Protein evaluation in fish:

African catfish as a case study

Folasade Esther Elesho

Propositions

1. The methionine requirement for African catfish is unaffected by feeding frequency. (this thesis)

2. Low-quality ingredients have a large variability in digestibility between individual amino acids. (this thesis)

3. The trend among authors to cite less from old publications impedes revolutionary scientific innovations.

4. Cellular meat makes animal nutritionists redundant.

5. Even if COVID-19 would be a visible alien invader, the world *still* would not fight united.

6. The ability to cook underrates the role women play in food security.

Propositions belonging to the thesis, entitled

Protein evaluation in fish: African catfish as a case study

Folasade Esther Elesho
Wageningen, 1 April 2022

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Thesis

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To my parents,

Elder J.T Oyawale
&
Deaconess C.W. Oyawale

….Without whom none of my success would be possible.

Contents

CHAPTER 1

General Introduction

1.1 Overview of aquaculture and aquafeed production

The rising global population coupled with a demand for cheap protein, has elevated aquaculture into the fastest growing food producing sector in the world, with an estimated annual growth rate of 4.5%, between 2011 and 2018 (FAO, 2020). More importantly, it supplies over half of the world's seafood, albeit at a high dependency on aquafeed. Therefore, the production of aquafeed must support the industrial expansion of aquaculture. On its part, aquafeed must be nutritionally balanced, as this depends on several sources of protein and energy ingredients (Hardy, 2010; Naylor et al., 2009). In fact, fishmeal (FM) is often used as a protein source in aquafeeds, due to the strong connection between the amino acid (AA) requirements of fish and their whole-body AA profile (Mambrini-Doudet and Kaushik, 1993). In this respect, a large percentage of FM produced is being used for aquafeed formulation (Figure 1.1) (Naylor et al., 2009). However, the rising demand for FM and consequently the depletion of wildlife resources has motivated researchers worldwide in a search for new alternative least-cost feedstuffs, that are both sustainable and can provide similar nutrients obtainable from FM for optimal fish growth and performance (Eyo, 2003). In light of this, plausible alternative ingredients are animal by-products and plant proteins (Che et al., 2017; Dam et al., 2019; Kitagima and Fracalossi, 2011; Lee et al., 2020; Solomon et al., 2017; Tomas-Vidal et al., 2019; Tran-Ngoc et al., 2019). Therefore, information on the nutrient profile and digestibility of these ingredients is essential to allow precise feed formulation. Such information is available for some species, but still lacking for new and emerging species.

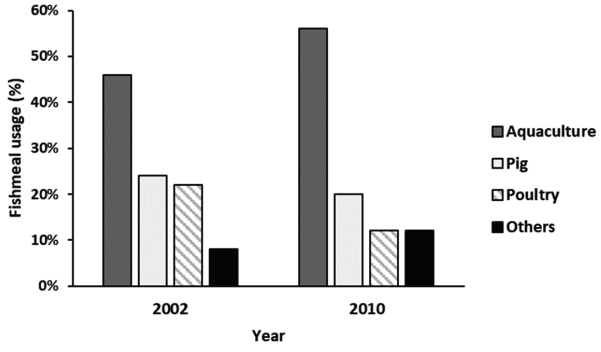

Figure 1.1 Percentage of fishmeal usage in feed production for aquaculture and other animals between 2002 and 2010. (Adapted from Miles and Chapman (2006))

Depending on the region, cultured species vary among continents and as aquaculture continues to expand, new species are being introduced for culture. As a result, it is crucial to understand their husbandry and nutritional requirements. In Europe, Atlantic salmon (*Salmo salar L.*), rainbow trout (*Oncorhynchus mykiss*) and European sea bass (*Dicentrarchus labrax*) are the most popular cultured species with relatively large information in literature about their nutritional needs. In Africa, aquaculture is dominated by freshwater indigenous species like African catfish (*Clarias gariepinus)* and tilapia (*Oreochromis niloticus*) (Adeleke et al., 2020). The African catfish ranks among the most important cultured fish after tilapia. Reportedly, Nigeria being the world's leading producer of African catfish in the sub-Saharan Africa region has a production output of 233,605 tonnes/year (Figure 1.2) (Dauda et al., 2018). This species is widely cultured in Africa due to its fast growth, resistance to diseases, wide geographical distribution and adaptation (Fagbenro et al., 1999). Considerably, its distribution is expanding as it has been introduced and cultured in other parts of the world, spanning

through South Europe, Asia, and South America (FAO, 2017). The euryphagic nature of the African catfish (Bruton, 1979) enables it to utilize different ingredients efficiently (Fagbenro et al., 1999; Wilson and Moreau, 1996). Although, the trend in aquaculture development shows a substantial growth, also in Africa, it seems difficult to attain its full potential, due to some challenges plaguing its developmental rythym. Among these problems are the unavailability of quality feeds and the limited information on the apparent digestibility of common ingredients, and if available, they are not easily accessible by local famers due to high costs (Moehl and Machena, 2000). Therefore, research efforts are now being directed into the development of nutrients–balanced diets for intensive fish culture. As such, the local industry would benefit from local ingredients if proper nutritional information is provided.

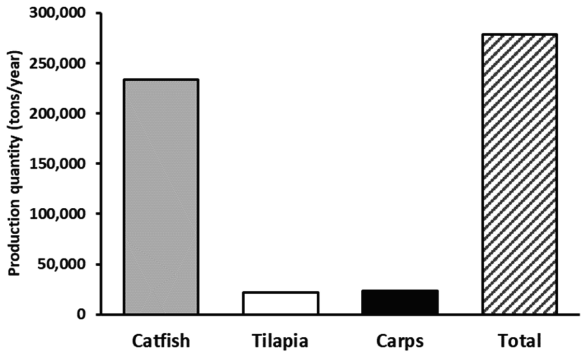

Figure 1.2 Quantity of aquaculture production in Nigeria in 2013. (Adapted from Adeleke et al. (2020))

1.2 Optimum diet formulation

Historically, the key components of ingredient evaluation encompass several steps, which include ingredient characterization, digestibility, palatability, nutrient utilization and functionality (Glencross et al., 2007). Primarily, the characterization of dietary ingredients provides information on their nutrients make-up which is needed for the feed formulation, while the palatability reveals the attractiveness and ingestion of a diet. As such, the formulation of an optimal diet requires information basically on the ingredient's characteristics and then, the nutrient requirements of the fish. In fish nutrition, feed ingredients are traditionally characterized by their nutrient profile (e.g., crude protein, crude fat, carbohydrate e.t.c.), with average values used in feed evaluation tables. (e.g., NRC (2011)). These analyzed values of a specific batch of feedstuffs, are used for diet formulation in such a way that dietary nutrient supply will match the daily nutrient requirements. The requirement for major essential macronutrients has been extensively studied in commonly cultured fish species (NRC, 2011), but less studied in African catfish. Protein appears to be the most important nutrient in fish diets due to the need for (muscle) growth. Therefore, the focus of this thesis is primarily on protein and amino acid requirements.

1.3 Digestibility of feed ingredients

Essentially, the assessment of the digestibility potential of ingredients in fish should succeed ingredients characterisation (Fornshell et al., 2016; Glencross et al., 2007). Evaluating the digestibility of feedstuff is crucial as it will expose nutrient bioavailability and thus the feasibility for their inclusion in fish diets (Fontes et al., 2019). In addition, the identification of the nutritional value through digestibility studies will reveal the efficiency and utilization capacity of these feedstuffs by animals

(Adéyèmi et al., 2020). In fish, this is commonly determined by quantifying the apparent digestibility coefficient (ADC) (Ribeiro et al., 2012; Solomon et al., 2017). Details on different methodologies used in digestibility studies have been extensively reported in literature. Ingredient digestibility values can be obtained with an assumption that, under normal conditions the digestibility of nutrients from different ingredients within a diet are additive. The digestibility of nutrients varies among different ingredients as well as between fish species (Davies et al., 2009). Therefore, it is important to characterise common and emerging ingredients regarding their digestibility for every fish species under culture. As aforementioned, a wider range of alternative ingredients have now been incorporated into aquafeed production. For instance, the use of insect and single cell protein (e.g., yeast, algae) as potential novel ingredients have gained more attention in recent years (Al-Hafedh and Alam, 2013; Gasco et al., 2014; Manoppo and Kolopita, 2016; Ovie and Eze, 2014; Piccolo et al., 2014; Pongpet et al., 2016; Sogbesan and Ugwumba, 2008; Solomon et al., 2017). However, variations in nutrient digestibility from these feedstuffs have been discovered in studies, owing primarily to differences in fish digestive physiology (Yuan et al., 2010b). Only few studies are available on the nutrient digestibility in African catfish. Therefore, the digestibility of alternative ingredients for African catfish feed needs to be investigated.

Studies exploring the digestibility in aquatic animals have reportedly affirmed that ADC can be affected by biological, nutritional, and environmental factors, such as water salinity, temperature, age, fish size, feeding time and feeding level (Halver and Hardy, 2002). Several studies have evaluated the impact of these factors in certain species. For instance, the impact of fish size and temperature on nutrient digestibility in rainbow trout was examined by (Windell et al., 1978), followed by the effect of salinity and nutritional status on growth of *Sparus sarba* by Woo and Kelly (1995), and much later, the impact of water salinity on nutrient digestibility in Atlantic salmon by Krogdahl et al. (2005). However, the effect of feeding level on nutrient digestibility is scarcely studied. Only a few studies have addressed the potential impact of increasing feeding level on the ADC in fish species. Haidar et al. (2016) and Yuan et al. (2010a) investigated the impact of feeding level on nutrient digestibility in Nile tilapia and *Myxocyprinus asiaticus,* respectively. Results from both studies showed a negative correlation between feeding level and ADC digestibility. Similarly, dry matter and crude protein ADC decreased with increasing feeding level in African catfish (Henken et al., 1985). However, Cho and Kaushik (1990) reported that the ADC of nutrients were neither affected by feeding level nor feeding rate. Apart from Henken et al. (1985) who determined the effect of feeding level on nutrient ADC in African catfish, no study so far has been carried out on the interaction between feeding level and AA ADC in African catfish. In the study by Haidar et al. (2016), the impact of feeding level was more pronounced for the NSP-rich diet compared to the non-NSP diet. This suggests that the effect of feeding level may be dependent on the quality of the diet and also on the ingredient composition.

Furthermore, since it has been established in other farm animals that the rate of transport of food in the digestive tract contributes to the time of release of nutrients to the body (Van den Borne, 2006), the currently accepted methods for measuring nutrient digestibility in fish do not consider the role of gastrointestinal tract in the digestion process. As such, faecal digestibility value is assumed to only account for the total amount of dietary nutrients that was apparently digested and absorbed along the GIT (Chen, 2017). In pigs and poultry, proteins are evaluated based on digestible AA, which is mostly detected in the ileal digestibility of the diets ingested. No appreciable absorption of amino acids from the large intestine of these animals seem to occur and the disappearance of AA in the hindgut is often associated to ammonia released from amino acid catabolism by microbial deaminases (Hendriks et al.,

2012). Therefore, protein evaluation in fish should expand beyond nutrient digestibility studies in faeces and incorporate the role of GIT during the process of digestion - an aspect that has received little or no attention in fish nutrition.

1.4 Determination of protein quality

The biological value of protein has been deemed substantially determined by the composition of AA that are digested and absorbed (Ruchimat et al., 1997). In other words, protein quality can be described as the ability of dietary protein to meet the body's metabolic needs for AA and nitrogen (Boye et al., 2012). In this respect, data on AA patterns in different ingredients and also the AA digestibility can be used for more precise feed formulation (Gomes et al., 1995a). Amino acids profile of commonly used ingredients in fish feeds are well documented but for many fish species, information on the ADC of AA in these ingredients is often still lacking. This is the case for African catfish, in which feeds are formulated based on protein requirement rather than amino acid requirement. In present days, the use of dietary protein requirement as a yardstick for feed formulation is losing significance, probably due to the increase of a wider variety of feed ingredients in the diets, each of which has a different AA composition. This can in turn cause a large variability in AA content of the diet as opposed to the actual requirements of the fish species. Next to AA content, good feed formulation requires information on ADC of AA for the important ingredients.

1.5 Protein requirement of fish

Basically, protein nutritional requirement signifies the lowest level of dietary protein intake neccesary to balance nitrogen losses from the body, in relation to maintenance and to support maximal protein gain (Boye et al., 2012). Data on dietary protein requirements are readily available for a wide range of species. In general, studies show that the dietary protein requirements range between 24 and 70% of the diet. However, this is dependent on a number of factors such as species, developmental stage, trophic level and size (Teles et al., 2020). Typically, herbivorous and omnivorous fish require less dietary protein in their feed than carnivorous fish, while smaller fish require more protein than larger fish, because protein requirements decrease as fish grow larger (Craigh and Helfrich, 2002). Furthermore, fish require a higher protein content in their diet, almost 2 to 4 times higher than terrestrial farm animals. This apparent difference in dietary protein requirements between fish and farm animals is due to fish's low energy requirements for maintenance (Cho, 1985). However, fish like other animals, do not have a true protein requirement, but requires a balanced supply of essential amino acid (EAA) and non-essential amino acid (NEAA) via their diet. Amino acids are derived from the breakdown of protein, which are then absorbed in the intestine. New proteins are then built from these absorbed amino acids, according to the needs of cells. As a matter of fact, about ten EAA (arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine) must be present in compound diets, as fish lack the metabolic mechanism needed to form the chemical structures of the carbon chain skeletons of these amino acids (Somsueb, 2017; Wagenmakers, 1998). A deficiency in one or two of these EAA can lead to reduced growth or even weight loss in fish due to the suboptimal utilization of the AA for protein synthesis (Wagenmakers, 1998). The unused AA are usually catabolized and lost for protein synthesis. During AA catabolism, ammonia is produced by the process of deamination, which is expelled from the body through the gills and urine (brachial and urinary losses; BUN). Nitrogen (N) is one of the most important nutrients responsible for water eutrophication, thus, excess of protein will negatively impact the environment.

From an ecological perspective, it is therefore important that dietary protein meet but do not exceed the requirements of the animals. This is especially true in fish, which have difficulty in controlling the amino acid catabolism and, as a result, N losses are significant even when dietary protein levels are low (Cowey, 1994; Kaushik and Seiliez, 2010). As such, knowledge of AA requirements is crucial for precise feed formulation.

AA requirements of fish are often expressed on dietary or crude protein basis with the assumption that all AAs react the same way, i.e., digestibility do not differ among AAs in protein. Although, the benefits of using 'digestible amino acid system' as mode of expression of AA requirement, are well known in e.g., pig and poultry's diet formulation (Lemme et al., 2004). However, the 'total amino acid system' is still widely used in fish nutrition. Besides, assessing the protein quality on digestible AA basis has been suggested in literature, especially with the array of cheaper but low-quality alternative ingredients in circulation. For example, the use of digestible AA data for FM will be negligible because its amino acid content is highly digestible compared to low-quality ingredients. Therefore, diets based on digestible AA will enable the use of alternative ingredients with low digestibility (Lemme et al., 2004). As research continues to evolve and different diets are being fed in various requirement studies, expressing requirement estimates on a digestible basis will reduce variability and ensure accurate comparison of values among species (NRC, 2011). This will also aid precise feed formulation, thereby minimizing economic losses and reducing the environmental footprint of aquaculture production.

1.6 Protein utilization efficiency

An essential approach during evaluation of dietary protein quality, should involve an assessment of the impact of those protein sources on utilization (Young and Pellett, 1989). However, the utilization of protein and other dietary nutrients (e.g., sugars, vitamins, and minerals) are never shown in feed analysis tables (Lall, 1991). It should be pointed out that utilization efficiency of AA can be affected by many factors; antinutritional factors (ANFs), digestive enzymes inhibitors, presence of dietary mycotoxins, naturally bounded resistant proteins, nutrients asynchrony and indigestible compounds formed during feed processing (e.g., maillard reaction) (Lall, 1991; Van den Borne et al., 2006). This implies that despite supplying a balanced AA profile, which meets the daily requirement of fish, the release of nutrient and absorption might be affected by these intrinsic factors.

Various factors have been reported to affect the protein utilization efficiency. The major one is the AA composition (profile) of the diets. For instance, plant proteins (mostly legumes) and some other animal by-products (e.g., feather meal) that are increasingly used as substitute for fishmeal, are deficient in some EAA needed for growth and development e.g., lysine and methionine (Ovie and Eze, 2010). In this case, crystalline AA are usually supplemented to the diet in order to overcome this deficiency problem. However, AA supplied in purified form are less utilized as compared to AA in intact protein (i.e., protein-bound AA). Basically, the absorbed free AA are quickly catabolised and lost rather than used for protein synthesis. The result is the asynchronous supply of nutrients and mismatch between the release and absorption of free AA and protein-bound AA, supplied within a day (figure 1.3a). Possibly, this effect may be more pronounced when factors like feeding management are not adequate (Van den Borne et al., 2006), which implies that nutrient synchronization/AA utilization can also be affected by feeding practices, such as feeding frequency. Feeding at low frequency may negate the optimum utilization of amino acids in a diet that contains combination of crystalline amino acids and purified ingredients. Asynchronous availability of crystalline AA and protein-bound AA will therefore occur as a consequence of feeding less than required. One way to improve the utilization of free AA is

feeding multiple times in a day (Ambardekar and Reigh, 2007), as this will complement the AA profile of intact protein present by providing more chances for timely absorption of free AA supplemented (figure 1.3b). The positive effect of multiple feeding was demonstrated in the study carried out by Zarate et al. (1999), on channel catfish fed diets containing free and protein-bound lysine twice and five times in a day, a better utilization of free lysine was achieved at increased frequency. Furthermore, Yamada et al. (1981) observed similar result in carp fed free AA from 3 to 18 times daily as growth increased in proportion to high frequency.

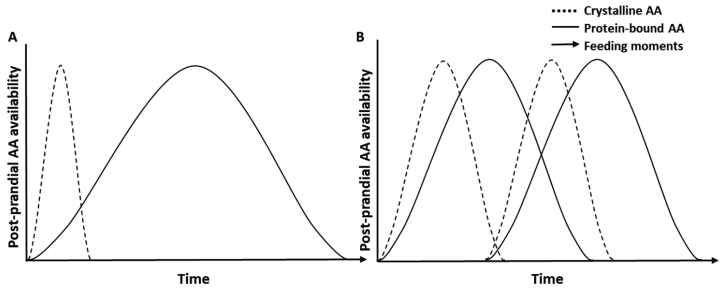

Figure 1.3 A) Post-prandial asynchronous availability of free amino acids (AA) and protein-bound AA due to low feeding frequency B) Overlapping in AA availability for protein synthesis due to multiple feeding time. (Adapted from (van den Borne, et al, 2006).

Another factor that can induce nutrient asynchrony is the difference between the digestion kinetics of dietary protein and non-protein energy sources (NPE). This usually happens when, for instance, a quickly digestible protein (e.g., hydrolysed protein), is supplied against slow digestible NPE sources (e.g., fat). Then, this can initiate a gap in-between the availability of protein and energy at the moment of protein synthesis (figure 1.4), and thus leading to a less efficient protein/AA use. Afterall, optimal protein synthesis is highly dependent on energy availability. Therefore, part of the available AA will be catabolised and used as energy. In monogastric animals, it has been reported that the physical state of the dietary proteins and carbohydrates can influence the digestion and passage rate of the nutrients before they are absorbed (Englyst and Englyst, 2005; Minekus, 1998; Weurding, 2002). This is evident in the study carried out by van den Borne et al. (2007) on synchronization between protein and carbohydrate availability in pre-ruminant calves. In fish, it is not clear if the characteristics of specific ingredients can impact the post-absorptive availability of AA. Moreover, there is relatively little information about the aspect of nutrient asynchrony.

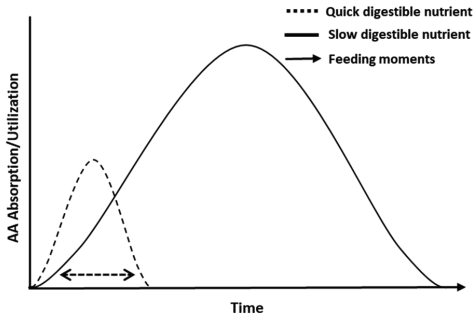

Figure 1.4 Post-prandial asynchronous availability of protein and energy constituents such as starch and AA (Adapted from Van den Borne (2006)).

1.7 Thesis aim and outline

The aim of this thesis was to assess factors that determine the quality of dietary protein in fish feeds in order to improve protein evaluation of fish feeds/ingredients. African catfish was used as a case study. According to NRC (2011), protein quality for most fish species is currently defined by; 1) the digestibility of crude protein and 2) the amino acid (AA) profile of the crude protein. **Chapter 2** of this thesis addressed the question whether the digestibility of AAs is equal among different AAs, as well as to the overall crude protein. Furthermore, this chapter investigated whether feeding level affects AA besides that of nutrient digestibility in fish. AA requirements are usually expressed per kg of feed or per kg of crude protein. However, it was shown in Chapter 2 that the ADC of individual AA can differ between ingredients and among other AAs constituents of protein. In **Chapter 3** the AA requirements of methionine was estimated, for the first time, on digestible methionine per digestible crude protein. Furthermore, the impact of the mathematical model on requirement estimates was studied.

The final part of the thesis (Chapter 4 and 5) investigated the factors that could hamper optimal protein digestion and utilization in fish. It was hypothesized that the timing of digestion and absorption of different amino acids can affect the utilization efficiency of total protein in fish. Therefore, the question whether asynchrony in nutrients digestion occur in fish was studied. Firstly, in **Chapter 4,** we investigated whether the kinetics of protein digestion can be altered by dietary composition. This was assessed by comparing two diets that differed in the type of fishmeal (FM): FM versus hydrolysed FM, and non-protein energy: starch versus fat. The question whether the hydrolysis of the protein source (FM) altered the faecal ADC, as well as the kinetics of digestion within the gastrointestinal tract (GIT) was studied. The observed differences in the kinetics of nutrient digestion in chapter 5 indicated that there may be asynchronous availability of nutrient during absorption. Therefore, **Chapter 5** studied if the utilization of crystalline AA is lowered due to asynchrony between different forms of AA in fish diets. Here, the impact of feeding frequency on protein utilization efficiency was assessed in two diets having different inclusion levels of crystalline methionine. The goal was to investigate if nutrient synchronization can be challenged with feeding frequency to improve AA utilization. Thus, we hypothesized that increasing feeding frequency will aid the utilization of CAA supplementation in diets deficient in AA, thereby leading to an increased protein deposition in fish

In **Chapter 6**, the main findings obtainable from different studies incorporated into this thesis were discussed. Apparent digestibility coefficient (ADC) of AAs are costly to estimate, therefore, the prediction of AA digestibility from the ADC of crude protein or the sum of total AA (SAA) might be an alternative option. Based on the observed variability in the individual AA digestibility compared to the crude protein ADC in chapter 2, a meta-analysis was performed in Chapter 6 to further check the relationship between the ADCs of AAs and the SAA in protein. It was hypothesized that the SAA can be used as predictor for individual AA ADC. The chapter ends with summary of the main conclusions theorized vis-a-vis the process of how protein quality can be better evaluated in fish.

CHAPTER 2

Effect of feeding level on the digestibility of alternative protein-rich ingredients for African catfish (*Clarias gariepinus*)

This chapter has been published as:

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Abstract

Apparent digestibility coefficients (ADC) of nutrients and individual amino acids (AA) for 13 feed ingredients as affected by feeding level were determined for African catfish, a species of economic importance in Africa. Results from two trials are reported. In each trial, ADC were determined using a reference diet and test diets with yttrium oxide as indicator. Juvenile African catfish (averaging 53.9 g, trial 1; 40.4 g, trial 2) were stocked in tanks connected to a common recirculation aquaculture system. Ingredients tested included hydrolysed feather meal (HFM), fishmeal (FM), insect meal (IM), soybean meal (SBM), sunflower meal (SFM), poultry meal (PM), corn dried distillers grains with solubles (CDDGS), faba beans (FB), lupine meal (LM), pea protein (PP), guar meal (GM), canola meal (CM) and yeast meal (YM). The effect of feeding level on ADC was determined by feeding fish restrictively (80% satiation) for 4 weeks and subsequently to apparent satiation for 3 weeks. Inclusion of yeast meal at 30% resulted in low palatability. ADC of nutrients were significantly affected by feeding level (except for fat and carbohydrate), but the effect was ingredient-dependent. African catfish was able to digest protein very effectively in almost all tested ingredients with ADC values ranging from 85.6 to 105.1% across feeding periods. Several ingredients tested, including animal protein ingredients and YM had similar high ADC for dry matter as FM. However, the ADC of AA differ among ingredients, indicating a need for digestible amino acid profile data. Methionine (Met) was the first limiting essential amino acid in HFM, FB, and LM with values ranging from 5-6 g/kg, expressed as digestible Met (dMet) per unit of digestible protein (DP), compared to FM (27 g dMet/kg DP). IM had comparable and sometimes higher overall digestible essential AA values compared to FM, except for methionine and lysine. For oilseeds and legumes, SBM tended to be the best quality AA source, as it had the highest digestible essential amino acid profile. These data provide information concerning nutrient and digestible AA values, which will allow a more efficient use of alternative ingredients in African catfish diets. Formulating diets based on the digestible AA in ingredients will aid precise feed formulation, thereby minimising economic losses and reducing the environmental footprint of aquaculture production.

2.1 Introduction

Due to the scarcity of fishmeal together with the increased production of aqua-feeds, numerous studies have been conducted on alternative ingredients in fish diets over the past decades. The vast majority of these studies have established the authenticity of fishmeal as the most suitable protein source for fish, due to its balanced amino acid (AA) profile, high digestibility and palatability (Che et al., 2017; Dam et al., 2019; Hardy, 2010). However, the global demand for fishmeal in aquaculture production has put a strain on the economic and environmental sustainability of this sector. Potential overfishing of marine fish species used for feed production conflicts with the demand for sustainable aquaculture and therefore it drives the reduction of fishmeal usage (Couto et al., 2016; FAO, 2018; Naylor et al., 2009). For these reasons, there has been a growing need for more insight into the potential of alternative protein sources in aquafeed to enable the increasing demand for aquafeeds (Kaushik et al., 2004; Taufek et al., 2016b).

Several alternative novel ingredients (animal and plant origin) of nutritional and economic benefits are now being investigated for the total or partial replacement of fishmeal in fish diet (Basto et al., 2020; Che et al., 2017; Davies and Ezenwa, 2010; dos Santos Cardoso et al., 2020; Fagbenro, 1998; Glencross, 2020; Goda et al., 2007; Lee et al., 2020; Nazzaro et al., 2021; Toko et al., 2008; Tomas-Vidal et al., 2019; Wang et al., 2008). In spite of these promising alternatives, it is quite difficult to get an ingredient with a complete AA profile that is not limiting in at least one essential AA (EAA) (Gomes et al., 1995b). Moreover, plant proteins contain anti-nutritional factors (NRC, 2011), which can reduce the availability of AA (Cai and Burtle, 1996; Ghosh et al., 2019). Therefore, to achieve an optimum diet that contains all EAAs, mixtures of plant and animal protein ingredients are used in formulating aquafeeds (Tomas-Vidal et al., 2019). Investigating the digestibility values of these ingredients is an essential step in formulating balanced practical diets (Glencross, 2020; Gomes et al., 1995a).

In terms of feed formulation, the quality of feed ingredient depends on their digestible amino acid profile, protein and energy (Fagbenro, 1996; Fagbenro, 1998; Glencross, 2020; Henken et al., 1985; Ovie and Eze, 2014). Since the larger part of feed formulation is based on the protein content, reliable data on the digestible AA content of these different ingredients for each species is considered a necessary prerequisite (Basto et al., 2020; Gomes et al., 1995a; Wolfe et al., 2016). Data on AA digestibility of feed ingredients for African catfish (*Clarias gariepinus*) are relatively limited, compared to other cultured fish species, like rainbow trout (*Oncorhynchus mykiss*), Nile tilapia (*Oreochromis niloticus*) and Atlantic salmon (*Salmo salar*) (NRC, 2011). Currently in Africa, the culture of African catfish is growing steadily due to the increasing local demand and high market price value. Moreover, the species voracious eating behaviour, fast growth rate, and ability to survive in adverse environmental conditions (Fagbenro et al., 1999), makes it relatively easy to be farmed in the natural inland freshwater areas. Evaluating the nutritional values of novel and array of ingredients for this species will play an important role in establishing how efficient African catfish is able to accept, digest and utilize the feedstuffs used (Allan et al., 2000; Udo and Umoren, 2011). Such information will be useful to simulate the ideal AA profile in the diets of African catfish and the production of least-cost feed, one of the problems to be resolved in its development.

There is considerable controversy regarding the effect of feeding level on the apparent digestibility coefficient (ADC) of nutrients in fish. Only a few studies have addressed the potential impact of increasing feeding level on the ADC in fish species. These include, Nile tilapia, rainbow trout and African catfish, with most results being negatively correlated to ADC (Haidar et al., 2016; Henken et al., 1985;

Staessen et al., 2020a). However, Cho and Kaushik (1990) came to a different conclusion in their study that ADC of nutrients were not affected by feeding level or feeding rate. Apart from Henken et al. (1985) who determined the effect of feeding level on nutrient ADC in African catfish, no study so far has been carried out to have a better understanding of the relationship between feeding level and AA ADC in African catfish. Therefore, the present study was undertaken (1) to obtain values for nutrients and AA digestibility of selected ingredients of plant and animal origin, (2) to evaluate the effect of feeding level on ADC of ingredients, and (3) to provide data on digestible AA of ingredients that will allow a greater accuracy in feed formulation for African catfish.

2.2 Materials and methods

This experiment was approved by the Animal Welfare Body of Wageningen University, The Netherlands. All procedures applied to the animals were in line with the Dutch legislation (Act on Animal Experiments) and were classified as not being an animal experiment according to Dutch legislation. The experiment was carried out at the Aquaculture Research Facility of Wageningen University (The Netherlands). Because of a limited number of aquaria equipped with settling units for faeces collection, the experiment was conducted in two trials: six test ingredients were tested in the first trial while seven ingredients were investigated in the second trial. Both trials were identical regarding the experimental set-up, housing and sampling procedure, only the initial weight was different and consequently also the stocking density.

2.2.1 Diet preparation

The ingredient composition of the test diets, the analysed nutrient composition of the ingredients and experimental diets are summarized in Table 2.1, 2.2 and 2.3 respectively. A reference diet (control) was formulated by combining information on the recommended amino acid requirements of Nile tilapia, common carp (*Cyprinus carpio*) and channel catfish (*Ictalurus punctatus)* (NRC, 2011), since information on amino acid requirement of African catfish is relatively scarce. Test ingredients were sourced from a wide range of protein rich ingredients of both animal and plant protein origin; hydrolysed feather meal (HFM), LT70 fishmeal (FM), insect meal (IM) from black soldier fly larvae (*Hermetia illucens*), soybean meal (SBM), sunflower meal (SFM), poultry meal (PM), corn dried distillers grain with solubles (CDDGS), faba beans (FB), lupine meal (LM), pea protein (PP), guar meal (GM), canola meal (CM) and single cell protein from brewer's yeast (*Saccharomyces cerevisiae*) (yeast meal, YM). The test diets are composed of 70% control diet with 30% test ingredient, except for HFM and GM, which were included at 15% in the mixture to prevent any negative effect that high inclusion levels may pose on digestibility. Yttrium oxide was added as inert marker for the determination of ADC. The diets were extruded floating pellets with sizes ranging from 3 to 3.5 mm, produced by Skretting ARC Norway using a twin-screw extruder (Wenger, Sabetha, KS, U.S.A). Diets were stored at 4 °C throughout the duration of the experiment.

2.2.2 Fish and housing conditions

Juvenile African catfish (*Clarias gariepinus*) of mixed sex were obtained from a commercial brood stock farm (Fleuren & Nooijen BV, Nederweert, The Netherlands) 2 weeks prior to the start of the experiment and were reared at the Wageningen University experimental facilities (Carus Aquatic Research Facility, Wageningen, The Netherlands). For the first trial, 630 fish with an average weight of 53.9 g were randomly allocated among 21 experimental tanks (30 fish per tank). For the second trial, 840 fish weighing on average 40.4 g were randomly assigned among 24 experimental tanks (35 fish per

tank). Each tank was equipped with air stones and swirl separators (AquaOptima AS, column height 44 cm; diameter 24.5 cm) for the collection of faeces and spilled pellets. The tanks were connected to a common recirculating water system equipped with a sump, a drum filter (Hydrotech 500®, Hydrotech Engineering, Italy) and a trickling filter for maintaining water quality parameters within a set range. The total water volume of the RAS system was 5 m^3 and water loss due to evaporation was continuously compensated by the addition of well water. Water quality parameters were monitored regularly and set at optimal levels for African catfish. Temperature, conductivity and pH were measured using digital probes (temperature: Testo 110, Testo B.V., Almere, The Netherlands; conductivity: WTW LF318 and pH: WTW pH340, WTW Wissenschaftlich-Technische Werkstätten GmbH, Weilheim, Germany). Merck tests (Merck KGaA, Darmstadt, Germany) were used for measuring ammonium (Aquamerck 1.11118.0001), nitrite (Aquamerck 1.0825.0001) and nitrate (Mercoquant 1.10020). Measured water quality parameters during trial 1 were as follows (mean ± SD): water temperature 27.3 ± 0.98 °C; pH, 7.3 ± 0.38; ammonium, 0.32 ± 0.24 mg/L; nitrite, 0.25 ± 0.14 mg/L; nitrate, 430 ± 66 mg/L and conductivity, 3802 ± 378 µS; and for trial 2 (mean ± SD): water temperature 27.9 ± 0.31 °C; pH, 7.4 ± 0.44; ammonium, 0.78 ± 0.59 mg/L; nitrite, 0.32 ± 0.34 mg/L; nitrate, 250 ± 0.0 mg/L and conductivity, 3257 ± 743 µS. Photoperiod was kept at 12 h light: 12 h dark.

Table 2.1 Ingredient composition of reference diet

Ingredient (%)	Reference diet
Wheat	20.5
Maize	19.9
Wheat gluten	12.0
Fishmeal	12.0
Soy protein concentrate	12.0
Pea protein	12.0
Soya oil	3.00
Fish oil	3.00
DL-Methionine	0.80
L-Lysine	0.80
Monocalciumphosphate	3.00
Yttrium premix	0.15
Vitamin & mineral premix	0.44
Calcium carbonate	0.36

2.2.3 Experimental procedures and sampling

At the start of each trail, total biomass and number of fish per tank were recorded. The diets within the two trials were studied with 3 replicates per treatment over a period of 4 weeks of restricted feeding followed by 3 weeks of satiation feeding. During the last 2 weeks of the restricted as well as the satiation feeding period, faeces were collected. During the 49-day experimental period, fish were fed twice daily (in the mornings and afternoons). For the restricted feeding period, the aim was to provide an equal amount of feed across diets. Therefore, the feeding level was fixed at 17.6 $g/kg^{0.8}/d$ (about 80% of satiation) based on the mean weight of fish at the start of the restricted feeding period

Table 2.2 Analyzed nutrient and amino acids composition of test ingredients[1]

	Test ingredients												
Nutrient (g/kg DM)	**FM**	**IM**	**PM**	**HFM**	**SBM**	**PP**	**FB**	**LM**	**GM**	**CM**	**SFM**	**CDDGS**	**YM**
Dry Matter	918	949	950	959	875	895	874	910	917	914	913	876	935
Ash	170	92	125	18	72	60	37	39	54	66	77	55	86
Crude protein	751	613	691	913	553	564	325	413	601	371	453	320	450
Fat	97	131	138	77	27	41	22	74	93	94	34	156	23
Starch	12	24	7	10	18	69	361	22	8	13	23	10	80
NSP[2]	-30[4]	140	39	-18[4]	330	267	255	452	245	456	413	338	361
Total carbohydrate[3]	-18[4]	164	46	-8[4]	348	336	615	474	253	469	436	348	441
Energy (kJ/g DM)	21.1	23.2	23.0	24.8	27.2	20.5	18.9	20.6	21.9	21.4	19.8	23.5	19.2
Phosphorus	24.7	8.5	18.9	2.4	7.0	9.4	4.7	7.2	7.9	10.8	13.6	9.9	10.7
Calcium	36.8	25.5	25.0	3.6	3.5	1.7	0.8	1.6	2.1	8.0	5.2	0.4	4.3
Magnesium	2.7	3.4	1.5	0.4	3.5	2.6	0.8	2.3	4.1	5.1	6.3	4.2	1.8
Essential AA (g/kg DM)													
Arginine	42.6	31.9	46.2	60.8	40.9	42.9	25.5	42.2	82.1	21.5	34.3	13.4	20.6
Histidine	14.8	18.1	15.9	5.9	14.3	12.9	7.3	10.9	16.5	10.0	11.1	8.7	9.5
Isoleucine	28.1	27.5	25.7	42.6	24.4	20.7	10.8	15.2	17.7	14.1	17.7	11.0	17.1
Leucine	50.6	45.1	49.8	73.3	42.8	36.2	19.9	26.1	33.0	25.5	28.9	35.5	27.1
Lysine	55.9	41.2	40.5	16.3	33.6	36.7	16.9	18.4	25.0	20.2	19.1	9.2	25.7
Methionine	20.0	13.3	13.1	4.5	7.2	4.6	1.9	2.4	6.3	7.1	9.8	6.0	6.2
Phenylalanine	29.2	27.0	29.2	43.1	30.3	22.9	11.2	15.0	24.4	14.8	20.3	14.6	18.1
Threonine	28.5	25.3	27.7	41.1	20.3	18.5	9.2	13.5	17.0	16.4	15.9	12.0	18.3
Valine	31.1	37.5	32.4	60.8	24.5	21.7	11.5	14.6	19.9	17.8	19.9	14.7	20.4
Non-essential AA (g/kg DM)													
Alanine	44.9	39.1	46.7	42.7	23.7	21.8	11.1	13.4	21.4	16.3	19.6	23.5	25.4
Aspartic acid	69.2	66.0	58.2	61.6	68.2	56.7	29.9	38.7	60.5	27.6	42.7	21.5	38.5
Glutamic acid	100	63.5	91.1	96.9	106	78.6	45.8	80.4	125	61.2	85.2	56.6	69.4
Cysteine	6.7	4.9	7.4	29.7	7.2	6.3	3.0	4.4	7.7	8.7	6.5	5.4	5.5
Glycine	46.6	32.5	64.3	68.8	22.8	20.7	11.1	16.3	29.2	18.5	24.0	13.2	18.6
Proline	28.1	35.6	45.0	100	27.6	22.1	11.9	15.8	20.4	23.4	19.4	27.1	28.0
Serine	29.1	25.8	29.5	104	27.6	24.8	13.1	18.4	27.4	16.0	18.8	15.4	21.3
Tyrosine	14.9	39.7	19.3	19.1	16.2	14.5	7.9	12.4	18.5	10.1	9.9	11.0	11.2
SAA (g/kg DM)	640	574	642	871	537	462	248	358	552	329	403	299	381

[1]AA, amino acid; SAA, sum of amino acid; DM, dry matter; FM, LT70 fish meal; IM, insect meal from black soldier fly larvae (*Hermetia illucens*); PM, poultry meal; HFM, hydrolysed feather meal; SBM, soybean meal; PP, pea protein; FB, faba beans; LM, lupine meal; GM, guar meal; CM, canola meal; SFM, sunflower meal; CDDGS, corn dried distillers grains with solubles; YM, yeast meal (*Saccharomyces cerevisiae*).

[2]NSP, non-starch polysaccharides were calculated as total carbohydrates – starch.

[3]Total carbohydrate was calculated as dry matter – crude protein – crude fat – ash content.

[4]The negative values for NSP and carbohydrates is most likely due to an overestimation of the calculation of crude protein as 6.25 times the measured N content. For FM and HFM, the Jones factor is lower than 6.25

The calculated daily feed ration per tank was set based on an expected growth using a FCR of 1 for all diets. Daily feed portions were divided into two equal parts and hand-fed twice a day at 9:00 and 15:30 h. During the first three days, the feeding level was gradually increased from 20% to 100% of the calculated ration to allow habituation to the diets. During the satiation period, fish were hand-fed twice daily at 9:00 and 15:30 h until voluntary feed ingestion stopped, with a maximum of 1 h per feeding. Mortality was checked twice a day, 30 minutes prior to feeding and dead fish were removed immediately. In case of mortality during the restricted feeding period, daily feeding rations were adjusted to the number of fish in the respective tank. Before feeding, a set of bottles was connected to the swirl separators in order to collect spilled pellets. Spilled and uneaten pellets were counted or weighed per tank 15 min after feeding was finished.

Faeces were collected overnight (17.00 h – 7.30 h) during the last two weeks of the restricted and satiation period, using detachable collection bottles (250 mL) connected to the settling tanks. The faecal collection bottles were submerged in ice-filled styrofoam boxes to reduce microbial degradation. Faeces were pooled per tank and stored at -20 °C for further analysis. At both end of the restricted and satiation period, fish were starved for 24 h and batch weighed per tank for final weight.

