisol, and tryptophan) at the same time point (three-way ANOVA, $p < 0.01$).

3.4. Kynurenine Aminotransferase 2 (KIAT 2) mRNA Gene Expression Changes

The mRNA gene expression of *KIAT 2* in head kidney cells stimulated with cortisol and tryptophan 5 and 50 µg/mL significantly increased at 12 h in *O. kisutch* (Figure 4A). The combined effect of cortisol and tryptophan 5 µg/mL presented an upregulation at 1, 3, 12, and 48 h, and also cortisol and tryptophan 50 µg/mL presented an upregulation at 12, 24, and 48 h (Figure 5A and Table 2).

In posterior kidney cells stimulated with cortisol, *KIAT 2* significantly increased at 12 h in *O. kisutch* (Figure 5B), while tryptophan stimulation with 5 µg/mL did not present changes compared to the control group; instead, stimulation with 50 µg/mL presented an upregulation at 3 h. The combined effect of cortisol and tryptophan 5 and 50 µg/mL presented an upregulation at 1, 3, 24, and 48 h; also, cortisol and tryptophan 50 µg/mL presented the highest level at 12 h (Figure 5B and Table 3).

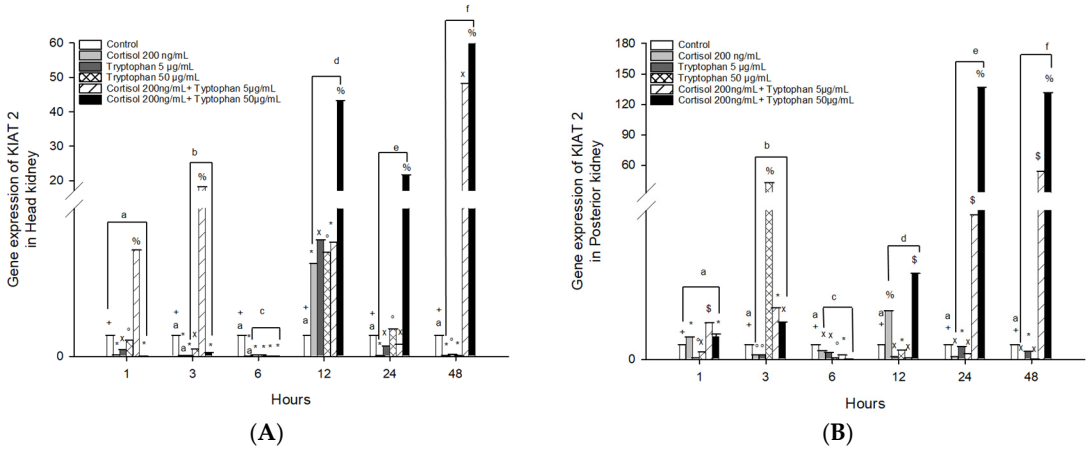


Figure 5. Gene expression of *KIAT 2* in the (**A**) head kidney primary cell culture (HKPCC) and (**B**) posterior kidney primary cell culture (PKPCC) of *O. kisutch* treated with cortisol, tryptophan, and a combination of cortisol and tryptophan in high and low concentrations. Each value represents the mean ± S.E.M. (n = 3). Different letters indicate statistical differences within the same treatment between time points. Symbols (*, °, %, x, $, and +) indicate statistical differences among different treatments (control, cortisol, and tryptophan) at the same time point (three-way ANOVA, $p < 0.01$).

4. Discussion

Cortisol is a product of HPI, derived from ACTH activation in the head kidney due to stress events, and tryptophan is an essential amino acid that needs to be incorporated into the diet [1,23,39]. In the last ten years, as recommendations to improve aquaculture have arisen, many articles suggest supplementing the diet with several products, such as tryptophan, since the levels of this essential amino acid are decreasing in fish affected by several stressors, as was mentioned by previous studies [40,41]. Nevertheless, Nile tilapia "*Oreochromis niloticus*" were fed with tryptophan food supplementation but the cortisol levels in tilapia stressed were not reduced [42], and although it reduced glucose levels as a secondary stress marker in Meagre "*Argyrosomus regius*" [41], the lowest growth rate was in fish with tryptophan supplementation.

Nualart et al. (2023) [34] indicated that the primary cell culture in tissue such as the kidney is an excellent approach to obtain results that are more specific than in vivo studies because the cells are not influenced by other tissues. In our study, we used both portions of the kidney, "head kidney and posterior kidney," which had different responses based on the stimulations. Our results regarding tryptophan hydroxylase (*TPH2*) and *5HT1α* receptor expression of mRNA showed patterns associated with stimulation with tryptophan and tryptophan + cortisol, especially *TPH2* where the high levels were at the earliest and latest points in primary cell cultures in both kidney portions. This enzyme converts tryptophan to serotonin and melatonin as the final products. Both hormones are involved in many physiological processes such as feeding intake, osmoregulation, and reducing stress [1,5,13,43], indicating that the serotonin system could have a dual role in the stress response, responding as an early signal during initiation but also as a late response during chronic stress as we mentioned in our results with two patterns of mRNA expression.

The IFN-γ cytokine is produced by natural killer cells (NK cells) and T lymphocytes to avoid virus replications [44]. The *IFNγ* in our study presented the highest levels of mRNA expression in the posterior kidney than the head kidney, and its response was highest in all of the kinetic time points except at 24 h. Tryptophan stimulation increased IFNγ expression levels in both tissues, but both stimulators were stimulated at the latest time points, which is contrary to the results obtained by the previous studies [45,46], where glucocorticoid and cortisol suppressed the immune response and, of course, IFNγ production, weakening the response against viruses. Saravia et al. (2022) [47] in *Harpagifer*

antarcticus indicated that both immunostimulants (LPS and Poly:C) were overexpressed, suggesting that interferon could play a role in antigen presentation in both types of infection for Antarctic Notothenioids.

Some authors [48] indicated that tryptophan is metabolized via the kynurenine pathway (see Figure 1). However, this pathway has two sub-pathways after kynurenine appears in two potential ways, quinolinic acid and kynurenic acid, where the latter has a kynurenine aminotransferase 2 (*KIAT 2*) enzyme that metabolizes kynurenic acid. Our results of *KIAT 2* mRNA expression showed that cortisol and tryptophan stimulate its overexpression, especially at the latest time point of the experiment [32]. Moreover, Badawy (2017) [30] indicated that IFNγ and glucocorticoids induce the expression of indoleamine 2,3-dioxygenase "IDO," which catalyzes the same step as tryptophan 2,3-dioxygenase "TDO" in the kynurenine pathway. Therefore, we stimulated with cortisol and tryptophan; as mentioned previously, IFNγ was overexpressed in the latest experimental time points; consequently, the kynurenine pathway can be induced, having high levels of KIAT2. Also, the IFNγ-stimulation of macrophages has been closely linked to inducing the mammalian macrophage IDO response [49]. *Lampetra japonicum* (lamprey) demonstrated that exogenous tryptophan could inhibit the expression of pro-inflammatory factors and promote the expression of anti-inflammatory factors by inhibiting the MyD88 signaling pathway and activating the IDO-KYN-AHR (aryl hydrocarbon receptor) signaling pathway [48]. In male BALB/c mice [50], exogenous kynurenic acid (KYNA) was indicated to have low levels of cytotoxicity toward murine splenocytes and exhibited immunotropic properties. In rainbow trout (*Oncorhynchus mykiss*), it was demonstrated that cortisol increased kynurenine levels at 48 h post-stress events in the liver and brain [51], which are in line with our results. In addition, in rainbow trout, the kidney was shown to present necrosis in epithelia, tubules, and glomerulus in practically all KYNA-supplemented groups; similar problems were presented in gills with fusion or disappearance of gill lamellae, of course affecting the osmoregulation and respiratory activity [33,52]. The TRP–KYN pathway in lamprey leukocytes (*Lampetra japonica*) was activated by adding TRP [53] and overexpression significantly reduced TNF-α and NF-κβ expression and clarified the ancestral features and functions of the TRP–KYN pathway. Meanwhile, Machado et al. (2021) [54] indicated that the tryptophan presented a clear role in the tolerance process responsible for restriction of the pro-inflammatory cluster of the immune response in Head Kidney Leucocytes (HKLs) of European seabass.

5. Conclusions

Supplementing tryptophan and cortisol in primary cell culture can be an excellent way to discover how the different pathways act. Le Floc'h et al. (2011) [55] and Michael et al. (1964) [56] indicated that tryptophan is degraded through three metabolic pathways, with 95% being degraded through the kynurenine pathway, indicating the relevance of knowing how this pathway is activated or not, and if stress levels associated with fish culture trigger its activation. It would also be essential to know in different species what the consequence is of increasing KYNA levels in the short and long term and even during the ontogeny of the fish.

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Effect of Tryptophan Dietary Content on Meagre, *Argyrosomus regius*, Juveniles Stress and Behavioral Response

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Simple Summary: Fish welfare is an opportunity to improve the standards and quality of aquaculture products and is crucial to ensure the sustainability of the industry. Tryptophan is thought to mitigate fish response to stress. In this study, different dietary tryptophan contents were tested in meagre juveniles. The results suggest, particularly in a higher dosage (0.8%), a reduction of anxiety-like behavior in meagre exposure to acute stress. Although the remaining results showed mild effects of tryptophan dietary supplementation on meagre resilience to stress, it provides some clues as to the potential of this amino acid as a stress mitigator in aquaculture.

Abstract: There are a high number of stressors present in aquaculture that can affect fish welfare and quality. One way of mitigating stress response is by increasing dietary tryptophan. In this study, three diets containing 0.5% (Tript1), 0.6% (Tript2), and 0.8% (Tript3) of tryptophan were tested in 32 g juvenile meagre for 56 days. At the end of the trial, survival, growth, and proximate composition were similar between treatments. Significant differences were found in the plasma parameters before and after a stress test consisting of 30 s of air exposure. Blood glucose levels were higher in the post-stress for all treatments (e.g., 63.9 and 76.7 mg/dL for Tript1 before and after the stress test), and the hemoglobin values were lower in the post-stress of Tript1 (1.9 g/dL compared to 3.0 and 2.4 g/dL for Tript2 and Tript3, respectively). In terms of behavior, three tests were carried out (novel tank diving and shoaling assays, and lateralization test), but no significant differences were found, except for the number of freezing episodes during the anxiety test (1.4 for Tript3 compared to 3.5 and 4.2 for the other treatments). This study suggests that supplementation with dietary tryptophan, particularly in higher dosage (0.8%), can reduce anxiety-like behavior in meagre exposure to acute stress (novel tank). Although the remaining results showed mild effects, they provide some clues as to the potential of this amino acid as a stress mitigator in aquaculture.

Keywords: *Argyrosomus regius*; stress; tryptophan; behavior; cortisol

1. Introduction

Fish welfare is not only an ethical concern but also a means to improve the standards and quality of aquaculture products due to its implications, not only for production but for the sustainability of the industry [1] (FAO, 2019). Good husbandry conditions reduce stress in fish stemming from various internal and external factors [2,3], consequently lowering disease susceptibility, reducing medication needs, benefiting farmers, consumers, and the fish itself [4,5]. Therefore, efforts to minimize stress and disease incidence should be intensified to ensure a safer final product. Intensive aquaculture practices often prioritize maximizing production, impacting fish welfare due to heightened stressors, such as high densities [6]. These stressors can compromise fish welfare and increase the susceptibility to diseases. To counteract these effects, investment in stress resilience methodologies and immune system enhancement is essential. This not only improves animal welfare but also augments aquaculture sustainability [6].

Several methods have been employed to alleviate stress in farmed fish, with dietary supplementation being prevalent [6]. Enriching diets with specific amino acids involved in stress mechanisms, like tryptophan, has emerged as a potential strategy. Tryptophan is an essential amino acid involved in the synthesis of monoamines such as serotonin and melatonin [5,7]. Serotonin influences stress response and immune regulation, while melatonin is involved in immunity, stress response, and antioxidant capacity [7]. Tryptophan also inhibits superoxide production, scavenges free radicals, and mitigates pro-inflammatory cytokines production [4,7]. In rainbow trout (*Oncorhynchus mykiss*) and Senegalese Sole (*Solea senegalensis*), tryptophan maintained or reduced the basal levels of blood cortisol [8,9]. Consequently, supplementing diets with tryptophan may enhance stress resilience and fish welfare [4,7].

Fish behavior in aquaculture settings is a valuable indicator of animal response and stress, often overlooked [10]. Acknowledging the role of fish behavior can facilitate credible and simple health monitoring. Over the last decades, several tests, like the novel tank diving and shoaling tests, have been developed in laboratory conditions to evaluate anxiety levels and social behaviors in fish [11,12]. Additionally, lateralization analyses (described by Bisazza [13]) provide insights into cognitive functions related to habitat exploration, foraging, and escape from predators, which are crucial to meet the ecological and social demands involved in natural selection [14–16]. Despite their importance, these tests are not frequently used in aquaculture studies, offering a different perspective on fish stress response beyond physiological responses like cortisol levels. In the novel tank diving test, it is possible to compare several behavioral parameters to assess anxiety [11]. The shoaling test allows an understanding of fish's individual response when near a small group of conspecific fish [12]. The lateralization test allows the assessment of brain lateralization that is involved in various routine actions, such as foraging [14–16].

This study aimed to evaluate the effect of dietary tryptophan supplementation on meagre behavior and physiological stress response. Meagre, chosen for its high commercial value and vulnerability to stress in intensive farming, was fed diets containing 0.5, 0.6, and 0.8% tryptophan to analyze meagre juveniles' physiological and behavioral responses.

2. Material and Methods

This study was carried out at the Aquaculture Research Station of the Portuguese Institute for the Sea and Atmosphere (IPMA) between September and November 2021.

2.1. Husbandry and Experimental Set-Up

In this trial, 112 days old meagre with an initial weight of 32.6 $\pm$ 3.4 g and 14.4 $\pm$ 0.5 cm length were placed in nine 1500 L round fiberglass tanks, each containing 100 fish. The water circulated in an open circuit, passing through a cartridge filter before entering the tank. The water flow was approximately 15.9 L/min, oxygen saturation, and water temperature were 5.6 $\pm$ 0.2 mg/L and 21.4 $\pm$ 0.2 °C, respectively, while salinity was 38 $\pm$ 1 ppt. A 14 L/10 D photoperiod was maintained.

Three diets with different tryptophan contents were tested in triplicates. The first diet (Tript1) contained 0.5% tryptophan content, approximating the estimated requirement for meagre of this age (preliminary trials suggested an optimal dietary tryptophan of approximately 0.5%). The other two diets (Tript2 and Tript3) were formulated with higher tryptophan levels (0.6 and 0.8, respectively).

The diets were formulated and produced by Sparos Lda (Olhão, Portugal). Powder ingredients were mixed in a double-helix mixer and ground twice in a micropulverizer hammer mill (SH1, Hosokawa-Alpine, Augsburg, Germany). Later, the oil fraction was added to the mixture, the diets were humidified, agglomerated via low-shear extrusion (Dominioni Group, Lurate Caccivio, Italy), and then dried in a convection oven (OP 750-UF, LTE Scientifics, Greenfield, UK) for 4 h at 60 °C. The diets were later crumbled (Neuero Farm, Melle, Germany) and sieved to 3 and 4 mm. The formulation of the three diets is presented in Table 1. Fish were hand-fed the diets ad libitum at 9 am, 11:30 am, 2 pm and 4:30 pm. The amount of feed given was quantified daily. The experimental trial lasted 56 days.

Table 1. Ingredients and proximate composition of the experimental diets. Tript 1—with 0.5% of dietary tryptophan; Tript 2—0.6%; Tript 3—0.8%.

Ingredients (%)	Tript1	Tript2	Tript3
Casein	5	5	5
Porcine gelatin	6	6	6
Soy protein concentrate	30	30	30
Pea protein concentrate	15	15	15
Wheat gluten	15	15	15
Potato starch	7	6.6	5.6
Fish oil	7	7	7
Rapeseed oil	7	7	7
Rapeseed lecithin	2	2	2
Vitamin and minerals	1	1	1
Vitamin C	0.1	0.1	0.1
Vitamin E	0.1	0.1	0.1
Betaine HCl	1	1	1
Antioxidant	0.3	0.3	0.3
Monoammonium phosphate	2	2	2
L-Lysine	0.1	0.1	0.1
L-Tryptophan	0.2	0.4	0.8
	Composition (% DW)		
Protein	52.0	52.3	52.2
Lipids	16.0	17.5	16.0
Ash	7.6	7.4	7.8
Energy (KJ)	23.9	24.1	24.1

2.2. Sampling and Biochemical Analysis

Before the start of the experimental trial, 60 fish were individually weighed and measured. From this group, 10 fish were randomly selected for fish proximate composition analysis. The initial biomass of each tank was determined by weighing all fish grouped in pools of 10. In the mid-trial sampling, 20 fish per tank (60 per treatment) were collected to assess growth based on wet weight and length. At the end of the experiment, the remaining fish were again weighed in groups to establish the final biomass. Additionally, 61 fish per tank were individually weighed and measured. To analyze the fish's final proximate composition, five fish per tank were collected (15 per treatment), and their samples were frozen at −20 °C.

Fish sampled for weight and length were sedated with 100 ppm 2-phenoxyethanol [17], while those collected for proximal composition and blood tests were euthanized using a higher concentration of phenoxyethanol. All handling of fish was carried out by trained

scientists following category B FELASA recommendations and adhered to the European guidelines for the protection of animals used for scientific purposes (Directive 2010/63/UE of the European Parliament and of the European Union Council).

2.3. Chemical Analysis of Fish and Feed

Chemical analysis of the diets and carcasses was carried out following the methods of the Official Association of Chemical Analysts [18] and was run in duplicates. All the collected fish and feed were grounded prior to the analysis. To determine the dry matter, the samples were dried at 105 °C for 24 h. The ash content was obtained by incinerating the previous samples in a muffle furnace at 450 °C for 16 h. The lipid content was determined using the Soxhlet modified method (1879). The energetic value was determined using direct combustion in an adiabatic pump calorimeter (PARR Instruments, Moline, IL, USA; PARR model 1261). Fish protein content was determined using the Kjedahl-modified method (1883).

2.3.1. Amino Acids Profile

Hydrolysis

The amino acid profiles of the three diets were determined according to the hydrolysis methods described in AOAC [19,20].

For the acidic hydrolysis, ca. 20 mg of the sample was hydrolyzed with 3 mL of 6 N HCl with 0.1% phenol, as described in Saavedra et al. [21].

For the alkaline hydrolysis, ca. 80 mg of sample was placed in 10 mL Nalgene Oak Ridge Teflon Fep tubes, with 3 mL of 4.2 N NaOH (deaerated by bubbling with N_2 for 10 min) and one drop of 1-octanol. The hydrolysis was performed under inert conditions at 110–115 °C for 20 h, and then the samples were neutralized with HCl (4.2 N, 3.5 mL) and quantitatively transferred into 20 mL volumetric flasks with ultrapure water.

Norvaline and sarcosine were added to samples (final concentration 500 pmol/μL) before both types of hydrolysis and used as internal standards. Samples were filtered (0.2 m pore size) and stored at −80 °C until amino acid separation. All samples were analyzed in triplicate.

HPLC Analysis

The chromatographic conditions used were in accordance with the Agilent method [22] (Henderson et al., 2000) and amino acids separation was performed by high-performance liquid chromatography (Agilent 1100 HPLC, Agilent Technologies, Palo Alto, CA, USA) in a Phenomenex Gemini ODS C18 guard column (4 mm × 3 mm), and a Phenomenex Gemini ODS C18 110 Å column (4.6 mm × 150 mm, 5 μm) (Phenomenex Inc., Torrence, CA, USA) and detection wavelengths set by fluorescence (340/450 nm and 266/305 nm).

Amino acids identification and quantification were assessed by comparison to the retention times and peak areas of standard amino acids (Sigma, St. Louis, MO, USA) within the range 9–900 pmol/μL (R^2 = 0.9999) with the software Agilent ChemStation for LC (Agilent Technologies, Palo Alto, CA, USA). All determinations were carried out in triplicate (repeatability 0.28–2.6% RSD; recovery 93–110%). Cysteine was not determined due to its destruction during acid hydrolysis.

2.4. Blood and Plasma Analysis

Blood was collected from 8 fish per tank (4 pre-stress and 4 post-stress) and analyzed individually. Between the stress test (30 s exposed to air) and the blood collection, an acclimation time of 30 min was given to the fish.

2.4.1. Hematological Analysis

Blood aliquots were placed into a micro-hematocrit capillary tube (75 mm Super Rior, Lauda-Königshofen, Germany) and then spun in a micro-hematocrit centrifuge (EBA 21 Hettich, Tuttlingen, Germany) at 10,000× g for 5 min for hematocrit determination.

For the hemoglobin analysis, a clinical analysis kit (ref. 1001239 SPINREACT) was used. After reagent and sample preparation, the absorbance was read at 540 nm in the microplate reader.

2.4.2. Plasmatic Parameters

First, blood was centrifuged at 2500× *g* for 10 min to obtain plasma and stored at −80 °C until further analysis. Cortisol was determined using a cortisol ELISA kit test (RE52611, IBL International, Hamburg, Germany). Plasma glucose and lactate were analyzed using a clinical diagnostic kit from QCA (ref. 998660) and SPINREACT (ref. 1001330), respectively, based on the following reactions: glucose was based on the reaction of glucose oxidase and peroxidase, while lactate analysis was based on the reaction of lactate oxidase and peroxidase. Absorbance was read in the microplate reader at 340 nm and at 505 nm for glucose and lactate, respectively. Glucose and lactate were determined using analytical duplicates of each individual sample and analyzed using a colorimetric reaction on a microplate reader (Thermo Scientific, Waltham, MA, USA). For cortisol, two analytical samples were done.

2.5. Stress and Behavioral Tests

To assess varying levels of stress in fish behavior, three tests were carried out: the novel tank diving assay (anxiety assay, [11]), the shoaling assay [12], and the lateralization assay [13], with slight modifications made to the first two tests. These assessments are not often applied in aquaculture, particularly in nutrition trials. Each test involved evaluating four fish per tank (12 per treatment) that had fasted for 12 h. To minimize the metabolic variability observed throughout the day, treatments were evaluated alternately. Fish were given a five-minute acclimatization period in the test tank before the trial started.

2.5.1. Novel Tank Diving and Shoaling Assays

The anxiety assay and the shoaling assay took place in a novel tank with 240 L capacity (120 cm × 72 cm × 36 cm) (Figure 1) [11,12]. Fish behavior was recorded using two cameras, one located above the water level and another underwater, to allow better observation of the fish movements. Later, recorded images were reviewed for behavior analysis. To carry the anxiety assay, the tank's right end was sectioned off with a transparent acrylic plate housing a group of six conspecific fish, while the tested fish occupied the remaining area. To prevent visual contact between the tested fish and the shoal, the acrylic was initially covered with an opaque plate until the trial began. For the anxiety assay, the tank was divided into four sectors (two upper and lower, and two left and right), using two lines. For the shoaling assay, the bottom of the tank was marked with two lines, creating three zones: near the shoal (NS), far from the shoal (FS), and very far from the shoal (VFS). Both tests had a duration of five minutes.

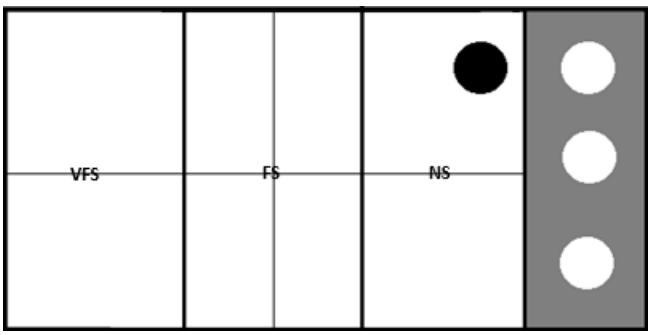

Figure 1. Schematic of the tank seen from above. Thicker lines—division of the three zones of the socialization test (NS, FS, VFS); thinner lines—division of the four sectors of the anxiety test (up/down, left/right); black circle represents the test individual, and white circles represent the shoal. VFS, very far from shoal; FS, far from shoal; NS, near shoal.

In the anxiety assay, the number of vertical and horizontal transitions were counted, as well as the time spent in the upper half of the tank and the number and time of freezing periods (time when the fish did not move). This test was carried out with the opaque plate on to avoid the interaction between the tested fish and the shoal, but once the anxiety assay was over, the opaque acrylic plate was removed, allowing the fish to have a full view of the shoal, and the start of the shoaling assay. In this trial, the time before the first approach to the shoal, the total time near and away from the shoal, the number of transitions towards or away from the shoal, the number of touches on the acrylic, and the number and time of freezing were quantified.

2.5.2. Lateralization Test

The lateralization assay was carried out in a novel tank with 480 L capacity (240 cm × 72 cm × 36 cm) featuring a maze and two barriers at opposite ends (Figure 2) [13]. Fish were introduced into the tank and placed in the center of the corridor, which remained closed with two grids during the acclimation time (5 min). Once the acclimation was over, the fish were allowed to swim freely, prompted by gentle water movement created using a fishing net, thus facing the obstacle and needing to choose between left and right.

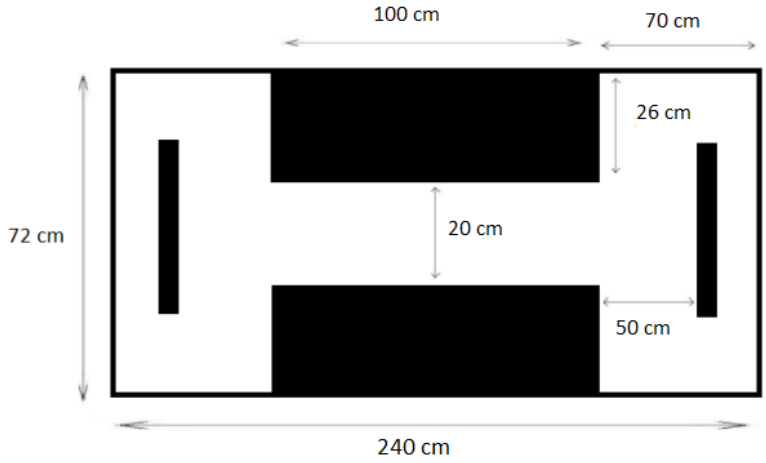

Figure 2. Schematic of the tank for the lateralization test (top view).

Following the initial choice, each fish was required to return to the corridor and proceed to the opposite end, making a new decision. The test consisted of recording ten runs per fish. Subsequently, the relative index (LR) for each fish was calculated according to Bisazza et al. [13]. Values close to 100 indicated a preference for turning right in most of the 10 runs, values close to −100 indicated a left-turn preference, while values near zero indicated a similar preference between left and right.

2.6. Statistical Analyses

The presence of significant differences ($p < 0.05$) in plasma parameters before and after the stress test was analyzed using a two-way ANOVA. Cortisol values were compared using a two-way ANOVA, considering dietary tryptophan content and stress conditions as fixed factors. To comply with normality assumptions, a logarithmic transformation was performed in cortisol values. *t*-tests were used to identify post hoc differences between pre and post-test cortisol levels within each treatment. For proximate composition, growth (except final length, initial biomass, and survival), novel tank diving assay (except erratic movements), and shoaling assay (except latency time), one-way ANOVAs were used. For the parameters that did not satisfy the ANOVA assumptions, such as lactate, final length, initial biomass, survival, erratic movements and latency time, a non-parametric analysis (Kruskal–Wallis) was used. For the lateralization assay, chi square for independent samples was used. The specific growth rate (*SGR*) was calculated as $SGR = \frac{\ln DWf - \ln DWi}{t} \times 100$,

where *DWf* and *DWi* are the final (*f*) and initial (*i*) dry weights (*DW*), respectively, and *t* the trial duration in days. Protein efficiency ratio (PER) was calculated as $PER = \frac{BIOf - BIOi}{Protein\ intake}$, where *BIOf* and *BIOi* are the final (*f*) and initial (*i*) biomass (*BIO*), respectively. The protein intake was calculated as $Feed\ intake \times Protein\ percentage$. The feed conversion ratio (*FCR*) was calculated as $FCR = \frac{Feed\ intake}{weight\ gain}$.

The relative index (*LR*) was calculated as $LR = \frac{turns\ to\ the\ right - turns\ to\ the\ left}{turns\ to\ the\ right + turns\ to\ the\ left} \times 100$.

3. Results

3.1. Amino Acid Diet Composition

The analysis of the three diets confirmed that the diets had three significantly different tryptophan contents (Table 2). Tript1 had a 0.5% tryptophan, Tript2 had 0.6%, and Tript3 had the highest content, 0.8%. When the AA profile of diets was analyzed, differences were observed in the content of aspartate, serine, threonine, and isoleucine, especially between diets Tript1 and Tript2 (Table 2).

Table 2. Amino acid composition (g/100 g) of the diets. Tript1—with 0.5% of dietary tryptophan; Tript2—0.6%; Tript3—0.8%. Values are mean and standard error. Different bold letters represent significant differences for $p < 0.05$.

Amino Acids	Tript1	Tript2	Tript3
Aspartate	2.7 ± 0.0 **a**	2.9 ± 0.0 **b**	2.9 ± 0.0 **ab**
Glutamate	8.1 ± 0.0	8.7 ± 0.0	8.6 ± 0.1
Serine	2.0 ± 0.0 **a**	2.1 ± 0.0 **b**	2.1 ± 0.0 **ab**
Histidine	0.6 ± 0.0	0.7 ± 0.0	0.7 ± 0.0
Glycine	3.4 ± 0.0	3.4 ± 0.0	3.1 ± 0.0
Threonine	2.1 ± 0.0 **a**	2.3 ± 0.0 **b**	2.2 ± 0.0 **ab**
Arginine	2.1 ± 0.0	2.3 ± 0.0	2.2 ± 0.0
Alanine	3.2 ± 0.0	3.4 ± 0.0	3.2 ± 0.0
Taurine	2.5 ± 0.0 **a**	2.3 ± 0.0 **b**	2.2 ± 0.0 **b**
Tyrosine	1.4 ± 0.0	1.6 ± 0.0	1.5 ± 0.0
Valine	1.6 ± 0.0 **a**	1.7 ± 0.0 **b**	1.6 ± 0.0 **a**
Methionine	0.7 ± 0.0	0.7 ± 0.0	0.7 ± 0.0
Tryptophan	0.5 ± 0.0 **a**	0.6 ± 0.0 **b**	0.8 ± 0.0 **c**
Phenylalanine	1.9 ± 0.0 **a**	2.0 ± 0.0 **b**	2.0 ± 0.0 **ab**
Isoleucine	1.3 ± 0.0 **a**	1.4 ± 0.0 **b**	1.3 ± 0.0 **ab**
Leucine	4.0 ± 0.0	4.3 ± 0.0	4.2 ± 0.0
Lysine	0.4 ± 0.0	0.3 ± 0.0	0.3 ± 0.0
Hydroxyproline	0.9 ± 0.0 **a**	1.0 ± 0.0 **b**	0.9 ± 0.0 **a**
Proline	4.0 ± 0.0 **a**	4.4 ± 0.0 **b**	4.1 ± 0.0 **ab**

3.2. Survival and Growth

Survival varied between 94% and 96%, and the values were not significantly different between treatments. At the end of the trial, no significant differences in fish length and weight between treatments were observed. The same was observed for parameters such as biomass, SGR, FCR, and PER, which did not show any significant differences between treatments (Table 3). Diet composition did not seem to affect fish, with proximate composition being approximately 15% protein and 4.7% lipid content (Table 4).

Table 3. Meagre juvenile biometry in the beginning (112 days), midterm (146 days), and end (174 days) of the experimental trial, which tested three diets with 0.5%, 0.6%, and 0.8% of tryptophan. SGR—specific growth rate; FCR—feed conversion rate; ER—protein efficiency rate. Tript1—with 0.5% of dietary tryptophan; Tript2—0.6%; Tript3—0.8%.Values are mean and standard error.

Treatment	Tript1	Tript2	Tript3
Survival (%)	95.3 ± 0.6	95.7 ± 0.2	94.3 ± 1.5
Weight (g)			
112 days	32.6 ± 0.1	32.6 ± 0.1	32.6 ± 0.1
146 days	47.3 ± 0.6	50.4 ± 0.4	48.9 ± 0.9
174 days	61.6 ± 1.1	63.5 ± 0.3	64.3 ± 0.6
Length (cm)			
112 days	14.4 ± 0.0	14.4 ± 0.0	14.4 ± 0.0
146 days	16.8 ± 0.0	17.1 ± 0.1	17.0 ± 0.0
174 days	18.5 ± 0.1	18.6 ± 0.0	18.5 ± 0.0
Biomass (g)			
112 days	3325.4 ± 0.8	3320.2 ± 0.6	3314.7 ± 2.9
146 days	946 ± 11.0	1008.7 ± 8.5	975.7 ± 6.0
174 days	5878.6 ± 128.7	6077.9 ± 32.6	6071.2 ± 126.6
SGR	1.1 ± 0.03	1.2 ± 0.01	1.2 ± 0.02
FCR	1.2 ± 0.03	1.1 ± 0.01	1.1 ± 0.03
PER	1.6 ± 0.04	1.7 ± 0.01	1.7 ± 0.05

Table 4. Proximate composition in a fresh matter of meagre juveniles at the end of the trial fed three diets with 0.5%, 0.6%, and 0.8% of tryptophan. Tript1—with 0.5% of dietary tryptophan; Tript2—0.6%; Tript3—0.8%.Values are mean and standard error.

