KÁTIA RODRIGUES BATISTA DE OLIVEIRA

INSECT MEAL INCLUSION AND NUTRITIONAL PROGRAMMING AFFECT THE DIGESTIVE PROCESS AND GROWTH PERFORMANCE OF NILE TILAPIA (*Oreochromis niloticus*)

LAVRAS - MG

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Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Zootecnia, área de concentração em Produção e Nutrição de Monogástricos, para a obtenção do título de Doutor.

Orientadora
Profa. Dra. Priscila Vieira Rosa

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2021

Ficha catalográfica elaborada pelo Sistema de Geração de Ficha Catalográfica da Biblioteca Universitária da UFLA, com dados informados pelo(a) próprio(a) autor(a).

Oliveira, Kátia Rodrigues Batista de.
Insect meal inclusion and nutritional programming affect the digestive process and growth performance of Nile tilapia (*Oreochromis niloticus*) / Kátia Rodrigues Batista de Oliveira. - 2021.
131 p. : il.

Orientador(a): Priscila Vieira Rosa.

Tese (doutorado) - Universidade Federal de Lavras, 2021.
Bibliografia.

1. Tilápia do Nilo. 2. Farinha de Inseto. 3. Enzimas Digestivas. I. Rosa, Priscila Vieira. II. Título.

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APROVADA em 26 de fevereiro de 2021

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Lavras – MG

2021

AGRADECIMENTOS

Ao Programa de Pós-graduação em Zootecnia da UFLA, todos os professores pelos ensinamentos e funcionários pelo suporte. Aos membros da banca, Dr. Aires, Dra. Luciana, Dr. Murgas e Dr. Renan, pela disponibilidade e contribuições para a melhoria dos trabalhos. À minha orientadora Priscila Vieira Rosa, pela amizade e confiança. Por sempre incentivar e participar tão ativamente na construção da minha carreira. Por todos os ensinamentos acadêmicos e acima de tudo pelos conselhos e conversas tão precisas e assertivas, que carregarei comigo por toda a vida.

Em especial ao meu pai José Maria, minhas irmãs Karina, Kênia e Layra, minha madrinha Marilene e à tia Direne que embarcaram comigo em todas as minhas aventuras, sempre me apoiando e incentivando a continuar, com muita coragem e humildade sempre. À minha sobrinha Eva que me agraciou nestes últimos dois anos com muita alegria.

Ao meu esposo Diego Zanetti por todo companheirismo, dedicação, carinho, amor e incentivo. Pela paciência incondicional e por todos os cafés da tarde super motivadores. Por sempre contribuir em meus projetos, incluindo pelas diversas vezes na construção desta tese. Obrigada por ter se aventurado comigo e por me fazer uma pessoa melhor.

À Tafanie por me receber tão bem como amiga e parceira de trabalho. Por partilhar da ideia e realização deste projeto. Às colegas e amigas Izabella e Tamira por todo apoio. Ao Elecir, por todo carinho, alegria, sabedoria e também pelos chás que sempre resolviam qualquer problema. Aos integrantes do NAQUA por toda ajuda e amizade, em especial ao Rafael Marcelino, Marcella e Matheus. Ao laboratório NUTRIMU da Universidade do Porto e a todos os seus membros pelo acolhimento, ensinamentos e parceria.

Aos amigos que tive o prazer de conhecer ao longo de toda essa minha jornada, agradeço pela amizade e por fazerem tão lindamente parte da minha história. Às minhas ex-orientadoras, Ana Lúcia Salaro e Elisabete Macedo por terem aberto tantas portas que ditaram meu caminho até aqui.

O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Código de Financiamento 001, e do Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

RESUMO

A presente tese trata de questões com alta demanda de conhecimento em quatro manuscritos. No primeiro manuscrito, dois ensaios avaliaram os efeitos da alimentação de alevinos de tilápia com farinha de *Zophobas morio* (ZM) sobre (i) a sobrevivência dos alevinos, desempenho produtivo, utilização do alimento e composição da carcaça e, (ii) desempenho produtivo, utilização do alimento, composição da carcaça e filé, nos juvenis alimentados com dietas contendo crescentes inclusões de ZM. Alimentar alevinos com ZM aumentou a sobrevivência, ganho de peso, eficiência na utilização do alimento além do conteúdo de proteína, energia e lipídios na carcaça. Juvenis alimentados com ZM durante a fase de alevinos tiveram melhor consumo de ração, taxa de conversão alimentar e peso de carcaça quando alimentados com a dieta contendo 300g/kg de ZM. No segundo manuscrito, três ensaios avaliaram a influencia da programação nutricional na capacidade de juvenis de tilápia em utilizar dietas contendo crescente concentração de farinha de *Z. morio*. Foram avaliados a atividade das enzimas digestivas e quitinase, histomorfologia do intestino e digestibilidade aparente das dietas. Os níveis crescentes de ZM afetam positivamente a atividade da quitinase. A introdução de ZM durante a fase de alevinos melhorou as atividades de tripsina e quimiotripsina, além da digestibilidade da matéria seca de juvenis alimentados com a dieta contendo 300 g/kg de ZM. Alimentar os peixes continuamente, de alevinos a juvenis, com farinha de *Z. morio*, em altos níveis de inclusão, melhora a capacidade de digestiva dos peixes e a utilização da farinha de insetos, permitindo inclusões de até 300g/kg. O terceiro estudo trata-se da adequação do protocolo de determinação da atividade de quitinase para a tilápia do Nilo. Após uma série de avaliações, uma metodologia foi proposta de forma a obter adequados valores de atividade de quitinase com redução dos custos, do tempo e com maior aplicabilidade. O quarto manuscrito discute sobre o procedimento de amostragem ideal para capturar a atividade das enzimas digestivas: amilase, lipase, tripsina e quimiotripsina. Glicose plasmática, colesterol e triglicerídeos também foram avaliados. No geral, amostrar a porção anterior do intestino no intervalo entre 10 e 12 horas pós-prandias é um procedimento adequado para ser usado em ensaios enzimáticos digestivos para tilápia do Nilo.

Palavras-chave: Enzimas. Quitinase. Horas pós-prandiais. Onívoros Tenébrio Gigante.

ABSTRACT

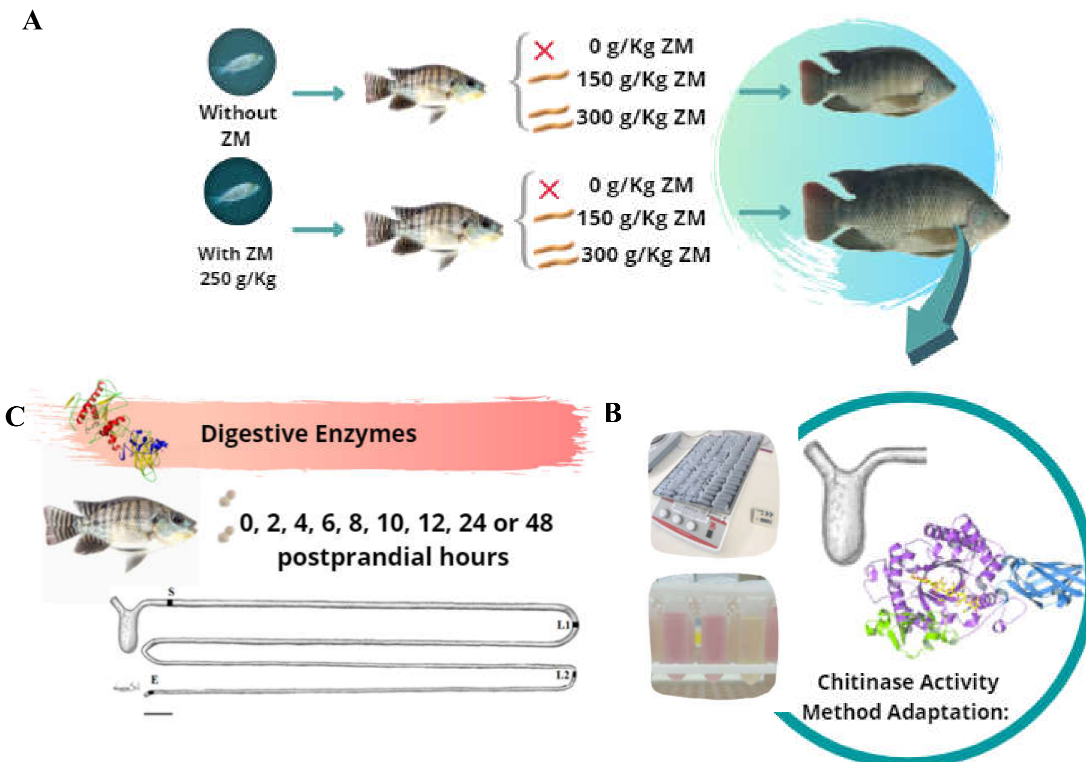
The present thesis concerns issues with a high demand for knowledge, in four manuscripts. In the first manuscript, two trials evaluated the effects of feeding tilapia fry with *Z. morio* meal on (i) fry survival, growth performance, feed utilization, and carcass composition; and on juveniles (ii) growth performance, feed utilization, carcass and fillet composition, after fed increasing *Z. morio* meal (ZM) diets. Feeding fry with ZM increased survival, weight gain, feed utilization and carcass protein, energy and lipids. Juveniles fed with ZM during fry stage had better feed intake, feed conversion ratio and carcass weight when fed with a diet containing 300g/kg of ZM. In the second manuscript, three trials evaluated the influence of nutritional programming on the ability of tilapia juveniles to use diets containing an increasing concentration of *Z. morio* meal. The activity of digestive enzymes and chitinase, histomorphology of the intestine and apparent digestibility of the diets were evaluated. Increasing levels of ZM positively affect chitinase activity. The introduction of ZM during the fry phase improved the activities of trypsin and chymotrypsin, in addition to the digestibility of dry matter of juveniles fed with a diet containing 300 g/kg of ZM. Feeding fish continuously, from fry to juveniles, with *Z. morio* meal at high levels of inclusion improves the digestive capacity of fish and the use of insect meal, allowing inclusions of up to 300g/kg. The third study deals with the adequacy of the chitinase activity determination protocol for Nile tilapia. After a series of evaluations, a methodology was proposed to obtain adequate chitinase activity values for tilapia with reduced costs, time and with greater applicability. The fourth study discussed the ideal sampling procedure to capture the activity of the digestive enzymes: amylase, lipase, trypsin, and chymotrypsin at their best. Plasmatic glucose, cholesterol, and triglycerides were also evaluated. Overall, to sample the anterior portion of the intestine into the interval between 10- and 12-hours post-feeding is an adequate sampling procedure to be used in digestive enzymatic assays for Nile tilapia.

Keywords: Chitinase. Enzymes. Postprandial hours. Omnivorous. Superworm

Insect meal inclusion and nutritional programming affect the digestive process and growth performance of Nile tilapia (*Oreochromis niloticus*)

Elaborado por **Kátia Rodrigues Batista de Oliveira** e orientado por **Profa Dra. Priscila Vieira Rosa**

Esta tese trata de duas questões com alta demanda de conhecimento na aquicultura. Em primeiro trata dos efeitos da programação nutricional quando utilizados altos níveis de inclusão de farinha de inseto em dietas para tilápias do Nilo. Foram realizados três ensaios *in vivo* e ensaios para adequação da metodologia de obtenção da atividade da quitinase para tilápia do Nilo. Foi observado que, alevinos de tilápia alimentados com farinha de tenébrio gigante apresentam melhor sobrevivência, ganho de peso, capacidade de utilização do alimento e maior conteúdo de proteína, energia e lipídios na carcaça. Além disso, a estratégia de alimentar continuamente os alevinos de tilápia até a fase de juvenis, com alta inclusão de farinha de inseto na dieta, permite otimizar a habilidade dos peixes de digestão da quitina e dos outros nutrientes da dieta, melhorando o desempenho produtivo e a utilização da dieta. Em seguida, a tese discute sobre o procedimento de amostragem ideal para avaliar a atividade das enzimas digestivas: amilase, lipase, tripsina e quimiotripsina para tilápia do Nilo. Para tal foram realizadas avaliações da atividade das enzimas além de glicose, colesterol e triglicerídeos plasmáticos ao longo de diferentes horas pós-prandiais. No geral, amostrar a porção anterior do intestino no intervalo entre 10 e 12 horas pós-prandiais é um procedimento de amostragem adequado para ser usado em ensaios enzimáticos digestivos para tilápia do Nilo.



Representações esquemáticas: A) Efeitos da alimentação contínua ou não de tilápia do Nilo com farinha de inseto *Zophobas morio* meal (ZM) de alevinos a juvenis. B) Adequação da metodologia para determinação da atividade de quitinase. C) Atividade enzimática determinada nos intestinos dos peixes coletados após diferentes horas pós-prandiais.

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FIRST SECTION - REVIEW

1. OVERVIEW

Nile tilapia (*Oreochromis niloticus*) is a fish natural from the African continent, widely distributed, adapted, with good acceptance of the consumer market and, produced all over the planet (Azevedo *et al.*, 2018). It is a rustic species, of omnivorous alimentary habit, docile, with easy reproduction, high carcass yield, and meat with good organoleptic characteristics, without thorns in "Y" (Boscolo *et al.*, 2001)).

It is the third most-produced fish in the world, over 4500 thousand tons, and Brazil is the fourth in tilapia production (FAO, 2020; PEIXE BR, 2020). Tilapia is the most produced fish species in Brazil representing 57% of fish-farming production in the country. The Southeast region is responsible for 27.5% of Brazil's tilapia production and Minas Gerais is responsible for 8.4% (PEIXE BR, 2020). It is a recognized source of protein in developing countries, containing essential amino acids, essential fatty acids, iron, iodine, vitamin D, and calcium (Mcconnell *et al.*, 2000).

Considering the importance of Nile tilapia in the aquaculture industry, the present thesis concerns two issues with a high demand of knowledge for researchers and producers. The first and third manuscripts highlight the effects of insect meal on Nile tilapia performance and how to continuously fed insect meal-based diets, from fry to juveniles, ameliorates the negative impacts. The second manuscript describe modifications on the original method of chitinase activity measurement, to reflect and be adequate to an omnivorous tropical fish species. The fourth manuscript is attached to the discussion about the ideal sampling procedure for Nile tilapia to capture the activity of digestive enzymes on its best. Thus, a brief theoretical framework was disposed to provide a background and enhance the discussion about the manuscripts.

2. THEORETICAL FRAMEWORK

2.1. Protein sources for Nile tilapia

The omnivorous food habit of the Nile tilapia (*Oreochromis niloticus*) allows the formulation of diets with high inclusions of alternative protein sources instead of fish meal. Even fish do not have requirements for protein but for amino acids, dietary protein content and quality are essential for good development and growth. Diets for initial

developmental phases usually contains high inclusions of protein even for omnivorous fish, over 40-45% for the tilapia for example (El-Sayed and Teshima, 1992; Hafedh, 1999) and, to replace total fishmeal in this phase is not recommended due to the high quality and biological availability of fishmeal amino acids and fatty acids. However, when considering price and sustainability, the replacement of fishmeal should be encouraged, even partially, depending on the fish developmental stage.

Between the ingredients candidates as fishmeal substitute, soybean meal has been used extensively due to its high protein content. Soybean meal is one of the predominant protein sources in commercial tilapia feed, averaging between 20-60% inclusion in the diets (Ng and Romano, 2013). However, its inclusion has adverse effects depending on fish species and food habit, including decrease in growth performance, protein utilization (Krogdahl and Bakke-Mckellep, 2001), nutrients digestibility and absorption (Tacon, 1993) and, intestine inflammation (Krogdahl *et al.*, 2000) and enteritis (Mahmoud *et al.*, 2014). The presence of antinutritional factors in soybean meal such as protease inhibitors, lectins, phytic acid, saponins, and allergens could interfere with food utilization and affect the health and production of animals (Francis *et al.*, 2001). Soybean meal has less favorable protein digestibility, fatty acids, and amino acids profile than fishmeal (Gatlin Iii *et al.*, 2007), being necessary to include synthetic amino acids in the diets, such methionine, and lysine, to prevent impairments on fish productive performance and health (Oliva-Teles, 2012).

Also, soybean production itself is not sustainable, with high costs and demand. Over 50% of grains production is used as a feed ingredient for livestock production, added the humans' consumption and the amount dispensed on exportations. Soybean is a high price commodity influenced by supply and demand. In Brazil, the price applied for soy grain increased over 25% in the last year in Mato Grosso, the main soy grain producer state in Brazil (CONAB, 2020). To avoid damages on fish productive performance and the dependence on feed formulations on an ingredient with such a variable price and market influence, new alternative protein sources for the aquaculture industry should be studied. Considering the nutritive value and, between the possible candidates, protein from animal sources should still be preferable. The inconsistency of by-products composition (Hardy, 1996) impairs at some point its use as a fishmeal substitute. Thus,

as an unconventional source, insect meals has appeared as a viable alternative protein ingredient for livestock feeding.

First of all, considering the environmental impact, to produce a ton of soybean meal it is necessary 2523 m^3 of water (Tschirner and Kloas, 2017). Replacing soybean meal with insect meal lead to over 10% of reduction in the footprint of water use, besides the reductions on land occupation, global warming, and aquatic acidification (AGRI-FOOTPRINT, 2019). In the economic aspect, insect production leads to a fast and high return of investments, and the demand exceeds the supply. Besides, insect farming does not require high technology, management, or training (Govorushko, 2019) and insects does not compete with humans for food. Instead, insects can convert low-grade organic substrates into high-quality protein (Van Huis *et al.*, 2013). Adding to the easy reproduction and high feed efficiency ratio, insects' nutritional composition also highlights its potential. Insects are an accessible source of protein, lipids, carbohydrates, vitamins, and an important source of minerals essential for animals' metabolism such as zinc, iron, and calcium (Payne *et al.*, 2016).

Several reviews have been published pointing out the nutritional value of insect meals for non-ruminants (Sánchez-Muros *et al.*, 2014; Gasco *et al.*, 2019), especially for fish species (Lock *et al.*, 2018; Nogales-Mérida *et al.*, 2018). Lower levels of insect meal inclusion can improve fish performance, especially for omnivorous species (Piccolo *et al.*, 2017; Bruni *et al.*, 2018; Rimoldi *et al.*, 2019) and levels higher than 250g/kg might negatively affect fish growth and digestive capacity (Gasco *et al.*, 2019). The inclusion of insect meals in fish diets is then limited, depending on fish species and food habits, protein ingredients replaced in the diet, and the insect's species and developmental stage.

Between insect species, *Zophobas morio* is a Coleoptera member abundant in tropical countries, that can be feed on byproducts (Costa *et al.*, 2006) with an important role in recycling materials. Its larvae, also known as "super worm", characterized by a leathery light brown exoskeleton with dark rings along the body. They hatch measuring up to 2.5 mm and can reach up to 50 mm before the pupa stage (Huis and Tomberlin, 2016; Kulma *et al.*, 2020). Even for commercial production, *Z. morio* diets can be based on grains such corn, and wheat. This species has great potential for usage as a vehicle to transport nutrients from alternative sources like algae, avoiding negative aspects of vegetal ingredients such as antinutritional factors (Nederlof *et al.*, 2017). Its chemical

composition varies among rearing conditions, but compounds of 92-96% of dry matter, 47-54% of crude protein, 33-40% of crude lipids, 2.5-3% of ash, and 4.5-6% of chitin (Finke, 2002; Barroso *et al.*, 2014; Benzertiha *et al.*, 2019; Fontes *et al.*, 2019). Distinctly from other insects, the chemical composition of superworm seems not to vary among larval stages what could allow its harvest at early stages without negative effects on its composition (Kulma *et al.*, 2020).

The interest in *Z. morio* meal (ZM) as a protein source for livestock feeding has increased nowadays, mainly in non-ruminants as poultry (Benzertiha *et al.*, 2020), piglets (Ji *et al.*, 2016), and most of all, aquatic species (Jabir, M. *et al.*, 2012; Jabir, 2012; Fontes *et al.*, 2019; Mikołajczak *et al.*, 2020). In farmed tilapia, ZM had shown good results of digestibility that, even not comparable with fishmeal in most of the cases ((Jabir, M. *et al.*, 2012), are compatible with soybean meal digestibility. Fontes *et al.* (2019) reported ZM digestibility of dry matter close to fishmeal and soybean meal and, protein and energy coefficients close to soybean meal. Evaluating ZM inclusion in fish diets, optimal growth was observed for Nile tilapia when ZM replaced 25% of fishmeal (Jabir, M. a. R. *et al.*, 2012), and no damages on growth performance were observed for sea trout (*Salmon trutta m. trutta*) fingerling fed with hydrolyzed ZM replacing 40% of fishmeal (Mikołajczak *et al.*, 2020). However, the utilization of ZM as a soybean meal substitute has not been evaluated until now. The closest is the inclusion of *Hermetia illucens* meal in Nile tilapia diets replacing soy protein concentrate (SPC), where levels over 185g/kg of *Hermetia* meal replacing over 50% of the SPC negatively affected feed conversion and protein efficiency ratios (Dietz and Liebert, 2018).

2.1.1. Impacts of insect meals inclusion in aquafeeds

Some authors have reported a reduction in fish growth performance and feed utilization with the dietary inclusion of insect meals. Gasco *et al.* (2019) observed that the substitution of fishmeal by *Hermetia illucens* meal is feasible since in low inclusion levels, without damages on growth performance or feed utilization, as observed for rainbow trout *Oncorhynchus mykiss* (Sealey *et al.*, 2015; Stadtlander *et al.*, 2017; Dumas *et al.*, 2018), Atlantic salmon *Salmon salar* (Belghit *et al.*, 2019), Jian carp *Cyprinus carpio* (Zhou *et al.*, 2018) and European seabass *Dicentrarchus labrax* (Magalhães *et al.*, 2017). Otherwise, fish seems to be more adaptable to moderate levels of *Tenebrio molitor* meal inclusions (Gasco *et al.*, 2019), showing different results between insect species.

The same was observed by Hua (2021) in a recent meta-analysis where those discrepancies were discussed and justified by overestimation of protein content in insect meal, and the influence of amino acids, fatty acids profiles and chitin content.

The amino acid profile of insects is suitable to vary according to insect species and developmental stage but, in general, are comparable to soybean meal but inferior to animal proteins (Barroso *et al.*, 2014; Payne *et al.*, 2016). The amino acid profile of *Z. morio* meal, for example, seems to not be enough to reach the nutritional requirement of most species evaluated in methionine and cystine, including Nile tilapia (Nogales-Mérida *et al.*, 2018). However, insects' amino acid profile can be modulated by the diet, as for *Z. morio* fed with algae concentrate (Nederlof *et al.*, 2017) for example. Even fish has requirements on amino acids and not on protein quantity, the concept of protein in insect meals needs to be actualized, since a considerable amount of the nitrogen measured by the standard method of KJEDAHL (1977) comes from chitin and should not be accounted as protein. The amount of chitin bounded to nitrogen correspond to 5-6% of total nitrogen (Barker *et al.*, 1998; Finke, 2002). A distinct nitrogen-to-protein correction factor was proposed by Janssen *et al.* (2017) considering insect species and stage of development, and its use may promote a drop of over 50% on the real protein content of the insect meal.

According to fatty acids composition, *Z. morio* is one of the insect species with higher content of lipids (Barroso *et al.*, 2014). Kulma *et al.* (2020) reported as the dominant fatty acids in superworms: oleic, palmitic, linoleic, and stearic acids, followed by linolenic, palmitoleic, myristic, and margaric acids. In general, insects are rich in n-6 polyunsaturated fatty acids, poor in the n-3 series and deficient on the metabolic essential fatty acids, eicosapentaenoic and docosahexaenoic acids (EPA and DHA). While total n-3 in fishmeal is around 16% and 5% in soybean meal, in the main insect meals it doesn't reach 3% (Nogales-Mérida *et al.*, 2018). However, the fatty acid profile of insects can be modulated by the composition of their diet. An increase in the content of n-3 fatty acids was obtained by feeding *H. illucens* larvae fed with fish offal (Pasotto *et al.*, 2020). Thus, it is necessary attention to the dietary amino acid composition and fatty acids profile when replacing protein sources with insect meals, since they are essential nutrients involved in several metabolic processes in fish, current affecting growth performance, nutrients digestibility, absorption, and utilization, and fish immunity.