2.2.4 Analytical methods

The faecal samples were freeze-dried (Scanvac FD8 Coolsafe Advanced, LaboGene A/S, Denmark), then manually pulverized through a 1 mm screen sieve. Feed pellets and ingredients were grinded by a grinding machine (Retsch ZM 200). Proximate composition of ingredients, feed, and faeces were assessed (in triplicate) according to ISO-standard analysis for determination of dry matter (ISO 6496, 1983), crude ash (ISO 5984, 1978), crude protein (ISO 5983, 1979); (crude protein = Kjeldahl- N × 6.25), crude fat (ISO 6492, 1999) and starch (ISO 6493: 2000). Energy content was measured bomb calorimetric by direct combustion (IKA® werke, C7000; IKA analysentechnik, Weitershem, Germany). Yttrium, phosphorus, calcium and magnesium in feed and faeces were determined from the ash by using inducted coupled plasma mass spectrometry according to the standard NEN 15510 (ICP-MS, 2007). Amino acids (excluding tryptophan) were analysed by Skretting ARC, Norway, using an automatic amino acid analyzer (Biochrom 30+, Biochrom Ltd, Cambridge, UK) and the methods described in the COMMISSION REGULATION (EC) No 152/2009 (Council, 2009). Diet, ingredient and faecal starch contents (incl. sugars) were determined via an enzymatic digestion as described by Goelema et al. (1998), excluding the ethanol washing step and was analysed by Nutricontrol (Veghel, The Netherlands). By excluding the ethanol step, sugars with less than 10 glucose units are included in the starch fraction and thereby gave a better calculation of the non-starch polysaccharide (NSP) content.

2.2.5 Calculation

Daily weight gain (g/d) was calculated as the differences between the average initial (W_i) and final (W_f) body weight of fish divided by the duration of the experiment (t). Feed conversion ratio (FCR; g/g) on dry matter (DM) basis was calculated as (feed intake × dry matter content of the feed)/(final weight of fish – initial weight of fish). Feed intake (FI; % BW/d) was calculated as FI/t/Wg × 100%, where FI is feed intake (g), t is the number of days, and Wg is the geometric mean BW (g) of each feeding period, respectively. The Wg was calculated as e ((ln W_t+ln W_0)/2), where W_0 and W_t are the initial and final BW (g) for each feeding period, respectively. Specific growth rate (SGR; %/d)

Table 2.3 Analysed nutrient and amino acids composition of test diets[1]

	Trial 1							Trial 2							
	CON 1	**FM**	**IM**	**HFM**	**SBM**	**YM**	**SFM**	**CON 2**	**PM**	**PP**	**FB**	**LM**	**GM**	**CM**	**CDDGS**
Inclusion level (%)															
Test ingredient	---	30	30	15	30	30	30	---	30	30	30	30	15	30	30
Reference diet	100	70	70	85	70	70	70	100	70	70	70	70	85	70	70
Nutrient (g/kg DM)															
Dry Matter	889	897	907	898	886	888	899	893	896	899	889	893	897	900	892
Ash	75	100	78	63	72	77	74	76	84	69	65	64	71	72	68
Crude protein	419	514	482	498	461	426	423	411	490	454	372	412	434	401	385
Fat	112	115	116	96	87	89	88	113	119	92	90	102	109	111	128
Starch	275	202	204	247	212	234	209	282	202	215	310	216	245	215	217
NSP[2]	113	67	114	85	162	171	197	118	105	170	164	205	141	201	203
Total carbohydrate[3]	394	271	324	342	381	408	414	400	306	385	474	421	386	416	419
Energy (kJ/g DM)	21.3	21.2	21.8	21.8	20.9	20.5	20.7	21.0	21.5	21.0	20.7	21.0	21.2	21.3	21.7
Phosphorus	14.3	17.2	12.7	11.9	11.7	13.2	14.2	14.8	15.7	13.4	12.5	12.5	13.7	13.9	13.3
Calcium	13.5	19.9	16.7	11.3	10.2	10.7	10.9	13.7	16.4	10.3	10.2	10.0	12.0	11.7	9.8
Magnesium	2.0	2.2	2.4	1.7	2.4	2.0	3.3	2.3	2.0	2.5	2.0	2.3	2.5	3.0	2.8
Essential AA (g/kg DM)															
Arginine	23.5	29.7	27.4	29.5	28.6	23.0	26.9	22.8	28.9	29.6	25.3	29.1	30.2	22.5	21.0
Histidine	9.8	12.3	12.7	9.1	11.2	9.9	10.1	9.4	10.6	10.6	9.1	9.7	9.9	9.3	8.9
Isoleucine	16.4	20.6	20.4	21.0	19.2	16.9	16.8	15.3	17.7	17.4	14.9	15.3	15.4	15.0	14.0
Leucine	30.2	37.1	35.2	37.1	34.2	29.4	29.5	28.9	33.7	31.9	27.8	28.2	28.8	27.8	29.8
Lysine	27.6	36.3	31.8	25.6	29.3	26.7	24.7	27.2	30.0	30.6	25.6	24.9	26.4	24.8	22.0
Methionine	14.9	16.5	14.6	13.6	12.9	12.3	13.3	15.0	13.8	12.0	11.2	11.4	13.4	12.4	12.1
Phenylalanine	18.8	21.1	20.4	22.6	21.5	19.1	19.1	18.0	20.5	20.8	17.8	17.7	19.0	17.7	17.8
Threonine	13.9	19.0	18.0	18.3	16.2	15.4	14.6	13.5	16.9	15.3	13.0	13.7	13.6	14.1	12.8
Valine	17.7	22.4	24.8	25.4	20.0	18.9	18.5	16.7	20.7	19.2	16.5	16.1	16.7	17.2	16.1
Non-essential AA (g/kg DM)															
Alanine	17.8	26.0	24.4	21.7	19.8	20.1	18.2	17.4	24.6	19.1	16.5	16.4	17.8	17.0	18.7
Aspartic acid	33.3	44.6	43.2	37.9	43.0	34.9	35.7	32.5	39.0	40.9	33.9	34.9	35.5	31.1	29.4
Glutamic acid	88.4	93.3	83.7	90.5	93.3	83.2	87.0	86.1	84.1	86.3	77.6	84.5	89.2	77.9	77.0
Cysteine	5.9	6.4	6.1	9.7	6.3	5.8	6.2	5.6	5.6	5.9	4.9	5.1	5.7	6.3	5.4
Glycine	17.8	26.4	22.7	26.0	19.4	18.1	19.6	17.5	28.7	19.0	16.6	17.3	18.8	17.5	16.2
Proline	28.0	29.4	32.0	39.6	28.5	28.7	25.8	28.1	31.3	27.0	24.4	24.5	26.9	26.4	26.2
Serine	18.8	23.1	22.7	32.1	22.1	19.9	19.1	18.5	21.1	20.9	18.0	18.7	19.2	17.8	17.6
Tyrosine	3.5	4.8	16.0	6.0	6.1	7.2	6.2	10.7	12.9	13.0	11.1	11.4	11.9	10.9	11.1
SAA (g/kg DM)	386	469	456	466	432	389	391	383	440	419	364	379	398	366	356

[1] AA, amino acid; SAA, sum of amino acid; DM, dry matter; CON, control; FM, LT70 fish meal; IM, insect meal from black soldier fly larvae (*Hermetia illucens*); HFM, hydrolysed feather meal; SBM, soybean meal; YM, yeast meal (*Saccharomyces cerevisiae*); SFM, sunflower meal; PM, poultry meal; PP, pea protein; FB, faba beans; LM, lupine meal; GM, guar meal; CM, canola meal; CDDGS, corn dried distillers grains with solubles.

[2]NSP, non-starch polysaccharides were calculated as total carbohydrates – starch.

[3]Total carbohydrate was calculated as dry matter – crude protein – crude fat – ash content.

was calculated as ($LnW_f - LnW_i \times 100$)/t, where t is the duration of the experiment in days (d). Fish survival (%) was calculated as number of fish at the beginning of the experiment divided by the number fish at the end of the experiment x 100.

The ADC of AA and macronutrients of diets were calculated according to the following formula described by Cheng and Hardy (2002) using yttrium oxide as inert marker, ADC (%) = 100 × [1 − (Yttrium concentration in the feed × concentration nutrient in faeces)/(Yttrium concentration in the faeces × concentration nutrient in feed)]. The dry matter ADC of the diets was calculated as, ADC (%) = 100 × [1 − (Yttrium concentration in the feed /Yttrium concentration in the faeces)]. The ADC of dietary component in the test ingredient were calculated using the following equation as described by Teuling et al. (2017); $ADC_{test\ ingredient} = ADC_{test\ diet} + (ADC_{test\ diet} - ADC_{reference\ diet})$ x (0.7 x $Nutrient_{reference\ diet}$/0.3 x $Nutrient_{test\ ingredient}$) x 100%, where $ADC_{test\ diet}$ and $ADC_{reference\ diet}$ are the apparent digestibility coefficient (%) of the dietary component in the test diet and the reference diet, respectively. $Nutrient_{reference\ diet}$ and $Nutrient_{test\ ingredient}$ are the nutrient contents (g/kg DM) or the gross energy (kJ/g) in the reference diet and test ingredient, respectively. The concentrations of yttrium and nutrients were expressed on DM basis. Total carbohydrate was calculated as dry matter minus crude protein minus crude fat minus ash content. The NSP fraction was calculated as total carbohydrates minus starch.

2.2.6 Statistical analysis

Tanks (trial 1 n = 21; trial 2 n = 24) were considered as experimental units. Due to differences in the start weight, performance data were subjected to a one-way analysis of variance (ANOVA) within trials. Furthermore, performance data were separately analysed per feeding period because the variance differed between the feeding periods (e.g., feeding level). Combined data regarding digestibility during restricted and satiation feeding were analysed using GLM procedure of repeated measurement to test the effect of feeding period (restricted vs. satiation feeding), ingredient and their interaction. The effect of ingredient was tested against the between tank variation. The level of significance adopted was 5%. Tukey's multiple range test was performed when finding significant interactions between factors. All data analysis were carried out using statistical analysis systems (SAS Institute) statistical software package version 9.1.

2.3 Results

The same reference diet was used in trial 1 and 2, however, the trails were conducted at different times using different batches of African catfish of the same origin. Differences between the respective ADC of the reference diets were examined using a one-way ANOVA to check if there was a trail effect. Result showed no significant difference in nutrients and AA ADC (P>0.05) of the reference diets between trials.

Performance parameters are presented in Supplementary Table 2.1. Fish were fed the same ration during the restricted period, therefore, feed intake did not differ (P>0.05; Table 2.4). In both trials at the start of the restricted feeding period, fish promptly accepted all the experimental diets with the exception of YM. YM feeds were rejected in the first two days, after which fish slowly adapted to this diet. This was reflected by an increase in feed intake over time. During satiation feeding, feed intake in trail 1 and 2 was respectively, 2.89 and 3.66% BW/d averaged over all diets. Within trial 1, satiation feed intake differed among diets (P<0.01; Table 2.4). In this trial, the lowest satiation feed intake (2.63% BW/d) was observed in fish fed the YM diet, whereas, SBM diet had

the highest intake of 3.11% BW/d, while all other test diets (FM, IM, HFM and SFM) had similar satiation feed intakes as the control diet. The ranking in feed intake expressed in g/d was slightly different. Feed intake expressed in g/d was highest for FM and IM and lowest for YM (Table 2.4). The ingredients tested in trial 2 (PM, PP, FB, LM, GM, CM and CDDGS) did not induce difference in satiation feed intake, both on g/d as well as % BW/d basis (P>0.05; Table 2.4).

The ADC of macro-nutrients, energy and minerals of experimental diets are presented in Supplementary table 2.1, because this study mainly focused on the ADC of ingredients. Ingredient ADC values of nutrients, which are placed in between brackets in Table 2.5 and 2.6, were excluded from the statistical analysis. This is because of the low contribution of these ingredients to the experimental diets (less than 8% of the total nutrient content in the test diet originating from the test ingredient), which amplifies the measurements errors in the calculated ingredient ADC values. This implication can lead to estimated ingredient ADC values < 0% and also > 100%, which occurred for some nutrients ADC of ingredients (Table 2.5). Except for fat and carbohydrate, feeding level had a significant effect on all macronutrients digestibilities. Feeding level influenced nutrients digestibility at a different degree, with the most significant response found in NSP digestibility (P<0.001), followed by DM, ash and starch (P<0.01). The least impact was observed in protein and energy (P<0.05) digestibility. Furthermore, an interaction effect was found between feeding level and dietary treatments on fat, NSP and starch digestibility. Feeding levels showed a contrasting trend on nutrient ADC among ingredients. For some ingredients (e.g., FM, IM and LM), protein digestibility increased with increased feeding level while others (e.g., PM and PP) decreased with feeding level.

Generally, ADC values for nutrients in ingredients tested were high, especially for ingredients of animal origin. DM digestibility of all ingredients differed significantly (P<0.01). For ingredients of animal origin, DM digestibility values exceeding 74% were recorded for FM, PM, IM, and HFM while values for legumes and oilseeds were above 60.4%. Fat in animal ingredients (95.1%) was better digested than that of vegetable ingredients (88.4%). In the same way, energy ADC was averagely 91.3% for animal protein ingredients and 81.7% for plant protein ingredients. The highest crude protein digestibility (105.1%) was recorded in GM, followed by SBM (96.5%) while the lowest values were found in IM and CDDGS (85.6% and 86.5%, respectively). FM displayed moderate value for crude protein ADC (averaged over both periods, 94.2%). Overall, ADC values for GM were exceptionally high (beyond 100%) for most of the nutrients analysed.

Apparent AA digestibility coefficients of the test ingredients are presented in Table 2.6. No significant differences in ADC for AA were observed between both feeding periods except for methionine and glutamic acid. The digestible essential AA content of each ingredient expressed per unit of digestible protein (dAA/DP) are visualized in figure 2.1-2.3 and digestible non-essential AA content in Supplementary figure 2.1-2.3. Overall, the ordering of ingredients from highest to lowest dAA/DP content varied strongly between the different amino acids. The dAA/DP content of Met in test ingredients of animal origin was highest for FM (27 g dMet/kg DP) and lowest for HFM (5 g dMet/kg DP). Also, all tested legumes had a low digestible Met content, which ranged from 5 to 14 g dMet/kg DP. IM and PM had a similar dAA/DP content of Met (20 and 19 g dMet/kg DP, respectively) though lower than FM (Figure 2.1). Except for HFM, the dAA/DP content of histidine in all ingredients was larger than that of FM (20 g dHis/kg DP). HFM had a histidine content of only 6 g dHis/DP (Figure 2.1). All legumes had a lower dAA/DP content of threonine compared to FM.

IM, HFM and CM had a higher dAA/DP content of threonine compared to FM. All other ingredients had a comparable digestible threonine content as FM (Figure 2.1). All ingredients had a lower dAA/CP content of lysine than that of FM (76 g dLys/kg DP), though the digestible lysine content of IM was only slightly lower. HFM had the lowest digestible lysine content (17 g dLys/kg DP) (Figure 2.2). Regarding the digestible arginine content, only CDDGS and YM had a value lower than FM. All other ingredients had an equal or higher dAA/DP content of arginine compared to FM (59 g dArg/kg DP). GM had a very high digestible arginine content, being 131 g Arg/kg DP (Figure 2.2). Regarding the digestible phenylalanine and isoleucine content, only some ingredients were below the content in FM (Figure 2.3). Excluding SBM, the tested legumes had a lower digestible valine content compared to FM (Figure 2.3). Considering all essential AA of tested ingredients, IM was the closest to FM regarding its dAA/DP profile.

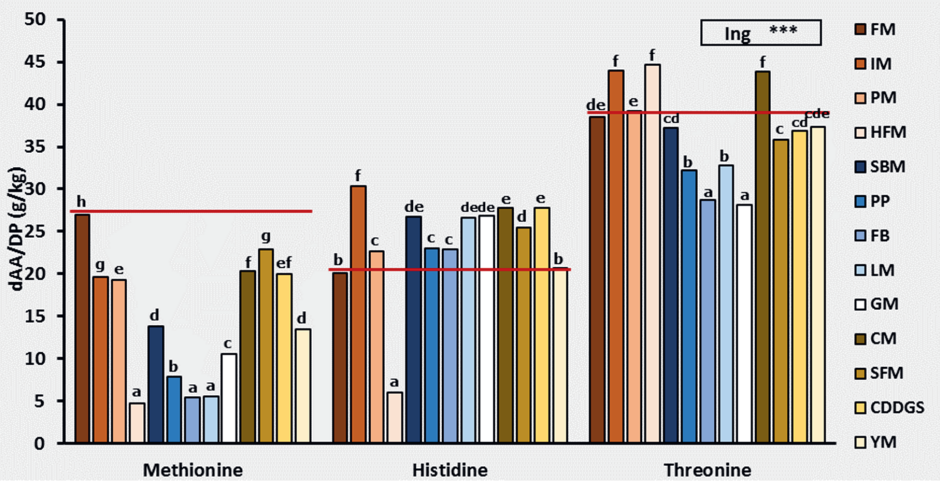

Figure 2.1 Digestible methionine, histidine and threonine expressed per digestible protein (dAA/DP) of various ingredients fed to African catfish. Red line showing fish meal dAA/DP compared to other ingredients. Ing, ingredients; FM, LT70 fish meal; IM, insect meal from black soldier fly larvae (*Hermetia illucens*); PM, poultry meal; HFM, hydrolysed feather meal; SBM, soybean meal; PP, pea protein; FB, faba beans; LM, lupine meal; GM, guar meal; CM, canola meal; SFM, sunflower meal; CDDGS, corn dried distillers grains with solubles; YM, yeast meal (*Saccharomyces cerevisiae*).

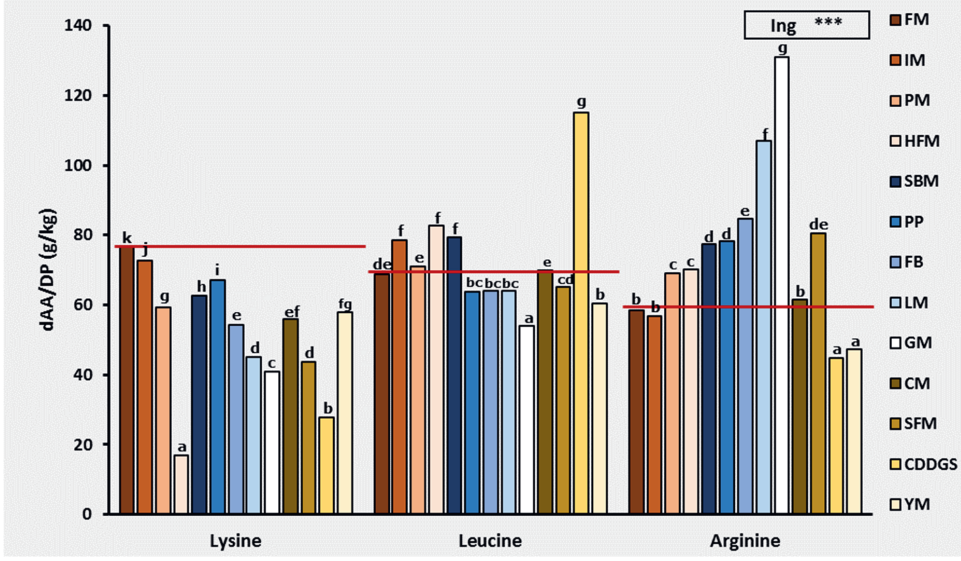

Figure 2.2 Digestible lysine, leucine and arginine expressed per digestible protein (dAA/DP) of various ingredients fed to African catfish. Red line showing fish meal dAA/DP compared to other ingredients. FM, LT70 fish meal; IM, insect meal from black soldier fly larvae (*Hermetia illucens*); PM, poultry meal; HFM, hydrolysed feather meal; SBM, soybean meal; PP, pea protein; FB, faba beans; LM, lupine meal; GM, guar meal; CM, canola meal; SFM, sunflower meal; CDDGS, corn dried distillers grains with solubles; YM, yeast meal (*Saccharomyces cerevisiae*).

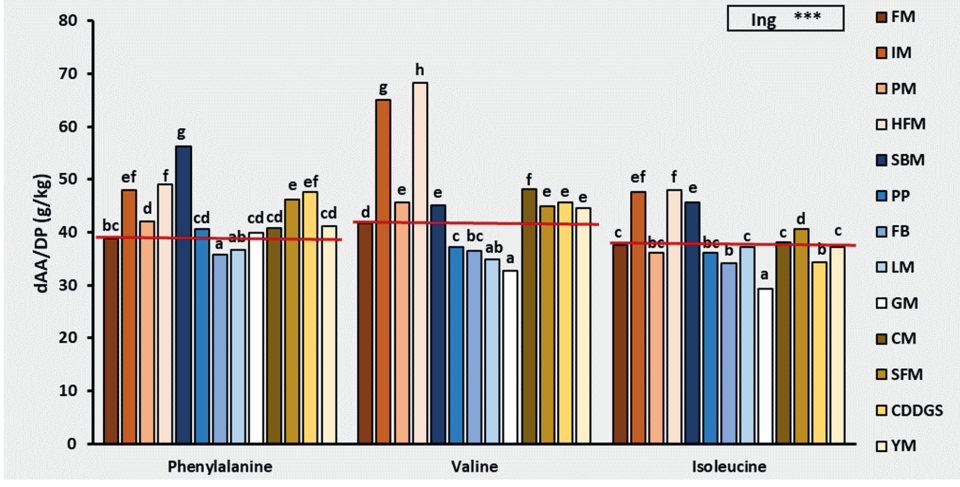

Figure 2.3 Digestible phenylalanine, valine, isoleucine expressed per digestible protein (dAA/DP) of various ingredients fed to African catfish. Red line showing fish meal dAA/DP compared to other ingredients. FM, LT70 fish meal; IM, insect meal from black soldier fly larvae (*Hermetia illucens*); PM, poultry meal; HFM, hydrolysed feather meal; SBM, soybean meal; PP, pea protein; FB, faba beans; LM, lupine meal; GM, guar meal; CM, canola meal; SFM, sunflower meal; CDDGS, corn dried distillers grains with solubles; YM, yeast meal (*Saccharomyces cerevisiae*).

Table 2.4 Effect of dietary ingredient on feed intake of African catfish during restricted feeding and satiation feeding[1]

		Trial 1									Trial 2									
Parameters	**FL**	**CON1**	**FM**	**IM**	**HFM**	**SBM**	**YM**	**SFM**	**SEM**	***P*-value[2]**	**CON2**	**PM**	**PP**	**FB**	**LM**	**GM**	**CM**	**CDDGS**	**SEM**	***P*-value[2]**
FI (g/d)	R	2.10	2.10	2.10	2.10	2.10	2.09	2.08	--	--	1.74	1.74	1.74	1.74	1.74	1.64	1.74	1.74	--	--
	S	5.23^{bc}	6.04^{c}	5.81^{c}	4.76^{ab}	5.45^{bc}	4.19^{a}	4.57^{ab}	0.21	***	5.50	5.42	5.50	5.87	5.50	5.01	5.28	5.10	0.21	NS
RFI (%/BW/d)	R	2.74	2.60	2.60	2.72	2.75	2.78	2.76	--	--	3.00	2.89	3.00	2.97	3.02	2.84	3.03	3.17	--	--
	S	2.99^{ab}	3.03^{ab}	2.95^{ab}	2.79^{ab}	3.11^{b}	2.63^{a}	2.76^{ab}	0.08	**	3.65	3.41	3.61	3.91	3.74	3.46	3.66	3.81	0.11	#

[1]FI, feed intake; RFI, relative feed intake; CON, control; FM, LT70 fish meal; IM, insect meal from black soldier fly larvae (*Hermetia illucens*); HFM, hydrolysed feather meal; SBM, soybean meal; YM, yeast meal (*Saccharomyces cerevisiae*); SFM, sunflower meal; PM, poultry meal; PP, pea protein; FB, faba beans; LM, lupine meal; GM, guar meal; CM, canola meal; CDDGS, corn dried distillers grains with solubles; SEM, standard error of mean; FL: feeding level (R or S); R, restricted feeding; S, satiation feeding;

[2]NS, not significant $P>0.1$; # $P<0.10$; ** $P<0.01$; *** $P<0.001$. Feed intake was not analysed during restricted period because feeding level was fixed.

[abc]Values in the same row within a trail lacking common superscripts are statistically different ($P<0.05$) according to Tukeys' multiple comparison test

Table 2.5 Apparent digestibility coefficient (ADC) of nutrients in ingredients fed to African catfish during the experimental period[1]

		Test Ingredients														*P*-value[2]		
ADC (%)	**FL**	**FM**	**IM**	**PM**	**HFM**	**SBM**	**PP**	**FB**	**LM**	**GM**	**CM**	**SFM**	**CDDGS**	**YM**	**Pooled SEM**	**I**	**FL**	**FL*I**
Dry matter	R	83.7	76.5	87.2	74.0	73.3	80.0	82.1	77.5	106.2	73.3	66.9	64.8	78.3	3.69	***	**	#
	S	95.3	89.3	89.9	93.4	77.3	80.8	80.3	74.6	111.1	75.8	60.4	72.2	87.2				
Ash	R	44.7	50.3	69.4	(-182.0)[4]	11.7	63.3	65.3	47.2	169.5	46.8	26.3	91.7	71.0	10.9	***	**	NS
	S	63.0	71.7	56.6	(-55.2)	54.3	59.7	75.8	83.4	166.1	72.0	44.4	124.3	83.5				
Crude protein	R	93.1	85.6	90.4	87.1	92.2	93.8	87.6	93.9	105.1	89.8	92.4	86.9	87.2	1.16	***	*	#
	S	95.4	90.1	89.2	91.5	96.5	92.7	87.3	94.8	103.1	89.9	92.5	86.5	87.8				
Fat	R	98.6	95.0	99.1	69.2	77.1	91.0	85.5	95.9	93.0	95.2	86.1	90.9	(84.1)	2.97	***	#	***
	S	103.1	100.5	98.7	96.9	89.7	88.6	84.2	95.2	91.1	97.0	67.6	90.6	(99.2)				
Starch	R	–[5]	–[5]	–[5]	–[5]	(101.6)	103.5	98.4	(105.3)	(112.7)	(113.4)	(98.2)	(83.4)	99.5	1.04	***	**	**
	S	–[5]	–[5]	–[5]	–[5]	(111.0)	112.8	95.4	(118.4)	(403.5)	(191.7)	(120.8)	(114.2)	103.9				
NSP	R	–[5]	28.4	–[5]	–[5]	39.7	41.0	29.8	50.6	119.7	49.2	37.2	31.8	51.9	8.61	***	***	***
	S	–[5]	71.0	–[5]	–[5]	36.1	57.3	55.1	42.6	123.3	47.1	21.0	46.8	72.9				
Total Carbohydrate	R	–[5]	51.5	–[5]	–[5]	52.4	58.5	77.9	60.7	111.4	56.4	44.7	36.2	69.1	7.23	***	#	NS
	S	–[5]	89.1	–[5]	–[5]	49.4	64.2	77.9	52.6	131.6	59.6	30.2	46.0	86.9				
Energy	R	93.4	81.5	91.3	83.8	82.0	83.8	84.2	81.4	104.3	78.0	71.7	68.2	78.4	2.93	***	*	NS
	S	101.1	91.6	93.4	94.7	82.7	84.6	82.5	79.0	108.0	79.9	63.2	73.6	87.6				
Calcium[3]	R	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	S	36.9	67.7	62.1	(49.3)	-16.8	(71.0)	(263.5)	(186.8)	(751.8)	70.9	29.2	(2296.0)	124.5	26.2	#	–	–
Phosphorous[3]	R	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	S	59.6	58.7	65.2	(37.0)	34.4	60.1	59.2	61.1	89.4	59.6	49.9	116.0	120.7	5.05	***	–	–
Magnesium[3]	R	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	S	89.7	85.1	79.5	(71.0)	58.3	65.2	34.4	64.8	94.9	67.7	51.4	98.9	105.3	4.71	***	–	–

[1]Presented values are means (n=3) per diet/ingredient within each experiment. FM, LT70 fish meal; IM, insect meal from black soldier fly larvae (*Hermetia illucens*); PM, poultry meal; HFM, hydrolysed feather meal; SBM, soybean meal; PP, pea protein; FB, faba beans; LM, lupine meal; GM, guar meal; CM, canola meal; SFM, sunflower meal; CDDGS, corn dried distillers grains with solubles; YM, yeast meal (*Saccharomyces cerevisiae*); SEM, standard error of mean; I, ingredients; FL, feeding level; IxFL, interaction between ingredients and feeding level; R, restricted feeding; S, satiation feeding; NSP, non-starch polysaccharide.

[2]NS, not significant $P>0.1$; # $P<0.10$; *$P<0.05$; **$P<0.01$; ***$P<0.001$.

[3]Chemical analysis was not performed for calcium, phosphorus and magnesium during for restricted period due to insufficient faecal materials.

[4]The values in the brackets were excluded from statistical analysis because the contribution of the nutrient originating from the ingredient was less than 8% of the nutrient content in the test diet.

[5]ADC values were not calculated for FM, IM, PM and HFM because these ingredients do not contain these nutrients.

Table 2.6 Apparent digestibility coefficient (ADC) of amino acids in ingredients fed to African catfish during the experimental period[1]

		Test Ingredients														*P*-value[2]		
ADC (%)	**FL**	**FM**	**IM**	**PM**	**HFM**	**SBM**	**PP**	**FB**	**LM**	**GM**	**CM**	**SFM**	**CDDGS**	**YM**	**Pooled SEM**	**I**	**FL**	**FL*I**
Essential AA																		
Arginine	R	96.4	95.9	92.9	93.1	97.5	97.1	94.7	98.6	100.6	95.7	97.9	93.6	90.3	0.69	***	NS	NS
	S	98.0	97.9	93.8	95.1	99.6	95.7	94.2	98.3	100.9	95.1	98.6	92.6	90.8				
Histidine	R	94.8	91.9	88.8	77.4	96.5	95.5	89.7	95.2	101.8	93.0	97.0	88.2	85.2	1.29	***	#	**
	S	97.3	91.7	89.4	89.3	98.1	93.4	89.7	94.2	102.5	92.6	95.1	89.0	86.3				
Isoleucine	R	94.1	93.2	87.9	91.8	95.5	94.0	89.4	95.7	104.5	90.0	95.8	86.7	85.5	1.36	***	NS	NS
	S	95.3	95.4	87.6	92.0	99.3	90.2	90.1	93.4	105.4	89.9	96.0	85.5	85.6				
Leucine	R	95.4	93.1	89.3	91.0	94.8	95.0	91.5	96.2	102.1	91.3	94.3	90.6	87.9	1.11	***	NS	NS
	S	96.5	96.1	89.1	92.5	98.6	91.5	91.3	94.3	104.4	91.4	94.5	89.6	87.8				
Lysine	R	96.2	95.9	91.2	78.1	96.4	97.3	91.4	96.3	104.4	93.0	96.2	84.7	87.7	1.26	***	NS	**
	S	97.5	96.7	91.8	91.0	98.3	95.7	92.2	94.4	102.1	91.4	95.5	83.9	89.2				
Methionine	R	95.1	96.0	92.7	(79.7)	98.0	95.1	(80.0)	(90.6)	(104.6)	95.9	97.7	94.5	84.5	0.88	***	*	***
	S	95.7	96.7	92.0	(88.8)	100.4	86.3	(79.0)	(89.8)	(104.9)	95.0	98.0	90.8	85.4				
Phenylalanine	R	93.4	95.6	90.4	91.9	95.4	95.6	91.7	96.1	102.5	93.4	95.0	92.0	89.3	1.18	***	NS	NS
	S	94.5	97.7	89.7	93.4	98.3	91.8	89.4	93.3	104.0	91.0	94.9	89.3	89.7				
Threonine	R	95.0	93.3	88.5	87.6	93.3	93.9	89.0	95.0	104.8	89.3	94.5	85.6	79.8	1.23	***	NS	NS
	S	96.1	95.2	88.7	89.5	98.1	90.0	88.9	93.4	104.3	88.9	93.8	85.2	81.0				
Valine	R	94.1	93.8	87.9	91.4	93.4	93.1	90.3	94.0	103.0	90.6	94.1	87.3	85.4	1.40	***	NS	NS
	S	95.7	95.1	88.0	91.7	98.3	88.9	89.3	91.2	105.3	90.1	94.8	85.3	86.0				

Table 2.6 (Contd.) Apparent digestibility coefficient (ADC) of amino acids in ingredients fed to African catfish during the experimental period[1]

		Test Ingredients														*P*-value[2]		
ADC (%)	**FL**	**FM**	**IM**	**PM**	**HFM**	**SBM**	**PP**	**FB**	**LM**	**GM**	**CM**	**SFM**	**CDDGS**	**YM**	**Pooled SEM**	**I**	**FL**	**FL*I**
Non-essential AA																		
Alanine	R	95.5	93.5	91.8	88.6	92.7	93.7	87.8	94.4	104.5	93.2	94.2	91.3	87.0	1.32	***	NS	NS
	S	96.2	95.2	91.7	90.9	98.4	89.0	88.9	93.0	105.0	92.3	95.4	90.5	87.2				
Aspartic acid	R	92.1	93.8	85.0	87.5	97.9	96.5	92.3	96.4	103.5	94.4	98.1	88.2	82.9	1.06	***	NS	NS
	S	94.7	95.5	88.9	91.0	99.2	94.4	92.9	95.0	102.9	94.4	96.6	87.9	83.3				
Glutamic acid	R	95.9	93.8	91.3	90.3	98.1	97.6	93.1	98.1	101.5	96.4	98.4	93.5	90.1	0.71	***	*	*
	S	97.8	96.3	92.6	94.8	99.3	96.3	93.2	97.7	101.5	96.3	97.6	93.4	90.7				
Cysteine	R	86.1	82.0	73.4	88.0	96.4	81.1	74.4	91.7	104.0	89.6	96.2	83.6	70.0	2.41	***	#	NS
	S	92.2	86.8	76.6	88.6	99.0	76.0	77.6	90.1	109.6	92.1	92.8	86.5	72.7				
Glycine	R	94.5	89.0	91.9	91.8	91.4	92.6	86.2	94.9	102.9	91.7	93.6	86.9	83.4	1.41	***	NS	NS
	S	96.1	91.2	93.4	92.6	96.6	88.8	87.4	93.5	103.4	91.7	93.9	87.4	84.3				
Proline	R	95.0	93.7	92.3	92.9	95.2	92.6	86.2	96.3	104.7	90.4	95.0	91.9	88.7	1.20	***	NS	NS
	S	97.2	95.3	92.3	94.2	98.1	89.4	87.1	94.6	106.4	90.8	94.3	92.0	88.9				
Serine	R	94.3	92.9	88.2	93.4	95.7	94.2	92.2	95.7	102.7	90.7	96.2	89.9	79.0	1.00	***	NS	NS
	S	96.6	94.9	89.5	93.6	98.6	91.2	91.3	94.6	103.5	91.2	95.0	89.6	81.4				
Tyrosine	R	84.7	99.5	90.0	98.1	98.1	98.6	96.1	97.0	97.2	95.7	99.2	92.1	88.1	1.41	***	NS	*
	S	85.2	100.5	92.7	89.9	98.1	96.4	94.0	94.9	102.0	93.0	99.2	91.9	91.4				
SAA	R	94.6	94.0	90.0	90.9	96.1	95.5	91.1	96.5	102.3	93.1	96.4	90.4	86.2	1.02	***	NS	NS
	S	96.3	95.8	90.9	93.0	98.7	92.8	91.1	95.3	102.9	92.7	96.0	89.8	87.0				

[1]FM, LT70 fish meal; IM, insect meal from black soldier fly larvae (*Hermetia illucens*); PM, poultry meal; HFM, hydrolysed feather meal; SBM, soybean meal; PP, pea protein; FB, faba beans; LM, lupine meal; GM, guar meal; CM, canola meal; SFM, sunflower meal; CDDGS, corn dried distillers grains with solubles; YM, yeast meal (*Saccharomyces cerevisiae*); SEM, standard error of mean; I, ingredients; FL, feeding level; IxFL, interaction between ingredients and feeding level; R, restricted feeding; S, satiation feeding.

[2]NS, not significant $P>0.1$; # $P<0.10$; *$P<0.05$; **$P<0.01$; ***$P<0.001$.

2.4 Discussion

Digestibility and palatability are fundamental measurements used in evaluating the nutrient availability and quality of feed ingredients for specific species and thus for formulating balanced diets. This research assessed the digestibility and satiation feed intake of 13 ingredients in African catfish, in which the protein contents were sourced from animal, plant, or single-cell protein origin.

Palatability is an important factor which determines the value and quality of an ingredient (Glencross, 2020). In trial 1, YM showed to have a lower palatability for African catfish compared to other ingredients based on the measured satiation feed intake (Table 2.4). Similarly, Solomon et al. (2017) observed reduced feed intakes in African catfish fed a yeast containing diet. The lower palatability of the YM diet was also observed during the first week of restricted feeding period, which was intended as an adaptation period to the experimental diets. During this adaptation period, all diets were well accepted by the fish, except for YM diet, which resulted in a longer feeding time for this diet. Fish fed the YM diet swallowed the pellets but often expelled them back into the water afterwards. A similar behaviour was observed in sunshine bass (*Morone chrysops × M. saxatilis*) fed yeast containing diets (Gause and Trushenski, 2011). The lower palatability of YM may be related to a bitter taste which can be present in fermented yeast products (In et al., 2005; Shotipruk et al., 2005). In trail 1, FM and IM resulted in the highest feed intake (in g/d) in African catfish. This is well in line with literature that FM (NRC, 2011) and insect meals (Makkar et al., 2014; Ng et al., 2001) are highly palatable for fish. Fish fed HFM and SFM diets had a lower satiation feed intake (in g/d) than FM and IM diets, but no expulsion after ingestion as seen with YM occurred during the adaptation period. Plant ingredients contains anti-nutritional substances that may affect palatability and reduce feed intake (Gatlin et al., 2007; Nazzaro et al., 2021; Teles et al., 2020). However, in the current study, diets were extruded and consequently heat liable anti-nutritional factors would have most likely be neutralized. This may explain the absence of difference in satiation feed intake between ingredients in trial 2. The impact of extrusion might also be involved in the observed high satiation feed intake at the SBM diet.

In the current study, the effect of feeding level on nutrient ADC was quite variable among feed ingredients (Table 2.5). A significant interaction effect between the ADC of ingredients and feeding level was observed for the digestibility of fat, NSP and starch. The differences in ADC between restricted and satiation feeding could be due to variability in the satiation feed intake between the test diets (i.e., ingredients). However, the change in ADC between both periods was not correlated with the realized satiation feeding level (data not shown). At satiation feeding level, the DM digestibility decreased for SFM, FB and LP but for all other ingredients, digestibility increased with increased feeding level. This is in contrast to what has been reported previously, as ADC appeared to decrease at high feeding level (Haidar et al., 2016; Henken et al., 1985). The higher transit of dietary material through the gastrointestinal tract with a high feeding level (satiation) was suggested as an explanation, as it may reduce the ability of the fish to digest/absorb the diet (Henken et al., 1985). The negative effect of increased feeding level on the ADC of most legumes and oilseeds may be due to the high fibre content in these ingredients (Table 2.2) (Haidar et al., 2016; Staessen et al., 2020a). This may also explain the interaction between feeding level and the ADC of NSP and starch in this study. Haidar et al. (2016) observed that diets with high amounts of NSP tend to be less well digested, especially at high feeding level. The explanation would be that NSP can hold high amounts of water and form gum-like masses in the intestine of fish, which may increase viscosity and reduce digestive enzyme activity (Francis et al., 2001). The differences between ingredients regarding the influence of feeding level

(restricted vs. satiation) on ADC in the current study might be also due to differences between the ingredients in water absorption capacity and viscosity. This may lead to altered gastric transit time, thereby affecting the effectiveness of enzymes. In contrast, Storebakken and Austreng (1987) found no significant difference in digestibility when feeding level was increased in rainbow trout. In another study, Cho and Kaushik (1990) demonstrated that neither feeding frequency nor feeding level affected the ADC of dry matter, crude protein, lipid and gross energy in rainbow trout. Differences in outcome is an indication that the effect of feeding level on nutrient ADC could be dependent on species, methodologies applied and ingredients used in diet formulation (Imtiaz, 2018). Findings from the present study suggest that the effect of feeding level on crude protein digestibility is dependent on the ingredient.

As expected, our results showed a consistent trend for a higher DM and CP digestibility among ingredients of animal origin and a lower DM and CP digestibility for several plant ingredients in African catfish. This result is in line with the observation reported for rainbow trout and yellowtails (both *Seriola lalandi* and *Seriola dumerili*) fed various feed ingredients (Dam et al., 2019; Lee et al., 2020; Tomas-Vidal et al., 2019). Generally, a low DM digestibility indicates the presence of a high quantity of indigestible substances or anti-nutritional factors in the feedstuffs (Dam et al., 2019; Lee et al., 2020; Li et al., 2013). Compared to animal protein ingredients, a lower ADC for plant protein ingredients has been reported in literature. This has generally been attributed to the negative effect of a high fibre content (Che et al., 2017; Lee et al., 2020; Luo et al., 2008; Zhou and Yue, 2012). On the other hand, Allan et al. (2000) reported a 99% nitrogen digestibility for wheat (which contains 15% protein, 80% carbohydrate) in the diet of silver perch (*Bidyanus bidyanus*). This is probably due to the omnivorous nature of this species (similar to African catfish). In evolution, both species may have developed mechanisms to digest and metabolize plant materials. These same mechanisms may explain why African catfish recorded high NSP digestibility in this study. Furthermore, for some ingredients (Table 2.5), ADC values are close to, or even above 100%, similar to what has been found in literature (Allan et al., 2000; Basto et al., 2020; Mo et al., 2019). In the current study, faeces egested into water was collected by settling units. The ADC > 100% might be an indication for the occurrence of leaching. Determination of ADC by faeces collection from water can lead to an overestimation compared to stripping of faeces (e.g., Storebakken et al. (1998)). Furthermore, the ADC > 100% for some ingredients, might be explained by the low nutrient contribution (less than 8%) from these ingredients to the experimental diet, thereby leading to higher uptake of this nutrient from the reference diet constituents to meet the species requirement (Basto et al., 2020). Another alternative explanation might be the presence of enzymes in some ingredients and/or other factors that improve the ADC of the basal part in the test diets.