Treatment	Tript1	Tript2	Tript3
Crude protein	15.7 ± 0.0	15.4 ± 0.2	15.4 ± 0.1
Crude fat	4.9 ± 0.1	4.7 ± 0.2	4.6 ± 0.1
% Dry matter	24.9 ± 0.0	25.0 ± 0.0	24.8 ± 0.0
% Ashes	3.8 ± 0.0	4.0 ± 0.0	3.6 ± 0.0
Energy kJ/g (FM)	5.6 ± 0.0	5.6 ± 0.0	5.6 ± 0.0

3.3. Blood Analysis

The hematocrit did not show significant differences between treatments or fish subjected/not subjected to a stress test. However, in meagre fed the Tript1 diet, hemoglobin decreased after fish were submitted to the stress test. In the other diets with higher tryptophan, hemoglobin levels were not affected by the stress test (Table 5).

Table 5. Blood parameters in meagre juveniles before and after being submitted to a stress test. Tript1—with 0.5% of dietary tryptophan; Tript2—0.6%; Tript3—0.8%. Values are mean and SE. Different letters represent significant differences for $p < 0.05$. HTC, hematocrit; Hgb, hemoglobin; Glu, glucose.

Treatment	Tript1		Tript2		Tript3	
Stress Test	Pre	Post	Pre	Post	Pre	Post
HTC (%)	20.4 ± 0.2	20.3 ± 0.1	19.8 ± 0.2	20.3 ± 0.2	20.3 ± 0.2	18.8 ± 0.1
Hgb (g/dL)	3.0 ± 0.1 ^{a}	1.9 ± 0.0 ^{b}	2.7 ± 0.1 ^{a}	3.0 ± 0.1 ^{a}	2.9 ± 0.1 ^{a}	2.4 ± 0.1 ^{ab}
Glu (mg/dL)	63.9 ± 1.0 ^{a}	76.7 ± 0.7 ^{b}	63.4 ± 0.8 ^{a}	74.1 ± 1.0 ^{bc}	63.5 ± 0.8 ^{a}	73.2 ± 0.7 ^{c}
Lactate (mg/dL)	3.5 ± 0.1 ^{a}	1.8 ± 0.0 ^{b}	3.1 ± 0.1 ^{a}	1.6 ± 0.0 ^{b}	3.1 ± 0.1 ^{a}	1.6 ± 0.0 ^{b}

For blood glucose levels, no significant differences were observed between treatments before the stress test. After the stress test, an increase in the glucose levels was observed in all treatments, being significantly higher in Tript1. Additionally, the glucose levels in the blood were significantly lower in Tript3 compared to Tript1, after the stress test (Table 5).

The concentration of lactate was not affected by the tryptophan level but was significantly affected by the stress test, with the levels decreasing after the stress test (Table 5).

The cortisol levels in the plasma did not seem to be affected by the diet but were affected by the stress test. Fish from Tript1 and Tript3 showed a significant increase in the concentration of cortisol after being subjected to the stress test. In Tript2, no significant differences were found before and after the stress test (Figure 3).

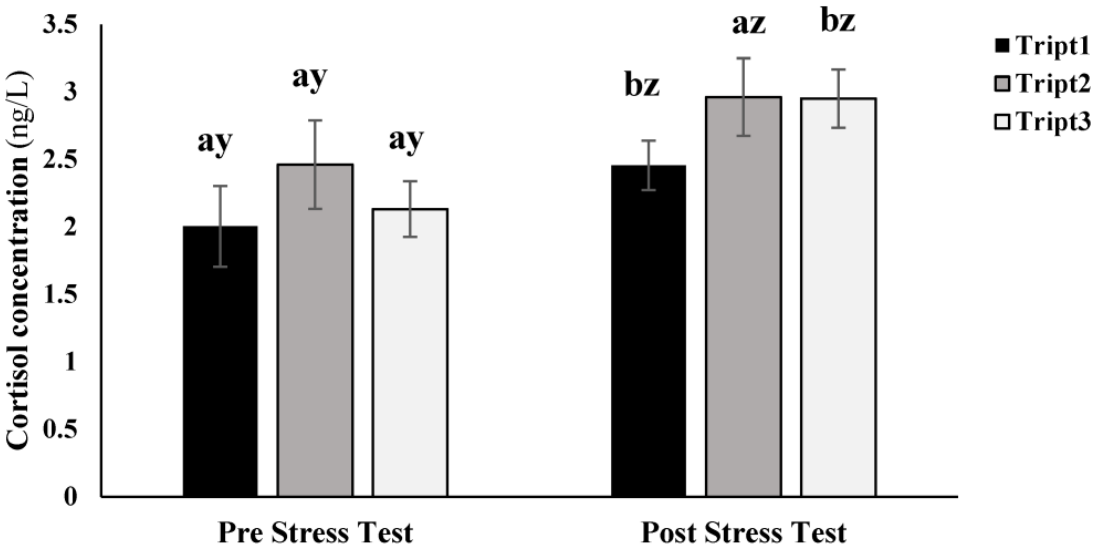

Figure 3. Cortisol blood concentration in the plasma before and after fish were submitted to a stress test consisting of 30 s outside water. Treatments tested three diets with 0.5% (Tript1), 0.6% (Tript2), and 0.8% (Tript 3) of tryptophan in meagre juveniles. Different letters correspond to significant differences between pre and poststress test for the same treatment (a/b) and between pre and poststress test for all treatments (y/z) for $p < 0.05$.

3.4. Stress and Behavioral Tests

To evaluate the effect of dietary tryptophan content, three tests were carried out: the novel tank diving assay (anxiety test), the shoaling assay, and the lateralization assay.

3.4.1. Novel Tank Diving Assay

In this assay, fish from Tript3 had more transitions between sectors and spent more time in the upper part of the tank, while fish from Tript1 and Tript2 had a similar number of transitions and meagre from treatment Tript2 spent less time in the upper half of the tank (Table 6). Fish from Tript1 and Tript2 had more episodes and longer periods of freezing (Table 6). Nevertheless, these differences were not significant.

Table 6. Results were obtained in the novel tank diving assay of meagre juveniles fed with three diets with 0.5%, 0.6%, and 0.8% of tryptophan. The freezing percentage corresponds to the time fish had no movement of the 5-min test. Tript1—with 0.5% of dietary tryptophan; Tript2—0.6%; Tript3—0.8%. Values are mean and standard error. Different letters correspond to significant differences for $p < 0.5$.

Treatment	Tript1	Tript2	Tript3
Total transitions	53.9 ± 2.5	57.0 ± 3.0	72.7 ± 3.8
Time on the upper half (%)	39.6 ± 2.7	31.5 ± 2.0	42.1 ± 2.8
Number of freezing	4.2 ± 0.3 ^{a}	3.5 ± 0.2 ^{ab}	1.4 ± 0.2 ^{b}
Freezing time (%)	20.3 ± 3.5	29.4 ± 0.8	16.8 ± 5.2

3.4.2. Shoaling Assay

In this assay, meagre from Tript2 took less time to approach the shoal, stayed next to the shoal for a longer period, and had fewer transitions and a lower number and duration of freezing episodes. On the other hand, fish from Tript3 took more time to approach the shoal, touched the acrylic more often, and had longer episodes of freezing (similar to Tript1). Meagre from Tript1 stayed next to the shoal for a shorter period, had more transitions, touched the acrylic less often, and had more episodes of freezing. However, these differences between treatments were not significantly different (Table 7).

Table 7. Results were obtained in the shoaling assay (5 min) of meagre juveniles fed with three diets with 0.5%, 0.6%, and 0.8% of tryptophan. Values are mean and standard error.

Treatment	Tript1	Tript2	Tript3
Time prior to 1st approach/300 s	0.4 ± 0.23	0.2 ± 0.2	2.2 ± 0.8
Time next to the shoal (%)	81.1 ± 3.5	86.5 ± 3.6	86.2 ± 2.6
Total transitions	17.0 ± 2.9	14.1 ± 3.7	15.7 ± 4.1
Touches on the acrylic/300 s	18.6 ± 2.6	23.8 ± 3.1	24.0 ± 1.4
Number of freezing/300 s	5.4 ± 1.6	3.8 ± 1.1	4.1 ± 0.9

3.4.3. Lateralization Assay

The lateralization assay evaluated the fish's choice of sides when it ran into an obstacle. Fish from Tript2 turned left most of the time. On the contrary, fish from treatment Tript1 chose right more. Nevertheless, there was a high variation between individuals, and no significant differences were found between treatments. For this, the relative lateralization index was used. The lateralization index was 18.3 for Tript1, −11.7 for Tript2 and 0.0 for Tript3 (Table 8).

Table 8. Results were obtained in the lateralization assay of meagre juveniles fed with three diets with 0.5%, 0.6%, and 0.8% of tryptophan. Values are mean and standard deviation.

Treatment	Tript1	Tript2	Tript3
Times choosing left	4.1 ± 0.2	5.6 ± 0.2	5.0 ± 0.2
Relative lateralization index	18.3 ± 3.1	-11.7 ± 4.2	0.0 ± 3.5

4. Discussion

In various countries, concerns regarding fish welfare in aquaculture are increasing, leading to intensified efforts to mitigate fish stress during the rearing process. Consequently, research is now focused on diverse strategies aimed at enhancing fish welfare. This endeavor seeks to bolster consumer trust in the industry and increase the value of aquaculture products. This study investigates the potential of dietary tryptophan supplementation- an amino acid implicated in fish stress response- to bolster resilience against stressors prevalent in daily aquaculture activities. Preliminary nutritional trials suggested an optimal dietary tryptophan level for meagre juveniles around 0.5%, corresponding to the tryptophan content in the Tript1 diet. Additionally, two other diets containing higher tryptophan contents were evaluated.

The survival rate obtained in this study ranged from 94 to 96%, aligning with expected levels for this species at this developmental stage [23–26]. Mortality primarily stemmed from fish jumping out of the tank, a behavior often linked to stress episodes. This emphasizes the need to mitigate stress in this species.

Regarding growth parameters, no significant differences were found in the midtrial or at the end of the experiment. This can be attributed to the diets' high similarity in composition and the absence of tryptophan deficiency, thus not limiting protein synthesis. These findings align with the SGR, FCR, and PER values, which also displayed no significant

differences among treatments. The SGR and FCR values obtained in this study are in the same range as those reported by Herrera et al. [27] and Saavedra et al. [24] using juveniles from the same species.

Likewise, fish proximate composition remained consistent across treatments. These outcomes agree with similar findings involving meagre juveniles [24,25]. Studies examining tryptophan supplementation have reported varied effects. For instance, Hoseini et al. [28] investigated different levels of tryptophan in rainbow trout juveniles (*Oncorhynchus mykiss*) diets, observing no significant differences in carcass composition. Conversely, Sharf and Khan [29] observed a significant increase in lipid and protein content when testing diets with up to 0.47% tryptophan fed to *Channa punctatus*.

Hematological parameters are good indicators of nutritional status [30] and are important in evaluating physiological and pathological changes in fish [24,31]. Tryptophan has been shown to mitigate fish response to stress in several aquaculture species, such as carps [32,33], totoaba [5] and striped bass [34]. In the present study, tryptophan supplementation did not significantly affect meagre response to the stress test, resulting in significant increases in cortisol levels after the stress test, except for fish fed 0.6% tryptophan, which did not exhibit a significant rise in plasma cortisol levels. However, due to considerable variability, confirming increased fish resilience is challenging. Moreover, cortisol levels after the stress test did not decrease compared to those from the other treatments. Similarly, glucose levels increased after the stress test, especially in fish fed 0.5% tryptophan, potentially associated with heightened energy resources catabolism. In the primary stress response, cortisol release triggers a secondary response, leading to increased glucose levels and its transport to energy-demanding tissues for restoration [5,35,36]. This is consistent with the results obtained for the treatment with lower tryptophan content and coincident with those reported by Saavedra et al. [24] for meagre, Abdel-Tawwab [35] for Nile tilapia and Hoseini et al. [37] for sturgeon. However, it was anticipated that a higher dietary tryptophan content could decrease blood glucose levels, as described by Herrera et al. [27], or at least maintain glucose levels before and after the stress test, as observed by Hoseini et al. [28]. The latter was witnessed in treatments with 0.6 and 0.8% of tryptophan.

Contrarily to glucose, lactate results are unexpected and complex to explain. Normally, exposure to air increases muscle glucose anaerobic catabolism, elevating lactate production [36]. Yet, in this study, lactate concentration decreased after the stress test, contrary to Saavedra et al. [24], where meagre juveniles showed increased lactate levels under similar stress conditions. Moreover, the lactate values in this study were considerably lower than those reported by Saavedra et al. [24]. According to Monteiro et al. [36], this discrepancy could be related to the nutritional balance of the diets, as fish-fed balanced diets could maintain lower lactate levels in the blood. Further, it might also indicate that the tryptophan levels in the diets had the desired effect.

Regarding other blood parameters like hematocrit, no significant differences were obtained between treatments or due to the stress test, with values within the previously published range for meagre [24]. However, hemoglobin concentration was affected by the stress test in meagre fed a diet with 0.5% tryptophan, resulting in reduced levels post-test. Some studies have reported similar decreases in hemoglobin during stress. Kpundeh et al. [38] observed reduced hemoglobin values in tilapia juveniles (*Oreochromis niloticus*) subjected to stressors. In this study, hemoglobin reduction was specific to the 0.5% tryptophan treatment, implying the potential positive effects of tryptophan supplementation on this parameter and its impact on the endocrine response to stress [5].

It was deemed important to analyze fish behavioral response, an aspect seldom explored in aquaculture studies. The focus was on three important components: fish anxiety in exploring a new environment (novel tank), fish social response, and fish brain lateralization. In the anxiety test, significant differences were not found overall, except for the number of freezing episodes. According to Maulvault et al. [14] and Egan et al. [11], an increase in these episodes is associated with higher levels of stress-related hormones (e.g., cortisol) and anxiety in general. This study revealed a decrease in the number of freezing

episodes in the treatment with the highest tryptophan content, suggesting that a higher dietary tryptophan might present an anxiolytic effect, potentially alleviating behavioral and endocrine effects of acute stress. These behaviors are typically characterized by higher cortisol levels in the blood and are influenced by individual fish traits [39] However, no other differences were observed in the other parameters analyzed, potentially due to high individual variability. Other studies [40] tested tryptophan administration through the water and did not observe differences in adult zebrafish behavior.

The shoaling test showed no differences between treatments. This outcome is unsurprising as meagre is a shoaling species, and it is expected for an individual to approach conspecifics within a few seconds. The waiting time until approaching the shoal was comparable among treatments (Tript1—6.4 s, Tript2—2.8 s, and Tript3—6.7 s), although high variability was noted in fish fed 0.8% tryptophan, which might have influenced the results. Maulvault et al. [14] observed significant differences in the time spent before the first approach to the shoal when individuals were exposed to venlafaxine via their feed. However, no significant differences were found in the time spent near the shoal, which suggests that individuals from different treatments had a similar eagerness to join the shoal, which is crucial for survival in nature.

Brain lateralization, driven by brain asymmetry, aids common animal behaviors like escaping from predators, offering a selective advantage [14]. In the lateralization assay, no significant differences were observed among the three tryptophan diets. The lateralization index (LR) in this study showed high variability, akin to Roche et al. [16], possibly masking potential differences between treatments.

5. Conclusions

In conclusion, this study adopted diverse approaches to meagre welfare, not solely relying on physiological parameters but also considering fish behavior, an uncommon approach in aquaculture studies. It suggests that dietary supplementation of tryptophan, particularly at a higher dosage (0.8% of tryptophan), can reduce anxiety-like behavior in meagre exposed to acute stress in a novel tank. Other results indicated mild effects of tryptophan dietary supplementation on meagre resilience to stress, potentially influenced by high variability in fish individual responses or by a nonexistent tryptophan depletion group. Nevertheless, this study provides insights into the potential of this amino acid as a stress mitigator in aquaculture.

Author Contributions: M.S. and M.C.S. designed the study and supervised A.V. A.V. and M.S. wrote the manuscript. A.V. and M.B. carried out the experimental trial and took care of the animal husbandry necessary. B.T. and R.M. were responsible for the amino acid analysis, fieldwork, data collection, and experiments. A.C. was responsible for the analysis of the proximate composition L.R. was responsible for the cortisol analysis. P.P.-F. is responsible for the Aquaculture Research Station of IPMA and provides the fish. All authors participated in the discussion of the results. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: All handling of fish was carried out by trained scientists following category B FELASA recommendations and adhered to the European guidelines for the protection of animals used for scientific purposes (Directive 2010/63/UE of the European Parliament and of the European Union Council).

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Article

Effects of Dietary Glycine Supplementation on Growth Performance, Immunological, and Erythrocyte Antioxidant Parameters in Common Carp, *Cyprinus carpio*

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Simple Summary: Amino acids have various crucial roles in fish growth and health. Among them, the non-essential amino acids have been less studied in aquaculture, but they have many physiological roles. Glycine is a non-essential amino acid that is involved in glutathione structure, and is also involved in the antioxidant system in fish. Moreover, glycine stimulates the immune system. This study shows that a period of 8-week feeding with a diets supplemented with 5 g/kg glycine can improve growth performance, erythrocyte stability, and humoral and mucosal immunity in common carp. So, the present results can be used in carp diet production to support higher growth and health.

Abstract: The effects of dietary glycine supplementation, 0 (control), 5 (5 GL), and 10 (10 GL) g/kg, have been investigated on growth performance, hematological parameters, erythrocyte antioxidant capacity, humoral and mucosal immunity in common carp, *Cyprinus carpio*. After eight weeks feeding, the 5 GL treatment exhibited significant improvement in growth performance and feed efficacy, compared to the control treatment. Red blood cell (RBC) and white blood cell (WBC) counts, hemoglobin, hematocrit, neutrophil and monocyte counts/percentages, RBC reduced glutathione (GSH) content, and skin mucosal alkaline phosphatase, peroxidase, protease, and lysozyme activities were similar in the glycine-treated fish and significantly higher than the control treatment. Blood lymphocyte percentage decreased in the glycine-treated fish, but lymphocyte count increased, compared to the control fish. RBC glutathione reductase activities in the glycine-treated fish were similar and significantly lower than the control treatment. The highest plasma lysozyme and alternative complement activities were observed in GL treatment. The glycine-treated fish, particularly 5 GL, exhibited significant improvement in RBC osmotic fragility resistance. Dietary glycine had no significant effects on RBC glutathione peroxidase activity, plasma immunoglobulin, eosinophil percentage/count, and hematological indices. In conclusion, most of the benefits of dietary glycine supplementation may be mediated by increased glutathione synthesis and antioxidant power.

Keywords: amino acid; glutathione; nutrition; blood cells; skin mucus

1. Introduction

Dietary amino acids have important roles in fish growth and health. Fish depends on dietary supply to meet the requirement of certain amino acids, called essential amino

acids. These amino acids cannot be synthetized in the fish body [1]. However, there are other amino acids that can be synthetized by fish, if certain substrates, enzymes, and cofactors are available. Most of the studies on amino acids' requirement in fish have focused on the essential amino acids, but the non-essential amino acids should be focused as well. These amino acids, although can be synthetized in fish body, have many important physiological roles vital for fish health and welfare [2,3]. Thus, the conventional definition on essential/non-essential amino acids has been recently challenged by researchers.

Glycine is one of the non-essential amino acids in fish like other animals, but has many important physiological functions [2]. Glycine participates in collagen formation and 33% of collagen is made from this amino acid [4]. Moreover, glycine, in collaboration with glutamate and cysteine, participates in synthesizing of glutathione, a powerful and important antioxidant molecule [5]. Thus, glycine modulates the antioxidant system. This amino acid has various roles in immune function. It has been established that a drop in the blood serum glycine concentration increases serum resistance of various human pathogens [6]. A study on Nile tilapia, *Oreochromis niloticus*, has revealed that glycine metabolism pathways are of the most important pathways in prevention of mortality in the fish infected by *Edwardsiella tarda* [7].

There are a few studies regarding the effects of dietary glycine on fish, but the results are not consistent. For example, Nile tilapia has exhibited significant increase in growth performance, when fed a diet supplemented with 5 g/kg glycine. Dietary glycine has failed to affect various plasma and/or hepatic metabolites' levels, antioxidant enzymes' activities, lysozyme/myeloperoxidase activities, and malondialdehyde (MDA) content. Experimental challenge with *Streptococcus iniae* has shown that glycine failed to improve the disease resistance, and the other antioxidant/immunological responses were similar to pre-challenge, except an increase in plasma superoxide dismutase (SOD), glutathione reductase (GR), and myeloperoxidase activities [8]. A study on largemouth bass, *Micropterus salmoides*, has revealed that a plant-based diet supplemented with 20 g/kg glycine was beneficial to prevent growth retardation and intestinal villi shortage, compared to a fishmeal-based diet. However, no change in the hepatic SOD and glutathione peroxidase (GPx) activities and increases in hepatic MDA content and plasma alanine aminotransferase activity suggest possible hepatic problems in the fish [9]. Hoseini, Moghaddam [10] have shown that glycine supplementation has no significant effects on growth performance of beluga, *Huso huso*. White blood cell (WBC) count, plasma reduced glutathione (GSH) level, GPx, lysozyme, and complement activities increased at 2.5 and/or 5 g/kg glycine levels, implying the antioxidant and immunomodulation effects of glycine; however, significant increase in plasma MDA content at 5 and 10 g/kg glycine makes it hard to believe the antioxidant effects of glycine on this species. Our previous study on common carp, *Cyprinus carpio*, has shown that a 3-week period of feeding with 2.5–10 g/kg glycine is beneficial in improving the plasma lysozyme (5 and 10 g/kg glycine), GPx (2.5–10 g/kg glycine), glutathione-s-transferase (GST; 10 g/kg glycine) activities, and GSH (5 and 10 g/kg glycine) content, but decreases the plasma catalase (CAT; 5 and 10 g/kg glycine) activity and has no significant effects on the plasma MDA [11]. An 8-week feeding with the same diets have shown that glycine had no growth-promoting effects on the fish, although numerical improvements of ~10.5 and 12.5% were observed in the fish growth rate and feed efficiency, respectively. Plasma SOD, CAT, GPx, and GST remained unchanged, but plasma GSH and MDA increased and decreased, respectively [12]. These studies indicate that further evaluations are needed to improve the current knowledge regarding the effects of dietary glycine in fish. For example, the above-mentioned studies have focused on limited numbers of humoral innate immune parameters (lysozyme, complement, myeloperoxidase, respiratory burst activities, and WBC) and neglected others, such as skin mucosal immune parameters. This is an important topic considering several immunological roles of glycine reported in mammals [13] and its particular role in mucosal immunity observed in pigs [14]. Moreover, antioxidant effects of glycine have been only assessed in fish blood and liver, but it has been demonstrated that red blood cells (RBC), both in mammals and fish, actively pump

glycine into their cytoplasm [15,16] to support glutathione synthesis and maintain internal antioxidant capacity. Glycine is also a key component of heme synthesis by participation in 5-aminolevulinic acid production; inhibition of glycine import by erythroids led to disrupted heme synthesis in mammals [17].

Considering limited information regarding the roles of dietary glycine in fish (compared to mammals), the present study was designed to assess the effects of dietary glycine on growth performance, the skin mucosal and humoral immune parameters, hematological parameters, RBC antioxidant capacity and propensity to hemolysis in common carp.

2. Materials and Methods

2.1. Diets

In light of the previous studies [11,12], we chose 5 (5 GL) and 10 (10 GL) g/kg glycine for supplementation, but reduced basal (endogenous) glycine level (from 19.8 to 16.1 g/kg) by reducing the amount of poultry by-product in the diet to find if glycine supplementation in plant-based diets (low poultry by-product) can support maximum growth. A control diet without glycine supplementation (CTL) was also included. Feedstuff (corn meal, wheat meal, soybean meal, cottonseed meal, fish canning by-product, and poultry by-product) were sieved (200 μ), mixed at the desired proportions (Table 1). Then, soybean oil, vitamin/mineral premixes, amino acids, cellulose, and glycine were added to the mixture. About 400 mL/kg water was added to the mixture and a dough was prepared, which was pelleted using a meat grinder. Dietary proximate composition was determined (three sample per diet) based on standard methods (kjeldahl method for protein; ether extraction for lipid; oven drying at 70 °C for moisture; furnace combustion for ash, and acid/base digestion for fiber) as described by Hoseini, Moghaddam [10]. Dietary amino acid profile was determined by an HPLC system (Waters Corporation, Milford, MA, USA). The samples were defatted with n-hexane and digested by HCl, followed by derivitization with phenyisothiocyanase, separation with PICO.TAG column, and detection in a dual λ absorbance detector [10].

Table 1. Compositions of the experimental diets containing grading levels of glycine.

Ingredients (g/kg)	CTL	5 GL	10 GL	Amino Acid Profile (%)	CTL	5 GL	10 GL
Corn meal	50	50	50	Glycine	1.61	2.07	2.74
Wheat meal	260	260	260	Arginine	2.47	2.35	2.56
Soybean meal [1]	350	350	350	Serine	1.83	1.63	1.75
Soybean oil	63	63	63	Glutamic acid	8.52	8.06	8.08
Fish canning by-product [2]	100	100	100	Histidine	0.87	0.92	0.80
Cotton seed meal [3]	60	60	60	Isoleucine	1.62	1.57	1.64
Poultry by-product [4]	80	80	80	Leucine	2.54	2.43	2.65
Vitamin premix [5]	5	5	5	Lysine	2.45	2.40	2.39
Mineral premix [6]	5	5	5	Methionine	1.10	1.19	1.11
Methionine [7]	6	6	6	Cyctein	0.56	0.53	0.49
Lysine [8]	6	6	6	Alanine	1.14	1.09	1.02
Cellulose [9]	15	10	5	Phenylalanine	1.58	1.46	1.49
Glycine [10]	0	5	10	Tyrosine	1.15	1.15	1.21
Proximate composition				Threonine	1.29	1.33	1.38
Moisture (g/kg)	106	102	107	Tryptophan	0.48	0.42	0.43

Table 1. *Cont.*

Ingredients (g/kg)	CTL	5 GL	10 GL	Amino Acid Profile (%)	CTL	5 GL	10 GL
Crude protein (g/kg dry matter)	338 ± 1.62	343 ± 0.99	347 ± 2.32	Valine	1.71	1.63	1.67
Crude fat (g/kg dry matter)	129 ± 1.35	132 ± 0.92	131 ± 0.37				
Crude ash (g/kg dry matter)	57.9 ± 0.13	58.4 ± 0.10	58.1 ± 0.23				
Crude fiber (g/kg dry matter)	51.3 ± 1.46	44.5 ± 0.87	39.1 ± 2.13				

[1] crude protein 42%; crude fat 1%; [2] crude protein 55%; crude fat 17%; [3] crude protein 39%; crude fat 2%; [4] crude protein 50%; crude fat 19%; [5] Amineh Gostar Co. (Tehran, Iran); the premix provided vitamins as follow (per kg diet): A: 1600 IU; D3: 500 IU; E: 20 mg; K: 24 mg; B3: 12 mg; B5: 40 mg; B2: 10 mg; B6: 5 mg; B1: 4 mg; H: 0.2 mg; B9: 2 mg; B12: 0.01 mg; C: 60 mg; Inositol: 50 mg; [6] Amineh Gostar Co. (Tehran, Iran); the premix provided minerals as follow (per kg diet): Se: 0.15 mg; Fe: 2.5 mg; Co: 0.04 mg; Mn: 5 mg; iodate: 0.05 mg; Cu: 0.5 mg; Zn: 6 mg; choline: 150 mg; [7] CheilJedang Co., Seul, Korea; [8] CheilJedang Co., Seul, Korea; [9] Sigma-Aldrich Co. (St. Louis, MO, USA); 99%; [10] Sigma-Aldrich Co. (St. Louis, MO, USA); 99%.

2.2. Fish Rearing

Two hundred common carp juveniles were purchased from a local farm and stocked in one 1 m^3 tank for 7 days, during which they were fed the CTL diet. After that, 90 healthy fish (24.5 ± 0.21 g) with a uniform size were selected and randomly distributed in 9 glass aquaria. The aquaria were filled with 50-L dechlorinated tap water, with continuous aeration. The stocking density was based on a previous study on the same fish species [12]. The fish were fed either of the above-mentioned diets, twice a day (2% of biomass), for 8 weeks. Every other week, the fish of each aquarium were bulk-caught and placed in a tank filled with water on a digital scale to record the tank biomass and the feed amounts adjustment. Water of the aquaria was renewed by 30% every day and the wastes were siphoned out. Water temperature, pH, dissolved oxygen, and total ammonia were 23.8 ± 0.85 °C, 7.74 ± 0.68, 6.78 ± 0.74 mg/L, and 1.53 ± 0.33 mg/L, respectively.

After 8 weeks rearing, weight gain (WG), specific growth rate (SGR), and feed conversion ratio (FCR) were recorded as follows:

$$\text{WG (\%)} = 100 \times [(\text{final weight} - \text{initial weight})/\text{initial weight}] \quad (1)$$

$$\text{FCR} = \text{feed intake}/(\text{final weight} - \text{initial weight}) \quad (2)$$

$$\text{SGR (\%/day)} = 100 \times [(\text{Ln final weight} - \text{Ln initial weight})/\text{rearing days}] \quad (3)$$

2.3. Sample Collection and Processing

At the end of the rearing period, three fish were caught from each aquarium and anesthetized in 100 µL/L eugenol. Then, blood samples (1 mL per fish) were taken from the fish caudal vein, using heparinized syringes. A portion of 300 µL of the fresh blood samples was used for hematological examinations. Another portion of 200 µL of the fresh blood samples was used for osmotic fragility test of the RBC. To make the cell lysate, the packed RBC of the samples were frozen at −70 °C and added with two volumes of phosphate buffer (pH 7.0). After vortexing, the suspensions were centrifuged (6000× *g*; 7 min; 4 °C) and the supernatants were kept at −70 °C until analysis of the RBC GSH, GR, and GPx. To obtain plasma, 350 µL of the fresh blood samples were centrifuged (6000× *g*; 7 min; 4 °C) and the plasma samples were kept at −70 °C until analysis of lysozyme, alternative complement (ACH50), and total immunoglobulin (Ig).

The skin mucus samples were collected from three fish per tanks. The fish were anesthetized as mentioned above and the mucus samples were collected from the dorsolateral surface by scrubbing a spatula. The collected mucus samples were mixed with equal volume of Tris-buffered saline (TBS, 50 mM Tris–HCl, 150 mM NaCl, pH 8.0). After vortexing, the mixtures were centrifuged (13,000× *g*; 15 min; 4 °C) and the supernatants were kept at −70 °C until the mucosal immunological assays.

2.4. Analysis

2.4.1. Hematological Examinations

RBC and WBC were counted using a Neubauer chamber, following dilution with the dacie solution. Hematocrit (Hct) and hemoglobin (Hb) were measured by centrifuging and cyanomethemoglobin methods, respectively. Mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were calculated according to Dacie and Lewis [18]. Leukocyte differential count was performed by preparing blood slides stained by Giemsa.

2.4.2. Plasma Immunological Parameters

Plasma lysozyme activity was determined based on a turbidimetric method, as described by Ellis [19]. *Micrococcus luteus* was used as the target and suspended in phosphate buffer (pH 6.2). To 1 mL of this suspension, 25 µL of the plasma sample was added and average decrease in optical density per minute was recorded over 5 min at 450 nm. Each 0.001 decrease in optical density per minute was considered as one unit of lysozyme activity.

Plasma ACH50 activity was measured based on a hemolytic method [20], using sheep RBC as the target in veronal buffer containing magnesium and gelatin (pH 7.0). Serially diluted plasma samples were added to the suspension of the RBC in the buffer and incubated at room temperature for 60 min. The plasma amount leading to 50% hemolysis was estimated and used to calculate ACH50 activity per mL plasma.