The negative results on fish growth performance due to insect meal dietary inclusions are also related with the lower nutrients digestibility of insect meal diets. The decrease in diet digestibility was observed by several authors with distinct fish and insect species, as for yellow catfish (*Pelteobagrus fulvidraco*) fed with up to 169 g/kg of black soldier fly (BSF) meal (Hu *et al.*, 2017), Siberian sturgeon (*Acipenser baerii Brandt*) fed diets with 185 to 375 g/kg of BSF meal (Caimi *et al.*, 2020), gilthead seabream (*Sparus aurata*) fed with mealworm meal at 250 and 500 g/kg (Piccolo *et al.*, 2017), red tilapia (*Oreochromis spp.*) fed 300g/kg of *Z. morio* meal (Jabir, M. *et al.*, 2012) and Atlantic salmon, European seabass, gilthead sea bream (*Sparus aurata*) and rainbow trout fed with *T. molitor* and *H. illucens* meals at 400-600g/kg (Gasco *et al.*, 2019). However no differences or even improvements in nutrients digestibility were observed by Belghit *et al.* (2018) in Atlantic salmon fed with 600 g/kg of BSF meal, *O. mossambicus* fed diets with up to 240g/kg of *Imbrasia belina* meal (Rapatsa and Moyo, 2017) and *S. salar, D. labrax* and *O. mykiss* fed with *T. molitor* and *H. illucens* meals at 147.5 - 250 g/kg of inclusion (Gasco *et al.*, 2019).

Besides differences among fish species and, the nutritive value of insect species, inclusions of high levels of insect meal generally promote negative effects on nutrients digestibility, mainly due to the chitin content (Henry *et al.*, 2015; Belforti *et al.*, 2016; Piccolo *et al.*, 2017; Belghit *et al.*, 2018; Li *et al.*, 2019). Chitin is a linear biopolymer polysaccharide with monomer β-1,4-N-acetyl-D-glucosamine unit, abundantly found in the cuticle of insects and, insoluble in most of the solvents (Kumirska *et al.*, 2010). The digestion of the chitin involves catalysis reactions promoted by a complex of chitinolytic enzymes. Endochitinases that breakdowns chitin releasing low size particles such as dimers, oligomers, and few monomers. The exochitinases chitobiosidases and 1-4-β-glucosaminidases realize the breakdown of those dimers and oligomers into the monomer N-acetylglucosamine. Between those enzymes, chitinase or endochitinase (E.C 3.2.1.14) is the key enzyme of this process since it realizes the first catalytic breakdown of dietary chitin.

Even some authors have reported the absence of chitinase in monogastric (Sánchez-Muros *et al.*, 2014; Piccolo *et al.*, 2017) its presence in Nile tilapia have already being reported (Molinari *et al.*, 2007) in the stomach, intestine, and serum. As an omnivorous species, tilapia naturally have sources of chitin in their diets such as aquatic

larvae, insects, and algae. Then, the existence of chitinolytic activity in tilapia is reasonable, and so its capacity to digest chitin from insect meals was already reported (Fontes *et al.*, 2019). Once chitin is digested, its biding propriety or undigested characteristic disappears and, its derivates are release into the fish gastrointestinal tract reacting with fish cells and microbiota.

The effects of chitin and its derivates as prebiotic modulating fish gut microbiota (Bruni *et al.*, 2018; Terova *et al.*, 2019) and as immunostimulants have been reported by other authors (Brinchmann *et al.*, 2011; Henry *et al.*, 2015; Henry *et al.*, 2018). In general, chitosan is associated with antimicrobial effects with mechanisms well described by (Verlee *et al.*, 2017). Insects contain also other polysaccharides such as silkose or dipterose which can exert immunostimulant activity in mammals (Ohta *et al.*, 2014; Ohta *et al.*, 2016). Antioxidant propriety of chitin and its derivates has also been reported (Ngo and Kim, 2014; Dutta, 2016; Li *et al.*, 2017; Zielińska *et al.*, 2018; Li *et al.*, 2019) where the inclusion of insect meal increased the activity of catalase, one of the enzymes responsible for the removal of reactive oxygen species (ROS) and to protect the fish organism against damages. Insects also have antimicrobial peptides (AMPs) that besides have a growth promoter effect (Xiao *et al.*, 2015), contribute to microbiota well balance acting against potentially pathogenic bacteria, fungi, parasites, and viruses (Yi *et al.*, 2014).

Hua (2021) highlight that chitin alone cannot explain the adverse results on nutrients digestibility of insect meal inclusions and that, the nutritional value of the insect meal, mainly amino acids and fatty acids composition, the use of previous treatments on the insect meal like defeating and, other components present on insect meals could be the major factors. Thus, it is important to highlight that insect species and developmental stage, fish species, and food habit, as well as the protein ingredient replaced in the diet, are factors capable of promoting adverse results on the parameters evaluated, such as growth performance, body composition, digestive enzymes activity, and dietary digestibility. At the present, the insect meals have an excessive cost and are not competitive in the market to the other protein sources as the soybean meal, mainly due to non-consistent industrial production. The European regulation that approves the use of processed animal protein derived from insect species in aquaculture is recent (EU 2017/893). However, after that date, the financial support from the government or private

sources for research and production of insect meal increased considerably, even more, due to the low footprint that the activity represents on the environment. Thus, more competitive prices are expected for the next years.

2.2. Digestive enzymes activity and its applications

The processes involving nutrient digestion, absorption and metabolism in fish are highly complex, and depend of a variety of factors such food habit, body temperature (Kapoor *et al.*, 1976), nutritional status, stress condition and diet composition (Duodu *et al.*, 2019). After ingestion, dietary nutrients undergo to physical and chemical processes before being absorbed and metabolized by fish organism. Whereas the physical process includes chewing, crushing, pulsating, and chyme churning, the chemical process involves the secretion of substances like hydrochloric acid and bicarbonate, and the action of many digestive enzymes. The digestive enzymes are nutrient-specific and catalyze the hydrolysis of nutrients into small size particles that would be later absorbed by fish intestine. The secretion and/or activation of those digestive enzymes are controlled by a complex diffuse neuroendocrine system and can be modulated by endogenous and exogenous factors, such as fish nutritional status (Pereira *et al.*, 2017; Tamadoni *et al.*, 2020), body temperature (Kapoor *et al.*, 1976), stress condition (Duodu *et al.*, 2019), intestinal transit time, food habit and diet composition (Fountoulaki *et al.*, 2005; Caruso *et al.*, 2009; Krogdahl *et al.*, 2011).

Considering the complexity of the fish digestive system and how it can be affected by intrinsic and extrinsic factors, the study of digestive enzymes become essential for understanding fish response against adversities such as changes in water temperature (Hani *et al.*, 2018), photoperiod (Guerra-Santos *et al.*, 2017), dietary composition (Mohammadi *et al.*, 2020) and feed management (Furne *et al.*, 2008; Thongprajukaew *et al.*, 2017; Tamadoni *et al.*, 2020). The activity of digestive enzymes has been investigated also to predict the digestibility of new ingredients (Oliveira *et al.*, 2020) and to understand the nutritional requirements of fish species (Kolkovski, 2001).

The presence of digestive enzymes in the mouth and esophagus is scarce and, even several digestive enzymes were isolated from fish stomachs, their efficacy remains unclear (Chakrabarti *et al.*, 1995). Then, the intestine is the main place of enzymatic digestion in fish. Intestine digestive enzymes can be classified into two groups: (i)

secreted by the pancreas as zymogens and released into fish intestine and, (ii) enzymes attached to the intestine microvilli. The enzymes belonging to the first group represent the main and first chemical breakdown of dietary nutrients. Amylase, lipase, trypsin, and chymotrypsin are examples of digestive enzymes synthesized by the pancreas and secreted into the proximate portion of the fish midgut (Formicki and Kirschbaum, 2019). Whereas amylase and lipase are secreted on their active form, trypsin and chymotrypsin are secreted as zymogens.

Amylase (E.C.3.2.1.1) is the most important enzyme on carbohydrate breakdown and is involved in the most challenged vial in aquaculture nutrition: the use of carbohydrates in fish diets (Maas *et al.*, 2020). Amylase acts attacking α 1, 4 linkages of starch and glycogen, converting them into small molecules of oligosaccharides, maltose, and isomaltose that will be later reduced to glucose by the brush-border enzymes. Fish seems to possess multiple amylase genes arranged into different amylase families (Krogdahl and Bakke-Mckellep, 2005). Besides the pancreas, amylase may also be produced by the microflora of the digestive tract (Sugita *et al.*, 1991). However, fish amylase cannot digest linkages β 1, 4 from cellulose, and α 1, 6 from starch what limits the utilization of carbohydrates in aquafeeds (Moraes and De Almeida, 2020). Also, the occurrence of amylase inhibitors has been reported and seems to be species-specific (Krogdahl and Bakke-Mckellep, 2005).

Lipases (EC 3.1.1.3) refer to a group of enzymes that catalyzes the breakdown of lipids together with colipases and after the formation of lipid droplets by bile salt (Smichi *et al.*, 2012). They act hydrolyzing the ester linkage of lipids molecules into triglycerides, phospholipids, cholesterol esters, and vitamins (Wong and Schotz, 2002), and have an affinity to the position *sn*-1,3 of acylglycerols. Added to the pancreatic lipase, the activity of other lipolytic enzymes were already described in fish such as phospholipases and esterase (Guillaume and Choubert, 2001). Thus, it is hard to determinates where fish lipases are secreted since they tend to be nonspecific and have a similar affinity for triacyl glycerides with different fatty acids (Moraes and De Almeida, 2020).

Trypsin and chymotrypsin are the major enzymes in the breakdown of proteins. Trypsin (EC 3.4.21.4) has specificity by the breakdown of peptide bonds of aromatic L-amino acids like lysine and arginine. The hydrolysis occurs by the attack of a serine hydroxyl group on the peptide with the formation of an ester bond between the carboxyl

group and the serine hydroxyl. The hydroxyl group is activated by histidine and aspartate, forming the intermediate "catalytic triad". This intermediate is hydrolyzed by a water molecule, regenerating the hydroxyl and releasing the carboxylic acid. At least six trypsin isozymes were observed in the Nile tilapia intestine, however, its correlation to dietary protein and fish age was not established (Unajak *et al.*, 2012). Besides the pancreas, the synthesis of trypsin was reported in the pyloric caecum of fish (Marcuschi *et al.*, 2010).

Chymotrypsin (EC 3.4.21.1) exists in three inactive forms (chymotrypsinogens A, B, and C) in the zymogen granules of the pancreas, however, only types A and B have been found in fish (Yang *et al.*, 2009). Chymotrypsin hydrolyzes peptides bonds of various α-amino acid carboxyl groups, with a preference for tyrosine, tryptophan, and phenylalanine residues (ZHOU (Zhou *et al.*, 2011). The hydrolysis starts with the substrate binding with the chymotrypsin structure. The side chain Ser-195 and His-57 hydrolyze the substrate forming an intermediate. Then, solvent water cleaves the intermediate to form carboxylic acid and reforms the enzyme (Kallies and Mitzner, 1996). The activity and concentration vary according to fish species and the environment in which the fish live (Von Elert *et al.*, 2004; De La Parra *et al.*, 2007). Even chymotrypsin acts in a large spectrum, trypsin activity is used as a nutritional indicator as it is responsible for the activation of other enzymes zymogens. The first trypsinogen is activated by enteropeptidase in fish intestine, and later, a small amount of activated trypsin activates more trypsinogens and all other pancreatic zymogens including chymotrypsinogen (Moraes and De Almeida, 2020).

The control of digestive enzymes release starts with the activation of the hormone cholecystokinin by the presence of amino acids and lipids into fish intestine that triggers the release of pancreatic enzymes and bile salts into the fish intestine (Krogdahl *et al.*, 2011). Thus, the activity of the digestive enzymes will be dependent on the gastric transit time, which in turn is dependent on fish species and food habit, fish size, amount of food, and type (Caruso *et al.*, 2009; Krogdahl *et al.*, 2011). Gastric evacuation, for example, takes longer time for carnivorous fish than herbivorous (Guillaume and Choubert, 2001). Both, activity patterns on the post-feeding period and distribution site influence the utilization of dietary nutrients. Therefore, it is extremely important to consider the differences existing among fish species when evaluating the activity of digestive enzymes.

2.2.1. Sampling procedures for enzymatic activity measurement

The study of digestive enzymes and information available for tropical fish species are very scarce when compared to the marine cold-water species, mostly with carnivorous food habits. The absence of basic knowledge regarding tropical fish species can negatively impact future researches and the whole production system. Understanding the interrelationship between nutrients, enzymatic activities and the digestive process would not only improve the profitability of fish farming but also would contribute to reducing environmental footprint. To determine the activity of digestive enzymes is a common procedure of a wide range of studies regarding physiological and nutritional aspects for aquatic animals. However, the inconsistence of sampling procedures such as the tissue sampled, fish nutritional status, and laboratory techniques (Hidalgo *et al.*, 1999), restrains the interpretation of the results in a better holistic and diffuse scientific view.

Recommendations of intestine sampling for enzymatic assays were done only for carnivorous species. Krogdahl and Bakke-Mckellep (2005) examined the effects of fasting and refeeding on digestive enzyme activity at different regions of the gastrointestinal tract of Atlantic salmon (*Salmo salar L.*). The authors have concluded that sampling should be performed only when intestines are feed-filled to avoid bias because of fasting. The presence of chyme into fish intestine allows maximum contact of the enzymes with their respective substrates. This recommendation has been confirmed later by Castro *et al.* (2015) that observed very low enzymatic activities for the carnivorous European sea bass (*Dicentrarchus labrax*) 24 hours postprandial and suggested the range between 2 and 6 hours postprandial for intestine enzymatic analysis. Suggestions of Krogdahl and Bakke-Mckellep (2005) and Castro *et al.* (2015) have been followed by trials with carnivorous fish (Diógenes *et al.*, 2018; Guerreiro *et al.*, 2018; De Moura *et al.*, 2019).

For omnivorous species, recent studies evaluating digestive enzymes activity have opted to adopt 24 hours post feeding (HPF) before intestine sampling, such as for common carp *Cyprinus carpio* (Zhao *et al.*, 2020), catfish *Ramdia quelen* (Ha *et al.*, 2019), and Nile tilapia (Santos *et al.*, 2016; Dawood *et al.*, 2020; Deng *et al.*, 2020; Mohammadi *et al.*, 2020). However, in Nile tilapia, gastric evacuation is around 8 HPF (Riche *et al.*, 2004; Heng *et al.*, 2007; Uscanga *et al.*, 2010). Then, considering Krogdahl and Bakke-Mckellep (2005) recommendations and, the existence and importance of interaction

between transit time and digestive enzymes activity, it is not possible to affirm that the approaches for intestine sampling adopted in previous literature for Nile tilapia reflect the real digestive enzymes' capability to breakdown dietary nutrients. Until now, no research was performed to evaluate the postprandial pattern and intestine site of amylase, lipase, trypsin, and chymotrypsin activities for Nile tilapia, which contributes to the difficulty of comparing enzymatic activities among studies and species.

The efficacy of the digestion process depends on the existence and activity of those digestive enzymes at proper sites in fish gastrointestinal tract. It is well established that the distribution of digestive enzymes along fish intestine varies among fish species and fish food habits (Onish, 1976; Takii, 1985; Uys *et al.*, 1987). The anatomy of the gastrointestinal tract is an important aspect that will lead to variations in digestive enzyme distribution and activity between species and food habits. For example, carnivorous fish have digestive enzymes highly activated along the whole intestine to overcome the short straight intestine, whereas in omnivorous species, with long-coiled intestine, the activity of digestive enzymes seems to concentrate in the anterior portion of the fish midgut (Jun-Sheng *et al.*, 2006; Castro *et al.*, 2015; Gioda *et al.*, 2017; Oliveira *et al.*, 2020). However, enzyme distribution can also vary according to the enzyme itself. Whereas the activity of amylase was reported throughout the whole gastrointestinal tract of many fish species (Krogdahl and Bakke-Mckellep, 2005), chymotrypsin and trypsin activities were much lower in the distal than in the proximate section of the Nile tilapia intestine (Uscanga *et al.*, 2010).

It is extremely difficult to differentiate intestine portions in fish without histological analysis. Early histological studies have reported only two divisions for Nile tilapia intestine: anterior portion referring to three-quarters of the total length of the intestine and, a posterior portion, including the presence of ileorectal valve (Morrison and Wright Jr, 1999). However, the inexistence of standard sampling procedures for digestive enzymatic assays led to variations on the intestine site used in such analysis. Some authors have adopted the division of fish intestine in half and expressed the results in anterior or posterior intestine sections for different fish species (Uscanga *et al.*, 2010; Castro *et al.*, 2015; Santos *et al.*, 2020). The division of fish intestine into three portions: anterior, mid, and distal, has also being adopted by several researchers (Gioda *et al.*, 2017; Diógenes *et al.*, 2018; De Moura *et al.*, 2019; Oliveira *et al.*, 2020), while others have opted by

sampling the entire intestine (Santos *et al.*, 2016; Guerra-Santos *et al.*, 2017; Thongprajukaew *et al.*, 2017; Ha *et al.*, 2019). The size of fish is also relevant and should be considered when determining the sampling location, as for small fish or in early developmental phases, it is necessary to use the entire intestine to reach significative sample amount (Hani *et al.*, 2018; Tamadoni *et al.*, 2020). Then, to perform an accurate evaluation of the activity of digestive enzymes it is necessary to understand fish digestive physiology and anatomy, to capture the right moment after feeding and, at the right sampling location where those enzymes will exhibit their maximum activity.

3. GENERAL CONSIDERATIONS

The idea of replacing non sustainable sources in aquatic feed formulation is a worldwide continuous and necessary search, mainly regarding fishmeal substitution. While advances have being made on the use of plant protein ingredients, negative impacts of their dietary use in high concentrations have also being reported. Not only for fish organism or growth performance but also for the environment. The production of those plant ingredients has behind it large footprint remains. Then, to be considered as good candidates nowadays, new alternative ingredients must have nutritional value and reduced environmental impact. That is the case of insect meals. To avoid the mainly limitation to insect meal use in aquafeeds, its chitin content, a feeding strategy will be presented in the first two manuscripts. Besides changes on dietary formulation or inclusion of feed additives, the main idea was to evaluate the capability of the fish itself to adapt its physiological systems to the new ingredient, allowing high dietary inclusions without negative impacts on fish productive performance.

Basic knowledge is always necessary and should be worked carefully and with more incentive between research groups. Even more the advance in technology and communication sciences are favoring the use of high tech in biological studies. However, standardization on experimental procedures according to the aquatic species are missing, even more because aquatic species have many anatomical and physiological differences promoted by environmental adaptation. So, the third and fourth manuscripts in the present thesis will show how important is to make adaptations on sampling procedures and laboratorial methodologies according to the fish species. The modifications proposed on sampling procedures for the determination of intestine digestive enzymes activity and on the methodology for chitinase activity measurement for Nile tilapia respects fish

physiological and anatomical differences allowing to express its greatest potential and truth reality.

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SECOND SECTION - MANUSCRIPTS

5. MANUSCRIPT 1

To be submitted to: **Aquaculture**, **ISSN: 0044-8486**

Nutritional programming improves *Zophobas morio* meal utilization by Nile tilapia (*Oreochromis niloticus*) juveniles

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Abstract

To understand the influence of nutritional programing on the growth performance and feed utilization of Nile tilapia juveniles feeding high insect meal diets, two trials evaluated the effects of feeding tilapia fry with *Z. morio* meal on fry survival, growth performance, feed utilization and carcass composition; and on juveniles feeding increasing *Z. morio* meal diets growth performance, feed utilization, carcasses and fillet composition. In experiment 1, triplicate groups of tilapia fry (0.30 ± 0.04 g mean weight) were fed experimental diets with or without ZM inclusion for 60 days. Then, three diets were formulated to contain 0, 150 and 300 g/kg of ZM inclusion replacing up to total dietary soybean meal. In experiment 2, male tilapia (3.1 ± 0.08 g mean weight) from both groups of experiment 1 were reallocated in a factorial design 2×3, fry feeding (with or without ZM) and experimental diets (0, 150 and 300 g/kg ZM) and fed with the experimental diets for 60 days. Feeding tilapia fry with ZM increased survival, weight gain, feed utilization and carcass protein, energy and lipids. Juveniles fed with ZM during fry stage had better feed intake and feed conversion ratio (FCR). The introduction of ZM during fry stage improved weight gain, FCR and carcass weight of juveniles fed the 300 ZM diet. The nutritional programming with early insect meal diets optimizes dietary utilization, improves fish productive performance and allows inclusions of up to 300 g/kg of *Z. morio* meal in Nile tilapia diets.

Keywords: chitin, nutritional programming, omnivorous, superworm

Introduction

Insect meals are alternative protein ingredients that have received attention in diets formulations due to the protein quantity and quality, potential prebiotic effects (Pinotti, Giromini et al. 2019) and absence of protease inhibitor, phytohemagglutinin and anti-vitamins (El-Sayed et al., 2006), potential antinutritional factors commonly present in plant protein ingrediets. Between the insect meals, *Zophobas morio* meal has an attractive amino acids profile capable to attend fish amino acid requirements (Barroso, de Haro et al. 2014, Henry, Gasco et al. 2015). Its chemical composition can alter depending on the feed and developmental stage but, in general, crude protein varies between 47-50%, crude fat between 33-40%, (Jabir, M. *et al.*, 2012; Fontes *et al.*, 2019; Benzertiha *et al.*, 2020) and chitin between 4-22% depending on the methodology applied (Fontes *et al.*, 2019; Benzertiha *et al.*, 2020). The high chitin is the main factor that limits the inclusion of insect meals in fish diets, leading to reductions on fish growth performance and feed utilization (Barroso, de Haro et al. 2014, Józefiak, Nogales-Mérida et al. 2019). The damages are usually linked to the absence or inefficiency of chitinolytic enzymes responsible to digest insects' chitin (Sánchez-Muros *et al.*, 2014; Piccolo *et al.*, 2017).

Nile tilapia is the third most-produced freshwater fish species worldwide and first in Brazil (FAO, 2018). It is a rustic tropical species, well accepted by the consumer market. Due to its omnivorous food habit, tilapia is adapted to several production systems and feed ingredients (Azevedo *et al.*, 2018). The presence of chitinase and the capacity to digest chitin have already being reported for this species (Molinari, Pedroso et al. 2007, Fontes, de Oliveira et al. 2019), potentialized by its natural feeding type, especially during early developmental stages (Muzzarelli 1999). Although positive results have been reported for Nile tilapia (*Oreochromis niloticus*) fed diets with inclusions up to 150g/kg of *Z. morio* meal replacing 50% of fishmeal, higher levels of insect meal inclusion were not well supported (Jabir *et al.*, 2012). As fish get bigger and start feeding exclusively dried formulated diets that do not contain any source of chitin, missing on chitinase activity might occur due to dismissed use.

Nutritional programming is based on the nutrition as an exogenous *stimuli* in which the animal is exposed during critical developmental stages with consequences on physiological functions, persistent in later life (Reynolds *et al.*, 2015). The use of nutritional programming strategies affects fish physiology specially in the early

developmental stages due to the high plasticity of some tissues (Mcmillen and Robinson, 2005). This plasticity allows changes on the animal epigenetics with consequent physiological and morphological modifications over exogenous or endogenous challenge conditions. In fish, recent studies indicate the possibility to tailoring metabolic pathways during young development stages for better use of new ingredient-based diets later in life (Panserat *et al.*, 2019; Hou and Fuiman, 2020). A recent study has successfully used nutritional programming to improve the metabolic use of carbohydrate for zebrafish (*Danio rerio*) (Kwasek *et al.*, 2020) however, the evaluation of nutritional programming on the usage of insect meal is inexistent until now.