In the current study, differences in AA digestibility values confirm the notion that the protein quality varies widely among the different ingredients. Among the animal protein ingredients used in this study, HFM had the lowest AA digestibility, whereas IM had a digestibility similar to FM. The high overall AA ADC values recorded for fish fed IM makes it a potential substitute for FM. However, fish fed IM recorded the lowest protein ADC during the restricted feeding period. This low protein digestibility could be linked to the presence of chitin in the insect exoskeleton. Decreased nutrient digestibility due to the presence of chitin in insect meal has been reported in Nile tilapia, turbot (*Psetta maxima*) and Atlantic salmon (Fontes et al., 2019; Karlsen et al., 2017; Kroeckel et al., 2012). It is interesting to note that even though IM showed a lower protein digestibility, it resulted in a similar growth performance as FM. In contrast, YM (considered to be a promising novel ingredient) resulted in the lowest AA

digestibility and growth. Similar observations were reported in several studies especially when using a high inclusion level of YM (Al-Hafedh and Alam, 2013; Manoppo and Kolopita, 2016; Ovie and Eze, 2014; Pongpet et al., 2016). In light of the consistently low feed intake, low digestibility and poor growth of fish fed YM in this and previous studies, it would appear prudent to limit the amount of YM in the diets of African catfish until better understanding of the reasons for the low digestibility are elucidated. However, the inclusion of yeast in feeds for other species was found to potentially improve the feed efficiency and enhance the immune responses (Eryalçin et al., 2017; Ortuño et al., 2002; Siwicki et al., 1994; Torrecillas et al., 2014). Regarding oilseeds and legumes, SBM had relatively high protein and AA digestibility in African catfish and may be a useful alternative to FM in aquafeeds. Protein ADC of soybean meal varies between species and falls within the range of 76-98% (Tomas-Vidal et al., 2019). The present study confirms that the ADC for SBM in African catfish falls towards the higher end of this range (94.4%).

The protein quality of an ingredient is mainly determined by its AA profile and their digestibility. Therefore, AA digestibility data for common feedstuffs is of paramount importance (Anderson et al., 1992; Glencross, 2020). In the current study, we calculated the digestible AA (expressed per unit of digestible protein [DP]) (Figure 2.1-2.3), in order to ascertain the potential values of various ingredients. Values for all ingredients were compared with the values obtained for digestible AA in FM. This is because FM has always been the preferred choice for protein source in aquafeeds due to its high nutrient and AA content (Hardy, 2010). Similar to most other studies (Che et al., 2017; Dam et al., 2019; Tomas-Vidal et al., 2019), a high digestible AA profile was recorded for FM in this study. However, FM was slightly lower in cysteine, serine and tyrosine compared to the other studied ingredients. Among the ingredients of animal origin, IM had comparable digestible AA values as FM indicating its potential for partial replacement of FM in an African catfish diet. A similar high amount in digestible EAA was reported for juvenile European sea bass (*Dicentrarchus labrax*) when fed insect larva (Basto et al., 2020). Similar to the result of Taufek et al. (2016a) for African catfish fed cricket meal, leucine was the most abundant EAA in IM used in the current study. Except for methionine, histidine, and lysine, HFM had higher digestible essential AA content compared to FM. Regarding the studied plant ingredients, SBM showed a high potential for FM replacement as it recorded a comparable essential amino acid profile. This has also been reported for other fish species, such as pacu (*Piaractus mesopotamicus*) (Abimorad et al., 2008), channel catfish (Lim et al., 1998) and Nile tilapia (Furuya et al., 2001b). With the exception of arginine, phenylalanine and histidine, the digestible EAA profile of other legumes was inferior to that of FM. This is consistent with the study on Atlantic salmon, where plant protein sources showed a lower lysine, methionine, threonine, and tryptophan content than fishmeal (Anderson et al., 1992). This implies that, for optimal utilization of these ingredients in diets, supplementation of crystalline amino acids is required to compensate for the amino acids' deficiencies. Methionine from yeast was the first limiting amino acid for pacu (Abimorad et al., 2008). In the current study, digestible methionine was moderately high in the yeast meal. This is in line with the findings of (Gaylord et al., 2004) on hybrid striped bass in which high availability values for methionine in brewer's yeast was recorded. Basto et al. (2020) suggested that the calculated sum of individual AA (SAA) should be regarded as the protein content of an ingredient (true protein). This is because analysed protein contains some other nitrogenous compounds that may contribute to the overall nitrogen estimate. In the current study, IM and SBM displayed the highest values for digestible SAA while PP, FB and YM had the lowest values. Conversely, European sea bass had higher sum of EAA for FM compared to other ingredients tested (Basto et al., 2020). This variation may be due to the fact that different species have

different capacity to digest and utilise nutrients in raw materials, due to differences in their natural trophic feeding habits (i.e., herbivore, omnivore or carnivore) (Dam et al., 2019).

The digestible methionine requirement of African catfish was determined as 18.7 g dMet/kg DP (Elesho et al., 2021). In this study, the digestible methionine values for FM, IM, PM, CM, SFM, and CDDGS met and surpassed the digestible methionine requirement for African catfish with values ranging from 19-27 g dMet/kg DP. Combination of two or more of these ingredients may be sufficient for balanced feed formulation for African catfish. However, due to the lack of reliable data of other AA requirement for this species, we could not further compare the digestible values of other AA with their requirements. More in general, the high amount of digestible EAA in IM makes this ingredient particularly valuable for African catfish since besides its high AA profile, it also improved the growth of African catfish. However, the negative effect of the chitin content on protein digestibility must be carefully evaluated.

In conclusion, the macro-nutrient digestibility in African catfish (*Clarias gariepinus*) is affected by feeding level, but this effect of feeding level is dependent on the type of ingredient. A decline in digestibility with feeding level is present for ingredients with high carbohydrate content. Results indicated that the amino acids digestibility of various ingredients tested in African catfish varies considerably. Therefore, the study provides data of more precise information concerning nutrient and amino acid digestibility in this species. This will allow fish meal substitutions in practical feed based on digestible amino acids in alternative ingredients.

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Supplemental table 2.1 Effect of diets on growth performance of African catfish during restricted feeding and satiation feeding.

		Trial 1									Trial 2									
Parameters	**FL**	**CON1**	**FM**	**IM**	**HFM**	**SBM**	**YM**	**SFM**	**SEM**	***P*-value**	**CON2**	**PM**	**PP**	**FB**	**LM**	**GM**	**CM**	**CDDGS**	**SEM**	***P*-value**
Initial BW (g)	R	53.9	54.1	53.8	54.1	54.0	54.0	53.1	0.8	NS	40.3	40.5	39.9	41.1	40.6	40.3	40.2	40.0	0.52	NS
	S	139^{ab}	150^{c}	146^{bc}	137^{a}	138^{a}	133^{a}	133^{a}	1.6	***	105^{b}	111^{c}	104^{b}	106^{b}	102^{b}	102^{b}	101^{b}	95^{a}	1.1	***
Final BW (g)	R	139^{ab}	150^{c}	146^{bc}	137^{ab}	138^{ab}	133^{a}	133^{a}	1.6	***	105^{b}	111^{c}	104^{b}	106^{b}	102^{b}	102^{b}	101^{b}	95^{a}	1.1	***
	S	278^{ab}	329^{d}	322^{cd}	263^{ab}	285^{bc}	241^{a}	254^{ab}	8.0	***	272^{bc}	283^{c}	275^{bc}	270^{bc}	264^{abc}	252^{ab}	254^{ab}	236^{a}	5.8	***
Survival (%)	R	96.7	97.8	95.6	98.9	94.4	95.6	91.1	2.1	NS	99.0	98.1	97.1	98.1	100	98.1	96.2	100	1.2	NS
	S	98.9	98.9	98.8	98.9	98.9	98.8	97.7	1.2	NS	99.0	99.0	99.0	99.0	99.0	100	99.0	100	0.8	NS
Growth (g/d)	R	2.97^{a}	3.42^{b}	3.2^{8b}	2.97^{a}	3.00^{a}	2.82^{a}	2.85^{a}	0.05	***	2.30^{b}	2.52^{c}	2.29^{b}	2.31^{b}	2.19^{b}	2.21^{b}	2.18^{b}	1.96^{a}	0.04	***
	S	6.66^{ab}	8.53^{c}	8.38^{c}	6.01^{ab}	6.99^{bc}	5.16^{a}	5.77^{ab}	0.34	***	7.59^{ab}	7.81^{b}	7.78^{b}	7.47^{ab}	7.39^{ab}	6.82^{ab}	6.93^{ab}	6.43^{a}	0.25	*
SGR (%/BW/d)	R	3.33^{a}	3.64^{b}	3.56^{b}	3.32^{a}	3.35^{a}	3.22^{a}	3.28^{a}	0.04	***	3.41^{bc}	3.61^{c}	3.42^{bc}	3.37^{bc}	3.29^{ab}	3.32^{ab}	3.30^{ab}	3.09^{a}	0.06	***
	S	3.32^{abc}	3.74^{c}	3.77^{c}	3.10^{ab}	3.45^{bc}	2.84^{a}	3.07^{ab}	0.12	***	4.33	4.25	4.43	4.26	4.34	4.11	4.18	4.14	0.09	NS
FCR (g/g)	R	0.71^{b}	0.61^{a}	0.64^{a}	0.71^{b}	0.70^{b}	0.74^{b}	0.73^{b}	0.012	***	0.76^{ab}	0.69^{a}	0.76^{ab}	0.75^{ab}	0.79^{b}	0.74^{ab}	0.80^{b}	0.89^{c}	0.02	***
	S	0.79^{c}	0.71^{ab}	0.70^{a}	0.80^{c}	0.78^{bc}	0.81^{c}	0.80^{c}	0.016	***	0.72^{abc}	0.69^{a}	0.71^{ab}	0.79^{de}	0.74^{bcd}	0.73^{abc}	0.76^{cde}	0.79^{e}	0.01	***

FI, feed intake; RFI, relative feed intake; CON, control; FM, LT70 fish meal; IM, insect meal from black soldier fly larvae (*Hermetia illucens*); HFM, hydrolysed feather meal; SBM, soybean meal; YM, yeast meal (*Saccharomyces cerevisiae*); SFM, sunflower meal; PM, poultry meal; PP, pea protein; FB, faba beans; LM, lupine meal; GM, guar meal; CM, canola meal; CDDGS, corn dried distillers grains with solubles; SEM, standard error of mean; FL: feeding level (R or S); R, restricted feeding; S, satiation feeding; NS, not significant $P>0.1$; # $P<0.10$; *$P<0.05$; ***$P<0.001$. Values in the same row within a trail lacking common superscripts are statistically different ($P<0.05$) according to Tukeys' multiple comparison test. Feed intake was not analysed during restricted period because feeding level was fixed.

Supplemental table 2.2 Apparent digestibility coefficient (ADC) of nutrients in experimental diets fed to African catfish

		Test Ingredients															*P*-value		
ADC (%)	**FL**	**CON**	**FM**	**IM**	**PM**	**HFM**	**SBM**	**PP**	**FB**	**LM**	**GM**	**CM**	**SFM**	**CDDGS**	**YM**	**Pooled SEM**	**D**	**FL**	**FL*D**
Dry matter	R	79.3	80.1	77.9	82.1	77.9	77.0	80.0	80.7	79.3	84.0	78.0	75.0	75.6	78.5	1.03	***	ns	*
	S	77.0	82.8	81.1	80.9	79.8	77.2	78.0	77.8	76.1	82.0	76.4	72.1	75.4	80.3				
Ash	R	37.0	37.7	37.8	53.8	21.9	25.5	48.1	47.0	43.9	57.2	44.1	29.5	54.5	44.4	4.13	***	***	ns
	S	41.4	49.7	49.0	50.6	32.7	41.6	49.5	51.2	52.9	59.6	53.3	39.0	64.2	52.3				
Crude protein	R	90.1	91.1	88.0	90.4	88.9	90.5	91.7	89.8	91.5	93.5	90.3	90.5	89.6	88.8	0.38	***	**	***
	S	90.3	92.6	90.3	89.7	90.8	92.6	91.1	89.4	91.5	92.8	90.1	91.1	89.2	89.6				
Fat	R	93.4	94.1	93.4	95.8	90.1	91.2	93.5	93.3	94.4	93.8	94.3	91.9	92.8	91.9	0.34	***	ns	***
	S	92.7	95.5	95.4	94.8	93.2	92.4	92.2	92.1	93.2	92.5	93.8	89.8	91.9	93.2				
Starch	R	98.7	98.8	98.9	99.2	98.8	98.7	99.3	98.7	99.1	98.9	99.2	98.6	98.7	98.7	0.33	***	***	***
	S	96.7	98.5	98.7	97.9	98.7	97.9	97.5	95.7	96.6	97.4	97.7	98.3	96.1	98.2				
Carbohydrate	R	71.7	68.8	68.7	70.4	68.2	66.7	67.7	74.3	67.9	74.9	66.8	63.1	61.0	71.0	1.72	***	***	*
	S	65.3	70.8	69.9	69.6	68.8	61.9	64.3	69.6	60.4	71.1	62.7	54.7	59.4	73.1				
Energy	R	84.9	87.6	84.0	86.6	84.9	84.1	84.6	84.5	83.9	87.7	82.6	81.4	79.9	83.3	0.81	***	***	*
	S	82.3	88.4	85.8	85.5	85.1	82.9	82.5	81.8	80.9	85.7	81.1	77.4	79.1	84.3				
NSP	R	9.5	-22.1	14.6	17.8	-20.6	24.8	26.9	21.4	35.2	35.2	33.6	25.4	22.9	33.2	4.47	***	*	*
	S	-8.3	-10.5	20.8	15.3	-9.2	16.6	22.3	20.0	22.3	25.1	25.2	10.3	20.2	39.3				
Phosphorous	R	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	S	60.8	58.0	57.2	64.9	56.2	53.0	63.7	64.1	64.1	66.8	63.5	54.8	75.8	72.7	1.46	***	–	–
Magnesium	R	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	S	47.4	54.0	55.4	65.6	34.6	43.8	62.6	57.9	62.4	69.5	64.5	43.7	77.7	53.6	3.92	***	–	–
Calcium	R	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	S	28.1	33.5	46.9	42.6	30.4	25.0	28.9	32.0	34.4	45.7	35.6	29.4	51.6	41.2	5.05	**	–	–

CON, control; FM, LT70 fish meal; IM, insect meal from black soldier fly larvae (*Hermetia illucens*); PM, poultry meal; HFM, hydrolysed feather meal; SBM, soybean meal; PP, pea protein; FB, faba beans; LM, lupine meal; GM, guar meal; CM, canola meal; SFM, sunflower meal; CDDGS, corn dried distillers grains with solubles; YM, yeast meal (*Saccharomyces cerevisiae*); SEM, standard error of mean; D, diet; FL, feeding level; DxFL, interaction between diet and feeding level; R, restricted feeding; S, satiation feeding; DM, dry matter; Carb, carbohydrate; NSP, non-starch polysaccharide level; NS, not significant P>0.1; *P<0.05; **P<0.01; ***P<0.001; # P<0.10. Values in the same row lacking common superscripts are statistically different (P<0.05) according to Tukeys' multiple comparison test. Chemical analysis was not performed for calcium, phosphorus and magnesium for restricted period due to insufficient faecal materials.

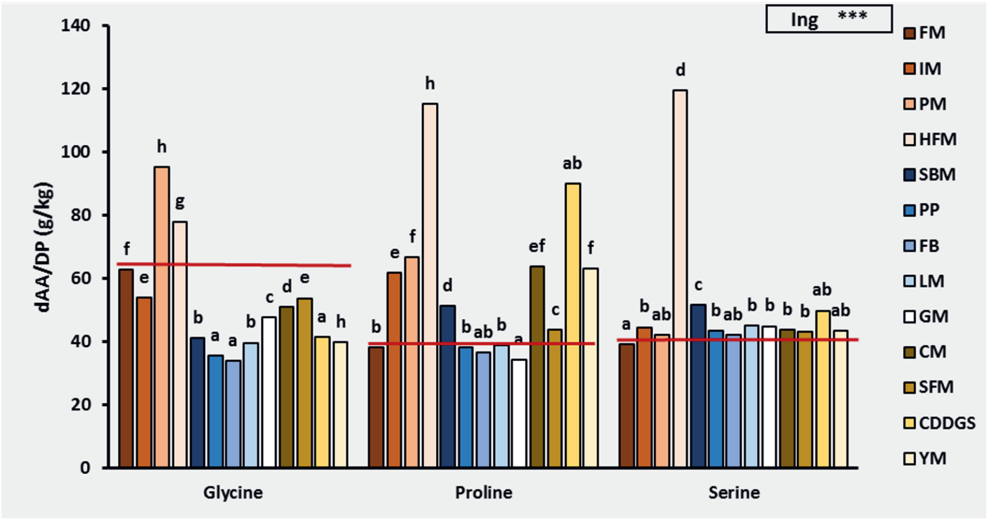
Supplemental table 2.3 Apparent digestibility coefficient (ADC) of amino acids in the experimental diets fed to African catfish

		Test Ingredients														*P*-value		
ADC (%)	**FL**	**CON**	**FM**	**IM**	**PM**	**HFM**	**SBM**	**PP**	**FB**	**LM**	**GM**	**CM**	**SFM**	**CDDGS**	**YM**	**Pooled SEM**	**D**	**FL**	**FL*D**
Essential																			
Arginine	R	95.2	95.7	95.5	94.2	94.5	96.1	96.1	95.0	96.7	97.3	95.3	96.2	94.9	93.8	0.27	***	***	ns
	S	93.8	95.5	95.2	94.1	94.4	96.0	95.0	94.3	96.1	96.9	94.5	95.5	94.0	92.8				
Histidine	R	93.3	93.9	92.7	91.5	91.7	94.5	94.2	92.4	94.0	95.3	93.2	94.5	92.0	90.9	0.37	***	***	ns
	S	92.1	93.9	91.7	91.1	91.2	94.1	92.7	91.6	92.9	94.7	92.4	92.8	91.4	90.1				
Isoleucine	R	91.5	92.6	92.2	89.9	91.6	93.0	92.3	90.8	92.6	93.5	90.9	92.8	90.3	89.6	0.43	***	***	ns
	S	89.6	91.9	91.9	89.2	90.8	93.1	90.2	90.2	91.2	92.8	90.2	91.4	89.2	88.1				
Leucine	R	93.1	94.1	93.1	91.5	92.4	93.7	93.7	92.6	93.9	94.5	92.5	93.4	92.2	91.6	0.38	***	***	ns
	S	90.9	93.1	92.8	90.7	91.6	93.5	91.7	91.7	92.5	93.9	91.7	91.7	91.1	89.8				
Lysine	R	94.6	95.3	95.1	93.4	93.0	95.2	95.7	94.0	95.1	96.1	94.3	95.0	93.5	92.6	0.28	***	ns	ns
	S	94.3	95.6	95.1	93.7	93.4	95.4	95.2	94.3	94.8	95.9	94.0	94.4	93.6	92.6				
Methionine	R	96.6	96.1	96.4	95.9	95.7	96.8	96.8	96.0	96.6	97.5	96.8	96.9	96.7	94.7	0.22	***	***	ns
	S	95.9	95.7	95.9	95.2	95.3	96.4	95.1	95.3	95.9	96.9	96.1	96.2	95.6	94.1				
Phenylalanine	R	93.4	93.4	94.2	91.5	92.9	94.2	93.5	92.1	93.3	94.2	92.6	93.9	92.2	92.2	0.35	***	***	ns
	S	91.7	92.6	93.9	90.7	92.4	94.1	91.5	91.0	91.9	93.8	91.3	92.5	90.9	90.9				
Threonine	R	89.6	92.1	91.3	89.3	88.9	91.0	91.4	89.7	91.4	92.6	89.7	91.2	88.8	86.0	0.48	***	***	ns
	S	88.0	91.5	90.9	89.1	88.6	91.5	89.6	89.3	90.6	92.1	89.2	89.6	88.3	85.1				
Valine	R	90.4	92.0	92.1	89.5	90.8	91.5	91.6	90.6	91.6	92.8	90.7	91.6	89.8	88.7	0.47	***	***	ns
	S	88.4	91.3	91.4	88.7	90.2	91.7	89.1	89.3	89.8	92.0	89.5	90.2	88.3	87.3				

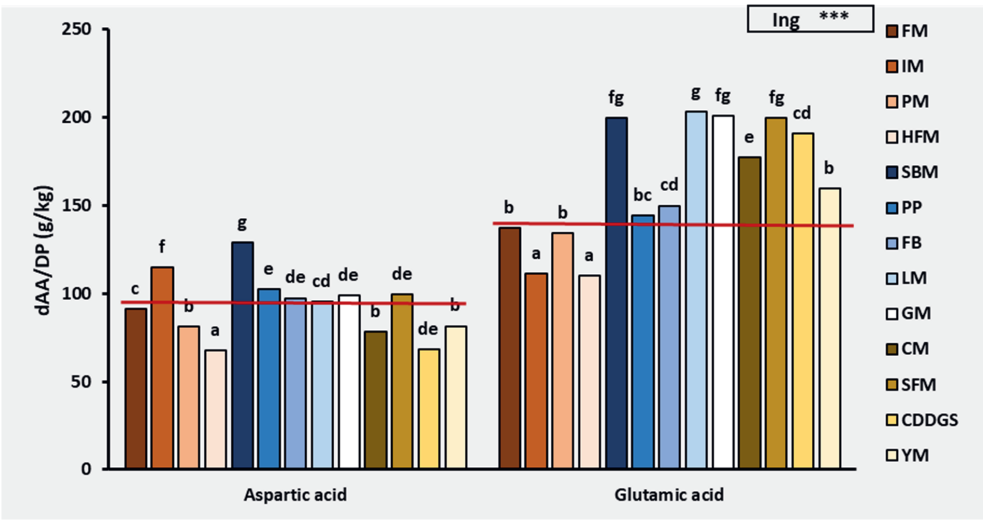
Supplemental table 2.3 (contd.). Apparent digestibility coefficient (ADC) of amino acids in the experimental diets fed to African catfish

		Test Ingredients															*P*-value		
ADC (%)	**FL**	**CON**	**FM**	**IM**	**PM**	**HFM**	**SBM**	**PP**	**FB**	**LM**	**GM**	**CM**	**SFM**	**CDDGS**	**YM**	**Pooled SEM**	**D**	**FL**	**FL*D**
Non-essential																			
Alanine	R	90.1	92.9	91.8	91.0	89.7	91.0	91.5	89.6	91.2	92.7	91.0	91.4	90.6	88.9	0.48	***	***	ns
	S	88.3	92.0	91.3	90.6	89.3	91.4	89.2	89.2	90.2	92.1	90.2	90.1	89.7	87.4				
Aspartic acid	R	91.2	91.6	92.4	88.8	90.2	94.2	93.7	91.8	93.2	94.5	92.3	93.6	90.8	88.4	0.42	***	#	ns
	S	90.5	92.3	92.7	90.5	90.5	94.3	92.9	92.0	92.8	94.4	92.4	92.4	90.9	87.9				
Glutamic acid	R	96.7	96.4	96.0	95.2	95.6	97.1	97.0	96.1	97.2	97.7	96.7	97.2	96.1	95.0	0.19	***	**	ns
	S	96.2	96.6	96.0	95.4	95.6	97.1	96.5	95.9	96.9	97.6	96.5	96.4	95.9	94.6				
Cysteine	R	88.2	87.5	86.5	83.6	88.1	91.0	86.3	86.1	89.7	91.9	89.2	90.8	87.5	82.9	0.64	***	**	ns
	S	86.0	88.0	86.1	83.8	87.8	90.3	83.7	85.6	88.3	91.9	89.4	88.1	87.3	82.1				
Glycine	R	90.2	92.5	89.7	91.2	90.9	90.6	91.1	89.4	91.6	93.2	90.7	91.4	89.5	88.1	0.47	***	*	ns
	S	88.7	92.4	89.5	92.0	91.0	91.0	89.5	89.3	90.9	92.9	90.5	90.2	89.3	86.9				
Proline	R	95.3	95.2	94.7	94.3	94.4	95.3	94.8	94.0	95.7	96.6	94.2	95.3	94.5	93.3	0.30	***	***	ns
	S	94.3	95.1	94.6	94.0	94.2	95.3	93.6	93.8	95.0	96.4	94.0	94.2	94.3	92.6				
Serine	R	92.6	93.3	92.7	91.0	93.0	93.8	93.4	92.7	93.7	94.9	92.3	93.7	92.1	88.1	0.37	***	***	ns
	S	91.0	93.0	92.2	91.1	92.8	93.7	91.8	91.9	92.8	94.4	91.9	92.0	91.5	87.6				
Tyrosine	R	84.6	84.7	97.1	92.3	91.4	93.4	95.7	94.5	95.0	94.7	94.5	92.6	93.4	86.6	1.96	***	*	#
	S	80.4	83.6	97.2	92.3	87.2	92.0	93.7	92.5	93.0	94.3	92.3	90.8	92.0	86.9				
SAA	R	93.5	94.0	93.7	92.2	92.8	94.5	94.4	93.1	94.5	95.5	93.6	94.4	92.9	91.3	0.34	***	***	ns
	S	92.4	93.8	93.5	92.2	92.5	94.5	93.0	92.7	93.8	95.1	93.0	93.3	92.4	90.5				

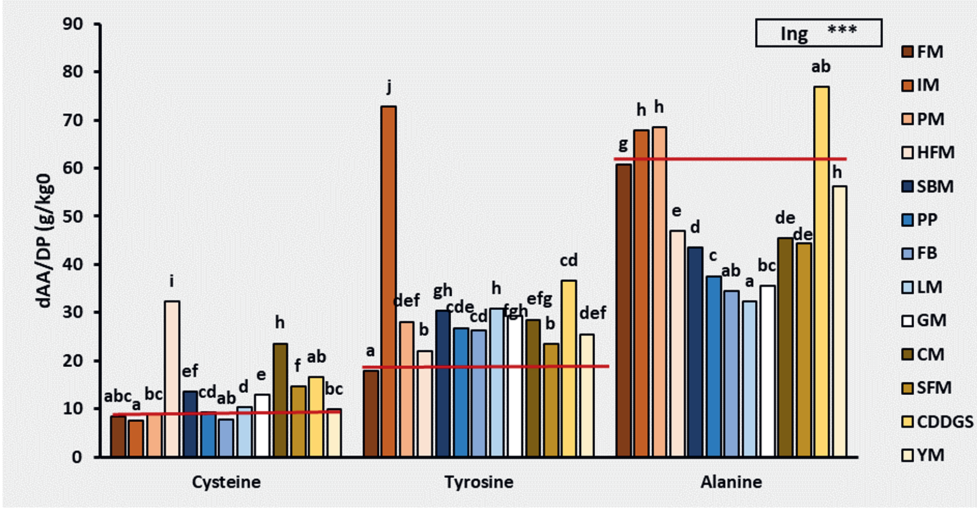
CON, control; FM, LT70 fish meal; IM, insect meal from black soldier fly larvae (*Hermetia illucens*); PM, poultry meal; HFM, hydrolysed feather meal; SBM, soybean meal; PP, pea protein; FB, faba beans; LM, lupine meal; GM, guar meal; CM, canola meal; SFM, sunflower meal; CDDGS, corn dried distillers grains with solubles; YM, yeast meal (*Saccharomyces cerevisiae*); SEM, standard error of mean; D, diet; FL, feeding level; DxFL, interaction between diet and feeding level; R, restricted feeding; S, satiation feeding; DM, dry matter; Carb, carbohydrate; NSP, non-starch polysaccharide level; NS, not significant P>0.1; *P<0.05; **P<0.01; ***P<0.001; # P<0.10. Values in the same row lacking common superscripts are statistically different (P<0.05) according to Tukeys' multiple comparison test.



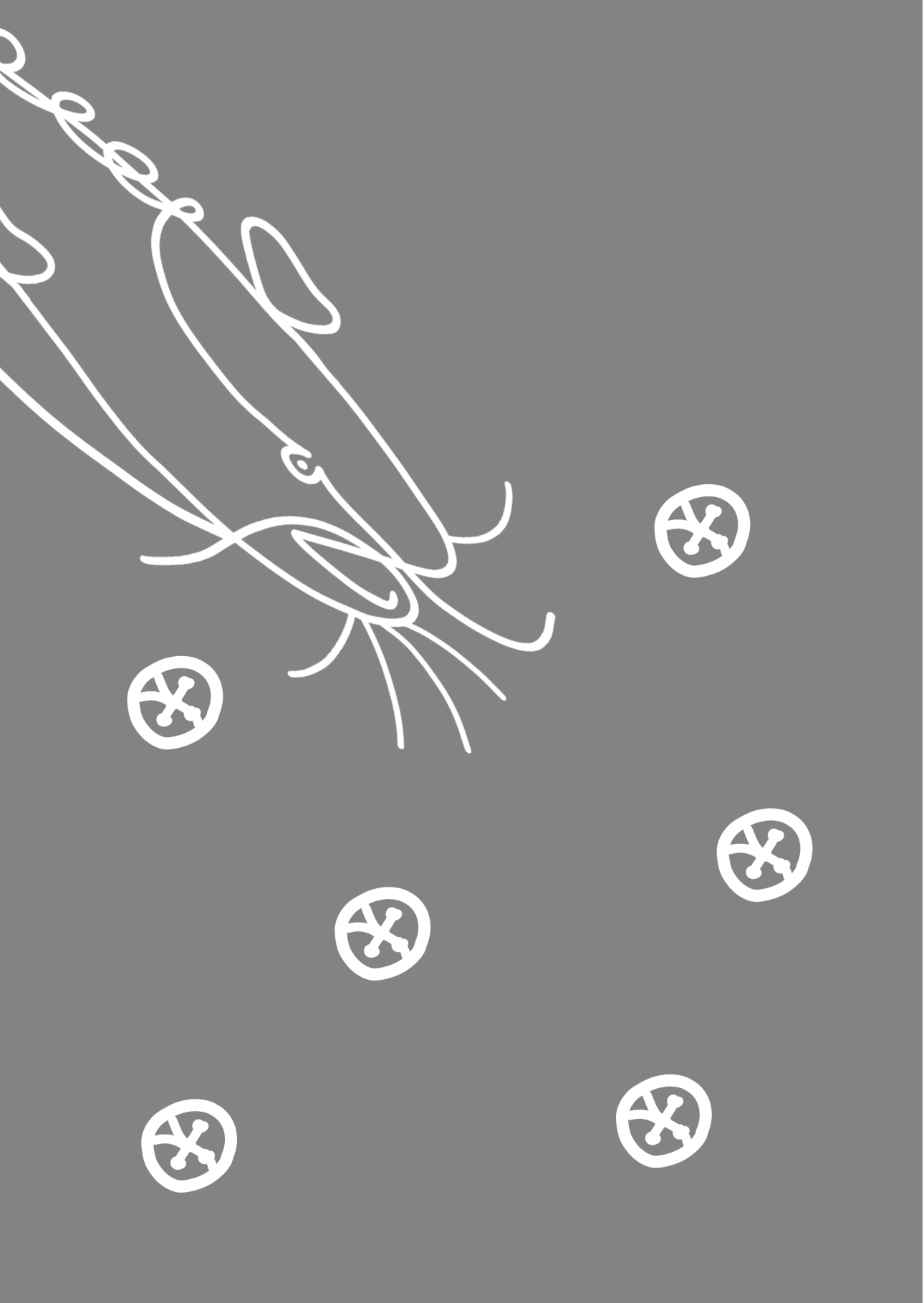
Supplemental figure 2.1 Digestible glycine, proline and serine expressed per digestible protein (dAA/DP) of various ingredients fed to African catfish. Red line showing fish meal dAA/DP compared to other ingredients. FM, LT70 fish meal; IM, insect meal from black soldier fly larvae (*Hermetia illucens*); PM, poultry meal; HFM, hydrolysed feather meal; SBM, soybean meal; PP, pea protein; FB, faba beans; LM, lupine meal; GM, guar meal; CM, canola meal; SFM, sunflower meal; CDDGS, corn dried distillers grains with solubles; YM, yeast meal (*Saccharomyces cerevisiae*).



Supplemental figure 2.2 Digestible aspartic acid and glutamic acid expressed per digestible protein (dAA/DP) of various ingredients fed to African catfish. Red line showing fish meal dAA/DP compared to other ingredients. FM, LT70 fish meal; IM, insect meal from black soldier fly larvae (*Hermetia illucens*); PM, poultry meal; HFM, hydrolysed feather meal; SBM, soybean meal; PP, pea protein; FB, faba beans; LM, lupine meal; GM, guar meal; CM, canola meal; SFM, sunflower meal; CDDGS, corn dried distillers grains with solubles; YM, yeast meal (*Saccharomyces cerevisiae*).



Supplemental figure 2.3 Digestible cysteine, tyrosine and alanine expressed per digestible protein (dAA/DP) of various ingredients fed to African catfish. Red line showing fish meal dAA/DP compared to other ingredients. FM, LT70 fish meal; IM, insect meal from black soldier fly larvae (*Hermetia illucens*); PM, poultry meal; HFM, hydrolysed feather meal; SBM, soybean meal; PP, pea protein; FB, faba beans; LM, lupine meal; GM, guar meal; CM, canola meal; SFM, sunflower meal; CDDGS, corn dried distillers grains with solubles; YM, yeast meal (*Saccharomyces cerevisiae*).



CHAPTER 3

Quantifying methionine requirement of juvenile African catfish (*Clarias gariepinus*)

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Abstract

This study was conducted to estimate the methionine (Met) requirement of African Catfish (*Clarias gariepinus*). A basal diet was formulated to contain 32% crude protein, 12% lipids, and 0.44% cysteine using only protein from legume ingredients as intact protein. This diet was supplemented with graded levels of crystalline DL- methionine (0, 0.12, 0.24, 0.36, 0.48, 0.60, and 0.84%), which resulted in seven dietary methionine levels ranging from 12.2 to 36.0 g/kg crude protein. Triplicate groups of 40 fish (78 g) were restrictively fed one of the seven diets for six weeks. Dietary methionine level significantly affected growth rate, feed conversion ratio, retained nitrogen, methionine efficiency and body composition. All parameters were fitted to dietary digestible methionine content expressed per unit of digestible protein (dMetDP) to estimate the Met requirement using; the linear plateau model (LP), broken line model (BL), or quadratic regression model (QR). LP and BL recorded similar values for requirement estimates while QR evidently recorded a 57% higher requirement estimates across different parameters. The digestible methionine requirement of African catfish for growth (using LP) ranges between 18.7 and 21.4 g/kg per unit of digestible protein. This equates to a minimum dietary methionine level of 6.3 g/kg diet (19.2 g/kg Crude protein), which is lower than was has been previously reported for this species.

3.1 Introduction

African catfish (*Clarias gariepinus*) is considered an excellent aquaculture species predominantly cultivated in Africa and some other countries in the world. African catfish is interesting for aquaculture due to its fast growth, resistance to diseases and wide geographical distribution (Fagbenro et al., 1999). The euryphagic nature of the species (Bruton, 1979) enables it to utilize different ingredients efficiently (Fagbenro, 1998). However, the dearth of nutritional knowledge specific to this species is one of the major problems mitigating against its successful culture. The economic viability of its culture is dependent on achieving the development of least-cost feeds, particularly using sustainable ingredients such as plant proteins.

Recent studies have pointed out that fish have no definite requirement for protein but rather a specific requirement for essential amino acids (EAA) (Miles and Chapman, 2007). This implies that, it is the essential amino acids in dietary protein that is most important for fish growth and development. However, the protein requirements (40-42%) of different life stages of African catfish has been previously reported (Uys, 1989), but the requirements for majority of the EAA are still unknown. Methionine is usually the first limiting amino acid in non-cereal plant products (Mai et al., 2006). Methionine is an indispensable sulphur amino acid that plays an essential functional role in initializing protein synthesis (Brosnan and Brosnan, 2006; Martinez et al., 2017; Wang et al., 2016). In addition, it participates in several metabolic processes including a precursor for S-adenosylmethionine (SAM) production, which serves as a principal methyl donor for ranges of molecules such as nucleic acids, choline, creatine and amines in vertebrates (Brosnan and Brosnan, 2006; Mato et al., 2002). Other methionine derivatives include cysteine, glutathione, taurine, sulphate, and some phospholipids (NRC, 2011). Methionine deprivation has been shown to cause growth reduction, decreased feed efficiency, reduced enzyme activities, intestinal development impairment, antioxidant degeneration and cataracts formation in various fish species (Espe et al., 2008; Harding et al., 1977; Jiang et al., 2017; Jiang et al., 2016; Poppi et al., 2017). To overcome this deficiency problem, crystalline methionine is usually supplemented in fish feed, based on the requirement of the species under culture.

The methionine requirements of commonly cultured fish species range from 13.0 to 45.3 g/kg crude protein (NRC, 2011; Zhou et al., 2011). For African catfish, Ovie and Eze (2010) and Fagbenro et al. (1999) reported a close range of dietary methionine requirement of 29.7 and 32.0 g/kg crude protein respectively. In these studies, highly digestible (e.g., fishmeal) and purified ingredients (e.g., casein and gelatine) supplemented with a large portion of synthetic (free) essential and non-essential amino acids were used for diet formulation, which may influence requirement outcome (Gorissen et al., 2016). There is debate about the validity of estimating amino acid requirement using purified ingredients with a large amount of synthetic amino acids to create the contrast in the amino acid being studied. For practical reasons, the reliance on these expensive ingredients as protein sources in aquafeeds are being reduced and replaced with plant proteins. Therefore, it is imperative to investigate the effects of crystalline methionine inclusion to practical diets that are mostly deficient in methionine (Salze et al., 2017). In this study, we fed African catfish a plant-based diet using only protein from legume ingredients, in which methionine is the first limiting amino acid. Crystalline methionine was supplemented to the diet in order to fill this knowledge gap on methionine requirements.

Generally, nutrient requirements are estimated using a dose-response approach, whereby mathematical models are fitted to a response variable (e.g., weight gain, nutrient deposition), against graded dietary levels of the nutrient (dose) under study (Salze et al., 2017). However, the selected

mathematical model and the mode of expression can largely influence the final requirement estimate (NRC, 2011; Zhou et al., 2011). Different authors have employed a wide range of models in requirement studies, but the unavailability of a concerted model has left a doubt in the outcome of various studies. As a result, it is easy to associate differences in values among the same species to the method of analysis (Baker, 1986; Salze et al., 2017; Shearer, 2000). In spite of the widespread use of different analytical models, the present work compared three models commonly used in fish nutrition: the linear plateau model (LP), the broken-line model (BL), and the quadratic regression model (QR). LP presumes that the response parameter (e.g., growth) increased linearly below the requirement and is constant above the requirement. However, for some nutrients, the response above the requirement might alter and still be dose dependent [i.e., when becoming toxic at high doses (Shearer, 2000)]. Beyond breakpoint, the response can either increase or decrease, which can be clearly shown by the BL model. The QR is also used in fish requirement studies, which take into account the curvilinear decrease in performance caused by the imbalance effects of toxicity of the studied nutrient after the requirement is attained. Here we present the estimated methionine (in the presence of cysteine) requirement of African catfish and explained the impact of using different analytical models on requirement estimates.

3.2 Materials and methods

3.2.1 Ethics statement and research facility

The study (project number 2018.W.0014.001) was carried out in accordance with the Dutch law on the use of animals (Act on Animal Experiments) for scientific purposes and was approved by the Central Animal Experiments Committee (CCD) of The Netherlands. This experiment was conducted in the research facility of CARUS-ARF at Wageningen University, The Netherlands. Fish were kept and handled in agreement with EU-legislation.

3.2.2 Fish and housing conditions

Mixed sex of juvenile African catfish (*Clarias gariepinus*) fingerlings were obtained from a commercial brood stock farm (Fleuren & Nooijen BV, Nederweert, The Netherlands). Upon arrival, fish were fed a commercial diet and adapted over 2 weeks to the experimental conditions. At the start of the experiment, 840 fish weighing on average 78 g were randomly assigned (40 fish per tank) into 21 experimental tanks. All tanks were connected to a common recirculation aquaculture system (RAS system). A trickling filter, sump and drum filter (Hydrotech 500®, Hydrotech Engineering, Italy) were connected to the RAS to help maintain the same water quality for all tanks. The water volume of the RAS system was 5 m^3 and water loss due to evaporation was continuously compensated with the addition of well water. Water refreshment was based on NO_3 removal from the system to keep NO_3 levels within limits (< 500 mg/L). Each tank was equipped with air stones and swirl separators (AquaOptima AS, column height 44 cm; diameter 24.5 cm) for the collection of faeces and spilled pellet. Water quality parameters were monitored regularly and set at optimal levels for African catfish. Average (SD) measured values over the experimental period were as follows: water temperature 27.6 ± 0.11 °C; pH, 7.2 ± 0.26; ammonium, 0.27 ± 0.14 mg/L; nitrite, 0.21 ± 0.13 mg/L; nitrate, 494 ± 16.2 mg/L; conductivity, 3109 ± 415 µS; and dissolved oxygen concentration, 6.90 ± 0.409 mg/L. Photoperiod was kept at 12 h light: 12 h dark.