Plasma total Ig concentration was determined after precipitation with polyethylene glycol [21]. Equal amounts of the plasma and polyethylene glycol solution (12%) were mixed and kept under agitation at room temperature. After 2 h, the mixture was centrifuged (6000× *g*; 7 min; 4 °C) to precipitate Ig. Difference in the sample protein before and after the centrifugation was equal to the sample total Ig concentration.

2.4.3. RBC Antioxidant Enzymes

The RBC antioxidant parameters were assessed according to Yousefi, Hoseini [22]. The RBC cell lysates were thawed and the solutions were centrifuged to precipitate any derbies. The supernatants were used for enzymatic assays. Commercial kits (Zellbio Co., GmbH, Deutschland, Germany) were used to assay the enzymes' activity. GSH concentration was measured based on reaction with 5,5′-dithiobis-(2-nitrobenzoic acid) at 412 nm. The principal of GPx assay was based on adding GSH to the samples and conversion of GSH to oxidized glutathione (GSSG) by the sample GPx. The resultant GSSG is recycled to GSH by GR activity, which uses NADPH. The decrease in NADPH concentration is proportional to GPx activity and measurable at 340 nm. The same protocol is used for GR activity determination, but GSSG is added to the samples instead of GSH. The solution hemoglobin level was measured and the enzymes' activities were expressed based on it.

2.4.4. Osmotic Fragility Test

Osmotic fragility test of RBC was conducted according to Gao, Liu [23]. In brief, 25 µL of the packed fresh RBC was added to 1 mL of 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.85% sodium chloride solution and left at room temperature for 90 min. Then, the suspensions were centrifuged (6000× *g*; 7 min; 4 °C) and the absorbance of the supernatants were measured at 492 nm. The optical densities were corrected based on the RBC count and MCH. The highest optical density was considered as 100% hemolysis and the other optical densities hemolysis rates were calculated based on it.

2.4.5. Skin Mucus Immunological Parameters

Soluble protein concentrations of the skin mucus samples were determined based on Bradford [24]. The skin mucus peroxidase activity was determined according to Quade and Roth [25], using 3,3′,5,5′-tetramethylbenzidine hydrochloride and hydrogen peroxide as the substrates at 450 nm. The skin mucus protease activity was determined according to Zhang, Hu [26], using AZOcasein as the substrate at 350 nm. The skin mucus lysozyme

activity was determined as described above. The skin mucus alkaline phosphatase (ALP) activity was determined using Parsazmun Co. kit (Tehran, Iran), according to Hoseinifar, Roosta [27].

2.5. Statistical Analysis

Before analysis, the averages of data per aquarium were calculated and used for statistical analysis (n = 3). After confirming normal distribution of the data by the Shapiro–Wilk test, and variance homogeneity by the Levene test, the data were subjected to one-way ANOVA. Significant differences among the treatments were determined by Tukey test. $p < 0.05$ was considered as significance and all analyses were performed in SPSS v.22.

3. Results

Growth performance of the fish is presented in Table 2. FCR in the 5 GL and 10 GL treatments were significantly lower than that of the CTL treatment. Final weight, WG, SGR in the 5 GL treatment were significantly higher than that of the CTL treatment. There was no mortality among the treatments.

Table 2. Growth performance of common carp following eight weeks feeding with diets containing graded levels of glycine. Different letters within a row indicate significant differences among the treatments (mean ± SE; n = 3; Tukey).

	CTL	5 GL	10 GL	p-Value
Initial weight (g)	24.4 ± 0.59 a	24.6 ± 0.35 a	24.6 ± 0.23 a	0.921
Final weight (g)	48.8 ± 2.45 a	56.9 ± 1.78 b	54.8 ± 0.98 ab	0.049
FCR	1.49 ± 0.06 b	1.21 ± 0.05 a	1.26 ± 0.03 a	0.025
WG (%)	99.9 ± 5.22 a	131 ± 8.02 b	123 ± 4.31 ab	0.022
SGR (%/d)	1.24 ± 0.05 a	1.50 ± 0.06 b	1.43 ± 0.03 ab	0.018
Survival (%)	100	100	100	1.00

RBC, Hct, and Hb of the 5 GL and 10 GL treatments were similar and significantly higher than those of the CTL treatment (Table 3). Blood MCV in the 10 GL treatment significantly decreased, compared to the CTL treatment. There were no significant differences in the blood MCH and MCHC among the treatments (Table 3).

Table 3. Hematological and erythrocytes' antioxidant parameters of common carp following eight weeks feeding with diets containing graded levels of glycine. Different letters indicate significant differences among the treatments (mean ± SE; n = 3; Tukey).

	CTL	5 GL	10 GL	p-Value
RBC (10^6 cell/μL)	1.33 ± 0.01 a	1.52 ± 0.01 b	1.51 ± 0.01 b	<0.001
Hct (%)	33.3 ± 0.40 a	37.3 ± 0.08 b	36.1 ± 0.32 b	<0.001
Hb (g/dL)	7.79 ± 0.12 a	8.78 ± 0.15 b	8.46 ± 0.11 b	0.005
MCV (fL)	251 ± 1.82 b	245 ± 1.34 ab	241 ± 1.64 a	0.013
MCH (pg)	58.7 ± 1.14 a	57.7 ± 1.13 a	56.3 ± 1.00 a	0.365
MCHC (mg/dL)	23.4 ± 0.53 a	23.5 ± 0.36 a	23.4 ± 0.45 a	0.983
GSH (nM/mg Hb)	6.21 ± 0.02 a	7.88 ± 0.04 b	7.59 ± 0.08 b	<0.001
GR (U/mg Hb)	91.5 ± 0.29 b	75.8 ± 0.35 a	79.3 ± 0.41 a	<0.001
GPx (U/mg Hb)	79.2 ± 0.55 a	72.2 ± 0.46 a	74.2 ± 1.02 a	0.593

Antioxidant parameters of RBC are shown in Table 3. GSH content significantly increased, as GR activity significantly decreased in the 5 GL and 10 GL treatments, compared to the CTL treatment. There was no significant difference in GPx activity among the treatments.

The results of osmotic fragility test are presented in Table 4. There were no significant differences in hemolysis rate among the treatments at NaCl concentrations of 0.1, 0.2, 0.7, and 0.85%. At NaCl concentration of 0.3–0.5%, hemolysis rate of the 5 GL treatment was

significantly lower than the CTL treatment. The hemolysis rates of glycine-treated fish at NaCl concentration of 0.6% were significantly lower than those of the CTL treatment.

Table 4. Blood hemolysis rate of common carp following eight weeks feeding with diets containing graded levels of glycine. The hemolysis rates were calculated as percentages of the highest hemolysis among the samples. Different letters indicate significant differences among the treatments (mean ± SE; *n* = 3; Tukey).

NaCl concentration (%)	CTL	5 GL	10 GL	*p*-Value
0.1	93.2 ± 1.57 a	93.4 ± 2.20 a	91.5 ± 2.22 a	0.769
0.2	89.0 ± 1.80 a	86.8 ± 0.74 a	84.3 ± 1.83 a	0.189
0.3	81.1 ± 2.22 b	68.0 ± 2.83 a	78.6 ± 2.69 ab	0.026
0.4	72.3 ± 2.14 b	53.1 ± 2.03 a	65.6 ± 2.17 b	0.002
0.5	52.4 ± 1.45 b	37.0 ± 1.46 a	43.7 ± 3.34 ab	0.009
0.6	34.3 ± 0.22 b	18.0 ± 0.93 a	20.7 ± 0.73 a	<0.001
0.7	17.7 ± 1.29 a	15.4 ± 0.63 a	15.7 ± 0.20 a	0.247
0.85	13.9 ± 0.51 a	14.1 ± 1.01 a	15.0 ± 0.42 a	0.555

WBC and differential leukocyte counts are presented in Table 5. Dietary glycine supplementation significantly increased WBC count, and neutrophil and monocyte percentages/counts, compared to the CTL treatment. On the other hand, the glycine-treated fish had significantly lower lymphocyte percentages, but higher lymphocyte count, compared to the CTL fish. There were no significant differences in WBC and leukocyte differential counts between the 5 GL and 10 GL treatments. The blood eosinophil percentages/counts were similar among the treatments. Plasma immunological parameters are presented in Table 5. The glycine-treated fish exhibited significantly higher plasma lysozyme and ACH50 activities, compared to the CTL fish. The highest plasma lysozyme and ACH50 activities were observed in the 5 GL treatment. Dietary glycine supplementation had no significant effects on the plasma total Ig concentrations.

Table 5. Humoral immunological parameters of common carp following eight weeks feeding with diets containing graded levels of glycine. Different letters within a row indicate significant differences among the treatments (mean ± SE; *n* = 3; Tukey).

	CTL	5 GL	10 GL	*p*-Value
WBC (10^3 cell/μL)	10.9 ± 0.08 a	14.3 ± 0.28 b	13.8 ± 0.24 b	<0.001
Lymphocyte (10^3 cell/μL)	9.49 ± 0.11 a	10.4 ± 0.21 b	10.4 ± 0.18 b	0.013
Neutrophil (10^3 cell/μL)	0.82 ± 0.04 a	2.65 ± 0.10 b	2.29 ± 0.11 b	<0.001
Monocyte (10^3 cell/μL)	0.42 ± 0.03 a	0.95 ± 0.08 b	0.92 ± 0.03 b	0.001
Eosinophil (10^3 cell/μL)	0.16 ± 0.01 a	0.21 ± 0.01 a	0.22 ± 0.04 a	0.327
Lymphocyte (%)	87.3 ± 0.51 b	73.4 ± 0.56 a	75.2 ± 0.73 a	<0.001
Neutrophil (%)	7.44 ± 0.48 a	18.4 ± 0.40 b	16.6 ± 0.62 b	<0.001
Monocyte (%)	3.78 ± 0.29 a	6.67 ± 0.58 b	6.67 ± 0.33 b	0.004
Eosinophil (%)	1.44 ± 0.11 a	1.44 ± 0.11 a	1.56 ± 0.29 a	0.897
Lysozyme (U/mL)	26.3 ± 0.47 a	50.4 ± 0.84 c	34.8 ± 0.29 b	<0.001
ACH50 (U/mL)	181 ± 4.56 a	397 ± 4.57 c	283 ± 2.47 b	<0.001
Total Ig (g/L)	7.87 ± 0.53 a	7.52 ± 0.65 a	8.17 ± 0.73 a	0.786

The skin mucosal immunological parameters of the fish are presented in Table 6. There were no significant differences in the mucosal soluble protein content, lysozyme, peroxidase, protease, and ALP activities between the 5 GL and 10 GL treatments; both exhibited significantly higher values, compared to the CTL treatment.

Table 6. The skin mucosal immunological parameters of common carp following eight weeks feeding with diets containing graded levels of glycine. Different letters indicate significant differences among the treatments (mean ± SE; n = 3; Tukey).

	CTL	5 GL	10 GL	p-Value
Soluble protein (mg/dL)	54.3 ± 1.26 a	77.1 ± 2.51 b	72.0 ± 2.03 b	<0.001
Proxidase (U/mg Pr)	3.96 ± 0.15 a	5.40 ± 0.13 b	5.66 ± 0.09 b	<0.001
Protease (U/mg Pr)	40.6 ± 0.29 a	63.6 ± 0.87 b	62.0 ± 0.19 b	<0.001
ALP (U/mg Pr)	120 ± 3.35 a	155 ± 3.09 b	155 ± 4.30 b	0.001
Lysozyme (U/mg Pr)	29.1 ± 0.53 a	38.7 ± 0.91 b	40.5 ± 1.16 b	<0.001

4. Discussion

Glycine contributes to synthesis of structural proteins and accounts for nearly 9% of the whole body protein in common carp [28]. In the present study, dietary glycine supplementation significantly improved growth performance and feed efficiency in common carp, which is not similar to our previous study on the same species [12]. The exact reasons for such a difference are not clear; it was expected that the lower glycine level in the control diet of the present study (1.61%), compared to the previous one (1.98%), made the difference. However, the growth performance of the control fish in the two studies was approximately similar. Thus, other factors might contribute to such a difference. One explanation may be change in the dietary amino acid compositions between the two studies. Lysine and methionine, two major growth-limiting amino acids, in the present study were higher than the previous one and sub-optimal dietary levels of these amino acids result in lower nitrogen, amino acid, and glycine retentions [29,30]. The present results are in line with the previous studies on Nile tilapia [8] and largemouth bass [9] that dietary glycine supplementation has improved the fish growth performance. Although non-essential amino acids can be synthetized in fish body, their synthesis rate may be insufficient to meet the biological requirements. Hence, the improved growth performance in glycine treatments in the present study can be due to higher glycine availability to meet growth and maintenance requirements.

Hematological studies give a sight regarding the fish health and gas mobilization status. Decrease in RBC count and hemoglobin content hinders tissue oxygenation and carbon dioxide clearance [31]. Therefore, a number of studies have assessed the role of nutritional manipulations [32–34], including amino acid supplementation [35–37], on hematological parameters in different fish species. Little is known regarding the role of glycine in hematological parameters in fish. A study on channel catfish, *Ictalurus punctatus*, has revealed that RBC actively transports glycine into the cytoplasm, which can be used for glutathione synthesis [15], as observed in mammals [17]. Due to the presence of hemoglobin, RBC are in constant threat of oxidation and glutathione has a crucial role in prevention of RBC oxidative stress [15]. According to the present results, the improvements in glutathione-related antioxidant parameters (increase in GSH content and decrease in GR activity) can be responsible for higher RBC count and resistance to hemolysis in the glycine-treated fish. It has been revealed that fish erythrocytes are very sensitive to oxidative conditions, under which a significant drop in GSH content (that has been due to oxidation) and increase in GPx, lipid peroxidation, and hemolysis occur [38–40]. No change in GPx activity in the present study suggests probably no difference in oxidative conditions among the treatment. Thus, higher GSH contents in 5 GL and 10 GL treatments can be due to improved glutathione synthesis under glycine availability. Increase in GSH synthesis can fulfil the cell biological requirement, so no need for further recycling of GSSG, which explains the decreases in GR activity in 5 GL and 10 GL treatments.

Leukocytes are important immune cells with diverse functions that help the host to combat foreign germs [41]. Increase in WBC after dietary supplementations has been found to augment disease resistance in different fish species [42,43]. Regarding glycine, dietary supplementation has been found to increase WBC in beluga [10], similar to the present results. Neutrophils are the first defending leukocytes, when fish encounter pathogen

attacks. They kill pathogens by phagocytosis, which leads to the formation of reactive molecules [44]. Monocytes are another immune cells with phagocytic functions [45]. Due to the production of various pro-oxidant molecules by these cells, they need a strong antioxidant system to survive. Studies on human cells have revealed that glutathione is crucially necessary for viability and function of neutrophils and monocytes [46,47], but there are limited information regarding this topic in fish. Studies on carp have revealed that glutathione content increases in neutrophils during phagocytosis or lipopolysaccharide induction [48]. Exposure of Nile tilapia monocytes to a pro-oxidant agent (copper nanoparticle) has induced apoptosis, oxidative stress, and depletion of GSH content [45]. Accordingly, it is speculated that higher blood neutrophil and monocyte number/percentage in the glycine-treated fish in the present study was a consequence of improvement in the cells GSH content. However, glycine has no significant effects on blood lymphocyte population and the decrease in lymphocyte percentages in the glycine-treated fish was as a result of increase in the percentages of other leukocyte types. All together, these results suggest that dietary glycine supplementation improves the strength of the innate immunity system.

Lysozyme acts as a bactericidal agent that is secreted by blood neutrophils in the circulation [49]. Therefore, it is speculated that the increase in plasma lysozyme in the glycine-treated fish was due to the increase in the blood neutrophil population or their lysozyme production. Similarly, elevations in the plasma lysozyme activity have been accompanied by elevations in the blood neutrophil population, after adding various feed additives to fish diets [50,51]. Similar to the present study, dietary glycine supplementation has significantly increased plasma lysozyme activity in beluga [10] and common carp [11]. The complement system acts as a germ-killing and opsonizing agent in fish that consists of several proteins produced in the liver [52]. Dietary glycine supplementation have been found to increase the plasma ACH50 activity in beluga [10]; however, a short-term period of feeding with glycine-supplemented diets induced no significant changes in the plasma ACH50 activity in common carp [11]. The exact mechanism by which glycine improves the plasma ACH50 activity is not clear, but GSH may contribute, as dietary glutathione supplementation has significantly increased concentration of complement proteins in hemolymph of mitten crab, *Eriocheir sinensis*, which may be as a results of higher hepatopancreas health and protein synthesis caused by GSH availability [53]. Therefore, it is speculated that dietary glycine can increase GSH concentration, which in turn may be responsible for higher ACH50 activity in the present study. Moreover, properdin, a glycine-rich molecule that up-regulates the complement activity, has been identified in various tissues of fish such as the liver, skin, neutrophil, and monocyte [54,55]. This molecule has been identified in common carp [56], so it is speculated that glycine availability might support higher properdin production and increased ACH50 activity in the present study.

Skin mucosal immunity is very important to prevent diseases in aquaculture practice, as water serves as the main pathogen transmitter and a strong mucosal immunity can prevent the entrance of pathogens to new fish [57]. Skin mucosal lysozyme acts as a bactericidal agent, similar to plasma lysozyme [49]. Protease in the skin mucus kills pathogens, decreases mucus integrity, increases mucus layer sloughing, and activates other immune parameters [58]. Skin mucosal peroxidase participates in the formation of peroxidase–H_2O_2–halide complex, a strong bactericidal and cytotoxic agent [58]. ALP activity in the skin mucus can detoxify pro-inflammatory compounds created by microbes [59]. There are no similar studies for comparison, but the present results suggest that dietary glycine supplementation can improve the skin mucosal immunity in common carp.

5. Conclusions

In conclusion, dietary glycine supplementation can support GSH synthesis, which improves antioxidant capacity and hemolysis resistance in RBC. Moreover, dietary glycine supplementation increase the population of blood neutrophils and monocytes that can be due to protective role of GSH in these cells. Interestingly, glycine can improve several immune-related factors in the fish skin mucus that can be helpful in inhibition of pathogen

entering fish body. Based on these benefits, dietary 5 g/kg glycine supplementation is recommended for common carp feed supplementation.

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Optimal Levels of Fish Meal and Methionine in Diets for Juvenile *Litopenaeus vannamei* to Support Maximum Growth Performance with Economic Efficiency

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Simple Summary: The shrimp feed industry is constantly looking for opportunities to minimize the dependency on expensive fish meal and to keep the industry profitable and sustainable. When fish meal is replaced with alternative protein sources available today, methionine (Met) is often the first limiting amino acid. This work investigated the optimal levels of fish meal (FML) and dietary Met required to optimize growth performance of juvenile *Litopenaeus vannamei* with economic efficiency. The study involved two feeding trials, one with outdoor tanks (1 m^3 volume) to evaluate shrimp growth performance over a 70-day feeding period and the other with indoor tanks (60 L) to evaluate feed digestibility over a 93-day period. The study used DL-methionyl-DL-methionine as the supplemental Met source. Under 0 and 6% FML conditions, total dietary Met levels of 0.69% and 0.82%, respectively, were required to maximize shrimp performance. In comparison, at 12% and 18% FML, a dietary Met content of only 0.58% was sufficient. Diets with 0 FML or with only 6% delivered the highest profit and return on investment compared to diets with higher levels. Overall, results indicated FML in shrimp feeds can be minimized or eliminated without impairing growth performance, providing Met requirement is met with appropriate sources.

Abstract: This work investigated the optimal levels of fish meal (FML) and dietary methionine (Met) required for maximum growth performance of juvenile *Litopenaeus vannamei* with economic efficiency. Four sets of diets were prepared to contain 0.00, 6.00, 12.00 and 18.00% FML. Each set was supplemented with DL-methionyl-DL-methionine (DL-Met-Met) to result in a total dietary Met (Met + Cys) content of 0.58 (1.05), 0.69 (1.16), and 0.82% (1.29%), on a fed basis. Shrimp of 1.00 ± 0.08 g were stocked in 60 outdoor tanks of 1 m^3 with 100 shrimp/m^2, allowing five replications per dietary group. Shrimp in all the groups were fed 10 times daily for 70 days. In a subsequent trial, dietary protein and amino acid digestibility of four FML groups, but only at high dietary Met levels (~0.82%), were evaluated in 40 60 L indoor tanks (11 replicates per diet) for 93 days with 70 shrimp/m^2. Final shrimp survival (92.85 ± 4.82%, mean ± standard deviation), weekly weight gain (1.17 ± 0.08 g), apparent feed intake (13.3 ± 0.5 g of feed per stocked shrimp), and feed conversion ratio (1.18 ± 0.06) were unaffected by dietary FML level and Met content. Gained yield was adversely affected when FML was reduced from 18% and 12% (1156 and 1167 g/m^2, respectively) to 0 (1090 g/m^2), but no change was observed at 6% (1121 g/m^2). A significant interaction was detected between FML level and dietary Met. Under 0 and 6% FML conditions, higher levels of total dietary Met, 0.69% and 0.82%, respectively, were required to maximize shrimp BW. In comparison, at 12% and 18% FML, a dietary Met content of only 0.58% was sufficient. Overall, results indicated the use of FML can be minimized or completely eliminated without major detrimental effects on feed digestibility or shrimp growth performance, as long as proper supplementation of Met is carried out. Diets with 0 FML or with only 6% delivered the highest profit and return on investment compared to diets with higher levels.

Keywords: fish meal; replacement; methionine; DL-methionyl-DL-methionine; supplementation

1. Introduction

Industrially compounded shrimp feeds are among the largest global consumers of fish meal within the aquaculture industry [1–4]. As a result, replacement of fish meal by other proteins has been the subject of a number of publications. Most studies have supported that dietary fish meal levels can be significantly reduced without adverse effects on shrimp growth performance. Consequently, there has been a progressive decrease in the inclusion of fish meal in shrimp feeds, from more than 25% in the 1990s [1,5] to 12% or less in the past decade [1,4]. This has led to a corresponding decline in the fish-in-fish-out (FIFO) ratio [2] from an estimated 2.81 in 2007 [5] to 0.82 in 2017 [4]. Further reduction or complete withdrawal of fish meal from shrimp feeds has been achieved under experimental culture conditions [6–11]. However, effective replacement levels vary according to culture conditions, substitute protein, feed formulation, and sources of supplemental amino acids.

The most common proteins adopted to replace fish meal in shrimp feeds are commodity by-products from the animal slaughtering industry (poultry by-product meal [11–14]; meat and bone meal [12,13,15]; porcine meat meal [16]) and agriculture (soybean meal [6,7,17–24]; soy protein concentrate [11,13,21,25–27]; canola meal [18,28,29]; corn meal [30]; cottonseed meal [31]; peanut meal [10,19]). Unconventional protein ingredients have also been evaluated with promising results (bacterial meal [32–34]; biofloc meal [26,35,36]; earthworm [37]; insect meal [38]; microalgae meal [39,40]). Soybean meal is by far the most common and preferred ingredient to support fish meal replacement in practical shrimp feeds due to its year-round availability and competitive and less volatile prices. Additionally, most studies have used a combination of protein sources to replace fish meal. Regardless of the protein sources chosen, studies have shown that formulation of low fish meal diets relies on a balanced supplementation of essential amino acids [20,27,41,42], fatty acids [27], and feed attractants [9,28,29,41].

Methionine (Met) is considered the most impacted essential amino acid (EAA) when fish meal is challenged. Recommended dietary Met levels in shrimp feeds have ranged between 0.7% and 1.0% of the diet (as-fed basis) depending on shrimp species (*Penaeus monodon* [43], *Marsupenaeus japonicus* [44]), source of supplemental Met [13,20,45–48], growth stage [49], culture conditions (stocking density [50], water exchange regime [51], feed allowance [52]), and dietary protein level [53,54]. However, very little information is available on the optimal dietary Met levels in response to graded levels of fish meal in order to support maximum shrimp growth performance with economic efficiency. The present work evaluated the growth performance of juvenile *Litopenaeus vannamei*, the feed digestibility, and the economics of fish meal (FML) reduction with the dietary supplementation of DL-methionyl-DL-methionine (DL-Met-Met) under intensive culture conditions.

2. Material and Methods

2.1. Rearing System and Water Preparation

This study consisted of two separate experimental stages. The 1st stage was designed to evaluate shrimp growth performance fed graded levels of FML and Met. A 2nd stage determined the apparent digestibility coefficients (ADCs) for crude protein (ACPDCs) and amino acids (AAADCs) of diets with graded levels of FML using a fixed dietary Met content.

For the growth performance evaluation, an outdoor rearing system as described by Façanha et al. [50–52] and Nunes et al. [54] was used. The system consisted of independent 1.0 m^3 outdoor tanks (1.02 m^2 bottom area $\times$ 0.74 m height), each equipped with a perforated lid on the top to avoid shrimp from escaping. The system operated with a continuous water recirculation at a rate of 100 mL/second (14.4% a day). Effluent water was drained into a 10 m^3 sump with constant aeration which pumped surface water back to two header tanks of 20 m^3 each. Water was then continuously distributed to rearing tanks with a 3-hp pump. No mechanical filtration was carried out in culture water during the study period.

Water was fertilized prior to shrimp stocking by applying liquid sugarcane molasses along with a 500 µm shrimp feed (minimum of 35% crude protein, CP) at a 1:1 ratio (20 g/m^3, as-is basis) over a five-day period. Culture water was then allowed to mix with strong aeration for three additional days before shrimp stocking.

The in vivo digestibility assay was carried out in rectangular 61 L indoor tanks (31.0 × 35.5 × 55.5 cm, height × width × length; bottom area of 0.19 m^2), each equipped with its own water inlet and outlet, aeration, and feeding tray. The rearing system was the same as described by Sabry-Neto et al. [55] and Vieira et al. [56]. It was operated with a continuous water recirculation regime at a rate between 15.65 and 21.81 L/h (26.1–36.4% of the tank volume/h). All culture water was chemically disinfected and filtered prior to shrimp stocking.

2.2. Shrimp Stocking

The shrimp species used in this study was the Pacific whiteleg shrimp, *L. vannamei*, purchased as post-larvae (PLs) from a commercial hatchery (Aquatec Aquacultura Ltda., Canguaretama, Brazil). A batch of 110,000 PL8 (245 PLs/g) was transported to the lab in 11 double-plastic sealed bags at a density of 667 animals/L (10,000 PLs/bag) under 35 g/L salinity, 24 °C temperature, 8.09 pH, and 148 mg/L $CaCO_3$ alkalinity conditions. At arrival, PLs were acclimated and stocked in five cylindrical nursery tanks of 23.85 m^3 each at about 22,000 animals per tank or 1 PL/L. Shrimp were fed a 40% CP commercial crumbled feed and nursery-reared until juvenile stage. In the end of the nursery stage, shrimp were size-graded to homogenize body weight (BW). Culling was carried out by weighing shrimp individually in a 0.01 g precision scale. A total of 6120 shrimp of 1.00 ± 0.08 g (mean ± standard deviation) were transferred to 60 outdoor tanks (five replicate tanks per diet) and stocked with 100 animals/m^2 (102 shrimp/tank) for the growth performance trial. Later, a total of 600 shrimp of 4.47 ± 0.14 g BW were stocked in 44 tanks (11 replicate tanks per diet) with 70 animals/m^2 (15 shrimp/tank) for the digestibility assay.

2.3. Experimental Diets

For the growth performance evaluation, three sets of pelleted diets were prepared, each containing three levels of total Met with four inclusion levels of FML (12 diets in total). Each set of diets was supplemented with DL-methionyl-DL-methionine (AQUAVI® DL-Met-Met, Evonik Operations GmbH, Hanau, Germany) to achieve the following mean (±standard deviation, SD) total dietary Met content (as-is basis): 0.58 ± 0.02%, (0.58% Met) 0.69 ± 0.02 (0.69% Met), and 0.82 ± 0.02% (0.82% Met), with a corresponding Met + Cys (cysteine) content of 1.05 ± 0.01, 1.16 ± 0.03, and 1.29 ± 0.02%, respectively (Table 1).

Table 1. Ingredient composition (%, as-is) and formula cost (USD/kg) of diets prepared to evaluate growth performance.

Ingredients (%, As-Is Basis)	Diets/Ingredient Composition (%, As-Is Basis)											
	0.58% Met (1.05% Met + Cys)				0.69% Met (1.16% Met + Cys)				0.82% Met (1.29% Met + Cys)			
% Fish Meal (FML) Level	0	6	12	18	0	6	12	18	0	6	12	18
Wheat flour [1]	30.00	30.00	30.00	30.00	30.00	30.00	30.00	30.00	30.00	30.00	30.00	30.00
Soybean meal [2]	46.08	37.80	28.95	20.73	46.08	37.80	28.95	20.73	46.08	37.80	28.95	20.73
Wheat gluten meal [3]	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00
Salmon meal [4]	-	6.00	12.00	18.00	-	6.00	12.00	18.00	-	6.00	12.00	18.00
Cassava starch [5]	0.26	1.61	4.46	6.20	0.13	1.49	4.34	6.09	-	1.35	4.20	5.95
Salmon oil	3.02	3.10	3.13	3.19	3.02	3.10	3.13	3.19	3.02	3.10	3.13	3.19
Soy lecithin	3.38	3.00	2.60	2.20	3.38	3.00	2.60	2.20	3.38	3.00	2.60	2.20
Yellow kaolin	-	2.00	4.00	5.00	-	2.00	4.00	5.00	-	2.00	4.00	5.00
Krill meal [6]	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Squid meal [7]	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00
MSP [8]	1.45	1.39	1.39	1.39	1.45	1.39	1.39	1.39	1.45	1.39	1.39	1.39
L-Lysine [9]	0.47	0.43	0.43	0.41	0.47	0.43	0.43	0.41	0.47	0.43	0.43	0.41
Magnesium sulphate	0.004	0.49	-	-	0.004	0.49	-	-	0.004	0.49	-	-
Calcium carbonate	1.97	1.00	-	-	1.97	1.00	-	-	1.97	1.00	-	-
Vitamin-mineral premix [10]	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00

Table 1. *Cont.*

Ingredients (%, As-Is Basis)	Diets/Ingredient Composition (%, As-Is Basis)											
	0.58% Met (1.05% Met + Cys)				0.69% Met (1.16% Met + Cys)				0.82% Met (1.29% Met + Cys)			
% Fish Meal (FML) Level	**0**	**6**	**12**	**18**	**0**	**6**	**12**	**18**	**0**	**6**	**12**	**18**
Potassium chloride	1.13	0.99	0.84	0.68	1.13	0.99	0.84	0.68	1.13	0.99	0.84	0.68
L-Arginine [11]	-	0.20	0.47	0.73	-	0.20	0.47	0.73	-	0.20	0.47	0.73
Synthetic binder [12]	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Salt	1.35	1.14	0.91	0.69	1.35	1.14	0.91	0.69	1.35	1.14	0.91	0.69
L-Threonine [13]	0.12	0.14	0.14	0.14	0.12	0.14	0.14	0.14	0.12	0.14	0.14	0.14
L-Trytophan [14]	0.04	0.06	0.09	0.11	0.04	0.06	0.09	0.11	0.04	0.06	0.09	0.11
DL-Met-Met [15]	0.14	0.09	0.05	-	0.25	0.20	0.16	0.11	0.39	0.34	0.30	0.25
Cholesterol [16]	0.07	0.04	0.02	-	0.07	0.04	0.02	-	0.07	0.04	0.02	-
Vitamin C [17]	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03
Formula cost (USD/kg)	0.756	0.822	0.878	0.932	0.761	0.827	0.883	0.937	0.767	0.833	0.889	0.943

[1] 10.82% crude protein (CP), 0.18% methionine (Met), 0.41% methionine + cysteine (Met + Cys), 0.23% lysine (Lys), 0.28% threonine (Thr), 0.40% arginine (Arg). [2] Bunge Alimentos S.A. (Luiz Eduardo Magalhães, Brazil). 47.88% CP, 0.62% Met, 1.30% M + C, 2.91% Lys, 1.83% Thr, 3.45% Arg. [3] Amytex 100. Tereos Syral S.A.S. (Marckolsheim, France). 78.71% CP, 1.22% Met, 2.80% M + C, 1.37% Lys, 2.01% Thr, 2.84% Arg. [4] Pesquera Pacific Star S.A. (Puerto Montt, Chile). 63.61% CP, 1.61% Met, 2.17% M + C, 4.30% Lys, 2.47% Thr, 0.59% Arg. [5] 0.26% CP. [6] Qrill^{TM} Antarctic krill meal, Aker BioMarine Antarctic AS (Lysaker, Norway). 57.05% CP, 1.63% Met, 2.08% M + C, 3.92% Lys, 2.54% Thr, 3.45% Arg. [7] 70.66% CP, 2.34% Met, 3.17% M + C, 5.06% Lys, 3.33% Thr, 5.69% Arg. [8] Monosodium phosphate. 0.60% calcium, 20.70% phosphorous, 14.12% available phosphorous. [9] Biolys®, L-lysine, Evonik Operations GmbH (Hanau, Germany). 54.6% Lys. [10] Rovimix Camarao Intensivo. DSM Produtos Nutricionais Brasil Ltda. (São Paulo, Brazil). Guarantee levels per kg of product: vitamin A, 1,250,000 IU; vit. D3, 350,000 IU; vit. E, 25,000 IU; vit. K3, 500 mg; vit. B1, 5000 mg; vit. B2, 4000 mg; vit. B6; 10 mg; nicotinic acid, 15,000 mg; pantothenic acid, 10,000 mg; biotin, 150 mg; folic acid, 1250 mg; vit. C, 25,000 mg; choline, 50,000 mg; inositol, 20,000 mg; Fe 2000 mg; Cu, 3500 mg; chelated Cu, 1500 mg; Zn, 10,500 mg; chelated Zn, 4500 mg; Mn, 4000 mg; Se, 15 mg; chelated Se, 15 mg; I, 150 mg; Co, 30 mg; Cr, 80 mg; filler, 1000 g. [11] L-Arginine HCl, Sigma-Aldrich Co. (St. Louis, MO, USA), 98.5% Arg. [12] Nutri-Bind Aqua Veg Dry, Nutri-Ad International NV (Dendermonde, Belgium). Synthetic pellet binder consisting of calcium lignosulfonate (94.00%) and guar gum (6.00%). [13] ThreAMINO®, L-Threonine, Evonik Operations GmbH (Hanau, Germany). Min. 98.5% Thr. [14] TrypAMINO®, L-Tryptophan, Evonik Operations GmbH (Hanau, Germany). 98% tryptophan. [15] Aquavi® Met-Met, DL-methionyl-DL-methionine, Evonik Operations GmbH (Hanau, Germany). 98% Met (min. 95% DL-Met-Met and max. 3% D, L-Met). [16] Cholesterol SF, Dishman Netherlands B.V. (Veenendaal, Netherlands). 91% cholesterol. [17] Rovimix® Stay C® 35, DSM Produtos Nutricionais Brasil Ltda. (São Paulo, Brazil). 350.0 g kg^{-1} phosphorylated vitamin C.