Therefore, the current study was designed to understand the influence of nutritional programing on the growth performance and feed utilization of Nile tilapia juveniles feeding high insect meal diets.

Material and Methods

The project was conducted at the Fish Culture Station at the Federal University of Lavras (UFLA) and the Central Laboratory of Animal Research (LCPA/DZO), in Lavras, Minas Gerais, Brazil. All experimental procedures were approved by the Ethical Commission of Animal Use (CEUA) of the Federal University of Lavras (UFLA), protocol number 041/18.

Two separated trials evaluated the effects of feeding tilapia fry with *Z. morio* meal on fry survival, growth performance, feed utilization and carcass composition; and on juveniles after fed increasing *Zophobas morio* meal diets, on growth performance, feed utilization, and carcasses and fillet chemical composition.

1.1.Experimental diets

In experiment 1, two isoproteic and isoenergetic diets (410 g/kg of crude protein and 19.3 KJ/kg of gross energy) were formulated to determinate the effects of *Z. morio* meal inclusion in diets for Nile Tilapia fry: a control diet, without *Z. morio* meal (Without ZM); and a stimulus diet containing 250 g/kg of *Z. morio* meal (With ZM). In experiment 2, three challenge diets were formulated to be isoproteic (270 g/kg digestible protein), isoenergetic (14.1 KJ/kg of digestible energy) and with increasing ZM levels (0, 150, and 300 g/kg) replacing up to total soybean meal and soybean oil. Apparent digestibility

coefficients of ingredients were based on data from Fracalossi and Cyrino (2013), Pezzato *et al.* (2002), and Fontes *et al.* (2019). The proximate and chemical composition of experimental diets are shown in Table 1.

The experimental diets were prepared by grinding all dry macro ingredients in a hammer mill (TRF-400 Trapp, Jaraguá do Sul, SC, Brazil) to a powder (0.5 mm sieve). Micro and macro ingredients were manually mixed. Hot water (50°C) was then blended into the mixture to attain a consistency appropriate for pelleting. The diets were pelleted in an electric meat grinder (1 mm diameter) (Tecnal, Piracicaba, SP, Brazil) and then dried in a forced recirculation oven at 50°C for 24 h. Pellets were ground in a hammer mill (TRF-400 Trapp), passed through a 2.0–2.5 mm sieve (Technal), and then stored at -20°C until used.

1.2.Fish and culture conditions

1200 Nile Tilapia (*O. niloticus*) fry were obtained from the Fish Culture Station – UFLA and acclimatized for 15 days upon arrival to the experimental facilities. During this period, fish were fed a commercial diet (Pira 40, Guabi, 400 g/kg of crude protein) three times daily.

1.2.1. Experiment 1

Nile Tilapia fry (0.30 ± 0.04 g mean weight) were randomly distributed in six circular tanks (500 L) at a density of 200 fry/tank. The tanks were maintained in a recirculated system supplied with aeration and the temperature was kept constant by heaters (26 °C). Each tank was considered as an experimental unit, arranged in a completely randomized design with two treatments (without ZM and with ZM) and three replicates. The water quality was maintained using supplemental aeration (central line and air diffusers), mechanical and biological filtration.

The dissolved oxygen and pH were monitored daily using a multiparameter (U-10, Horiba, Kyoto, Japan). Total ammonia and nitrite were measured using commercial kits (Labcon Test Fresh Water Toxic Ammonia and Labcon Test Nitrite NO^{2-}). The fish were fed with experimental diets for 60 days, three times daily (8:00, 12:00, and 16:00h), until apparent satiation.

1.2.2. Experiment 2

To set up the trial, male juveniles (3.12 ± 0.08 g mean weight) from both groups of experiment 1 (without ZM and with ZM), were randomly distributed in 24 circular tanks (150 L) at a density of 12 fish/tank. Each tank was considered as an experimental unit, and the experiment was lain out in factorial design 2×3 (two fry feeding and three experimental diets) and four replicates.

The water quality was maintained using supplemental aeration (central line and air diffusers), mechanical and biological filtration, and UV lamps. The water temperature was controlled with a heat exchanger (26 °C) and measured twice daily. Water quality was monitored daily, as described in experiment 1.

Fish were fed a fixed ration close to satiation two times per day (at 8:00h and 14:00h). The daily ration was adjusted throughout the trial to account for growth (fish were bulk weighed by tank biweekly), decreasing from 70 to 50 g/kg body weight/day (BW/d) over 60 days.

1.3.Sampling

At the end of experiment 1, fish were fasted for 24 hours and, the number of fish left in each tank were counted and weighed to measure the parameters: Weight Gain = 100*(Final Body Weight (FBW) - Initial Body Weight (IBW))/IBW; Feed Intake (FI) = Total Feed Intake (g dry matter); Feed Efficiency (FE) = Wet Weight Gain/ Dry Feed Intake; Specific Growth Rate (SGR) = [(log FBW – log IBW) / Period (days)]*100; Protein Efficiency Ratio (PER) = Wet Weight Gain/Crude Protein Intake; Survival Rate (SR %) = 100 – [(Number of fish stocked – Number of fish harvested/Number of fish stocked*100)]. Fish carcasses were sampled from three fish from each tank and stored at -20 °C until future analysis.

At the end of experiment 2, all the fish were fasted for 24 hours and weighed. Six fish from each tank were gutted for measurement of Weight Gain = 100*(Final Body Weight (FBW) - Initial Body Weight (IBW))/IBW; Feed Intake (FI) = Total Feed Intake (g dry matter); Feed Conversion Ratio (FCR) = Dry Feed Intake/ Wet Weight Gain; Specific Growth Rate (SGR) = [(log FBW – log IBW) / Period (days)]*100; Daily Growth Index (DGI) = 100*($FBW^{1/3}$ - $IBW^{1/3}$)*$Days)^{-1}$.Protein Efficiency Ratio (PER) = Wet

Weight Gain/Crude Protein Intake; Carcass Yield (CY) = (Carcass Weight/FBW) *100; Viscera Somatic Index (VSI) = (Viscera Weight/FBW) *100; Hepatic Somatic Index (HSI) = (Liver Weight/FBW) *100; Vicera Fat Index (VFI) = (Vicera Fat Weight/FBW) *100. Fillet and carcasses were sampled from three fish from each tank and stored at -20 °C until future analysis.

1.4. Chemical Analysis

Chemical analyses of the ingredients, diets, carcasses, and fillet were determined according to the Association of Official Agricultural Chemists methodology (Horwitz *et al.*, 2012) for dry matter (930.15), crude protein (968.06), and ash (942.05). The chemical composition of the diets is shown in Table 1 and, amino acids composition of ingredients in Table 2. Crude lipid was quantified following Folch's (1957) methodology. Total carbohydrates and gross energy were estimated using the Atwater general factor system (Jobling, 2012) in which gross energy (KJ/Kg) was defined as the sum of the total protein, lipid, and carbohydrate multiplied by their respective conversion factor, based on the heat generated after combustion. Crude fiber and neutral detergent fiber were calculated based on data obtained from INRA (2020), Finke (2002) and Benzertiha *et al.* (2019). Chitin was determined following the spectrophotometric method from Tsuji et al. (1969) and Han and Heinonen (2020). The glucosamine (GlcN) from chitin hydrolysate was deaminated and color reacted with MBTH. The absorbance was read at 650 nm and the content of chitin calculated using an external standard curve with GlcN-HCl.

1.5. Statistical Analysis

Data are expressed as mean ± pooled standard error of the mean (SEM). Normality and homogeneity of variances were tested using the Shapiro-Wilk and Levene tests, respectively. All statistical procedures were performed using the SPSS software package for Windows (IBM® SPSS® Statistics, New York, USA) and probability level of 0.05 was used for rejection of the null hypothesis. In experiment 1, data was analyzed by Student's *t*-test. Statistical analysis of experiments 2 was done by two-way ANCOVA, with fry feeding and experimental diets as factors and the initial body weight as covariable. When necessary, Tukey's test was used to detect differences in fry feeding among experimental diets within each dietary treatment and among dietary ZM levels within each fry feeding group.

2. Results

2.1.Experiment 1

The growth parameters and feed efficiency are presented in Table 3. Feeding Nile tilapia fry with 250 g/kg ZM improved the FBW, WG, FE, PER and SR. No differences were observed for FI and SGR. The FBW, WG, FE and SR were all higher for fish fed with ZM than for those fed the diet without ZM.

The proximate composition of the fish carcass is shown in Table 4. The dietary inclusion of ZM improved the content of protein, energy, and lipids in tilapia carcasses. No differences were observed for dry matter and ash contents.

2.2.Experiment 2

2.2.1. Growth performance and feed utilization

The data of juveniles' productive performance and feed utilization are presented in Table 5. The parameters FBW, FI, WG, SGR, DGI, FCR and PER were affected by both fry feeding and ZM levels. The inclusion of ZM at 150 g/kg improved FBW, FI, WG, SGR, DGI, FCR and PER of Nile tilapia juveniles from both fry feeding groups, except the feed intake of fish fed with ZM at fry stage, that remained the same from 0 to 150ZM diet. However, the high insect meal diet (300 g/kg ZM) promoted distinct results in fish depending on the fry feeding. Fish that were not fed with ZM during fry stage had reduced FBW, WG, SGR, DGI, PER, and worse FCR. Otherwise, the introduction of ZM during fry stage improved FBW, FI, WG and DGI, and did not influenced the parameters of SGR, FCR and PER that did not differ between the 150 and 300ZM diet. The parameters of CY, VSI, HSI and VFI were not affected by fry feeding or dietary ZM levels.

The intake of high insect meal diet (300ZM) had increased the content of dry matter in fish fillet in both fry feeding groups (Table 6). Carcass composition was altered only on the content of protein. The increasing levels of ZM inclusion improved the content of protein in fish carcasses within the group not fed with ZM during fry stage. Otherwise, the content of protein in the carcasses of fish that were fed with ZM during fry stage were not influenced by dietary inclusion of ZM, however were higher than the non-fed group for the 0 and 150ZM diets (Table 6).

3. Discussion

3.1. Experiment 1

To the best of our knowledge, this is the first study that evaluated the effects of insect meal on fish in the early developmental stage. The results obtained affirm the potential use of *Z. morio* meal as a protein source for Nile tilapia fry, improving growth performance, feed utilization, carcass composition and fish survival. Tilapia fry survival is one of the most important traits that affects productivity and economic return for the aquaculture industry. Fish at this stage are more susceptible to various diseases associated with high mortality which generally averages 40% (Boyd, 2004). In the present study, fry survival was improved on 7.4% when fish were fed the diet with ZM. Using the bio-economic model proposed by Kankainen *et al.* (2012), that improvement could lead to a significant extra of $46.500, for the production of thousand tons of tilapia per year. The presence of specific substances in insect meals associated with immunity and gut health benefits may have improved fish survival and development.

Several studies have reported the immunostimulant effect of insect meal for different fish species and the reasons are related to specific substances found on insect composition like chitin, antimicrobial peptides and lauric acid (Gasco *et al.*, 2018). These components can stimulate the immune system and/or exert antimicrobial effects improving fish defense against pathogens (Mahlapuu *et al.*, 2016; Elieh Ali Komi *et al.*, 2018; Han *et al.*, 2018). In addition, insect meal has shown to influence stress gene response in fish (Stenberg *et al.*, 2019), however further studies are needed to elucidate the bioactive compounds and pathways.

According to the effects of insect meal on intestine health, modulation of gut microbiota has been reported in fish fed diets with insect meal (Bruni *et al.*, 2018; Rimoldi *et al.*, 2019). These modifications were observed to be beneficial by protecting fish from pathogens and enhancing welfare, physiological functions and digestion (Bruni *et al.*, 2018). One of the pathways would be through fermentation of undigested chitin by fish microbiota and synthesis of short-chain fatty acids (Ghanbari *et al.*, 2015; Terova *et al.*, 2019). The enhancement of intestine health could benefit the utilization of dietary nutrients, improve digestion and absorption processes and positively affect fish productive performance, once immune system, gut microbiota and diet can interact with each other and impact on animal growth performance (Kogut and Arsenault, 2016).

Results of the effects of dietary insect meal on growth performance and feed utilization of fish in early stages are very scarce. In the present study, fish fed with ZM had better FCR and WG comparing to the control (without ZM). Besides other components, the presence of antimicrobial peptide in insect meals could have positively influenced fish growth performance (Xiao et al., 2015). Added to the benefits promoted by insect meal, the partial removal of soybean meal on the diet with ZM may also have potentialized the results since soybean meal has antinutritional factors responsible for injuries on fish intestine, reduction on diet digestibility and absorptive capacity (Mahmoud *et al.*, 2014).

Regarding dietary composition, there was an increase in 23% of lipid for the diet with ZM and, although diets had similar protein content, their differ on amino acid profile. The diet with ZM had an expressive higher content of some amino acids involved on fish growth and development such as arginine, leucine, aspartic and glutamic acids (Jabir, M. a. R. *et al.*, 2012). The differences in dietary nutrients composition could have also contributed with improvements on growth performance and reflected on increases of protein, lipids, and energy contents in the carcass of fish fed the diet with insect meal.

3.2.Experiment 2

The efficiency of nutritional programming on fish physiology and metabolic responses has been observed for several carnivorous fish species exposed to a wide range of nutritional challenges, such as the intake of plant-based diets (Geurden *et al.*, 2013; Clarkson *et al.*, 2017), high-carbohydrates inclusions (Gong *et al.*, 2015; Liang *et al.*, 2017), and low metabolic essential fatty acids availability (Vagner *et al.*, 2009). The pathways or the molecular level effects that justify the improvements observed are still not very clear, but early stimulus with challenge diets seems to impact on sensory and cognitive perception, intestinal gut microbiota, digestion, and intermediary metabolism of carbohydrates and lipids (Panserat *et al.*, 2019). Nutritional programming for omnivorous fish is recent, with few studies with zebrafish (*Danio rerio*) (Fang *et al.*, 2014; Rocha *et al.*, 2014; 2015; Kwasek *et al.*, 2020) and Nile tilapia (Kumkhong, Marandel, Plagnes-Juan, Veron, Boonanuntanasarn*, et al.*, 2020; Kumkhong, Marandel, Plagnes-Juan, Veron, Panserat*, et al.*, 2020). This is the first study that demonstrated the nutritional programming as a tool to improve the use of high insect meal-based diets for fish.

The effects of nutritional programming were more evident on the high insect meal-based diet (300g/kg), since inclusions at lower levels seems to not promote any damages on Nile tilapia performance (Jabir, M. a. R. *et al.*, 2012). Indeed, the inclusion of insect meal has been performed in fish diets in no more than 150 or 200 g/kg to replace fishmeal or soybean meal (Sánchez-Muros *et al.*, 2014; Gasco *et al.*, 2016; Sánchez-Muros *et al.*, 2016; Muin *et al.*, 2017) due to differences between ingredients' amino acids profile, fatty acids composition and nutrients digestibility. The high chitin content of insect meals is usually related to a reduction in fish productive performance (Józefiak *et al.*, 2019). In the present study, the inclusion of ZM meal up to 150 g/kg in replacement of soybean meal improved tilapia growth, independently of the fry feeding (Table 5). In addition, the already discussed beneficial effects of insect meal on digestive and immune systems and the high palatability of insect meal diets (Benzertiha *et al.*, 2020) may have positively affected fish growth performance and feed utilization, with improvements on FCR, PER, SGR, DGI and feed intake.

When comparing the two groups of fry feeding, fish fed with ZM during fry stage and later control and 150 ZM diets had worse FCR and PER. Reduction on feed utilization was previously reported for tilapia fed 150 g/kg of black soldier fly (Muin *et al.*, 2017) possibly as a result of lower dietary digestibility or protein quality. However, in the present study, the dietary reduction of ZM content from fry to juveniles (from 250g/kg to 0 and 150g/kg) may have interfered on the results, since fish would lose the benefits of nutrients digestibility and absorptive capacity once promoted by the consumption of the diet with high inclusion of ZM during fry stage. Probably as a reflection of worse feed utilization, the superior initial BW of tilapia fed with ZM during fry stage did not influenced the growth performance of juveniles fed diets up to 150 g/kg ZM inclusion. Although fish fed with ZM at fry stage started the second trial almost 12% heavier than the control, the final BW and weight gain were similar between groups, showing the ability of compensatory growth against dietary manipulation, as reported by Liu *et al.* (2019).

The same was not true for fish fed the diet with the highest ZM content (300 g/kg). Fish fed the 300 ZM diet had been directly influenced by the consumption of diets with or without ZM during the fry stage. Considering chitin content, it was expected a reduction in growth performance and feed utilization for both groups when fed the 300

ZM diet. Instead, the group of fish that were fed with ZM during fry stage had considerably better growth performance, FCR and feed intake. The reduction in growth performance only occurred for juveniles fed without ZM during fry stage, which could not compensate for the low initial body weight and when fed the diet containing the highest ZM inclusion (300 g/kg) could not reach the weight gain observed for the other group. Not only for final BW or weight gain, the better feed intake and feed conversion ratio also affirm that fish fed the ZM diet during early stage were more efficient on the usage of high insect meal diets, probably due to the earlier adaptation of the new ingredient and of the chitin highly present on insect meal diets.

Until now, physiological adaptations of chitin have been reported only for humans. Early feeding with insect meal has been suggested as an alternative to allergies provoked by chitin from parasites, due to chitin effects on the immunomodulatory system and, thus, the possible adaptation of the human immune system to this component (Van Huis *et al.*, 2013). In the present study, adapted fish fed with insect meal during early stages turned into juveniles with better growth performance when fed a high insect meal diet (300 ZM) compared with the non-fed group. Chitin physiological adaptations could include modifications not only on the immune system but also on fish digestive performance.

The improvements on final BW and weight gain occurred by an increase in fish carcass weight only, since there was no difference in viscera weight between treatments. Carcass and fillet nutritional composition did not change over dietary treatments in the present study and the same was observed by other authors. Gasco *et al.* (2019) reported no significant changes in fillet, muscle or body composition of different fish species and shrimp fed diets with several insect meal inclusions. The whole-body composition of tilapia juveniles also did not change over increasing levels of black soldier fly in the diets (Devic *et al.*, 2018). Although Belforti *et al.* (2016) observed an increase on protein content and reduction on muscle dry matter and lipids contents of rainbow trout fed diets with *T. molitor*, specific changes on fish fatty acids profile are more suitable to occur when including insect meal in fish diets (Belforti *et al.*, 2016; Sánchez-Muros *et al.*, 2016).

At low inclusion levels, replacing fishmeal or other conventional protein sources by insect meal is not a problem because fish seem to adapt well to this substitution.

However, when the inclusion levels of insect meal are higher the acceptance by the fish is more challenging. In the present study, feeding fish continuously with insect meal, from fry to juveniles, is presented as alternative feed management capable to optimize fish growth performance and allow inclusions of insect meal up to 30%. Further studies should be performed to determinate the metabolic pathway of chitin and elucidate the mechanisms in which the digested chitin products reacts with fish organism, benefiting survival, growth performance and nutrients utilization, as observed in the present study.

4. *Conclusion*

Feeding fry with ZM increased survival, weight gain, feed utilization and carcass protein, energy, and lipids. To feed fish continuously with insect meal, from fry to juveniles, is an alternative feed management capable to optimize fish insect meal utilization, allowing inclusions of up to 300 g/kg of *Z. morio* meal and improving fish productive performance.

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Table 1. Formulation and proximate composition of experimental diets

Ingredients	Experiment 1		Experiment 2		
	Without ZM	With ZM	Control	150 ZM	300 ZM
Fish Meal	232.4	235.2	130.0	130.0	130.0
Zophobas morio Meal	-	247.4	-	150.0	300.0
Soybean Meal	297.8	150.8	195.0	97.5	-
Inert	-	-	58.0	50.0	40.0
Soybean Oil	127.9	64.7	87.0	42.5	-
Corn	51.3	51.9	174.5	174.5	174.5
Rice Bran	188.1	147.0	100.0	100.0	100.0
Corn Gluten	10.1	40.8	95.0	95.0	95.0
Wheat Bran	82.7	52.3	150.0	150.0	150.0
BHT[1]	0.5	0.5	0.5	0.5	0.5
Premix[2]	9.2	9.4	10.0	10.0	10.0
Total	1000.0	1000.0	1000.0	1000.0	1000.0
Proximate analysis (g/kg of dry matter)					
Dry matter (g/kg as fed)	893.2	834.7	940.1	938.0	930.1
Crude protein	408.8	412.0	308.1	303.9	309.6
Ether extract	139.3	181.0	98.0	102.7	112.3
Ash	11.2	10.9	150.1	120.8	119.7
Crude Fiber*	51.31	47.40	45.62	46.25	46.88
Neutral Detergent Fiber*	148,33	126,96	155,21	154,67	154,13
Gross energy (kcal/kg)	4564.0	4875.0	4324.9	4448.6	4591.9
Digestible Protein	371.0	371.0	278.1	274.3	279.8
Digestible Energy (kcal/kg)	3574.5	3801.7	3378.6	3373.4	3385.8
Total Carbohydrates**	538.1	494.2	516.9	545.0	532.1
Chitin	-	56.2	27.5	43.8	60.0
DE/DP	9.6	10.2	12.8	12.8	12.9

[2] Vitamin and Mineral Premix: vitamin A - 500.000 UI; vitamin D3 - 250.000 UI; vitamin E - 5.000 mg; vitamin K3 - 500 mg; vitamin B1 - 1.500 mg; vitamin B2 - 1.500 mg; vitamin B6 - 1.500 mg; vitamin B12 - 4.000 mg; folic acid - 500 mg; pantothenate Ca - 4.000 mg; vitamin C - 10.000 mg; biotin - 10 mg; Inositol - 1.000; nicotinamide - 7.000; choline - 10.000 mg; Co - 10 mg; Cu - 1.000 mg; Fe - 5.000 mg; I - 200 mg; Mn - 1500 mg; Se - 30 mg; Zn - 9.000 mg3. (Agromix LTDA, Sao Paulo, Brazil).

*Calculated according to INRA (2020), Finke (2002) and Benzertiha et al. (2019)

**Total Carbohydrates = 100 - (protein + fat + ash) according to Atwater System.

Digestible protein and energy were calculated by using apparent digestibility coefficients for *Z. morio* meal obtained by Fontes et al. (2019).

Table 2. Amino acids profile[1] (g/100g dry matter) of diets from experiment 1 and experiment 2.

	Experiment1		Experiment 2		
	Without ZM	With ZM	Control	150 ZM	300 ZM
Amino acids profile					
Essential					
Arginine	18.20	21.81	14.18	16.20	18.22
Histidine	10.73	10.83	8.28	8.14	8.00
Isoleucine	14.14	15.27	11.59	11.79	12.00
Leucine	24.23	28.78	25.54	26.50	27.47
Lysine	21.07	22.86	15.49	16.16	16.82
Methionine	6.60	7.07	5.32	5.39	5.47
Phenylalanine	14.14	16.83	12.63	13.71	14.78
Threonine	13.65	14.74	10.68	11.05	11.41
Valine	18.18	18.32	14.88	14.42	13.96
Nonessential					
Aspartic acid	25.73	31.68	20.31	23.42	26.53
Glutamic acid	44.93	57.72	43.36	49.06	54.76
Alanine	25.14	25.14	23.46	21.99	20.52
Cystine	3.18	3.79	3.35	3.60	3.84
Glycine	19.36	20.18	14.18	14.45	14.73
Serine	12.87	15.40	11.37	12.49	13.60
Tyrosine	17.11	15.42	13.58	11.95	10.31

[1]The amino acid profile of experimental diets were calculated based on data obtained from: Nogales-Mérida et al. (2018) for fishmeal, soybean and *Zophobas morio* meal; Guimaraes et al. (2008) for corn gluten meal; Ribeiro et al. (2012) for corn meal and wheat bran; and Guimaraes et al. (2008a) for rice bran

Table 3. Growth parameters, feed utilization a survival of Nile tilapia fry fed with or without *Z. morio* meal[1]

	Without ZM	With ZM	SEM	p-value
IBW (g)	0.28	0.32	0.04	ns
FBW (g)	2.96	3.35	0.08	*
FI (g DM)	282.4	288.0	9.90	ns
WG (g)	470.3	576.1	24.41	*
FE	1.68	2.01	0.08	*
SGR	3.97	4.02	0.22	ns
PER	4.09	4.87	0.20	*
SR (%)	89.1	95.7	1.53	*

[1]Values presented as means (n = 600) and pooled standard error of the mean (SEM). Means with different lower case represents significant differences between diets ($p < 0.05$). ns: no significance ($p > 0.05$); * $p < 0.05$. ZM (*Zophobas* morio meal), IBW (Initial Body Weight), FBW (final body weight), FI (feed intake), WG (weight gain), FE (feed efficiency), SGR (specific growth rate), PER (protein efficiency ratio), SR (survival).