3.2.3 Diet preparation

The ingredient composition and proximate analysis of the seven experimental diets are given in Table 3.1 & 3.2. These experimental diets were formulated to be identical regarding ingredient composition and thus macro-nutrient content except for the amount of crystalline DL-methionine supplementation and cellulose. The basal diet (Diet A), without methionine supplementation was formulated to have as low as possible methionine content using commonly used plant protein ingredients. Protein originated solely from soy protein concentrate and faba beans and resulted in a methionine content of 12.2 g/kg crude protein (CP). Between the experimental diets, cellulose was exchanged by crystalline DL-methionine in a dose-response manner: 0, 0.12, 0.24, 0.36, 0.48, 0.60, and 0.84%, respectively. This resulted in an analyzed methionine content ranging between 12.2 and 36.0 g/kg CP. This range was chosen based on the expected methionine requirement being around 25 g/kg CP, of other species summarized by NRC (2011). The diets were formulated to meet the nutrient requirements of African catfish (NRC, 2011), except for dietary methionine. As cysteine synthesis is dependent on the level of methionine in the diet and interferes with the methionine requirement, the cysteine levels were kept constant in all the seven diets. The analyzed dietary cysteine content was 0.44% (4.4 g/kg; Table 3.2). Yttrium oxide was added as a marker for the determination of apparent nutrient digestibility (ADC). The extruded floating pellets with sizes ranging from 3 to 3.5 mm, were produced by Skretting ARC Norway using a twin-screw extruder (Wenger, Sabetha, KS, U.S.A). Diets were sealed and stored at 4°C throughout the experimental period.

Table 3.1 Ingredient composition of the basal diet (Diet A) without methionine supplementation.

Basal Ingredients	%
Faba beans	15.00
Soy protein concentrate	35.76
Wheat	20.00
Wheat starch	15.00
Cellulose[1]	0.84
Fish oil	2.00
Rapeseed oil	8.00
Moisture loss[2]	0.50
Calcium carbonate	0.50
Monocalcium phosphate	2.50
Mineral premix	0.10
Vitamin premix	0.10
Yttrium oxide	0.10
DL-Methionine supplementation[1]	0.00
L-Lysine	0.40
L-Threonine	0.20

[1]The ingredient composition of the other 6 experimental diets (Diet B to G) was identical to the basal diet except for the content of cellulose and DL-methionine. In Diet B, C, D, E, F and G, respectively, 0.12, 0.24, 0.36, 0.48, 0.60, and 0.84% cellulose was replaced by crystalline DL-methionine.

[2]The pellet production was targeted at a dry matter content of 93%, which resulted in an expected water loss during extrusion of 0.5%.

3.2.4 Feeding and Sampling

At the start of the experiment, 20 fish were selected at random and euthanized by an overdose of phenoxy-ethanol (1.0 mL/L) for initial body composition analysis. During stocking of the tanks, total biomass and number of fish per tank were recorded while being sedated (0.25 mL/L phenoxy-ethanol). Each diet was randomly assigned to the experimental tanks in triplicate. During the 42-day experimental period, fish were fed restrictively in order to provide the same amount of CP to all fish across all diets. This is to minimize the variation in response parameters due to variability in feed intake. Feeding level was fixed at 19.8 g/kg$^{0.8}$/d (about 90% of satiation) based on the mean initial weight over all diets. Daily feed ration per tank was increased based on an expected growth using a FCR of 1 for all diets, again to ensure that the feeding levels per fish were equal at all diets. In the case of mortality, the daily feeding rations was adjusted for the number of fish in the tank. Daily feed portions were hand-fed twice a day at 8:00 h and 16:00 h. During the first three days, feeding level was gradually increased from 20% to 100% of the intended ration. After each meal, the uneaten feed was weighted and the spilled pellets counted per tank, which were collected by the swirl separators 15min after feeding was finished. For proximate analysis of the feed, a representative sample from each diet was taken and stored at 4 °C weekly.

Faeces were collected overnight (17.00h – 7.30h) for digestibility studies from week 2 onwards (Monday - Friday), using detachable collection bottles (250 mL) connected to settling tanks. The faecal collection bottles were submerged in ice-filled styrofoam box, to reduce microbial degradation. Faeces were pooled every week and per tank using aluminum trays, then stored at -20 °C for further analysis. Faeces from weeks 2, 4 and 6 were pooled per tank for analysis. Throughout the experiment, fish behavior was monitored, and fish were visually inspected for discernible signs of cataract and deformity that may arise from methionine deficiency. Mortality was checked twice a day, 30 minutes prior to feeding. At the end of the experiment, fish were batch weighed per tank for final weight under mild sedation and 10 fish per tank were randomly selected and killed by an overdose of phenoxy-ethanol (1.0 mL/L) for final body composition analysis.

3.2.5 Chemical analyses on feed, faeces and fish body composition

Analyses were performed on the diets, whole fish samples, and faeces samples. Before chemical analysis, frozen fish samples were sawed into small pieces, and homogenized by mincing twice through a 4.5 mm-screen grinder (Gastromaschinen, GmbH model TW-R 70; Feuma). A portion of the minced fish were freshly sampled for CP analysis. Minced fish samples for AA, crude fat and energy determination were freeze-dried before further analysis. Faecal samples were freeze-dried, then manually pulverized through a 1 mm screen sieve. Feed pellets were grinded by a grinding machine. Fish, faeces, and feed samples were analyzed in triplicate, using the same analytical method. DM content was determined by drying the samples to constant weight at 103 °C for at least 4 h (ISO 6496, 1983). Ash content by incineration in a muffle furnace at 550 °C overnight (ISO 5984, 1978). The Kjeldahl method was used for CP analysis (ISO 5983, 1979). Crude fat analysis was determined using the Soxhlet method (ISO 6492, 1999). Energy was measured using adiabatic bomb-calorimeter (C7000 IKA®, IKA analysentechnik, Weitershem, Germany; ISO 9831, 1998). Yttrium, phosphorus, calcium and magnesium in feed and faeces were determined from the ash by using inducted coupled plasma mass spectrometry according to the standard NEN 15510 (ICP-MS, 2007). Amino acids (excluding tryptophan) were analyzed by Skretting ARC, Norway, using an automatic amino acid analyzer

(Biochrom 30+, Biochrom Ltd, Cambridge, UK) and the methods described in the COMMISSION REGULATION (EC) No 152/2009 (Council, 2009).

3.2.6 Calculation

Daily weight gain (g/d) was calculated as the differences between average initial (W_i) and final (W_f) body weight of fish divided by the duration of the experiment (t). Feed conversion ratio (FCR; g/g) on dry matter (DM) basis was calculated as (feed intake × dry matter content of the feed)/(final weight of fish – initial weight of fish). Feed intake (FI; % BW/d) was calculated as feed intake divided by mean body weight x (100%). Specific growth rate (SGR; % BW/day) was calculated as (LnWf − LnWi × 100)/t, where t is the duration of the experiment in days (d). Fish survival (%) was calculated as number of fish at the beginning of the experiment divided by the number fish at the end of the experiment x 100.

Table 3.2 Analysed proximate composition of experimental diets (on dry matter basis)

	Diet code						
	Diet A	**Diet B**	**Diet C**	**Diet D**	**Diet E**	**Diet F**	**Diet G**
Methionine level (g/kg)	**4.0**	**5.4**	**6.3**	**8.0**	**9.2**	**10.3**	**12.0**
EAA (g/kg)							
Arginine	23.2	23.2	23.7	23.2	23.1	23.3	22.9
Histidine	8.8	8.6	8.8	8.6	8.7	8.6	8.5
Isoleucine	14.2	14.2	14.4	14.5	14.3	14.5	14.1
Leucine	24.2	24.3	24.3	24.3	24.2	24.4	23.9
Lysine	22.0	22.0	21.9	21.9	21.5	22.1	21.8
Methionine	4.0	5.4	6.3	8.0	9.2	10.3	12.0
Phenylalanine	16.3	16.1	16.2	16.3	15.6	15.8	15.1
Threonine	13.9	13.9	14.0	13.8	13.7	13.8	13.5
Valine	14.4	14.4	14.5	14.7	14.3	14.5	14.3
NEAA (g/kg)							
Alanine	13.7	13.7	13.8	13.8	13.6	13.6	13.5
Aspartic acid	35.6	35.7	35.5	35.2	35.2	35.3	34.8
Glutamic acid	60.1	59.9	60.6	59.3	59.9	60.9	59.1
Cysteine	4.4	4.4	4.4	4.4	4.4	4.5	4.3
Glycine	13.4	13.5	13.5	13.4	13.3	13.5	13.2
Proline	16.6	16.5	16.4	16.8	16.7	17.2	16.3
Serine	15.8	16.0	16.1	16.1	15.8	16.1	15.6
Tyrosine	9.7	9.9	9.8	9.7	9.9	10.1	9.4
Sum of AA	310	312	314	314	313	319	312
Nutrients							
Dry matter (g/kg)	932	930	933	929	932	929	932
Crude protein (g/kg)	326	333	331	332	335	337	332
Crude fat (g/kg)	129	128	131	131	132	129	132
Ash (g/kg)	61.1	61.5	60.0	61.5	60.3	60.1	60.7
Energy (kJ/g)	21.1	21.2	21.2	21.2	21.1	21.3	21.1

EAA: Essential amino acid

NEAA: Non-essential amino acid

SAA is without tryptophan since tryptophan was not analyzed, Amino acid composition was determined by the Skretting (ARC) laboratory Norway.

The apparent digestibility coefficient (ADC) of AA and macronutrients were calculated according to the following formula described by Cheng and Hardy (2002) using Yttrium oxide as inert marker; ADC (%) = 100 × [1 − (Yttrium concentration in the feed × concentration nutrient in faeces)/(Yttrium concentration in the faeces × concentration nutrient in feed)]. The concentrations of Yttrium and nutrients were expressed on DM basis.

Methionine (Met), nitrogen (N) and energy balance parameters were calculated per treatment and expressed as; mg/kg/d, mg/kg/d and kJ/kg/d, respectively (summarized in supplementary Table 3.1). Detailed description of calculation of balance parameters have been previously described by Saravanan et al. (2012). Met and protein efficiency ratio was calculated as the amount of nutrients retained as percentage of the digestible nutrient intake.

3.2.7 Statistical analysis

All measured parameters were tested for the effect of diet using one-way ANOVA. If significant ($p < 0.05$), means were compared by Tukey's honest significant difference (HSD, using IBM SPSS Statistics, version 23.0 for Windows (IBM Corp., Armonk, NY, USA). Thereafter, all parameters were subjected to regression analysis, to test for the linear and quadratic effect of dietary level of digestible methionine content expressed per unit of digestible protein ($dMet_{DP}$; X-variable). Estimation of methionine requirements was done by three different regression models: Linear plateau (LP), broken-line regression (BL) and quadratic regression (QR). These regression analyses were done for different response parameters (weight gain, retained nitrogen, etc.) against $dMet_{DP}$ (X-variable). Estimation of methionine requirements by LP and BL models were done by the procedure NLIN of SAS. All statistical analysis were performed using the Statistical Analysis System (SAS) statistical software package version 9.2 (SAS institute, Cary, NC, USA).

3.3 Results

The mean values of each experimental diet and the one-way ANOVA analysis of all parameters are presented in Tables 3.3-3.6. In supplementary tables 3.2 and 3.3, the regression analysis of all parameters in relation to the dietary digestible methionine (Met) content expressed per unit of digestible protein ($dMet_{DP}$) are presented. When being significant, the linear or quadratic relationship is given. At visual inspection, no cataracts or any other pathological sign were observed in fish fed Met-deficient diet in this study. Met supplementation did not affect the survival of African catfish, as survival rate averaged at 96% over treatments (Table 3.3; $P>0.05$).

Fish were fed restrictively, the same ration, which resulted in minimal differences in feed intake among diets. Despite the equal feed intake, final body weight (BW) and growth increased with increasing Met supplementation, and were curvilinearly related to $dMet_{DP}$ (Figure 3.1I; $P<0.01$). Consequently, FCR reduced with $dMet_{DP}$ (Table 3.3 and Figure 3.1C).

In this study, the impact of $dMet_{DP}$ on efficiency parameters were quantified. Growth and efficiency data paralleled each other. Feed conversion ratio (FCR), Met efficiency and protein efficiency ratio (PER) were all quadratically related to $dMet_{DP}$ ($P<0.001$). Fish fed the diet without DL- Met supplementation had the highest FCR (0.99), which differ significantly from all other treatments (Table 3.3). Relatable effect of low dietary Met was seen in the PER values as fish fed the Met-deficient diet recorded the lowest PER. In contrast, Met efficiency was reduced with increasing $dMet_{DP}$.

Table 3.3 Growth performance and feed utilization of African catfish fed the experimental diets

	Diet code								
	Diet A	**Diet B**	**Diet C**	**Diet D**	**Diet E**	**Diet F**	**Diet G**		
Methionine level (g/kg)	**4.0**	**5.4**	**6.3**	**8.0**	**9.2**	**10.3**	**12.0**	**SEM**	***P*-value**
Experimental period (d)	42	42	42	42	42	42	42		
Tanks (n)	3	3	3	3	3	3	3		
Fish per tank (n)	40	40	40	40	40	40	40		
Survival (%)	95.8	98.3	95.8	97.5	97.5	94.2	94.2	2.02	ns
Initial BW (g)	78.8	78.4	78.2	77.9	77.6	77.2	77.4	0.52	ns
Final BW (g)	196^{a}	207^{ab}	211^{b}	211^{b}	210^{b}	210^{b}	208^{b}	2.45	**
Feed Intake (g/d)	2.77	2.77	2.77	2.77	2.77	2.77	2.77	-	ns
Growth (g/d)	2.80^{a}	3.06^{ab}	3.15^{b}	3.17^{b}	3.16^{b}	3.16^{b}	3.12^{b}	0.06	**
SGR (%/d)	2.18^{a}	2.31^{ab}	2.36^{b}	2.37^{b}	2.37^{b}	2.38^{b}	2.36^{b}	0.03	**
FCR (g/g)	0.99^{b}	0.91^{a}	0.88^{a}	0.87^{a}	0.88^{a}	0.88^{a}	0.89^{a}	0.02	**

SGR, specific growth rate; FCR, feed conversion ratio; BW, body weight; SEM, standard error of means; ns, not significant P<0.1; * P<0.05; ** P<0.01; ***, P<0.001; values in the row with different superscripts are significantly different (P<0.05) according to Tukeys' multiple comparison test.

Digestibility of macro nutrients was not influenced by the dietary treatments (One-way ANOVA; P>0.10, table 3.4). However, almost all AA ADC were affected by $dMet_{DP}$. This was seen in the result of Met ADC, which increased with increasing Met supplementation (Figure 3.2). Except for Met ADC, no consistent pattern (dose-response) was present for AA ADC values between diets (Table 3.4 and Supplementary figure 3.1).

Regarding body composition, both protein and fat content were linearly affected by $dMet_{DP}$ (P<0.001; Table 3.5 and Supplementary table 3.2). Protein content in African catfish was low at low $dMet_{DP}$ and high at high $dMet_{DP}$. Body Met content paralleled the pattern of body protein content and increased linearly with increasing $dMet_{DP}$ (P<0.001). In contrast, body fat content declined with increasing $dMet_{DP}$, which resulted to leaner African catfish at high $dMet_{DP}$.

Energy retention (ER), nitrogen retention and Met retention were measured as alternative growth indices (Table 3.6). The nitrogen retention showed a similar response as growth. It increased with $dMet_{DP}$ at low levels and the increase in response levelled off at higher levels of $dMet_{DP}$ (Figure 3.1F; quadratically P<0.001). In contrast, ER and Met retention were unaffected by $dMet_{DP}$ (P>0.1; Table 3.6).

Table 3.7 highlights the digestible Met requirements of African catfish, which was estimated by fitting three different analytical models to the dose-response relationship: linear plateau (LP), broken line (BL) and quadratic regression (QR). Dietary digestible Met content expressed per unit of digestible protein ($dMet_{DP}$) was used as independent variable, while different outcome parameters as shown in table 3.7 were applied as dependent variables. Figure 3.1 shows the relationship between $dMet_{DP}$ and FCR (panel A, B and C), retained nitrogen (panel D, E and F) and growth (panel G, H and I) respectively, and the respective estimated methionine requirements.

In Table 3.7, the estimated methionine requirement of African catfish based on selected parameters using different analytical models (linear plateau, LP; Broken line, BL; Quadratic regression QR) is given. Irrespective of the dependent variables, the LP and BL models resulted in similar values obtained for

Table 3.4 Apparent digestibility coefficient (ADC) of AA and nutrients in African catfish fed the experimental diets

	Diet code								
	Diet A	**Diet B**	**Diet C**	**Diet D**	**Diet E**	**Diet F**	**Diet G**		
Methionine level (g/kg)	**4.0**	**5.4**	**6.3**	**8.0**	**9.2**	**10.3**	**12.0**	**SEM**	***P*-value**
EAA (%)									
Arginine	95.0^{ab}	95.0^{ab}	95.5^{b}	94.9^{ab}	95.2^{ab}	95.7^{b}	94.2^{a}	0.37	*
Histidine	92.1^{ab}	92.4^{ab}	92.9^{b}	92.0^{ab}	92.3^{ab}	93.2^{b}	90.9^{a}	0.56	*
Isoleucine	90.5^{ab}	90.6^{ab}	91.4^{ab}	90.5^{ab}	90.2^{ab}	91.8^{b}	89.4^{a}	0.60	*
Leucine	90.8^{ab}	91.2^{ab}	91.8^{ab}	90.8^{ab}	90.9^{ab}	92.2^{b}	90.0^{a}	0.54	*
Lysine	93.9^{ab}	94.0^{ab}	94.4^{ab}	93.6^{ab}	93.7^{ab}	94.6^{b}	92.9^{a}	0.49	#
Methionine	89.9^{a}	92.6^{b}	94.0^{bc}	94.7^{cd}	95.3^{cde}	96.4^{e}	96.1^{de}	0.50	***
Phenylalanine	91.1^{ab}	91.6^{b}	92.2^{b}	91.3^{ab}	91.0^{ab}	92.2^{b}	89.7^{a}	0.55	**
Threonine	90.1^{ab}	90.3^{ab}	90.9^{b}	89.8^{ab}	89.7^{ab}	91.4^{b}	88.6^{a}	0.61	*
Valine	88.6^{ab}	89.0^{ab}	89.7^{ab}	88.7^{ab}	88.4^{ab}	90.1^{b}	87.4^{a}	0.69	*
NEAA (%)									
Alanine	87.6^{ab}	87.7^{ab}	88.4^{ab}	87.4^{ab}	87.1^{ab}	88.9^{b}	85.8^{a}	0.87	#
Aspartic acid	93.2^{ab}	93.3^{ab}	93.8^{b}	92.9^{ab}	93.0^{ab}	94.0^{b}	92.0^{a}	0.49	*
Glutamic acid	95.6^{ab}	95.8^{ab}	96.1^{b}	95.5^{ab}	95.7^{ab}	96.2^{b}	94.9^{a}	0.31	*
Cysteine	86.9^{ab}	87.5^{ab}	88.3^{b}	87.0^{ab}	87.0^{ab}	89.0^{b}	85.6^{a}	0.76	*
Glycine	87.4^{ab}	87.6^{ab}	88.5^{b}	87.2^{ab}	87.3^{ab}	89.0^{b}	85.5^{a}	0.88	*
Proline	92.1^{ab}	92.3^{ab}	92.8^{b}	92.3^{ab}	92.2^{ab}	93.5^{b}	90.9^{a}	0.46	**
Serine	91.4^{ab}	91.7^{ab}	92.3^{b}	91.5^{ab}	91.4^{ab}	92.7^{b}	90.3^{a}	0.53	**
Tyrosine	92.9^{ab}	93.2^{ab}	93.6^{ab}	92.5^{ab}	93.2^{ab}	93.8^{b}	91.5^{a}	0.66	#
Sum of AA	92.3^{ab}	92.5^{ab}	93.0^{ab}	92.2^{ab}	92.3^{ab}	93.4^{b}	91.4^{a}	0.50	*
Nutrients (%)									
Dry matter	74.1	73.45	75.71	73.54	76.35	74.11	72.33	1.14	ns
Protein	87.69	88.04	88.37	88.04	89.11	87.98	87.42	0.53	ns
Fat	93.93	94.19	94.77	94.39	95.13	94.08	94.01	0.29	ns
Ash	39.53	39.10	41.56	37.69	38.71	37.82	31.72	4.93	ns
Phosphorus	60.40	59.87	60.96	59.68	61.26	60.15	56.94	2.03	ns
Energy kJ/g	79.68	79.40	80.89	79.63	82.15	80.20	78.48	0.84	ns

AA, amino acid; DM, dry matter; SGR, specific growth rate; FCR, feed conversion ratio; BW, body weight; SEM, standard error of means; NS, not significant $P<0.1$; * $P<0.05$; ** $P<0.01$; ***, $P<0.001$; values in the row with different superscripts are significantly different ($P<0.05$) according to Tukeys' multiple comparison test.

the Met requirement for African catfish. On the other hand, QR model estimated consistently higher values for all parameters. Only parameters such as Met ADC and Met efficiency showed disparity in their outcome, with high values recorded when all three models were applied. Growth parameters displayed similar values for optimal Met requirement, averaging at a $dMet_{DP}$ content of 19.7 g/kg, when LP model was applied. Quadratic regression analysis indicated a $dMet_{DP}$ requirement of 30.8 g/kg for SGR, 29.2 g/kg for daily growth, and 31.3 g/kg for retained nitrogen. As for the efficiency indices, Met efficiency depicted larger variation compared to other parameters. Similarly, ADC Met projected high values for $dMet_{DP}$ breakpoints: LP, 31.0 g/kg; BL, 31.9 g/kg; and QR, 39.0 g/kg. In contrast, protein efficiency followed a similar pattern as growth, which plateaued at a $dMet_{DP}$ value of 19.4 g/kg, for Met requirement. Based on the R^2 values of the three models and the model, which depicted the least

SSE, the BL model was shown to estimate the dose-response relationship more accurately, but the difference in R^2 was marginal compared to the LP model. In contrast to the LP model, the outcomes of the NLIN procedure of SAS were not fully stable for the BL model. Small differences in the starting values of the NLIN procedure affected the estimated breakpoint by BL for growth and retained nitrogen. Therefore, LP seemed to be the most appropriate model due to its stability. Using this LP model, the estimated digestible methionine requirement for optimal growth in African catfish is a $dMet_{DP}$ value ranging between 18.7 and 21.4 g/kg.

Table 3.5 Whole body composition (g/kg) of African catfish fed with the experiemntal diets

		Diet code								
		Diet A	Diet B	Diet C	Diet D	Diet E	Diet F	Diet G		
Methionine level (g/kg)	**Initial**	**4.0**	**5.4**	**6.3**	**8.0**	**9.2**	**10.3**	**12.0**	**SEM**	***P*-value**
EAA (g/kg dry matter)										
Arginine	37.8	30.5^{a}	33.2^{b}	34.2^{b}	32.8^{b}	33.6^{b}	33.8^{b}	33.7^{b}	0.40	***
Histidine	16.0	11.9^{a}	13.6^{b}	14.2^{bc}	14.1^{bc}	14.3^{bc}	14.4^{c}	14.4^{c}	0.16	***
Isoleucine	24.8	19.3^{a}	21.2^{b}	22.0^{b}	21.4^{b}	21.8^{b}	22.0^{b}	22.0^{b}	0.25	***
Leucine	43.4	33.6^{a}	37.0^{b}	38.4^{b}	37.3^{b}	38.0^{b}	38.5^{b}	38.4^{b}	0.44	***
Lysine	47.6	37.9^{a}	41.8^{b}	44.0^{b}	42.6^{b}	43.3^{b}	44.1^{b}	44.1^{b}	0.53	***
Methionine	15.2	11.8^{a}	13.2^{b}	13.6^{b}	13.4^{b}	13.7^{b}	13.7^{b}	13.7^{b}	0.15	***
Phenylalanine	24.9	20.2^{a}	22.0^{ab}	22.7^{b}	21.8^{ab}	22.5^{b}	22.6^{b}	22.7^{b}	0.39	**
Threonine	25.8	20.2^{a}	22.1^{b}	22.7^{b}	22.2^{b}	22.5^{b}	22.7^{b}	22.6^{b}	0.26	***
Valine	27.0	21.5^{a}	23.5^{b}	24.5^{b}	23.7^{b}	24.2^{b}	24.3^{b}	24.4^{b}	0.31	***
NEAA (g/kg dry matter)										
Alanine	38.8	31.9^{a}	34.7^{b}	35.5^{b}	33.8^{ab}	34.7^{b}	34.7^{b}	34.4^{b}	0.44	**
Aspartic acid	57.8	45.4^{a}	50.0^{b}	52.2^{b}	50.4^{b}	51.4^{b}	52.0^{b}	51.4^{b}	0.71	***
Glutamic acid	84.1	66.2^{a}	73.1^{b}	76.3^{b}	73.4^{b}	75.0^{b}	75.6^{b}	74.7^{b}	0.92	***
Cysteine	5.39	4.25^{a}	4.60^{ab}	4.69^{b}	4.53^{ab}	4.68^{b}	4.74^{b}	4.75^{b}	0.08	**
Glycine	49.1	43.5	46.5	46.5	43.4	44.7	44.3	44.0	0.93	ns
Proline	30.6	26.0	27.8	28.0	26.6	27.2	26.9	26.9	0.56	ns
Serine	24.8	19.7^{a}	21.4^{b}	21.8^{b}	21.1^{b}	21.5^{b}	21.7^{b}	21.7^{b}	0.26	***
Tyrosine	15.1	11.8^{a}	12.8^{ab}	13.6^{b}	12.6^{ab}	13.1^{ab}	13.2^{ab}	13.4^{b}	0.30	**
Sum of AA	568	456^{a}	499^{b}	515^{b}	495^{b}	506^{b}	509^{b}	507^{b}	5.2	***
Proximate composition (g/kg dry matter)										
Dry matter	243	293	282	285	287	290	287	287	3.0	ns
Crude protein	645	501^{a}	547^{b}	546^{b}	553^{b}	557^{b}	551^{b}	560^{b}	5.9	***
Crude fat	236	387^{b}	353^{a}	351^{a}	350^{a}	345^{a}	353^{a}	347^{a}	6.7	***
Ash	121	105	99.0	99.1	100	102	100	105	3.1	ns
Phosphorus	17.0	18.6	15.7	15.8	17.7	17.3	17.2	18.2	1.0	ns
Energy (kJ/kg DM)	24.9	28.1^{b}	27.1^{ab}	27.0^{ab}	26.9^{ab}	26.8^{a}	27.0^{ab}	26.9^{ab}	0.3	*

Amino acid composition was determined by the Skretting (ARC) laboratory Norway. EAA, essential amino acid; NEAA, non-essential amino acid; SAA, sum of amino acid (SAA is without tryptophan, no tryptophan was detected after acid hydrolysis); DM, dry matter; SEM, standard error of means; NS, not significant $P<0.1$; * $P<0.05$; ** $P<0.01$; ***, $P<0.001$; values in the row with different superscripts are significantly different ($P<0.05$) according to Tukeys' multiple comparison test.

Table 3.6 Effect of methionine inclusion on nitrogen and energy balances of African catfish

	Diet codes								
	Diet A	**Diet B**	**Diet C**	**Diet D**	**Diet E**	**Diet F**	**Diet G**		
Methionine level (g/kg)	**4.0**	**5.4**	**6.3**	**8.0**	**9.2**	**10.3**	**12.0**	**SEM**	***P*-value**
Methionine balance (mg/d)									
Gross methionine intake	11.06^{a}	14.96^{b}	17.60^{c}	22.28^{d}	25.68^{e}	28.51^{f}	33.25^{g}	0.01	***
Digestible methionine intake	9.95^{a}	13.86^{b}	16.54^{c}	21.10^{d}	24.48^{e}	27.49^{f}	31.95^{g}	0.07	***
Retained methionine	10.79	12.02	12.60	11.64	13.08	11.77	12.37	0.61	ns
Methionine efficiency	108.47^{d}	86.73^{c}	76.19^{bc}	55.19^{ab}	53.44^{a}	42.81^{a}	38.71^{a}	4.43	***
Nitrogen balance (mg/d)									
Gross nitrogen intake	144.7^{a}	147.9^{c}	146.8^{b}	148.3^{d}	148.8^{e}	149.5^{f}	147.8^{c}	0.05	***
Digestible nitrogen intake	126.9^{a}	130.2^{ab}	129.7^{ab}	130.5^{ab}	132.6^{b}	131.5^{b}	129.2^{ab}	0.80	**
Branchial urinary losses	63.9^{b}	55.2^{ab}	51.4^{a}	49.3^{a}	49.6^{a}	50.9^{a}	47.9^{a}	1.62	***
Retained nitrogen	63.0^{a}	75.0^{b}	78.4^{bc}	81.3^{bc}	83.0^{c}	80.7^{bc}	81.3^{bc}	1.86	***
Protein efficiency	49.6^{a}	57.6^{b}	60.4^{b}	62.3^{b}	62.6^{b}	61.3^{b}	63.0^{b}	1.33	***
Energy balance (KJ/d)									
Energy intake	58.60^{a}	58.80^{b}	58.80^{b}	58.96^{c}	58.57^{a}	59.10^{d}	58.67^{a}	0.02	***
Digestible energy intake	46.69	46.69	47.57	46.95	48.12	47.40	46.05	0.50	ns
Branchial urinary losses	1.59^{b}	1.37^{ab}	1.28^{b}	1.23^{b}	1.24^{b}	1.27^{b}	1.19^{b}	0.05	***
Metabolizable energy intake	45.10	45.32	46.29	45.72	46.88	46.13	44.85	0.48	#
Heat production	17.85	18.97	18.89	18.10	19.24	18.53	17.67	1.04	ns
Retained energy	27.25	26.34	27.40	27.63	27.65	27.60	27.19	0.72	ns
Retained energy as protein	9.33^{a}	11.11^{b}	11.61^{bc}	12.03^{bc}	12.29^{c}	11.95^{bc}	12.05^{bc}	0.24	***
Retained energy as fat	17.92	15.23	15.79	15.59	15.36	15.65	15.14	0.66	ns
Maintenance energy requirement (kJ/kg$^{-0.8}$/day)	41.94	40.67	37.56	31.75	36.66	34.42	29.83	6.23	ns

SEM, standard error of means; NS, not significant $P<0.1$; * $P<0.05$; ** $P<0.01$; ***, $P<0.001$; values in the row with different superscripts are significantly different ($P<0.05$) according to Tukeys' multiple comparison test.

Table 3.7 Estimated methionine requirement of African catfish on selected parameters using different analytical models (linear plateau, LP; Broken line, BL; Quadratic regression QR).

Traits	Breakpoint LP (SE)	Breakpoint BL (SE)	Maximum QR
SGR (%/d)	19.1 (1.46)	19.0 (1.66)	30.8
Growth (g/d)	18.7 (1.35)	19.0 (1.57)	29.2
Retained nitrogen (mg/d)	21.4 (0.91)	18.7 (1.06)	31.3
FCR (g/g)	18.5 (1.16)	18.8 (1.35)	29.7
Methionine efficiency (mg/d)	31.7 (1.55)	24.6 (2.57)	40.9
Protein efficiency (%)	19.4 (1.19)	18.8 (1.26)	32.0
ADC methionine (%)	31.0 (1.7)	31. 9 (2.03)	39.0
Branchial urinary losses (mg/d)	19.8 (1.59)	19.0 (1.66)	33.3

SGR, specific growth rate; FCR, feed conversion ratio; ADC, apparent digestibility coefficient; SE, standard error of the estimated breakpoint is given between brackets. The full relationships for the models; LP and BL are given in Supplementary table 3.5 and for the QR model in Supplementary table 3.2 and 3.3.

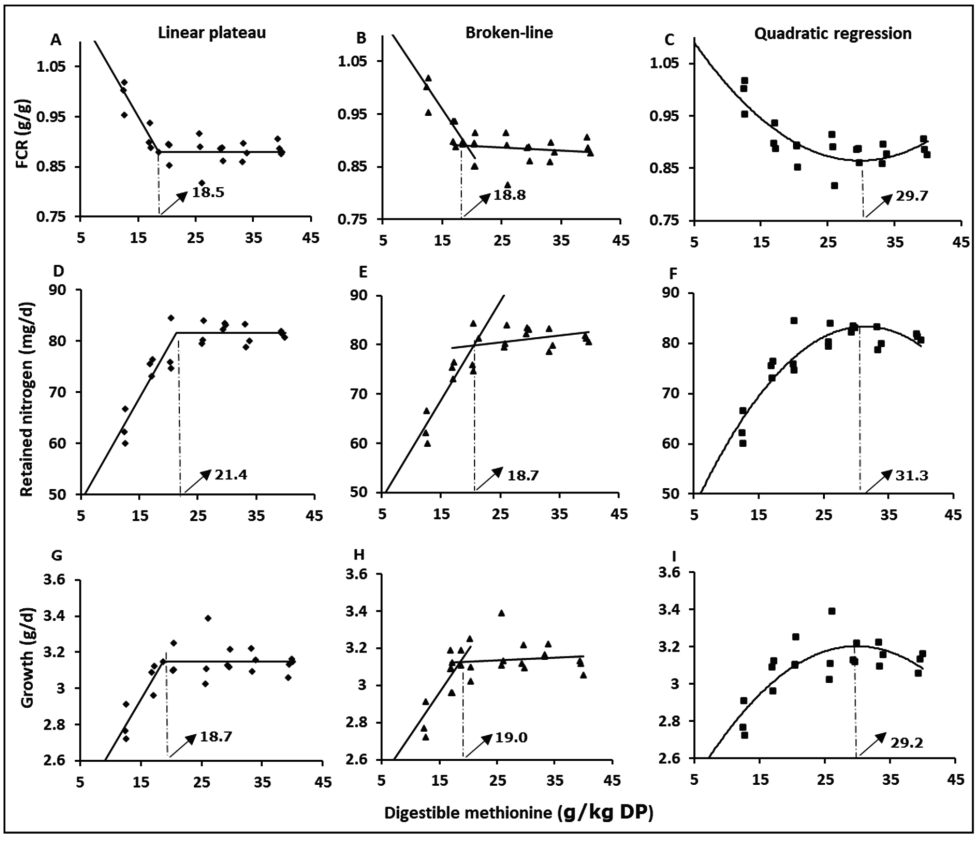

Figure 3.1 The relationship between dietary digestible methionine content (expressed in g/kg digestible protein [DP]) and FCR (panel a, b and c); retained nitrogen (d, e and f); growth (g, h and I), in African catfish. Three models for estimating the optimal dietary digestible methionine content were compared: linear plateau model (panel a, d and g); broken line model (panel b, e and h); and quadratic regression (panel c, f and i). The estimated equations and R^2 for the linear plateau and broken line model are provided in supplementary table 3.5 and for quadratic regression in Table 3.3 and 3.4.

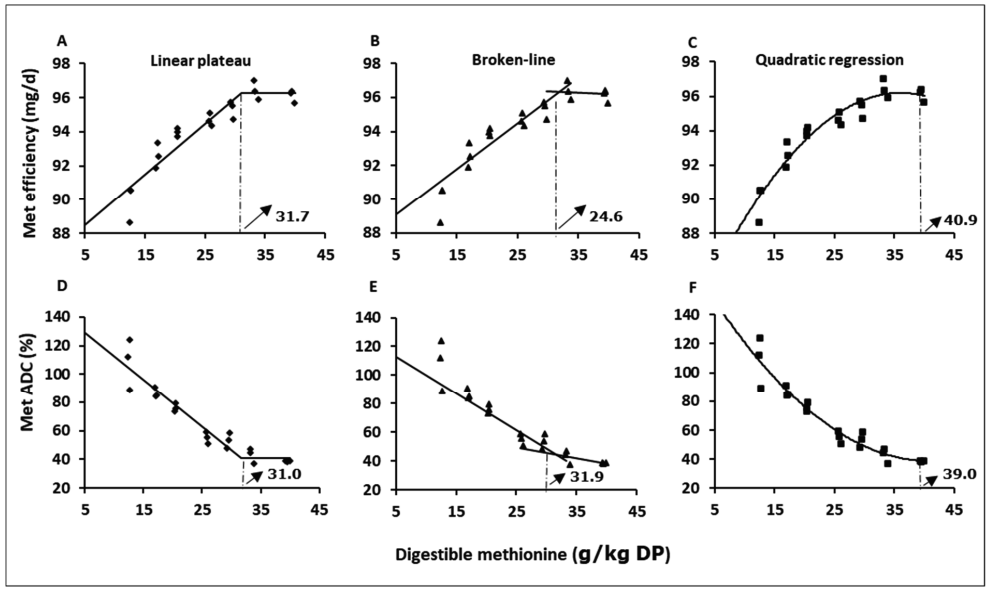

Figure 3.2 The relationship between dietary digestible methionine content (expressed in g/kg digestible protein [DP]) and methionine (Met) efficiency (panel a. b and c) and methionine (Met) digestibility (d, e and f), in African catfish. Three models for estimating the optimal dietary digestible methionine content were compared: linear plateau model (panel a and d); broken line model (panel b and e); and quadratic regression (panel c and f). The estimated equations and R^2 for the linear plateau and broken line model are provided in supplementary table 3.5 and for quadratic regression in supplementary table 3.2 and 3.3.

3.4 Discussion

In this study, methionine (Met) supplementation resulted in improved growth and better feed utilization of juvenile African catfish. The results suggest that Met was the first limiting amino acid in the basal diet used in the current study (a leguminous plant-based diet). This reflected in the poor feed efficiency, and reduced growth reported for fish fed the low-Met diet. However, African catfish were able to utilize crystalline Met, as supplementation improved growth performance. The general observation that low dietary Met level can limit growth performance has been demonstrated in various studies (Fagbenro et al., 1999; Liang et al., 2016; Yan et al., 2007; Zhou et al., 2011). Reduced growth in fish fed Met-deficient diets may be attributed to: reduced feed intake; reduced protein deposition (i.e., N-retention); and increased fat deposition (i.e., enhanced deamination of non- Met AA). All these factors contribute to an altered FCR, PER and protein utilization efficiency.

In the current study, the decline in performance was not related to feed intake (FI) because fish were fed restrictively. In literature, the majority of requirement studies often apply satiation feeding strategy. This may lead to variation in nutrient intake, as loss of appetite has been reported in species fed Met-deficient diets under satiation feeding (Alam et al., 2000; Elmada et al., 2016; Wu et al., 2017). Methionine is known to play a crucial role in the modification of the gene and endocrine pathways responsible for appetite regulation (Fontagné-Dicharry et al., 2017; Sourabié et al., 2018). Restricted feeding prevents differences in FI between treatments, therefore, variations in growth rate will only result from differences in metabolic efficiency (Cowey, 1994). Moreover, the total consumption of feed

supplied will improve the use of available nutrients, thereby increase feed efficiency (Coloso et al., 1999). Our results suggest that the major reason for the hampered performance in the low-Met group was the reduction in N retention (protein deposition) coinciding with a reduced protein efficiency. However, methionine efficiency was higher in fish fed the Met-deficient diets. This high efficiency (slightly higher but not different from 100%) is an indication that Met was the first limiting AA. Consequently, the available Met was completely utilized. This is because the first limiting AA in a diet often has the highest marginal efficiency leading to the complete utilization of available AA rather than being catabolised (Gerrits et al., 1998).

As expected, body protein contents (on DM basis) increased with increasing Met level and lowest values were recorded for fish fed the Met-deficient diet (Table 3.5). High body protein contents in the Met-balance and Met-excess group is an indication of increased nitrogen retention due to the supply of diets with balanced AA. Nwanna (2016) and Ovie and Eze (2010) reported that DL-methionine inclusion in the diet resulted in a significant increase of African catfish carcass protein, which improved carcass quality. The body fat content showed an opposite trend to the body protein content as it decreased with increasing Met supplementation. This inverse response of body protein and fat to Met deficiency have been reported in some studies (Powell et al., 2017). Since protein synthesis and accretion depend on a balanced AA supply (De la Higuera et al., 1998), a deficiency in one essential amino acid (EAA) will disrupt this process. As a result, shortage of Met (e.g., in Met-deficient diet) at the synthesis sites will cause the remaining AA to be catabolized and or used as energy source for fat synthesis. This explains the higher fat content of fish fed the Met-deficient diet. Our results show a positive contribution of Met inclusion on overall protein efficiency (PER). Improved PER in response to Met supplementation has been observed in other fish species (Alam et al., 2000; Luo et al., 2005; Ruchimat et al., 1997; Wu et al., 2017; Yan et al., 2007). The retained nitrogen supports the growth trend reported in this study, suggesting increased protein synthesis due to Met inclusion (Elmada et al., 2016).