The dietary inclusions of FML ranged from a maximum of 18.00% (18% FML) to 12.00% (12% FML), 6.00% (6% FML), and no FML at all (0% FML). FML was replaced for soybean meal (SBM) which increased from a minimum of 20.73 (diet 18% FML) to 28.95 (12% FML), 37.8 (6% FML), and 46.08% (0% FML), respectively. All diets required DL-Met-Met supplementation to meet the targeted Met levels, except diets with 18.00% FML and a final total Met content of 0.58%. In this case, Met was only derived from protein-bound sources.

Diets reached a mean CP and total lipid content of 34.57 ± 0.37% and 7.73 ± 0.44% on a fed basis, respectively (Table 2). To maximize protein utilization and keep a well-balanced amino acid (AA) profile, diets were formulated on an ideal protein basis using lysine (Lys) as the first limiting and reference AA [57]. Total dietary Lys, threonine (Thr), and arginine (Arg) content reached 1.78 ± 0.04, 1.30 ± 0.02, and 2.25 ± 0.15% (as-is), respectively. A commercial grower shrimp feed with 39.25% CP and 6.90% total lipids was used as a reference (CTL). Total Met (Met + Cys), Lys, Thr, and Arg in the CTL diet were 0.86 (1.29), 2.21, 1.41, and 2.30%, respectively. Crude ash content was 12.17%, equivalent to the experimental diets containing the highest level of FML, i.e., 18%. However, total fiber content was 2.99%, higher than experimental diets with 46.08% SBM.

For the digestibility assay, a new set of four diets containing 0, 6, 12, and 18% FML and a constant level of total dietary Met was formulated (Table 3). Formulas were similar to the ones used in the growth performance evaluation but included 1.00% of chromic oxide to act as an inert marker. DL-Met-Met was supplemented at 0.41, 0.34, 0.27, and 0.20% (as-is) in diets with 0, 6, 12, and 18% FML, respectively. Finished diets reached a total CP, Met (Met + Cys), Lys, Thr, and Arg content of 33.99 ± 0.33, 0.81 ± 0.01 (1.27 ± 0.03), 1.73 ± 0.02, 1.24 ± 0.01, and 1.99 ± 0.02% (as-is), respectively (Table 4).

Table 2. Proximate and amino acid composition (% of the diet, as-is) of experimental diets to evaluate growth performance.

Nutrient	Diets/Nutrient Composition (%, As-Is Basis)												
	0.58% Met (1.05% Met + Cys)				0.69% Met (1.16% Met + Cys)				0.82% Met (1.29% Met + Cys)				
% Fish Meal (FML) Level	0	6	12	18	0	6	12	18	0	6	12	18	CTL [1]
Dry matter	90.35	88.75	89.02	88.80	90.11	90.48	88.88	87.73	90.13	90.17	88.85	88.98	90.47
Crude protein	34.57	34.20	34.36	34.94	34.40	35.21	34.41	34.39	34.03	34.87	34.39	35.07	39.25
Ether extract	7.62	7.90	8.89	8.11	7.45	7.88	7.52	7.21	7.34	7.48	7.67	7.63	6.90
Crude ash	8.96	10.96	11.45	11.90	8.90	10.39	10.89	11.86	9.28	10.61	11.23	12.24	12.17
Fiber	3.25	2.30	1.91	1.48	3.27	2.56	2.37	2.16	3.21	2.53	2.30	1.58	2.99
NFE [2]	35.95	33.39	32.41	32.37	36.09	34.44	33.69	32.11	36.27	34.68	33.26	32.46	29.16
Gross energy (MJ/kg) [3]	17.35	16.93	17.19	17.01	17.27	17.34	16.88	16.48	17.17	17.15	16.86	16.87	17.00
Essential amino acids (EAAs)													
Arginine	2.09	2.14	2.32	2.48	2.06	2.22	2.31	2.39	2.06	2.22	2.29	2.48	2.30
Histidine	0.75	0.73	0.73	0.73	0.76	0.76	0.72	0.71	0.76	0.76	0.74	0.72	1.02
Isoleucine	2.43	2.35	2.30	2.26	2.41	2.41	2.29	2.22	2.40	2.40	2.26	2.25	2.84
Leucine	2.43	2.35	2.30	2.26	2.41	2.41	2.29	2.22	2.40	2.40	2.26	2.25	2.84
Lysine	1.74	1.72	1.81	1.85	1.74	1.78	1.78	1.76	1.75	1.77	1.76	1.85	2.21
Methionine	0.57	0.56	0.59	0.62	0.67	0.71	0.71	0.69	0.80	0.83	0.83	0.83	0.86
Met + Cys	1.07	1.04	1.04	1.04	1.17	1.20	1.17	1.12	1.29	1.31	1.28	1.27	1.29
Phenylalanine	1.66	1.58	1.51	1.45	1.65	1.63	1.50	1.43	1.65	1.61	1.48	1.43	1.79
Threonine	1.29	1.29	1.31	1.33	1.29	1.33	1.30	1.29	1.29	1.33	1.29	1.31	1.41
Valine	1.52	1.48	1.46	1.47	1.49	1.51	1.46	1.44	1.49	1.50	1.45	1.45	1.93
Non-essential amino acids (NEAAs)													
Alanine	1.35	1.40	1.46	1.56	1.33	1.43	1.46	1.51	1.33	1.42	1.45	1.53	2.19
Cysteine	0.50	0.47	0.46	0.42	0.50	0.49	0.45	0.43	0.49	0.49	0.45	0.43	0.43
Glycine	1.39	1.53	1.69	1.88	1.38	1.57	1.69	1.83	1.38	1.57	1.68	1.86	2.60
Serine	1.60	1.55	1.50	1.47	1.61	1.58	1.50	1.43	1.61	1.59	1.48	1.47	1.68
Proline	2.17	2.06	2.16	2.18	2.08	2.20	2.13	2.12	2.08	2.16	2.15	2.15	2.22
Aspartic acid	3.15	2.97	2.85	2.76	3.11	3.08	2.85	2.69	3.11	3.05	2.83	2.72	3.47
Glutamic acid	7.05	6.61	6.48	6.27	6.86	6.88	6.43	6.16	6.85	6.86	6.41	6.23	5.84
Sum EAA [4]	14.47	14.22	14.32	14.44	14.48	14.75	14.35	14.14	14.60	14.81	14.35	14.56	17.20
Sum NEAA	17.20	16.59	16.60	16.53	16.88	17.23	16.52	16.15	16.84	17.13	16.43	16.38	18.42
Sum EAA + NEAA	31.67	30.81	30.92	30.97	31.36	31.98	30.87	30.30	31.44	31.94	30.78	30.94	35.62

[1] Commercial control. [2] Nitrogen free extract. Calculated by subtraction (dry matter − (crude protein + ether extract + crude fiber + ash)). [3] Gross energy (GE) given on a DM basis. Calculated as GE = (4143 + (56 × ether extract (DM)) + (15 × crude protein (DM)) − (44 × crude ash (DM))) × 0.0041868. [4] Tryptophan not included.

Table 3. Ingredient composition (%, as-is) of diets used to evaluate digestibility.

Ingredients	Diets/Ingredient Composition (%, As-Is)			
% Fish Meal (FML) Level	0	6	12	18
Wheat flour [1]	28.60	29.48	31.17	34.39
Soybean meal [2]	45.02	37.32	28.84	20.02
Wheat gluten meal [3]	5.00	5.00	5.00	5.00
Salmon meal [4]	-	6.00	12.00	18.00
Kaolin	1.24	3.13	4.81	5.00
Salmon oil	3.41	3.32	3.35	3.39
Soy lecithin	3.28	2.88	2.47	2.05
Calcium carbonate	1.95	2.02	2.09	2.50
Krill meal [5]	2.00	2.00	2.00	2.00
Squid meal [6]	2.00	2.00	2.00	2.00
MSP [7]	1.70	1.63	1.55	1.39
Vitamin-mineral premix [8]	1.00	1.00	1.00	1.00
Potassium chloride	0.984	0.89	0.79	0.68
Salt	0.99	0.89	0.78	0.69

Table 3. *Cont.*

Ingredients	Diets/Ingredient Composition (%, As-Is)			
% Fish Meal (FML) Level	**0**	**6**	**12**	**18**
Chromic oxide III [9]	0.50	0.50	0.50	0.50
Synthetic binder [7]	0.50	0.50	0.50	0.50
L-Lysine [7]	0.55	0.40	0.30	0.20
DL-Met-Met [7]	0.41	0.34	0.27	0.20
Magnesium sulphate	0.57	0.37	0.17	-
L-Tryptophan [7]	0.01	0.06	0.12	0.17
Cholesterol [7]	0.10	0.10	0.10	0.10
L-Threonine [7]	0.13	0.10	0.09	0.07
L-Arginine HCl [7]		0.03	0.08	0.10
Vitamin C [7]	0.04	0.04	0.04	0.04

[1] 12.80% moisture, 11.44% crude protein (CP), 0.97% ether extract (EE), 0.19% crude fiber (CF), 0.68% ash, 0.18% methionine (Met), 0.27% lysine (Lys), 0.44% methionine + cysteine (M + C). [2] Bunge Alimentos S.A. (Luiz Eduardo Magalhães, Brazil). 10.30% moisture, 47.38% CP, 2.28% EE, 5.99% CF, 6.05% ash, 0.61% Met, 2.88% Lys, 1.28% M + C. [3] Amytex 100. Tereos Syral S.A.S. (Marckolsheim, France). 6.75% moisture, 79.68% CP, 2.44% EE, 0.41% CF, 1.87% ash, 1.16% Met, 1.35% Lys, 2.68% M + C. [4] Pesquera Pacific-Star (Puerto Montt, Chile). 10.89% moisture, 64.44% CP, 8.71% EE, 0.21% CF, 16.12% ash, 1.87% Met, 4.97% Lys, 2.70% M + C. [5] Qrill™ Antarctic krill meal, Aker BioMarine Antarctic AS (Lysaker, Norway). 6.61% moisture, 57.05% CP, 18.47% EE, 11.82% ash, 1.63% Met, 3.92% Lys, 2.08% M + C. [6] 9.75% moisture, 83.13% CP, 5.65% EE, 2.34% Met, 5.06% Lys, 3.17% M + C. [7] Check Table 1 for composition. [8] Vaccinar Industria e Comercio Ltda. (Pinhais, Brazil). Guarantee levels per kg of product: vitamin A, 1,200,000 IU; vit. D3, 200,000 IU; vit. E, 60,000 mg; vit. K3, 1000 mg; vit. B1, 2400 mg; vit. B2, 2400 mg; vit. B6, 6000 mg; vit. B12, 4 mg; nicotinic acid, 10,000 mg; pantothenic acid, 5200 mg; biotin, 20 mg; folic acid, 400 mg; vit. C, 30,000 mg; choline, 50,000 mg; inositol, 80,000 mg; Fe 26,000 mg; Cu, 2000 mg; Zn, 20,000 mg; Mn, 5000 mg; Se, 100 mg; I, 600 mg; Co, 105 mg; Cr, 60 mg. [9] Vetec Química Fina Ltda. (Rio de Janeiro, Brazil). Minimum of 99.0% of Cr_2O_3.

Table 4. Crude protein and amino acid composition (% of the diet, as-is) of experimental diets used in the digestibility assay.

Composition	Diets/Amino Acid Composition (%, As-Is)				
% Fish Meal (FML) Level	**0**	**6**	**12**	**18**	**CV (%) [1]**
Dry matter	91.04	90.67	90.51	90.20	0.39
Crude protein	34.29	34.20	33.93	33.54	0.98
Essential amino acids (EAAs)					
Arginine	1.96	2.00	2.01	2.01	1.19
Histidine	0.68	0.70	0.71	0.72	2.59
Isoleucine	1.32	1.35	1.36	1.39	2.21
Leucine	2.24	2.27	2.30	2.31	1.43
Lysine	1.71	1.70	1.73	1.75	1.28
Methionine	0.80	0.81	0.81	0.82	1.34
Met + Cys	1.22	1.26	1.28	1.30	2.66
Phenylalanine	1.44	1.50	1.54	1.58	4.12
Threonine	1.23	1.24	1.24	1.25	0.58
Valine	1.47	1.48	1.45	1.45	0.83
Non-essential amino acids (NEAAs)					
Alanine	1.53	1.47	1.39	1.32	6.51
Cysteine	0.43	0.45	0.47	0.48	5.23
Glycine	1.87	1.70	1.54	1.35	13.68
Serine	1.43	1.48	1.52	1.53	2.92
Proline	2.15	2.12	2.09	2.06	1.82
Aspartic acid	2.68	2.82	2.91	2.99	4.69
Glutamic acid	6.22	6.38	6.51	6.64	2.81
Sum EAA [2]	12.84	13.05	13.16	13.28	1.46
Sum NEAA	16.31	16.42	16.42	16.37	0.32
Sum EAA + NEAA	29.14	29.47	29.58	29.66	0.77

[1] Coefficient of variation. [2] Tryptophan not included.

All experimental diets were produced using a laboratory extruder following the methodology described by Nunes et al. [58]. Finished diets (pellets of 2.0 mm in diameter by 5 mm in length) were stored at 16 °C in sealed containers. For diets used in the growth performance, physical water stability measured by the orbital shaker method was 87.44 ± 2.23% (n = 75) for the treatments and 93.14 ± 0.32% (n = 5) for the CTL.

2.4. Shrimp Feeding, Feces Collection, and Water Quality

2.4.1. Growth Performance Trial

In outdoor tanks, shrimp were fed 10 times during daylight using an automatic feeder [59]. The daily rations were calculated based on the equation MM = $0.0931BW^{0.6200}$, where MM is the maximum amount of feed that can be consumed daily by an individual with a specific BW [60]. Previous work had shown that the MM can be restricted by 28.8% without any detrimental effect on shrimp growth performance [61]. Therefore, the daily meals were reduced by 30% across all treatments to control feed conversion ratio (FCR). Meals were adjusted daily, assuming a fixed weekly drop in shrimp survival (by 0.38%) and a BW gain of 100 mg/shrimp/day. Starting from the 11th day of rearing, every two weeks, 10 shrimp/tank were sampled to determine their mean BW gain. Until the next sampling, meals were adjusted assuming an average daily weight gain achieved in the previous week for each specific rearing tank, maintaining a fixed 0.38% weekly drop in shrimp survival across all diets. No feed leftovers were collected during the rearing period. Dead animals were not replaced throughout the culture period.

2.4.2. Digestibility

In the digestibility assay, shrimp were fed in excess at 07:00 a.m., 01:00, and 04:00 p.m. Diets were delivered manually and exclusively in feeding trays (diameter of 9.75 cm). Daily meals were adjusted according to the amount of feed leftovers collected from feeding trays, which, when present, were collected, dried in a convection oven, weighed, and discarded. Feces were collected by siphoning four times daily [56]. Feces samples were gently rinsed with distilled water for salt removal and stored at −23 °C. All samples were freeze-dried prior to chemical analysis.

Water pH, temperature, and salinity were measured once daily starting at 09:00 a.m. in all tanks. In outdoor tanks, mean values reached 7.87 ± 0.20 (7.00–8.90, n = 4452), 28.5 ± 0.6 °C (n = 4452), and 39 ± 3 g/L (n = 4452), respectively. In the indoor tanks, mean pH, temperature, and salinity reached 7.81 ± 0.29 (n = 3468), 30.6 ± 0.9 °C (n = 3400), and 39 ± 4 g/L (n = 3264), respectively. In both rearing systems, dissolved oxygen was kept saturated over the complete rearing period.

2.5. Chemical and Physical Analyses of Feeds and Feces

Feed chemical analyses followed standard methods [62]. DM was determined in a convection oven for 24 h at 105 °C. CP was analyzed with the Kjeldahl method of nitrogen estimation [62]. Ash content was determined by burning samples in a muffle at 600 °C for 2 h (AOAC 942.05) and crude fiber by enzymatic-gravimetric determination (AOAC 992.16). Amino acid content in diets and feces samples was analyzed with wet chemistry (AMINOLab®, Evonik Operations GmbH, Hanau, Germany) using ion exchange chromatography [63,64]. Experimental diets and the CTL feed were analyzed for physical stability in seawater (35 g/L salinity) using the orbital shaker method described by Nunes et al. [59]. Readings were determined for five replicate samples of each diet and CTL. Chromium oxide content in shrimp feces and experimental diets used to determine digestibility was determined in duplicate using electrothermal atomic absorption spectrometry (ETAAS) by SGS Analytics Germany GmbH (Jena, Germany).

2.6. Shrimp Growth Performance

Shrimp were reared for 70 and 93 days in outdoor and indoor tanks, respectively. At harvest, all live shrimp were collected, counted, and individually weighed to a 0.01 g

precision scale. Final shrimp survival (S, %) was calculated as S = (POPf/POPi) × 100, where POPi = number of stocked shrimp and POPf = number of shrimp at harvest. The weekly weight gain (WWG, g/week) was determined by the formula: WWG = [(BWf − Bwi)/t] × 7, where Bwi = wet shrimp body weight (BW, g) at stocking, BWf = final shrimp BW at harvest, and t = number of days in culture. The gain in shrimp yield (YIE, g of shrimp biomass gained/m^2) was determined as YIE = (BIOf − BIOi) ÷ tank bottom area (m^2), where BIOi = initial shrimp biomass (g) per tank, BIOf = final shrimp biomass (g) per tank, and tank bottom area = 1.02 and 0.19 m^2 (outdoor and indoor tanks, respectively). FCR was calculated on a DM basis, by dividing the total inputs of feed (g, dry-matter basis, DM) delivered during the entire rearing period by the total harvested shrimp biomass (g, as-is basis) from each tank. The apparent feed intake (AFI, g of feed delivered divided by the number of stocked shrimp) was calculated by dividing the total amount of feed delivered (g, DM basis) by the number of stocked shrimp.

2.7. In Vivo Digestibility

The concentration of Cr_2O_3 in the finished diets and in shrimp feces was used to determine the ADC, according to the formula (Cho et al. 1982):

$$\text{ADC} = 100 - \left[100\left(\frac{\%Cr_2O_3d}{\%Cr_2O_3f}\right) \times \left(\frac{\%Nf}{\%Nd}\right)\right] \quad (1)$$

where, ADC = apparent digestibility coefficient of CP (in %) and (or) AA (in %); Cr_2O_3d = concentration (in %) of chromic oxide in the diet; Cr_2O_3f = concentration (in %) of chromic oxide in shrimp feces; Nd = concentration (in %) of CP and AA in the diets; Nf = concentration (in %) of CP and AA in shrimp feces.

2.8. Economic Analysis

The cost of formulation of each individual diet was first calculated by using local market prices of each ingredient and feed additives (Table 1). The price of FML, SBM, and DL-Met-Met were USD 1.300, 0.415, and 5.000 per kg, respectively. Feed sale price was calculated by increasing 30% of the formula costs to account for feed mill margins, manufacturing, packaging, marketing, and other miscellaneous costs involved in feed production and sales. Feeds accounted for 40% of the total shrimp production costs (USD/kg) with the remainder attributed to other variable (PLs, amendments, sediment removal, electricity, fuel, labor) and fixed costs [65]. The total production cost (USD/kg) was determined by multiplying the feed sale price (USD/kg) by the FCR and the gained shrimp yield (kg). The farm gate price for shell-on, head-on shrimp was estimated at USD 3.42/kg. An excess of USD 0.19/kg was added on top of the final price for every one gram of shrimp in excess of 10 g BW at harvest (Brazilian Shrimp Farmers Association, February, 2022). The gross revenue (USD/kg) was determined by multiplying the farm gate shrimp price (USD/kg) with the gained shrimp yield (kg) from each tank. The gross profit (USD/kg) was given as the gross revenue subtracted by the total production cost. The return on investment (ROI, %) was calculated by subtracting the gross revenue by the total production cost and then dividing the result by the total production cost multiplied by 100.

2.9. Statistical Analysis

One-way and two-way analyses of variance (ANOVAs) were used to compare the means of shrimp growth and economic performance as a function of the dietary FML inclusion and (or) Met content. One-way ANOVA was used to compare the means of economic efficiency. When significant differences were detected, they were compared two-by-two with Tukey's HSD test. The significant level of 5% was set in all statistical analyses. The statistical package IBM® SPSS® Statistics 23.0 (SPSS Inc., Chicago, IL, USA) was used.

3. Results

3.1. Growth Performance

In outdoor tanks, shrimp final survival was high (92.7 ± 4.7%) and unaffected by dietary Met content, inclusion level of FML, or their interaction ($p > 0.05$, Table 5). Additionally, final survival did not differ between shrimp fed the experimental diets and the CTL (89.7 ± 2.8%). Gained shrimp yield increased progressively with higher levels of FML, from a low of 1090 ± 54 (0% FML) to a high of 1166 ± 66 g/m^2 (12% FML). However, yield did not differ statistically when shrimp were fed diets with 18, 12, or 6% FML, regardless of the dietary Met content. The elimination of FML did not affect yield when compared to shrimp fed 6% FML (1121 ± 68 g/m^2), but it was significantly lower than those fed 12% and 18% FML. The increase in the dietary Met levels from 0.58 (1127 ± 56 g/m^2) to 0.69 (1145 ± 45 g/m^2) or 0.82% (1135 ± 84 g/m^2) had no statistical effect on gained yield or a significant interaction with FML level. Shrimp fed the CTL (1040 ± 33 g/m^2) achieved a lower yield compared to those fed diets containing 0.69% Met at all levels of FML, except the highest (18%). At 0.58% Met, higher FML levels were required (12 and 18% FML) to significantly increase yield beyond the CTL. In comparison, at 0.82% Met, there was no significant difference in yield between shrimp fed the CTL and the experimental diets, except when 18% FML was used. In this case, yield was higher for the latter compared to the CTL.

Table 5. Final shrimp growth performance (mean ± SD) in outdoor 1 m^3 tanks as a function of dietary methionine (Met) and fish meal (FML) inclusion levels. Shrimp of 1.00 ± 0.08 g were raised with 100 animals/m^2 for 70 days. Asterisks (*) refer to statistically significant differences between the commercial control (CTL) and the experimental diets ($p < 0.05$, Student's *t*-test). Common letters in each column indicate non-statistically significant differences between different dietary fish meal levels according to Tukey's HSD test ($p > 0.05$).

Variable	% Met	Mean ± SD	Dietary Fish Meal Level (%, As-Is)				CTL
			0	6	12	18	
Final survival (%)	0.58	92.1 ± 4.08	93.3 ± 2.1	92.4 ± 2.0	91.7 ± 1.9	92.5 ± 1.7	89.7 ± 2.8
	0.69	94.2 ± 4.06	94.1 ± 1.9	95.5 ± 2.0	95.1 ± 2.6	92.6 ± 1.9	-
	0.82	92.5 ± 5.46	90.6 ± 3.4	88.2 ± 2.9	93.1 ± 1.4	94.4 ± 1.7	-
	Mean ± SD	-	92.7 ± 5.52	92.0 ± 5.72	93.4 ± 4.37	93.2 ± 3.84	-
Gained yield (g/m^2)	0.58	1127 ± 56	1077 ± 20	1098 ± 20	1177 ± 30 *	1162 ± 20 *	1044 ± 33 *
	0.69	1145 ± 45	1131 ± 7 *	1142 ± 19 *	1149 ± 26 *	1132 ± 26	-
	0.82	1135 ± 84	1062 ± 30	1123 ± 47	1177 ± 47	1168 ± 28 *	-
	Mean ± SD	-	1090 ± 54 ^{a}	1121 ± 68 ^{ab}	1166 ± 66 ^{b}	1151 ± 55 ^{b}	-
Growth (g/week)	0.58	1.17 ± 0.09	1.10 ± 0.04	1.14 ± 0.05	1.23 ± 0.01	1.20 ± 0.03	1.12 ± 0.06
	0.69	1.16 ± 0.07	1.15 ± 0.03	1.14 ± 0.05	1.15 ± 0.04	1.17 ± 0.03	-
	0.82	1.17 ± 0.09	1.12 ± 0.01	1.22 ± 0.03	1.21 ± 0.06	1.18 ± 0.04	-
	Mean ± SD	-	1.12 ± 0.06	1.17 ± 0.10	1.19 ± 0.08	1.18 ± 0.08	
AFI (g/shrimp)	0.58	13.4 ± 0.5	12.9 ± 0.2	13.4 ± 0.3	13.9 ± 0.2 *	13.4 ± 0.1 *	12.8 ± 0.2 *
	0.69	13.2 ± 0.5	13.3 ± 0.2	13.1 ± 0.3	13.3 ± 0.3	13.3 ± 0.1	-
	0.82	13.4 ± 0.5	13.2 ± 0.2	13.5 ± 0.3	13.5 ± <0.01 *	13.6 ± 0.2 *	-
	Mean ± SD	-	13.1 ± 0.4	13.3 ± 0.7	13.5 ± 0.5	13.4 ± 0.5	
FCR	0.58	1.19 ± 0.04	1.20 ± 0.02	1.22 ± 0.01	1.18 ± 0.02	1.16 ± 0.01 *	1.23 ± 0.02 *
	0.69	1.15 ± 0.05	1.17 ± 0.02	1.15 ± 0.02 *	1.16 ± 0.03	1.17 ± 0.02	-
	0.82	1.19 ± 0.08	1.24 ± 0.05	1.21 ± 0.04	1.16 ± 0.05	1.16 ± 0.01	-
	Mean ± SD	-	1.21 ± 0.08	1.19 ± 0.06	1.16 ± 0.06	1.16 ± 0.05	-
Two-Way ANOVA		Survival	Yield		Growth	AFI	FCR
FML		0.891	0.007; 0 < 12, 18%, 6 = 0%, 6 = 12, 18%		0.122	0.132	0.175
Met		0.221	0.880		0.558	0.367	0.253
FML x Met		0.551	0.451		0.574	0.502	0.538

Shrimp weekly growth rate exceeded 1.1 g regardless of the dietary treatment. No difference or significant interaction was detected in growth between shrimp fed different levels of dietary Met and/or FML ($p > 0.05$). Shrimp fed experimental diets also grew at

a similar rate compared to those fed the CTL. Apparent feed intake (AFI) did not differ statistically as a result of FML level or dietary Met content ($p > 0.05$). However, some of the experimental diets showed a higher AFI than the CTL. FCR was generally low, between 1.15 to 1.22. FCR was also not affected by variations in FML or dietary Met. FCR for experimental diets was within the range of the CTL (1.23 ± 0.02). Exceptions were diets with 6% FML containing 0.69% Met and 18% FML with 0.58% Met, which resulted in statistically lower values compared to the CTL.

Final shrimp BW at harvest ranged between 12.61 ± 0.92 (0% FML and 0.58% Met) and 13.92 ± 0.51 g (12% FML and 0.58% Met; Figure 1). Final BW was affected by both FML levels and dietary Met content, with a strong interaction between the two ($p < 0.0001$). With 0 and 6% FML, higher levels of dietary Met, i.e., 0.69 and 0.82%, respectively, were required to maximize shrimp BW. However, a complete withdrawal of FML was detrimental to shrimp BW even at 0.82% Met. In comparison, at 12 and 18% FML, a dietary Met content of only 0.58% was sufficient to enhance shrimp BW.

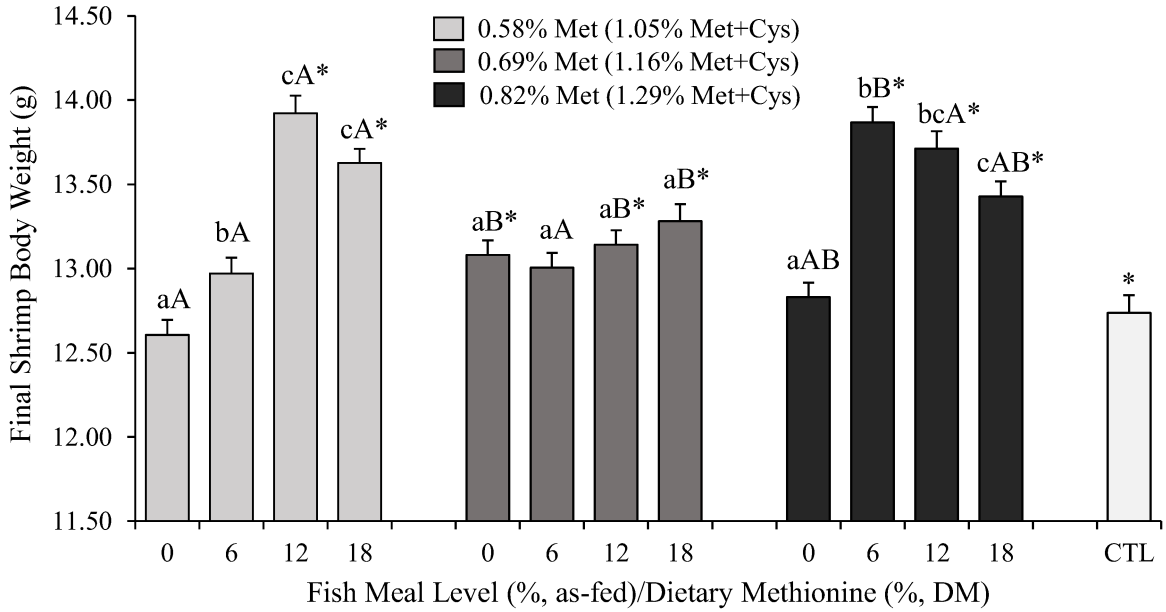

Figure 1. Mean (±standard error) body weight (g) of *L. vannamei* after 70 days of rearing in green water tanks of 1 m^3. Different lowercase letters indicate statistically significant differences between fish meal (FML) levels within the same dietary methionine (Met) content at the $\alpha = 0.05$ level according to Tukey's HSD. Different capital letters refer to significant differences ($p < 0.05$) between Met levels within each FML dietary inclusion. Asterisks (*) indicate statistically significant differences between the commercial reference (CTL) and the experimental diets ($p < 0.05$, Student's *t*-test).

The reduction in the dietary inclusion of FML resulted in a significantly lower BW at harvest, especially with diets containing 0 and 6% FML. The highest BW was achieved when shrimp were fed 12% and 18% FML ($p < 0.05$), but the latter did not differ from 6% ($p > 0.05$). Similarly, reducing dietary Met content from 0.82 to 0.58 and 0.69% resulted in a significantly lower BW in *L. vannamei* ($p < 0.05$). However, no difference was detected in BW between shrimp fed 0.58% and 0.69% dietary Met ($p > 0.05$). At the lowest level of dietary Met, i.e., 0.58%, diets containing 12 and 18% FML outperformed the diets with 6% and 0% FML ($p < 0.05$). A similar but non-statistically significant trend was detected with higher levels of dietary Met, i.e., 0.69% and 0.82% ($p > 0.05$).