Table 4. Proximate composition of Nile Tilapia fry carcass fed experimental diets with or without *Z. morio* meal[1].

	Without ZM	With ZM	SEM	p-value
Dry matter	86.4	87.0	1.47	ns
Ash	14.2	15.6	0.44	ns
Protein	58.0[a]	62.2[b]	0,91	*
Energy	2432.8[a]	2633.0[b]	40.8	*
Lipids	14.8[a]	17.3[b]	0.58	*

[1]Values presented as means (n = 15) and pooled standard error of the mean (SEM). ns: no significance ($p > 0.05$); * $p < 0.05$. ZM (*Zophobas morio* meal)

Table 5. Growth parameters and feed utilization efficiency of Nile Tilapia juveniles fed experimental diets after fed with or without *Z. morio* meal during fry stage [1]

	Without ZM			With ZM			SEM	FF	D	FF x D
	0ZM	150ZM	300ZM	0ZM	150ZM	300ZM				
Final body weight (g)	20.02 ^{aA}	23.76 ^{bA}	20.84 ^{aA}	17.65 ^{aA}	21.11 ^{bA}	23.36 ^{cA}	0.479	0.5359	0.0001	**0.0037**
Feed intake (g/dry matter)	22.82 ^{aA}	25.32 ^{bA}	25.92 ^{bA}	26.12 ^{aB}	24.94 ^{aA}	28.91 ^{bB}	0.508	0.1352	0.0011	**0.0266**
Weight gain (%)	533.86 ^{aA}	662.00 ^{bA}	562.59 ^{aA}	468.37 ^{aA}	570.95^{bA}	639.73 ^{cA}	16.669	0.5305	0.0001	**0.0040**
Specific growth rate	3.08 ^{aA}	3.38 ^{bA}	3.15 ^{aA}	2.88 ^{aA}	3.17 ^{bA}	3.33 ^{bA}	0.042	0.4681	0.0001	**0.0045**
Daily growth index	2.08 ^{aA}	2.35 ^{bA}	2.14 ^{aA}	1.90 ^{aA}	2.16 ^{bA}	2.32 ^{cA}	0.035	0.5043	0.0001	**0.0044**
Feed conversion ratio	1.32^{bA}	1.19 ^{aA}	1.49^{cA}	1.77 ^{bB}	1.42 ^{aB}	1.41 ^{aA}	0.034	0.0061	<.0001	**<.0001**
Carcass yield (%)	86.36	87.14	87.53	86.16	86.13	86.53	0.270	0.5984	0.5942	0.8242
Visceral somatic index (%)	11.95	11.33	10.17	10.22	10.55	10.77	0.185	0.4433	0.3564	0.0566
Hepatosomatic index (%)	1.46	1.15	1.21	1.52	1.65	1.61	0.048	0.1522	0.6782	0.1500
Visceral fat index	1.01	1.14	1.25	1.07	1.23	1.26	0.059	0.8430	0.3551	0.9707
Protein efficiency ratio	2.21 ^{aB}	2.48^{bA}	2.02 ^{aA}	1.66 ^{aA}	2.18 ^{bA}	2.06 ^{bA}	0.054	0.0613	0.0001	**0.0038**

[1]Values presented as means (n = 48) and pooled standard error of the mean (SEM). Means with different lower case represents significant differences between fry feeding ($p < 0.05$); FF (fry feeding), D (diets), ZM (*Zophobas morio* meal).

Table 6. Proximate composition of Nile Tilapia juveniles fillet fed experimental diets after fed with or without *Z. morio* meal during fry stage[1]

	Without ZM			With ZM			SEM	FF	D	FF x D
	0ZM	150ZM	300ZM	0ZM	150ZM	300ZM				
Fillet										
Dry Matter	88.40 ^{a}	88.71 ^{a}	90.10 ^{b}	88.67 ^{a}	89.77 ^{ab}	90.00 ^{b}	0.114	0.1094	**0.0262**	0.2699
Ash	5.69	5.74	6.50	5.74	6.43	6.21	0.062	0.3061	0.1272	0.0758
Protein	83.72	85.40	80.07	83.71	85.95	84.35	0.965	0.5159	0.452	0.7013
Lipids	8.54	8.17	7.02	8.12	6.90	7.12	0.226	0.3774	0.6059	0.7134
Energy	2300.57	2325.63	2155.27	2283.82	2288.48	2259.82	23.899	0.7823	0.4346	0.5848
Carcass										
Dry Matter	89.96	89.58	89.98	86.93	89.05	89.60	0.409	0.5106	0.4542	0.3981
Ash	16.27	15.77	15.90	17.71	17.11	15.57	0.290	0.5636	0.2664	0.4507
Protein	49.48 ^{aA}	50.65 ^{abA}	52.62 ^{bA}	55.41 ^{aB}	56.18 ^{aB}	54.15 ^{aA}	0.398	0.0136	0.4424	**0.0414**
Lipids	29.34	27.88	25.46	25.65	24.76	25.91	0.466	0.3305	0.3004	0.1921
Energy	2316.25	2286.32	2237.35	2310.24	2293.68	2291.15	15.456	0.8098	0.4959	0.7577

[1]Values presented as means (n = 12) and pooled standard error of the mean (SEM). ns: no significance (p > 0.05). FF (fry feeding), D (diets), ZM (*Zophobas morio* meal)

6. MANUSCRIPT 2.

To be submitted to: **Aquaculture**, **ISSN: 0044-848**

Nutritional programming optimizes digestive performance and intestine histomorphology of Nile tilapia (*Oreochromis niloticus*) fed high insect meal diets

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Abstract

The capacity of tilapia juveniles to utilize high insect meal diets after consume *Z. morio* meal during fry stage was analyzed using the concept of nutritional programming. For that, in the *nutritional stimulus* trial, triplicate groups of tilapia fry (0.30 ± 0.04 g) were fed experimental diets with or without ZM inclusion for 60 days. After that, male juveniles from both groups (without ZM and with ZM), were selected and reallocated in two separate *challenge* trials (experiments 1 and 2). The diets for experiments 1 and 2 were formulated to contain 0, 150, and 300 g/kg of ZM inclusion replacing up to total dietary soybean meal. Experiment 1 was designed in a factorial 2×3: fry feeding (with or without ZM) and experimental diets (0, 150, and 300 g/kg ZM). Quadruplicate groups of male tilapia (3.1 ± 0.08 g) were fed the experimental diets for 60 days to evaluate digestive enzyme activity and intestine histomorphology. In experiment 2, triplicate groups of male tilapia (3.0 ± 0.3 g) were fed the experimental diets added 50 g/kg of chromium oxide III to determine apparent digestibility coefficients. The increasing levels of ZM positively affect chitinase activity. The introduction of ZM during fry stage improved amylase, lipase, and trypsin activities and the ratios A/L and A/P. The activity of total proteases was higher for fish fed without ZM during fry stage, excepted for fish fed the 150ZM diet. The activity of chymotrypsin was higher for fish fed without ZM during fry stage and later the control diet. Fish fed with ZM during fry stage had better chitin digestibility than fish that did not consume chitin as a fry. Dry matter and protein digestibility were higher for fish fed with ZM and later the 300 ZM diet. Total carbohydrates digestibility was higher for fish fed without ZM during fry stage for the 300 ZM diet. In fish fed with ZM during fry stage the histomorphology changes were characterized by increased total villus height, villus height, the width of lamina propria, and mucosa, and villus and lamina propria area. The width of mucosa increased over 40% for fish fed with ZM during fry stage and later the 300ZM diet. The strategy of feeding Nile tilapia continuously, from fry to juveniles, with *Z. morio* meal at high inclusion levels is presented as alternative feed management capable to optimize fish digestive performance and allow inclusions of insect meal up to 300 g/kg of *Zophobas morio* meal.

Keywords: nutritional programming; histomorphology; digestive enzymes; digestibility; chitinase

1. Introduction

The interest of insect meals especially as an alternative protein source in aquafeeds has been driven by its absence of antinutritional factors, the potential use as prebiotic and, protein quantity and quality (Pinotti *et al.*, 2019). Some species such as the *Zophobas morio* can even exceed fish amino acid requirements (Barroso *et al.*, 2014; Henry *et al.*, 2015). *Z. morio*, also known as superworms, belongs to the Coleoptera order and characterizes as a thick larva of 2-4 cm at harvest. Its chemical composition can alter depending on the feed (St-Hilaire *et al.*, 2007; Kroeckel *et al.*, 2012; Henry *et al.*, 2015), and developmental stage (Barroso *et al.*, 2014; Nogales-Mérida *et al.*, 2018) but, in general, dry matter varies between 38-95%, crude protein in 40-68%, crude fat in 14-40%, the neutral detergent fiber in 13-50%, ash in 2.7-6.2% (Jabir, M. *et al.*, 2012; Fontes *et al.*, 2019) and chitin between 13-50% (Oonincx and Dierenfeld, 2012).

The high chitin content is the main factor that limits the inclusion of insect meals in fish diets, due to reductions in fish growth performance and feed utilization (Barroso *et al.*, 2014; Józefiak *et al.*, 2019). Chitinase (E.C 3.2.1.14) is the key enzyme of chitin digestion and its presence in Nile tilapia and, the capacity to digest chitin have already being reported (Molinari *et al.*, 2007; Fontes *et al.*, 2019). The natural feeding type, especially during early developmental stages, seems to have positively influenced the chitinolytic ability of this species. Nile tilapia (*Oreochromis niloticus*) has an omnivorous food habit, adapted to several production systems and a variety of feed ingredients as animal or vegetal sources, usually present in commercial diets (Azevedo *et al.*, 2018). During the fry stage, planktons are still the primary source of nutrients and, especially the zooplanktons have considerable amounts of chitin on their composition (Muzzarelli, 1999). Once chitin is naturally present in fish diets, the existence of chitinolytic activity at this stage is reasonable. However, as soon as fish get bigger, and are transferred to intensive production systems, they are fed exclusively with dried formulated diets that do not contain any source of chitin. The drastic drop on fish chitin intake could lead on missing chitinase activity due to dismissed use.

Positive results have been reported for *Z. morio* meal inclusion in omnivorous red tilapia (*Oreochromis spp.*) and Nile tilapia (*Oreochromis niloticus*) diets. The digestibility of *Z. morio* meal fulfilled the nutritional requirements of these fish species (Jabir, M. *et al.*, 2012; Fontes *et al.*, 2019) and, inclusions up to 150g/kg successfully

replaced 50% of fishmeal and increased Nile tilapia growth performance (Jabir, M. a. R. *et al.*, 2012). Therefore, studies evaluating soybean meal substitution by *Z. morio* meal are scarce in fish nutrition, especially for the Nile tilapia in early developmental phases.

Then, the current study was designed to evaluate the capacity of tilapia juveniles to utilize high insect meal diets after consume *Z. morio* meal continuously from fry stage by measuring digestive enzyme activity, intestine histomorphology, and nutrients digestibility.

2. Material and Methods

The project was conducted at the Fish Culture Station at the Federal University of Lavras (UFLA) and the Central Laboratory of Animal Research (LCPA/DZO), in Lavras, Minas Gerais, Brazil. All experimental procedures were approved by the Ethical Commission of Animal Use (CEUA) of the Federal University of Lavras (UFLA), protocol number 041/18.

Two separate trials evaluated the effects of feeding increasing *Z. morio* meal (ZM) diets on Nile tilapia juveniles digestive enzymes activity, intestine histomorphology, and *in vivo* apparent digestibility coefficients, after feeding diets with or without ZM inclusion during fry stage.

2.1.Experimental diets

In the *nutritional stimulus* trial, two isoproteic and isoenergetic diets (410 g/kg of crude protein and 4600 kcal/kg of gross energy) were formulated to promote the effects of ZM inclusion in diets for Nile Tilapia fry: a control diet, without *Z. morio* meal (Without ZM); and the *stimulus* diet containing 250 g/kg of *Z. morio* meal (With ZM). Thereafter, in the challenge trials (experiment 1 and 2) three diets were formulated to be isoproteic (270 g/kg digestible protein), isoenergetic (3370 kcal/kg of digestible energy), and with increasing ZM levels (0, 150, and 300 g/kg) replacing up to total soybean meal and soybean oil. Apparent digestibility coefficients of ingredients were based on data from Fracalossi and Cyrino (2013), Pezzato *et al.* (2002), and Fontes *et al.* (2019). In experiment 2, the digestibility trial, diets were as described added 50 g/kg of chromium oxide III. The proximate and chemical composition of experimental diets is shown in Table 1.

The experimental diets were prepared by grinding all dry macro ingredients in a hammer mill (TRF-400 Trapp, Jaraguá do Sul, SC, Brazil) to a powder (0.5 mm sieve). Micro and macro ingredients were manually mixed. Hot water (50°C) was then blended into the mixture to attain a consistency appropriate for pelleting. The diets were pelleted in an electric meat grinder (1 mm diameter) (Tecnal, Piracicaba, SP, Brazil) and then dried in a forced recirculation oven at 50°C for 24 h. Pellets were ground in a hammer mill (TRF-400 Trapp), passed through a 2.0–2.5 mm sieve (Technal), and then stored at -20°C until used.

2.2.Fish and culture conditions

1200 Nile Tilapia (*O. niloticus*) fry were obtained from the Fish Culture Station – UFLA and acclimatized for 15 days upon arrival to the experimental facilities. During this period, fish were fed a commercial diet (Pira 40, Guabi, 400 g/kg of crude protein) three times daily.

2.2.1. Nutritional stimulus

Nile Tilapia fry (0.30 ± 0.04 g mean weight) were randomly distributed in six circular tanks (500 L) at a density of 200 fry/tank. The tanks were maintained in a recirculated system supplied with aeration and the temperature was kept constant by heaters (26 °C). Each tank was considered as an experimental unit, arranged in a completely randomized design with two treatments (without ZM and with ZM) and three replicates. The water quality was maintained using supplemental aeration (central line and air diffusers), mechanical and biological filtration. The fish were fed with experimental diets for 60 days, three times daily (8:00, 12:00, and 16:00h), until apparent satiation.

2.2.2. Challenge Trials

After this period, male juveniles from both groups (without ZM and with ZM), were selected and reallocated into the experimental facilities for evaluation of digestive enzyme activity, intestine histomorphology (experiment 1), and *in vivo* digestibility (experiment 2).

In experiment 1, fish (3.2 ± 0.3 g mean weight) were randomly distributed in 24 circular tanks (150 L) at a density of 12 fish/tank. Each tank was considered as an experimental unit, and the experiment was lain out in factorial design 2×3 (two fry feeding and three experimental diets) and four replicates. Fish were fed a fixed ration close to satiation two times per day (at 8:00h and 14:00h). The daily ration was adjusted

throughout the trial to account for growth (fish were bulk-weighed by tank biweekly), decreasing from 70 to 50 g/kg body weight/day (BW/d) over 60 days.

In experiment 2, triplicate groups of male Nile Tilapia juveniles (3.0 ± 0.3 g mean weight), from both groups (without ZM and with ZM), were distributed randomly in 18 digestibility tanks (250 L) at a density of 50 fish/tank. Each tank was considered as an experimental unit, and the trial was laid out in factorial design 2×3 (two fry feeding and three experimental diets) in triplicate. The digestibility assay was conducted in a water recirculation system with fiberglass tanks adapted to a modified Guelph system. The fish were fed twice a day (8:00 and 14:00h), until apparent satiation seven days before feces collection for diet adaptation and continued to be fed during the feces collection. During this period, the tanks were cleaned every day after the last feeding. Then, in the water exit of each tank, tubes were fixed for the feces collection. Daily, at 8:00h the tubes were withdrawn, the feces were dried in a forced circulation heater at 60 ºC for 36 hours and stored in refrigeration. The feces were collected for 15 days.

The water quality for both experiments 1 and 2, was maintained using supplemental aeration (central line and air diffusers), mechanical and biological filtration, and UV lamps. The water temperature was controlled with a heat exchanger (26 °C) and measured twice daily. The dissolved oxygen and pH were monitored daily using a multiparameter (U-10, Horiba, Kyoto, Japan). Total ammonia and nitrite were measured using commercial kits (Labcon Test Fresh Water Toxic Ammonia and Labcon Test Nitrite NO^{2-}).

2.3.Sampling

At the end of experiment 1, the fish fasted for six hours. The intestines and stomachs were carefully removed from six fish from each tank, freed from the adjacent adipose tissue. Each intestine and stomach was individually stored in 2 ml microtubes, immediately frozen in liquid nitrogen, and then stored at -80 °C until enzymatic analyses.

Anterior intestine samples were also collected from two other fish per tank, gently washed with saline water (0.9%) to remove biological fragments, fixed in Bouin's solution for 12 hours. Bouin's solution was made following the proportions: 75 ml of picric acid (2.1 %), 25 ml of formaldehyde (40 %), and 5 ml of acetic acid glacial.

2.4.Chemical Analysis

Chemical analyses of the diets and feces were determined according to the Association of Official Agricultural Chemists (Horwitz *et al.*, 2012) methodology for dry matter (930.15), crude protein (968.06), and ash (942.05). Crude lipid was quantified following Folch's (1957) methodology. Total carbohydrates and gross energy were estimated using the Atwater general factor system (Jobling, 2012) in which gross energy (MJ/Kg) was defined as the sum of the total protein, lipid, and carbohydrate multiplied by their respective conversion factor, based on the heat generated after combustion: 4.0 kcal/g for protein, 9.0 kcal/g for lipids, and 4.0 kcal/g for carbohydrates. Crude fiber and neutral detergent fiber were calculated based on data obtained from INRA (2020), Finke (2002), and Benzertiha *et al.* (2019). Chitin was determined following the spectrophotometric method from Tsuji *et al.* (1969) and described in (Han and Heinonen, 2020). The glucosamine (GlcN) from chitin hydrolysate was deaminated and color reacted with MBTH. The absorbance was read at 650 nm and the content of chitin calculated using an external standard curve with GlcN-HCl.

2.5.Digestive Enzymes Activity

Fish intestines and stomachs were weighted, and pH measured. Then, samples were homogenized on ice (1:5 dilution), centrifuged at 3300 *g* for 30 min at 4 °C, and the supernatant collected and stored at -80 °C until analyses. All enzyme activities were determined using a PowerWavex microplate scanning spectrophotometer (Bio-Tek Instruments, USA).

Total protease activity was determined by the casein-hydrolysis method described by Walter (1984) and adapted by Hidalgo *et al.* (1999). The enzymatic determination was made using several pH values. Buffers for each pH assay were: 0.1 M citrate–0.2 M phosphate (pH 7.0); 0.1 M Tris–HCl (pH 8.5) and 0.1 M glycine–NaOH (pH 10.0). The reaction mixture containing casein (1% w/v; 0.125 ml), buffer (0.125 ml), and homogenate supernatant were incubated for 1 hour at 37 °C and stopped by adding 0.6 ml trichloroacetic acid (8% w/v) solution. After being kept for 1 h at 2 °C, samples were centrifuged at 1800 *g* for 10 min and the absorbance read at 280 nm against blanks. A blank for each sample was prepared by adding the homogenate supernatant after incubation.

Chymotrypsin activity was determined following Hummel (1959), with Rao and Lombardi (1975) modifications, using BTEE (N-benzoyl-L-tyrosine ethyl ester) as a substrate in Tris-HCl 41.4 mM and CaCl2 10.4 mM (pH 8.1) and the absorbance read at 256 nm. Trypsin activity was measured according to Bergmeyer et al. (1974), using 10nM TAME ester (Nα-p-toluenesulfonyl-L-arginine methyl ester) as substrate in Tris-HCl 41.4 mM and CaCl2 10.4 mM (pH 8.1) and the absorbance read at 247 nm. Amylase (E.C.3.2.1.1) and lipase (EC 3.1.1.3) activities were measured with kits (Spinreact ref. 41201 and ref. 1001274, respectively), adapted for fish intestine samples. The substrate 2-chloro-4-nitrophenyl-α-D-maltotrioside was used for amylase and absorbance read at 405 nm. Lipase was determined using 1-2-O-dilauryl-rac-glycero-3-glutaric acid-(6'-methylresorufin)-ester as substrates and absorbance read at 580 nm. Chitinase activity was determined using a modification of the standard procedure of Abro *et al.* (2014), the detailed description of the protocol is present elsewhere.

Enzyme activity was expressed as specific activity, defined as μmol of product generated per minute. Soluble protein concentration was determined according to Bradford (1976) with bovine serum albumin solution as standard.

2.6.Histological Assays and Analysis

Samples were washed and stored in alcohol at 70% until processing by classical histological techniques and stained with hematoxylin and eosin. Briefly dehydration with ethanol, paraffin inclusion, obtention of histological sections with a microtome, deparaffinization, hydration, and staining. Histological preparations were photographed using a light microscope (Carl Zeiss, Germany) for analysis of histological modifications. Points of measurement are shown in Figure 1. Six villi per slide (slides with HE – 40x) were selected respecting full visibility from tip to the submucosa and absence of shattered edges. For each suitable villus, the following data were collected: total fold height (from top to the base of the mucosal fold), enterocytes height, villus, lamina propria and mucosa width (measured at the midpoint of each villus), villus and lamina propria area (area within the perimeter for each fold). The values correspond to the measurement of 48 villi per treatment, totaling 288 villi. Variables were measured by using Image J software (National Institutes of Health, Bethesda, Maryland, USA).

2.7.Statistical Analysis

Data are expressed as mean ± pooled standard error of the mean (SEM). Normality and homogeneity of variances were tested using the Shapiro-Wilk and Levene tests, respectively. All statistical procedures were performed using the SPSS software package for Windows (IBM® SPSS® Statistics, New York, USA) and the probability level of 0.05 was used for rejection of the null hypothesis. Statistical analysis of experiments 1 and 2 was done by two-way ANOVA, with fry feeding and experimental diets as factors. When necessary, Tukey's test was used to detect differences in fry feeding among experimental diets within each dietary treatment. Significant differences among dietary ZM levels within each fry feeding group were determined by orthogonal polynomial contrast to identify linear and quadratic effects. For the effect of pH values over total proteases activity the one-way ANOVA was performed followed by Tukey's test ($p < 0.05$).

3. Results

The effect of the treatments on growth performance and feed utilization were not the object of the present study and are present elsewhere. In short, feeding Nile tilapia continuously with insect meal diets improved weight gain, feed conversion ratio, and carcass weight.

3.1.1. Digestive enzymes

Chitinase activity was higher for fish previously fed with ZM for all the treatments and, increased linearly from diet 0ZM to 300ZM. The activity of amylase, lipase, chymotrypsin, and the ratios amylase/lipase and amylase/proteases were not affected by fry feeding (Table 2).

Digestive enzyme activities were influenced by both fry feeding and the dietary inclusion of ZM (Table 2). Amylase activity was higher in the group fed without ZM during fry stage and later the 150ZM diet. However, when juveniles were fed with the 300ZM diet, fish that did not receive ZM during fry stage had lower amylase activity than the other group. An increase in the activity of lipase was also observed for fish fed earlier with ZM and later the 300ZM diet. No differences were observed for lipase activity between fry feeding for the other treatments.