In the current study, the dietary digestible Met requirement expressed per digestible protein ($dMet_{DP}$) for the growth of African catfish (*Clarias gariepinus*) was 18.7 g/kg (based on the linear plateau model). This is equivalent to a dietary Met content of 6.3 g/kg diet and 19.2 g/kg crude protein (CP) (Supplementary table 3.4). These estimates fall in the range of dietary Met values (4.9 – 7.1 g/kg diet) reported for different fish in literature, with no clear pattern across species (Espe et al., 2008; Furuya et al., 2001a; Harding et al., 1977; Ren et al., 2017). Only few studies reported slightly lower dietary Met values ranging between 4.9 and 5.3 g/kg diet (Jackson and Capper, 1982; Kim et al., 1992; Nguyen and Davis, 2009). Quite a number of identified factors such as experimental design, method of analysis and the choice of statistical models, may largely influence the requirement estimate (Figueiredo-Silva et al., 2015; Shearer, 2000). Furthermore, cysteine and methionine make up the total sulphur amino acid (TSAA) of fish (Ahmed et al., 2003; Wilson, 1986; Zhou et al., 2011). Cysteine inclusion in the diet has been shown to spare up to 40–60% Met in meeting the requirements for TSAA (NRC, 2011). This will replace Met in the synthesis of cysteine and its derivatives (Brosnan and Brosnan, 2006; Fagbenro et al., 1999). High requirement for Met reported for some species could be attributed to low inclusion level of cysteine in the diet. In the current study, cysteine was supplied at a constant level (4.3 – 4.5 g/kg diet) in the diet.

The reported Met requirement value in the current study, though slightly lower, has a clear consistent pattern with the whole-body Met composition (24.4 g/kg CP) of African catfish (Table 3.5). In general,

most essential AA have an estimated requirement by dose-response study that is lower than the body composition of the respective AA (NRC, 2011). Previously, amino acid requirements estimates were based on the amino acid profiles of the fish and whole-body protein (NRC, 2011; Wilson, 1986). This is still used when limited information is available for a new species.

In this study, 3 models; quadratic regression (QR), linear plateau (LP) and broken-line (BL), were applied to estimate the Met requirement of African catfish. Estimated requirements for $dMet_{DP}$ were similar for the LP and BL models whereas estimates for the QR, averaged over all parameters were 57% higher (Table 3.7). There is an ongoing debate in the field of fish nutrition as to which model is most appropriate for nutrient requirements estimation. Some authors argued that fish response to nutrient dosage is curvilinear (Figueiredo-Silva et al., 2015), whereby they often opt for quadratic regression (QR) model as method of analysis (Elmada et al., 2016; Zhou et al., 2006) (Supplementary table 3.4). I.e., they believe that animals gradually (smoothly) transition from one state (deficiency) to another (balanced diet). On the contrary, others favour a sharp transition and therefore apply models with a distinct/sharp inflection point by using e.g., BL or LP analysis (Chi et al., 2020; Harding et al., 1977; Nguyen and Davis, 2009). A major downside of QR for estimating requirement is the fact that the estimated value is dependent upon the width of the dosing (x-variable) applied in the study. When we applied a stepwise reduction in the range (width) of Met doses in our study by excluding first diet G (with the highest dose), then diet F and G and finally E, F and G, this strongly reduced the estimated $dMet_{DP}$ requirement from 29.2 to 23.6 g/kg DP (see Figure 3.3). The estimated requirement for growth by BL analysis altered the estimation only from 19 to 18.4 g/kg DP (data not shown), whereas LP remained nearly unchanged. This implies that using values below or above required nutrient needed for maximum response would have greater impact on predictions near maximal responses when QR is used (Pesti et al., 2009). Given this arbitrary sensitivity for the width of the applied dosing, favours us to choose LP or BL for Met requirement estimation.

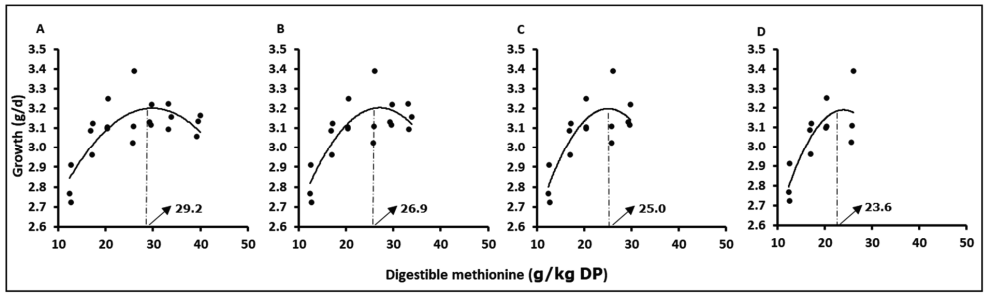

Figure 3.3. Methionine requirement of African catfish in response to varying nutrient dose range, using quadratic regression A) All diets ($y = -0.0012x^2 + 0.07x + 2.1578$ $R^2 = 0.6161$) , B) all diets excluding diet G, ($y = -0.0018x^2 + 0.0968x + 1.894$ $R^2 = 0.6641$) C) all diets excluding diet F and G, ($y = -0.0025x^2 + 0.1248x + 1.6383$ $R^2 = 0.6875$) D) all diets excluding diets E, F and G ($y = -0.003x^2 + 0.1416x + 1.4942$ $R^2 = 0.6813$).

As previously mentioned, LP and BP gave almost identical estimates in the current study. Regarding most criteria used, the slope of the regression above the breakpoint in BL analysis was never significantly different from zero (data not shown). This suggests that under the current experimental condition, the Met level (I.e., highest level; Diet G) did not affect the performance parameters. In literature, methionine has been reported to be toxic in excessive dosage (Choo et al., 1991; Murthy and Varghese, 1998). This toxicity is induced by the over accumulation of S-Adenosylmethionine SAM in the liver, a product formed in the Met metabolism pathway (Ahmed, 2014). In addition, excessive

methionine intake has been reported to negatively affect FI in some fish species (NRC, 2011). At this toxic level, protein deposition is reduced and a fall in growth slope is often observed. Using LP model in such case will only display the linear component of the dose-response variables until a plateau is reached (Hermesch et al., 1998). However, it does not enable the means to deviate after the inflection point, which is generally not the case. Hypothetically, departure from linearity may occur, whereby the slope descends at higher dose concentration above the requirement. BL allows an ascending or descending slope and show the clear fall in response to toxicity that may arise from excess nutrient dosage (Gonçalves et al., 2016; Shearer, 2000). This model depicts clearly the theoretical ideas of the pattern of nutritional responses exhibited by animals compared to LP (Pesti et al., 2009). However, for BL, instability of the estimated breakpoint was observed when the input values for growth and retained nitrogen of the NLIN procedure was alternated in this current study, whereas LP remained stable.

Comparing the X variables used differs among studies. In most studies (Supplementary table 3.4), Met requirement are often expressed as g/kg diet or g/kg CP. AA requirements expressed as g/kg diet have the disadvantage that these requirements change if the applied dietary CP content alters. As an example, tilapia diets for pond culture where the natural food web contributes to the fish diet, have a lower optimal dietary protein content than tilapia diets for cage culture without a food web (Kabir et al., 2019). Applying the Met requirement of 19.2 g/kg CP found in the current study would imply an optimal dietary Met content of 5.8 and 7.7 g/kg feed for a diet having respectively a CP content of 300 and 400 g/kg feed. In the current study, we expressed Met requirement as dietary digestible methionine content in g/kg digestible protein (dMet g/kg DP) in order to make the estimated requirement independent upon differences in protein and AA digestibility. Although, AA are formed from protein hydrolysis, individual AA digestibility can differ from the overall protein digestibility (unpublished data). Since different diets are fed in different requirement studies, expressing estimates on digestible basis will reduce variability and ensure precise comparison of values among species (NRC, 2011). Moreover, estimating methionine requirement based on digestible methionine has been previously suggested (Figueiredo-Silva et al., 2015; NRC, 2011; Ren et al., 2017). Furthermore, evidences suggest that protein deposition may be a more robust and rational criterion for response (y) variables, compared to weight gain, FCR and SGR that are commonly used (NRC, 2011). Different parameters (Table 3.7) tested in this study, gave dMet estimates that fall within the same range (18.7 – 21.4 g/kg DP). This response is in line with literature as e.g., Zhou et al. (2011) observed only slight differences in the Met requirement for both SGR and protein productive value (PPV) in black sea bream (*Sparus macrocephalus*). Similar observations were made in blunt snout bream (*Megalobrama amblycephala*) (Liang et al., 2016) and Indian major carp (*Cirrhinus mrigala*) (Ahmed et al., 2003).

It is worthy to note that the existing Met requirement estimates; 32.0 g/kg CP (Fagbenro et al., 1999) and 29.7 g/kg CP (Ovie and Eze, 2010), for African catfish in literature are higher than what we found in the current study (19.2 g/kg CP). This may be due to a number of factors; firstly, both studies did not take the cysteine level into account. Secondly, variability in analytical models applied, e.g., Ovie and Eze (2010) employed QR for data analysis (Supplementary table 3.4). Thirdly, choice of ingredients used in diet formulation. For example, Fagbenro et al. (1999), used casein and gelatine as intact protein, which are considered as highly digestible ingredients. Using such purified products in requirement studies may influence requirement values compared to when practical diets are used (Nguyen and Davis, 2009). For practical reasons, faba beans and soy protein concentrate were solely used as intact protein in our study. Lastly, the initial body weights of fish used in these studies greatly differ. it has been reported that the intestinal transporters capacity and whole-body activity of

enzymes for AA catabolism vary with developmental stage (Segner and Verreth, 1995). Although, Fagbenro et al. (1999) used broken-line as the method of analysis, the differences in estimates may also arise from differing experimental duration, and genetic variation in African catfish used (Figueiredo-Silva et al., 2015; Shearer, 1995; 2000).

In conclusion, the low-methionine plant-based diet (not supplemented with crystalline methionine) used in the present study resulted in methionine deficiency, indicated by poor feed efficiency and reduced growth of fish. However, crystalline methionine supplementation (0.12% to 0.84%) alleviated this deficiency problem. Based on linear plateau model, the digestible methionine requirement of juvenile African catfish (80-210 g) for growth ranges between 18.7 and 21.4 g/kg expressed per unit of digestible protein ($dMet_{DP}$), depending on the response criteria. This equates to a minimum dietary methionine level of 6.3 g/kg diet (19.2 g/kg crude protein) in the presence of 4.4 g/kg cysteine. Furthermore, the current study demonstrated that quadratic regression can lead to an overestimation of nutrient requirements.

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Supplemental tables

Supplemental table 3.1 Nutrient balances calculations

Parameters	Formula
Methionine (Met) balance (mg/d)	
Gross Met intake (GMI)	$GMI = Feed\ intake\ x\ Met\ content\ of\ feed$
Digestible Met intake (DMI)	$DMI = (GMI\ x\ ADCmet)/100$
Retained Met (RM)	$RM = Wf\ x\ Mf - Wi\ x\ Mi$
Met efficiency (%)	$Met\ efficiency\ (\%) = RM/DMI\ x\ 100$
Nitrogen balance (mg/d)	
Gross nitrogen intake (GNI)	$GNI = Feed\ Intake\ x\ Nitrogen\ content\ of\ feed$
Digestible nitrogen intake (DNI)	$DNI = (GNI\ x\ ADCcp)/100$
Branchial and urinary loses (BUN)	$BUN = DNI - RN$
Retained nitrogen (RN)	$RN = ((Wf\ x\ CPf)/\ 6.25) - ((Wi\ x\ CPi)/6.25)$
Protein efficiency (PER; %)	$PER =\ RN/DNI\ x\ 100$
Energy balance (KJ/d)	
Gross energy intake (GEI)	$GEI = FI\ x\ EF$
Digestible energy intake (DEI)	$DEI = (GE\ x\ ADCe)/100$
Metabolizable energy intake (MEI)	$\mathrm{MEI}\ =\ \mathrm{DE} - \mathrm{BUE}$
Branchial and urinary energy losses (BUE)	$BUE =\ (BUN\ \times\ 24.9)\ /\ 1000$
Retained energy (RE)	$RE\ =\ Wf\ \times\ Ef - Wi\ \times\ Ei$
Heat production (HP)	$HP =\ RE\ -\ ME$
Retained energy as protein (REpro)	$REpro =\ (RN\ x\ 6.25)\ x\ 23.7$
Retained energy as fat (REfat)	$REfat\ =\ RE\ -\ REpro.$

ADCmet (%), apparent digestibility coefficient of methionine in the feed; Wi, initial weight in gram per fish; Wf, final weight in gram per fish; Mi, initial methionine content of the fish; Mf, final methionine content of the fish; ADCcp (%), apparent digestibility coefficient of crude protein; CPf and CPi, respectively final and initial crude protein content of the fish (g/kg); DMfeed, dry matter content of feed (g/g); Nfeed, nitrogen content of feed (mg/g); Ef, energy content of feed; ADCe (%), apparent digestibility coefficient of the energy; Ef, energy content of the fish at the end of the experiment; Ei, energy content of the fish at the beginning of the experiment; 23.7 kj/g, energy content of protein; 24.9 kj N g^{-1}, energy concentration of NH_3-N as calculated by Bureau et al. (2003), assuming all N was excreted as NH_3-N.

Supplementary table 3.2 Relationship between dietary digestible methionine content (X-variable, expressed in g/kg digestible protein, $dMet_{DP}$) with different measured parameters (performance and fish body composition traits being Y-variable) in African catfish.

Variables	N	Mean	Range	SD	Equation (X = $dMet_{DP}$)	R^2	Effect
Initial BW (g)	21	78	77-80	0.92	-	-	-
Final BW (g)	21	208	193-220	6.1	$Y = 171\ (8.1) + 2.8\ (0.67)\ X - 0.048\ (0.013)\ X^2$	57.1	Q^{**}
Survival (%)	21	96	90-100	3.3	-	-	ns
Feed Intake (g/d)	21	2.8	2.8-2.8	0.00	-	-	ns
FCR (g/g)	21	0.90	0.82-1.02	0.046	$Y = 1.19\ (0.057) - 0.022\ (0.0047)\ X + 0.00037\ (0.00009)\ X^2$	64.3	Q^{***}
Growth (g/d)	21	3.1	2.7-3.4	0.15	$Y = 2.2\ (0.19) + 0.07\ (0.016)\ X - 0.0012\ (0.0003)\ X^2$	61.6	Q^{**}
SGR (%/d)	21	2.33	2.12-2.48	0.082	$Y = 1.83\ (0.099) + 0.037\ (0.0082)\ X - 0.0006\ (0.0002)\ X^2$	65.4	Q^{**}
Analyzed body composition							
Dry matter (g/kg DM)	21	288	277-295	5.4	-	-	ns
Methionine (g/kg DM)	21	13.3	12.0-14.3	0.68	$Y = 11.9\ (0.32) + 0.055\ (0.012)\ X$	52.9	L^{***}
Crude protein (g/kg DM)	21	545	498-578	20.9	$Y = 505\ (10.5) + 1.6\ (0.39)\ X$	46.2	L^{***}
Fat (g/kg DM)	21	355	328-400	16.8	$Y = 381\ (9.6) - 1.0\ (0.36)\ X$	30.2	L^{**}
Ash (g/kg DM)	21	101	93-112	5.0	-	-	ns
Phosphorus (g/kg DM)	21	17.2	12.0-19.4	1.8	-	-	ns
Energy (kJ/g)	21	27.1	26.4-29.8	0.58	$Y = 30.1\ (0.88) - 0.22\ (0.073)\ X + 0.0037\ (0.0014)\ X^2$	46.5	Q^{*}

$dMet_{DP}$, dietary digestible methionine content per unit of digestible protein; CP, crude protein; DM, dry matter; SGR, specific growth rate; FCR, feed conversion ratio; BW, body weight; SD, standard deviation; L, linear effect; Q, quadratic effect; ns, not significant $P<0.1$; * $P<0.05$; ** $P<0.01$; ***, $P<0.001$.

Supplementary table 3.3 Relationship between dietary digestible methionine content (X-variable, expressed in g/kg digestible protein; $dMet_{DP}$) with different measured parameters (methionine, nitrogen and energy balances, traits being Y-variable) in African catfish.

Variables	N	Mean	Range	SD	Equation (X = $dMet_{DP}$)	R^2	Effect
Methionine balance (mg/d)							
Gross methionine intake	21	21.9	11.0-33.3	7.5	Y = 0.87 (0.19) + 0.83 (0.0069) X	99.9	L^{***}
Digestible methionine intake	21	20.7	9.8-32.1	7.4	Y = -0.15 (0.23) + 0.82 (0.0083) X	99.8	L^{***}
Retained methionine	21	12.0	8.9-14.3	1.1	-	-	ns
Methionine efficiency	21	66	37-124	25.0	Y = 183 (13.6) – 7.03 (1.13) X + 0.086 (0.021) X^2	93.0	Q^{***}
Nitrogen balance (mg/d)							
Gross nitrogen intake	21	148	145-150	1.5	Y = 138 (1.33) + 0.70 (0.11) X – 0.011 (0.0021) X^2	80.5	Q^{***}
Digestible nitrogen intake	21	130	127-134	2.07	Y = 117 (2.75) + 1.05 (0.23) X – 0.018 (0.0044) X^2	57.8	Q^{***}
Branchial and urinary nitrogen losses	21	52.5	45.0-66.8	5.9	Y = 64.7 (2.76) – 0.47 (0.10) X	53.0	L^{***}
Retained nitrogen	21	77.5	60.0-84.5	7.0	Y = 32.4 (5.91) + 3.25 (0.49) X – 0.052 (0.009) X^2	82.9	Q^{***}
Protein efficiency (%)	21	59.5	47.3-65.2	4.9	Y = 30.5 (4.85) + 2.05 (0.4) X – 0.032 (0.0077) X^2	76.6	Q^{***}
Energy balance (kJ/d)							
Gross energy intake	21	58.8	58.5-59.1	0.18	Y = 58.0 (0.33) + 0.062 (0.0028) X – 0.0011 (0.00053) X^2	23.2	Q^{*}
Digestible energy intake	21	47.1	45.3-48.8	0.97	Y = 43.0 (1.69) + 0.36 (0.14) X – 0.007 (0.0027) X^2	27.9	Q^{*}
Metabolizable energy intake	21	45.8	44.2-47.6	0.97	Y = 40.9 (1.61) + 0.41 (0.13) X – 0.0078 (0.0026) X^2	34.7	Q^{**}
Heat production	21	18.5	14.7-20.7	1.6	-	-	ns
Retained energy	21	27.3	25.3-29.9	1.1	-	-	ns
Retained energy as protein	21	11.5	8.9-12.5	1.03	Y = 4.8 (0.87) + 0.48 (0.073) X – 0.0077 (0.0014) X^2	82.9	Q^{***}
Retained energy as fat	21	15.8	13.3-18.4	1.3	Y = 17 (0.8) – 0.06 (0.03) X	18.8	L^{*}

$dMet_{DP}$, dietary digestible methionine content per unit of digestible protein; SD, standard deviation; L, linear effect; Q, quadratic effect; ns, not significant *P*<0.1; * *P*<0.05; ** *P*<0.01; ***, *P*<0.001.

Supplementary table 3.4 Comparison of different analytical models used in estimating methionine requirements for growth of some selected commonly cultured species.

		Estimated methionine requirement				
Species	**Cysteine level (g/kg)**	**g/kg diet**	**g/kg CP**	**dMet g/kg DP**	**Analytical model**	**Reference**
African catfish (*Clarias gariepinus*)	4.4	6.3	19.2	18.7	Linear plateau	This study
	4.4	6.3	19.2	19.0	Broken-line regression	
	4.4	9.2	27.6	29.2	Quadratic regression	
Channel catfish (*Ictalurus punctatus*)	4	9.4	34.1	-	Broken-line regression	(Cai and Burtle, 1996)
	ND	6.0	23.0	-	Linear regression	(Harding et al., 1977)
African catfish (*Clarias gariepinus*)	ND	12.6	32.0	-	Break point analysis	(Fagbenro et al., 1999)
	ND	12.1	29.7	-	Second-order polynomial regression	(Ovie and Eze, 2010)
Nile Tilapia (*Oreochromis niloticus*)	TSSA	11.0	21.0	-	polynomial regression	(Furuya et al., 2001a)
	4.5	4.9	17.5	-	Broken-line regression	(Nguyen and Davis, 2009)
Indian major carp (*Labeo rohita (Hamilton)*)	1.4	11.5	28.8	-	Break-point regression	(Murthy and Varghese, 1998
Indian major carp (*Cirrhinus mrigala (Hamilton)*)	10.0	12.0	30.0	-	Second-degree polynomial regression	(Ahmed et al., 2003)
Rainbow trout (*Oncorhynchus mykiss*)	5.0	5.2	15.0	-	Broken line regression	(Kim et al., 1992)
Yellow catfish (*Pelteobagrus fulvidraco*)	4.0	11.5	23.5	-	Quadratic regression	(Elmada et al., 2016)
Cobia (*Rachycentron canadum*)	6.7	11.9	26.4	-	Quadratic regression	(Zhou et al., 2006)
Silver catfish (*Rhamdia quelen*)	0.7	12. 5	34.42	-	polynomial regression	(Rotili et al., 2018)

DP, digestible protein; dMet, dietary digestible methionine; TSSA, total sulphur amino acid; ND, not determined.

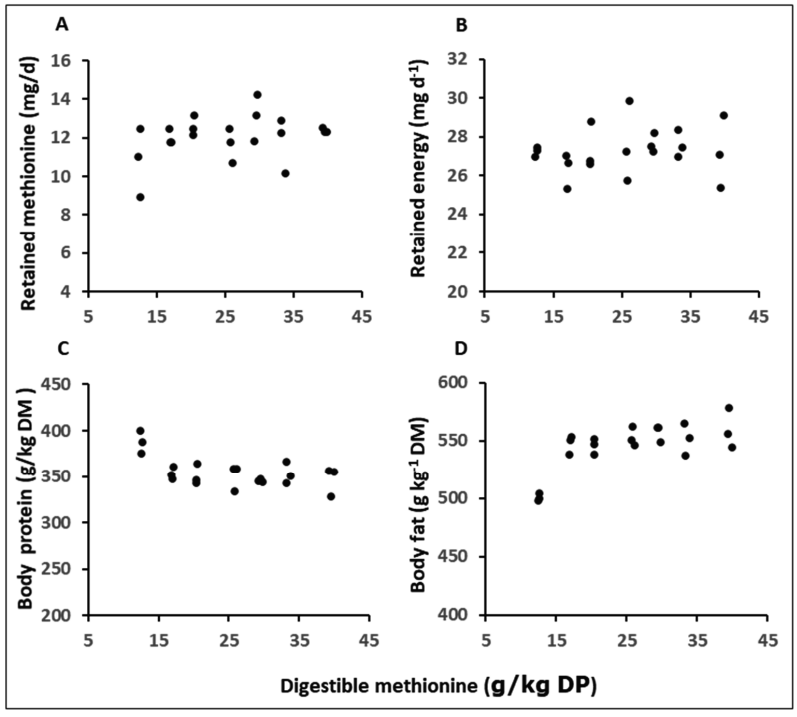
Supplementary table 3.5 Estimated relationship between dietary digestible methionine content (X-variable, expressed in g/kg digestible protein, $dMet_{DP}$) with different measured parameters in African catfish. The relationships were estimated by different analytical models (linear plateau, LP and Broken line, BL) using the NLIN procedure in SAS.

Traits		Linear plateau equation	R^2		Broken line equation	R^2
SGR (%/d)	X < 19.1	Y = 2.40 (0.012) + 0.030$^{\$}$ (0.0086) * (19.1 - X)	0.7012	X < 19.0	Y = 2.37 (0.024) + 0.030$^{\$}$ (0.0089) * (19.0 - X)	0.701
	X > 19.1	Y = 2.40		X > 19.0	Y = 2.37 (0.024) + 0.00011 (0.0019) * (X - 19.0)	
Growth (g/d)#	X < 18.7	Y = 3.15 (0.023) + 0.057$^{\$}$ (0.016) * (18.7 - X)	0.68	X < 19.0	Y = 3.17 (0.045) + 0.057$^{\$}$ (0.017) * (19.0 - X)	0.684
	X > 18.7	Y = 3.15		X > 19.0	Y = 3.17 (0.045) - 0.0017 (0.0037) * (X -19.0)	
Retained nitrogen (mg/d)#	X < 21.4	Y = 81.55 (0.80) + 2.0$^{\$}$ (0.29) * (21.4 - X)	0.858	X < 18.7	Y = 79.6 (1.49) + 2.68$^{\$}$ (0.51) * (21.6 - X)	0.862
	X > 21.4	Y = 81.55		X > 18.7	Y = 79.6 (1.49) + 0.12 (0.11) * (X - 21.6)	
FCR (g/g)	X < 18.5	Y = 0.88 (0.0067) - 0.019$^{\$}$ (0.0047) * (18.5 - X)	0.722	X < 18.8	Y = 0.88 (0.013) - 0.019$^{\$}$ (0.0048) * (18.8 - X)	0.725
	X > 18.5	Y = 0.88		X > 18.8	Y = 0.88 (0.013) + 0.00046 (0.0011) * (X - 18.8)	
Methionine efficiency (mg/d)	X < 31.7	Y = 40.76 (3.18) -3.32$^{\$}$ (0.33) * (31.7 - X)	0.913	X < 24.6	Y = 57.4 (6.1) – 4.147$^{\$}$ (0.74) * (24.6 - X)	0.930
	X > 31.7	Y = 40.76		X > 24.6	Y = 57.4 (6.1) - 1.32 (0.41) * (X - 24.6)	
Protein efficiency (%)	X < 19.4	Y = 61.91 (0.57) + 1.79$^{\$}$ (0.40) * (19.4 - X)	0.818	X < 18.8	Y = 60.80 (1.17) + 1.79 (0.40) * (18.8 - X)	0.831
	X > 19.4	Y = 61.9		X > 18.8	Y = 60.80 (1.17) + 0.1008$^{\$}$ (0.086) * (X - 18.8)	
ADC methionine (%)	X < 31.0	Y = 96.26 (0.32) + 0.30$^{\$}$ (0.034) * (31.0 - X)	0.885	X < 32.0	Y = 97.92 (0.78) + 0.28$^{\$}$ (0.027) * (32.0 - X)	0.881
	X > 31.0	Y = 96.26		X > 32.0	Y = 97.92 (0.78) -1.017 (1.79) * (X -32.0)	
Branchial urinary losses (mg/d)	X < 19.8	Y = 49.80 (0.79) - 1.96$^{\$}$ (0.55) * (19.8 - X)	0.758	X < 19.0	Y = 51.28 (1.63) -1.96$^{\$}$ (0.55) * (19.0 - X)	0.776
	X > 19.8	Y = 49.80		X > 19.0	Y = 51.28 (1.63) -0.14 (0.12) * (X - 19.0)	

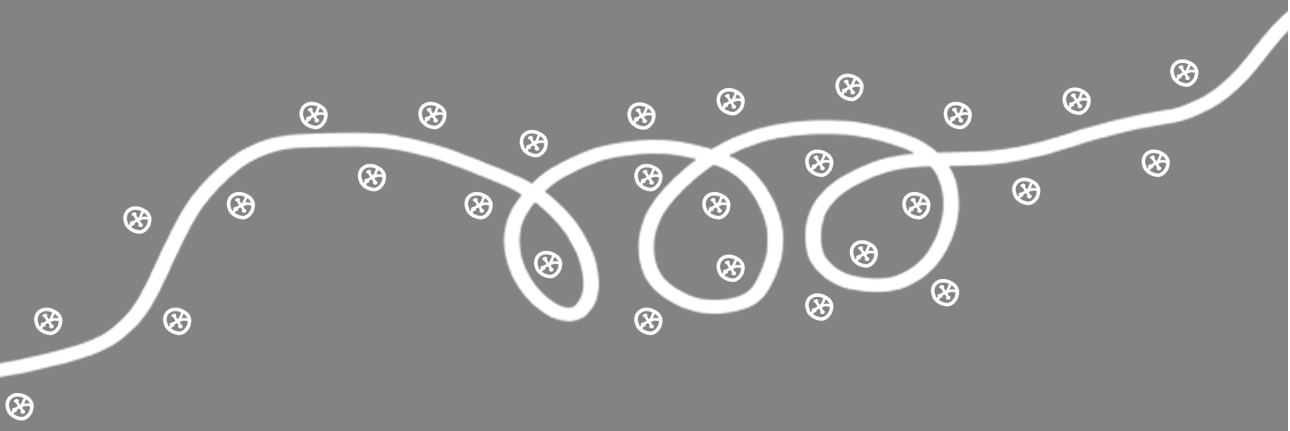
$ Beta values having this superscript are different from zero (P<0.05).

The broken line analysis by the SAS NLIN procedure for the parameters growth and retained nitrogen did not give a stable convergence.The estimated breakpoint and slopes for these parameters was sensitive to the starting iteration values and gave small differences in R2 but quite large range in estimated breakpoints (estimated dMetDP for growth ranged between 19.0 and 20.8 g/kg and for retained nitrogen between 18.7 and 21.6 g/kg)

Supplementary figures



Supplementary Figure 3.1 The relationship between dietary digestible methionine content (expressed in g/kg digestible protein [DP]) and retained methionine (RM), retained energy (RE), body protein, and body protein in African catfish.



CHAPTER 4

Fishmeal hydrolysation and non-protein energy sources affect the kinetics of nutrient digestion in the gastrointestinal tract of African catfish (*Clarias gariepinus*)

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Abstract

The kinetics of nutrients digestion and development of chyme characteristics in African catfish (*Clarias gariepinus*) were assessed in response to fishmeal hydrolysation and non-protein energy (NPE) sources. Four diets were formulated to contain starch or fat as NPE source, and fishmeal or hydrolysed fishmeal as protein source in a 2x2 factorial design. Juvenile African catfish (average weight, 63 g) were stocked in glass experimental tanks connected to a common recirculation aquaculture system and were fed restrictively for 3 weeks. Four hours after the consumption of a single meal, fish were dissected to collect chyme from the gastrointestinal tract (GIT). Chyme was collected from stomach, proximal- and distal intestine and analysed for dry matter (DM) content, crude protein (CP) and marker concentration. Postprandial water fluxes to the GIT and stomach evacuation were calculated using yttrium oxide (Y_2O_3) as an inert marker. Faecal DM and CP apparent digestibility coefficient (ADC) were determined by a marker method. Results showed that fishmeal hydrolysation had no effect (P>0.1) on the DM content in all the GIT compartments. However, replacing dietary fat by starch resulted in a higher DM content in the stomach (P<0.01). In the proximal intestine, NPE did not influence the chyme DM content (P>0.1) but in the distal intestine, chyme DM was higher at the "fat diets" (P<0.01). "Starch-diets" had a larger water influx into the stomach compared to "fat diets" (P<0.001), but also a larger water re-absorption in the distal intestine (P<0.05). The inert marker and DM evacuation rate from the stomach was affected by NPE source (P<0.05) and was slower in the starch-fed fish. Hydrolysation of fishmeal increased the digestibility of CP in the stomach, but this effect of hydrolysation was dependent on the energy source, indicated by the interaction effect (P<0.05). The increase in digestibility of CP in the stomach of the diets containing hydrolysed fishmeal was larger at the "fat diets". In the other GIT compartments, CP digestibility were similar between diets (P>0.1). However, hydrolysation of fishmeal had no effect on faecal ADC of CP. Our results suggest that the hydrolysation of fishmeal can alter the process of digestion along the GIT. In addition, dietary macronutrient composition can alter the postprandial digestion of nutrients in the GIT without being reflected in the faecal digestibility.

4.1 Introduction

Fish diets are usually formulated based on the faecal nutrient digestibility of ingredients, which only accounts for the total amount of dietary nutrients that was apparently digested and assumed to be absorbed along the gastrointestinal tract (GIT) (Chen, 2017; NRC, 2011). This does not take into consideration the kinetics of nutrient digestion along the GIT. In pig and poultry nutrition, more attention is now directed towards assessing chyme characteristics, nutrient passage rates and the degree of absorption along the GIT after feed ingestion. This is because ileal and faecal digestibility of nutrients differ among feed ingredients (Chen, 2017). Next to nutrient digestibility, it has been shown in poultry that the digestion kinetics of nutrient influences feed utilization (Liu et al., 2013). A poultry study revealed that the starch digestion rate is higher than the protein digestion rate. In the same study, reducing the starch digestion rate by altering dietary composition increased nitrogen retention and reduced FCR without affecting the total starch digestibility (Liu et al., 2013). It was hypothesised that slowly digestible starch reduced the catabolism of amino acids by enterocytes (Liu and Selle, 2015). Knowledge on the dynamics of nutrients could be applied by nutritionist to formulate balanced diets that can improve nitrogen retention and better FCR.

In fish, only few studies have investigated diet-elicited effects on digestion of nutrients in different compartments of the GIT (i.e., stomach, proximal-, mid- and distal intestine) (Harter et al., 2015; Harter et al., 2013; Leenhouwers et al., 2007a; Maas, 2021; Tran-Tu et al., 2019). Dietary fat replacement by starch reduced protein disappearance in the stomach (Harter et al., 2015), which coincided with a lower stomach dry matter content and increased water influxes in the stomach of African catfish (*Clarias gariepinus*) (Harter et al., 2015). Increasing the dietary viscosity (induced by guar gum addition) decreased the digestibility of protein and dry matter in all GIT compartments of striped catfish (*Pangasianodon hypophthalmus*), while chyme dry matter content declined and chyme viscosity increased only in the stomach (Tran-Tu et al., 2019). In Nile tilapia (*Oreochromis niloticus)*, diets supplemented with an enzyme cocktail (phytase and xylanase) had an improved faecal protein digestibility, which occurred from the proximal intestine onward (Maas et al., 2021). In this same study on Nile tilapia, dietary probiotic supplementation increased the disappearance of protein in the stomach while it reduced the faecal protein digestibility, but the differences were small (Maas et al., 2021). Starch is now increasingly used to replace fat as a cheap non-protein energy source due to its availability and low cost (Harter et al., 2015). African catfish and several other omnivorous species can readily handle high inclusions of carbohydrates (Belal, 1999; Bureau et al., 1995; Kirchgessner et al., 1986). However, during starch digestion, a large amount of osmotically active mono- and disaccharides are produced (Harter et al., 2013), but quantitative information on the starch hydrolysis throughout the GIT is lacking. Bucking and Wood (2006) have suggested that these compounds drive the addition of water to the GIT, which is reflected in an increased water influx in the stomach of fish (Harter et al., 2013). Furthermore, starch induces viscosity in the stomach of fish, which often goes together with a lower chyme dry matter content but mainly in the stomach and proximal intestine (Amirkolaie et al., 2006a; Harter et al., 2015; Leenhouwers et al., 2006; Leenhouwers et al., 2007a). Despite these research efforts in addressing the impact of dietary characteristics on digestion, most studies have mainly focused on dietary non-protein energy sources. They, however, do not provide information on the kinetics of protein digestion as affected by dietary protein sources in the GIT of fish. Such information is important to understand the interaction of dietary protein and energy on nutrient passage dynamics in the GIT, especially now that increasing amounts of less expensive and more sustainable alternative protein ingredients are used to replace fishmeal in aquafeeds.

Fishmeal is in many cases superior regarding attractability, palatability and bioavailability of nutrients compared to alternative protein sources in aquafeeds. Hydrolysation of protein sources can increase the attractability, palatability and/or bioavailability of nutrients in ingredients (Silva et al., 2017). Several studies have demonstrated that hydrolysation of protein sources increases the nutritional values as it enhances growth without adversely affecting protein quality. This positive effect of hydrolysation has been shown for both animal (e.g., fish, shrimp, milk & feather meal) and plant protein sources (e.g., rapeseed, cottonseed, wheat gluten and soybean meal) (Gui et al., 2010; Leal et al., 2010; Muranova et al., 2017; Siddik et al., 2021; Xu et al., 2017; Yuan et al., 2019). Fish protein hydrolysates are products that originated from the conversion of inexpensive and underutilized fish by-products into a commercially valuable protein ingredient by the action of enzymatic hydrolysis. The use of fish protein hydrolysate has gained great attention by fish nutritionists due to its nutritional composition, amino acid profile and antioxidant properties (Chalamaiah et al., 2012; Swanepoel and Goosen, 2018). Partial replacement of fishmeal by fishmeal hydrolysate in the diet of African catfish improved growth and feed efficiency (Swanepoel and Goosen, 2018). In humans, protein hydrolysates resulted in a faster postprandial increase of plasma AAs than their non-hydrolysed equivalents, which suggests a quicker absorption of AA in the GIT (Morifuji et al., 2010). This implies that the passage rate of fishmeal can be altered by the process of hydrolysation thereby influencing chyme characteristics and faecal digestibility.

The overall kinetics of dietary protein digestion is related to the passage rate of digesta along the GIT, which depends on the physicochemical properties (e.g., solubility, viscosity, water binding capacity) of the digesta (Chen, 2017). Therefore, understanding the difference in digestion and absorption rates of nutrients (e.g., energy and protein) along the GIT as affected by ingredient characteristics is important for understanding the differences in faecal ADC and thus for formulating balanced aquafeeds. In relation to the observed effects of fat replacement with starch, we hypothesised that the hydrolysation of dietary protein (fishmeal in this study) increases the protein digestion in the proximal intestine. It was expected that hydrolysation of fishmeal would lead to a more rapid stomach evacuation as compared to the non-hydrolysed fishmeal, which might relate to alteration in chyme characteristics. Furthermore, we proposed that these effects might be affected by the type dietary non-protein energy (i.e., fat vs starch).

To substantiate these assumptions, four diets were formulated to contain two types of energy sources (fat vs starch) and protein sources (fishmeal vs hydrolysed fishmeal). These diets were fed to African catfish with the aim to (1) assess the effect of fishmeal hydrolysation on chyme characteristics in different segments of the GIT, (2) investigate the water balance in the GIT in response to dietary macronutrients, and (3) determine potential interactions between fishmeal hydrolysation and dietary non-protein energy sources on the kinetics of digestion in African catfish.

4.2 Materials and methods

4.2.1 Ethics statement and research facility

The study (project number 2018.W.0014.003) was carried out in accordance with the Dutch law on the use of animals (Act on Animal Experiments) for scientific purposes and was approved by the Central Animal Experiments Committee (CCD) of The Netherlands. This experiment was conducted in the research facility of CARUS-ARF at Wageningen University, The Netherlands. Fish were kept and handled in agreement with EU-legislation.

4.2.2 Experimental diets

This study aimed to examine the effect of fishmeal hydrolyzation and non-protein energy sources on the chyme characteristics and digestion kinetics in African catfish. Therefore, four diets were formulated according to a 2 by 2 factorial design which differed in protein sources (fishmeal or fishmeal hydrolysate, respectively NH-FM versus H-FM)) and type of non-protein energy sources (fat or starch, respectively FD versus SD). The four experimental diets were extruded with a 1.7 die size into 3 to 3.5 mm pellets by Skretting ARC Norway using a twin-screw extruder (Wenger, Sabetha, KS, U.S.A). Meal mixes were preconditioned for 80 sec resulting in an outlet dough temperature of 70 °C for all diets. Die and barrel temperature were equal within each diet production run and was 65, 70, 60 and 70 °C for diets "NH-FM + SD", "NH-FM + FD", "H-FM + SD" and "H-FM + FD", respectively. The extrusion resulted in a pellet width of 3.5, 2.1, 3.5 and 2.2 mm, a pellet length of 2.7, 3.0, 2.9 and 3.8 mm and a bulk density (directly after extrusion) of 380, 390, 420 and 420 g/L for the "NH-FM + SD", "NH-FM + FD", "H-FM + SD" and "H-FM + FD" diet, respectively. Diets were formulated to be iso-nitrogenous and iso-energetic. The difference between the starch and fat diets was created by replacing 320 g wheat starch by 121.5 g rapeseed oil. These amounts of both ingredients provide the same amount of gross energy (GE). Cellulose was added to the fat diets to compensate for the higher energy content of rapeseed oil. The ingredients and analysed chemical composition of the diets are given in Table 4.1. Hydrolysis of the fishmeal was performed using a proprietary enzymatic process. After undergoing hydrolysis, the hydrolysate was transferred to a storage tank from where it was continuously pumped into the preconditioner. Enzyme activity was completely stopped once the enzymes were exposed to the high temperatures in the preconditioner. Yttrium oxide was included in all diets as inert marker for measuring water fluxes, DM and protein digestion along the GIT. Feeds were kept in cold storage at 4 °C throughout the experiment and a representative sample was taken for analysis.

4.2.3 Fish and housing conditions

Juvenile African catfish (*Clarias gariepinus*) of an average individual body weight of 63 g were obtained from a commercial hatchery (Fleuren & Nooijen BV, Nederweert, The Netherlands). The fish comprised of a mixed-sex population. Two weeks prior to the experiment, fish were fed a commercial diet to adapt to the experimental conditions. At the start of the experiment, fish were randomly stocked (40 fish per tank) into 12 aquaria (200 L) connected to a common recirculation aquaculture system (comprising of a trickling filter, sump and drum filter). Each tank was equipped with air stones and swirl separators (AquaOptima AS, column height 44 cm; diameter 24.5 cm) for the collection of faeces and spilled pellets. The water flow rate was set at 7 L/min, temperature was 28 °C and the photoperiod regime was kept at 12 h light: 12 h dark. Water quality parameters were monitored regularly and maintained at the optimal levels for African catfish: pH, 7.5 ± 0.30; ammonium, 0 ± 0.00 mg/L; nitrite, 0.12 ± 0.075 mg/L; nitrate, 175 ± 82 mg/L; conductivity, 3287 ± 848 µS; and dissolved oxygen concentration, 6.21 ± 0.37 mg/L. Water refreshment was performed based on NO_3 removal from the system to keep NO_3 levels within limits (< 500 mg/L).