The amount of dietary Met required to maximize shrimp BW was also a function of FML level. When FML was decreased to 6% or 0%, the highest BW was achieved with 0.69–0.82% Met, but at 12% and 18%, a minimum of 0.58% Met was sufficient. Thus, in general, as FML levels were challenged, higher levels of total dietary Met were required to maximize shrimp BW. Finally, shrimp fed the CTL achieved an equivalent BW compared to

those fed 0 and 6% FML. Levels of FML higher than 6% regardless of dietary Met content resulted in significantly higher BW compared to the CTL ($p < 0.05$).

3.2. Dietary Protein and Amino Acid Digestibility

There was an increasing trend towards in apparent crude protein digestibility (ACPDC) with higher dietary inclusion levels of FML (Table 6). ACPDCs varied from a minimum of 81.1 ± 1.7% for shrimp fed a diet without FML to a high of 88.6 ± 2.1% for 18% FML. The apparent amino acid digestibility coefficients (AAADCs) followed a similar fashion for both EAAs and non-essential AAs (NEAAs). An exception was detected in ADCs for Lys, Met, and Met + Cys, which all fell within the same range in diets containing 0, 6, and 12% FML (91.0–91.8, 90.4–91.3, and 88.0–89.2%, respectively). However, higher ADCs values for these EAAs were found for the 18% FML diet (93.6, 93.2, and 91.6%, respectively).

Table 6. Mean (±SD) apparent digestibility coefficient (ADC, %) of protein and amino acids in diets containing graded levels of fish meal.

Nutrient	**Diets/Apparent Digestibility Coefficient (ADC, %)**			
% Fish Meal (FML) Level	**0**	**6**	**12**	**18**
Crude protein	81.1 ± 1.7	83.1 ± 9.2	84.7 ± 1.3	88.6 ± 2.1
Essential amino acids (EAAs)				
Arginine	91.9 ± 1.2	92.7 ± 4.0	93.1 ± 0.8	94.8 ± 0.8
Histidine	87.4 ± 1.6	88.1 ± 6.5	88.9 ± 1.2	90.9 ± 1.6
Isoleucine	87.8 ± 0.9	89.0 ± 5.1	89.7 ± 1.5	92.0 ± 1.3
Leucine	87.5 ± 1.5	88.9 ± 6.1	89.6 ± 1.4	92.2 ± 1.3
Lysine	91.0 ± 1.3	91.8 ± 4.1	91.8 ± 1.0	93.6 ± 1.1
Methionine	91.1 ± 0.9	90.4 ± 4.5	91.3 ± 1.5	93.2 ± 0.7
Met + Cys	88.0 ± 1.4	88.2 ± 5.9	89.2 ± 1.6	91.6 ± 1.1
Phenylalanine	88.6 ± 1.2	89.4 ± 5.6	89.9 ± 1.3	92.3 ± 1.3
Threonine	85.4 ± 2.3	87.2 ± 6.7	87.8 ± 1.5	90.5 ± 1.6
Valine	85.7 ± 0.8	87.2 ± 5.9	88.1 ± 1.3	90.9 ± 1.7
Non-essential amino acids (NEAAs)				
Alanine	81.5 ± 1.4	84.6 ± 7.0	86.3 ± 0.3	89.9 ± 1.7
Cysteine	82.6 ± 2.1	84.4 ± 8.1	85.6 ± 1.7	88.5 ± 1.8
Glycine	80.5 ± 2.0	84.2 ± 7.8	86.2 ± 0.5	89.7 ± 1.7
Serine	87.0 ± 1.9	88.2 ± 6.1	88.8 ± 1.1	91.4 ± 1.4
Proline	87.4 ± 1.7	89.7 ± 5.3	90.4 ± 1.1	93.2 ± 1.3
Aspartic acid	86.4 ± 1.5	87.7 ± 5.9	88.2 ± 1.2	90.6 ± 1.7
Glutamic acid	92.2 ± 1.0	93.2 ± 3.5	93.6 ± 0.6	95.2 ± 0.8

In indoor tanks, shrimp fed a diet deprived of FML achieved the highest final survival compared to other dietary treatments (Table 7, $p < 0.005$). Survival reached a mean of 87.5 ± 7.8% ($p > 0.05$). At harvest, shrimp fed 0% FML grew at 0.62 ± 0.04 g/week and achieved 12.69 ± 0.55 g final BW, both significantly lower than other dietary treatments ($p < 0.05$). However, no statistically significant differences were detected for gained shrimp yield (557 ± 80 g/m^2) and FCR (2.68 ± 0.37). There was a significantly lower AFI when shrimp were fed 0 and 6% FML, but the later did not differ statistically from 12% and 18% FML ($p > 0.05$).

Table 7. Final shrimp growth performance (mean ± SD) in a clear-water recirculating indoor tank system as a function of dietary fish meal (FML) level. Shrimp were raised for 93 days with 70 shrimp/m^2 in 60 L tanks to determine feed digestibility. Different letters in the same line indicate statistically significant differences according to Tukey's HSD test at $\alpha = 0.05$.

Performance Parameter	Dietary Fish Meal Level (%, As-Is)					One-Way ANOVA
	0	6	12	18	Mean ± SD	
Initial body weight (g)	4.41 ± 0.14	4.52 ± 0.21	4.46 ± 0.09	4.47 ± 0.07	4.47 ± 0.14	0.368
Final survival (%)	91.7 ± 7.6	86.0 ± 8.0	83.0 ± 5.9	88.0 ± 7.6	87.5 ± 7.8	0.068
Final body weight (g)	12.69 ± 0.55 [a]	14.13 ± 1.29 [b]	14.28 ± 0.81 [b]	14.35 ± 0.49 [b]	-	<0.0001
Growth (g/week)	0.62 ± 0.04 [a]	0.72 ± 0.09 [b]	0.74 ± 0.06 [b]	0.74 ± 0.04 [b]	-	<0.0001
Gained yield (g/m^2)	540 ± 61	532 ± 84	553 ± 75	611 ± 86	557 ± 80	0.093
AFI (g/shrimp)	18.7 ± 1.3 [a]	19.1 ± 1.3 [ab]	20.6 ± 1.3 [c]	20.3 ± 1.0 [bc]	-	0.003
FCR	2.63 ± 0.32	2.76 ± 0.47	2.83 ± 0.30	2.53 ± 0.34	2.68 ± 0.37	0.289

3.3. Economic Efficiency

Total shrimp production cost, gross revenue, profit, and return on investment (ROI) were driven by FCR, yield, shrimp final BW, and formula costs, i.e., feed sales price. Formulation costs ranged from a minimum of 0.706 (0% FML with 0.56% Met) to a maximum of 0.943 USD/kg (18% FML with 0.82% Met; Table 1). The dietary inclusion of FML and total Met content both affected formula costs. The higher the dietary inclusion of FML, the higher the formula costs within the same level of dietary Met. Although a reduction in FML required higher levels of crystalline amino acid (CAA) supplementation, including DL-Met-Met, costs were offset by higher inclusions of SBM, which is a cheaper source of protein compared to FML. A reduction in FML from 18 to 12, 6%, and 0 at 0.69% dietary Met resulted in formula savings of 6.2, 13.3, and 23.1%, respectively. The increase in dietary Met content within the same level of FML also raised formula costs. However, the increase was less critical. Raising total Met content from 0.58 to 0.69 and 0.82% in diets deprived of FML impacted formula costs at 0.7 and 1.5%, respectively. A similar increase in formula cost was observed with 18% FML.

By taking into account the total production costs and shrimp performance data, it was possible to determine the ROI for each individual rearing tank and dietary treatment (Table 8). Economic analysis has indicated that FML levels significantly affected all parameters analyzed ($p < 0.05$), as opposed to the dietary levels of Met which showed no effect ($p > 0.05$). There was also no significant interaction between FML levels and dietary Met content for total production cost, gross revenue, profit, and ROI ($p > 0.05$).

Table 8. Economic analysis of experimental diets as a function of fish meal (FML) and dietary methionine (Met) content. Common letters indicate non-statistically significant differences between dietary FML inclusion levels according to Tukey's HSD test ($p < 0.05$).

Diets	Economic Analysis (USD/kg)			ROI (%) [1]
	Production Cost	Gross Revenue	Profit	
0% FML	3.24 ± 0.10 [a]	4.32 ± 0.28 [a]	1.07 ± 0.27 [a]	33.2 ± 8.4 [a]
6% FML	3.59 ± 0.19 [b]	4.54 ± 0.41 [ab]	0.95 ± 0.30 [a]	26.5 ± 7.9 [ab]
12% FML	3.88 ± 0.13 [c]	4.78 ± 0.42 [b]	0.90 ± 0.38 [ac]	23.1 ± 9.5 [b]
18% FML	4.10 ± 0.13 [d]	4.69 ± 0.38 [ab]	0.59 ± 0.28 [c]	14.3 ± 6.4 [c]
0.58% Met	3.68 ± 0.61	4.55 ± 0.38	0.87 ± 0.41	24.2 ± 8.2
0.69% Met	3.64 ± 0.72	4.55 ± 0.32	0.91 ± 0.25	25.7 ± 11.4
0.82% Met	3.76 ± 0.85	4.62 ± 0.37	0.86 ± 0.42	23.1 ± 11.8
Two-Way ANOVA	Production Cost	Gross Revenue	Profit	ROI (%)
FML	<0.0001	0.011	0.001	<0.0001
Met	0.130	0.784	0.997	0.889
FML × Met	0.530	0.354	0.352	0.339

[1] Return on investment.

Production cost reduced progressively with lower dietary inclusions of FML, from a high of 4.10 ± 0.13 (18% FML) to a low of 3.24 ± 0.10 USD/kg (0% FML). There was on average a reduction in 0.86 USD/kg in production cost when FML was completely eliminated, i.e., from 18% to 0 FML. Although gross revenue was impacted with a reduction in FML, it was not significantly different between diets containing 6, 12, and 18% FML ($p > 0.05$). Gross revenue with 12% FML (4.78 ± 0.42 USD/kg) was higher than 0% FML (4.32 ± 0.28 USD/kg), but it did not differ from 6% (4.54 ± 0.41 USD/kg). On the other hand, the lowest profits were achieved with higher levels of FML, i.e., 12% (0.90 ± 0.38 USD/kg) and 18% (0.59 ± 0.28 USD/kg), although the former did not differ statistically from 0% (1.07 ± 0.27 USD/kg) and 6% FML (0.95 ± 0.30 USD/kg).

Raising shrimp with a diet containing 18% FML led to the lowest ROI, at 14.3 ± 6.4%. Interestingly, the highest ROI was obtained with diets containing no or only 6% FML (33.2 ± 8.4% and 26.5 ± 7.9%, respectively). At no FML, ROI was significantly higher than 12% and 18% FML. At moderate levels of dietary inclusion, i.e., 6% and 12% FML, no differences in ROI could be observed.

4. Discussion

This study has demonstrated that dietary FML levels and Met (Met + Cys) content and their interaction significantly impact final shrimp BW. The responses in BW as a function of FML level varied according to the dietary Met (Met + Cys) content. At the lowest dietary Met (Met + Cys), i.e., 0.58% (1.05%), we observed that FML could only be reduced from 18% to 12%. Further reductions led to a reduced shrimp BW at harvest. At moderate levels of dietary Met, i.e., 0.69% (1.16%), FML could be completely eliminated without any impact to shrimp BW, but with an adverse effect on yield. In comparison, at the highest dietary Met, i.e., 0.82% (1.29%), FML could be reduced from 18% to 6%, but complete withdrawal negatively impacted BW. Therefore, the levels of dietary Met (Met + Cys) required to maximize shrimp BW at 0 and 6% FML ranged between 0.69 (1.16) and 0.82% (1.29%), while at 12% and 18%, only 0.58% (1.05%) was needed. Notably, Met apparent digestibility coefficient was not affected by the FML across diets, which means that even on a digestibility basis this difference will hold true. Therefore, it is clear that an effective reduction in FML in diets for *L. vannamei* is dependent on appropriate dietary Met (Met + Cys) levels. This can be achieved through the supplementation with crystalline Met or by raising protein ingredients rich in these intact AAs. This is consistent with other work which indicates the importance of supplemental Met while challenging FML levels [20,42].

We found that under outdoor and indoor tank conditions, the optimal dietary FML level without an adverse effect on the growth performance of *L. vannamei* was reached at 6%. This finding is corroborated with the work of Suárez et al. [18], who raised juvenile *L. vannamei* (0.3 ± 0.1 g) under clear-water conditions for 95 days. The authors replaced FML with SBM while keeping the dietary levels of Met + Cys (1.0–1.2%) consistent with the use of canola meal, a natural source of sulfur-containing AAs. They found that a combination of SBM and canola meal was able to reduce FML from 30 to 6% but not beyond that. In the current study, to completely withdraw FML from the diets, SBM levels were increased to 46.08%. In this case, most of the dietary protein came from plant sources (wheat flour, wheat gluten) with only a small portion (4%) derived from animals (squid and krill). We found that even at higher levels of dietary SBM (45% of the diet), the ADCs for dietary protein and AAs were adequate (>80%). Thus, the reduced shrimp growth performance with 0% FML was likely driven by other factors, including a poorer feed attractability. We could not observe any negative response in AFI with the outdoor rearing system likely because shrimp were fed with feed restrictions. However, in the indoor systems, when shrimp were fed in excess, there was a reduced AFI when shrimp were fed diets containing 0 and 6% FML.

In other work, plant-based feeds [6,7] and feeds containing in excess of 50% SBM have been advocated for juvenile *L. vannamei* [8,66–69]. Some of these studies report that a complete replacement of FML for SBM can be achieved with or without the use of proteins

from terrestrial animals, such as poultry meal. These studies also adopted green-water conditions, but shrimp were raised with a much lower stocking density (25 shrimp/m^2 [66]; 35 shrimp/m^2 [7,8,67]; 37.5 shrimp/m^2 [6] compared to the present work (100 shrimp/m^2). With a lower density, the nutrient contribution of naturally available food sources to shrimp growth can be significant and can lead to much higher levels of FML replacement. Their formulas also combined other protein sources for FML replacement in addition to SBM. For example, Amaya et al. [6] were able to reduce FML from 9% to 0 by increasing SBM from 32.58% to 39.08%. All of their diets contained a fixed level of 16.0% poultry by-product meal, except one which only contained plant ingredients (SBM, sorghum meal, corn gluten meal, fermented corn solubles). Therefore, FML is more likely to be fully replaced when feed formulation can rely on more than one or on a combination of substitute protein sources.

In the current study, from the economic point of view, eliminating FML was as competitive as including 6% FML, with both being more advantageous than 12 and 18% FML. The production costs of diets containing lower levels of FML exceeded the benefits of a higher revenue, resulting in greater profit and ROI. The cost of FML was the main driver for a higher production cost recorded for diets 12 and 18% FML. In comparison, total dietary Met content had no effect over economical parameters, including production costs.

5. Conclusions

Results from the present study show that a correct balance of FML and dietary Met has a critical effect on whiteleg shrimp performance. Shrimp final survival, growth, FCR, and the dietary protein and amino acid digestibility were not negatively affected by a reduction or complete elimination of FML with a proper balance with CAAs, including Met. However, while gained shrimp yield is reduced when FML is withdrawn, dietary inclusion levels of 12% or higher leads to increased costs, which are not offset by higher revenues. In conclusion, the total amount of dietary Met needed to maximize shrimp growth performance depends on the amount of dietary FML. Higher amounts of supplemental DL-methionyl-DL-methionine reduces the reliance on FML. A total dietary supplementation of DL-Met-Met of 0.34% can reduce FML inclusion from 18 to 6% without any negative effect on shrimp performance. Overall, results indicate that the use of FML can be minimized or completely eliminated without major detrimental effects on shrimp performance, as long as methionine requirement is met with proper supplementation of CAAs. Feeds with 0 FML or with only 6% with levels of dietary Met (as-fed basis), i.e., 0.69 and 0.82%, respectively, deliver the highest shrimp growth performance, profit, and return on investment compared to diets with higher levels.

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animals

MDPI

Article

Taurine Supplementation to Plant-Based Diets Improves Lipid Metabolism in Senegalese Sole

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Simple Summary: In contemporary dietary formulations for aquaculture, the amounts of fishmeal are being constantly reduced, and the inclusion of plant-protein sources is now a standard procedure. This approach may result in an unbalanced supply of selected nutrients, from which taurine was identified. Taurine is an amino acid that plays important physiological roles and is included in bile salts, which are essential for the emulsion, digestion, and absorption of dietary lipids and fat-soluble vitamins. In mammals, a hypolipidemic effect of taurine has been described. Senegalese sole (*Solea senegalensis*) is a marine fish species with increasing importance for aquaculture production in Southern European countries, with a high market value and low tolerance to dietary lipid levels. Thus, this study tested the effects of taurine supplementation to low-fishmeal diets on the physiological responses of Senegalese sole, with an emphasis on lipid metabolism. The results show that dietary inclusion of high levels of plant-protein sources to replace marine ingredients resulted in negative effects on lipid metabolism, due to the resultant low bile-acid concentration and/or the limited availability of taurine for bile-salt emulsification. Taurine supplementation mitigated part of the negative effects of plant-based diets, leading to better lipid utilisation.

Abstract: Taurine is a sulphur-containing amino acid with important physiological roles and a key compound for the synthesis of bile salts, which are essential for the emulsion and absorption of dietary lipids. This study aimed to evaluate the effects of taurine supplementation to low-fishmeal diets on the metabolism of taurine, bile acids, and lipids of Senegalese sole. A fishmeal (FM) and a plant-protein-based (PP0) diet were formulated, and the latter was supplemented with taurine at 0.5 and 1.5% (diets PP0.5 and PP1.5). Diets were assigned to triplicate tanks containing 35 fish (initial weight ~14 g) for 6 weeks. Fish from the PP0 treatment presented lower taurine and bile-acid concentrations compared with the FM treatment, and a downregulation of *cyp7a1* and *abcb11* was observed. Triolein catabolism decreased in PP0-fed fish, resulting in increased hepatic fat content and plasma triglycerides, while no effects on plasma cholesterol were observed. Taurine supplementation to plant-based diets resulted in a higher taurine accumulation in fish tissues, increased bile-acid concentration, and upregulation of *cyp7a1* and *abcb11*. Hepatic fat content and plasma triglycerides decreased with increasing dietary taurine supplementation. Taurine supplementation mitigated part of the negative effects of plant-based diets, leading to better lipid utilisation.

Keywords: aquaculture; *Solea senegalensis*; flatfish; fishmeal replacement; taurine; bile acids; lipid utilisation; metabolic trials

1. Introduction

Aquaculture has grown rapidly during the last few years, fuelled by the rising per capita consumption of seafood [1]. This growth was supported by a significant research effort to increase the industry's sustainability. During the last two decades, a lot of research was focused on the potential of plant-derived ingredients as an alternative to substitute fishmeal and fish oil in aquafeed formulations [1]. However, the dietary replacement of fishmeal with plant ingredients may result in an unbalanced supply of key nutrients. Among these, taurine (2-aminoethanesulfonic acid) was identified, since it is virtually absent in terrestrial plant ingredients, while it is particularly abundant in the marine food chain [2]. Taurine is an end product of the metabolism of sulphur-containing amino acids. Taurine synthesis occurs in the liver, but it seems to be limited in fish, especially in marine fish, due to the low levels or absence of rate-limiting enzymes in taurine biosynthesis [2,3]. Therefore, taurine is considered a conditionally essential nutrient for marine fish [4], and when feeding these species with terrestrial plant-protein-based diets, taurine requirements may not be fulfilled. Although considered a sulphur amino acid, taurine is not incorporated into proteins, remaining free in the cytosol, and it takes part in many important physiological processes, such as osmotic regulation, membrane stabilisation, antioxidation, eye development, and control of plasma cholesterol levels [2,3]. Taurine is also involved in the synthesis of bile salts, which are essential for the emulsion, digestion, and absorption of dietary lipids and fat-soluble vitamins. Bile acids are synthesised from cholesterol in the liver, with cholesterol 7α-hydroxylase acting as the rate-limiting enzyme [5]. Bile acids are then conjugated with an amino acid, which in fish is mainly taurine, to form bile salts that are secreted into the bile [6]. The secretion of compounds into the bile canaliculi is mediated by several ATP-binding cassette (ABC) transporters expressed at the canalicular membrane of hepatocytes [7]. Considering the key role of taurine in bile-acid synthesis, its importance in lipid metabolism is evident.

Several works studied the effects of dietary taurine supplementation, with contradictory results found across the literature depending on the species, developmental stage, and diet formulation (marine or plant-based). Dietary supplementation to fishmeal-based diets had no effects on the growth performance of several freshwater and marine fish species [8–10]. Although it was suggested that taurine-synthesis capacity was more limited in marine than in freshwater fish species [11], some studies showed an improved growth performance in carps (*Mylopharyngodon piceus* and *Ctenopharyngodon idella*), catfishes (*Clarias gariepinus* and *Pelteobagrus fulvidraco*), Nile tilapia (*Oreochromis niloticus*), and rainbow trout (*Oncorhynchus mykiss*) when taurine was included in high- or all-plant diets [8,12–16]. However, even when feeding low-fishmeal diets, no effects on growth performance were found in zebrafish (*Danio rerio*) and Atlantic salmon (*Salmo salar*) [17,18]. In marine fish species, several studies reported an increase in growth performance and/or feed efficiency with an increase in dietary taurine content, especially when using low-fishmeal diets [9,10,19–26].

Even when no effects were observed on growth performance, taurine supplementation may enhance the physiological condition of the fish. Several works report that dietary taurine supplementation to plant-based diets enhanced antioxidant status, immunity, and health in several freshwater and marine fish species [12,14,15,17,21,27–29]. To the best of our knowledge, negative effects on liver health were only reported for Persian sturgeon (*Acipenser persicus*) fed high-plant diets supplemented with taurine [30]. However, further studies demonstrated that dietary taurine inclusion levels in that study were supraoptimal [31]. Furthermore, taurine deficiency impaired bile pigment (biliverdin and bilirubin) production and secretion, inducing green liver syndrome in species such as red seabream (*Pagrus major*) and yellowtail (*Seriola quinqueradiata*) [22,23,32].

In mammals, taurine exerts a hypolipidemic effect, with the prevention of increasing levels of cholesterol and triglycerides [33]. As taurine is the only amino acid to conjugate with bile acids in teleost fish [6], it is not surprising that several studies report an increase in conjugated or total bile-acid concentration concomitantly with an increase in dietary taurine content [20,28,34–36]. Since bile-acid synthesis results from cholesterol catabolism [5], a

decrease in plasma cholesterol levels was correlated with increasing bile-acid synthesis in fish fed low-fishmeal diets supplemented with taurine [13,14,28]. However, in other studies, taurine supplementation to low-fishmeal diets had no effects on cholesterol levels, even if an increased concentration of bile acids was found [19,20]. Additionally, increased cholesterol levels were observed in fish fed fishmeal-based diets supplemented with taurine [34,37], but also in fish fed taurine-supplemented low-fishmeal diets [12,31,36]. In some studies, the potential hypolipidemic effects of taurine were translated into lower whole-body lipid deposition and/or lower hepatosomatic index [13,14,17,18], but the potential of taurine in accelerating lipogenesis was also described [34]. Thus, dietary taurine supplementation seems to regulate lipid metabolism in multiple ways.

Considering the previous studies, it seems that fish, especially marine carnivorous fish, require a supplemental taurine source when fed plant-protein-based diets to properly modulate physiological functions. Senegalese sole (*Solea senegalensis*) is a marine fish species with high market value and increasing importance for aquaculture production in Southern European countries [38]. Research studies demonstrated tolerance to high dietary inclusion levels of plant-protein sources [39–43], but it was suggested that taurine inclusion in these diets may be beneficial [43]. Furthermore, Senegalese sole has a low tolerance to high levels of dietary lipids [44], and as mentioned before, taurine assumes an important role in lipid digestion and metabolism in fish. Therefore, this study aims to evaluate the effects of taurine supplementation to low-fishmeal diets on the physiological responses of Senegalese sole, with an emphasis on lipid metabolism.

2. Materials and Methods

2.1. Experimental Diets

Two basal isonitrogenous (~55% crude protein) and isolipidic (8.6% crude fat) diets were formulated (Table 1): a fishmeal-based diet (FM), similar to a commercial diet, and a plant-protein-based diet (PP0), in which plant-protein sources replaced 85% of marine ingredients, as previously used by Aragão [45] and Richard [46]. FM and PP diets contained 0.4 and 0.08% of taurine, respectively (Table 1). Based on the PP0 formulation, two additional diets were further supplemented with microencapsulated taurine (as previously described in Aragão [45]) to obtain dietary taurine levels similar to the FM diet and to the level found in polychaetes, the natural food of Senegalese sole in the wild [47]: diets PP0.5 and PP1.5, respectively. The detailed formulations are depicted in Table 1. All diets were formulated to fulfil the known nutritional requirements (indispensable amino acids and phosphorus) of juvenile Senegalese sole.

Table 1. Formulation and analysed proximate composition of experimental diets.

	Diets			
Ingredients	**FM**	**PP0**	**PP0.5**	**PP1.5**
Fishmeal Super Prime [1]	37.00	3.00	3.00	3.00
Fishmeal 60 [2]	15.00	0.00	0.00	0.00
Fish soluble protein concentrate [3]	7.50	3.00	3.00	3.00
Squid meal [4]	7.50	3.00	3.00	3.00
Fish gelatine [5]	2.00	2.00	2.00	2.00
Pea protein concentrate [6]	0.00	20.00	20.00	20.00
Potato protein concentrate [7]	0.00	12.00	12.00	12.00
Wheat gluten [8]	0.00	10.00	9.45	7.70
Corn gluten meal [9]	0.00	8.00	8.00	8.00
Soybean meal [10]	5.00	5.00	5.00	5.00
Soy protein concentrate [11]	5.00	5.00	5.00	5.00

Table 1. *Cont.*

Ingredients	Diets			
	FM	**PP0**	**PP0.5**	**PP1.5**
Wheat meal [12]	6.40	5.10	5.10	5.10
Pea starch [13]	7.00	7.00	7.00	7.00
Fish oil [14]	2.50	6.80	6.80	6.80
Vitamin and mineral premix [15]	0.20	0.20	0.20	0.20
Lutavit C35 [16]	0.10	0.10	0.10	0.10
Lutavit E50 [17]	0.05	0.05	0.05	0.05
Monocalcium phosphate [18]	0.00	4.00	4.00	4.00
Glycerol [19]	2.50	2.50	2.50	2.50
Binder [20]	2.00	2.00	2.00	2.00
Antioxidant [21]	0.25	0.25	0.25	0.25
Lysine [22,*]	0.00	0.50	0.50	0.50
Methionine [23,*]	0.00	0.50	0.50	0.50
Taurine [24,*]	0.00	0.00	0.55	2.30
Proximate composition (% as fed)				
Dry matter	92.7	92.4	92.6	92.5
Ash	9.2	9.2	9.3	9.1
Crude protein	55.2	54.9	55.1	55.0
Crude fat	8.6	8.6	8.6	8.6
Taurine	0.40	0.08	0.43	1.40

All values are reported as the mean of duplicate analysis. [1] Super Prime: 66.3% crude protein (CP), 11.5% crude fat (CF), Pesquera Diamante, Lima, Peru. [2] Fair Average Quality (FAQ) fishmeal: 62% CP, 12% CF, COFACO, Lisboa, Portugal. [3] CPSP 90: 84% CP, 12% CF, Sopropêche, Wimille, France. [4] Super prime without guts: 84% CP, 4.7% CF, Sopropêche, Wimille, France. [5] Fish gelatine: 95% CP, WEISHARDT International, Liptovský Mikuláš, Slovakia. [6] NUTRALYS F85F: 83% CP, 1% CF, Roquette Frères, Lestrem, France. [7] Potato protein concentrate: 76% CP, 1.3% CF, AgroKorn, Videbæk, Denmark. [8] VITAL: 85.7% CP, 1.3% CF, Roquette Frères, Lestrem, France. [9] GLUTALYS: 61% CP, 6% CF, Roquette Frères, Lestrem, France. [10] Micronised soybean meal: 51% CP, 2.9% CF, SORGAL SA, Aveiro, Portugal. [11] Soycomil P: 65% CP, 0.8% CF, ADM, Amsterdam, The Netherlands. [12] Wheat meal: 10.2% CP, 1.2% CF, Casa Lanchinha, Alhos Vedros, Portugal. [13] NASTAR: 90% starch, COSUCRA, Warcoing, Belgium. [14] Marine oil omega 3: Henry Lamotte Oils GmbH, Bremen, Germany. [15] PVO40.01 Premix for marine fish, PREMIX Lda, Neiva, Portugal. Vitamins (per kg diet): 100 mg DL-alpha tocopherol acetate, 25 mg sodium menadione bisulphate, 20,000 IU retinyl acetate, 2000 IU DL-cholecalciferol, 30 mg thiamine, 30 mg riboflavin, 20 mg pyridoxine, 0.1 mg B12, 200 mg nicotinic acid, 15 mg folic acid, 3 mg biotin, 100 mg calcium pantothenate, 1 g ascorbic acid, 0.5 g inositol, 1 g choline chloride, and 0.5 g betaine. Minerals (per kg diet): 2.5 mg cobalt sulphate, 1.1 mg copper sulphate, 0.2 g ferric citrate, 5 mg potassium iodide, 15 mg manganese sulphate, 0.2 mg sodium selenite, 40 mg zinc sulphate, 0.6 g magnesium hydroxide, 1.1 g potassium chloride, 0.5 g sodium chloride, and 4 g calcium carbonate. [16] Lutavit C35, BASF, Ludwigshafen am Rhein, Germany. [17] Lutavit E50, BASF, Ludwigshafen am Rhein, Germany. [18] MCP: 220 g kg^{-1} P, 180 g kg^{-1} Ca, Beernem Fosfitalia, Ravenna, Italy. [19] From rapeseed, BELGOSUC, Beernem, Belgium. [20] Kieselguhr, LIGRANA GmbH, Eilsleben, Germany. [21] Paramega PX, Kemin Europe NV, Herentals, Belgium. [22] L-Lysine HCl 99%: Ajinomoto Eurolysine SAS, Paris, France. [23] DL-Methionine 99%: EVONIK Nutrition & Care GmbH, Krefeld, Germany. [24] L-Taurine 98.5%: Ajinomoto Eurolysine SAS, Paris, France. * Supplemented amino acids were microencapsulated in gelatine, according to Aragão [45].

All diets were manufactured and extruded at SPAROS Lda. (Olhão, Portugal). Briefly, powdered ingredients were mixed in a double-helix mixer, and diets were extruded (twin-screw extruder, model BC45 (Clextral, Firminy, France)) and dried in a convection oven (OP 750-UF, LTE Scientific, Oldham, UK). After cooling, the oils were added to the pellets by vacuum coating (model PG-10VCLAB (Dinnisen, Sevenum, The Netherlands)). Samples from all diets were collected and analysed for proximate composition, taurine content (Table 1), and amino acid (Table 2) profile, as described in Section 2.4. Throughout the duration of the trial, experimental diets were stored at room temperature in a cool and aerated storage room.

Table 2. Analysed amino acid composition of experimental diets.

Amino Acids (% as Fed)	Diets			
	FM	**PP0**	**PP0.5**	**PP1.5**
Arginine	3.9	3.6	3.7	3.7
Histidine	1.4	1.1	1.2	1.2
Lysine	4.2	4.1	4.1	4.1
Threonine	2.1	1.8	1.9	1.9
Isoleucine	2.3	2.2	2.3	2.3
Leucine	3.5	4.2	4.2	4.2
Valine	2.6	2.6	2.6	2.5
Methionine	1.7	1.3	1.2	1.2
Phenylalanine	2.2	2.6	2.5	2.6
Cystine	0.2	0.3	0.3	0.3
Tyrosine	1.8	1.9	1.9	2.0
Aspartic acid	4.3	4.0	4.1	4.1
Glutamic acid	6.8	10.5	10.4	10.6
Alanine	2.7	2.2	2.2	2.2
Glycine	3.3	2.1	2.1	2.1
Proline	2.3	3.5	3.5	3.5
Serine	2.2	2.5	2.5	2.5

All values are reported as the mean of duplicate analysis.

2.2. Rearing Trial

Senegalese sole juveniles were acquired from a commercial aquaculture and transported to the Centre of Marine Sciences (CCMAR) facilities (Faro, Portugal). Fish were acclimated to the new rearing conditions (recirculated aquaculture system with flat-bottomed tanks equipped with a mechanical filter, a submerged and a trickling biological filter, a protein skimmer, and a UV steriliser) for three weeks, during which they were fed with the FM diet.