For trypsin, fish fed ZM during fry stage and later control and 150ZM diets had reduced trypsin activity. Otherwise, when fed with diet 300 ZM, trypsin activity

recovered and was higher than the observed for juveniles not fed with ZM during fry stage (without ZM). The activity of chymotrypsin was higher for fish fed without ZM during fry stage and later the control diet. No differences were observed between fry feeding and other treatments. The activity if total proteases were higher for fish fed without ZM during fry stage, excepted for fish fed the 150ZM diet, where fish fed with ZM during fry stage had high total proteases activity. The ratio amylase: lipase (A/L), amylase: proteases (A/P), and lipase: proteases (A/P) were higher for fish fed without ZM during fry stage and later the 150ZM diet, however, when fed with the diet containing 300g/kg of ZM, the A/L and A/P ratios were higher for fish fed with ZM during fry stage.

There was a linear increase of lipase activity in all fry feeding groups with the increase of dietary ZM levels, whereas protease activity was reduced linearly. Quadratic effects were observed for amylase and A/P. No differences were observed for chymotrypsin, trypsin activities, and A/L ratio with the increasing inclusion of ZM in fish diets.

The pH influenced proteolytic activity in the Nile tilapia intestine and with few exceptions, total protease activity was higher at pH 10 (Figure 3).

3.1.2. Histomorphometry

Histological changes were noted in intestine samples influenced by fry feeding and the levels of ZM in the diets (Table 3, Figure 2). In fish fed with ZM during fry stage the changes were characterized by increased total villus height (hTV), villus height, (hV) width of lamina propria (wLP), and mucosa (wM), and villus (aV) and lamina propria (aLP) area. Quadratic effects were observed for hTV, hV, and height of lamina propria (hLP) with increasing ZM inclusion levels. There was observed to be linear the effect of ZM diets on increasing wLP in fish intestines and, quadratic for wM and lamina propria height: villus (hLP:hV). Even the hLP and hLP:hV had increased as an effect of fry feeding and ZM diets, fish fed the 300ZM diet from both fry feeding groups (with and without ZM) had a similar and reduced height of lamina propria and consequently ratio hLP:hV.

The quadratic effect was observed for ZM inclusion levels on the width of mucosa (wM), which had increased from 0 to 150ZM diet, independently of fry feeding. However, for fish fed with ZM during fry stage and later the 300ZM diet, wM increased over 40%.

There were no reported differences also on the enterocytes heigh (hE), lamina propria width: villus (wLP:wV), and lamina propria area: villus (aLP: aV) ratios.

3.1.3. Apparent digestible coefficients

The apparent digestibility coefficients (ADC) of the experimental diets are presented in Table 4. The digestibility of chitin was influenced by fry feeding and ZM inclusion levels. Linear increase of chitin digestibility was observed with ZM inclusion for fish fed with ZM during fry stage (with ZM). However, for the fish fed without ZM earlier, the intake of a high insect meal diet (300ZM) resulted in a reduction in the digestibility of chitin. Independently of ZM dietary inclusion, fish fed with ZM during fry stage had better chitin digestibility than fish that did not consume chitin as a fry.

The inclusion of ZM up to 300 g/kg decreased in quadratic effect the ADCs of dry matter, lipids, energy, and total carbohydrates. Although diet digestibility was not influenced by fry feeding, a significant interaction was observed for dry matter, protein, and total carbohydrates. The inclusion of ZM up to 150 g/kg increased the ADCs of dry matter and protein, however, for the 300 ZM diet, fish were fed without ZM during fry stage had reduced ADCs when compared to the fish fed with ZM in fry stage. Total carbohydrates digestibility was higher for fish fed without ZM during fry stage for the 300 ZM diet, whereas fish fed with ZM during fry stage had higher carbohydrates digestibility at the 0 ZM diet.

4. Discussion

4.1.1. Chitinase activity

The damages promoted by insect meal intake are generally linked to the incapacity or inefficiency of some fish species to digest insects' chitin (Sánchez-Muros *et al.*, 2014; Piccolo *et al.*, 2017). Chitin digestion requires the existence of a specific enzyme complex with chitinolytic characteristics, where the chitinase (E.C 3.2.1.14) is the key enzyme of chitin digestion. Chitinase does the first catalytic breakdown of dietary chitin, releasing low size particles of dimers, oligomers, and few monomers of N-acetylglucosamine. Despite wide discussion among its existence, activity, and site of action in productive animals, chitinolytic activity has been identified for Nile tilapia in the stomach, intestine, and serum (Molinari *et al.*, 2007). In the present study, chitinase activity was determined by incubation of stomach and intestine samples with pure chitin and, no activity of chitinase was observed in tilapia intestine. The absence of intestinal activity of chitinase was also reported for *Oreochromis mossambicus* fed mopane worm (*Imbrasia belina*)

meal (Rapatsa and Moyo, 2017) and *Psetta maxima* fed black soldier fly meal (Kroeckel *et al.*, 2012).

The ability of Nile tilapia on chitin digestion can be linked to its omnivorous food habit and the characteristics of the natural food consumed, which includes insects and zooplankton containing high concentrations of chitin (Gutowska *et al.*, 2004). Considering chitinase substrate specificity, it is reasonable to find its activity on the stomach, the first major site of digestion in fish, where chitinase starts the breakdown of chitin and frees food particles to other enzymes attack. Stomach chitinase seems to have adapted to fish feeding habits and food according to substrate specificity (Ikeda *et al.*, 2017), becoming an efficient mechanism to digest the chitin ingested through the diet. Whereas high endochitinase activity had been reported in the stomach of several fish species, chitin seems not to be hydrolyzed in the intestinal tract of most fish because of the absence of chitinase (Rapatsa and Moyo, 2017). Only exochitinases activity has been observed in the intestine of fish (Ikeda *et al.*, 2017).

In the present study, stomach chitinase activity was influenced by the diet in two aspects: the early administration of ZM in tilapia fry diets and, secondly the increase of ZM inclusion in juveniles diets. Fish that were fed the diet without ZM during fry stage had lower chitinase activity than the group of fish fed with ZM. Although both groups have had contact with zooplanktons during larval stage, once started to fed diets that do not contain any source of chitin, the control group (without ZM) may have dismissed the ability to digest chitin by inactivation of chitinolytic enzymes synthesis, since it was no longer needed. Also, the increasing dietary intake of chitin, proportional to the levels of ZM inclusion in the diets, positively affects chitinase activity. The stimuli of chitinolytic activity by dietary chitin content have been reported by other fish organisms (Krogdahl and Bakke-Mckellep, 2005; Abro *et al.*, 2014; Pohls *et al.*, 2016) and may reflect the control that dietary components exert on the gene expression of digestive enzymes. The existence of a relationship between diet and gene-regulation of digestive enzymes has been pointed out by several authors (Zambonino Infante and Cahu, 2007; Panserat and Kaushik, 2010; Santos *et al.*, 2020).

4.1.2. Intestine Digestive enzymes

Modifications of digestive enzyme activity following diet composition are expected however are not a rule (Sabat *et al.*, 1999). The variations on the effects of insect meal inclusion on fish digestive enzyme activity seem to be influenced by fish species and developmental stage, insect meal composition, and the products released during chitin degradation into the gastrointestinal tract. While the increase in chitinase activity is associated with reduced activity of other digestive enzymes as observed for *Macrobranchium tenellum* prawns (Santos-Romero et al., 2017), no changes were reported for Jian carp (Li et al., 2017), Atlantic salmon (Belghit et al., 2018) and European seabass (Magalhães et al., 2017) fed black soldier fly diets. Inversely, the inclusion of chitosan oligosaccharides in diets for loach *Paramisgurnus dabryanus* improved pancreatic enzymes (Zhang et al., 2019) and authors have suggested a reflection of the reduction of bacterial digestive enzymes by the antibacterial activity effects of chitosan.

The activity of amylase may have been influenced by changes in dietary carbohydrate profile and intestine microbiota. Although total carbohydrates did not vary among diets, the levels of carbohydrates excluding chitin, dropped from 520 to 460 g/kg from 0 to 300 ZM diets. Also, the capability of insect meal on changing intestine microbiota has being reported by several authors (Bruni et al., 2018; Terova et al., 2019; Benzertiha et al., 2019; Józefiak et al., 2019; Rimoldi et al., 2019), and both, carbohydrate and gut microbiota profile, can promote changes in fish amylase activity (Nayak, 2010; Clements et al., 2014; Wang et al., 2017).

The activity of chitinase seemed to have directly influenced the activity of trypsin. Fish fed with ZM during fry stage had reduced trypsin activity and increased chitinase activity when fed the diets 0 and 150 ZM. Therefore, trypsin seems to have an inverse effect with chitinase activity, as reported by Santos-Romero et al. (2017). However, when fed with the highest chitin diet (300 ZM) that consequently promoted high chitinase activity, fish fed ZM during fry stage had better activity of trypsin, probably a response of chitin physiological adaptations. Santos-Romero et al. (2017) also observed reduction on the activity of chymotrypsin with increasing chitinase activity and chitin levels into the diet. The drop on the activity of chymotrypsin from fish fed without ZM to fish fed with ZM during fry stage and later the control diet could be a reflect of the higher chitinase activity in the last group, however, as no differences were observed for the other

treatments, the influence of chitinase or chitin on chymotrypsin activity seems to be independent of the increase in activity or dietary content.

Although total protease activity is known to be influenced also by the quality of the nutrients (Le Moullac et al., 1996), chitin may interfere with dietary utilization of protein (Longvah et al., 2011). Total protease activity reflects the total activity of alkaline proteases in fish intestine, including brush border enzymes. Reduction of leucine aminopeptidase was reported for Atlantic salmon fed insect meal-based diets (Belghit et al., 2018) and the author suggested an interference of chitin on intestinal turnover or sloughing.

Besides chitinase, the dietary composition had directly affected lipase and total protease activities. Whereas the slight linear increase of lipase activity may be related to differences in the lipid content of experimental diets (< 2 %), the linear reduction of total proteases activity with increasing ZM inclusion did not follow dietary protein composition.

The higher activity of total protease in the intestine of Nile tilapia at the pH 10.0 demonstrated an optimum pH for proteolytic activity measurement. Otherwise, for the carnivorous fish meagre (*Argyrosomus regius*) and white seabream (*Diplodus sargus*) total protease activity in the intestine did not vary the activity in distinct pH (Castro et al., 2013) however, in the pyloric ceca protease was higher for pH 10 than pH 7 in both species. Alkaline pH has been reported to allow the maximum activity of trypsin (Unajak et al., 2012) and chymotrypsin (Zhou et al., 2011) in fish intestine, two important proteolytic enzymes accounted in the analysis of total proteases.

4.2.Histomorphology

Soybean meal is one of the predominant protein sources in commercial tilapia diets, averaging 20-60% of inclusion (Ng and Romano, 2013). However, its antinutritional compounds can cause damages to the integrity of intestinal mucosa (Dumas *et al.*, 2018) and affect nutrients absorption and digestibility (Mahmoud *et al.*, 2014). Some of the histopathological changes observed in fish gut after soybean administrations include shortening of villus height and reduced villus thickness (Zhang et al., 2018) as a typical sign of intestinal inflammation (Rimoldi et al., 2016). Even the

effects of soybean meal intake can vary among fish species (Zhang et al., 2018), a reduction in the levels of soybean meal in the diet in replacement by another protein source could lead to better intestinal morphology and functionality. The improvements on fish intestine observed in the present trial can be associated with the replacement of soybean meal by insect meal. The main modifications included an increase in villus height and in villus area. Intestinal villus height is considered the main aspect to characterize fish absorption ability, intestinal development, health, and functionality (Cerezuela et al., 2012), which influence nutrient digestion and absorption (Wang et al., 2008). Villus increase is generally associated with the increase of total luminal absorptive area and can lead to more absorption of nutrients (Laudadio et al., 2012).

Besides the benefits of soybean meal replacement, the differences in the amino acid composition between soybean and *Z. morio* meal and the presence of chitin might impact the construction of intestinal microvilli. The effects of distinct protein sources on intestine morphology were verified in rainbow trout (Caballero et al., 2002), gilthead seabream (Caballero et al., 2003), and common carp (Ostaszewska et al., 2010). In general soybean meal is deficient in some essential amino acids for fish and in contrast, insects can exceed some fish requirements. An increase in villus height was observed in rainbow trout fed 200 g/kg of *Blatta lateralis* full-fat meal (Josefiak et al., 2019). Unlikely, the chitin and its derivates present in insect meals are reported to decrease nutrients absorption in the intestine (Alegbeleye et al., 2012) and were associated with damages on intestine histopathology of Jian carp fed diets with defeated black soldier fly (BSF) over 7.9% in fishmeal replacement (Li et al., 2017) and reduction on villus height in hens intestine fed up to 14% of BSF (Moniello et al., 2019). However, absence of gut inflammation and morphology damages were reported for rainbow trout (Cardinaletti et al., 2019; Elia et al., 2017), clownfish, *Amphiprion ocellaris* (VargasAbúndez et al., 2019), zebrafish *Danio rerio* (Zarantoniello et al., 2019) and Atlantic salmon (Li et al., 2020) fed BSF larvae meal; and for *Oreochromis mossambicus* (Rapatsa et al., 2017) fed *Imbrasia belina* meal.

4.3.Digestibility

The evaluation of nutrients digestibility allows determining the potential nutritive value of the diet, even more considering the inclusion of new ingredients, with direct effects on fish productive performance. Several studies have been conducted evaluating

the digestibility of insect meals for a wide range of aquatic species. Gasco *et al.* (2019), in systematic analysis, pointed out that, low levels of *T. molitor* and *H. illucens* meals inclusion (147.5 - 250 g/kg) have not changed nutrients digestibility for the carnivorous *S. salar, D. labrax*, and *O. mykiss*. In the present study, an increase in dietary digestibility was observed for tilapia fed diets up to 150 g/kg of ZM and the same was observed for *O. mossambicus* fed diets with up to 240g/kg of *Imbrasia belina* meal (Rapatsa and Moyo, 2017). Thus, seems that lower levels of insect meal or chitin inclusion not only do not negatively affect diet digestibility for carnivorous fish but improve it for omnivorous species. Besides positive effects on intestine health and modulation of gut microbiota (Bruni *et al.*, 2018; Rimoldi *et al.*, 2019), an increase in the relative length of fish intestine could also improve digestion and absorption capacity (Piccolo *et al.*, 2017).

Instead, high inclusions of *T. molitor* and *Hermetia illucens* meals (400-600g/kg) led to a reduction of nutrients digestibility for the carnivorous *Salmo salar, Dicentrarchus labrax, Sparus aurata,* and *Oncorhynchus mykiss* (Gasco *et al.*, 2019). Reduction in nutrients digestibility was also observed for the omnivorous red tilapia (*Oreochromis spp.*) fed *Zophobas morio* meal at 300g/kg of inclusion (Jabir, M. a. R. *et al.*, 2012). The reduction in dietary digestibility has been related to the high content of chitin in insect meal diets (Henry *et al.*, 2015; Belforti *et al.*, 2016; Piccolo *et al.*, 2017; Belghit *et al.*, 2018; Li *et al.*, 2019). Studies evaluating the inclusion of pure chitin in Nile tilapia (*O. niloticus* x *O. aureus*) diets have also reported a reduction in nutrients digestibility (Shiau and Yu, 1999). Chitin can bide with other molecules forming complex structures and reducing the digestibility of protein (Longvah *et al.*, 2011; Schiavone *et al.*, 2018) and lipids (Kroeckel *et al.*, 2012).

A decrease in protein digestibility in special has been reported by fish fed increasing insect meal diets without changing on growth performance or even with better results on feed utilization (Belforti *et al.*, 2016; Gasco *et al.*, 2016; Piccolo *et al.*, 2017). The same was observed in the present study however, by using the nitrogen-to protein conversion ratio factor of 4.76 proposed by Janssen *et al.* (2017), the digestibility of protein remained unchanged. To estimate protein content of meals, diets, or feces in trials with insect meals using the conventional conversion ratio factor of 6.25 (Kjeldahl, 1883) overestimates the protein values and might ended up in misleading results, for example, the reduction in protein digestibility, as described by other authors (Belforti *et al.*, 2016; Belghit *et al.*, 2019).

Dry matter digestibility is usually used to give a general idea about the digestibility of an ingredient or a diet and, when fish fed the 300 g/kg ZM diet in the present study, the dry matter digestibility decreased as an example of high chitin effects. However, for fish fed with ZM during fry stage, the digestibility of dry matter was maintained high. Besides the reduction of energy and lipids were observed, the results of dry matter are consistent with no changes in fish growth performance and allow to affirm that fish fed continuously with ZM had physiological advantages that contributed to the digestion and absorption of high ZM diet, without compromising digestibility and fish performance.

One advantage was the expressive increase of 15-20% on chitinase activity in fish fed with ZM during fry stage comparing to the control group. However, when fed the 150 ZM diet, fish fed without ZM during fry stage exhibited the same dry matter and nutrients digestibility as the other group even presented lower chitinase activity. The dietary digestibility could have been achieved by distinct mechanisms instead. For fish fed without ZM as fry, chitin might have acted more as a prebiotic and, whereas for fish fed with ZM as fry, chitin might have exerted also the function of a nutrient, since fish had a higher capacity to digest this component.

It is important to highlight that insect species and developmental stage, fish species, and food habit, as well as the protein ingredient replaced in the diet, are factors capable of promoting adverse results on the parameters evaluated, such as intestine histomorphology, digestive enzymes activity, and dietary digestibility. At low inclusion levels, replacing fishmeal or other conventional protein sources with insect meal is not a problem because fish seem to adapt well to this substitution, however, when the inclusion levels of insect meal are higher this acceptance by the fish is more challenging. In the present study, the strategy of feeding Nile tilapia continuously, from fry to juveniles, with *Z. morio* meal at high inclusion levels is presented as alternative feed management capable to optimize fish digestive performance and allow inclusions of insect meal up to 30% of *Zophobas morio* meal. Further studies should be performed to determinate the metabolic pathway of chitin and elucidate the mechanisms in which the digested chitin products reacts with fish organism.

5. *Conclusion*

To feed fish continuously with insect meal, from fry to juveniles, is alternative feed management capable to optimize fish digestive performance, allowing inclusions of up to 300 g/kg of *Z. morio* meal and improving insect meal utilization.

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Table 1. Formulation and proximate composition of experimental diets

Ingredients	Experiment 1		Experiment 2		
	Without ZM	With ZM	Control	150 ZM	300 ZM
Fish Meal	232.4	235.2	130.0	130.0	130.0
Zophobas morio Meal	-	247.4	-	150.0	300.0
Soybean Meal	297.8	150.8	195.0	97.5	-
Inert	-	-	58.0	50.0	40.0
Soybean Oil	127.9	64.7	87.0	42.5	-
Corn	51.3	51.9	174.5	174.5	174.5
Rice Bran	188.1	147.0	100.0	100.0	100.0
Corn Gluten	10.1	40.8	95.0	95.0	95.0
Wheat Bran	82.7	52.3	150.0	150.0	150.0
BHT[1]	0.5	0.5	0.5	0.5	0.5
Premix[2]	9.2	9.4	10.0	10.0	10.0
Total	1000.0	1000.0	1000.0	1000.0	1000.0
Proximate analysis (g/kg of dry matter)					
Dry matter (g/kg as fed)	893.2	834.7	940.1	938.0	930.1
Crude protein	408.8	412.0	308.1	303.9	309.6
Ether extract	139.3	181.0	98.0	102.7	112.3
Ash	11.2	10.9	150.1	120.8	119.7
Crude Fiber*	51,31	47,40	45,62	46,25	46,88
Neutral Detergent Fiber*	148,33	126,96	155,21	154,67	154,13
Gross energy (kcal/kg)	4564.0	4875.0	4324.9	4448.6	4591.9
Digestible Protein	371.0	371.0	278.1	274.3	279.8
Digestible Energy (kcal/kg)	3574.5	3801.7	3378.6	3373.4	3385.8
Total Carbohydrates**	538.1	494.2	516.9	545.0	532.1
Chitin	-	56.2	27.5	43.8	60.0
DE/DP	9.6	10.2	12.8	12.8	12.9

[2] Vitamin and Mineral Premix: vitamin A - 500.000 UI; vitamin D3 - 250.000 UI; vitamin E - 5.000 mg; vitamin K3 - 500 mg; vitamin B1 - 1.500 mg; vitamin B2 - 1.500 mg; vitamin B6 - 1.500 mg; vitamin B12 - 4.000 mg; folic acid - 500 mg; pantothenate Ca - 4.000 mg; vitamin C - 10.000 mg; biotin - 10 mg; Inositol - 1.000; nicotinamide - 7.000; choline - 10.000 mg; Co - 10 mg; Cu - 1.000 mg; Fe - 5.000 mg; I - 200 mg; Mn - 1500 mg; Se - 30 mg; Zn - 9.000 mg3. (Agromix LTDA, Sao Paulo, Brazil).

*Calculated according to INRA (2020), Finke (2002) and Benzertiha et al. (2019)

**Total Carbohydrates = 100 - (protein + fat + ash) according to Atwater System.

Digestible protein and energy were calculated by using apparent digestibility coefficients for *Z. morio* meal obtained by Fontes et al. (2019).

Table 2. Intestine digestive enzymes activities (mg/U) and stomach chitinase (U/ml) of Nile Tilapia juveniles fed experimental diets after fed with or without *Z. morio* meal during fry stage[1]

	Without ZM			With ZM			SEM	FF	D	FF x D	Linear	Quadratic
	0ZM	150ZM	300ZM	0ZM	150ZM	300ZM						
Chitinase	0.51	0.57	0.74	0.62	0.67	0.87	0.02	*	*	ns	*	ns
Amylase	7815.9[a]	11940.6[b]	7765.2[a]	7487.4[a]	8540.7[a]	11584.4[b]	343.06	ns	*	*	*	*
Lipase	1.00[a]	1.11[a]	1.00[a]	0.89[a]	1.06[a]	1.15[b]	0.02	ns	*	*	*	ns
Total Protease	117.6[a]	53.1[a]	55.6[a]	120.2[a]	93.8[b]	54.2[a]	5.09	*	*	*	*	ns
Chymotrypsin	21167.8[b]	17708.5[a]	16339.3[a]	16294.4[a]	16887.9[a]	19910.1[a]	480.21	ns	ns	*	ns	ns
Trypsin	368.9[b]	344.8[b]	279.8[a]	268.1[a]	273.2[a]	329.4[b]	7.34	*	ns	*	ns	ns
A/L	7873.3	10908.6[b]	7860.8[a]	8467.6	8158.5[a]	10148.1[b]	289.78	ns	ns	*	ns	ns
A/P	67.2	246.1[b]	152.2[a]	64.8	101.7[a]	224.7[b]	12.60	ns	*	*	*	*
L/P	0.009	0.023[b]	0.019	0.008	0.013[a]	0.022	0.001	*	*	*	*	ns

[1]Values presented as means (n = 8) and pooled standard error of the mean (SEM). Polynomial Contrasts: *p < .05. Polynomial Contrasts: *p < .05. 0ZM: 0 g kg^{-1} of *Z. morio* meal; 150ZM: 150 g kg^{-1} of *Z. morio* meal; 300ZM: 300 g kg^{-1} of ZM; ZM: *Zophobas morio* meal; FF: fry feeding; D: diet; A/L: amylase/lipase; A/P: amylase/proteases; L/P: lipase/proteases.