4.2.4 Experimental procedure

The four diets were randomly assigned (in triplicate) to the twelve aquaria. To prevent variability in measurements due to differences in feed intake, fish were hand-fed restrictively in the morning (8:00 h) and afternoon (16:00 h) for 3 weeks. Feeding level was fixed at 19.8 g/kg$^{0.8}$/d to rule out the effects of feeding level on the chyme characteristics. The daily ration was increased throughout the

Table 4.1 Formulation and proximate composition of the experimental diets

%	NH-FM + SD	NH-FM + FD	H-FM + SD	H-FM + FD
Fishmeal	35.17	35.17	17.59	17.59
Fishmeal hydrolysate	0.00	0.00	17.59	17.59
Wheat	15.09	15.10	15.09	15.10
Gelatinized wheat starch	32.00	0.00	32.00	0.00
Rapeseed oil	0.00	12.15	0.00	12.15
Fish oil	3.00	3.00	3.00	3.00
Wheat bran	15.00	15.00	15.00	15.00
Moisture loss[1]	-2.58	0.00	-2.58	0.00
Yttrium oxide	0.10	0.10	0.10	0.10
Mineral & vitamin premix	0.22	0.22	0.22	0.22
Cellulose[2]	2.00	19.26	2.00	19.26
Analysed proximate composition (g/kg dry matter)				
Dry matter	911	927	891	914
Crude protein	325	312	311	306
Crude fat	88	218	85	227
Ash	62	61	61	61
Phosphorus	11.1	10.7	10.7	10.4
Calcium	11.0	11.0	10.8	10.5
Magnesium	1.9	1.8	1.8	1.8
Total carbohydrate	525	409	543	407
Energy (kJ/g)	20.3	23.4	20.0	23.3
Energy excluding cellulose (kJ/g)[3]	20.0	20.1	19.7	20.0

NH-FM, non-hydrolysed fishmeal; H-FM, hydrolysed fishmeal; SD, starch diet; FD, fat diet.
[1]The production of NH-FM + SD and H-FM + SD diets was targeted at a dry matter content of 92%, which resulted in an expected water loss during extrusion of 2.58%.
[2]Qualicel® pc 150 (CFF GmbH & Co. KG, Gehren, Germany).
[3]The calculated energy content excluding the energy from the added cellulose.

experimental period by predicting growth using a FCR of 1. During the first week, feeding level was gradually increased from 20% to 100% of the intended ration to allow adaptation to diets. In the case of mortality, the feeding list of the respective tank was adjusted for the remaining number of fish to maintain equal feed intake among treatments. After each meal, the uneaten and spilled pellets were collected and counted for the accurate determination of feed intake. Faeces were collected twice daily from week 2 till the end of the trial, using swirl separators attached to each experimental tank. The faecal collection bottles underneath the swirl separators were suspended in ice to minimize bacterial degradation. Faeces samples were pooled per tank and frozen (-20 °C) until chemical analysis.

At the end of the experiment, thirteen fish were randomly selected from each tank for collecting chyme samples. Sampling was done 4 hours after feeding a single meal. These fish were euthanized using an overdose of 2-phenoxy-ethanol (1.0mL/L). Fish were individually weighed and dissected to collect chyme from the gastrointestinal tract (GIT). For this sampling, the GIT was divided into three compartments: stomach and two equal parts of the gut, representing proximal and distal intestine. Chyme samples were pooled per tank (i.e., 13 fish) and per compartments, weighed (to determine weight wet) and frozen (-20 °C) for further analysis.

4.2.5 Sample analysis

Analyses were performed on the diets, chyme and faeces samples. Feed pellets were grinded. Faecal and chyme samples were freeze-dried, then manually pulverized through a 1 mm screen sieve. The chemical analysis of the feed and faeces were performed in triplicate using the same methods while chyme was analysed in duplicate. The dry matter (DM) was determined by drying at 103 °C in the oven (ISO 6496, 1983) while ash was determined after furnacing for 4 hours at 550 °C (ISO 5984, 1978). Crude protein of feed and faeces was quantified according to the Kjeldahl method (ISO 5983, 1979), while the crude protein content of chyme was quantified using the DUMAS method due to the limited amount of chyme samples. Crude fat was determined by differences following extraction with petroleum ether at 40 °C – 60 °C in a Soxhlet apparatus (ISO 6492, 1999) and energy content was quantified using an adiabatic bomb-calorimeter (C7000 IKA®, IKA analysentechnik, Weitershem, Germany; ISO 9831, 1998). Yttrium, phosphorus, calcium and magnesium were detected by using inducted coupled plasma mass spectrometry according to the standard NEN 15510 (ICP-MS, 2007).

4.2.6 Calculations

The apparent digestibility coefficient (ADC) of nutrients in faeces were calculated according to the formula described by Cheng and Hardy (2002) using yttrium oxide as inert marker; ADC (%) = 100 × [1 − (yttrium concentration in the feed × nutrient concentration in the faeces)/(yttrium concentration in the faeces × nutrient concentration in feed)]. Nutrient ADC per compartment was calculated as follows; ADC (%) = 100 × [1 − (yttrium concentration in the feed × nutrient concentration in the chyme)/(yttrium concentration in the chyme × nutrient concentration in feed)]. The concentrations of yttrium and nutrients were expressed on DM basis. Total carbohydrate was calculated as dry matter minus crude protein minus crude fat minus ash content.

Stomach evacuation rates (g DM/h) were calculated as the amount of dry matter of the ingested feed minus the chyme content (DM) of the stomach divided by the time since the last feeding. The chyme dry matter (g DM mg/yttrium) was calculated as ingested DM on the sampling day divided by the yttrium content (mg) of the ingested feed. Water flux was calculated according the formula described by Harter et al. (2013). The relative water fluxes (mL/g of ingested DM) were calculated from the relative water content of the chyme in one compartment minus the relative water content in the previous compartment divided by the relative ingested DM. Where, relative water content of chyme (g) was expressed in relation to its marker content (mg). This was calculated from the wet weight and DM content of chyme and then converted into a volumetric measure (mL) (assuming that 1 g of water had a volume of 1 mL under the tested conditions).

4.2.7 Statistical analysis

Data analyses were performed by using SPSS Statistics, version 23.0 for Windows (IBM Corp., Armonk, NY, USA). Nutrient ADCs, chyme characteristics within compartments and stomach evacuation were analysed by two-way ANOVA. Following a significant ANOVA result, means were compared by a multiple comparisons test using Tukey's honest significant difference (HSD).

4.3. Results

Results for the dry matter (DM) content of chyme in each GIT compartment are depicted in Figure 4.1. The average DM content of "starch-diets" was highest in the stomach (0.18 kg/kg); decreased towards the proximal intestine (0.11 kg/kg) and slightly increased in the distal intestine (0.14 kg/kg). Likewise, the DM content of "fat-diets" was highest in the stomach (0.22 kg/kg); decreased towards the proximal intestine (0.12 kg/kg) and slightly increased in the distal intestine (0.16 kg/kg). The stomach chyme DM differed between diets having different non-protein energy sources (NPE) (P<0.01). Fish fed "fat-diets" had higher chyme DM in the stomach compared to those fed "starch-diets". However, NPE did not influence the chyme DM content (P>0.1) in the proximal intestine. In the distal intestine, the DM content was higher (P<0.05) in the fish fed "fat-diets" compared to "starch-diets". The hydrolysation of fishmeal had no effect (P>0.1) on the DM content in all the GIT compartments. Overall, there was no significant interaction between fishmeal hydrolysation and NPE on DM contents of both diets in all the compartments.

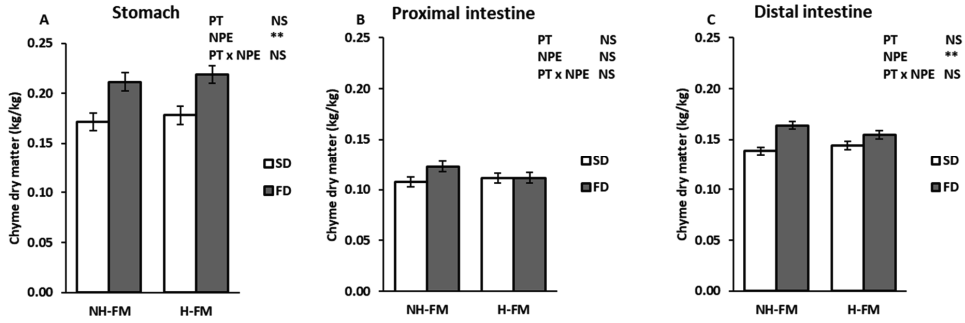

Figure 4.1 Dry matter (DM) content of chyme (kg/kg), measured in the stomach, proximal intestine and distal intestine of African catfish. NH-FM, control fishmeal; H-FM, hydrolysed fishmeal; SD, starch diet; FD, fat diet. Presented values are means (n=3) per dietary treatment within each compartment, except for the NH-FM+FD diet in the proximal and distal intestine (n=2). Error bars indicate standard error of means; PT, protein type (non-hydrolysed fishmeal and hydrolysed fishmeal); NPE, non-protein energy source (starch and fat); NS, not significant; **, P<0.01.

Results for the relative water fluxes (RWF) in the GIT are illustrated in Figure 4.2. Water fluxes differ between compartments. Within each compartment, no significant interaction between fishmeal hydrolysation and NPE was detected. Water fluxes to the stomach was influenced by the NPE (P<0.001), while a tendency (P<0.1) of the effect of fishmeal hydrolysation was observed. Relative water additions to the stomach were higher (P<0.001) in fish fed "starch-diets" compared to "fat-diets", having 4.07 and 3.15 mL of water added per g of ingested DM, respectively. Both dietary treatments did not influence the water fluxes (P>0.1) in the proximal intestine. When comparing water fluxes in the stomach with proximal intestine, results suggested that the water addition decreased as the chyme progressed distally. The sum of relative water influx to the proximal intestine was not significantly different between diets but numerically higher for "starch-diets" (3.01 mL/g of ingested DM) compared to "fat-diets" (2.24 mL/g of ingested DM). NPE affected (P<0.05) the water fluxes in the distal intestine. A higher water re-absorption in the distal part of the intestine was observed in fish fed "starch-diets" compared to "fat-diets", which was reflected by the relative water flux of -4.64 and -3.05 mL/g of ingested DM, respectively.

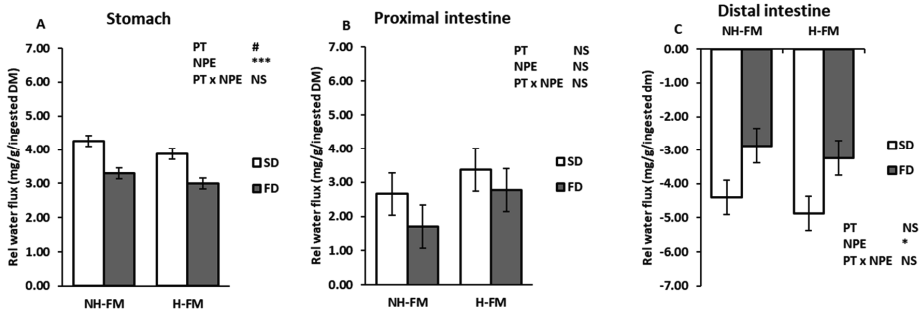

Figure 4.2 Relative water flux (mL/g ingested DM), measured in the stomach, proximal intestine and distal intestine of African catfish; NH-FM, control fishmeal; H-FM, hydrolysed fishmeal; SD, starch diet; FD, fat diet. Presented values are means (n=3) per dietary treatment within each compartment, except for the NH-FM+FD diet in the proximal and distal intestine (n=2). Error bars indicate standard error of means; PT, protein type (non-hydrolysed fishmeal and hydrolysed fishmeal); NPE, non-protein energy source (starch and fat); NS, not significant; #, P<0.1; *, P<0.05; ***, P<0.001.

The results for stomach evacuation rate are presented in Table 4.2. During the first 4 hours post-prandial, the rate of yttrium evacuation from the stomach was higher (P<0.05) for "fat-diets" (15.3% Y/h) compared to "starch-diets" (12.6% Y/h). Crude protein (CP) evacuation rate from the stomach, was affected by the fishmeal hydrolysation (P<0.05), but not by the type of NPE (P>0.1). The evacuation rate of CP was higher for the hydrolysed fishmeal diets (23.5% CP/h) than for the non-hydrolysed fishmeal (23.0% CP/h). CP left the stomach at a faster rate compared to yttrium (average over all diets, 23.3 CP/h vs. 14.0% Y/h). Similarly, a slightly higher evacuation rate was recorded for DM (average over all diets, 16% DM/h) compared to yttrium. Furthermore, NPE had an effect (P<0.05) on the DM evacuation from the stomach, with "fat-diets" having a higher evacuation rate than "starch-diets". The total yttrium loss (% feed excreted) was calculated (Supplementary Table 4.3). Averaged over diets, 71.40% of the yttrium consumed was found in all compartment after 4h postprandial. This implies that 4hrs after feeding, 28.59% of the consumed yttrium was already excreted via the faeces. However, the amount of faecal yttrium loss was not affected by fishmeal hydrolysation but differed between NPE sources (P<0.05), which was higher for "fat-diets" (37.00% feed excreted) compared to "starch-diets" (20.18% feed excreted).

Table 4.2 Stomach evacuation rate of African catfish after 4h of feeding diets containing different protein and energy sources.

	Diet							
	Control fishmeal		Hydrolysed fishmeal			*P*-value		
Evacuation	**SD**	**FD**	**SD**	**FD**	**SEM**	**PT**	**NPE**	**PT x NPE**
DM (% DM/h)	14.35	16.70	15.47	17.39	0.88	NS	*	NS
CP (% CP/h)	23.17	22.85	23.55	23.49	0.21	*	NS	NS
Yttrium (% Y/h)	12.16	15.10	13.03	15.52	1.08	NS	*	NS

DM. dry matter; Y, yttrium; h, hour; SD, starch diet; FD, fat diet; PT, protein type (non-hydrolysed fishmeal and hydrolysed fishmeal); NPE, non-protein energy source (starch and fat); SEM, standard error of means; NS, not significant; *, P<0.05.

Apparent digestibility coefficients (ADC) of DM and CP along the GIT are visualised in Figure 4.3 & 4.4 respectively. Hydrolysation of fishmeal increased the digestibility of CP in the stomach and this was dependent on the energy source, as indicated by the interaction between NPE and fishmeal hydrolysation (P<0.05). Whereas, both dietary treatments had no significant effect on ADC of CP in the proximal and distal intestine (P>0.1). The fish fed "starch-diets" had higher (38.99%) CP digestibility in the stomach compared to the "fat-diets" (26.76%). Regarding DM digestibility, no interaction effect

(*P*>0.1) of fishmeal hydrolysation and NPE was detected in the stomach. However, NPE sources independently showed a significant effect on ADC of DM in the proximal (*P*<0.05) and distal intestine (*P*<0.01). In the proximal intestine, a significantly lower DM digestibility was observed for "starch-diets" (12.18%) compared to the "fat-diets" (26.32%). However, the reverse was the case in the distal intestine with "starch-diets" showing higher values (58.10%) than "fat-diets" (51.25%). Kinetics of digestion of crude ash, phosphorous, calcium and magnesium are provided in Supplementary Table 4.1. The ADC of P increased from stomach towards the distal intestine, but the major part of the digested P already disappeared in the stomach. ADC of P was not different between diets in any gut segments. ADC of Ca averaged over diets was 47, 12 and 25% in stomach, proximal and distal intestine, respectively. ADC of Ca in the stomach was higher for "starch-diets" than for "fat diets" (*P*<0.01), while in the proximal intestine, the ADC of Ca was highest at the "fat diets" (*P*<0.05). In the distal intestine, no differences in ADC of Ca between diets were present. The ADC of Mg in the different gut segments fully paralleled the pattern in ADC of Ca (Supplementary Table 4.1). ADC of P, Ca, and Mg was unaffected by fishmeal hydrolysation in all gut segments (*P*>0.1).

The result of faecal digestibility showed some similarities with the outcome of the proximal and distal intestine, with no effect (*P*>0.1) of fishmeal hydrolysation on both ADC of DM and CP (Figure 4.3D and 4.4D). However, the ADC of DM was significantly affected by the dietary energy sources where "fat diets" yielded a lower DM faecal ADC (*P*<0.01). In contrast, fish fed "fat-diets" showed a higher faecal ADC of CP (*P*<0.001) but the difference was minor (86.86% for starch and 88.89% for fat diets) (Figure 4.4D). There was no interaction effect of fishmeal hydrolysation and energy type on faecal ADC of DM and CP (*P*>0.1). ADC data of fat, crude ash, energy, calcium, phosphorous and magnesium are given in Supplementary Table 4.2.

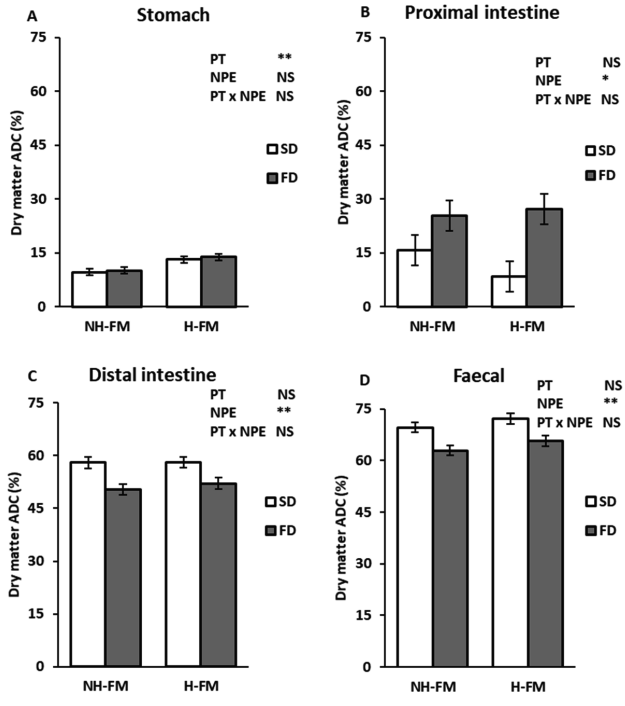

Figure 4.3 Apparent digestibility coefficient (ADC) of dry matter in the stomach, proximal intestine, distal intestine and faeces of African catfish; NH-FM, control fishmeal; H-FM, hydrolysed fishmeal; SD, starch diet; FD, fat diet. Presented values are means (n=3) per dietary treatment within each compartment, except for the NH-FM+FD diet in the proximal intestine (n=2). Error bars indicate standard error of means; PT, protein type (non-hydrolysed fishmeal and hydrolysed fishmeal); NPE, non-protein energy source (starch and fat); NS, not significant; *, *P*<0.05; **, *P*<0.01.

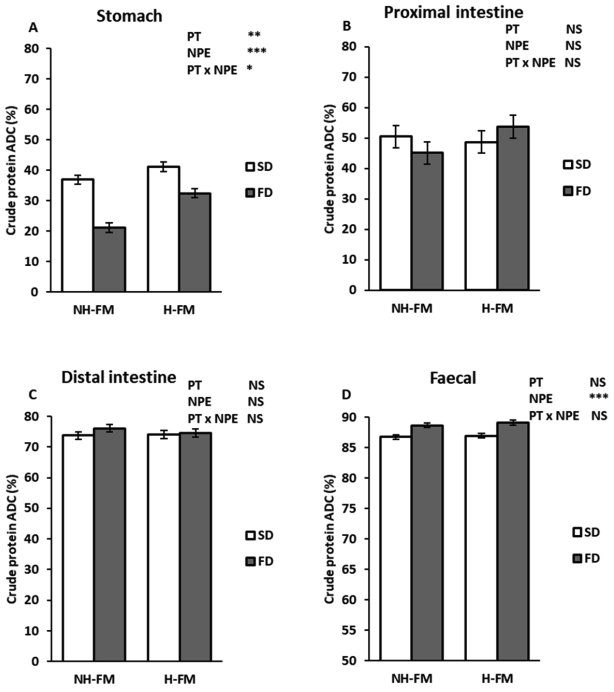

Figure 4.4 Apparent digestibility coefficient (ADC) of crude protein (CP) in the stomach, proximal intestine, distal intestine and faeces of African catfish; NH-FM, control fishmeal; H-FM, hydrolysed fishmeal; SD, starch diet; FD, fat diet. Presented values are means (n=3) per dietary treatment within each compartment, except for the NH-FM+FD diet in the proximal intestine (n=2). Error bars indicate standard error of means; PT, protein type (non-hydrolysed fishmeal and hydrolysed fishmeal); NPE, non-protein energy source (starch and fat); NS, not significant; *, $P<0.05$; *, $P<0.01$; ***, $P<0.001$.

4.4 Discussion

DM content of the chyme was highest in the stomach, decreased in the proximal intestine and slightly increased in the distal intestine. In the same light, most water was added to the stomach and less to the proximal intestine while in the distal intestine, water was re-absorbed. Harter et al. (2013) also reported similar results for African catfish as well as Bucking and Wood (2006) for rainbow trout (*Oncorhynchus mykiss*). The study of Bucking and Wood (2006) on water fluxes in the GIT of rainbow trout revealed a large addition of water to the stomach over the first 12h after feeding a single meal. Addition of large amounts of water to the stomach is due to the physiological demands resulting from the consumption of dry diets by fish (Bucking and Wood, 2006). This water influx may be of exogenous (postprandial drinking) or endogenous (addition of digestive juices) origin. Although, freshwater (FW) fish are known to be hyperosmotic to their environment and to drink less water than marine fish (Perrott et al., 1992), water uptake in their intestine can be controlled in the same way as marine species do. In addition, FW fish are able to regulate the influx of interstitial water to the stomach based on their nutritional demands (Harter et al., 2013). The lower addition of water to the proximal intestine can be explained by the previous water influx to the stomach. In a study investigating water fluxes in African catfish (Harter et al., 2013), 59% of water in the proximal intestine originated from the stomach, which led to a decreased water addition in this compartment to compensate for the initial surplus influx. Another part of the water in the proximal intestine originates from water that is secreted together with bicarbonate. The latter being secreted to neutralize the acidic stomach chyme entering the proximal intestine. It is very likely that this also occurs in African catfish as this is supported by the

current observation of a drop in ADC of Ca from 47% in the stomach to 17% in the proximal intestine, which indicates that there is an influx of Ca into the proximal intestine. Also, intestinal fluids such as, bile and pancreatic enzymes released to this compartment after feeding contributes to the influx of water in the proximal intestine (Grosell et al., 2000). In the current study, the flux of water was negative in the distal part of the intestine, which was also observed in rainbow trout (Bucking and Wood, 2006). According to Bucking and Wood (2006), the negative result could be due to the net reabsorption of water that was added in the previous compartments. However, a more logical explanation might be the net reabsorption of digestive fluids gained from the previous compartments. There is a possibility of passive absorption of water with the fluids at the distal end of the gut. However, the mechanism that surrounds the interstitial reabsorption of digestive fluids and water in the GIT of fish requires further investigation.

The observed higher stomach DM content for fish fed “fat-diets” than for “starch-diets” is in line with the study of Harter et al. (2015) on African catfish. Harter et al. (2015) suggested that the hydrophobic properties in a high fat diet would interfere with water mixing with chyme. This is also a likely explanation for the observed differences in the water balance results observed in this study, in which more water was added to “starch-diets” than “fat-diets”. Hydrolysis of starch produces large amounts of osmotically active mono- and disaccharides, which are thought to result in the addition of water to the GIT (Harter et al., 2013), but also the water binding capacity of starch itself might contribute to the lower DM content in the stomach. The higher ADC of Ca and Mg in the stomach at the “starch-diets” compared to the “fat-diets” might suggest that there is a difference in pH in the stomach resulting in an increased dissolving of Ca and Mg. As the drop in ADC of Ca between stomach and proximal intestine was larger at the “starch-diets” than at the “fat-diets”, this implies that the influx of Ca in the proximal intestine was higher at the “starch-diets” compared to the “fat-diets”. These observations about the ADC of Ca might be an indication that the increased acid-secretion in the stomach requires more bicarbonate secretion in the proximal intestine. However, this hypothesis requires further testing. The present study showed also a large reabsorption of water at the distal intestine when fish were fed “starch-diets”. Regarding the protein source, non-hydrolysed fishmeal diets sparked a tendency of more water addition to the stomach of African catfish compared to the hydrolysed fishmeal diets. The hydrolysis process required by fishmeal diets might have increased the need for both endo- and exogenous water addition. Nevertheless, water addition and re-absorption was not significantly different among the protein diets in subsequent compartments. However, a good explanation for our observation of the water fluxes along the GIT for both type of fishmeal diets is lacking.

In the current study, the DM evacuation rate of “fat-diets” was higher in the stomach of African catfish than “starch-diets”. This observation does not substantiate that a higher chyme DM content of “fat-diets” would prolong its retention time in the stomach thereby delaying evacuation time, as suggested by Harter et al. (2015). Chyme with a higher DM is expected to remain in the stomach until appropriate liquefaction is achieved, until then will evacuation occur. However, the improper mixture of water and ‘fat-diet’ due to its high lipid content may have led to the quicker evacuation of the chyme, especially the liquid portion. On the other hand, it appears that the high viscous nature of starch upon reaction with water in the stomach may be associated with a longer passage rate (Amirkolaie et al., 2006a). Literature shows that the kinetics of DM along the GIT is negatively related to the viscosity of the diet (Leenhouwers et al., 2006; Leenhouwers et al., 2007a; Leenhouwers et al., 2007b). Starch has been shown to increase chyme viscosity in the stomach of African catfish, which can slow down the passage rate (Harter et al., 2015). Amirkolaie et al. (2006a) reported that gelatinized starch as used in the

present study increased chyme viscosity in the stomach more than in other segments in Nile tilapia. Furthermore, the longer retention time of the starch diets in the stomach may also be related to the fish needing to achieve a more natural degree of liquefaction of the diet, in an attempt to reach the water content of natural prey (Bucking and Wood, 2006). This may also explain why more water addition was required by the "starch-diets". The passage rate of CP through the stomach in fish fed the non-hydrolysed fishmeal diets was lower than for hydrolysed fishmeal diets. This can be explained by the need to undergo further hydrolysis by the action of stomach acid and enzymes prior to absorption in contrast to the fishmeal hydrolysate that had been partially broken down. Consequently, it is expected that fishmeal hydrolysate will display higher solubility and to some extent, the liquid phase with solved protein will evacuate from the stomach at a faster rate.

The hydrolysation of fishmeal resulted in higher DM and CP digestibility in the stomach, although, absorption of nutrient is not expected to take place in this compartment, rather compounds are broken down by the action of enzymes. This observation is striking and may be explained by differences in evacuation times between the protein and inert marker. The CP in the stomach had a higher evacuation rate (23.52%) compared to the inert marker (14.27%), which could have led to inaccurate calculations. Calculation errors may occur when dissociation between chyme and marker happens as they proceed along the GIT (Bucking and Wood, 2006). When proteins are hydrolysed in the stomach, peptides can/will move to the next segment of the GIT. However, if the dissolved peptides exit the stomach before the marker (here: yttrium) as suggested by results from the current study, the ADC may be overestimated (Harter et al., 2015). This is because the peptides are no longer present in the stomach and neither absorbed in this compartment. Since fish stomachs are unable to absorb larger molecules such as peptides (Uys and Hecht, 1987), the high ADC of CP in the stomach indicates that a fraction of the peptides had already moved into the next section of the GIT. It has been reported that the liquid and solid fraction of chyme may not always move at the same pace. Bucking and Wood (2006) used ballotini beads and polyethylene PEG to investigate the movement of substances in rainbow trout. They observed a continuous association of marker and chyme as they transit along the GIT in their study. However, the liquid portion of the chyme was reported to slightly move faster than the solid part from the stomach.

There is an overall indication of improved nutrients digestibility due to fishmeal hydrolysation in the current study (Supplementary table 4.2). The positive effect of using protein hydrolysate on fish growth has been discussed in many studies (Cahu et al., 1999; Chalamaiah et al., 2012). The growth of crucian carp (*Carassius auratus gibelio*) was significantly higher in fish fed cottonseed hydrolysate diet compared to a diet containing unprocessed cottonseed meal (Gui et al., 2010). In another study on Nile tilapia, shrimp protein hydrolysate was shown to be a good protein source with no adverse effects on growth and nutrient utilization (Leal et al., 2010). In a study with humans, it was demonstrated that protein hydrolysates show a faster and greater postprandial increase of plasma AAs than their non-hydrolysed equivalents (Morifuji et al., 2010). Pre-digested fish protein was seen to be absorbed quicker by Atlantic salmon (*Salmo salar*) than intact fish protein, which resulted in a faster and higher postprandial peak of essential amino acids in the plasma (Espe and Lied, 1994; Espe et al., 1993). Hydrolysed fishmeal contains protein with short peptides, which can easily dissolve in water, are highly digestible (Chalamaiah et al., 2012) and well utilised for growth (Khieokhajonkhet and Surapon, 2020; Refstie et al., 2004) and thus can be regarded as fast digestible protein.

In the current study, the ADC of CP in the stomach was higher for the fish fed the “starch-diets”. This is in contrast to the study of Harter et al. (2015) in which the fat diet had a higher ADC of CP in the stomach than the starch diet. This contrast between both studies, might relate to various aspects. Firstly, digestion is a dynamic process that relates with the time after consuming a meal. In other words, sampling time after feeding will be important especially for the conditions occurring in the stomach; like the postprandial decline in stomach chyme DM (Bucking and Wood, 2006; Harter et al., 2013), in stomach chyme pH (Saravanan et al., 2013) and in stomach chyme osmolality (Bucking and Wood, 2006), all these observations concerned freshwater fish. In addition, time related differences in digestive enzyme activity might be involved. Furthermore, a hampered mixing of stomach chyme with gastric fluids containing enzymes at the ”fat-diets” may be an explanation for the reduced digestibility in the stomach in current study. This hampered mixing at the “fat-diets” might be related to a high lipophilic characteristic but also to the supplementation of cellulose. Cellulose was added to the “fat-diets” in order to have an equal stomach filing directly after given an equal meal (equal protein and energy consumption), as decreasing stomach pH and DM content is dependent on meal size. In contrast to the current study, Harter et al. (2013; 2015) did not add a dietary filler to their fat-diet. The difference between the “starch-diets” and “fat-diets” regarding DM content and water influx in the stomach were fully comparable. Still though, the difference in stomach ADC of CP between these studies might be related to the addition of a filler in the current study. However, this seems not to be a plausible explanation as non-viscous dietary carbohydrate fillers increase stomach emptying rate of DM, like carboxymethylcellulose in tilapia (Shiau et al., 1988) and wheat bran in rainbow trout (Hilton and Slinger, 1983). However, the addition of indigestible cellulose in the “fat-diets” in the current study is the major reason for the lower ADC of DM in the distal intestine and faeces. As the chyme progresses to the proximal intestine, bile and other pancreatic digestive fluids are released for further digestion, especially for fat. As such, the hydrophobic problem will be solved and the proper interaction between digestive enzymes and nutrients present in the diet is enabled. This is supported by the increased ADC of CP of “fat-diets” in the subsequent compartments as well as the faecal digestibility. This observation is in line with the study of Harter et al. (2015), whereby CP in the fat diet was better digested at the distal part of the GIT of African catfish. However, it is noteworthy that “fat-diets” had the highest overall faecal ADC for CP, fat and ash, a reflection of what occurred at the posterior end of the GIT, which did not previously occur in the anterior part. This implies that absorption took place throughout the whole intestine of African catfish in accordance with previous studies (Bucking and Wood, 2006; Harter et al., 2013), but differences in faecal ADC can be created in the distal part of the intestine.

The effect of fishmeal hydrolysation on CP digestibility observed in the stomach did not directly reflect in the CP digestibility in other GIT compartments and faecal ADC of CP. Considering this outcome, it can be speculated that the effect of dietary composition on the digestion rate of CP in the stomach could be compensated in other compartments of the GIT as the chyme passes through the gut. Thus, lack of differences among the ADC of CP of the different protein diets in the proximal and distal intestine (also faecal ADC) indicate that a prolonged retention time of the non-hydrolysed fishmeal in the stomach resulted in the slow release of AAs and di- and tri-peptides. This resulted in high digestibility (similar to hydrolysed fishmeal) up to the end of the GIT (Chen, 2017). These results indicate that the mechanism of hydrolysis and absorption of proteins was rather similar among both diets in these compartments compared to the stomach.

Currently, fish diets are mostly formulated based of the faecal digestibility of nutrients, which only accounts for the total quantity of dietary nutrients that was apparently absorbed in the GIT (NRC,

2011). This does not take into account the kinetics of protein/nutrient digestion along the gut. As such, it provides less information (compared to kinetics of protein digestion study) on the timing of release and absorption of AAs and nutrients along the GIT after meal ingestion (Liu and Selle, 2015). Information on the kinetics of protein and energy digestion in fish is scarce. Results of the current study showed that the hydrolysation of fishmeal increased the crude protein digestibility in the stomach of African catfish, but this was dependent on the dietary non-protein energy. However, this effect did not translate into other compartments in the gastrointestinal tract and overall faecal digestibility. Replacement of dietary fat by starch increased water addition to the stomach but reduced the passage rate of chyme from the stomach. In African catfish (Harter et al., 2015) and Nile tilapia (Amirkolaie et al., 2006b), replacing dietary fat by starch increased stomach chyme viscosity. Dietary supplementation with viscous non-starch polysaccharides (NSP) reduced the stomach evacuation rate of DM (Nikolopoulou et al., 2011; Storebakken, 1985). In contrast, non-viscous NSP, like cellulose, stimulate gastric emptying (Hilton and Slinger, 1983; Shiau et al., 1988). Exchanging fat by starch in African catfish without cellulose being used as diet filler, strongly increased stomach chyme viscosity coinciding with a reduced increased gastric emptying rate of DM at 2 h postprandial (Harter et al., 2015). Therefore, the viscous nature of starch after water solubility together with the inclusion of cellulose in the "fat-diets" likely caused the lower stomach evacuation rate at the "starch-diets" compared to the "fat-diets", while the "starch-diets" had lower dry matter content compared to the "fat-diets".

High water fluxes inside the GIT are often considered as being not ideal. This might relate to the fact of an association with "diarrheal" like faeces. E.g., Enteritis induced by soybean meal in the distal intestine of salmon often coincides with reduced faecal DM content (Refstie et al., 1999). However, for a proper function of the digestive tract in fish fed dry pellets with a DM content >90%, a proper influx of fluid is essential. Bucking and Wood (2006) clearly showed that in rainbow trout, stomach emptying only starts if stomach chyme DM content is smaller than ~45%. Thus, a proper influx of water is needed to start gut emptying as well as to enable proper mixing of chyme with digestive enzymes, bile etc. From the current study, it was observed that dietary macro-nutrient composition influences the water influx in the stomach. Therefore, it can be concluded that water fluxes, digesta passage rate and the kinetics of digestion along the GIT are dependent on dietary macronutrient composition. Next to dietary composition, it can further be hypothesised that physical pellet characteristics, like water solubility and pellet hydration time, can influence water fluxed in the stomach and also digestive kinetics. As extrusion process conditions determine such pellet characteristics (e.g., Wang et al., 2021), it is worthwhile to assess the relationship between feed process conditions (i.e., physical pellet characteristics) and digestion kinetics.

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Supplemental tables

Supplementary table 4.1 Effect of protein and energy type on the apparent digestibility coefficient (ADC) of nutrients in the stomach proximal and distal intestine of African catfish.

	Diet							
	Non-hydrolysed fishmeal		Hydrolysed fishmeal			*P*-value		
%	SD	FD	SD	FD	SEM	PT	NPE	PT x NPE
Stomach								
Dry matter	9.61	10.08	13.23	13.95	0.92	**	NS	NS
Crude protein	36.89	21.07	41.08	32.46	1.56	**	***	*
Ash	57.10	37.32	57.82	46.96	2.23	*	***	#
Phosphorus	49.81	32.35	41.53	39.51	5.46	NS	NS	NS
Calcium	63.74	32.17	52.30	39.18	6.36	NS	**	NS
Magnesium	79.98	63.13	77.59	68.85	2.95	NS	**	NS
Proximal intestine								
Dry matter	15.85	25.39	8.50	27.24	4.24	NS	*	NS
Crude protein	50.49	45.05	48.70	53.75	3.70	NS	NS	NS
Ash	-35.33	-28.63	-29.18	-29.83	7.42	NS	NS	NS
Phosphorus	49.93	45.85	52.31	55.52	2.73	#	NS	NS
Calcium	1.02	15.67	9.36	20.18	4.93	NS	*	NS
Magnesium	-11.71	2.11	-11.59	-4.55	4.56	NS	#	NS
Distal intestine								
Dry matter	58.05	50.37	58.15	52.13	1.61	NS	**	NS
Crude protein	73.72	76.12	74.16	74.61	1.31	NS	NS	NS
Ash	21.93	20.01	28.72	21.19	4.00	NS	NS	NS
Phosphorus	51.77	49.94	52.45	47.47	2.66	NS	NS	NS
Calcium	18.49	29.17	26.60	24.27	4.66	NS	NS	NS
Magnesium	-25.76	-19.96	-15.40	-35.46	7.70	NS	NS	NS

Presented values are means (n=3) per dietary treatment within each compartment, except for the NH-FM+FD diet in the proximal intestine (n=2). SD, starch diet; FD, fat diet. Error bars indicate standard error of means; PT, protein type (non-hydrolysed fishmeal and hydrolysed fishmeal); NPE, non-protein energy source (starch and fat); NS, not significant; #, $P<0.1$; *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

Supplementary table 4.2 Effect of protein and energy type on the faecal nutrient apparent digestibility coefficient (ADC) of African catfish

	Diet							
	Non-hydrolysed fishmeal		Hydrolysed fishmeal			*P*-value		
%	SD	FD	SD	FD	SEM	PT	NPE	PT x NPE
Dry matter	69.63	62.93	72.17	65.67	1.50	NS	**	NS
Crude protein	86.78	88.67	86.93	89.12	0.38	NS	***	NS
Crude fat	83.57	93.85	85.60	94.51	0.86	NS	***	NS
Ash	47.72	47.30	46.88	53.29	2.28	NS	NS	NS
Phosphorus	68.64	64.27	68.10	68.12	1.60	NS	NS	NS
Energy	72.03	71.06	74.16	73.25	1.29	NS	NS	NS
Calcium	48.48	40.20	49.11	49.50	3.49	NS	NS	NS
Magnesium	59.87	57.12	63.90	61.61	2.43	NS	NS	NS
Total carbohydrate	59.26	29.13	64.48	33.79	2.81	NS	***	NS

Presented values are means (n=3) per dietary treatment. SD, starch diet; FD, fat diet. Error bars indicate standard error of means; PT, protein type (non-hydrolysed fishmeal and hydrolysed fishmeal); NPE, non-protein energy source (starch and fat); NS, not significant; **, $P<0.01$; ***, $P<0.001$.

Supplementary table 4.3 The effect of protein and energy types on the water flux of the chyme in the stomach, proximal and distal intestine of African catfish.