After the three weeks of acclimation, fish were individually weighed under light anaesthesia (300 μL L^{-1} 2-phenoxyethanol (Sigma, Madrid, Spain)), and 35 fish (initial body weight: 13.7 ± 5.0 g) were distributed per tank (area: 0.18 m^2; volume: 18 L; initial fish density: 2.7 kg m^{-2}). A short rearing trial was performed to analyse the impacts of taurine supplementation to plant-based diets on the metabolism of taurine, bile acids, and lipids. For this, fish were kept for six weeks under controlled abiotic conditions (temperature: 19.0 ± 1.1 °C; salinity: 31.7 ± 1.7 ‰; dissolved oxygen: 94.3 ± 5.8% of saturation; ammonia < 0.1 mg L^{-1}; nitrites < 0.25 mg L^{-1}; photoperiod 12 h L:12 h D). Each diet was randomly assigned to triplicate tanks, and fish were fed at 1.0–1.5% body weight with automatic feeders 12 times per day (from 10h00 to 22h00). The feeding ration was adjusted daily based on the fish feed intake. Water-quality parameters and fish mortality were monitored daily.

At the end of the six weeks, twelve fish per tank were sampled after being deprived of feed for 24 h. All fish were anaesthetised (500 μL L^{-1} 2-phenoxyethanol) and individually weighed. From three fish, blood was collected from the caudal vein using heparinised syringes. Plasma samples were snap-frozen in liquid nitrogen and kept at −20 °C until triglycerides and total cholesterol analyses. These fish were then euthanised (1500 μL L^{-1} 2-phenoxyethanol), and the liver was collected and rapidly transferred to RNAlater (Sigma, Madrid, Spain). Samples were maintained at 4 °C for 24 h and then kept at −80 °C until gene expression analysis. Six fish were euthanised and used for the analysis of somatic indexes. The gallbladders were sampled, snap-frozen in liquid nitrogen, and kept at −80 °C for bile-acid-content analysis. Three fish were used for proximate composition and taurine content, and samples were kept at −20 °C until analysis. The liver of the other three fish were sampled and fixed with 10% buffered formaldehyde at pH 7.2 for histological analysis.

All animal manipulations were carried out in compliance with the European (Directive 2010/63/EU) and Portuguese (Decreto-Lei no. 113/2013 de 7 de Agosto) legislation for

the use of laboratory animals and were performed by trained scientists under Group-C licenses from the Direção-Geral de Alimentação e Veterinária, Portugal.

2.3. Metabolic Trial

At the end of the six weeks, fish from the same treatment were transferred to one tank and kept for two weeks under the same feeding regime and rearing conditions. Fish were deprived of feed for 24 h, and six fish from each treatment were randomly chosen and transferred to the metabolic flux laboratory at CCMAR. Metabolic trials using radiolabelled triolein were performed to better understand the effects of dietary taurine supplementation on Senegalese sole lipid metabolism.

The metabolic trials used the in vivo method of tube-feeding described in detail previously [46,48–50]. Fish were anaesthetised (300 μL L^{-1} 2-phenoxyethanol) and tube-fed at 0.5% body weight with the experimental diets previously labelled with ^{14}C-triolein ([U-^{14}C]-triolein,1.85 MBq, Perkin Elmer, Waltham, MA, USA). After tube feeding, fish were allowed to recover from anaesthesia in clean seawater with aeration and then were transferred into individual incubation chambers containing 2 L of clean seawater. Each chamber was hermetically sealed, provided with a gentle oxygen flow, and connected to CO_2 traps (containing 0.5 M potassium hydroxide) to collect $^{14}CO_2$ produced by the fish from the catabolism of ^{14}C-triolein. After 24 h of incubation, oxygen flow was stopped, and fish were euthanised (1000 mg L^{-1} MS-222 buffered with sodium bicarbonate, Sigma, Madrid, Spain) inside the chambers. Fish were then taken for sampling. After fish removal, incubation chambers were immediately resealed, and acidification of incubation water was conducted gradually, resulting in the diffusion of any remaining $^{14}CO_2$ from the water into the CO_2 traps. Samples from the incubation chambers (considered to contain ^{14}C resulting from fish evacuation) and from the CO_2 traps were collected for radioactivity counting. Fish liver and viscera were carefully sampled, and the rest of the fish was considered to be the fish body.

Fish samples were completely dissolved in Solvable^{TM} (Perkin Elmer, Waltham, MA, USA) at 50 °C for 24 h. A scintillation cocktail (Ultima Gold XR^{TM}; Perkin Elmer, Waltham, MA, USA) was added to all samples (water, traps, and fish tissues), and disintegrations per minute (DPM) were determined in a TriCarb 2910TR low-activity Liquid Scintillation Analyser (Perkin Elmer, Waltham, MA, USA). All samples were corrected for quench and lumex. All the results were expressed as a percentage of the sum of the total DPM [51].

2.4. Chemical and Biochemical Analysis

Fish bodies, liver, and viscera from each tank were pooled together (n = 3 per treatment), and all samples (diets and fish tissues) were ground before analysis. The contents of moisture (105 °C for 24 h) and ash (combustion at 550 °C for 12 h) were determined in diets and fish bodies. Crude protein (N × 6.25) content was determined in freeze-dried-diet and fish-body samples using an elemental analyser (Elementar Vario EL III, Elementar, Stockport, UK). Crude fat was analysed by petroleum ether extraction using a Soxtherm Multistat/SX PC (Gerhardt, Königswinte, Germany) on freeze-dried-diet, fish-body, liver, and viscera samples.

Freeze-dried samples from experimental diets were analysed for total amino acid content after 48 h acid hydrolysis. Taurine content was analysed in freeze-dried-diet, fish-body, and liver samples. Amino acid and taurine analyses were performed according to the procedures described in Aragão [52].

Bile was collected from gallbladders using sterilised syringes. Due to the very low volumes of bile available, the bile from three fish per tank was pooled before analysis (n = 3 per treatment). Total bile-acid content in bile was analysed after acid hydrolysis (6 M HCl for 2 h) using a commercial kit (Trinity Biotech, Co. Wicklow, Ireland). Plasma triglycerides and cholesterol concentrations were analysed using commercial kits (Spinreact, St. Esteve de Bas, Spain) in individual fish plasma samples.

2.5. Histological Analyses

The fixed-liver samples were embedded in paraffin, and sections of 5 µm were obtained in a microtome Leica© RM-2155 (Leica, Vienna, Austria) and stained with hematoxylin-eosin for general histological observations. Mounted slides were scanned with a Hamamatsu NanoZoomer C13140-01 (Hamamatsu, Hamamatsu City, Japan), and images were visualised with the NDP.view 2 (Hamamatsu, Japan). Based on Ribeiro [53], liver-tissue integrity and hepatocyte vacuolisation (representing glycogen deposits and/or fat storage normally dissolved during the routine histological process) were compared among treatments.

2.6. Gene Expression

2.6.1. RNA Extraction and cDNA Synthesis

Total RNA from the fish liver was extracted using Tri reagent (Sigma, Madrid, Spain) following the manufacturer's specifications. RNA was purified using the High Pure RNA Isolation Kit (Roche, Basel, Switzerland) and treated with DNase I to avoid genomic DNA amplification during real-time PCR. Total RNA was quantified based on absorbance at 260 nm with a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and integrity was assessed by agarose gel electrophoresis. cDNA was synthesised from 500 ng of purified RNA using the M-MLV Reverse Transcriptase Kit (Invitrogen). Negative control reactions were run without the enzyme. For each sample, reverse transcription was performed in duplicate.

2.6.2. Real-Time PCR (RT-PCR)

Quantification of gene expression by RT-PCR was performed using the StepOnePlus™ Real-Time PCR system (Applied Biosystems, Waltham, MA, USA) with SsoFast EvaGreen chemistry (Bio-Rad, Hercules, CA, USA). Transcriptomic analysis was focused on the expression of target genes related to bile-acid and lipid metabolism. The transcripts analysed were: cytochrome P450 family 7 subfamily A member 1 (*cyp7a1*), solute carrier family 6 member 6 (*taut*), ATP-binding cassette subfamily C member 2 (*abcc2*), and ATP-binding cassette subfamily B member 11 (*abcb11*) for bile-acid metabolism; apolipoprotein A1 (*apoa1*), A4 (*apoa4*), and B (*apob100*), microsomal triacylglycerol transfer protein (*mtp*), very-low-density lipoprotein receptor (*vldlr*), and perilipin 2 (*plin2*) for lipid metabolism. Primers' sequences for glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) were taken from Infante [54], the ones for *taut* from Pinto [55], and the ones for *apoa1*, *mtp,* and *vldlr* from Borges [56]. The remaining primers were defined with the Beacon designer 7.9 software, and forward primers were designed so that they overlap with an intron. Information on specific primers (GenBank accession numbers, forward and reverse primer sequences, and annealing temperatures) is described in Table 3.

The efficiency of the PCR reaction assay for each gene was previously evaluated to assure it was close to 100%. For the *gapdh*, *abcc2*, *vldlr,* and *plin2* genes, five-point standard curves of a 5-fold dilution series (1:5–1:3125) of pooled cDNA were used for PCR efficiency calculation. In the case of the *cyp7a1*, *taut*, *abcb11*, *apoa1*, *apoa4*, *apob100,* and *mtp* genes, PCR efficiency was calculated using five-point standard curves of a 2-fold dilution series (1:10–1:160) of pooled cDNA. Minus reverse transcriptase controls were checked for every gene. Thermal cycling was initiated with the incubation at 95 °C for 30 s for hot-start polymerase activation. Forty-five cycles of PCR were performed, each consisting of heating at 95 °C for 5 s for denaturing and at a specific temperature depending on the primer pair used (see Table 3) for 10 s for annealing and extension. The specificity of the reactions was assessed by analysis of the melting curves with ramping rates of 0.5 °C every 10 s over a temperature range of 55 to 95 °C. Real-time PCR was performed in duplicates for each gene, and negative controls were run for each reaction. Ct values were determined using the baseline subtracted curve fit method using the StepOnePlus™ Real-Time PCR System Software (Applied Biosystems, Waltham, MA, USA) with a fluorescence threshold automatically set. Relative quantification of the target genes' transcripts was made following the

ΔΔCt method [57]. *Gadph* was tested for gene expression stability using RefFinder [58], and it was used as a housekeeping gene in the normalisation procedure. The mRNA expression of the target genes was compared among dietary treatments in reference to the expression level of *gadph* in fish fed the FM diet, which was arbitrarily assigned a value of 1. All data are presented after log2 transformation.

Table 3. Forward and reverse primers for real-time PCR.

Gene	Genbank/Unigene * Accession Number	Forward Primer (5′-3′)	Reverse Primer (5′-3′)	AT [1] (°C)
gapdh	AB291587	AGCCACCGTGTCGCCGACCT	AAAAGAGGAGATGGTGGGGGGTGGT	64
cyp7a1	416791 *	GCCTACAGTGCCAGAGAGAAC	GCGAAGCCCAAAGCAGTG	64
taut	HQ148721.1	CCGAAAGCTGTGTCCATGATG	CAATAGAGGTGATCTGTCCTTCCA	63
abbc2	XM_044046365.1	GCTTACATCCACGACTGCTTCCAA	ACATCCTGACTGACGCCTTCCTT	60
abcb11	29210 *	AAGCAGAACAACCAGCCATCAGG	CCACCACCATCATCAGCACATCTT	60
apoa1	FF283994	TTGAGGCTAATCGTGCCAAA	CCTGCGTGCTTGTCCTTGTA	62
apoa4	KP842775.1	AGGAACTCCAGCAGAACCTG	CCTGCGTGCTTGTCCTTGTA	60
apob100	14427 *	CCGCTGAGATGGAGAGATA	CTGGGTCATCTTGGAGAAGG	64
mtp	KC888960	TGGCACGTTACTGTGGACAT	CCAGGGCAGAGATGATTC	63
vldlr	AJ_879619.2	CTGTGTTTGAGGACCGAGTGTT	GACCTGCGTCTTCTTGCTCT	64
plin2	185823 *	CTGTCTGGTCCTTGTCTC	GCCTTGCTGAAGTTAGTG	56

gapdh: glyceraldehyde-3-phosphate dehydrogenase; *cyp7a1*: cytochrome P450 family 7 subfamily A member 1; *taut*: solute carrier family 6 member 6; *abcc2*: ATP-binding cassette subfamily C member 2; *abcb11*: ATP-binding cassette subfamily B member 11; *apoa1*: apolipoprotein A1; *apoa4*: apolipoprotein A4; *apob100*: apolipoprotein B; *mtp*: microsomal triacylglycerol transfer protein; *vldlr*: very-low-density lipoprotein receptor; *plin2*: perilipin 2. [1] AT: annealing temperature.

2.7. Statistical and Data Analysis

Data from the rearing trial were used to calculate key performance indicators—weight gain (WG, 1), daily voluntary feed intake (VFI, 2), feed conversion ratio (FCR, 3), and hepatosomatic (HSI, 4) and viscerosomatic (VSI, 5) indexes:

$$\text{WG (\%)} = 100 \times (\text{final fish weight} - \text{initial fish weight}) \times \text{initial fish weight}^{-1} \quad (1)$$

$$\text{VFI (\% day}^{-1}\text{)} = 100 \times \text{apparent feed intake} \times ((\text{initial fish weight} + \text{final fish weight})/2)^{-1} \times \text{days}^{-1} \quad (2)$$

$$\text{FCR} = \text{apparent feed intake} \times (\text{final fish weight} - \text{initial fish weight})^{-1} \quad (3)$$

$$\text{HSI (\%)} = 100 \times \text{liver weight} \times \text{fish weight}^{-1} \quad (4)$$

$$\text{VSI (\%)} = 100 \times \text{viscera weight} \times \text{fish weight}^{-1} \quad (5)$$

All data were expressed as means with standard deviation (SD). Before statistical analysis, data expressed as a percentage were transformed (arcsin square root [59]), and all data were checked for normality and homogeneity of variances. Significant differences among groups were assessed by one-way ANOVA when data fulfilled the assumptions for analysis. A Kruskal–Wallis test was performed if data failed the assumptions of normality and homogeneity of variances. When statistically significant variations were found ($p < 0.05$), pairwise comparisons of means were performed with Tukey HSD tests or with Bonferroni corrections, respectively. The control group was set as the FM treatment for the relative quantification of gene expression. Regression analyses between the content of taurine in diets and the fish liver, body, or bile-acid concentration were performed using a linear model. All statistical analyses were performed in IBM SPSS Statistics version 27 (Armonk, NY, USA).

Gene expression data were subjected to a principal component analysis (PCA) to verify differences between fish fed the distinct diet formulations and find potential clusters of observations. The standard *prcomp* R function in the auto-scaled matrices was used for PCA, and score plots were produced for the two first principal components (PC1 and

PC2) using the *ggbiplot* and *factoextra* packages for R. Confidence ellipses were included, representing 95% confidence intervals around the centroid value of each data cluster. The function *fviz_cos2* was used to visualise the quality of representation (cos2) of the variables in the PCs. PCA analyses were carried out using the open-source software R version 4.2.1 (R Core team, Vienna, Austria).

3. Results

Diet analysis revealed that taurine content (Table 1) in the PP0 diet (0.08%) was five-times lower than in the FM diet (0.40%). Taurine contents were similar in FM and PP0.5 (0.43%) diets, while the PP1.5 diet (1.40%) presented 3.5-times more taurine than the FM diet.

At the end of the short-term rearing trial, fish fed the FM diet doubled their initial weight (Table 4). The ANOVA results indicate that the final weight of fish presents significant differences among dietary treatments ($p = 0.046$); however, post hoc tests were unable to identify those differences. Furthermore, the weight gain of fish fed the PP0 and the PP0.5 diets was significantly inferior ($p = 0.002$) to that of fish fed the FM diet. However, when fish were fed the PP1.5 diet, the weight gain was significantly higher than that of fish from the PP0 and PP0.5 treatments and not significantly different from fish fed the FM diet. Voluntary feed intake, FCR, and survival were similar among the dietary treatments ($p > 0.05$; Table 4).

Table 4. Growth, feed utilisation, and survival of Senegalese sole juveniles fed a fishmeal (FM), a plant-based (PP0), or a PP diet supplemented with 0.5% (PP0.5) or 1.5% (PP1.5) of taurine.

Dietary Treatments	FM	PP0	PP0.5	PP1.5
IBW (g) [1]	13.7 ± 5.0	13.7 ± 5.7	13.6 ± 4.6	13.6 ± 4.9
FBW (g) [2]	27.6 ± 6.8	24.2 ± 7.0	24.2 ± 5.3	27.1 ± 7.3
WG (%) [3]	102.6 ± 10.9 [a]	77.2 ± 4.8 [b]	77.5 ± 4.5 [b]	98.9 ± 5.2 [a]
VFI (% day^{-1}) [4]	1.2 ± 0.01	1.2 ± 0.04	1.2 ± 0.03	1.2 ± 0.01
FCR [5]	1.7 ± 0.2	2.3 ± 0.4	1.7 ± 0.3	2.0 ± 0.1
Survival (%)	83.8 ± 7.2	85.7 ± 7.6	83.8 ± 3.3	75.2 ± 5.9

Values are means ± standard deviation ($n = 3$, except for fish weight). Different superscripts within the same row indicate significant differences ($p < 0.05$) among dietary treatments. The absence of superscripts indicates no significant differences (except for final body weight, see text). [1] Initial body weight ($n = 35$), [2] final body weight ($n = 36$), [3] weight gain, [4] daily voluntary feed intake, [5] feed conversion ratio.

Fish somatic indexes (Figure 1) were significantly affected by the dietary protein source. In fish fed plant-based diets, both HSI (Figure 1a, $p < 0.001$) and VSI (Figure 1b, $p < 0.001$) were significantly higher than in fish fed the FM diet, irrespective of taurine supplementation.

Ash content in fish bodies (Table 5) significantly increased in fish fed the PP0 diet compared with FM-fed fish ($p = 0.014$), while intermediate values were found for fish fed the taurine-supplemented diets (PP0.5 and PP1.5). No significant differences ($p > 0.05$) in dry matter and protein contents were observed among dietary treatments. Fat content in fish bodies decreased significantly ($p < 0.001$) in fish fed all the plant-based diets (PP0, PP0.5, PP1.5) compared with FM-fed fish (Table 5). A significant increase ($p < 0.001$) in hepatic fat content was observed in fish fed the PP0 diet compared with FM-fed fish (Figure 2a). Dietary taurine supplementation decreased hepatic fat content, leading to results not significantly different between fish fed the PP1.5 and the FM diets (Figure 2a). As for viscera, an increase in fat content was observed in fish fed the plant-based diets ($p = 0.009$), although results were not significantly different between fish fed the PP0.5 and the FM diets (Figure 2b). Histological analysis revealed no significant differences that could be attributed to diet (Figure 3). Hepatocytes exhibited a polygonal-like shape, disposed along sinusoids in a manner that was similar among dietary treatments. The hepatocyte nucleus was displaced laterally among dietary treatments because of the storage area

(vacuolisation-like area), but the variability observed within each treatment was higher than among treatments.

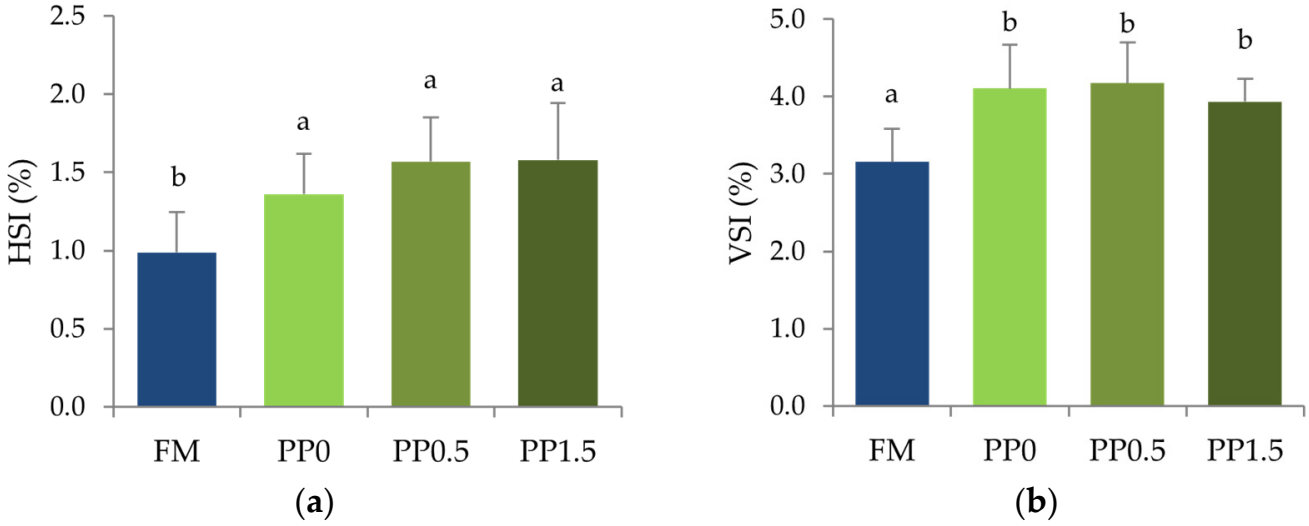

Figure 1. Hepatosomatic (**a—HSI**) and viscerosomatic (**b—VSI**) indexes of Senegalese sole juveniles fed a fishmeal (FM), a plant-based (PP0), or a PP diet supplemented with 0.5% (PP0.5) or 1.5% (PP1.5) of taurine. Values are means + standard deviation (n = 6). Different letters indicate significant differences ($p < 0.05$) among dietary treatments.

Table 5. Body proximate composition of Senegalese sole juveniles fed a fishmeal (FM), a plant-based (PP0), or a PP diet supplemented with 0.5% (PP0.5) or 1.5% (PP1.5) of taurine.

Dietary Treatments	FM	PP0	PP0.5	PP1.5
Dry matter (%)	74.8 ± 1.1	75.0 ± 0.4	75.5 ± 0.7	76.0 ± 1.3
Ash (% DM)	7.0 ± 0.2 ^{b}	9.3 ± 1.2 ^{a}	7.9 ± 0.4 ^{ab}	8.2 ± 0.6 ^{ab}
Protein (% DM)	67.2 ± 1.4	68.2 ± 0.7	69.3 ± 1.0	69.4 ± 0.7
Fat (% DM)	19.9 ± 1.9 ^{a}	16.3 ± 1.6 ^{b}	15.3 ± 0.7 ^{b}	13.7 ± 1.0 ^{b}

Values are means ± standard deviation (n = 3). Different superscripts within the same row indicate significant differences ($p < 0.05$) among dietary treatments. The absence of superscripts indicates no significant differences. DM = dry matter.

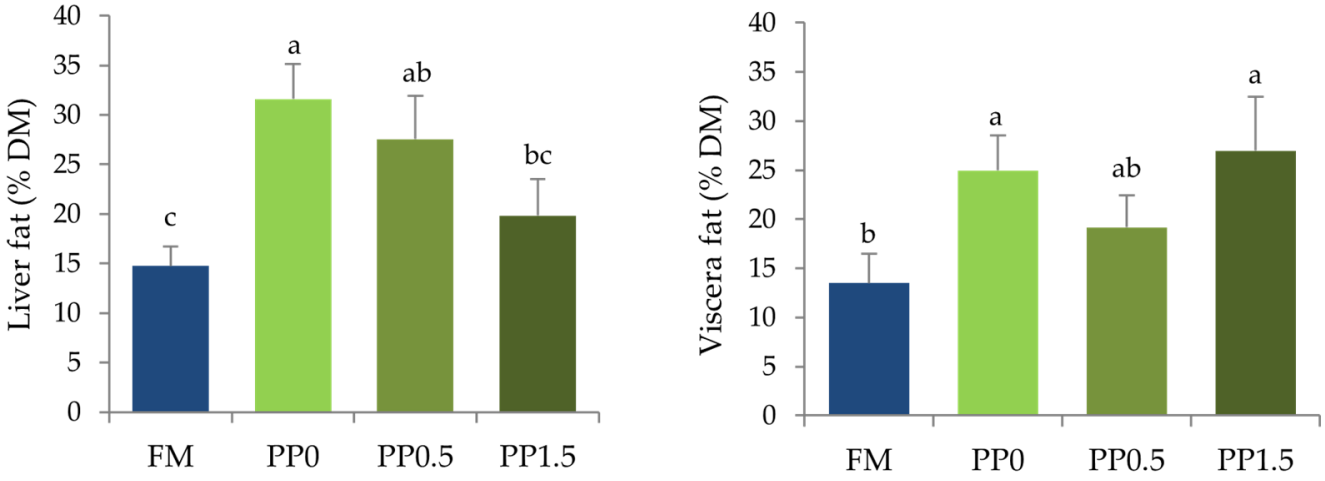

Figure 2. Fat content in the liver (**a**) and viscera (**b**) of Senegalese sole juveniles fed a fishmeal (FM), a plant-based (PP0), or a PP diet supplemented with 0.5% (PP0.5) or 1.5% (PP1.5) of taurine. Values are means + standard deviation (n = 3). Different letters indicate significant differences ($p < 0.05$) among dietary treatments. DM = dry matter.

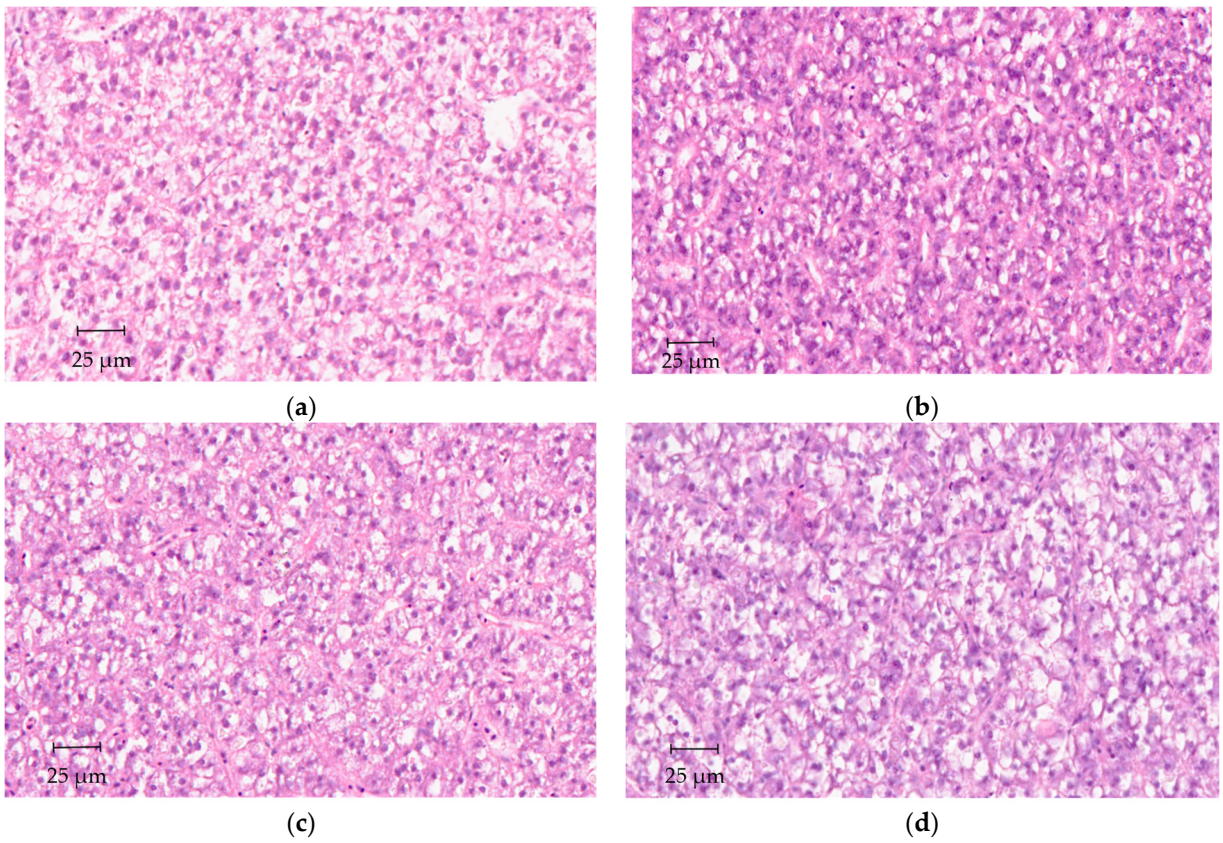

Figure 3. Histological sections (H&E staining) of the liver of Senegalese sole juveniles fed a fishmeal (**FM—a**), a plant-based (**PP—b**), or a PP diet supplemented with 0.5% (**PP0.5—c**) or 1.5% (**PP1.5—d**) of taurine.

Feeding fish with the PP diet significantly decreased ($p < 0.05$) taurine contents in fish liver and body (Figure 4). Dietary taurine supplementation linearly increased taurine contents in both fish liver and body, as confirmed by regression analysis ($p < 0.001$, R^2 = 0.975 and 0.984, respectively). The same pattern was found for bile-acid concentration

in fish bile (Figure 5), and this increased linearly with the dietary taurine concentration, as confirmed by regression analysis ($p < 0.001$; $R^2 = 0.973$).

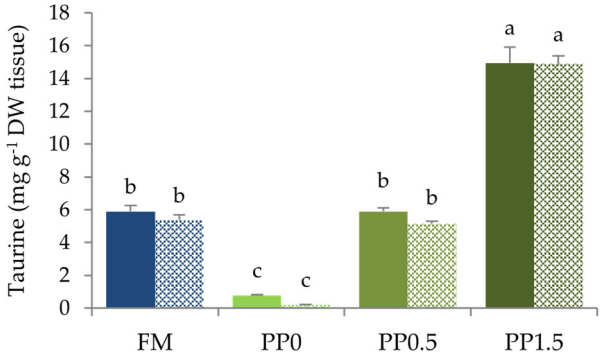

Figure 4. Taurine content in the liver (filled bars) and body (shaded bars) of Senegalese sole juveniles fed a fishmeal (FM), a plant-based (PP0), or a PP diet supplemented with 0.5% (PP0.5) or 1.5% (PP1.5) of taurine. Values are means + standard deviation ($n = 3$). Different letters indicate significant differences ($p < 0.05$) among dietary treatments. DM = dry matter.

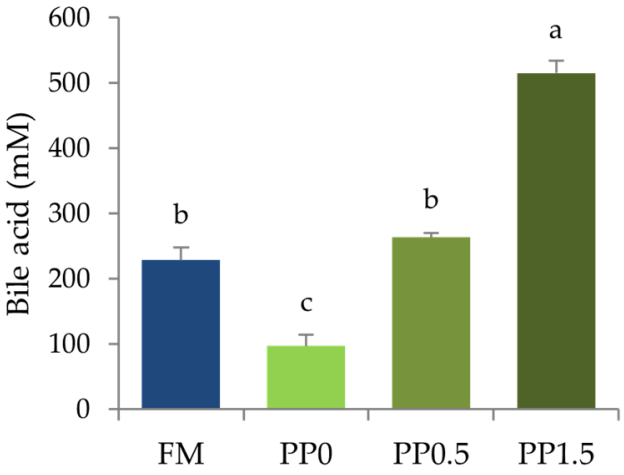

Figure 5. Bile-acid concentration in the bile of Senegalese sole juveniles fed a fishmeal (FM), a plant-based (PP0), or a PP diet supplemented with 0.5% (PP0.5) or 1.5% (PP1.5) of taurine. Values are means + standard deviation ($n = 3$). Different letters indicate significant differences ($p < 0.05$) among dietary treatments.

Triglycerides levels in plasma were significantly higher ($p = 0.004$) in fish fed the PP0 diet compared with FM-fed fish (Figure 6a). A decrease in plasma triglyceride concentration with an increase in dietary taurine supplementation was observed, thus resulting in no significant differences between fish fed the PP1.5 and the FM diets (Figure 6a). Total cholesterol levels in plasma were not significantly affected ($p = 0.853$) by the dietary treatments (Figure 6b).