Table 3. Histomorphometry of intestine of Nile Tilapia juveniles fed experimental diets after fed with or without *Z. morio* meal during fry stage[1]

	Without ZM			With ZM			SEM				Contrast	
	0	150	300	0	150	300		FF	D	FF x D	Linear	Quadratic
hVF	244.32	265.63	230.66	268.92	315.04	250.18	3.53	*	*	ns	*	*
hV	220.75	246.22	209.72	239.67	276.51	220.47	3.36	*	*	ns	*	*
hLP	190.00[a]	215.04[a]	184.29[a]	219.69[b]	255.93[b]	187.70[a]	3.44	*	*	*	*	*
wV	80.92	82.38	82.38	81.47	86.73	91.46	1.17	*	ns	ns	ns	ns
wLP	51.73	52.21	58.01	53.70	57.78	63.50	0.99	*	*	ns	*	ns
aV	165.28	172.92	169.64	193.39	216.83	212.21	4.28	*	ns	ns	ns	ns
aLP	100.85	102.31	102.08	110.51	125.11	132.49	2.87	*	ns	ns	ns	ns
hLP_hV	0.86[a]	0.87[a]	0.87[a]	0.91[b]	0.92[b]	0.85[a]	0.01	*	*	*	ns	*
wLP_wV	0.65	0.64	0.71	0.68	0.67	0.69	0.01	ns	ns	ns	ns	ns
aLP_aV	0.61	0.58	0.60	0.57	0.58	0.62	0.01	ns	ns	ns	ns	ns

[1]Values presented as means (n = 48) and pooled standard error of the mean (SEM). Polynomial Contrasts: *p < .05. 0ZM: 0 g kg^{-1} of *Z. morio* meal; 150ZM: 150 g kg^{-1} of *Z. morio* meal; 300ZM: 300 g kg^{-1} of ZM; ZM: *Zophobas morio* meal; FF: fry feeding; D: diet; hTF: total villus height; hV: villus height; hLP: lamina propria height; wV: villus width; wLP: lamina propria width; aV: villus area; aLP: lamina propria area; hLP_hV; lamina propria height/villus height; wLP_wV: lamina propria width/villus width; aLP_aV: lamina propria area/villus area.

Table 4. Apparent digestible coefficients (%) of Nile Tilapia juveniles fed experimental diets after fed with or without *Z. morio* meal during fry stage[1]

	Without ZM			With ZM			SEM	Contrast				
	0ZM	150ZM	300ZM	0ZM	150ZM	300ZM		FF	D	FF x D	Linear	Quadratic
Chitin	32.3[a]	62.9[a]	54.9 [a]	41.4[a]	73.2[a]	83.9 [b]	4.87	*	*	*	*	*
Dry Matter	65.6[a]	81.1[a]	65.1[a]	63.4[a]	78.1[a]	77.7[b]	2.04	ns	*	*	ns	*
Lipids	90.2	92.0	85.2	88.6	93.2	90.0	0.85	ns	*	ns	ns	*
Protein	89.6[b]	89.7[a]	84.4[a]	83.9[a]	90.8[a]	89.1[a]	0.89	ns	ns	*	ns	ns
Energy	73.9	84.5	75.4	72.0	84.2	81.3	1.56	ns	*	ns	ns	*
Carbohydrates	59.4[a]	56.4[a]	77.8[b]	82.8[b]	63.2[a]	65.9[a]	2.97	ns	*	*	ns	*

[1]Values presented as means (n = 9) and pooled standard error of the mean (SEM). Polynomial Contrasts: *$p < .05$. 0ZM: 0 g kg^{-1} of *Z. morio* meal; 150ZM: 150 g kg^{-1} of *Z. morio* meal; 300ZM: 300 g kg^{-1} of ZM; ZM: *Zophobas morio* meal; FF: fry feeding; D: diet.

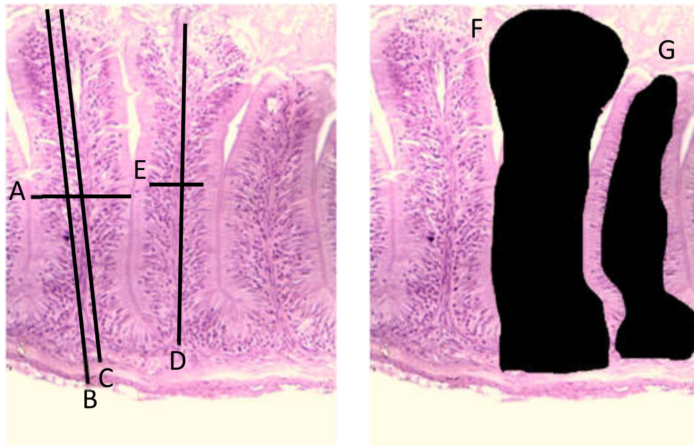

Figure 1. Method used to measure the height, width (μm), and area (μm^2) of intestinal villi of Nile tilapia juveniles. Images H&E 40x. A (villus width), B (total villus height), C, (villus height), D (lamina propria height), E (lamina propria width), F (villus area), G (lamina propria area)

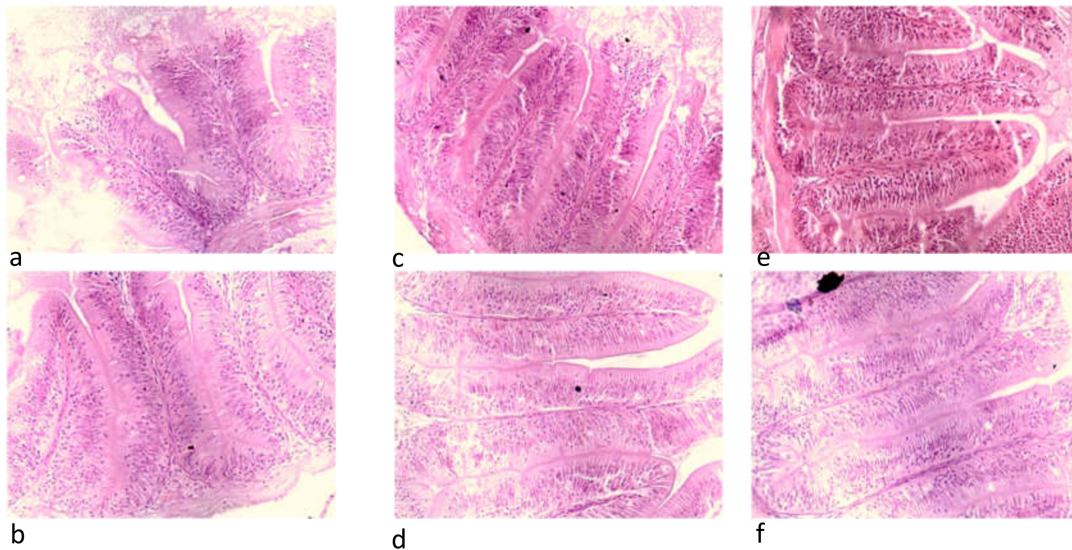

Figure 2. Histological modifications on intestines of Nile tilapia juveniles feeding diets with increasing levels of *Z. morio* meal inclusion and soybean meal replacement after fed with or without *Z. morio* meal during fry stage. Images H&E 40x. Fish fed without ZM (a, c, e) and fish fed with ZM (b, d, f) during fry stage. 0g/kg of ZM (a, b), 150 g/kg ZM (c, d), 300 g/kg ZM (e, f).

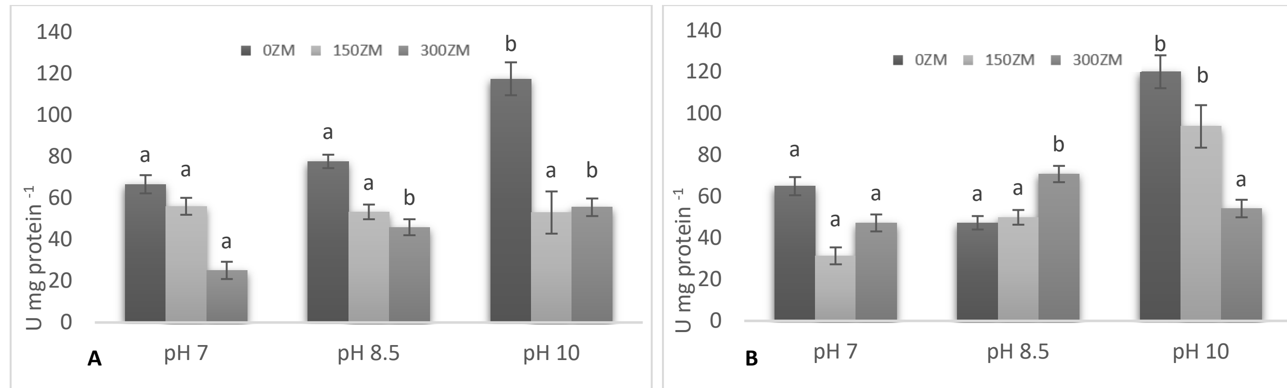

Fig. 3. Effect of pH on protease specific activity in the intestine of tilapia fingerlings, fed without (A) or with (B) ZM diets during fry stage and, later, 0, 150 or 300 g/kg of ZM diets (0ZM, 150ZM and 300ZM). Values are mean ± SEM (n = 8). Distinct small letters indicate differences ($p < 0.05$) between pH values for each diet.

7. MANUSCRIPT 3.

To be submitted to: **MethodsX, ISSN: 2215-0161**

An optimized method for chitinase activity measurement in tropical omnivorous fish

Title	*A modified method for chitinase activity measurement in tropical omnivorous fish*
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Keywords	• *Insect meal* • *Tilapia* • *Chitin*
Direct Submission or Co-Submission	*Direct SubmissionDirect Submission*

ABSTRACT

To measure the activity of chitinolytic enzymes are extremely important since it can reflect the capacity of a tissue or organism on the breakdown of chitin. The measurement of the endochitinase activity itself dependent on the protocol adopted. Up to now, one of the most adopted protocols to determinate total chitinolytic activity is adapted for marine fish species. As chitinase type and activity vary between fish species, it is necessary to validate the methodology for tropical fish species. We propose a modified method easy to follow, with reduced cost, and applicable in case of a limited amount of sample, that allows the measurement of reasonable and consistent chitinolytic activity in Nile tilapia stomach.

SPECIFICATIONS TABLE

Subject Area	Agricultural and Biological Sciences Agricultural and Biological Sciences
More specific subject area	*Enzymatic analysis applied to aquatic science*
Method name	*Spectrophotometer method for chitinase analysis*
Name and reference of original method	*Abro, R., et al., Evaluation of chitinolytic activities and membrane integrity in gut tissues of Arctic charr (Salvelinus alpinus) fed fish meal and zygomycete biomass. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology, 2014. 175: p. 1-8.*
Resource availability	

Method details

Background

Chitinase (E.C 3.2.1.14) is the key enzyme of chitin digestion as does the first catalytic breakdown of dietary chitin. However, adopting the correct description and nomenclature in studies involving chitinolytic enzymes are extremely important since it can reflect the activity of a specific enzyme or the total chitinolytic activity of a tissue or organism. According to the International Union of Biochemistry enzyme nomenclature, endochitinases (EC 3.2.1.14) solubilizes the chitin by cleaving its internal site at random positions and producing N-acetyl glucosamine multimers. The degradation of multimers into monomers of N-acetyl glucosamine is realized by two exochitinases: chitobiosidases (E.C. 3.2.1.29) and, 1-4-β-glucosaminidases (E.C. 3.2.1.30) (Harman *et al.*, 1993; Sahai and Manocha, 1993). The practical method to verify the specific activity of chitinolytic enzymes involves the use of Chitinase Assay Kit Protocol (Sigma-Aldrich) where the substrates are specific for certain chitinolytic enzymes: exochitinases, N-acetyl glucosaminidase, and chitobiosidase, and endochitinases. However, considering the large amounts of samples analyzed in a *in vivo* trial and the high costs of the kits, protocols with lower financial inputs are preferable.

Most studies use the term activity of chitinase or endochitinase (EC 3.2.1.14), however, the measurement of the endochitinase activity itself dependent on the protocol adopted. If specific substrates are not used, the measurement reflects the total chitinolytic activity since the product will be released through a conjunct action of endo and exochitinases. One of the most adopted protocols to determinate total chitinolytic activity is the one

proposed by Jeuniaux (1966), with recent modifications proposed by Gutowska *et al.* (2004), and Abro *et al.* (2014) in fish trials. As chitobiose does not react, this protocol involves the quantification of the monomer N-acetyl glucosamine (NAG), released after samples incubation with chitin. The monomer is linked with a color reagent and the absorbance is read in a spectrophotometer. The chitinolytic activity is calculated using a standard curve of NAG (Sigma # G7274).

However, several fish species possess multiple and even novel active chitinases (Matsumiya *et al.*, 2006; Ikeda *et al.*, 2009; Ikeda *et al.*, 2013). Thus, differences among species should be considered, since multiple chitinase isomers with different or similar functions are expressed (Adrangi and Faramarzi, 2013). The activity and function of chitinolytic enzymes vary between fish species, food habit, feed frequency, and so on the methodologies used to determinate them (Fänge *et al.*, 1979; Lindsay, 1984; Gutowska *et al.*, 2004). The method proposed by Jeuniaux (1966) and later modified by Gutowska *et al.* (2004), and Abro *et al.* (2014) was developed using carnivorous marine fish species as a model. So, it is necessary to questions its applicability for different fish species, especially for tropical omnivorous fish, as is the case of the Nile tilapia (*Oreochromis niloticus*), the third species most produced worldwide (FAO 2020). The potential of the use of insect meal in Nile tilapia diets revolves around not only the high plasticity of food type but also the necessity of reducing the footprint and improve fish performance and healthy at the same time.

Location and ethical approval

The *in vivo* experiment was conducted at the Fish Culture Station at the Federal University of Lavras, in Lavras, Minas Gerais, Brazil. The *in vitro* experiment was carried out in the Fish Nutrition Laboratory at Porto University, Porto, Portugal. All experimental procedures were approved by the Ethical Commission of Animal Use (CEUA) of the Federal University of Lavras (UFLA), protocol number 041/18.

Original method

The present study proposes modifications to the method described by Abro *et al.* (2014) for the determination of endo-chitinase activity in fish. The original method is based on modifications for microplate reader of the procedures described by Jeuniaux (1966) and Gutowska *et al.* (2004). The method described in Abro *et al.* (2014) has the following steps:

1. Homogenize the samples with the buffer (1 M malic acid, 40 mM sodium hydroxide, 40 mM sodium chloride, 40 mM calcium chloride, 0.1% sodium azide) and adjust the pH to 5 for the stomach.
2. Centrifuge the homogenates for 10 min at 15,800 *g*.
3. Take 250 µL supernatant from the centrifuged homogenate, 250 µL chitin suspension, and 250 µL distilled water into glass tubes for the test assay.
4. Place the glass tubes on a rotary shaker for incubation at 20 °C for 2 h.
5. Boil the tubes with the assay solution for 10 min to stop the reaction, cooled to room temperature, and centrifuge for 30 min at 13,600 g.
6. Transferred a sample of the supernatant (0.5 mL) to a new tube containing 0.1 mL 0.8 M borate buffer ($K_2B_4O_7$) to maintain pH 9.8 in the solution.
7. Boil the solution was boiled for 3 min and immediately cooled in tap water.
8. Add 3 mL of p-dimethylamino-benzaldehyde (DMAB) solution
9. After mixing the solution, incubate at 37 °C for 20 min to promote color reaction.
10. Measure the formation of NAG at 550 nm at room temperature on a microtiter plate reader.
11. The chitinolytic activity is calculated by using the absorbance values obtained and a standard curve of NAG (Sigma# G7274) with a concentration of 5–200 µM.

The methods described in Abro *et al.* (2014) and the studies used to support it are regarding marine fish species. Thus, the adequacy of the procedure described above was evaluated for the tropical Nile tilapia (*Oreochromis niloticus*), and concerns about particular steps will be discussed in sequence.

Experimental procedures

The endo-chitinase activity was measured in stomach samples collected from Nile tilapia juveniles (21 ± 2 grams mean weight) fed experimental diets for 60 days. The diets were isoproteic, isoenergetic, and isolipidic (270 g/kg of digestible protein, 3370 kcal/kg of digestible energy, and 112 g/kg of lipids) and in brief, contained fish meal, corn, rice bran, corn gluten, wheat bran, soybean meal (control), and 150 or 300 g/kg of *Zophobas morio* meal replacing the total of soybean meal. The samples were collected and stored at -80˚ C until further analysis.

Total chitinolytic activity assay and statistical analysis

In the present study, we will use the concept of total chitinolytic activity since the enzymatic activity was measured by the content of the monomer NAG released, following the methodology described in Abro *et al.* (2014) and detailed above. The standard curve of NAG (Sigma # G7274) was constructed from zero to 500μM. The total chitinolytic activity was calculated by including the absorbance measured on the standard curve equation and converting the values from μM of NAG to U/ml of enzyme activity.

Normality and homogeneity of variances were tested using the Shapiro-Wilk and Levene tests, respectively. All statistical procedures were performed using the SPSS software package for Windows (IBM® SPSS® Statistics, New York, USA) and probability level of 0.05 was used for rejection of the null hypothesis. Data were analyzed by Student's t-test in case of two treatments, and by one-way ANOVA for superior number of treatments. When necessary, Tukey's test was used to detect differences between treatments. For method validation the results were evaluated using a simple linear regression according to the following model:

$$Y_{ij} = \beta 0 + \beta 1 \times Xi + e_{(i)j},$$

where Y_{ij} is the observed total chitinolytic activity of the ith level of insect meal inclusion in the jth replicate; β0 is the intercept and β1 is the slope; Xi is the level of of insect meal inclusion; and $e_{(i)j}$ is the random error assumed.

Modified method evaluation

To evaluate the modified method, it was tested only samples of Nile tilapia fed the highest insect meal diet (300 g/kg of *Zophobas morio* meal) and so chitin (67.4 g/kg of diet) to identify a high chitinolytic activity.

In the step 1 of the original method we opted to utilize as a buffer the 0.15 M citric acid, 0.3 M Na_2HPO_4 (pH 5) described in Gutowska *et al.* (2004) for homogenization of the samples, due to the availability of the components and the facility to prepare it. The samples were homogenized in 1:6 dilution with an Ultra Turrax. To standardize the homogenate and allows its use for chitinase and for the other digestive enzymes currently evaluated in our lab, the same protocol for homogenization was adopted. The

homogenates were then centrifugated at 4˚C for 30 minutes at 30000 *g* instead the 10 min at 15,800 *g* described in the original protocol.

Then, the original method of Abro *et al.* (2014) was tested in the stomach of Nile tilapia fed the insect meal diet in three different dilutions of homogenate:buffer (1:10, 1:20 and 1:50) in six replicates. Neither the absorbance values nor chitinase activity (U/ml) responded to the dilutions and, the activity of chitinase obtained had negative values (data not shown).

The samples were then tested in a concentrated state, without being diluted. Considering that water can be used for samples homogenization in the study of other digestive enzymes as it was in several studies (Gisbert *et al.*, 2009; Hassaan *et al.*, 2019; Jesus *et al.*, 2019) we decided to test if water could be used, instead of the buffer, to homogenize Nile tilapia samples for chitinase. So, samples from the same fish were homogenized with water mili Q. (4 ˚C) or with the 0.15 M citric acid, 0.3 M Na_2HPO_4 (pH 5) buffer, and the activity of chitinase was measured (Figure 1).

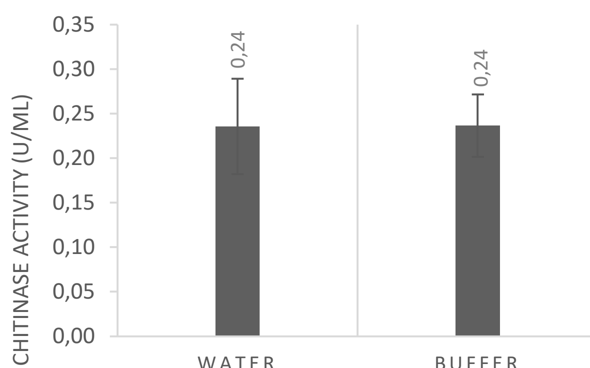

Figure 1. Specific activity of stomach chitinase of tilapia fed 300 g/Kg of insect meal after homogenization with water miliQ (4 ˚C) or 0.15 M citric acid, 0.3 M Na_2HPO_4 (pH 5) buffer.

There was obtained a small but positive activity of chitinase in fish stomach, and no differences were observed between water and buffer homogenization. As it was used the whole organ in the homogenization the ideal pH for chitinase was maintained and like the buffer pH (4.98±0.02). The substitution of buffer by water mili Q. (4 ˚C) for samples homogenization did not negatively impact the reaction. Then, from this moment on the samples were homogenized in water miliQ (4 ˚C).

According to the differences in food habits between species and so the differences in the time of food permanence into digestive organs, where omnivorous fish have longer

digestive transit time, the chitinase activity was evaluated in two distinct incubation times (original protocol – 2H and, overnight – 16H) and for fish fed diet with insect meal inclusion. The overnight incubation was choose considering the long transit time and the easy of execution in the laboratory. The results showed increasing the time of incubation led to higher activity of chitinase (Figure 2).

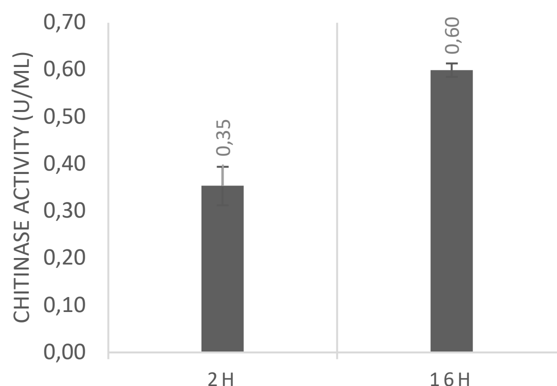

Figure 2. Specific activity of stomach chitinase of tilapia fed 300 g/Kg of insect meal after sample homogenization with water miliQ (4 ˚C) and incubation with chitin for 2 (2H) or 16 (16H) hours at room temperature (25 ˚C).

In the step 3, the original method described the reaction as being 250µl of sample extract, 250µl of substrate and 250µl of distilled water, however Gutowska *et al.* (2004) run the analysis using the proportion of 2:1 of samples extract and substrate. Considering that is currently on biological trials limited amount of samples, the use of reduced volumes in laboratory analysis should be tested and applied when possible. Thus, using the incubation period of 16 hours, we tested different volumes of samples extract and substrate into three different options:

1. 500µl of sample extract, 250µl of substrate and 250µl of distilled water (Gutowska *et al.*, 2004);
2. 250µl of sample extract, 250µl of substrate and 250µl of distilled water (original method of Abro *et al.* (2014));
3. 200µl of sample extract, 100µl of substrate, and 100µl of distilled water (original method of Abro *et al.* (2014) with reduced volume).

The results are in Figure 3, and it was observed that option 3 promotes the highest chitinase activity. The use of smaller volumes is beneficial for the cases where the amount of sample is limited, and reduces the costs with other reagents, as the quantity of reagents used is proportional to the quantity of sample. However, further studies should be done to better explain the higher chitinase activity when using lower volumes compered to bigger volumes in the same dilution.

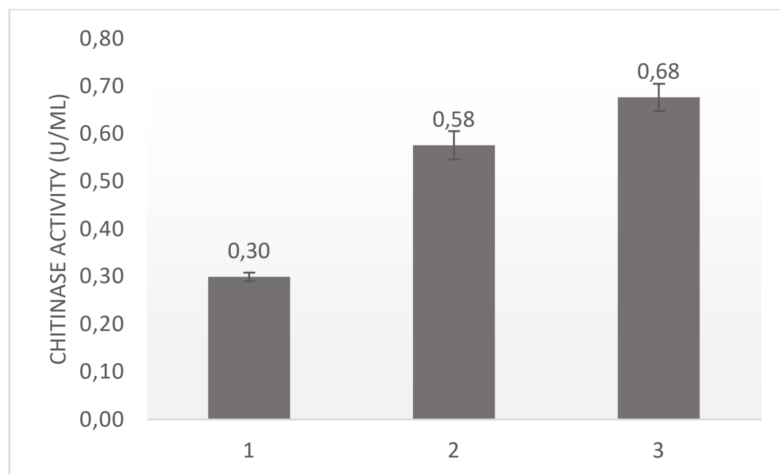

Figure 3. Specific activity of stomach chitinase of tilapia fed 300 g/Kg of insect meal in using different volumes of sample extract. Different letters indicate differences among treatments ($p<0.05$) detected after Tukey´s Test. **1.** Gutowska *et al*. (2004); **2.** original method of Abro *et al.* (2014); **3.** original method of Abro *et al.* (2014) with reduced volume

In step 4, considering the increase in chitinase activity previously reported, the incubation time was deeper evaluated by testing other incubation hours (2, 4, 6, 12, 16 and 24, 16 and 48 hours). An additional treatment of 10 minutes of incubation was tested to permit the calculation of the delta at the 2 hours treatment. However, 10 minutes was not enough to detect the activity of chitinase, and the values were negative. As negative values for enzyme activity have no biological acceptance and explanation, we decided to exclude the specific activity at 2 hours from the analysis.