	Diet							
	Non-hydrolysed fishmeal		Hydrolysed fishmeal			*P*-value		
	SD	FD	SD	FD	SEM	PT	NPE	PT x NPE
Stomach								
Chyme dry matter (kg/kg)	0.17	0.21	0.18	0.22	0.01	NS	**	NS
Relative water flux (mL/g of ingested DM)	4.26	3.31	3.88	2.99	0.16	#	***	NS
Amount of Yttrium present (%)[1]	51.4	39.6	47.9	37.9	4.33	NS	*	NS
Proximal intestine								
Chyme dry matter (kg/kg)	0.11	0.12	0.11	0.11	0.01	NS	NS	NS
Relative water flux (mL/g of ingested DM)	2.65	1.70	3.37	2.78	0.63	NS	NS	NS
Total Yttrium in compartment (mg/tank)	0.10	0.15	0.13	0.17	0.02	NS	#	NS
Yttrium content per FI (%)	5.47	8.61	6.69	9.11	1.18	NS	#	NS
Distal intestine								
Chyme dry matter (kg/kg)	0.14	0.16	0.14	0.15	0.00	NS	**	NS
Relative water flux (mL/g of ingested DM)	-4.40	-2.87	-4.88	-3.23	0.51	NS	*	NS
Total Yttrium in compartment (mg/tank)	0.43	0.32	0.48	0.29	0.05	NS	*	NS
Yttrium content per FI (%)	22.9	17.8	25.3	16.0	2.44	NS	*	NS
Total intestine								
Amount of Yttrium present (%)	79.74	62.94	79.90	63.06	5.54	NS	*	NS
Amount of Yttrium excreted (%)	20.26	37.06	20.10	36.94	5.54	NS	*	NS

[1]Amount of Yttrium per compartement expressed as percentage of the amount of Yt consumed during feeding prior to sampling. SD, starch diet; FD, fat diet. Presented values are means (n=3) per dietary treatment within each compartment, except for the NH-FM+FD diet in the proximal and distal intestine (n=2). FI, feed intake; PT, protein type (non-hydrolysed fishmeal and hydrolysed fishmeal); NPE, non-protein energy source (starch and fat); SEM, standard error of means; NS, not significant; #, $P<0.1$; *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

CHAPTER 5

Effect of feeding frequency on performance, nutrient digestibility, energy and nitrogen balances in juvenile African catfish (*Clarias gariepinus*) fed diets with two levels of crystalline methionine

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Abstract

In this study, the effect of feeding frequency and its interaction with crystalline methionine supplementation level on performance, digestion, energy and nitrogen balances was assessed. The experiment had a 2 x 4 factorial design, testing two levels of crystalline methionine (Met) and four feeding frequencies. The two diets contained Met either just fulfilling or exceeding the Met requirement of African catfish (*Clarias gariepinus*). African catfish with initial mean weight of 44 g were allocated to one of four feeding frequencies (six, two, one time (s) per day and two times out of three days) in a recirculation aquaculture system. Fish were fed an equal daily ration for 32 days. Performance parameters, nutrient digestibility, body composition, and nutrient balances were evaluated. Except for digestible nitrogen intake and dry matter of body content, none of the tested parameters was affected by the interaction between dietary Met levels and feeding frequencies. Growth, energy and nitrogen gain were unaffected by feeding frequency as well as dietary Met level. FCR was low, being 0.84 averaged over all treatments. However, feeding frequency affected feed intake and the apparent digestibility coefficient (ADC) of nutrients. Feeding at lowest frequency was accompanied by a lower feed consumption compared to other frequencies (P<0.001). At higher frequency, ADC was higher for macronutrients but lower for phosphorus and magnesium. It was concluded that the asynchronous availability of AA for protein synthesis, which is often suggested to cause a sub-optimal utilization of crystalline AA, was not influenced by feeding frequency. However, feeding at a low frequency hampered daily feed intake of African catfish. Whereas, higher frequency improved nutrient digestibility, though it did not result in improved growth probably due to the higher energy required for maintenance related to physical activities at higher frequency.

5.1 Introduction

Plant protein sources are increasingly used as alternative to marine protein sources in fish diets. However, plant ingredients contain antinutritional factors, which may hamper digestion and metabolism (NRC, 1993). In addition, the amino acid (AA) composition of plant ingredients is less favourable for fish (Ambardekar and Reigh, 2007), because they are often deficient in one or more essential AA (e.g., lysine and methionine). To overcome imbalances in AA composition, crystalline amino acids (CAA) are usually supplemented to diets. Dietary addition of crystalline methionine improved the growth of African catfish when fed diets based on plant-protein (Elesho et al., 2021). Nevertheless, species such as channel catfish (*Ictalurus punctatus*), carp (*Cyprinus carpio*), hybrid striped bass (*Morone saxatalis F X Morone chrysops M*) and tilapia (*Oreochromis niloticus*) have been reported to utilize CAA less efficiently compared to protein-bound AA in the diet (Lumbard, 1997; Nose et al., 1974; Teshima, 1990; Yamada et al., 1981; Zarate et al., 1999). This sub-optimal utilization has been ascribed to the rapid absorption of the CAA in the gastrointestinal tract compared to protein-bound AA, which are more slowly released (Nwanna et al., 2012; Plakas and Katayama, 1981; Zarate et al., 1999). Basically, it is suggested that this difference in moment of absorption may result in quick catabolization of the absorbed CAA and thus lost rather than used for protein synthesis (Ambardekar and Reigh, 2007; Nwanna et al., 2012).

It is assumed that among other factors, feeding frequency can affect the utilization efficiency of CAA. For instance, feeding at a low frequency may negate the optimum utilization of AA in a diet that contains a combination of CAA and purified ingredients (Van den Borne et al., 2006). One way to improve utilization of CAA is feeding multiple times in a day (Ambardekar and Reigh, 2007; Barroso et al., 1999), as this will complement the temporal release of protein-bound AA and at the same time providing more chances for the timely absorption of CAA (Lanna et al., 2016). It was thus hypothesized that increasing feeding frequency will aid the utilization of CAA supplementation in diets deficient in AA, thereby leading to an increased protein deposition in fish. The positive effect of multiple feeding was demonstrated in a study carried out by Zarate et al. (1999) on channel catfish fed diets containing free and protein-bound lysine. A better utilization of free lysine was achieved when the animals were fed five times a day compared to twice a day. Yamada et al. (1981) observed similar results in carp fed free AA from 3 to 18 times daily, as growth increased in proportion to high frequency.

Methionine (Met) is often the first limiting AA in many vegetable proteins, especially legumes (Mai et al., 2006), therefore, its supplementation in fish diets is usually inevitable. In a recent study, we estimated Met requirement of African catfish to range between 18.7 and 21.4 g/kg digestible methionine per unit of digestible protein by feeding fish restrictively twice per day (Elesho et al., 2021). Despite providing nutrients to meet the average daily requirement of animals, the circadian fluctuation in nutrient requirement and availability can be disrupted within a day (Van den Borne et al., 2006). Feeding at low frequency may diminish the match between nutrient supply and requirement within a day, especially when nutrients with low storage capacity in the body are fed e.g., AA (Van den Borne et al., 2006). For instance, when plant diets supplemented with CAA are fed, there may be a temporal excess supply of CAA relative to the requirement and thus a sub-optimal utilization for protein deposition may occur.

African catfish is a species of great and still increasing economic importance in Africa and worldwide (Eyo and Ekanem, 2011; Fagbenro et al., 1999). Several authors have suggested that feeding twice or three times per day is sufficient for the optimum growth of African catfish (Aderolu et al., 2010; Eyo

and Ekanem, 2011; Marimuthu and Muralikrishnan S, 2010). However, none of these studies assessed whether the effect of feeding frequency on growth is dependent on diet composition (crystalline methionine supplementation). Therefore, the objective of the current study was to examine the influence of feeding frequency on digestibility, growth, nitrogen and energy balances of African catfish fed diets supplemented with crystalline methionine. Furthermore, it was assessed if the effect of feeding frequency was dependent on the level of methionine supplementation.

5.2 Materials and methods

5.2.1 Ethics statement and research facility

The study (project number 2018.W.0014.002) was in accordance with the Dutch law on the use of animals (Act on Animal Experiments) for scientific purposes and was approved by the Central Animal Experiments Committee (CCD) of The Netherlands. This experiment was conducted in the research facility of CARUS-ARF at Wageningen University, The Netherlands. Fish were kept and handled in agreement with EU-legislation.

5.2.2 Fish and housing conditions

Mixed sex of African catfish (*Clarias gariepinus*) fingerlings were obtained from a commercial hatchery (Fleuren & Nooijen BV, Nederweert, The Netherlands). At the start of the experiment, 980 fish weighing on average 44 g were randomly assigned (40 fish per tank) into 24 experimental tanks. All tanks were part of a recirculating water system sharing a common reservoir. The system water volume was 5 m^3. Water loss due to evaporation was continuously compensated with the addition of well water. Additional water refreshment was based on NO_3^- removal from the system to keep NO_3^- levels below 500 mg/L. Each tank was equipped with air stones and the water outlet of each tank was connected to a separate swirl separator (AquaOptima AS, column height 44 cm; diameter 24.5 cm) for collection of faeces and spilled feed pellets. Water quality parameters were monitored regularly and maintained within optimal levels for African catfish. Average (SD) measured values over the experimental period were as follows: water temperature 27.3 ± 0.15°C; pH, 7.4 ± 0.19; ammonium, 0.17 ± 0.138 mg/L; nitrite, 0.13 ± 0.019 mg/L; nitrate, 381 ± 33 mg/L; conductivity, 3454 ± 307 mS; and dissolved oxygen concentration, 6.6 ± 0.43 mg/L. Photoperiod was kept at 12 h light: 12 h dark.

5.2.3 Experimental diets

This experiment was designed to assess the effect of feeding frequency and its interaction with crystalline methionine supplementation level on digestion, energy and nitrogen balances. The ingredient composition and proximate analysis of the experimental diets are given in Table 5.1 and 5.2. Two diets were formulated to represent minimal but sufficient supply (required) and oversupply of Met. These experimental diets were formulated to be identical regarding ingredient composition and nutrient concentrations except for the amount of crystalline DL-methionine supplementation and cellulose. The basal diet (Adq-Met diet) was formulated to meet exactly the Met requirement of African catfish (19.2 g/kg crude protein) based on the results of a previous study (Elesho et al., 2021). Since Met can be converted into cysteine (Cys), a small amount of hydrolysed feather meal was included in the Adq-Met diet to avoid low Cys levels. The analysed Cys level was 6.0 g/kg dry matter (DM). Methionine was then added to create a High-Met diet representing an oversupply of AA. In both diets, protein originated mostly from plant protein ingredients (i.e., fishmeal-free diet). The contrast between the Adq-Met and High-Met diet was created by exchanging 3.0 g/kg cellulose for crystalline

DL-methionine. This resulted in an analyzed methionine content of 6.7 g/kg DM (19.3 g/kg crude protein) for the Adq-Met diet and 9.8 g/kg DM (29.1 g/kg crude protein) for the High-Met diet. The contrast between both diets in Met content was equal to the contrast in Met+Cys content. The Met+Cys content was 12.7 and 15.9 g/kg DM at the Adq-Met and High-Met diet respectively. This range in dietary Met content was chosen to test the hypothesis that at low feeding frequencies, the AA utilization is reduced and consequently the amount of Met available for growth at low feeding frequencies is insufficient at the Adq-Met diet but not at the High-Met diet. Yttrium oxide was added as a marker for the determination of the apparent digestibility coefficient (ADC) of nutrients. The experimental diets were extruded as floating pellets (3 to 3.5 mm diameter) using a twin-screw extruder (Wenger, Sabetha, KS, U.S.A) and were produced by the Skretting ARC Norway. After the extrusion process, pellets were coated with 40 g/kg palm oil in order to prevent leaching of the crystalline Met before uptake. Diets were stored at 4 °C throughout the experimental period.

Table 5.1 Ingredient composition of the experimental diets[a]

	Adq Met	High Met
Test ingredients (g/kg)		
DL-Methionine	2.8	5.8
Cellulose	3.0	0.0
Basal ingredients (g/kg)		
Soybean meal	50	
Sunflower meal	25	
Faba bean (dehulled)	150	
Lupine meal	100	
Pea meal	150	
Canola meal	75	
Hydrolysed feather meal	70.7	
Wheat	100	
Wheat flour	27.7	
Gelatinized wheat starch	100	
Fish oil	45	
Palm oil	40	
Mono calcium phosphate	36	
Calcium carbonate	10	
Yttrium oxide	1.0	
Vitamin and minerals[b]	4.8	
Lysine HCl	5.2	
L-Threonine	2.8	
L-Tryptophan	1.0	

[a]The ingredient composition of the 2 experimental diets were similar except for the content of cellulose and DL-methionine.
[b]Skretting ARC closed formula for vitamin and trace mineral premix to meet requirements specified for freshwater fish, according to NRC (2011) recommendation.

5.2.4 Feeding and Sampling

At the beginning of the experiment, 20 fish were randomly selected and euthanized by an overdose of phenoxy-ethanol (1.0 mL/L), to determine proximate composition. Before stocking of the tanks, fish were counted while being sedated (0.25 mL/L phenoxy-ethanol) and the total biomass was recorded. The response of African catfish to both Met diets was compared under four feeding frequencies (FF).

These were (A) “6/1d-FF”, feeding six times per day, at 8:00, 12:00, 16:00, 20:00, 24:00 and 4:00 h; (B) “2/1d-FF”, feeding two times per day, at 8:00 and 16:00 h; (C) “1/1d-FF”, feeding one time per day at 12:00 h; (D) “2/3d-FF”, feeding two times out of three days, at either 8:00 h or 16:00 h with a 36 h interval. These feeding frequencies resulted in an interval between successive feeding of 4, 12, 24 and 36 h. Each combination of diet and feeding frequency was randomly assigned to the experimental tanks in triplicate. During the 32-day experimental period, the daily food allowance was divided into the appropriate number of equal amounts for each feeding frequency. This was done to minimize the variation in response parameters due to variability in feed intake. Fish were hand-fed, except for the feedings of the “6/1d-FF” treatment at 20:00, 24:00 and 4:00 h, where feed was provided using a belt feeder (each feeding period lasted for 30 minutes). Fish were fed based on their metabolic body weight. Metabolic body weight was calculated as $BW^{0.8}$ with BW expressed in kg. The feeding level was fixed at 14.5 $g/kg^{0.8}$/d based on the mean initial weight over all diets. Furthermore, to ensure that the feeding levels per fish were equal for all treatments, daily feed rations per tank were increased based on an expected growth using a FCR of 1. In the case of mortality, the daily feeding rations were adjusted for the number of fish in the tank. During the first three days, the feeding level was gradually increased from 20% to 100% of the intended ration. After each meal, the uneaten feed was weighed and the spilled pellets, which were collected by the swirl separators 15min after feeding was counted per tank. For proximate analysis of the feed, a representative sample from each diet was taken and stored at 4 °C.

Faeces were collected for digestibility measurements from week 2 till the end of the trial, 5 days per week (Monday - Friday), using detachable collection bottles (250 mL) connected to settling tanks. The faecal collection bottles were submerged in ice-filled styrofoam boxes to reduce microbial degradation. Faeces were collected overnight and stored daily in the morning in aluminum trays at -20 °C for further analysis. Faeces were pooled per tank. Throughout the experiment, fish behavior was monitored and visually inspected for discernible signs of deformities. Mortality was checked 30 min prior to each hand-feeding period. At the end of the feeding trial, fish were sedated and batch weighed after 24 h of food deprivation. Ten fish per tank were randomly selected and euthanized by an overdose of phenoxy-ethanol (1.0 mL/L) for final body composition analysis.

5.2.5 Chemical analyses on feed, faeces and fish body composition

Analyses were performed on the diets, whole fish samples and faeces samples. Before chemical analysis, frozen fish samples were sawed into small pieces and homogenized by mincing twice through a 4.5 mm-screen grinder (Gastromaschinen, GmbH model TW-R 70; Feuma). Dry matter (DM), ash and crude protein (CP) were analyzed using a portion of the freshly sampled minced fish. The remainder of the samples were freeze-dried for later determination of crude fat and energy. Faecal samples were freeze-dried, then manually pulverized through a 1 mm screen sieve. Feed pellets were grinded by a grinding machine. Fish, faeces, and feed samples were analyzed in triplicate using the same analytical method. DM content was determined by drying the samples to constant weight at 103 °C for at least 4 h (ISO 6496, 1983) and ash content by incineration in a muffle furnace at 550 °C overnight (ISO 5984, 1978). The Kjeldahl method was used for nitrogen analysis (ISO 5983, 1979) and CP contents calculated as N content times 6.25. Crude fat analysis was determined using the Soxhlet method (ISO 6492, 1999). Energy was measured using an adiabatic bomb-calorimeter (C7000 IKA®, IKA analysentechnik, Weitershem, Germany; ISO 9831, 1998). Yttrium, phosphorus, calcium and magnesium in feed and faeces were determined from the ash by using inducted coupled plasma mass spectrometry according

to the standard NEN 15510 (ICP-MS, 2007). Amino acids (excluding tryptophan) were analyzed by Skretting ARC, Norway, using an automatic amino acid analyzer (Biochrom 30+, Biochrom Ltd, Cambridge, UK) and the methods described in the COMMISSION REGULATION (EC) No 152/2009 (Council, 2009).

Table 5.2 Analysed amino acid and nutrients composition of experimental diets (g/kg DM)

	Diets	
	Adq Met	**High Met**
EAA		
Arginine	27.2	27.0
Histidine	7.9	7.7
Isoleucine	14.8	14.5
Leucine	25.2	24.8
Lysine	22.2	21.9
Methionine	6.7	9.8
Phenylalanine	15.4	15.3
Threonine	16.3	15.9
Valine	17.2	16.9
NEAA		
Alanine	14.8	14.1
Aspartic acid	32.5	31.9
Glutamic acid	57.7	56.6
Cysteine	6.0	6.1
Glycine	17.5	17.0
Proline	19.7	19.5
Serine	20.5	20.6
Tyrosine	8.7	9.0
SAA	330	329
Nutrients		
Dry matter	914	911
Crude protein	346	336
Crude fat	124	127
Ash	75.0	75.1
Phosphorus	14.7	14.7
Energy (kJ/g)	21.1	20.8

Amino acid composition was determined by the Skretting (ARC) laboratory Norway. DM: dry matter; EAA: Essential amino acids; NEAA: Non-essential amino acids; SAA: Sum of amino acids, which is without tryptophan since tryptophan was not analyzed.

5.2.6 Calculations

Calculations of performance parameters (daily weight gain, specific growth rate, feed conversion ratio on DM basis and survival) are given in Table 5.3. The apparent digestibility coefficient (ADC) of macronutrients were calculated according to the following formula described by Cheng and Hardy (2002) using yttrium oxide as inert marker; ADC (%) = 100 × [1 − (yttrium concentration in the feed × concentration nutrient in faeces)/(yttrium concentration in the faeces × concentration nutrient in feed)]. The concentrations of yttrium and nutrients were expressed on DM basis. Nitrogen (N) and energy balance parameters were calculated per treatment and expressed as; mg/d and kJ/d, respectively (summarized in Table 5.3). A detailed description of the calculation of balance parameters

was previously provided by Saravanan et al. (2012). The utilization efficiency of digested protein was calculated as the amount of nitrogen retained as percentage of the digestible nitrogen intake.

Table 5.3 Fish performance and nutrient balances calculations

Parameters	Formula
Performance parameters	
Growth (g/d)	$Growth = (Wf - Wi)/t$
Specific growth rate (SGR, %/d)	$SGR = 100\ x\ (ln(Wf) - ln\ (Wi))/t$
Feed conversion ratio on DM basis (FCR)	$FCR = (FI\ x\ DMfeed)/growth$
Survival (%)	$Survival = NoFishi/NoFishf\ \ x\ 100$
Nitrogen balance (mg/d)	
Gross nitrogen intake (GNI)	$GNI = FI\ x\ Nfeed$
Digestible nitrogen intake (DNI)	$DNI = (GNI\ x\ ADCcp)/100$
Branchial and urinary loses (BUN)	$BUN = DNI - RN$
Retained nitrogen (RN)	$RN = ((Wf\ x\ CPf)/\ 6.25) - ((Wi\ x\ CPi)/6.25)$
Utilization efficiency of digested protein (EDP; %)	$EDP = \ RN/DNI\ x\ 100$
Energy balance (kJ/d)	
Gross energy intake (GEI)	$GEI = FI\ x\ Efeed$
Digestible energy intake (DEI)	$DEI = (GE\ x\ ADCe)/100$
Metabolizable energy intake (MEI)	$\mathrm{MEI}\ =\ \mathrm{DE} - \mathrm{BUE}$
Branchial and urinary energy losses (BUE)	$BUE = \ (BUN\ \times\ 24.9)\ /\ 1000$
Retained energy (RE)	$RE\ =\ Wf\ x\ Ef - Wi\ x\ Ei$
Heat production (HP)	$HP = \ RE\ -\ ME$
Retained energy as protein (REpro)	$REpro = \ (RN\ x\ 6.25)\ x\ 23.7$
Retained energy as fat (REfat)	$REfat\ =\ RE\ -\ REpro$

Wi, initial fish weight (g); Wf, final fish weight (g); t, duration of experiment (d); FI, feed intake (g/d); NoFishi and NoFishf, respectively initial and final number of fish per tank; ADCcp, apparent digestibility coefficient of crude protein (%); CPf and CPi, respectively final and initial crude protein content of the fish (g/kg); DMfeed, dry matter content of feed (g/g); Nfeed, nitrogen content of feed (mg/g); Efeed, energy content of feed (kJ/g); Ef and Ei, respectively final and initial energy content of feed (kJ/g); ADCe, apparent digestibility coefficient of the energy (%); 23.7 kJ/g, energy content of protein; 24.9 kJ/g N, energy concentration of NH_3-N as calculated by (Bureau et al., 2003), assuming all N was excreted as NH_3-N.

5.2.7 Statistical analysis

Data were analysed by two-way ANOVA to test for the effects of feeding frequency and dietary methionine level. Following a significant ANOVA result, means were compared by a multiple comparisons test using Tukey's honest significant difference (HSD). All statistical analyses were performed by using SPSS Statistics, version 23.0 for Windows (IBM Corp., Armonk, NY, USA).

5.3 Result

This experiment was designed to assess the effect of feeding frequency and its interaction with crystalline methionine supplementation level on digestion, energy and nitrogen balances. Therefore, two diets with different amounts of CAA were fed to African catfish at four different FFs. The experiment was aimed to have equal feed intakes (FI) in all treatments. However, during the first week, some treatment groups were unable to cope with the set feeding level, especially fish fed at 2/3d-FF. As a consequence, we reduced the rate of increase in feeding level to allow equal ration among treatments. However, only three treatments (1/1d-FF, 2/1d-FF and 6/1d-FF) were able to reach the

targeted feeding level after 12 days of the start of the experiment. But the group of fish at 2/3d-FF still lagged behind the other groups and still had substantial feed rejections. These treatments were able to consume only 75% of the targeted feed ration. Consequently, the 2/3d-FF treatment was excluded from the two-way ANOVA and the effect of the diet within the 2/3d-FF was tested by one-way ANOVA. Figure 5.1 shows the mean values of FI per FF. Averaged over diets, the group fed 2/3d ate 10.9 g/kg$^{0.8}$/d of feed and the other FF treatments ate 13.1 g/kg$^{0.8}$/d.

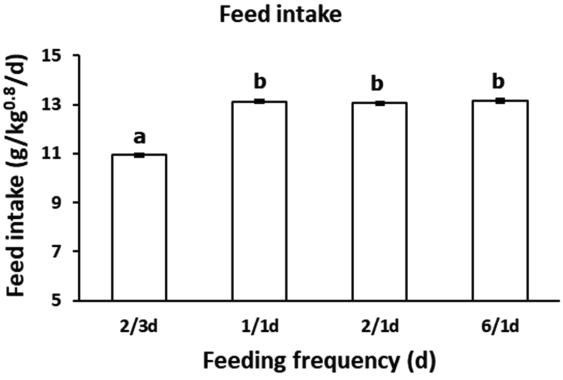

Figure 5.1 Effect of feeding frequency (FF) on feed intake in African catfish. Presented values are means over both experimental diets. Means lacking a common letter differ (P<0.05). The applied feeding frequencies were: 2/3d, feeding two times in three days; 1/1d, feeding one time per day; 2/1d, feeding two times per day; 6/1d, feeding six times per day.

Except for digestible nitrogen intake and dry matter body content, no interaction effect between dietary Met and FF was observed for any of the measured parameters (Table 5.4-5.6). The growth performance of African catfish fed the two diets under varying FF is presented in Table 5.4. Daily gain and specific growth rate were neither affected by the dietary Met treatment nor the FF treatment. Similarly, FCR on DM basis was unaffected by the applied treatments. Averaged over all treatments, FCR was 0.84. Although, no effect of treatment occurred on survival, fish fed 6 times a day had the highest survival rate.

Means of apparent digestibility coefficient (ADC) of nutrients of all experimental treatments are given in Supplementary Table 5.1. For all the measured nutrient ADCs, there was no effect of the dietary Met treatment (P>0.1). In contrast, all nutrient ADCs were affected by FF (P<0.05; Supplementary Table 5.1). The main effect of feeding frequency on nutrient ADCs is visualized in Figure 5.2. Increasing the feeding frequency from 2/3d to 6/1d increased the digestibility of fat from 90.9% to 94.2% (P<0.01) and of protein from 86.0% to 88.7% (P<0.01). In contrast, phosphorus and magnesium digestibility decreased with increasing feeding frequency (P<0.05).

For the three highest feeding frequencies, there was no effect of dietary Met and FF on fish body composition (Table 5.5) except for energy content. Feeding frequency had a significant effect on energy content with slightly higher value recorded for fish fed at 6/1d. This was also reflected in a tendency for a higher fat content at 6/1d-FF. At the lowest feeding frequency, 2/3d-FF, dietary Met level affected the ash content (P<0.001), but at the other FF, dietary Met level had no impact on the ash content.

Table 5.4 Growth performance of African catfish during the experimental period.

	Diets										Diets			
	Adq-Met			High-Met							Adq-Met	High-Met		
Feeding frequency	**6/1d**	**2/1d**	**1/1d**	**6/1d**	**2/1d**	**1/1d**		***P*-value**			**2/3d**	**2/3d**		***P*-value**
Feeding interval	**4h**	**12h**	**24h**	**4h**	**12h**	**24h**	**SEM**	**D**	**FF**	**D x FF**	**36h**	**36h**	**SEM**	**D**
Initial BW (g)	43.7	43.6	43.5	43.7	43.7	43.5	0.074	ns	ns	ns	43.4	43.3	0.25	ns
Final BW (g)	103	103	103	101	98	102	1.23	ns	ns	ns	87	85	0.48	#
Survival (%)	97.5	94.2	92.5	95.0	95.0	93.3	1.30	ns	ns	ns	92.5	94.2	1.18	ns
Feed Intake DM (g/d)[a]	1.52	1.49	1.49	1.50	1.48	1.52	-	-	-	-	1.19	1.14	-	-
Growth (g/d)	1.85	1.86	1.86	1.78	1.71	1.82	0.039	ns	ns	ns	1.35	1.31	0.013	#
SGR (%/d)	2.76	2.77	2.78	2.69	2.61	2.74	0.043	ns	ns	ns	2.23	2.18	0.018	ns
FCR (g/g)	0.82	0.81	0.80	0.84	0.88	0.84	0.022	ns	ns	ns	0.88	0.88	0.009	ns

d, day; D, main effect of diet; FF, main effect of feeding frequency; D x FF, interaction effect between diet and feeding frequency; 6/1d, feeding six times per day; 2/1d, feeding two times per day; 1/1d, feeding one time per day; 2/3d, feeding two times in three days; SGR, specific growth rate; FCR, feed conversion ratio; BW, body weight; DM, dry matter; SEM, standard error of mean; ns, not significant $P>0.1$; #, $P<0.1$.
[a]Feed intake was not statistically analyzed because fish were fed restrictively.

Table 5.5 The effect of dietary methionine and feeding frequency on body composition of African catfish on dry mater basis (DM)

		Diets										Diets			
		Adq-Met			High-Met							Adq-Met	High-Met		
Feeding frequency	**Initial**	**6/1d**	**2/1d**	**1/1d**	**6/1d**	**2/1d**	**1/1d**		***P*-value**			**2/3d**	**2/3d**		***P*-value**
Feeding interval		**4h**	**12h**	**24h**	**4h**	**12h**	**24h**	**SEM**	**D**	**FF**	**D x FF**	**36h**	**36h**	**SEM**	**D**
Dry matter	229	257	262	259	261	260	262	1.24	#	ns	*	258	253	2.61	ns
Protein (g/kg DM)	647	604	586	597	601	595	597	5.84	ns	ns	ns	594	610	6.09	ns
Fat (g/kg DM)	246	300	302	294	304	300	294	3.32	ns	#	ns	292	285	3.59	ns
Ash (g/kg DM)	114	111	110	114	112	116	114	2.19	ns	ns	ns	118	113	0.35	***
Energy (KJ/g DM)	24.3	25.8	25.7	25.3	25.9	25.6	25.4	0.14	ns	*	ns	25.3	25.4	0.16	ns

d, day; D, main effect of diet; FF, main effect of feeding frequency; D x FF, interaction effect between diet and feeding frequency; 6/1d, feeding six times per day; 2/1d, feeding two times per day; 1/1d, feeding one time per day; 2/3d, feeding two times in three days; SEM, standard error of mean; ns, not significant $P>0.1$; #, $P<0.1$; *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

Nitrogen and energy balances of African catfish fed experimental diets under different FFs are shown in Table 5.6. Digestible nitrogen, energy and metabolisable energy intake were significantly higher in the 6/1d-FF compared to other FF (P<0.001). This effect was more pronounced in the fish fed the Adq-Met diet. With the increase in dietary Met level, BUN significantly decreased in fish fed High-Met diet at 2/3d-FF (P<0.01). In contrast, both dietary Met and FF did not affect retained nitrogen and energy (P>0.05). The utilization efficiency of digested protein was not influenced by FF. Only a tendency for a reduced utilization efficiency of digested protein was observed in fish fed the Adq-Met diet compared to the High-Met diet in the 2/3d-FF treatment (Table 5.6).

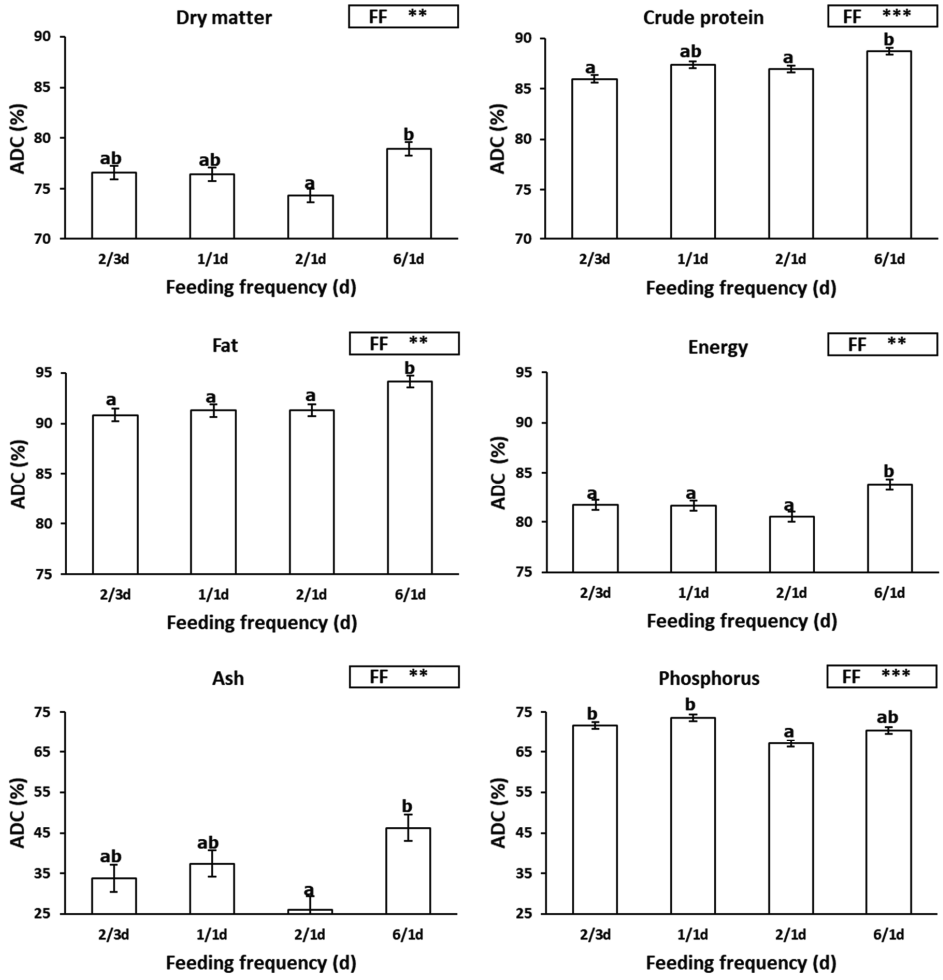

Figure 5.2. Effect of feeding frequency (FF) on apparent digestibility coefficient (ADC) (%) of nutrients in African catfish. Presented values are means over both experimental diets. Means lacking a common letter differ significantly (P<0.05). The applied feeding frequencies were: 2/3d, feeding two times in three days; 1/1d, feeding one time per day; 2/1d, feeding two times per day; 6/1d, feeding six times per day.

Table 5.6 The relationship between dietary treatments and nitrogen and energy balances in African catfish.

	Diets										Diets			
	Adq-Met			High-Met							Adq-Met	High-Met		
Feeding frequency	6/1d	2/1d	1/1d	6/1d	2/1d	1/1d		*P*-value			2/3d	2/3d		*P*-value
Feeding interval	4h	12h	24h	4h	12h	24h	SEM	D	FF	D x FF	36h	36h	SEM	D
Nitrogen balance (mg/d)														
Gross nitrogen intake[a]	83.7	82.6	82.2	80.6	79.7	81.7	0.21	-	-	-	65.9	61.5	0.07	-
Digestible nitrogen intake	74.5	72.0	72.1	71.3	69.1	71.1	0.23	***	***	*	56.6	52.9	0.41	**
Branchial urinary losses	25.7	23.8	23.4	23.1	23.9	22.5	0.99	ns	ns	ns	21.2	18.2	0.44	**
Retained nitrogen	48.7	48.2	48.7	48.2	45.2	48.6	1.01	ns	ns	ns	35.4	34.7	0.68	ns
Utilization efficiency of digested protein (%)	65.4	66.9	67.5	67.6	65.4	68.4	1.44	ns	ns	ns	62.6	65.5	0.90	#
Energy balance (kJ/d)														
Energy intake[a]	31.9	31.4	31.3	31.1	30.8	31.6	0.08	-	-	-	25.1	23.8	0.03	-
Digestible energy intake	26.8	25.5	25.6	25.9	24.6	25.6	0.13	**	***	#	20.6	19.3	0.20	*
Metabolisable energy intake	26.2	24.9	25.0	25.4	24.0	25.1	0.13	**	***	ns	20.0	18.9	0.21	*
Heat production	12.3	10.5	11.3	11.4	10.9	11.3	0.26	ns	#	ns	9.74	9.22	0.29	ns
Retained energy	13.9	14.4	13.7	13.9	13.1	13.8	0.29	ns	ns	ns	10.31	9.64	0.21	#
Retained energy as protein	7.22	7.14	7.22	7.15	6.70	7.20	0.15	ns	ns	ns	5.25	5.13	0.10	ns
Retained energy as fat	6.65	7.25	6.51	6.78	6.44	6.62	0.18	ns	ns	ns	5.06	4.51	0.23	ns
Maintenance energy requirement (kJ/kg$^{0.8}$/d)	47.1	31.6	38.8	40.4	40.1	38.9	3.53	ns	ns	ns	43.9	40.9	2.41	ns

d, day; D, main effect of diet; FF, main effect of feeding frequency; D x FF, interaction effect between diet and feeding frequency; 6/1d, feeding six times per day; 2/1d, feeding two times per day; 1/1d, feeding one time per day; 2/3d, feeding two times in three days; SEM, standard error of mean; ns, not significant $P>0.1$; #, $P<0.1$; *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.[a]Nitrogen and energy intake were not statistically analyzed because restricted feed intake was applied.

5.4 Discussion

This study was conducted to assess the interaction effect of feeding frequency and dietary crystalline methionine level on nutrient digestibility, nitrogen and energy balances in African catfish. Thereby, it investigated the underlying factors causing sub-optimal AA utilization in fish, especially when plant ingredients are used as protein source. Some authors have reported that this problem is often caused by the asynchronous availability of dietary protein-bound AA and CAA supplemented to an AA-deficient diet (Ambardekar and Reigh, 2007; Zarate et al., 1999). Dietary CAA tend to be more quickly released and absorbed by the gastro-intestinal tract than protein-bound AA. This is believed to result in the premature catabolism of CAA (Batterham, 1974), thereby reducing their utilization.

It has been reported that fluctuations in nutrient availability may be influenced by the feeding patterns (Van den Borne et al., 2006). This led to the hypothesis that CAA utilization might not be optimal if the feeding frequency is low. To test this hypothesis, two diets with methionine contents, which is adequate or exceeded the methionine requirement of African catfish (Adq-Met versus High-Met diet), were tested under varying feeding frequencies. Contrary to our expectation, a low feeding frequency did not hamper the utilization efficiency of digested protein of African catfish when fed the Adq-Met diet (Table 5.6). This observation was supported by the absence of an interaction effect between FF and dietary CAA supplementation for all tested parameters. Furthermore, at the lowest FF (2/3d-FF with a feeding interval of 36h), none of the growth, energy and nitrogen balance parameters were improved by the supplementation of Met with the exception of utilization efficiency of digested protein, which tended to be higher for the High-Met diet. This implies that synchrony of dietary CAA with protein-bound AA is not affected by feeding frequency in African catfish. In contrast to the current observation, positive effects of feeding frequency on nutrient synchronization have been demonstrated in other farmed animals (Batterham, 1974; Van den Borne et al., 2006). For example, increasing feeding frequency increased the efficiency of digestible protein in pre-ruminant calves when fed non-clotting protein sources under varying feeding frequencies (Van den Borne et al., 2006). In pigs, increasing the feeding frequency from once daily to six times per day improved their response to a lysine-deficient diet supplemented with free lysine (Batterham, 1974). Similar to the results of the current study, channel catfish and common carp have shown that feeding frequency did not improve utilization efficiency of supplemental lysine and methionine respectively (Nwanna et al., 2012; Zarate et al., 1999).

Fish in the present study received similar amounts of feed in all treatments except for the 2/3d-FF treatment group. Feed rejection was observed in this group during the first part of the experiment. In the original design, it was planned to give the same ration across treatments and thus, the amount of feed per feeding moment (i.e., meal) increases as FF reduces. Consequently, this increased meal size may have led to the overloading of the gut by the bulk supply of feeds. The current feed intake results of African catfish (Figure 5.1) suggest that feed intake is hampered when the FF is reduced below once daily. This was unexpected beforehand, as fish are known to be capable of adjusting their stomach volume to accomodate food in order to compensate for the period of starvation (Jobling, 1982), when fish are fed to apparent satiation under different regimes. Feeding fish less frequently for a longer time can lead to an increased gut capacity, which will result in hyperphagia (Jobling, 1982; Rouhani, 1993). This is a situation whereby fish try to adapt and adjust to reduced access to feed by consuming more feed per meal (Eyo and Ekanem, 2011). To which extent hyperphagia may occur varies among species, ages and sizes (Okomoda et al., 2019). Furthermore, the response of fish to varying feeding frequencies

has been linked to the size of its stomach (Pillay and Kutty, 2005). This is because smaller fish size with a smaller stomach do require more frequent feeding for maximum growth to be attained compared to larger fishes (Okomoda et al., 2019). The fact that juvenile fish were used in the current study might explain the feed rejection at the low FF. Studies conducted on other fish species have shown that feed consumption and growth generally increased with feeding frequency up to a given limit (Aderolu et al., 2010; Basçinar et al., 2001; Lanna et al., 2016; Wang et al., 1998). However, more studies are required to investigate the interaction between feed intake and feeding frequency in fish.

In the current study, increasing the feeding frequency improved nutrient digestibility regardless of the dietary Met level. Usually, nutrient digestibility is improved when fish are exposed to challenging conditions, which often relates to a reduced feed intake. For example, in Nile tilapia (*Oreochormis niloticus*), exposure to hypoxia (Tran-Duy et al., 2012) as well as exposure to brackish conditions (Tran-Ngoc et al., 2017) leads to higher nutrient digestibility. However, in the current study the opposite was found, feed intake was lower at the lowest feeding frequency, which coincided with the lowest macronutrient ADCs. In general, macronutrients ADCs were highest in the fish fed six times per day (Figure 5.2). This may be due to close intervals in feed availability, which resulted in increased enzymatic activities for optimum digestion (Zhao et al., 2016). In line with our result, a study on common carp where two feeding frequencies (twice daily vs. continuous feeding) were applied observed increased protein digestibility under continuous feeding (Nwanna et al., 2012). Furthermore, Zhou et al. (2003) reported an improved protein and energy digestibility in gibel carp (*Carassius auratus gibelio*) when feeding frequency was increased from two to four meals per day. In contrast to these findings, there are several studies in which feeding frequency had no effect on nutrient digestibility (Amadou et al., 2019; Charles et al., 1984; Marian et al., 1982; Zhao et al., 2016). These discrepancies between studies can stem from both differences in the range of applied feeding frequencies or diet formulations. In contrast to macronutrients, the digestibility of minerals such as phosphorus and magnesium was lower at higher frequencies when compared to lower feeding frequencies (Figure 5.2). The reason for this observation is unclear but may be due to changes in stomach pH induced by feeding frequency. Postprandial stomach pH declines with time after meal (Saravanan et al., 2013). Moreover, stomach pH is important in dissolving minerals for absorption. Therefore, it can be suggested that frequent feeding might result in higher average pH throughout the day. However, the impact of feeding frequency on stomach chyme characteristics of African catfish requires further assessment.

African catfish fed 2/3d-FF in this study showed low nutrient digestibility. This could be as a result of an overload of the digestive enzymes with bulk amounts of feed, resulting in a reduction of nutrient absorption capacity and digestibility (Staessen et al., 2020b). Only a few studies have examined the effects of feeding fish less than once per day on nutrient digestibility (Li and Lucas, 2017). Feeding channel catfish every other day was not found to significantly influence nutrient ADC (Li and Lucas, 2017). In the current study, fish fed at 2/1d-FF had the lowest nutrient digestibility (average over both diets). This implies that feeding more frequently than 2/1d is optimal for a good digestibility. Earlier studies on performance in African catfish suggested that feeding twice or three times per day is optimum for growth (Aderolu et al., 2010; Eyo and Ekanem, 2011; Marimuthu and Muralikrishnan S, 2010). However, these studies were done by applying satiation feeding and no digestibility measurements were carried out. In addition, the frequencies applied in those studies ranged from one time to three times per day. Feeding intervals have been reported to strongly correlate with gastric

evacuation time (Huebner and Langton, 1982; Liu and Liao, 1999; Zhao et al., 2016), as such, increasing the frequency would have probably influenced their final outcome by feed intake.