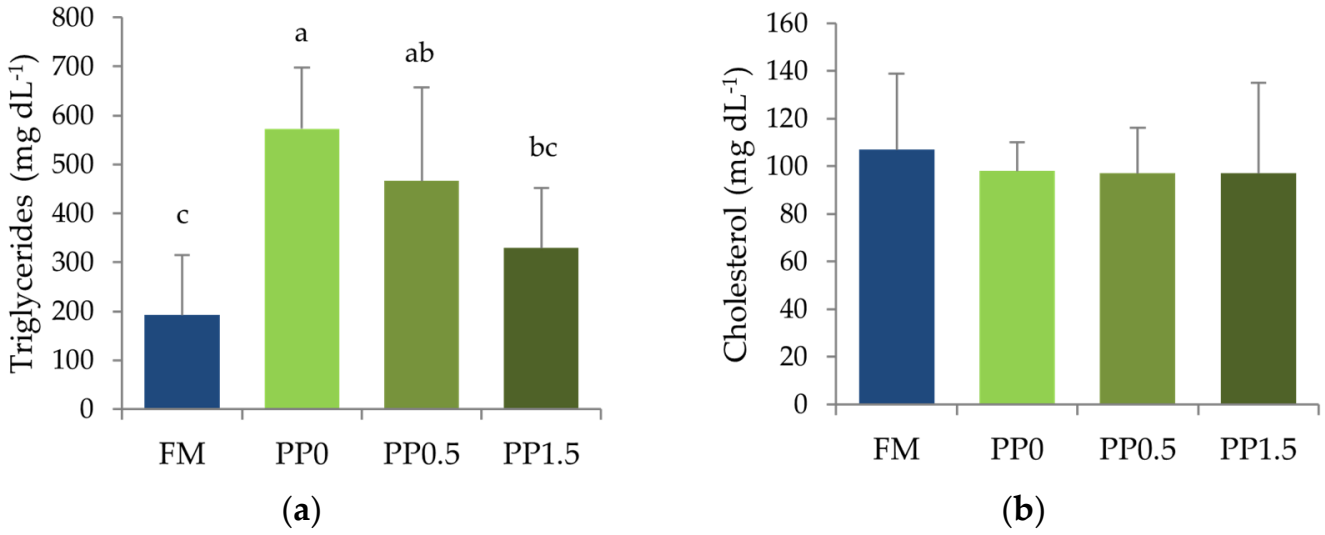

Figure 6. Triglycerides (**a**) and total cholesterol (**b**) concentration in plasma of Senegalese sole juveniles fed a fishmeal (FM), a plant-based (PP), or a PP diet supplemented with 0.5% (PP0.5) or 1.5% (PP1.5) of taurine. Values are means + standard deviation (n = 9). Different letters indicate significant differences ($p < 0.05$) among dietary treatments. The absence of letters indicates no significant differences.

Fish from the PP0 treatment displayed a downregulation of genes involved in bile-acid metabolism, such as *cyp7a1* and *abcb11*, compared to FM-fed fish (Figure 7a). The mRNA expression levels of these genes significantly increased ($p > 0.05$) in fish fed the PP0.5 diet when compared with animals from the PP0 treatment but were still significantly different from the levels found in the FM treatment. At the highest dose of taurine tested (PP1.5), the expression of *cyp7a1* and *abcb11* presented values not significantly different from those found either in fish fed the PP0 or the PP0.5 diet. Concerning the genes involved in lipid metabolism (Figure 7b), a downregulation of *vldlr* was found in fish fed the PP0 diet compared with the FM-fed fish ($p = 0.001$). mRNA expression levels of *vldlr* significantly increased in fish fed the PP diets supplemented with taurine compared with nonsupplemented ones, and this result was not significantly different from the FM treatment. The mRNA expression levels of the other genes analysed were not significantly different ($p > 0.05$) among treatments.

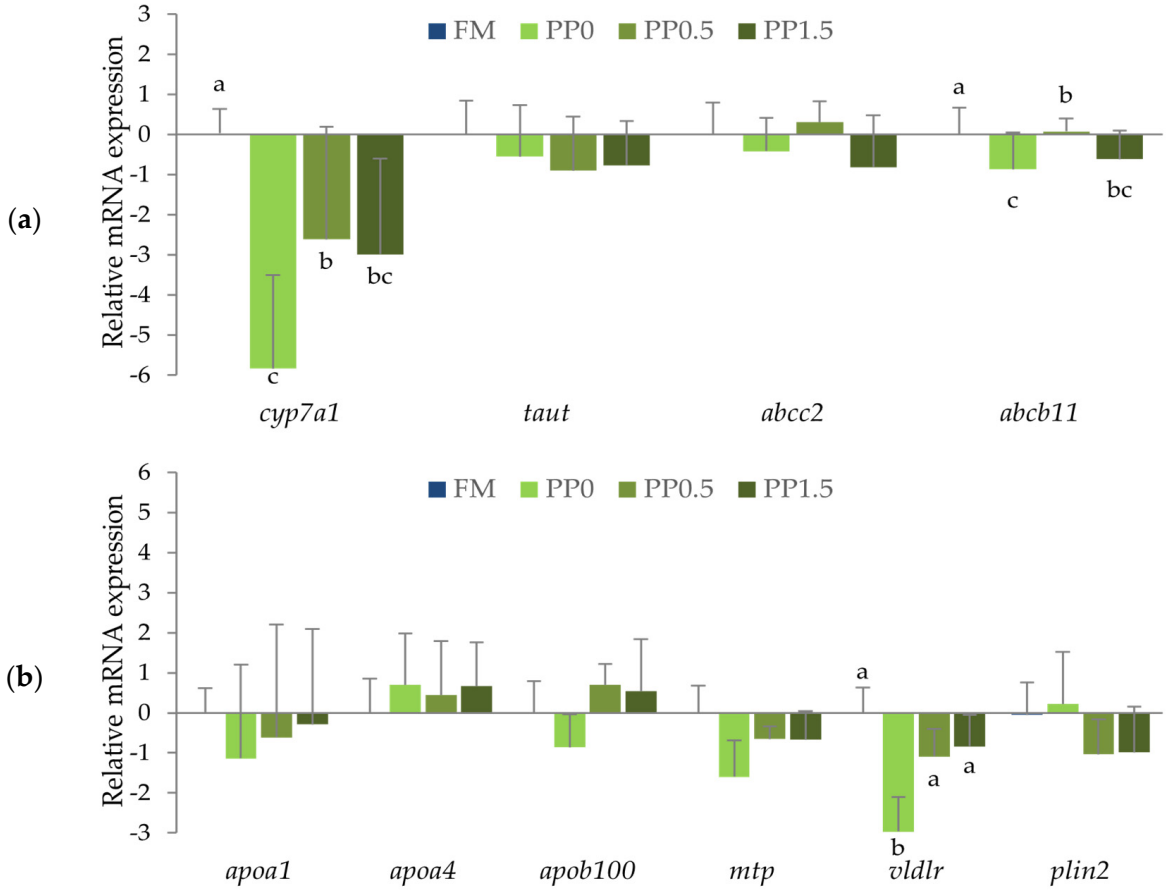

Figure 7. Relative mRNA expression of genes involved in bile-acid metabolism (**a**): *cyp7a1* (cytochrome P450 family 7 subfamily A member 1), *taut* (solute carrier family 6 member 6), *abcc2* (ATP-binding cassette subfamily C member 2), and *abcb11* (ATP-binding cassette subfamily B member 11); or in lipid metabolism (**b**): *apoa1* (apolipoprotein A1), *apoa4* (apolipoprotein A4), *apob100* (apolipoprotein B100), *mtp* (microsomal triacylglycerol transfer protein), *vldlr* (very-low-density lipoprotein receptor), and *plin2* (perilipin 2) of Senegalese sole juveniles fed a fishmeal (FM), a plant-based (PP0), or a PP diet supplemented with 0.5% (PP0.5) or 1.5% (PP1.5) of taurine. Values are means + standard deviation (n = 7–9). Different letters indicate significant differences ($p < 0.05$) among dietary treatments. The absence of letters indicates no significant differences.

Principal component analysis (PCA) was used to reduce the complexity of the data from gene expression analysis (Figure 8). Relative mRNA expression of the target genes allowed the differentiation of FM- and PP0-fed fish as two distinct clusters along the PC1 axis. Conversely, it was observed that in the taurine-supplemented treatments (PP0.5 and PP1.5), the clusters were not so distinct and intermingled among the others. The differential expression of the *vldlr* and *abcb11* genes were the most responsible for the obtained dissimilarities, as observed by the darker colour in the cos2 scale.

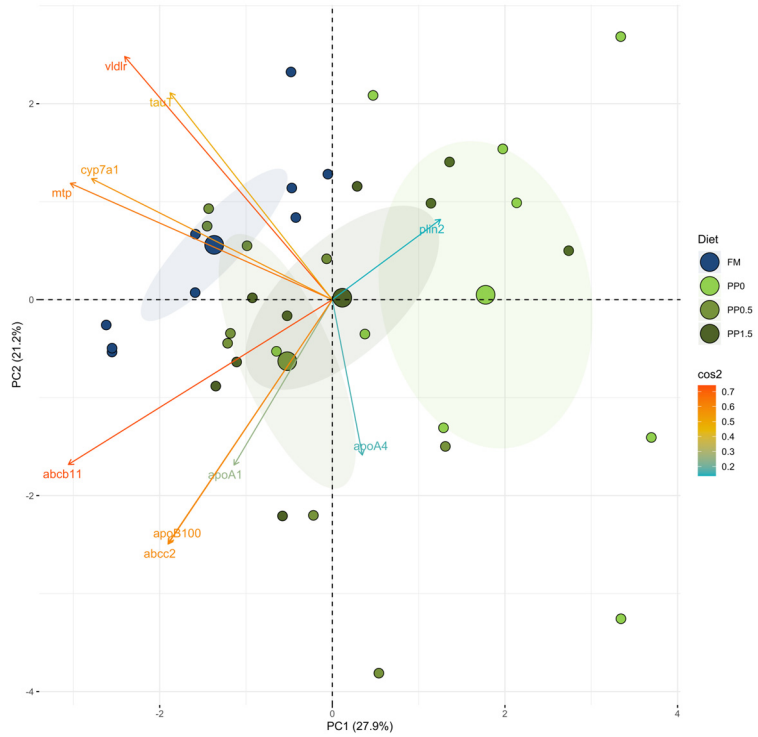

Figure 8. Principal component analysis (PCA) of the relative mRNA expression data in the liver of Senegalese sole juveniles fed the different experimental diets (FM, PP, PP0.5, and PP1.5). Each point represents the projection of an individual sample in the PC1 and PC2 axes. The ellipses represent 95% confidence intervals around the centroid (larger point) of each data cluster.

The results from the metabolic trials showed that a significant part (21 ± 12%) of the ^{14}C-triolein was not absorbed by the Senegalese sole and was evacuated (Figure 9a) without a significant impact of the dietary treatments ($p = 0.757$). Catabolism of ^{14}C-triolein was significantly reduced ($p = 0.045$) in fish fed the PP0 diet compared with FM-fed fish (Figure 9b), while taurine-supplemented treatments presented intermediate values. Hepatic ^{14}C-triolein retention was significantly higher ($p = 0.011$) in the PP1.5 treatment compared with the other treatments (Figure 9c). The retention of ^{14}C-triolein in fish viscera (Figure 9d) and body (Figure 9e) was not significantly affected ($p > 0.05$) by the dietary treatments, and the results indicate that the major proportion (53 ± 16%) of ^{14}C-triolein was retained in the fish viscera (Figure 9d).

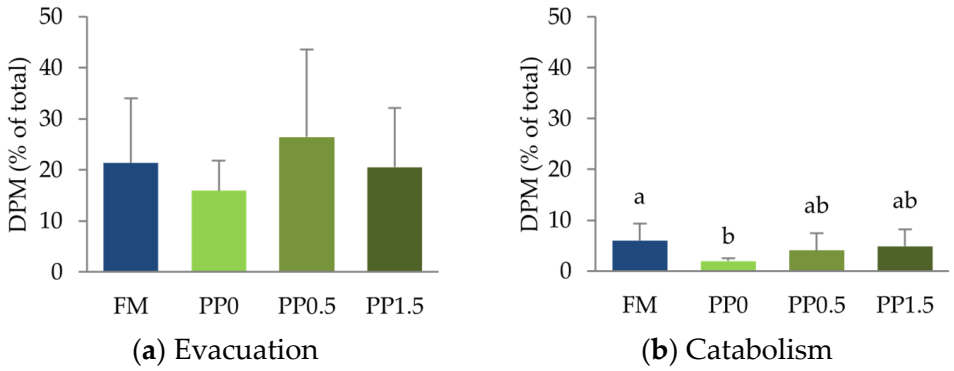

Figure 9. *Cont.*

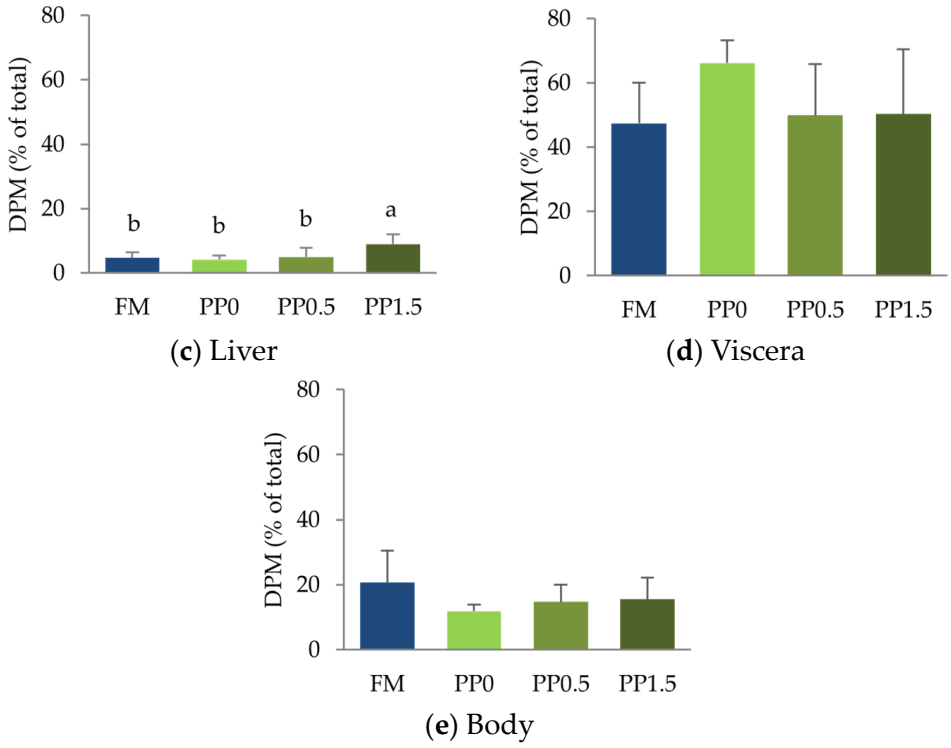

Figure 9. ^{14}C-Triolein evacuation (**a**), catabolism (**b**), and retention in the liver (**c**), viscera (**d**), and body (**e**) of Senegalese sole juveniles fed a fishmeal (FM), a plant-based (PP0), or a PP diet supplemented with 0.5% (PP0.5) or 1.5% (PP1.5) of taurine. Values are means + standard deviation (n = 6). Different letters indicate significant differences ($p < 0.05$) among dietary treatments. The absence of letters indicates no significant differences. DPM = disintegrations per minute.

4. Discussion

In contemporary aquafeed formulations, the amounts of fishmeal are being constantly reduced, and the inclusion of plant-protein sources is now a standard procedure. Given the important physiological role of taurine and its minimal content in high-plant diets, the importance of testing the effects of its dietary supplementation for marine carnivorous fish seems fundamental. The current study was a short-term feeding trial to analyse the effects of taurine supplementation to plant-based diets on the metabolism and physiological responses of Senegalese sole.

Dietary taurine levels analysed went according to what was expected, as a clear reduction in taurine levels was found in the PP0 diet compared with the FM diet. However, by reducing the dietary fishmeal and increasing the plant-protein sources, some changes in the dietary amino acid profile were observed. Since taurine is the end product of sulphur amino acid metabolism, dietary methionine and cysteine levels may present interactive effects with taurine. However, it should be emphasised that the diets were formulated to fulfil the known indispensable amino acid requirements of the species. Published works indicate no interaction between dietary taurine and methionine levels on meagre performance and metabolism [36,60], while a possible sparing effect of taurine on methionine requirements has also been described [16,61]. Though taurine is virtually absent in terrestrial plants, the residual content found in the PP diet is the result of the inclusion of marine ingredients known to be rich in taurine, such as fishmeal, fish soluble protein concentrate, and even squid meal [62]. Taurine supplementation to plant-based diets resulted as planned, since the PP0.5 diet presented taurine levels similar to the FM diet, while in the PP1.5 diet, taurine content was within the range found in polychaetes [63], which Senegalese sole feed on in the wild [47]. The above-mentioned marine ingredients were included at low levels in the plant-based diets to act as feed attractants and ensure good diet acceptability. This objective was attained since no significant differences were

found in voluntary feed intake among treatments. Although taurine is considered a feeding attractant, the low inclusion levels of marine ingredients in the plant diets may explain the absence of effects of taurine supplementation on fish-feed intake, contrary to other studies that reported its increase in fish fed plant-based diets supplemented with taurine [15].

Even though the short duration of the trial does not allow for robust considerations on the effects of dietary taurine supplementation on growth performance, these results seem pertinent, as it is possible to verify that the fish were in good rearing conditions and doubled their weight when fed the FM diet for 6 weeks. Taking into consideration the limitations previously acknowledged, it is possible to describe a negative effect of the high dietary replacement of fishmeal by plant-protein sources on the growth performance of Senegalese sole. This negative effect should be analysed with caution due to the short-term nature of the trial since previous studies have shown a good acceptance of high-plant diets [39–43]. It should be noted that in this study, 85% of marine ingredients were replaced by plant ingredients, resulting in a low inclusion level of the former (9% of fishmeal, fish soluble protein concentrate, and squid meal) compared with the previous studies (min. 15% of marine ingredients). The negative effects of the PP0 diet on growth performance were at least partially mitigated through taurine supplementation. In similar short-term trials with sturgeon, taurine inclusion in high-plant diets increased growth performance, but only at low levels, resulting in negative effects on growth performance when included at supraoptimal levels [30,31]. Interestingly, with Senegalese sole, growth performance in the PP0.5 treatment presented intermediary values between fish fed the FM and the PP0 diets, and only at the highest dietary taurine supplementation (PP1.5) was the fish growth performance similar to the FM treatment. This does not seem to be related to increasing feed palatability, as mentioned before, since no effects of taurine supplementation were observed in the fish voluntary feed intake. Previous studies with Japanese flounder (*Paralichthys olivaceus*) and turbot (*Scophthalmus maximus*) showed that the optimal dietary taurine level decreased as fish grew [64,65]. This suggests that in high-plant diets for young Senegalese sole, taurine supplementation should be above the levels found in commercial fishmeal-based diets.

The improved fish growth performance as a result of dietary taurine supplementation is probably the reflection of the several effects observed on fish physiology and metabolism. The dietary taurine content was strongly correlated with the taurine levels in tissues (liver and body), as previously observed in other fish species, such as Japanese flounder, turbot, cobia (*Rachycentron canadum*), white grouper (*Epinephelus aeneus*), red seabream, Nile tilapia, and African catfish, irrespectively of the main dietary protein source [12,16,24,25,37,64,65]. The increase in taurine levels in the fish muscle (which comprises the major portion of the fish body) may represent a potential health benefit for consumers, as suggested by Watson [37], providing an additional source of a nutrient known for its hypolipidemic and antiatherogenic effects in humans [33].

The different hepatic taurine contents had no effect on the HSI, as previously observed in other fish species fed low fishmeal diets [8,25,27,60,66]. Additionally, the somatic indexes were only affected by the dietary protein source, similar to other works that found an increased HSI and VSI in Senegalese sole fed high-plant diets [42,43]. No histological alterations in hepatocytes were observed in the current study, contrary to the increased hepatic vacuolisation and necrosis found previously in Senegalese sole fed high-plant diets [43]. This might be related to the low dietary fat levels used in this study (~8%), since previous works indicated moderate hepatic steatosis and cellular necrosis when Senegalese sole were fed fishmeal-based diets with 15% of crude fat, but not with 8% [43]. The dietary protein source also affected fat deposition in fish tissues. As in other studies with Senegalese sole fed high-plant diets [42] and also with black carp [13], body fat levels were lower in PP-fed fish compared with FM-fed fish, and no effects of taurine were observed. Senegalese sole is a lean fish, presenting low fat content in muscle, with the liver and viscera functioning as important fat storers [44]. The results from the metabolic trial corroborate that triolein retention occurred mostly in the viscera, irrespective of the dietary protein

source. Furthermore, lipid content in the liver and viscera increased in fish fed the plant-based diets, which is in line with the observed increase in somatic indexes. Previously, it has been suggested that this increase seems to be due to augmented triglyceride storage [43], and metabolic trials have demonstrated a lower capacity of PP0-fed fish to catabolise triolein, although it was further mitigated by taurine supplementation.

A similar metabolic trial with ^{14}C-triolein was previously performed with Senegalese sole juveniles [46]. Interestingly, while in the current study dietary taurine supplementation to plant-based diets increased triolein catabolism, in the previous study, triolein absorption augmented concomitantly with increasing dietary taurine levels, without effects on catabolism or retention. These differences may be related to the timeframe of exposure to the experimental diets, since in the previous study Senegalese sole were fed the experimental diets only for five days and after a period of taurine deprivation. The short-time habituation to the diets resulted in evacuation of almost 60% of ^{14}C-triolein in fish fed a high-plant-based diet without taurine supplementation. This probably impaired the further metabolism of triolein, since in the present study triolein evacuation was only 20% for all dietary treatments. These effects of taurine on lipid digestion and metabolism seem to be linked with the impacts on bile-acid metabolism. Bile-acid concentration in Senegalese sole's bile was linearly related to dietary taurine content. Thus, the replacement of fishmeal by plant proteins led to a significant reduction in bile-acid content, which was also observed in white seabream (*Diplodus sargus*) [20]. However, as in other studies using low-fishmeal diets [20,25,28,36], taurine supplementation to the plant-based diet led to a significant increase in bile-acid content. Bile acids are synthesised from cholesterol and conjugated with taurine to form bile salts, and the rate-limiting enzyme is *cyp7a1* [5], which was downregulated in fish fed the PP diet and significantly increased with taurine supplementation, as previously observed in black seabream (*Acanthopagrus schlegelii*) [26]. As cholesterol circulating levels were not affected by the dietary treatments while taurine concentrations were linearly affected by the dietary taurine content, bile-acid synthesis in the current study seems to be limited by taurine availability. Similar results were observed in white seabream fed low-fishmeal diets, in which the increased taurine supplementation led to a higher concentration of bile salts, with concomitant lower levels of plasma triglyceride but without effects on cholesterol [20]. Although a relation between hepatic triglyceride accumulation and increased fat content seems to be supported by the current results, at this point, a possible hepatic accumulation of cholesterol in fish fed the PP0 diet cannot be excluded, as a result of the downregulation of *cyp7a1* and, to some extent, of *vldlr*. The increase in hepatic cholesterol when lowering fishmeal contents in diets for yellowtail was previously observed, and this effect was reverted by taurine supplementation [67].

The expression levels of *abcc2* and especially of *abcb11* (which encode for the transporter proteins that actively transport secreted molecules into bile [6]) followed a similar pattern to that of *cyp7a1*, indicating an increased bile-acid secretion in fish fed PP diets supplemented with taurine in comparison to nonsupplemented ones. Disturbances in bile-acid excretion were previously related to the appearance of green liver [22,23,32]. In this study, hepatic appearance was not affected by dietary treatments, but the possible effects of long-term feeding should not be disregarded, since, as previously mentioned, Senegalese sole reared to the market size with high-plant diets presented histopathological signs [43]. The hepatic *taut* mRNA expression levels were not affected, which is similar to what was observed in cobia [37]. In the latter, despite an increase in taurine contents in the fillet and liver with increasing dietary taurine levels, hepatic *taut* transcript levels did not vary. The authors suggested that the transporter may be upregulated in other tissues to facilitate the recycling of taurine at the lower levels of dietary input, which can also be a possibility in the current study. For instance, it was previously identified that *taut* in the Senegalese sole intestine was more expressed in the hindgut, suggesting an enterohepatic recycling pathway for maintaining taurine levels in the body [55], with implications for bile-salt synthesis.

The gallbladder bile-acid content was inversely related to hepatic (and to some extent to body) fat accumulation and to the plasma triglycerides levels. In line with this, the metabolic trials showed a decrease in triolein catabolism in the PP treatment, which was partially reverted through taurine supplementation. Surprisingly, hepatic triolein retention was higher in PP1.5 treatment, but this seems to be a transient effect, as the hepatic fat content was significantly lower than in PP0-fed fish. This suggests a better utilisation of triglycerides in the presence of increased levels of taurine and/or bile acids. The effects of taurine on circulating triglycerides levels were not so evident in other fish species, even if comparing only with studies using low-fishmeal diets, with no effects observed in European seabass (*Dicentrarchus labrax*) [28,29], increased plasma levels found in meagre (*Argyrosomus regius*) and African catfish [12,36], but, like in this study, decreased levels found in white seabream, black carp, and yellow catfish [13,14,20]. The expression levels of *vldlr* and up to some level of *mtp* genes indicate a disruption in lipid transport in fish fed the PP diet, which was at least partially reverted by taurine supplementation. The results of the metabolic trials indicate that lipid digestion was not affected by the different bile-acid content, but these seem to lead to better lipid utilisation. The hypolipidemic effects of taurine found in mammals [33] and in some fish species [2] were thus also found in Senegalese sole.

The effects of taurine supplementation to plant-based diets on the mRNA expression levels of the genes involved in bile-acid and lipid metabolism were mostly mild, but the PCA analysis of these data clearly demonstrated a high separation between clusters from fish fed the FM and the PP0 diets. Even more interesting is that the clusters of fish fed taurine-supplemented diets were located in between. Therefore, the increased taurine concentrations in Senegalese sole tissues with a concomitant increase in bile-acid concentrations led to impacts on bile-acid and lipid metabolism that may explain the positive effects of taurine supplementation to plant-based diets. This is in line with the previous discussion indicating that taurine supplementation partially reverted the negative effects of plant-protein-based diets and explains the potential beneficial effects on fish growth performance.

In this study, low-lipid diets were used, since it was previously established that Senegalese sole does not tolerate high dietary lipid levels [44]. Given the positive impact of dietary taurine on lipid metabolism, further studies looking at the effects of dietary taurine supplementation on high-lipid diets for Senegalese sole seem to be worthy of investigation.

5. Conclusions

This study showed that dietary inclusion of high levels of plant ingredients to replace fishmeal and other marine ingredients resulted in disturbances in the lipid metabolism of Senegalese sole, due to the resultant low bile-acid concentration and/or the limited availability of taurine for bile-salt emulsification. On the opposite side, increasing levels of taurine in plant-based diets resulted in a higher accumulation of taurine in tissues (liver and body) and in increasing bile-acid concentration. This was associated with an upregulation of *cyp7a1* and *abcb11*, indicating an increase in bile-acid production and secretion, respectively. Thus, taurine supplementation mitigated part of the negative effects of plant-based diets, leading to better lipid utilisation. Additionally, although taurine supplementation in plant-based diets had a positive effect on growth performance, this seems to be necessary at levels that go beyond those found in commercial diets based on fishmeal. These positive effects may eventually have a higher impact on growth performance and physiological condition of Senegalese sole in long-term trials.

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Institutional Review Board Statement: The experiment was carried out in compliance with European laws (2010/63/EU) and Portuguese legislation for the use of laboratory animals (DL no.113/2013, 7th of August). This study was approved by the ORBEA Animal Welfare Committee of the Centre of Marine Sciences of Algarve (CCMAR), was performed by trained scientists, and followed the European Directive 2010/63/EU of the European Union Council on the protection of animals used for scientific purposes under authorisation reference number 0421/000/000/2016. CCMAR facilities and their staff are certified to house and conduct experiments with live animals (license by the 'Direção Geral de Veterinária', Ministry of Agriculture, Rural Development and Fisheries of Portugal).

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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animals

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Article

Health-Promoting Additives Supplemented in Inert Microdiets for Whiteleg Shrimp (*Penaeus vannamei*) Post-Larvae: Effects on Growth, Survival, and Health Status

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Simple Summary: The whiteleg shrimp (*Penaeus vannamei*) is currently the most produced species in aquaculture. However, the species larviculture is frequently associated with sub-optimal growth, high size dispersion, and low survival due to cannibalism and reduced disease resistance to pathogens. There is evidence that dietary additives can stimulate the shrimp immune system, but few studies have focused on the initial developmental stages. Therefore, this study aimed to evaluate the potential beneficial effects of several nutrients/additives (i.e., vitamins C and E, *β*-glucans, taurine, and methionine) supplemented in microdiets for whiteleg shrimp post larvae. The additives tested had no effect on growth performance and survival, but results suggest that vitamins C and E and *β*-glucans may impact the shrimp post-larvae antioxidant capacity and robustness, especially when coupled together. These findings suggest that tailored diets including these health-promoting additives may address some of the larviculture problems and may contribute to the success of whiteleg shrimp farming in the long term, affecting the downstream production of high-quality juveniles and adults.

Abstract: Dietary additives have the potential to stimulate the whiteleg shrimp immune system, but information is scarce on their use in diets for larval/post-larval stages. The potential beneficial effects of vitamins C and E, *β*-glucans, taurine, and methionine were evaluated. Four experimental microdiets were tested: a positive control diet (PC); the PC with decreased levels of vitamin C and E as negative control (NC); the PC with increased taurine and methionine levels (T + M); and the PC supplemented with *β*-glucans (BG). No changes in growth performance and survival were observed. However, post-larvae shrimp fed the NC had lower relative expressions of *pen-3* than those fed the PC, suggesting that lower levels of vitamins C and E may impact the shrimp immune status. Lipid peroxidation levels dropped significantly in the BG compared to the PC, indicating that *β*-glucans improved the post-larvae antioxidant mechanisms. Furthermore, when compared with the NC diet, PL fed with BG showed significant increases in tGSH levels and in the relative expression of *crus* and *pen-3*, suggesting a synergistic effect between vitamins C and E and *β*-glucans. Amongst the additives tested, *β*-glucans seems to be the most promising even when compared to a high-quality control diet.

Keywords: *Penaeus vannamei*; whiteleg shrimp; post larvae; microdiets; dietary additives; immunostimulants; antioxidants; health status

1. Introduction

The whiteleg shrimp (*Penaeus vannamei*) is currently the most representative animal species in aquaculture, constituting in 2020 a share of 4.7% in global production [1]. To meet the market demands, whiteleg shrimp larvae and post-larvae (PL) yields in hatcheries has increased intensively. However, problems in larviculture can have an enormous impact on shrimp performance in the long-term, affecting the downstream production of high-quality juveniles and adults. Initial developmental stages are frequently associated with sub-optimal growth, high size dispersion, and low survival due to cannibalism and reduced disease resistance to opportunistic pathogens. The latter results from a high dependence on optimal zootechnical conditions and nutrition, as shrimp lack an adaptive immune response and depend uniquely on their innate immune system to maintain a good health status and avoid pathogenic outbreaks that can result in disastrous consequences to production and significant economic losses [2–4]. Besides the fact that shrimp cannot be vaccinated due to the lack of an adaptive immune system, the use of antibiotics in the aquaculture industry is limited due to inherent food safety concerns, environmental issues, and the increased antimicrobial resistance [5]. Consequently, the use of functional dietary additives to stimulate the shrimp immune system has been studied as a prophylactic alternative and is regarded as an extremely important strategy to overcome the constraints of intensive shrimp farming. Organic acids, plant/algae extracts, nucleotides, functional amino acids, vitamins, and naturally occurring immunostimulant compounds such as β-glucans have been studied thoroughly in diets for fish and crustaceans, as they may improve growth performance, survival, stress, and disease resistance, as recently reviewed by Dawood et al. [6]. Their potential to be included in diets for juvenile and/or adult whiteleg shrimp has also been demonstrated to some extent by several authors [7–18]. Nevertheless, far fewer studies are available on the use of these supplements in diets for whiteleg shrimp initial developmental stages. There is evidence that vitamin C supplemented in diets for PL can be an effective antioxidant at the tissue level [19]. More recently, increases in growth performance, digestive enzymes activity, and improvement of immune condition were verified when incorporating commercial prebiotic and probiotic blends in diets for PL [20–22].

Hence, innovative nutritional solutions that enhance development and resistance to stress and pathogenic factors during these critical stages and thus improve shrimp quality in posterior phases of production have tremendous potential to reinforce the success of shrimp farming. Therefore, this study aimed to evaluate the effects of several health promoting nutrients/additives (i.e., vitamins C and E, β-glucans, taurine, and methionine) supplemented in inert microdiets on the growth performance and health status of whiteleg shrimp post larvae.