The results in Figure 4 had shown that incubating the stomach samples of Nile tilapia with chitin for 6 hours at room temperature provided the higher chitinase activity. That means that after 6 hours of incubation the value of chitinase activity increased in better proportionality between activity and time, which characterizes as the moment where the enzyme activity was more efficiently expressed.

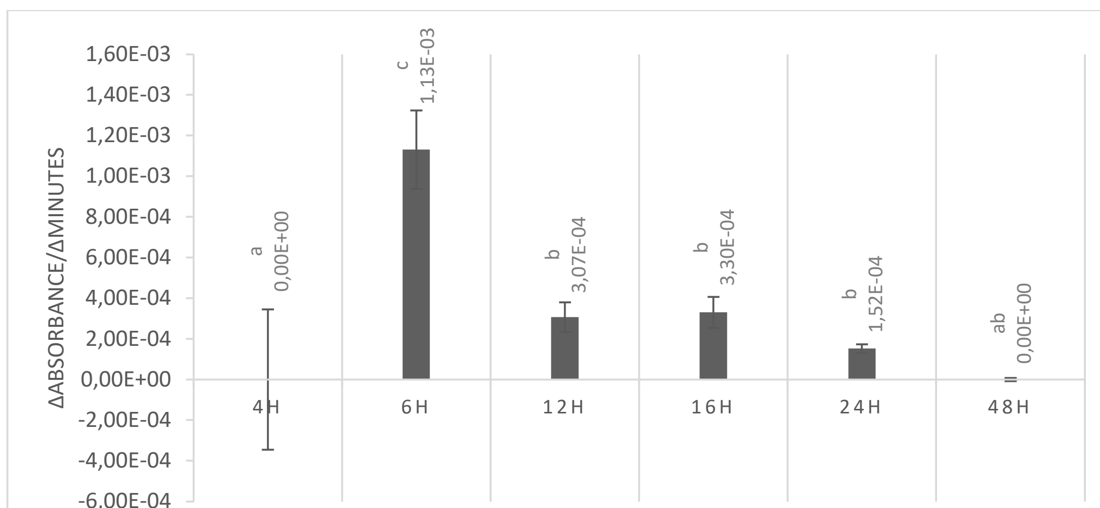

Figure 4. Chitinase specific activity (Δabs/Δmin) calculated from Nile tilapia stomach samples after increasing hours of incubation with chitin. Different letters indicate differences among treatments (p < 0.05) detected after Tukey's test.

Comparison between methods

The activity of chitinase obtained by both the original and modified method was quite different (Figure 5). While the original method measured 0.32 U/ml of chitinase activity, the modified method obtained a value almost 260% higher. The presence of chitinolytic activity in Nile tilapia was previously reported by (Molinari *et al.*, 2007) using a fluorescence spectrophotometer and the fluorogenic substrates according to the Chitinase Assay Kit Protocol (Sigma-Aldrich). The chitinolytic activity reported in the present study using the modified method was different to the endochitinase activity reported by Molinari et al. (2007), which certifies the necessity of standardization of the protocol for measuring Nile tilapia chitinolytic enzymes.

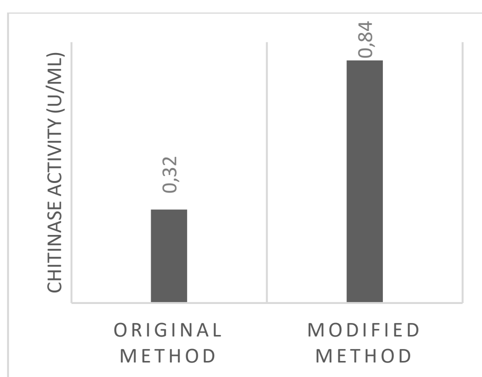

Figure 5: Specific activity of stomach chitinase of of Nile tilapia fed 300 g/Kg of insect meal using the original and the modified method.

It is known that in omnivorous fish the food remains in the digestive tube for a longer period than for carnivorous fish due to the bigger size of the intestine. The time of stomach emptiness in Nile tilapia was reported to be around 8 hours post-feeding (Riche et al. 2004; Heng et al. 2007; Uscanga et al. 2010), demonstrating that for the Nile tilapia and in real biological conditions, the chime reacts with the stomach chitinase for a period longer than 2 hours.

Method validation

The validation of the modified method proposed for chitinolytic activity determination was performed by evaluating the chitinolytic activity of the stomach of Nile tilapia fed diets with increasing content of insect meal (0, 150, and 300 g/kg of *Zophobas morio* meal). The response of the chitinolytic enzyme activity on the increasing insect meal content into the experimental diets was linear (chitinolytic activity = 0.0006 × insect meal + 0.6778) with an R^2 = 0.87. The increase of chitinolytic activity in fish stomach was very similar to the increase of insect meal in the diets: 11% for the 150g/kg diet and 28% for the 300g/kg diet (Figure 6).

Figure 6. Relationship between the increasing levels of insect meal inclusion (0, 150, and 300 g/kg on specific activity of stomach chitinase of Nile tilapia.

Conclusion

We presented a method for the measurement of total chitinolytic activity in the Nile tilapia stomach. The proposed modifications allowed to achieve a reasonable and consistent chitinolytic activity in Nile tilapia samples. Also, the method is easy to follow, with reduced cost, and applicable in case of a limited amount of sample. Furthermore, this method

saves technicians labor by removing the necessity of a specific buffer and allowing the use of homogenates for other analyses that also do not require a specific buffer.

Acknowledgments

Thanks to the National Council for Technological and Scientific Development (CNPq) (Grant number 148553/2016-8). Coordination for the Improvement of Higher Education Personnel (CAPES) (Grant number 88881.190287/2018-0; 88887.508821/2020-00)

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Supplementary materials

Supplementary material associated with this article can be found, in the appendix section.

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Appendix A: Supplementary material and/or Additional information:

Description of the modified method and calculation example

Reagents

- Hydrochloric acid (HCl – UN1789)
- Potassium tetraborate ($B4K_2O_7 \bullet 4H_2O$ – Sigma P5754, Sigma Chemical Co., St. Louis, MO)
- 4-(Dimethylamino)benzaldehyde (DMAB – Sigma D2004, Sigma Chemical Co., St. Louis, MO)
- Glacial acetic acid ($C_2H_4O_2$)
- Purified chitin (Sigma C9752, Sigma Chemical Co., St. Louis, MO)
- N-Acetylglucosamine (NAG – Supelco)

Solution preparation

- Borate buffer 0.8M: Prepare the initial solutions of 12.22g of B4K2O7 • 2H2O to each 50mL of H2Odd. Perform the mix using a goblet with a magnet and a heating plate (as it is a supersaturated solution it needs to reach 80°C to completely dissolve). Adjust the final pH to 9.3 using HCl. Adjust the final volume in a volumetric flask.
- DMAB: Must be freshly prepared. Prepare the initial solution of HCl 12N; Perform the mix using a goblet with a magnet; Add to the goblet the 1.5g of DMAB with a small volume of glacial acetic acid; Add the correspondent volume of HCl 12N; Adjust the final volume in a volumetric flask. Note: Prepare, if possible, under the hood.
- Chitin suspension: Perform the mix using a goblet with a magnet. Prepare the mix using H2Odd; The solution must be continuously mixed on a magnetic stir plate during pipetting to maintain a uniform colloidal suspension
- Standard solution of NAG 5mM: Perform the mix using a goblet with a magnet. Weight 5.53 mg of NAG into a goblet and add 5 mL of distilled water.

Procedures

- *Homogenates preparation:*
  1. Immediately weight the samples in a 50ml Falcon tube after removing them from the -80 ˚C storage.
  2. Perform a dilution by adding distilled water to the 50ml Falcon tube. Example: to do a 1:6 dilution, multiply the weight of the sample (g) by 5x and add the volume of distilled water (ml) to the sample.
  3. With the tubes immersed on ice, homogenize the samples with an Ultra Turrax.

4. Centrifuge the homogenates for 30 min at 4˚C, at 30000 *xg*. Collect the supernatant, divide it into 0.5 ml microtubes and store them at -80 ˚C until perform the next steps.

- *Chitinase assay:*

1. Unfreeze the homogenates at room temperature. Gently shake and take samples for the test. In a 2 ml microtube, take 200 µL from the homogenate. Add 100 µL chitin suspension and 100 µL distilled water.
2. Three blanks solutions are necessary. Using a 2 ml microtube:
   Control blank: take 200 µL of the homogenate + 200 µL distilled water.
   Reagent blank: take 400 µL of distilled water (do no need to incubate);
   Substrate blank: take 100 µL of chitin suspension + 300 µL distilled water.
3. Placed the microtubes on a rotary shaker for incubation at room temperature for six hours.
4. Boil the microtubes 10 min to stop the reaction.
5. Cool the microtubes at room temperature.
6. Centrifuge at 13600g for 30 min (This step is not needed for the NAG standard curve);
7. To 2 ml microtubes, collect 0.25 mL of the chitinase assay solution (samples, blanks and standards). Add 0.05 mL of Borate Buffer 0.8M.
8. Place the microtubes in a boiling water bath for three minutes. Then, rapidly cool the microtubes in water.
9. Add 1.5 mL of DMAB and incubate the microtubes at 37°C for 20 minutes using a water bath.
10. Load the microtiter plate and read at 585 nm.

- *Standard curve:*

1. Take the standard solution of NAG 5mM and perform dilutions to achieve the desired concentrations for the calibration curve.
2. Execute the protocol starting at the step 7.

Notes:

i. The chitin solution was continuously mixed on a magnetic stir plate during pipetting to maintain a uniform colloidal suspension.
ii. The test was performed in duplicates for samples and blanks.

iii. The 2 mL microtube allows the solution to be continuously mixed when placed in the rotary shaker; Tubes with irregular bottle did not cause sufficient agitation, allowing the chitin to settle on the tube.

Calculation example

An example of NAG standard curve is present in Supplementary Table 1. The standard curve must be constructed with the absorbance read minus the absorbance of the blank or control.

Supplementary Table 1. Example of standards absorbance

	µM of NAG								
	0	10	25	50	75	100	150	250	500
Absorbance (nm)	0.000	0.0102	0.0203	0.0451	0.0644	0.0756	0.1332	0.2348	0.5225

Standard equation: Y = 0.001x – 0.0112, R^2 0.995

- Example: **Sample absorbance average equal to 0.1819**.

Control Blank: 0.0714; Reagent Blank: 0.0483; Substrate Blank: 0.0808

Incubation Time: 360 minutes

Extract volume: 0.2 mL

1. *Calculate the µM of NAG of the sample.*

First the blanks must be removed from the sample absorbance read:

$ABS_{sample} = 0.1819 – ((0.0808 - 0.0483) + (0.0714 - 0.0483) + 0.0483)$

$ABS_{sample} = 0.0780$

Then to calculate the [NAG] substitute the x in the standard equation by the sample absorbance:

$NAG_{\mu M} = (0.0780 – b)/a$

$NAG_{\mu M} = (0.0780 + 0.0112)/0.001$

$NAG_{\mu M} = 89.15$

2. *Calculate the activity of chitinase in U/ml*

To convert the [NAG] in U/ml divide it by the time of incubation in minutes:

$U_{\mu M/min} = 89.15/360$

$U_{\mu M/min} = 0.25$

Then, divide the value obtained by the total volume of homogenate extract used in the assay:

$U/ml_{\mu M/min/ml} = 0.25/0.2$

$\mathbf{U/ml_{\mu M/min/ml} = 1.24}$

8. MANUSCRIPT 4

To be submitted to: **Journal of World Aquaculture Society, ISSN: 1749-7345**

Optimal fasting time and intestine sampling in digestive enzymatic assays for Nile tilapia (*Oreochromis niloticus*)

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Abstract

To determine the optimal fasting time and intestine sampling procedures in Nile tilapia (*Oreochromis niloticus*) enzymatic assays, the activity of amylase, lipase, trypsin, and chymotrypsin on each intestine portion of Nile tilapia, and plasmatic glucose, cholesterol, and triglycerides were evaluated varying the postprandial hours. 108 juveniles (87.6 ± 1.7 g) were randomly distributed in nine circular tanks, in a completely randomized design with nine treatments (0, 2, 4, 6, 8, 10, 12, 24, and 48 postprandial hours) and twelve replicates, fed with commercial diet for 30 days. Digestive enzymes activities were affected by intestine portion and, postprandial hours or hours post-feeding (HPF). With few exceptions, amylase, lipase, trypsin and chymotrypsin had shown the highest activity in anterior portion of fish intestine followed by mid and posterior portion. Anterior and mid intestine portion had similar patterns of activity along postprandial hours. The peak of amylase occurred from 8 to 12 HPF, lipase at 6 HPF, trypsin from 10 to 12 HPF, and chymotrypsin from 8 to 10 HPF. Otherwise, the highest activity in posterior portion of amylase at 0, 2, 4, and 48 HPF, as so lipase and chymotrypsin after 48 HPF, reflects the enzyme-producing bacteria contribution to the host enzymes activities. Plasmatic triglycerides peaked at 6 HPF, glucose from 8 to 12 HPF, and cholesterol at 4 HPF. Overall, to sample the anterior portion of the intestine into the interval between 10 and 12 HPF is an adequate sampling procedure to be used in digestive enzymatic assays for Nile tilapia.

Keywords: enzymes; gastric emptying; glucose; intestine portion; postprandial hours; omnivorous

Declarations: Not applicable

1. Introduction

Amylase, lipase, trypsin, and chymotrypsin are digestive enzymes synthesized by the pancreas and secreted into the proximate portion of fish midgut through the pancreatic duct (Formicki and Kirschbaum 2019), with substantial importance on the breakdown of carbohydrates, lipids, and proteins delivered into the gastrointestinal tract through dietary intake. The study of these enzymes is essential for understanding fish physiology in front of factors that could modify physiological mechanisms, such as changes on dietary composition (Mohammadi et al. 2019), the inclusion of feed additives (Guerreiro et al. 2017; Ha et al. 2019; Hassan et al. 2020), fish development and environmental changes (Hani et al. 2018; Guerra-Santos et al. 2017), fish nutritional status (Furné et al. 2008; Tomadoni et al. 2020), digestive capacity (Dawood et al. 2020; Pradhan et al. 2020) and feed management (Thongprajukaew et al. 2017).

Furthermore, the release and activity of the digestive enzymes are variable and can be modulated by several factors like dietary composition, intestinal transit time, chime arrival in the intestine, and/or fish species (Fountoulaki et al. 2005; Caruso et al. 2008; Krogdahl et al. 2011). Thus, to perform an accurate evaluation of the activity of digestive enzymes it is necessary to understand fish digestive physiology and anatomy, to capture the right moment after feeding and, the right sampling location where those enzymes will exhibit their maximum activity.

Nile tilapia (*Oreochromis niloticus*) is a rustic species, widely distributed and produced over the world. It is the third specie most produced worldwide and the first specie most produced in Brazil (FAO 2020). It has omnivorous food habits adaptable to a variety of feed ingredients as animal or vegetal sources, usually present in commercial diets (El-Sayed 2006). Studies with Nile tilapia involving digestive enzymes analysis have adopted distinct and long starvation time prior intestine sampling (Santos et al. 2020; Santos et al. 2016; Deng et al. 2019; Mohammadi et al. 2019; Dawood, et al. 2020), probably a reflect of the lack of studies regarding the postprandial activity of digestive enzymes for this species. Due to the absence of researches and standardization of sampling procedures, it is not possible to affirm that the approaches for

intestine sampling adopted in previous literature reflect the real digestive enzymes' capability on the breakdown of dietary nutrients.

Considering the extension of the usage of digestive enzymes analysis, variations on the procedures and/or the use of no empiric methods should not be encouraged. Thus, the aim was to determine the optimal fasting time and intestine sampling procedures for enzymatic assays, evaluating the activity of the digestive enzymes amylase, lipase, trypsin, and chymotrypsin on each intestine portion of Nile tilapia and plasmatic glucose, cholesterol, and triglycerides, varying the postprandial hours.

2. Material and Methods

The project was conducted at the Fish Culture Station at the Federal University of Lavras (UFLA) and the Central Laboratory of Animal Research (LCPA/DZO), in Lavras, Minas Gerais, Brazil. All experimental procedures were approved by the Ethical Commission of Animal Use (CEUA) of the Federal University of Lavras (UFLA), protocol number 006/ 2015.

2.1. Fish and Culture Conditions

Nile Tilapia (*O. niloticus*) were obtained from the Fish Culture Station – UFLA and acclimatized for 15 days upon arrival to the experimental facilities. After that, 108 juveniles (87.6 ± 1.7 g mean weight) were randomly distributed in nine circular tanks (280 L of capacity) at a density of 12 fish/tank. The trial was carried out in a completely randomized design with nine treatments (0, 2, 4, 6, 8, 10, 12, 24, and 48 postprandial hours) and twelve replicates. Each fish was considered as an experimental unit. All fish were weighed and microchipped at the beginning of the trial. During the trial, fish were fed with an extruded commercial diet (Aquos Juvenil 36, Neovia, 360 g/kg of crude protein and 80 g/kg ether extract) three times daily (8:00, 12:00, and 16:00h), until apparent satiation.

The tanks were placed in a recirculated system supplied with aeration and constant temperature kept by heaters. The water quality was maintained using supplemental aeration (central line and air diffusers), mechanical and biological filtration, and UV lamps. The dissolved oxygen and pH

were monitored daily using a multiparameter (U-10, Horiba, Kyoto, Japan). Total ammonia and nitrite were measured using commercial kits (Labcon Test Fresh Water Toxic Ammonia and Labcon Test Nitrite NO_{2^-}). Average water temperature was 26 ± 1 ºC, dissolved oxygen 6.5 ± 2 mg L^{-1}, toxic ammonia 0.03 ± 0.01 mg L^{-1}, and nitrite 0.01 ± 0.01 mg L^{-1}.

The basic composition of the commercial diet provided by the company includes as major ingredients: rice bran, soybean meal, wheat meal, meat, and bone meal, fishmeal, blood meal, poultry meal, cornmeal, and soybean oil; and eventual substitutes: broken rice, cotton meal, corn gluten meal 60, corn gluten meal 21, corn bran, salmon meal, hydrolyzed feather meal, wheat bran, and soy lecithin.

2.2. Sampling

The trial lasted 30 days, and at the end, all fish (131.0 ± 2.27 g mean weight) from each tank were fed one last time. After the postprandial period previously determined, fish from each treatment were anesthetized with benzocaine (50 mg/L). Blood was obtained from the caudal vein with heparinized needles and plasma recovered after centrifugation at 1,000 *g* for 10 min and stored at -80 ºC until analysis. Fish were then euthanized by the spinal cord section and dissected on chilled trays. The intestines were carefully removed freed from the adjacent adipose tissue and divided into three portions (anterior, mid, and posterior) as illustrated in Fig. 1.

Fish intestines were disposed into three loops, with equal length, respecting the differences of intestine size between individuals. Anterior portion was defined as the segment corresponding to 2 cm after the fish stomach (S) until the end of the first intestinal loop (L1). The mid portion was defined as the section between L1 and the third intestine loop (L2), whereas the posterior portion was defined from L2 until the end of the fish intestine (E). Each intestine portion was individually stored in Eppendorf tubes, immediately frozen in liquid nitrogen, and then stored at -80 °C until enzymatic analysis.

2.3. Digestive Enzymes Activity

Fish intestines were weighted, and the pH was measured. Then, samples were homogenized on ice (1:5 dilution), centrifuged at 3300 *g* for 30 min at 4 °C, and the supernatant collected and stored at -80 °C until analyses. All enzyme activities were determined using a microplate scanning spectrophotometer (Multskan GO, Thermo Scientific, USA).

Amylase (E.C.3.2.1.1) and lipase (E.C. 3.1.1.3) activities were measured with kits (Gold Analisa Cat. 311 and 304, respectively), adapted for fish intestine samples. Chymotrypsin activity was determined following Hummel (1959), with Rao and Lombardi (1975) modifications, using BTEE (N-benzoyl-L-tyrosine ethyl ester) as substrate and the absorbance read at 256 nm. Trypsin activity was measured according to Bergmeyer et al. (1974), using 10nM TAME ester (Nα-p-toluenesulfonyl-L-arginine methyl ester) as a substrate in Tris-HCl 41.4 mM and CaCl2 10.4 mM (pH 8.1) and the absorbance read at 247 nm.

Enzyme activity was expressed as specific activity, defined as µmol of product generated per minute. Soluble protein concentration was determined according to Bradford (1976) with bovine serum albumin solution as standard.

2.4. Blood chemical analysis

Commercial kits (Labtest Diagnóstica SA) were used to quantify triglycerides (Triglycerides Liquiform, Cat. 87), glucose (Glucose HK Liquiform, Cat. 137) and cholesterol (Cholesterol Liquiform, Cat. 76) in plasma. The analyses were done in triplicate in 96-well plates and absorbance read in a spectrophotometer (Multskan GO, Thermo Scientific, USA).

2.5. Statistical Analysis

Data are expressed as mean ± pooled standard error of the mean (SEM). Normality and homogeneity of variances were tested using the Shapiro-Wilk and Levene tests, respectively. All statistical procedures were performed using SPSS software package for Windows (IBM® SPSS® Statistics, New York, USA). Statistical analysis of digestive enzymes was done by two-

way ANOVA, with postprandial hours and intestine portions as factors. When necessary, Tukey's test was used on two occasions: 1) to detect differences in digestive enzymes activities within each intestine portion and, 2) to detect differences in digestive enzymes activity, for each intestine portion, among postprandial hours. Data from blood parameters were analyzed by one-way ANOVA, and, in case of significance, Tukey's test was applied to identify differences between treatments.

3. Results

3.1. Digestive Enzymes Activity

Digestive enzymes activities were affected by both, intestine portion and, postprandial hours or hours post-feeding (HPF) (Table 1, Figure 2). With few exceptions and for all postprandial hours evaluated the enzymes lipase, trypsin and chymotrypsin had shown the highest activity in the anterior portion of fish intestine followed by mid and posterior portion. The same was not true for amylase where, at 0, 2, 4, and 48 HPF, the highest activity was observed in the posterior portion of fish intestine and the lowest in the anterior portion. At 10 and 12 HPF, an increase in the activity of amylase in the mid portion was observed, even slightly higher than the anterior portion.

For all digestive enzymes evaluated there was observed similar patterns of activity between anterior and mid intestine portion along postprandial hours. The increases in the activity of amylase in anterior and mid intestine portions were observed at 6 HPF, reached the peak at 8, and kept it until 12 HPF. There was an increase in amylase activity in the posterior intestine portion at 48 HPF. Lipase had the highest activity in the anterior intestine at 6 hours post-feeding. Otherwise, the activity in the mid intestine was increased only 48 hours post-feeding and, the posterior intestine had not shown differences in lipase activity between postprandial hours.

The activity of trypsin on anterior and mid intestine portions reached the highest values at 10, and 12 HPF. The activity in the posterior intestine was higher than the other portions only at 0

hours post-feeding. Chymotrypsin activity in the anterior intestine portion had the highest value at 10 HPF. An increase in activity on the mid intestine was observed at 8 and 10 HPF. At 48 HPF, the posterior intestine portion showed its highest chymotrypsin activity.

3.2. Plasma parameters

Plasma triglycerides (TG), glucose (GLU), and total cholesterol (TC) were affected by the increasing of postprandial hours (Table 2) and, the effects are present in Figure 3. The increase in plasmatic TG started at 4 HPF and reached a peak at 6 HPF. At 8 hours the concentration of TG in plasma decreased and was equal to those at 0 and 2 hours. At 24 and 48 hours post-feeding, TG was lower than the other treatments.

Glucose raise began at 8 HPF and lasted until 12 HPF. At 24 hours, GLU concentration lowered and was equal to the observed at 0, 2, 4, and 6 HPF; however, at 48 HPF GLU reached its highest plasmatic concentration. Total cholesterol peak was observed at 4 HPF, followed by a decrease in the subsequent hours, reaching the lowest concentration at 24 HPF. There was observed an increase in plasmatic cholesterol after 48 HPF, with concentrations similar to those observed at 4 HPF.

4. Discussion

The interaction between transit time, digestive enzymes activity, and nutrients absorption are known to determinates the efficiency of the whole digestive process itself. The present study explores the interrelation between those three factors in Nile tilapia and, based on the discoveries, elucidates a new methodology approach for intestine sampling when evaluating pancreatic digestive enzymes activity.

After ingestion, the diet is stored into fish stomach where undergoes the first representative physical and enzymatic breakdown of nutrients (Bakke et al. 2011). The time on what the chime is going to remain in the stomach will be dependent on factors such as food habits, feed frequency, and food composition (Lee et al. 2000; Riche et al. 2004). Gastric evacuation in Nile tilapia has been earlier reported to be around 8 hours post-feeding (HPF) (Riche et al. 2004;

Heng et al. 2007; Uscanga et al. 2010). In the present study, it was possible to follow diet breakdown in tilapia stomach during each hour of sampling and, excluding some common variations between individuals (Heng et al. 2007), the time of gastric evacuation observed was in agreement with previous reports (8 HPF - data not shown). That information is quite important as it will reflects on the arrival of the chime into fish intestine and exert influence on the peak of digestive enzymes activities and the absorption of dietary nutrients.

4.1. *Digestive enzymes*

The activity of digestive enzymes on the postprandial period has been shown to differ between fish food habits (Onish et al. 1976; Takii et al. 1985; Uys et al. 1987), however, no research was performed for Nile tilapia, what makes impossible to propose sampling procedures for enzymatic assays. Until now, recommendations for intestine sampling for digestive enzymes analysis have been performed only for carnivorous fish. Castro et al. (2015) proposed the time between 2 to 6 hours postprandial for sampling sea bass (*Dicentrarchus labrax*) intestines instead of 24 hours, due to the high enzymatic activities in the presence of chime into the fish intestine, in agreement with Krogdahl and Bakke (2005) recommendations. After that, this fasting time has been followed by several trials with carnivorous fish (Diogenes et al. 2017; Guerreiro et al. 2017; De Moura et al. 2019).

Considering anatomical and physiological differences between fish with distinct food habits it is unsustainable to adopt sampling strategies recommended for carnivorous fish when evaluating digestive enzymes for omnivorous species. Thus, as for omnivorous species, recent studies evaluating digestive enzymes activity have opted to adopt 24 HPF before intestine sampling, such as for common carp *Cyprinus carpio* (Zhao et al. 2020), catfish *Ramdia quelen* (Ha et al. 2019), pacu *Piaractus mesopotamicus* (Oliveira et al. 2020) and Nile tilapia (Santos et al. 2016; Deng et al. 2019; Mohammadi et al. 2019; Dawood et al. 2020). However, as observed in the present study, tilapia intestine is already empty after 24 hours post-feeding, suggesting that the activity measured is residual and does not corroborate with the fulfilled intestine, recommended by Krogdahl and Bakke, 2005.

Similar patterns of activity were observed between anterior and mid intestine portions along the postprandial hours. The peaks of activity of most of the digestive enzymes evaluated in this trial were between 8 and 12 HPF, which agrees with the time took for gastric emptiness (8 HPF), except for lipase that reached the peak at 6 HPF. Despite that, it is unquestionable that at 24 HPF all the enzymes had the activity reduced. This reduction differs between enzymes and was observed to be around 44% for amylase, 46% for lipase, 18% for trypsin, and 16% for chymotrypsin. This variation may be dependent on dietary composition, nutrients digestibility, and the specificity for substrates of each enzyme, as reported for lipase (Castro et al. 2015) which is more dependable of fatty acids profile than lipid content.

Amylase, lipase, trypsin and, chymotrypsin are pancreatic digestive enzymes released necessarily at the same time into the intestine, even if its concentration depends on dietary nutrients composition (Santigosa et al. 2008). Thus, it was expected a similarity on the time after feeding taken for those enzymes to reach the peak of activity. In truth, that similarity was observed for most of the enzymes, excepted for lipase. In the present study, there was observed that at 10 and 12 hours postprandial the enzymes amylase, trypsin, and chymotrypsin have, in common, exhibited their maximum activity. Although, at those hours, lipase activity was 30 and 48% reduced, respectively, when compared to the peak attached at 6 HPF. The early peak of lipase might be a junction of the activities of pancreatic lipase and esterase since the substrate used (tributyrate) is not specific for pancreatic lipase. Tributyrate also reacts with esterase which is known to have high activity in fish intestine (Tengjaroenkul et al. 2000). Esterase performs the hydrolyze of water-soluble carboxylic esters into free fatty acids and glycerol without dependence of bile salt, even faster than pancreatic lipase (Olsen and Ringo 1997; Hui and Howles 2002).

Even trypsin and chymotrypsin rose the peak of activity at similar time post-feeding (10 HPF), the response of trypsin seems to be more aggressive, with a rapid increase from 8 to 10 HPF. Unlikely that, the activity of chymotrypsin continuously increased from 6 to 10 HPF. Differences in the response of postprandial activity between trypsin and chymotrypsin might

reflect differences in amino acid specificity. Trypsin is a more specific enzyme, hydrolyzing preferentially aromatic L-amino acids like arginine and lysine (Unajack et al. 2012), whereas chymotrypsin acts on various α-amino acid groups, including tyrosine, phenylalanine and tryptophan, larger nonpolar aromatic amino acids (Zhou et al. 2011). Uscanga et al. (2010) also observed differences between trypsin and chymotrypsin activities after food ingestion for Nile tilapia, with the peak of trypsin estimated to be around 13 HPF whereas the activity of chymotrypsin remained constant.

The higher activity of amylase at 0, 2, 4 and 48 HPF and, lipase and chymotrypsin at 48 HPF in the posterior intestine of Nile tilapia, may reflect the contribution of enzymes produced by gut microbiota on nutrients digestion (Sugita et al. 1997), as the posterior intestine is the local with the highest abundance of gut bacteria in this fish species (Molinari et al. 2003). Several enzyme-producing bacteria were detected from Nile tilapia intestinal tracts such as amylase, lipase, and protease (Ray et al. 2012), and, the ability of those enzymes to contribute to the host enzymes activities have been discussed by several authors and for several fish species (Nayak 2010; Clements et al. 2014; Wang et al. 2017). Thus, the posterior intestine, besides relatively low digestive enzymes activity and none influence by postprandial hours, could also be a source of data contamination, since the activities reported could be from another source instead of fish endogenous production, as reported above.

With few exceptions, the distribution of digestive enzymes activity along the intestinal tract for Nile tilapia followed the sequence anterior-mid-posterior for most of the treatments and enzymes, with anterior portion presenting the highest enzyme activity. These higher activities in the anterior portion of fish intestine agree with the location where all the fluids and enzymes produced by the pancreas are released by the pancreatic duct, including the digestive enzymes studied. This same pattern was observed for Nile tilapia with similar size (Jun-Sheng et al. 2006) and for several fish species (Castro et al. 2015; Gioda et al. 2017; Oliveira et al. 2020) however, it may not occur in early phases (Chackrabarti et al. 1995). Further studies involving fish with distinct size and/or developmental stages should however be recommended.

4.2. *Blood chemical analysis*

In the present study, blood parameters were within the desirable range for Nile tilapia (Hrubec et al. 2000; Duodo et al. 2019) showing that fish were not under stress conditions or physiological problems. In general, the profile of digestive enzymes activity and plasmatic metabolites are in accordance (Castro et al. 2015a). Glucose increased in fish blood until 8 HPF and continued to be high until 12 HPF. That agrees with gastric emptiness and, most important, with the peak of amylase activity, since pancreatic amylase is the first enzyme in fish that acts on the breakdown of carbohydrates (Hemre et al. 2002). Similarly, Guerra-Santos et al. (2017) observed maximum glucose concentration at 9 HPF for Nile tilapia.

Plasmatic glucose remained high from 8 to 12 HPF and, returned to basal levels after 24 HPF, the same returning time observed by Feng et al. (2018) and Chen et al. (2017). The longtime of hyperglycemia was already expected since fish is known to have an intolerance to glucose and the degree of intolerance depends on food habit. Omnivorous fish are more efficient on the use of glucose than carnivorous due to factors like higher number of insulin receptors, better capacity to phosphorylate glucose in the muscle (Parrizas et al. 1995; Capilla et al. 2004b), and most efficient inhibition of hepatic gluconeogenesis (Panserat et al. 2002b), however other factors such as the existence of antagonism of hormones such as glucagon-like peptide and somatostatin and so on (Polakof et al. 2012) also promotes inefficient effects of insulin on lowering blood glucose in omnivorous species.

The peak of triglycerides (TG) at 6 HPF followed the peak of lipase activity, which reaffirm that the concentration of TG in fish blood is mainly a consequence of liver metabolism. After the synthesis of chylomicron in the intestinal lumen, lipids products are delivered to the lymphatic system and then to the liver. Even some free fatty acids can be incorporated directly into the blood circulation it is not usual (Tocher 2003). However, some characteristics of fish stomach may have also contributed to the plasmatic TG peak, such as the presence of gastric lipase (Tocher 2003), the capacity of lipids absorption (Al- ghandi 1998) and the release of lipids from the diet due to the low pH (Olsen and Ringo 1997). The reduction in TG after 24 HPF is

associated with suppression of lipogenesis as observed for several species of fish (Pérez-Jiménez et al. 2007; Nebo et al. 2018; Li et al. 2018). During starvation, free fatty acids are primarily used for ketone bodies synthesis to obtain glucose and maintain fish glycemia (lipolysis).

According to cholesterol, the early peak observed is most related to tissue mobilization than dietary intake, since up to 4 HPF there was no evidence of enough absorption of nutrients capable to maintain fish homeostasis. Besides, plasmatic cholesterol derivates mostly from endogenous biosynthesis (Wen and Wang 2002) and the most of cholesterol absorbed through the intestine is also endogenous and derived from biliary cholesterol (Turley and Dietschy 2003).

Adding, the observed increase in glucose and cholesterol after 48 HPF reflects fish tissue mobilization due to a long time of food deprivation. To maintain glucose homeostasis fish organism activates mechanisms responsible for the increase of glucose into the blood, such as glycogenolysis (from glycogen) and gluconeogenesis (from lactate, glycerol and amino acids) (Polakof et al. 2012). The raise of cholesterol after 48 hours of food deprivation appears as a result of reverse cholesterol transport (Liao et al. 2017) and was early reported by omnivorous fish in fasting situations (Peterson and Small 2004; Gimbo et al. 2015; Favero et al. 2017). Besides a component of high-density protein (HDL) on the transport of cholesterol from peripheric tissues to the liver, cholesterol is involved in the synthesis of cortisol, a lipolytic hormone that regulates several processes including energetic metabolism (Mommsen et al., 1999) and might be, as well, a response for stress conditions promoted by starvation (Favero et al. 2017).

It became evident that using the fasting time applied nowadays for sampling Nile tilapia intestine is not accurate and does not represent the full capacity and reality of digestive enzymes. Considering the results observed, to sample only the anterior portion of Nile tilapia intestine for enzymatic assays seems to be a better approach unless specific situations like the small size of the anterior intestine or studies involving intestinal microbiota. In case of absence

of enough sample material for analysis, anterior and mid intestine portions could be used together, as similar patterns of activity were observed between them along the postprandial hours.

5. Conclusion

Compiling the data of transit time, digestive enzymes activities, and nutrients plasmatic concentrations, to sample the anterior portion of the intestine into the interval between 10 to 12 hours post-feeding, is an adequate sampling procedure that should be used in digestive enzymatic assays for Nile tilapia (*Oreochromis niloticus*).

6. Acknowledgments

Thanks to the National Council for Technological and Scientific Development (CNPq) (Process 148553/2016-8).

7. Conflict of interest: The authors declare that they have no competing interest.

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9. Figure Legends

Figure 1. Schematic drawing Nile tilapia intestine section in three portions for enzymatic analysis. Anterior (S - L1), Mid (L1 – L2), Posterior (L2 – E). Bar = 1 cm. Adapted from Pereira et al. (2017)

Figure 2. Activities of Nile tilapia digestive enzymes (amylase, lipase, trypsin and chymotrypsin expressed as U/mg protein) in different intestine portions, varying postprandial hours. a: amylase; b: lipase; c: trypsin; d: chymotrypsin. Anterior (A, black), mid (M, dashed), posterior (P, grey). Data are presented as means ($n = 12$) and bars of standard error of the mean. Values sharing different low case letters are significantly different at Tukey's test between the same intestine portion within postprandial hours ($p < .05$). While the absence of letter means not significantly different ($p > .05$).

Figure 3. Effect of postprandial hours on plasma biochemical indices for Nile tilapia. Data are presented as means ($n = 12$) and bars of standard error of the mean. Values sharing different low case letters are significantly different at Tukey's test within postprandial hours ($p < .05$). TC: total cholesterol; TG: triglycerides; GLU: glycose

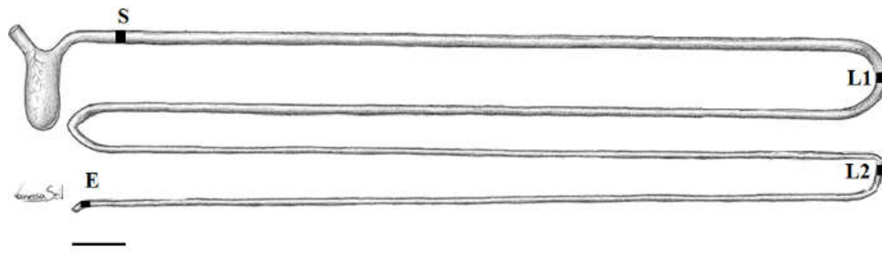

Figure 2.

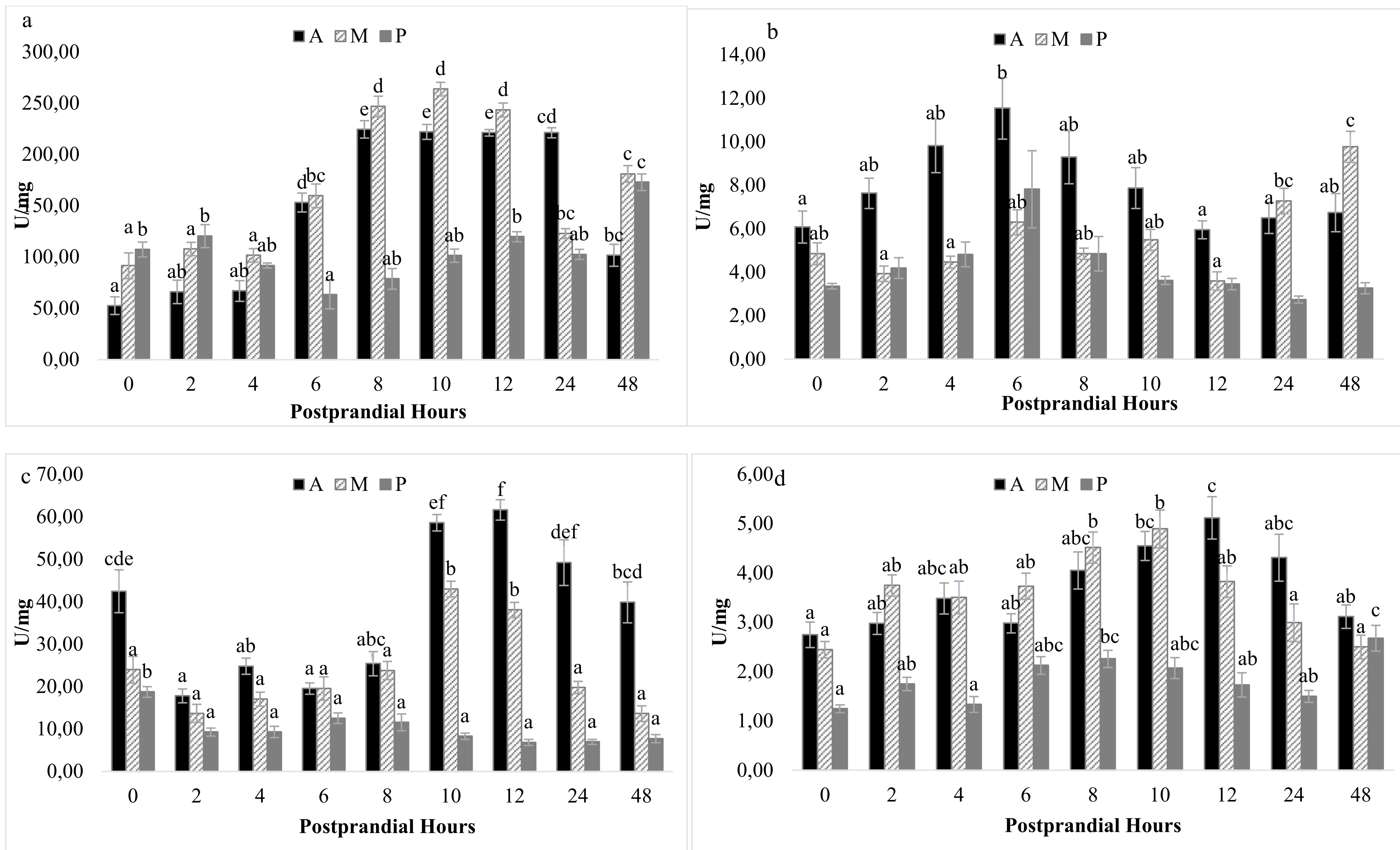

Figure 2.

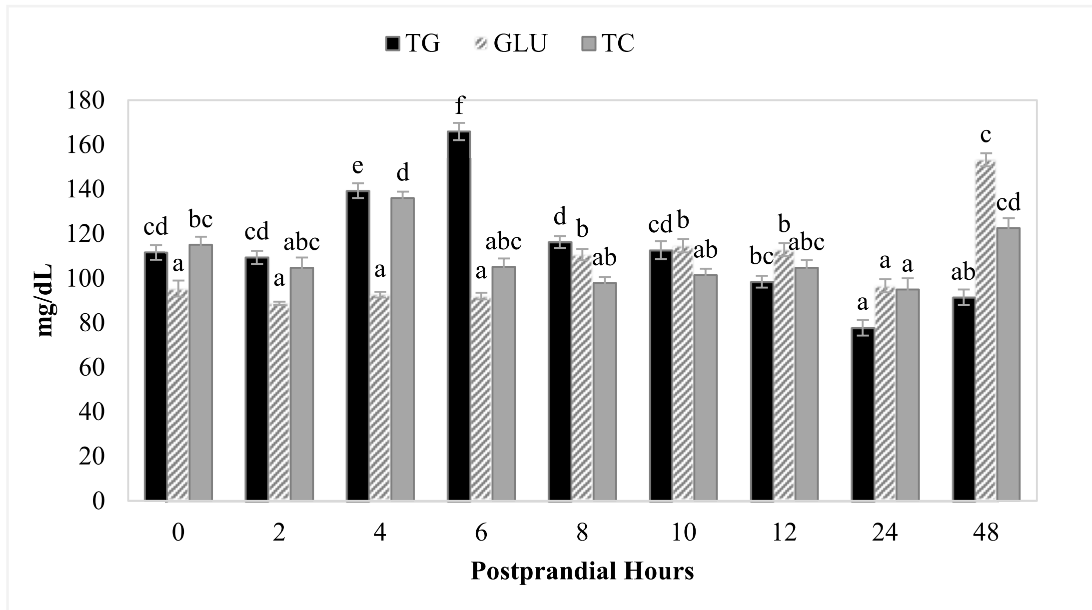

Figure 3

Table 1. Postprandial specific digestive enzymes activities (U/mg protein) in different intestine portions of tilapia

	Postprandial Hours									SEM	Main Effects		
	0	2	4	6	8	10	12	24	48		H	IP	H x IP
Amylase											*	*	*
Anterior	52.5^{A}	66.0^{A}	66.9^{A}	153.1^{B}	224.5^{B}	222.0^{B}	221.2^{B}	119.0	101.6^{A}	9.73			
Mid	91.5^{AB}	107.7^{B}	191.7^{B}	159.6^{B}	246.9^{B}	263.7^{C}	243.4^{C}	122.9	181.0^{B}	9.29			
Posterior	107.3^{B}	120.5^{B}	91.7^{AB}	63.2^{A}	78.7^{A}	101.3^{A}	119.8^{A}	102.5	173.0^{B}	4.95			
Lipase											*	*	*
Anterior	6.1^{B}	7.6^{B}	9.8^{B}	11.6^{B}	9.3^{B}	7.9^{B}	6.0^{B}	6.5^{B}	6.7^{B}	0.42			
Mid	4.9^{AB}	3.9^{A}	4.5^{A}	6.3^{A}	4.9^{A}	5.5^{AB}	3.6^{A}	7.3^{B}	9.8^{C}	0.33			
Posterior	3.4^{A}	4.2^{A}	4.8^{A}	7.8^{A}	4.9^{A}	3.6^{A}	3.5^{A}	2.7^{A}	3.3^{A}	0.312			
Trypsin											*	*	*
Anterior	42.5^{B}	17.8^{B}	24.8^{C}	19.5	25.4^{B}	58.6^{C}	61.7^{C}	49.2^{B}	39.9^{B}	2.51			
Mid	24.0^{A}	13.7^{AB}	17.1^{B}	19.5	23.8^{B}	43.0^{B}	38.1^{B}	19.8^{A}	13.6^{A}	1.58			
Posterior	18.7^{A}	9.3^{A}	9.3^{A}	12.5	11.6^{A}	8.3^{A}	6.8^{A}	7.0^{A}	7.7^{A}	0.64			
Chymotrypsin											*	*	*
Anterior	2.8^{B}	3.0^{B}	3.5^{B}	3.0^{AB}	4.1^{B}	4.6^{B}	5.1^{B}	4.3^{B}	3.1	0.16			
Mid	2.5^{B}	3.8^{B}	3.5^{B}	3.7^{B}	4.5^{B}	4.9^{B}	3.8^{B}	3.0^{B}	2.5	0.15			
Posterior	1.3^{A}	1.8^{A}	1.3^{A}	2.1^{A}	2.3^{A}	2.1^{A}	1.7^{A}	1.5^{A}	2.7	0.09			

Values expressed as U/mg protein and presented as means (n = 12) and pooled standard error of the mean (SEM). Two-way ANOVA: *$p < .001$. Values sharing a common superscript letter are not significantly different at Tukey's test within intestine portion ($p > .05$). H: postprandial hours; IP: intestine portion

Table 2. Plasma biochemical indices (mg/dL) of Nile tilapia varying postprandial hours

	Postprandial Hours									SEM	p-value
	0	2	4	6	8	10	12	24	48		
TC	115.1	104.8	136.0	105.1	97.9	101.4	104.8	95.0	122.6	1.96	*
TG	111.7	109.5	139.4	165.9	116.4	112.7	98.5	77.9	91.4	3.12	*
GLU	95.4	88.8	92.7	92.1	96.7	114.7	112.9	96.7	153.2	2.41	*

Values presented as means (n = 12) and pooled standard error of the mean (SEM). One-way ANOVA: *$p < .001$. TC: total cholesterol; TG: triglycerides; GLU: glycose