2. Materials and Methods

2.1. Dietary Treatments

Four experimental microdiets were evaluated in triplicates. A positive control diet (PC) was formulated to meet the nutritional requirements of whiteleg shrimp post larvae, containing 515 g kg^{-1} of SPAROS proprietary marine protein mix, 160 g kg^{-1} of SPAROS proprietary plant protein mix, 103 g kg^{-1} of fish protein hydrolysate, 19 g kg^{-1} of fish oil, 28 g kg^{-1} of marine phospholipids, and 57 g kg^{-1} of SPAROS proprietary vitamins and minerals premix. On the remaining treatments, three experimental variants based on the PC were used, differing only in the ingredient formulation by the following: (1) decreasing inclusion levels of the vitamins and minerals premix by 7 g kg^{-1} to reduce vitamin C and E contents in the negative control diet (NC); (2) supplementing 5 g kg^{-1} of taurine and 10 g kg^{-1} of methionine to increase the levels of both molecules in the T + M diet; and (3) supplementing the PC diet with 1 g kg^{-1} of *Saccharomyces cerevisiae* β-(1, 3)/(1, 6)-glucans (BG). The experimental diets formulation can be seen in Table 1. The proximate composition of the experimental diets was analyzed by Eurofins Food Testing Portugal following their standard procedures (Table 2).

Table 1. Dietary formulation of the experimental diets used to culture whiteleg shrimp (*P. vannamei*) PL for 18 days.

Ingredients (g kg^{-1})	NC	PC	T + M	BG
Marine protein mix [1]	515	515	510	515
Fish protein hydrolysate [2]	103	103	103	103
Plant protein mix [3]	160	160	160	160
Cellulose [4]	17	10	0	9
Fish oil [5]	19	19	19	19
Marine phospholipids [6]	28	28	28	28
Lecithin [7]	56	56	56	56
Vitamins and minerals [8]	50	57	57	57
Cholesterol [9]	10	10	10	10
Antioxidant [10]	4	4	4	4
Monoammonium phosphate [11]	38	38	38	38
β-(1, 3)/(1, 6)-glucans [12]	0	0	0	1
DL-Methionine [13]	0	0	5	0
Taurine [14]	0	0	10	0

[1] Proprietary product for shrimp: 37% crude protein, 5% crude fat—SPAROS, Portugal
[2] Sopropêche, France
[3] Proprietary product for shrimp: 13% crude protein, 1% crude fat—SPAROS, Portugal
[4] Disproquimica, Portugal
[5] Sopropêche, France
[6] Triple nine, Denmark
[7] Lecico, Germany
[8] Proprietary premixes/products for shrimp—SPAROS, Portugal
[9] Carbogen, The Netherlands
[10] Kemin, Italy
[11] Timab Iberica, Spain
[12] MacroGard—Orffa, The Netherlands
[13] Premix—Especialidades Agricolas e Pecuárias Lda, Portugal
[14] Proprietary product for marine fish and shrimp—SPAROS, Portugal

Table 2. Proximate composition of the experimental diets used to culture whiteleg shrimp (*P. vannamei*) PL for 18 days.

	NC	PC	T + M	BG
Dry matter (DM, %)	94.0 ± 0.5	93.7 ± 0.5	94.3 ± 0.5	94.3 ± 0.5
Crude protein (% DM)	66.2 ± 1.7	66.4 ± 1.7	67.4 ± 1.7	66.1 ± 1.7
Crude fat (% DM)	15.9 ± 1.0	16.2 ± 1.0	16.3 ± 1.0	15.6 ± 1.0
Fiber (% DM)	1.4 ± 0.7	1.2 ± 0.7	1.1 ± 0.7	1.2 ± 0.7
Ash (% DM)	11.4 ± 0.4	11.7 ± 0.4	11.7 ± 0.4	11.8 ± 0.4
Phosphorous (% DM)	1.9 ± 0.4	2.0 ± 0.4	1.9 ± 0.5	2.0 ± 0.4
Energy (MJ/Kg DM)	23.0 ± 0.0	23.0 ± 0.0	23.1 ± 0.0	22.9 ± 0.0
Vitamin C (mg/kg DM)	159.6 ± 0.0	2027.7 ± 0.0	2014.8 ± 0.0	2014.8 ± 0.0
Vitamin E (mg/kg DM)	42.6 ± 0.0	1067.2 ± 0.0	1060.4 ± 0.0	1060.4 ± 0.0
Taurine (g/100 g DM)	0.31 ± 0.3	0.31 ± 0.3	0.94 ± 0.9	0.31 ± 0.3
Methionine (g/100 g DM)	1.5 ± 0.2	1.5 ± 0.2	2.0 ± 0.3	0.6 ± 0.1

Results expressed as mean ± standard deviation (n = 2 experimental units).

All diets were produced at Sparos Lda facilities (Olhão, Portugal), using extrusion at low temperature as the main production process, as follows: powder ingredient mixing according to target formulation using a double-helix mixer; grinding in a micropulverizer hammer mill (SH1, Hosokawa-Alpine, Augsburg, Germany); addition of the oil fraction; humidification and agglomeration through low-temperature extrusion (Dominioni Group, Lurate Caccivio, Italy); drying of resultant pellets in a convection oven (OP 750-UF, LTE Scientifics, Oldham, UK) for 4 h at 60 °C; crumbling (Neuero Farm, Melle, Germany); and sieving to desired size ranges.

2.2. Shrimp Rearing and Sampling

Whiteleg shrimp post larvae (PL16), originated from Blue Genetics (La Paz, Mexico), were reared for 18 days at Riasearch Lda facilities (Murtosa, Portugal). Shrimp were

randomly distributed to 12 tanks with approximately 50 L that were part of a clear water-recirculating system. Each tank was stocked with 200 individuals averaging 9 mg of wet weight. These were kept under a 12 h light:12 h dark photoperiod and were fed close to ad libitum with automatic feeders that supplied eight meals a day. Feeders were cleaned daily and charged with adjusted feed quantities based on the observation of the tanks and the presence/absence of remnants from the previous day. Feed size was 400–600 µm for the first week and 600–800 µm for the remaining feeding period. Water temperature was maintained at 28.8 ± 0.3 °C, dissolved oxygen concentration at 7.5 ± 0.4 mg L^{-1}, salinity at 20.3 ± 1.2, pH at 7.96 ± 0.1, NH_3 at 0.0 ± 0.0 mg L^{-1}, and NO_2 at 0.36 ± 0.3 mg L^{-1}.

At the start of the trial, a total of 60 shrimp from the initial stock were randomly selected and group weighed for initial wet weight determination. At the end of the experiment, all shrimp were weighed in groups of 20 individuals for the final wet weight determination of each tank. Additionally, 40 shrimp were randomly selected from each tank for oxidative stress and immune parameters analysis and 10 shrimp for analysis of gene expression. Shrimp were fasted for 12 h prior to samplings to ensure their guts were empty at collection. Shrimp sampled for oxidative stress and immune parameters were stored at −80 °C for subsequent analysis. Shrimp sampled for molecular biology analysis were kept in RNAlater (Sigma, St. Louis, MO, USA) at >1:5 volume ratio, at 4 °C, for 24 h prior to being stored at −20 °C. Relative growth rate (RGR), feed conversion ratio (FCR), and survival for each treatment were assessed at the end of the experiment.

2.3. Oxidative Stress and Immunity-Related Biomarkers

2.3.1. Sample Preparation

A total of 40 whole whiteleg shrimp post larvae from each tank sampled at end of the trial were weighed and homogenized in quadruple groups of 10 individuals for oxidative stress and immune parameters analysis. Potassium phosphate buffer (0.1 M) was added to each group in a 1/10 (w/v) proportion followed by homogenization using a high-performance dispersing instrument (SilentCrusher M, Heidolph Instruments, Schwabach, Germany). An aliquot for lipid peroxidation (LPO) with butylated hydroxytoluene was reserved prior to centrifugation. After centrifugation (5500 rpm for 20 min), sample supernatant was collected and distributed in separate aliquots for oxidative stress parameters and immune parameters. The remaining 10 shrimp sampled for molecular biology analysis were homogenized in NZYol (Nzytech, w/v proportion according to the manufacturer's instructions) using a Precellys 24 tissue homogenizer (Bertin instruments, Montigny-le-Bretonneux, France).

2.3.2. Determination of Oxidative Stress Biomarkers

Catalase (CAT), lipid peroxidation (LPO), and total glutathione (tGSH) activities as well as total proteins content were determined in the homogenized samples. Total proteins were measured by using PierceTM BCA Protein Assay Kit, as described by Costas et al. [23]. Samples were diluted in K-phosphate buffer (0.1 M; pH 7.4), and bovine serum albumin (BSA, 2 mg mL^{-1}) was used as standard. Afterwards, 25 µL of each diluted sample and standards were plated in triplicate and read at 562 nm in a Synergy HT microplate reader. Results were calculated using a standard curve and expressed as mg mL^{-1}.

CAT activity levels were determined measuring the decrease of hydrogen peroxide (H_2O_2 30%, Sigma) concentration as described by Clairborne [24]. The reaction mixture was composed of K-phosphate buffer (0.05 M pH 7.0) and H_2O_2 (30%) as substrate, and 10 µL of homogenate sample was added to the reaction mixture, effecting a total volume of 300 µL. Absorbance was read at 240 nm in UV microplates for 2 min (1 reading every 15 s) in a Synergy HT microplate reader and results expressed as enzyme units per milligram of total protein (U mg^{-1} protein). One enzyme unit is the amount of enzyme needed to catalyze one micromole of substrate per minute.

Endogenous LPO was assessed by measuring thiobarbituric acid-reactive substances (TBARS), preventing artefactual lipid oxidation by adding butylhydroxytoluene (4%; Sigma) [25].

Homogenate samples incubated for 60 min at 100 °C with a 100 µL of trichloroacetic acid 100% solution and 1 mL of 2-thiobarbituric acid 0.73% (Sigma), trizma hydrochloride (Sigma), and diethylenetriaminepentaacetic acid (Fluka) solution in polystyrene microtubes. Afterwards, these were centrifuged for 5 min at 11,500 rpm, and supernatant (200 µL) was added to the microplate wells. Absorbance was read at 535 nm and results expressed as nmol g wt^{-1}.

Total glutathione content in post-larvae homogenate samples was measured based on the oxidation of glutathione by 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB; Sigma) as described by Rodrigues et al. [26]. Samples were diluted in K-phosphate buffer (0.1 M pH 7.4) to obtain 0.7 mg mL^{-1} of protein. Thereafter, 50 µL of each diluted sample was added to microplate wells, followed by the addition of 250 µL of a reaction solution composed by DTNB, K-phosphate buffer (0.1 M, pH 7.4), NADPH (ß-Nicotanimide adenine dinucleotide 2′-phosphate reduced tetrasodium salt; Alpha Aesar), and glutathione reductase (Sigma). Absorbance was read at 412 nm for 3 min (1 reading every 20 s) in a Synergy HT microplate reader and results expressed as nmol mg of $protein^{-1}$.

2.3.3. Analysis of Immune Parameters

Lysozyme, pro-phenoloxidase, and bactericidal activities were determined in the homogenized samples. Lysozyme activity was measured using a turbidimetric assay as described by Costas et al. [27]. Briefly, a solution of *Micrococcus lysodeikticus* (0.25 mg mL^{-1}, 0.05 M sodium phosphate buffer, pH 6.2) was prepared and 40 µL of homogenized samples, and 130 µL of this suspension were added to a microplate, effecting a final volume of 170 µL. The reaction was carried out at 25 °C, and absorbance (450 nm) measured after 0.5 and 30 min in a Synergy HT microplate reader. Lyophilized hen egg white lysozyme (Sigma) was diluted in sodium phosphate buffer (0.05 M, pH 6.2) and used to develop a standard curve. The amount of lysozyme in the sample was calculated using a standard curve. Lysozyme was expressed as µg mg $protein^{-1}$.

Pro-phenoloxidase activity was measured spectrophotometrically using L-DOPA (L-3,4- dihydroxyphenylalanine) as substrate and trypsin (Sigma) as activator following the method described by Ji et al. [28] with modifications. Homogenate samples of 50 µL were diluted in 100 µL of trypsin solution (0.1% in cacodylate solution) in a 96-well microplate and incubated for 30 min at room temperature. Afterwards, 100 µL L-DOPA solution (0.3% in cacodylate solution) was added. The absorbance was measured every minute during 5 min at 490 nm using a Synergy HT microplate reader. Results were calculated using the Beer–Lambert law using the molar extinction coefficient of the L-DOPA (3700). Results were expressed as units of pro-phenoloxidase mL^{-1} of sample.

Vibrio harveyi was used in the bactericidal activity assay. Exponentially growing bacteria were resuspended in sterile HBSS and adjusted to 3.1×10^9 cfu mL^{-1}. Plating serial dilutions of the suspensions onto TSA-2 plates and counting the number of cfu following incubation at 22 °C confirmed bacterial concentration of the inoculum. Homogenized samples' bactericidal activity was then determined following the method described by Machado et al. [29]. Briefly, 60 µL of homogenized samples were added to a U-shaped 96-well plate. HBSS was added to some wells instead of homogenized samples and served as positive control. To each well, 20 µL of *V. harveyi* (3.1×10^9 cfu mL^{-1}) were added, and the plate was incubated for 2.5 h at 25 °C. To each well, 25 µL of 3-(4, 5 dimethyl-2-yl)-2, 5-diphenyl tetrazolium bromide (1 mg mL^{-1}; Sigma) was added and incubated for 10 min at 25 °C to allow the formation of formazan. Plates were then centrifuged at 2000× *g* for 10 min, and the precipitate was dissolved in 200 µL of dimethyl sulfoxide (Sigma). The absorbance of the dissolved formazan was measured at 560 nm. Bactericidal activity was expressed as percentage calculated from the difference between the surviving bacteria compared to the number of bacteria from positive controls (100%).

2.3.4. Gene Expression Analysis

Extraction of RNA was performed using the NZY total RNA isolation kit (NZYTech, Lisboa, Portugal) according to the manufacturer's instructions. RNA concentration and purity

was analyzed by spectrophotometry using DeNovix DS-11 FX (Wilmington, NC, USA). RNA concentration varied from 123.9 to 2180.7 ng μL^{-1} and 260:280 ratios between 1.99 and 2.17, respectively. The integrity of the RNA samples was verified through a 2% agarose gel. The cDNA was obtained using the NZY first-strand cDNA synthesis kit (NZYTech). This step was used to standardize the concentration of the samples. Reverse transcription was carried out in a Veriti DX 96-well thermal cycler (Applied Biosystems, Waltham, MA, USA), using 4.4 µL of diluted cDNA (20 ng μL^{-1}) mixed with 5 µL of NZYSpeedy qPCR Green Master Mix® (NZYTech) and 0.3 µL (10 µM) of each specific primer in a final volume of 10 µL. Real-time quantitative PCR was performed, in duplicate for each sample, using a CFX384 Touch Real-Time PCR Detection System (Biorad, Hercules, CA, USA). Nine genes were selected and analyzed due to their role in the immune response. Primer efficiency was tested for each gene (Table 3). Cycling conditions were the same between the different genes, consisting of one cycle of 95 °C for 10 min, followed by 40 cycles of 2 steps of 95 °C for 15 s and 62 °C for 1 min, with a final cycle at 95 °C for 1 min, followed by 35 s at 62 °C and ending at 95 °C for 0.5 s. The Pfaffl method [30] was used to perform gene expression analyses, and target genes were normalized using *bactn* and *rpl-8* as housekeeping.

Table 3. Selected genes and specific primers used to evaluate the immune status of whiteleg shrimp (*P. vannamei*) PL at the end of the experimental period.

Gene	Acronym	Efficiency (%)	Annealing Temperature (°C)	Accession n°	Amplicon Length (bp)	Primer Sequence (5′-3′)
Cytoplasmic-type actin 4	*bactn*	83.2	62	MF627841.1	260	F: CACGAGACCACCTACAACTCCATC R: TCCTGCTTGCTGATCCACATCTG
Ribosomal protein L8	*rpl-8*	90	62	DQ316258.1	219	F: AGCCAAGCAAGATGGGTCG R: TGTAACGATAAGGGTCACGGAAG
PvHm117 crustin P	*crus*	81	62	AY488497.1	109	F: GAAACCACCACCAACACCTACTCC R: TCTGTGCGGCCTCTTTACGG
Penaeidin-3a	*pen-3*	86.1	62	Y14926.1	137	F: ATACCCAGGCCACCACCCTT R: TGACAGCAACGCCCTAACC
Hemocyanin	*hmc*	92	62	KY695246.1	124	F: GTCTTAGTGGTTCTTGGGCTTGTC R: GGTCTCCGTCCTGAATGTCTCC
Lysozyme C-like	*lys*	73	62	XM_027352857	82	F: CGGGAAAGGCTATTCTGCCT R: CCAGCACTCTGCCATGTACT
C-type lectin 2-like	*lect*	83	62	DQ858899.2	138	F: GCTTCTGTTGGTGCTGTTGGC R: GTTCCCTTCCCGTATGTGGC
Thioredoxin 1	*trd*	85.3	62	EU499301.1	116	F: TTAACGAGGCTGGAAACA R: AACGACATCGCTCATAGA
Glutathione transferase	*gst*	99	62	AY573381	146	F: AAGATAACGCAGAGCAAGG R: TCGTAGGTGACGGTAAAGA
Glutathione peroxidase	*gpx*	86.6	62	XM_027372127.1	117	F: AGGGACTTCCACCAGATG R: CAACAACTCCCCTTCGGTA
Caspase 3	*casp-3*	93.6	62	KC660103.1	182	F: ACATTTCTGGGCGGAACACC R: GTGACACCCGTGCTTGTACA

2.4. Data Analysis

Relative growth rate (RGR, % weight day^{-1}) was calculated as follows: RGR = (e^g − 1) × 100, where e = exponential and g = (lnWf − lnWi) × t^{-1}. Wf and Wi correspond to the final and initial weights, respectively. Feed conversion ratio (FCR) was calculated as follows: FCR = (Fi/Wg), where Fi corresponds to feed given (g) and Wg to the mean weight gain (g). Survival was expressed as percentage and calculated as follows: S = (Lf/Li) × 100, where Li and Lf correspond to the initial and final number of post larvae in the tanks, respectively. Differences in growth performance, FCR, survival, oxidative stress, immune condition, and gene expression between dietary treatments were evaluated using one-way ANOVAs, followed by Tukey multiple comparison tests. Kruskal–Wallis one way analysis of variance tests followed by Wilcoxon pairwise comparison tests were used when data did not comply with the one-way ANOVA's assumptions. Results were expressed as means ± standard deviation (SD). In results expressed as percentage, an arcsine transformation was

performed prior to any statistical test: T = ASIN (SQRT (value/100)). The significance level considered was $p < 0.05$ for all tests performed.

All activities were undertaken within the clear boundaries of national and EU legal frameworks directed by qualified scientists/technicians and conducted according to the European guidelines on the protection of animals used for scientific purposes (Directive 2010/63/UE of the European Parliament and on the European Union Council) and under strict monitoring and control of DGAV—(Direção Geral de Alimentação e Veterinária), Animal Welfare Division, which is the competent authority responsible for implementing the legislation on the "protection of animals used for scientific purposes".

3. Results

3.1. Growth Performance

No significant differences in growth performance and survival were observed among dietary treatments. Final wet weight averaged around 100 mg, RGR values 15% day^{-1}, FCR was close to 1, and survival ranged between 86 to 88% for all treatments (Table 4).

Table 4. Initial and final weight, relative growth rate (RGR), feed conversation ratio (FCR), and survival of whiteleg shrimp (*P. vannamei*) PL during the experimental period.

	NC	PC	T + M	BG
Initial weight (mg)		8.8 ± 0.0		
Final weight (mg)	110.8 ± 19.3	110.8 ± 18.4	114.0 ± 9.5	94.4 ± 9.2
RGR (% day^{-1})	15.0 ± 1.1	15.0 ± 1.1	15.3 ± 0.5	14.0 ± 0.6
FCR	0.9 ± 0.0	0.9 ± 0.2	0.9 ± 0.1	1.0 ± 0.2
Survival (%)	87.0 ± 6.6	86.2 ± 7.6	85.5 ± 6.1	87.5 ± 5.0

Results expressed as mean ± standard deviation. For initial weight, n = 60 observational units; for final weight, FCR, RGR, and survival, n = 3 experimental units.

3.2. Oxidative Stress and Immune Status Related Biomarkers

Regarding the oxidative stress parameters measured, CAT levels were similar, with no significant differences being detected across treatments; LPO levels were significantly lower in shrimp PL fed the BG dietary treatment than those fed the PC diet, with no significant differences between the remaining treatments; tGSH levels were significantly higher in shrimp PL fed the BG treatment than in their counterparts fed the NC diet, with no significant differences between the remaining treatments. As for the immune condition, no significant differences between treatments were observed regarding the parameters measured (Table 5).

Table 5. Catalase (CAT), lipid peroxidation (LPO), total glutathione (tGSH), lysozyme, pro-phenoloxidase, and bactericidal activity levels in whiteleg shrimp (*P. vannamei*) PL fed the experimental diets for 18 days.

	NC	PC	T + M	BG	*p*-Value
CAT (U mg^{-1} protein)	22.9 ± 8.0	22.4 ± 6.3	28.9 ± 19.3	21.4 ± 7.2	0.675
LPO (nmol g wt^{-1})	14.0 ± 2.2 ^{ab}	15.6 ± 3.1 ^{a}	14.6 ± 2.6 ^{ab}	12.7 ± 2.1 ^{b}	0.039
tGSH (nmol mg protein^{-1})	4.7 ± 0.9 ^{a}	5.0 ± 0.7 ^{ab}	5.0 ± 0.8 ^{ab}	5.7 ± 1.1 ^{b}	0.018
Lysozyme (µg mg protein^{-1})	1.2 ± 0.5	1.5 ± 0.6	1.1 ± 0.3	1.2 ± 0.4	0.165
Pro-phenoloxidase ($\times 10^{-3}$ U mL^{-1})	12.1 ± 6.8	14.0 ± 9.3	12.4 ± 3.8	13.0 ± 6.5	0.854
Bactericidal activity (%)	12.6 ± 6.9	12.9 ± 8.1	14.6 ± 11.9	14.5 ± 7.8	0.551

Results expressed as mean ± standard deviation (n = 3 experimental units). Represented are also the p-values for a one-way ANOVA. Different superscript letters indicate statistical differences ($p < 0.05$) between treatments in a post hoc Tukey multiple comparison test.

3.3. Gene Expression Analysis

The normalized relative mRNA expression of the PvHm117 crustin P gene decreased significantly in shrimp PL fed the NC diet compared to those fed the T + M and BG dietary treatments. Similarly, the penaeidin-3a mRNA expression level decreased significantly

in shrimp PL fed the NC diet compared to their counterparts fed the PC and BG dietary treatments. Hemocyanin transcripts increased significantly in shrimp PL fed the NC diet compared to PL fed the T + M dietary treatment. As for the normalized relative mRNA expression of the remaining genes, no significant differences between treatments were observed (Table 6).

Table 6. Relative expression to housekeeping (*bactn* and *rpl-8*) of target immune related genes of whiteleg shrimp (*P. vannamei*) PL fed the experimental diets for 18 days.

Gene	Acronym	Relative Expression				*p*-Value
		NC	PC	T + M	BG	
PvHm117 crustin P	*crus*	0.6 ± 0.2^{a}	1.1 ± 0.5^{ab}	1.3 ± 0.4^{b}	1.5 ± 0.8^{b}	0.003
Penaeidin-3a	*pen-3*	0.4 ± 0.3^{a}	1.1 ± 0.4^{b}	0.7 ± 0.5^{ab}	1.2 ± 0.8^{b}	0.001
Hemocyanin	*hmc*	1.1 ± 0.7^{b}	1.9 ± 2.4^{ab}	0.2 ± 0.2^{a}	0.7 ± 0.7^{ab}	0.029
Lysozyme C-like	*lys*	0.7 ± 0.4	1.1 ± 0.5	1.3 ± 0.6	1.3 ± 0.9	0.212
C-type lectin 2-like	*lect*	0.7 ± 0.4	1.1 ± 0.5	1.3 ± 0.7	1.3 ± 1.0	0.236
Thioredoxin 1	*trd*	1.0 ± 0.5	1.0 ± 0.2	1.0 ± 0.3	0.90 ± 0.3	0.819
Glutathione transferase	*gst*	0.9 ± 0.5	0.9 ± 0.4	0.6 ± 0.3	0.4 ± 0.1	0.218
Glutathione peroxidase	*gpx*	1.0 ± 0.4	1.1 ± 0.4	0.9 ± 0.1	1.0 ± 0.3	0.622
Caspase 3	*casp-3*	0.6 ± 0.3	0.8 ± 0.4	0.6 ± 0.5	1.9 ± 2.5	0.410

Results expressed as mean ± standard deviation ($n = 3$ experimental units). Represented are also the *p*-values for a one-Way ANOVA. Different superscript letters indicate statistical differences ($p < 0.05$) between treatments in a post hoc Tukey multiple comparison test.

4. Discussion

This study aimed at evaluating the potential health-promoting effects of including several dietary supplements in inert microdiets for whiteleg shrimp PL. Vitamin C and E, methionine, taurine, and β-glucans were selected for this purpose since their potential ability to enhance the health status of whiteleg shrimp in the initial stages of development is still promising but yet to be experimentally validated. A control diet formulated to fulfil the nutritional requirements of whiteleg shrimp PL was used as positive control, and the remaining experimental diets were based on it, differing only in the reduction or addition of the previously mentioned nutrients. In overall, the formulation changes in the diets did not compromise their adequacy, as good growth performances and survival results were obtained in all experimental treatments, also revealing that good zootechnical conditions were maintained during the trial. Growth results were similar to those reported by Wang et al. when using graded levels of *Schizochytrium* meal [31] and as a replacement of fish oil [32] in practical diets for whitleg shrimp PL, but survival results were considerably inferior in those studies (40.3–44.5% and 42.7–45.6%, respectively) than in the current trial.

Increasing the vitamin C and E supplementation levels in the PC dietary treatment did not produce any changes in growth performance and survival when compared with the NC, suggesting that the levels of these vitamins present in the NC diet still allowed the shrimp post larvae to maintain an adequate development. Like other vitamins, vitamin C and E are essential nutrients, as animals are unable to synthesize sufficient amounts to meet their physiological needs, and a deficient supply in the diet often results in poor growth, possibly leading to severe health issues and even compromising survival [6,33]. Additionally, no changes were verified in the activity levels among most of the immune and antioxidant parameters measured in this study when increasing the levels of these vitamins in the diets. Accordingly, other studies reported thresholds in inclusion levels for these vitamins in diets for whiteleg shrimp juveniles at which their beneficial effects did not increase after a certain incorporation percentage [7,14]. The only significant dissimilarity detected was the lower relative expression of *pen-3* in shrimp fed the NC diet compared to those fed the PC diet, suggesting that lower levels of these vitamins may impact the shrimp immune status. Penaeidins, a key group of antimicrobial peptides in penaeid shrimp, have antibacterial and antifungal activities, which are particularly effective against Gram+ bacteria and filamentous fungi [34,35]. These findings indicate that higher supplementation levels of

vitamins C and E in microdiets for whiteleg shrimp PL did not directly enhance growth and survival in the current study but may have improved their robustness. Although not confirmed in the current experiment, this may be an indication that shrimp may have a higher survival capacity in the long term and particularly in a potentially challenging husbandry situation. Therefore, inert diets with adequate levels of vitamins C and E can be vital during critical stages of production, particularly in farms where a nursery system is employed (intermediate step between the early PL stage and the grow out phase), in which PL are kept at extremely high stocking densities that can induce stress and vulnerability to opportunistic pathogens [36].

The supplementation of methionine to balance the nutritional profiles of aquafeeds rich in plant-based proteins has become a common practice [16,37]. Traditionally, methionine supply was ensured by fish meal, but continuous efforts are underway to reduce the industry dependence on this ingredient and replace it by plant-based proteins, where methionine and lysine are generally low in the amino acid profile. It has been shown by several authors that when dietary requirements are not met, generally, when low-fish-meal diets are concomitantly used, growth performances and survival of whiteleg shrimp can be affected [13,16,38–40]. Additionally, methionine has a recognized role in the immune system and has recently been used to improve the antioxidant capacity, innate immune response, and/or disease resistance of whiteleg shrimp juveniles [16,18,41]. Besides that, methionine is also a precursor for taurine. The supplementation of this nutrient in diets for whiteleg shrimp is also recommended since it can provide beneficial effects on their growth and immune response [12,17]. Yet, the evaluation of the supplementation of both molecules in diets for the initial developmental stages of shrimp is still necessary. In this study, whiteleg shrimp PL fed with the T + M diet showed similar growth performances, survival, oxidative status, and immune condition to those fed the PC dietary treatment, suggesting that the ingredient formulation of the control diet was capable of covering the shrimp PL requirements for taurine and methionine, and no extra benefits were obtained through the supplementation of these amino acids. These results can probably be explained by the fact that the PC was a high-quality diet with considerable levels of protein of marine origin. However, shrimp PL fed the T + M diet showed significantly higher relative expressions of *crus* and significantly lower *hmc* transcripts when compared with those fed the NC dietary treatment. Both PvHm117 crustin P and hemocyanin are associated with important broad-spectrum antimicrobial peptides involved in the first line of the shrimp defense [42–44]. These results could be considered contradictory, as it would be expected that variations in the expressions of both genes would follow the same trend. Still, it is important to bear in mind that hemocyanin is a multifunctional protein involved in several physiological processes beyond innate immunity, such as oxygen transport, protein storage, molt cycle, exoskeleton formation, and osmoregulation [45,46]. Concomitantly, taurine is also one of the main organic osmolytes in osmoregulation for decapods [47,48], and it has been shown that increases in dietary taurine inclusion levels increases the molecule contents in different tissues and hemolymph of whiteleg shrimp [17]. Therefore, the hemocyanin levels needed to maintain osmolality were probably lower in shrimp fed the T + M diet, which may have caused a downregulation of the *hmc* gene. Still, it should be noted that the analysis only focused on measuring mRNA transcripts of *hmc* and not the taurine molecule levels. Therefore, to better understand these interactions and clarify if the supplementation of taurine and methionine in diets for whiteleg shrimp PL is beneficial when lower vitamin C and E inclusion levels are used, further studies should be conducted.

The Inclusion of β-glucans in the diets did not significantly affect the shrimp PL growth performance and survival. Still, shrimp PL fed the BG diet tended to grow less and achieved final weights around 15% lower than those fed the PC, although it was not supported by the statistical analysis. Nonetheless, lipid peroxidation levels dropped significantly in shrimp PL fed the BG dietary treatment compared to those fed the PC diet, suggesting that β-glucans improved the antioxidant mechanisms of the animals. In fact, the immunostimulatory and antioxidant-boosting properties of β-glucans as aquafeed addi-

tives have been reported for several species, as recently reviewed by Pogue et al. [49]. These can be tremendously valuable in the larval/PL stages, where shrimp undergo extremely fast development, as accelerated growth is likely to produce excess reactive oxygen species that can result in oxidative stress, damaging key physiological structures [50]. Although there are reports of the enhancement of whiteleg shrimp disease resistance through the dietary supplementation of β-glucans [51–53], in the present study, no significant improvements in immune condition were observed in the shrimp PL fed the BG diet compared with those fed the PC diet. Bai et al. [54] suggested that discontinuous feeding, changing between a basal diet and one with the inclusion of β-glucans, is the most suitable strategy to enhance the immunity of whiteleg shrimp, as continuous feeding for long periods of time with the supplemented diet can cause immune fatigue, mitigating the beneficial effects provided in the short term. Considering this hypothesis, the BG diet potential to improve the whiteleg shrimp PL immunity could have been clearer if a different feeding strategy had been employed. When compared with the NC diet, PL fed with BG showed significant increases in tGSH levels as well as in the relative expression of *crus* and *pen-3*. This suggests that the β-glucans supplementation coupled with higher levels of vitamin C and E can boost the antioxidant capacity and immune status of whiteleg shrimp PL. In fact, Wu et al. [14] proposed that there is an interaction between β-glucans and vitamin C that is capable of increasing the nonspecific immune response of the whiteleg shrimp. The results obtained in that study corroborate this hypothesis, as the addition of β-glucans to the PC diet amplified the differences in the shrimp's immune condition and antioxidant capacity relative to the NC diet.

5. Conclusions

In conclusion, the results obtained in this study suggest that although no improvements in growth performances and survival were observed at the end of the experimental period, all dietary additives tested have the potential to add value to inert microdiets for whiteleg shrimp PL. Benefits to the antioxidant capacity and robustness of the shrimp PL were clearer when the vitamin C and E levels were higher than those used in the NC, similar to those used in the PC. However, the control diet can be considered a premium option, and it should be expected that the positive effects provided by these supplements are augmented when incorporated into more economical alternatives. Amongst the additives tested, the inclusion of β-glucans in the diets seems to be the most promising, as it reduced lipid peroxidation in the shrimp PL even when compared to a high-quality control diet. When compared to the NC, the interaction between the supplementation of β-glucans and higher levels of vitamins C and E also seems beneficial to the antioxidant capacity of whiteleg shrimp PL.

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