Although, a high FF improved digestibility, growth remained nearly unchanged (Table 5.4). Yet, fish doubled in size at the end of the experiment, indicating a favourable rearing condition for improved growth. The absence of a FF effect on growth might be due to the impact of FF on endogenous faecal losses. If endogenous faecal losses were higher in less frequently fed fish, the true nutrient ADC might have been similar and consequently also growth. However, this hypothesis needs further testing. Lack of differences in growth among treatments may also be explained by the activities of the fish during feeding. At the lower FFs, African catfish were seldomly resting with their tail at the bottom of the tank, and this behaviour was frequently recorded for fish in the 2/3d-FF group. Subjective observations during this experiment suggested that fish fed at the highest FF tended to be more active compared to fish from the other FFs. This might also explain the higher numerical values for maintenance energy requirement recorded for this group (Table 5.6), which is an indication that more nutrients and energy were diverted into physical activity rather than growth. Furthermore, heat production was higher for the fish fed on a 6/1d regime, which may be related to energy losses due to swimming activities (NRC, 1993). In line with this study, several studies have reported that increasing feeding frequency had no significant effect on the growth of fish (Amadou et al., 2019; Lanna et al., 2016; Sveier and Lied, 1998). Lanna et al. (2016) reported that feeding Nile tilapia (*Oreochromis niloticus*) a low-protein diet supplemented with CAA under different feeding regimes had no effect on growth. Similarly, Atlantic Salmon (*Salmo salar*) fed under two feeding regimes showed no differences in growth and feed efficiency (Sveier and Lied, 1998). In contrast, a GIFT Nile tilapia fed 6 times per day grew better than when fed only two times per day (Zhao et al., 2010). Likewise, Zarate et al. (1999) confirmed that increasing the feeding frequency from two to five times per day significantly increased the growth of channel catfish fed a diet supplemented with free lysine. Differences in the outcome of studies could be attributed to the differences in duration of the experiments and quantity of feed consumed in each frequency group (Lanna et al., 2016), but most likely due to differences across species and feed intake behaviour (e.g., filter feeders versus prey swallowers).

At both applied dietary methionine supplementation levels, performance, nitrogen and energy balance parameters were unaffected by feeding frequency. However, feeding frequency had an impact on feed intake and nutrient digestibility in African catfish. A higher feeding frequency increased the digestibility of nutrients, but this did not translate into increased growth, most likely due to increased maintenance energy requirements because of higher physical activity of the fish.

Acknowledgment

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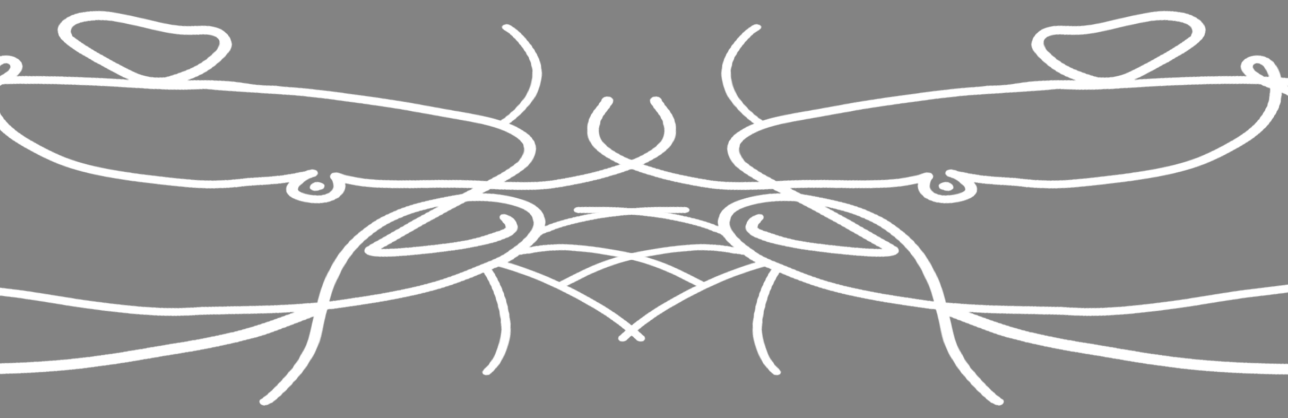


CHAPTER 6

General Discussion

6.1 Introduction

Protein is an expensive component of fish feed and also an important macronutrient for growth and development. Therefore, it is important to accurately determine the protein requirements for each species and life stage cultured. Historically, the supply of optimum dietary protein requires knowledge on 1) the protein requirements of the fish 2) the protein content of feedstuffs. In recent times, this process has shifted to quantifying the amino acid requirement of fish and the AA content of feedstuffs, respectively. This is because fish, like other animals, do not have a true protein requirement but require a well-balanced mixture of essential and non-essential amino acids. Consequently, the quality of protein depends on its AA content and digestibility. Currently, the protein evaluation in fish feeds is mostly based on the concentration and digestibility of protein in the selected ingredients. This may not be the accurate representation of fish needs due to the variability in the digestibility of crude protein and that of individual amino acids. Dietary protein quality may vary when different ingredients are used in the feed formulation as both the AA composition and digestibility may vary among different ingredients used for feed formulation. Compared to other farm animals, fish diets are rarely evaluated using digestible AA data. The latter method of evaluation has been adopted in pigs and poultry for decades but is yet to be fully explored in fish. Therefore, this thesis assessed the digestibility of the amino acids in ingredients in order to improve protein evaluation of fish feeds. Furthermore, the digestible AA requirement and the factors that affect optimum AA utilization in fish were explored. For the former, a study (**chapter 3**) was designed to compare the currently used approach (i.e., AA requirement based on crude protein) with the digestible AA requirement. **Chapter 2** of this thesis addressed the question whether the digestibility of AAs is equal among different AAs, as well as to the overall crude protein. Because of the similarity between the digestibility of crude protein and the total sum of AAs found in **chapter 2**, the feasibility of estimating/predicting AA digestibility by using the CP ADC data will be further explored in this chapter.

As aforementioned (**chapter 1**), an essential aspect of protein evaluation is the assessment of the impact of protein sources on the protein utilization efficiency. To what extent digestible nutrients are ultimately utilised by fish has remained unclear. Indeed, obtaining maximum use from a feed supplied is of utmost importance to fish culturists and the environment. Therefore, increasing protein utilization is considered an important strategy to optimize the use of dietary amino acids thereby reducing the aquaculture footprint. In **chapter 1**, it was hypothesized that the sub-optimal utilization of dietary AA is caused by nutrient asynchrony. Our study touched on some aspects to verify this hypothesis (**chapter 4 and 5**). However, some unresolved questions about the factors that can affect the utilization efficiency of dietary AA will be further discussed in this section. Finally, insight is provided for future research, including implications of research outcomes for current and future fish and aquafeed production.

6.2 Determination of amino acid requirements

The AA digestibility of a diet is not 100%, yet, in spite of this, the amino acid requirements as reported by NRC (2011) are based on the assumption that dietary AAs content are completely digestible, hence, a safety margin was excluded. This assumption spread through the notion that EAA requirement values in literature are digestible amino acid requirements (NRC, 1993). This statement may be true only if the whole diet contains synthetic substances, and indeed, the majority of these studies used synthetic protein ingredients which are highly digestible (e.g., caseine and gelatine). However, in practical

feedstuffs, AA are usually not only synthetic and thus amino acids digestibility may be significantly lower than in experimental diets (Cho and Bureau, 1997). Therefore, the best unit to express requirement estimates (i.e., either digestible amino acid per crude protein (mg DAA/CP) or amino acid per digestible crude protein (mg AA/DCP)) remains unclear. This information is critical for precise feed formulation.

In this thesis, we investigated whether there is a significant variation in how AA requirement estimates are expressed, thereby using methionine as a case study. Results revealed only a slight difference in methionine requirement expressed on a digestible methionine or on crude protein basis (**chapter 3**). For instance, the methionine requirement of African catfish for optimum growth was 18.7 g/kg when expressed as g digested methionine per kg of digestible protein (dMetDP). Within the same experiment, this requirement was 19.2 g/kg when expressed as g methionine per kg of crude protein (AA/CP). Although, the estimations for dMetDP and AA/CP are nearly identical, the observed variability cannot be overlooked. These differences may occur when there is a small difference in methionine digestibility and crude protein digestibility. In addition, the quality of ingredients may influence such outcomes, a topic that will be further discussed in this chapter. Therefore, such heterogeneity still supports the need to introduce a protein evaluation method that estimates AA requirement based on digestible AA per unit of digestible crude protein.

Furthermore, this method of protein evaluation should take into account the impact of the experimental design on requirement estimates. For instance, the mathematical models used for estimating requirements could pose a huge impact on the outcome. Common models used in the field of fish nutrition include broken-line, regression (quadratic, sigmoid, and saturation kinetics models), linear, ANOVA, and nonparametric models. The benefits and drawbacks of using these models have been extensively discussed in literature (Dougall et al., 1996; Koshio et al., 1993; NRC, 2011; Robbins, 1986; Robbins et al., 1979; Shearer, 2000; Zeitoun et al., 1976). Compared to literature, the methionine requirement of African catfish (19.2 g/kg CP) observed in this study (**Chapter 3**) was strongly different from what was reported in the literature (32.0 g/kg CP; Fagbenro et al. (1999) and 29.7 g/kg CP; Ovie and Eze (2010)). This might be partly due to the differences in the mathematical model used. In **chapter 3**, it was shown that the quadratic regression (QR) model gave higher value estimates than the broken-line and linear plateau model. Furthermore, it was demonstrated that when the QR model is used, the estimate of the optimum level was dependent on the width of methionine doses applied. Reducing the width (see chapter 3, figure 3.3) strongly lowered the initial estimated methionine requirement. This may be one explanation for the high differences between studies, especially those that applied a polynomial model. This aspect of estimation should also be considered in presenting AA requirements in fish nutrition (e.g., NRC), where some of their recommendations might be affected by the methods used. An indication for the usage of this model (i.e., QR) can be seen in NRC (2011), where up to 5 studies (out of 22 studies) applied the quadratic regression model for methionine requirement, also for lysine (6 out of 32 studies). For future studies, it is important to include all aforementioned factors in the process of evaluation whereby a consensus agreement will be reached on how to estimate nutrient requirements.

6.3 Formulating fish diets on the basis of digestible AA

The majority of literature reports and also the current study have clearly demonstrated a large variation in the digestibility of individual amino acids within protein (Boisen and Fernández, 1995). For instance, lysine and cysteine which are known to be important AAs in poultry nutrition usually have a lower digestibility than other AAs within the same ingredient (Parsons, 2020). In **chapter 2**, we demonstrated that cysteine, methionine, histidine, and tyrosine have profound lower ADC values than others AA in African catfish. A low ADC of a particular AA may be the result of high endogenous losses of that particular AA and may also depend on the general chemical composition of the diet (Boisen and Fernández, 1995; Knabe et al., 1989). The exact causes of the discrepancies in apparent amino acid digestibility among feedstuffs and within proteins are unclear (Knabe et al., 1989). As studies evolve, numerous environmental and management factors have been reported to cause this variability in fish, one of which is feeding level (Halver and Hardy, 2002). However, in our study, feeding level had only a small impact on the AA digestibility in African catfish (**chapter 2**). Other constituents of the diet may also affect the AA digestibility. For instance, anti-nutritional factors (ANFs), often present in plant protein ingredients, may reduce nutrient digestibility (Cai and Burtle, 1996; NRC, 1993). On the other hand, the excessive heat treatment applied during the processing of raw materials to reduce the negative effects of these ANFs could lead to protein damage (Abimorad et al., 2008; Masumoto et al., 1996; Portz and Cyrino, 2004; Yamamoto et al., 1998). In addition, excessive heat could induce covalent cross-linking of protein especially among AAs (e.g., lysine) that are susceptible to heat damage (Cho et al., 1982). According to Batterham (1992), heat-damaged amino acids like methionine, threonine, tryptophan and lysine, might create inaccuracy in apparent amino acid digestibility estimations since they are in a state that can be absorbed but cannot be used for protein synthesis. An example is Maillard reaction products; when this happens, a high total lysine estimate is given, but biologically, they will be unavailable to the animal for protein synthesis (Yamamoto et al., 1998). On the other hand, Amezcua and Parsons (2007) hypothesized that the susceptibility of different ingredients to heat-induced damage varies. Also, other compounds in the diet may play a role e.g., crude fibre, chitin etc. In our study, evidence of how chitin could affect the protein quality was also found. The CP digestibility of insect meal was lower than that of all other ingredients (**Chapter 2**). This low protein digestibility could be linked to the presence of chitin in the insect exoskeleton. Decreased nutrient digestibility due to the presence of chitin in insect meal has also been reported in Nile tilapia, turbot (*Psetta maxima*) and Atlantic salmon (Fontes et al., 2019; Karlsen et al., 2017; Kroeckel et al., 2012).

Fish diets have been and are still formulated based on crude protein content or on the basis of digestible protein instead of AA content or digestible AA content. When high-quality ingredients (e.g., fishmeal) are used, it may be safe to formulate a feed based on digestible protein rather than on digestible AA as the difference in utilization efficiency is small. This is because fishmeal has a balanced AA content and is highly digestible. However, when non-fishmeal ingredients are used, more proteins are being added in a bid to create a safe margin to compensate for the requirement of specific AA. This may not be economically and environmentally sustainable. Moreover, the majority of these ingredients (e.g., plant ingredients and animal by-products) are low/deficient in certain essential AA (**chapter 2**). Furthermore, some AAs (e.g., cysteine) are less digestible than the others. As such, formulating a diet based on digestible AA becomes more important in order to account for the variation in digestibility among AAs. Moreover, the economic value of AA supplements (e.g., DL-methionine, L-lysine, and L-threonine) in feed is mostly underestimated when formulated on a crude protein or total AA basis. This is because crystalline AAs are 100% digestible compared to protein-

bound AA in the ingredients (Parsons, 2020). On this basis, it is important to evaluate the AA digestibility of existing and emerging ingredients in various aquaculture species. In this way, one can calculate digestible AA per digestible CP. Moreover, Parsons (2020) proposed the application of a digestible AA system in formulating poultry diets, especially when simple formulas (i.e., non-corn or non-soybean meal) are used. He emphasized the advantage of using this method over the total AA concept specifically when these low-quality ingredients are used. Based on these observations, a method for evaluating dietary protein by quantifying individual digestible AA based on digestible protein, as described in this thesis **(chapters 2 and 3)**, should be implemented in the field of fish nutrition. This way, the portion of AA that will be available for absorption will be taken into account during feed formulation, which will aid precise feed formulation.

6.4 Protein digestibility as a proxy for total amino acid digestibility

In spite of our previous conclusion (*op cit*), the use of protein ADC to predict AA ADC remains an important question in fish nutrition. Since ideally, the combination of AA makes the whole protein, it is expected that the digestibility of individual AA or the sum of AA is equivalent to crude protein digestibility. Many authors have reported the similarity in the digestibility values of CP and AA (Koprucu and Ozdemir, 2005; Lin et al., 2004; Luo et al., 2009; Yuan et al., 2010b; Zhou et al., 2004), although the majority of these studies revealed some variability in the individual AA digestibility. Masumoto et al. (1996) observed that the ADC of EAA were not different from the protein ADC. However, histidine ADC of the full-fat soybean meal used in their study differs from the overall protein digestibility. In pigs, the in-vitro digestibility of amino acids (measured in nine feedstuffs) was in general closely related to that of protein, although there were exceptions (e.g., cysteine, arginine, aspartic acid, glutamic acid and proline). Furthermore, Sales (2008) showed the reliability of using CP as the sole predictor of AA digestibility compared to using other nutrient components like lipid and/or ash. However, despite the various reports on this subject, the question remained unresolved in the field of fish nutrition. Feedstuffs are acquired in batches, with each batch differing in nutritional make-up and thus requiring a proper evaluation of their nutritional contents. For practical reasons, it is important to have the digestible AA information of each batch for precise feed formulation. However, evaluating the protein quality through the analysis of AA ADC of each batch of ingredients is expensive and time-consuming (Boisen and Fernández, 1995; Sales, 2008). Therefore, alternative methods, such as in-vitro techniques and methods focusing on the dietary chemical composition (Sales, 2008) have been developed. However, nutritionists demand a more rapid, simple and cheaper method that could reduce labor, expense, and save time. Therefore, more and better evidence have been searched to support the use of CP digestibility for routine prediction of ADC of AA. Moreover, a multiple regression analysis revealed that dietary CP and fat content are good predictors (R^2=0.87) of the apparent digestible protein content of feed ingredients in hybrid tilapia (Sklan et al., 2004).

Based on the measured ADC values for 13 ingredients in African catfish (**chapter 2**), it was checked if the ADC of crude protein can be used to predict the ADC of total sum of amino acid (SAA). This was done by linear regression analysis of ADC SAA as a function of the ADC of CP (Figure 6.1A). By linear regression, 70% of the variation in SAA ADC between ingredients was explained by the variation in CP ADC. The estimated linear relationship revealed that high quality ingredients with CP ADC values close to 100% had similar ADC values for SAA. For low quality ingredients, the CP ADC was however, lower than the SAA ADC (Figure 6.1A). This was also reflected by the estimated slope of 0.74 for the linear relationship. This estimated slope was below 1, though not significant different from 1 (P>0.1).

Based on this observation, a literature review was conducted to construct a data set on ingredients for which ADC of CP and all AAs (excluding tryptophan) were reported. Studies included into the data set have calculated the ADC at ingredient level according to the method described by Teuling et al. (2017); $ADC_{test\ ingredient} = ADC_{test\ diet} + (ADC_{test\ diet} - ADC_{reference\ diet}) \times (0.7 \times Nutrient_{reference\ diet}/0.3 \times Nutrient_{test\ ingredient}) \times 100\%$, where $ADC_{test\ diet}$ and $ADC_{reference\ diet}$ are the apparent digestibility coefficient (%) of the dietary component in the test diet and the reference diet, respectively. $Nutrient_{reference\ diet}$ and $Nutrient_{test\ ingredient}$ are the nutrient contents (g/kg DM) in the reference diet and test ingredient, respectively. Furthermore, the CP content and also the AA content in the ingredients needed to be reported as criteria to be entered into the database (see explanation below). This resulted in a dataset containing 178 ingredients tested in 18 fish species (originated from the following sources: Abimorad et al. (2008); Allan et al. (2000); Anderson et al. (1992); Basto et al. (2020); Campos et al. (2018); Che et al. (2017); Dam et al. (2019); dos Santos Cardoso et al. (2020); (Glencross et al., 2017); Koprucu and Ozdemir (2005); Lee et al. (2020); Masumoto et al. (1996); Mo et al. (2019); Portz and Cyrino (2004); Stone et al. (2000); Tomas-Vidal et al. (2019) and Yamamoto et al. (1998). The enlargement of the number of observations from 13 to 178 ingredients slightly increased the R^2 of the linear relationship between CP ADC and SAA ADC from 70 to 78% (Figure 6.1). Across species, 78% of the variation in SAA ADC of ingredients is related to variation in CP ADC. Considering the high number of ingredients in the dataset and R^2 of 78%, one can predict the ADC of SAA from the ADC of CP but not with a very high accuracy. The biggest change in the relationship when expanding the dataset, was the estimated beta being 0.92, which is closer to 1. However, even in the larger data set, the slope of the relationship was significantly different from 1 (P<0.05).

Potential reasons for this observation could be explained by the presence of other nitrogenous compounds in CP which did not influence the digestibility of SAA. Protein is estimated by multiplying its N content by 6.25, thus, non-protein nitrogen (NPN) is included in the estimates. As a result, it seems that the NPN digestibility differs from that of true protein (Lupatsch et al., 1997). Furthermore, our results revealed that certain ingredients were deviating from the slope, which can be attributed to ingredients of low protein quality (e.g., HFM). The other reason might be that low-quality ingredients have a negative impact on some AA that might be sensitive to (for instance) heat damage. Thus, it seems that also across species, the ADC of SAA in low quality ingredients is higher than the ADC of CP. It can be concluded that crude protein digestibility can be used as a predictor for the total sum of AA, although this approach is not fully perfect.

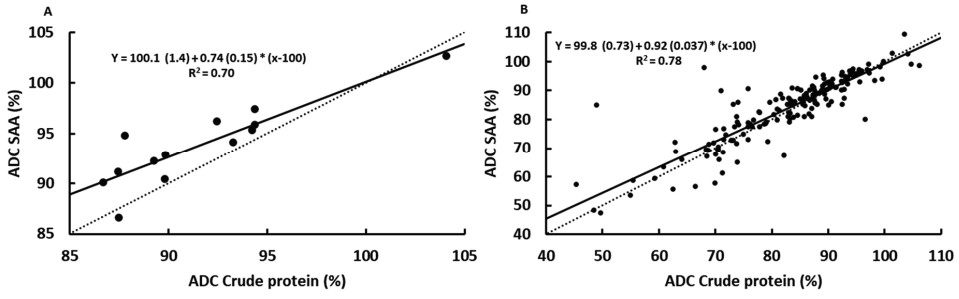


Figure 6.1 Relationship between crude protein digestibility and sum of amino acid digestibility (solid lines). Panel A; this thesis (n=13), panel B; constructed dataset by literature review (see main text), (n=178). Dotted lines in both panels indicates the line Y=X.

6.5 Total AA ADC as a proxy for individual AA ADCs

Considering the possible relationship between CP and SAA discussed above, we also studied the predictability of individual AA ADC by using SAA digestibility values. This is to ascertain if individual AA are showing similar functionalities as the overall SAA. Yamamoto et al. (1998) reported that the digestibility of certain AA such as glycine was notably lower than the average digestibility of total amino acids (i.e., SAA), even though the individual AA digestibility within each protein source approximated the ADC of CP. In **chapter 2**, we showed that the digestibility of AA is not always equal. For instance, the ADC of histidine in poultry meal is lower compared to the ADC of arginine in the same ingredient.

Relating the ADC of individual AA to the ADC of SAA for the 13 ingredients reported in **chapter 2,** it was revealed that the response differed between the individual AA. In Figure 6.2 A and C, the 2 extreme relationships are given. Arginine ADC was strongly related to SAA ADC with an R^2 of 95%, but having a linear line slope of only 0.66, which was different from 1 (P<0.001). Thus, ingredients with a lower protein quality had a relatively higher ADC of arginine compared to the ADC of SAA. In contrast, the ADC of cysteine was less strongly related to SAA ADC (R^2=70%) and a linear line slope of 2.1, which was different from 1 (P<0.05). For cysteine, its ADC declined stronger with reducing SAA ADC. In other words, for low quality ingredients, the cysteine ADC reduced stronger than the ADC of the sum of AA. In Figure 6.2B and D the relationships for arginine and cysteine are also given for the total 178 ingredients in the dataset constructed (see above). The relationship between ADC of individual ingredients and the ADC of SAA was done by PROC MIXED in SAS using the absolute amount of the individual AA in the test diets originating from the test ingredient (in g/kg) as weight factor. In this way, ingredients with a very low individual AA content or a very low inclusion level (into the test diet) were considered less accurate compared to ingredients with a high AA content and inclusion level. The observed trends in the individual AA ADC relationships with SAA ADC were the same in the large dataset (Figure 6.2B & D) as for the ingredients reported in **chapter 2** (Figure 6.2A & C). The slopes for both arginine and cysteine still deviated from 1 but less strong in the African catfish dataset (n=13). Moreover, in the large dataset (n=178), the ADC of SAA explained a lower percentage of the variation in arginine ADC (76%) as well as cysteine (56%).

In Figure 6.2, arginine and cysteine were given as examples for the relationship between the ADC of individual AA with the ADC of SAA. In figure 6.3, the slope of the linear relationship for the ADC of each individual AA with the ADC of SAA is given for the large dataset (n=178). This figure shows that arginine, methionine, phenylalanine and glutamic acid have a slope that is significantly lower than 1. Thus, for low quality ingredients, the ADC for these AA are higher than the ADC of the total sum of AA. Moreover, valine, glycine asparagine and cysteine have a slope being significantly larger than 1. For these amino acids, the ADC is strongly reduced in low quality ingredients compared to the ADC of SAA. This generally indicate that, for low quality ingredients, the differences in ADC of individual amino acids are not equal and becomes larger with declining total AA ADC.

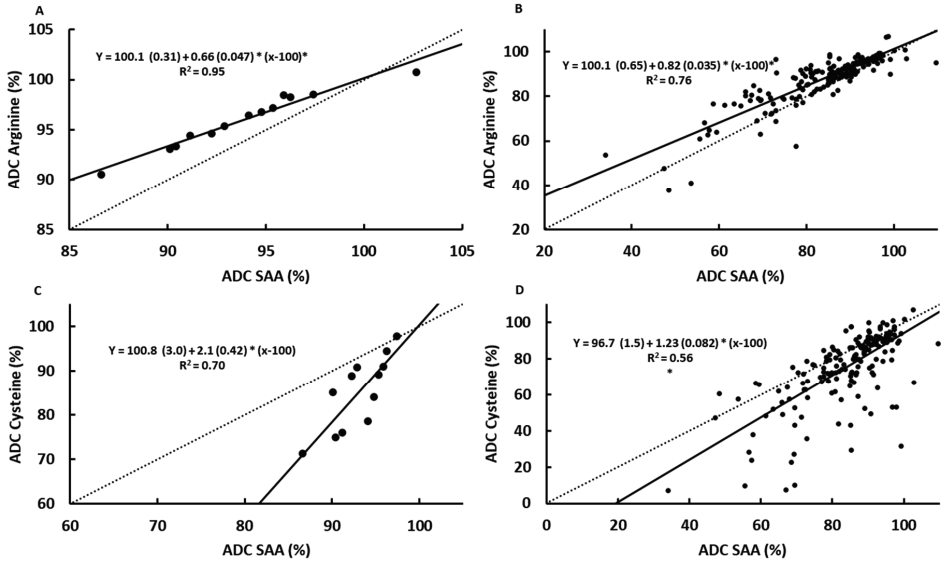


Figure 6.2 Relationship between the sum of amino acid digestibility and individual AA (arginine and cysteine) digestibility (solid lines). Panel A; arginine in this thesis (n=13), panel B; constructed dataset of arginine by literature (see main text), (n=178), panel C; cysteine in this thesis (n=13), panel D; constructed dataset of cysteine by literature (see main text), (n=178). Dotted lines in both panels indicates the line Y=X.

In a study by Zhou et al. (2004) on juvenile cobia (*Rachycentron canadum*), it was discovered that amino acid digestibility coefficients tend to only reflect protein digestibility coefficients among highly digestible ingredients like Peruvian fish meal and corn gluten meal. In the same study, meat and bone meal had a lower lysine digestibility compared to the other animal- or plant-based ingredients tested. This observation was attributed to the reduced digestibility of protein in bone fragments which lowered the quality of the ingredient (Zhou et al., 2004). Another study concluded that the ADCs of protein in yeast and corn gluten meal should not be used as AA digestibility indicators because they displayed up to 6.7% differences between the protein and AA digestibility (Abimorad et al., 2008). The rate at which amino acids are released during digestion is determined by the availability of dietary proteins. In the current study, the tendency for a stronger relationship of arginine and methionine relative to other amino acids, is in accordance with the explanation from (Low, 1979), e.g., that these amino acids would be among the first to be absorbed, based on the specificities of the enzymes involved in the proteolytic breakdown of feed proteins. On the other hand, the low performance of cysteine could be due to the fact that cysteine is incorporated in small protein molecules of some ingredients especially in most legume seed like soybean meal and pea protein (**chapter 2**) (Boisen and Fernández, 1995). They have often been shown to be protease inhibitors and are highly resistant to proteolysis by forming stable disulphide bonds. (Boisen, 1983). Although the effect of disulphide bond formation on protein utilization is unknown (Ei and Kavas, 1996), some experimental data suggest that it may decrease protein digestibility (Opstvedt et al., 1984). According to Mauron (1982), when foods are boiled at high temperatures, development of complex chemical (crosslinking) reactions such as protein interactions or protein-fat interactions can reduce protein digestibility. It has also been reported that smoking conditions (time, temperature, and wood smoke compounds) reduced protein digestibility (Opstvedt, 1988). There is also a possibility that methionine is converting to cysteine by microbes for the *de novo* synthesis of the latter. From the above discussion, it can be concluded that

the digestibility of SAA can be used to predict the digestibility of individual AA but that it may vary considerably per individual AA which also depends largely on the quality of protein ingredient used.

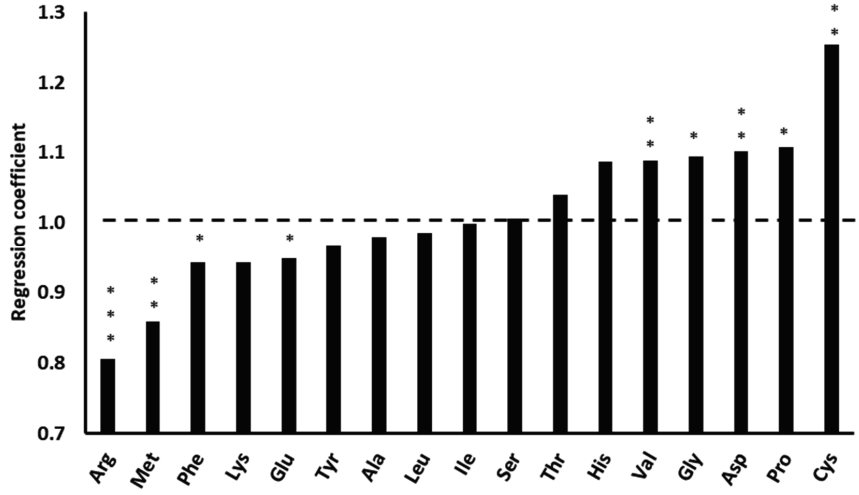


Figure 6.3 Estimated regression coefficient of the relationship between the digestibility (ADC) of individual AA and the ADC of the sum of the amino acids. This was done on a dataset of 178 ingredients (see main text).

Furthermore, the strength of this prediction may be dependent on the type of ingredients (e.g., plant or animal ingredients). This is demonstrated in Table 6.1, where the large dataset was split into ingredients of plant origin versus animal origin (animals both from marine and terrestrial origin). Within each type of ingredient source, the relationship between the individual AA ADC with the ADC of SAA was made. This analysis showed that for various AA the slope was significantly different between the type of ingredients. For instance, methionine and lysine digestibility in animal sources are less affected by ingredient quality which is indicated by the slope being smaller than 1 compared to the sum of all AA. Whereas, in plant sources, the digestibility of methionine and lysine respond quite similar to that of the ADC of SAA (a beta of 1). Cysteine and histidine responses are also different between the type of ingredients. Here the slopes of cysteine and histidine are larger than 1 in animal sources while it is closer to 1 for plant ingredients. Overall, it seems that the variability in ADC between the individual AA is larger at low quality ingredients in animal sources compared to plant protein sources. This observed effect of ingredient type on the relationship of the ADC of various individual AA with the ADC of SAA make predictions of ADC of AA from the ADC of SAA and thus also from ADC of CP difficult. Next to that one can question whether these relationships are different between fish species and or trophic levels of fish species. This requires further assessment.

Table 6.1 Relationship between the AA and sum of AA in different sources.

	Animal sources	Plant sources	P-value
	Beta ± se	Beta ± se	type of source
Methionine	0.772 ± 0.062	0.946 ± 0.061	*
Lysine	0.884 ± 0.047	1.002 ± 0.054	#
Arginine	0.735 ± 0.053	0.876 ± 0.045	*
Histidine	1.303 ± 0.077	0.870 ± 0.073	***
Isoleucine	0.919 ± 0.056	1.077 ± 0.047	*
Asparagine	1.197 ± 0.048	1.007 ± 0.043	**
Cysteine	1.458 ± 0.123	1.049 ± 0.111	*
Glycine	0.969 ± 0.058	1.220 ± 0.069	**

Given in table (n=173 because SCP were excluded). For all AA not included in this table the regression coefficient (Beta) of animal sources did not differ from that of plant sources.

6.6 Cross-link between protein digestion and nutrient synchrony

For proper evaluation of dietary protein quality, an assessment of the impact of protein sources on the protein utilization efficiency should be included (Young and Pellett, 1989). Indeed, the utilization efficiency of AA in protein can be affected by many factors such as the presence of antinutritional factors (ANFs), mycotoxins, naturally bounded resistant proteins, and nutrients asynchrony (Lall, 1991; Van den Borne et al., 2006). In other words, the supply of the right amount of AA needed to meet the daily requirement of fish may not guarantee its optimal utilization for protein synthesis.

Earlier in the general introduction, we speculated that nutrient asynchrony could be the reason for sub-optimal utilization of dietary amino acids in fish. Indeed, literature evidence has indicated disproportionate absorption rates of amino acids from a diet containing both free amino acids and protein-bound AA. Some authors have reported that this problem is often caused by the asynchronous availability of protein-bound AA and crystalline AA supplied to diets that would otherwise be deficient in essential AA (Ambardekar and Reigh, 2007; Zarate et al., 1999). Studies with rats (Rolls et al., 1972), prawns (Kangsen et al., 1988), carp (Plakas and Katayama, 1981) and channel catfish (Zarate et al., 1999) have indicated that free amino acids are quickly released and absorbed from the gastro-intestinal tract in contrast to protein-bound AA, thereby leading to an imbalance in the amino acids profile in the tissues at different moments postprandial. This leads to diverting amino acids into catabolic rather than anabolic processes (Batterham, 1974; Cowey et al., 1979). Our results (**chapter 4**) showed a different passage rate of nutrients in the GIT of African catfish which may be an indication that there are differences in nutrient uptake in the gut. An improved utilization efficiency of dietary protein and supplemented AA is an effective way of solving this problem. The concept of balancing the dietary inputs with the nutrient requirements, e.g., ”Nutrient synchronization” can be used to harmonize the different sources of AA supplied in such a way that it will favour optimal utilization. In literature, different strategies of dietary nutrients synchronization have been evaluated in farm animals. Some examples are: i) Harmonisation of energy or protein sources ii); change in feeding frequency or pattern; iii) Matching the different forms of AA; iv) Controlling the timing of feed offering;

v) Balancing the form of supplied nutrients and supplement types and vi) Exchange of feedstuffs (Hersom, 2008; Van den Borne et al., 2006; Yang et al., 2010). In this thesis, we looked at nutrient synchronization as affected by feeding frequency in African catfish, but no beneficial impact was observed (**chapter 5)**. However, another study (**chapter 4)** showed that the sources of dietary nutrients (i.e., protein and energy) can impact the nutrient digestion kinetics in the GIT. Based on this observation, it might be interesting to further assess the impact of this type of synchrony on utilization efficiency in fish. There are supportive evidences to proof that the synchronization of protein and energy is beneficial in terms of improved efficiency of microbial protein synthesis, improved protein utilization and decreased urinary N excretion in ruminants (Cole and Todd, 2008; Kaswari et al., 2007) and in preruminant calves (van den Borne et al., 2007). In contrast, no improvement was seen in both performance and breast muscle yield of broilers, when fed both rice starch and soy protein isolate or pea starch and soybean meal simultaneously (Chen, 2017). Although the concept of nutrient synchrony is common and has been applied for decades in many farm animals (e.g., pig and poultry), it has gained little attention in fish nutrition. This may be related to the limited positive effects of this concept in fish as seen in this thesis. Moreover, asynchrony might be less pronounced in fish due to the use of high-quality protein ingredients in fish feeds. For instance, if fishmeal is used as protein source, one could expect a rapid digestion and uptake due to its high-quality, therefore, asynchrony between supplemented AA and the protein-bound AA therein would not be triggered. This suggest that the asynchrony in the supply of the nutrients (AA and glucose) might be more substantial in low-quality ingredients, especially when nutrients are supplemented to compensate for the deficiency.

According to Hersom (2008), one of the intriguing aspects of employing dietary supplements to influence synchronization in animals is that a positive synchronistic response is more likely to occur in low-quality ingredients supplemented. In a number of experiments using mature cows, forage quality was an important factor for successful nutrient synchrony effects. Low quality forage with frequent supplementation tended to increase the positive effect of nutrient synchrony (Hersom, 2008) more than with high quality forages (Yang et al., 2010).

Besides the quality of ingredients and supplemented AA, feeding frequency is also known to affect nutrient synchrony (Chen, 2017; Hersom, 2008; 2009). We earlier speculated that a higher feeding frequency might reduce the postprandial asynchronous availability of nutrient (e.g., glucose and AAs). In addition, a high feeding frequency was expected to improve protein efficiency especially when the required level of methionine (**chapter 3**) is fed. This is because a low feeding frequency might induce a more pronounced postprandial fluctuation in nutrient availability due to a larger gap in the release of various forms of dietary nutrients in the GIT. Chen (2017) proposed that meal-feeding could increase the concentration of glucose and AAs in the plasma with a larger gap in-between postprandial availability of these nutrients. In contrast, they assumed that continuous feeding would allow a steady flow of nutrients in the GIT and a smaller postprandial increase in plasma glucose and AA concentrations. In this thesis, feeding frequency had no impact on protein utilization efficiency (**chapter 5**). This substantiate the earlier submission that asynchrony does not occur in African catfish and if it does, feeding frequency is not the responsible factor. Similar to our study, feeding frequency did not improve utilization efficiency of supplemental lysine and methionine in channel catfish and common carp respectively (Nwanna et al., 2012; Zarate et al., 1999). This is an indication that the efficiency of crystalline AA supplementation in some fish species is not hampered by nutrient asynchrony or feeding frequency. However, it might also be related to the use of high-quality protein sources in fish feeds leading to rapid digestion which reduces the time lag between the absorption of

supplemented AA and the protein-bound AA. When synchronization is achieved, an increase in intake and digestibility is excepted (Haag, 2008). Indeed, increasing feeding frequency increased nutrient digestibility in African catfish but feeding 6 times a day did not improve the overall growth performance. This might be related to a number of factors; for example, multiple feeding can trigger increased activities among fish. Fish can be active throughout the day, unlike animals such as broilers whose feeding pattern can be influenced by lighting schedule (Savory, 1976; Weaver Jr and Siegel, 1968). Such increased activity might result in increased heat production (**chapter 5**), increased gill frequency (more oxygen needed), which may be the reason for the unpronounced effect on performance. In a study on grazing beef cows fed feed supplemented with cottonseed meal at various feeding frequencies (daily, three times a week, once a week), animals which received no cottonseed meal (the control group), lost more body weight and lower body condition score (BSC) than those that received cottonseed meal, however, considerably less variability was observed in weight and condition between the grazing cows that received the supplementation on a daily basis and those that received it less frequently (Haag, 2008). Although nutrient synchronization can be regulated by changing feedstuffs or nutrient supplementation, these strategies have some inherent drawbacks (Yang et al., 2010). The majority of experiments that have applied such methods have been unable to distinguish between the effects of synchronization and those induced by various properties of the individual feedstuffs. To reduce the impact of feedstuffs, different feeding frequencies or feeding pattern should be applied to form a contrast. Indeed, an altered feeding pattern would clearly reveal any change in metabolite since the same ingredients were used.

6.7 Factors responsible for the sub-optimal utilization of amino acids.

The sub-optimal utilization of AA in most fish may be due to other factors aside nutrient asynchrony. Leaching for example has been linked to nutrient loss even before the uptake of feed pellets by fish. This often happens when crystalline AA, which are susceptible to leaching are supplied to deficient diets. Zarate and Lovell (1997) reported a leaching loss of about 12% of crystalline lysine compared to a loss of 2% of protein-bound lysine, indicating that the crystalline form is significantly less efficient. However, this may be minimal in fast-eating fish like African catfish, which often rapidly (within 5min) consume their food (**this thesis**). Moreover, diets are sometimes coated to prevent nutrient leaching. For instance, the experimental diets used in **chapter 5** were sealed with palm oil to prevent the supplemented crystalline methionine from leaching. Encapsulation of the diet has also been suggested to be effective in preventing rapid leaching of free amino acids, especially in the GIT (Ambardekar and Reigh, 2007).

Furthermore, the forms of ingredients used during feed formulation might contribute to sub-optimal utilization of amino acids. In other words, an optimum utilization of AA may be achieved by continuous feeding of the right synchronised diet, in terms of the ingredient's constituents (i.e., slow-slow digested nutrients or fast-fast digested nutrients). Amino acid supplementation may be more beneficial to fish fed diets containing quickly digested proteins than to fish provided diets containing proteins that are digested slowly. In the latter situation, amino acid supplements are likely absorbed too quickly. Purified amino acids are more likely to be effective if dietary protein is quickly digested. As a result, efficient usage of amino acid supplements in fish feeds may necessitate the consideration of the used ingredient. Despite the likely increase in cost, it may be sometimes better to formulate a diet with exclusively intact proteins. In other situations, a combination of intact proteins and purified amino acids may be appropriate, provided that the ingredient composition of the diet favours the efficient

utilization of amino acids from both sources (Ambardekar and Reigh, 2007). For instance, since hydrolysed protein ingredients (e.g., hydrolysed fishmeal) have been partially broken down, which is mostly accompanied by a more rapid evacuation in the stomach (**chapter 4**), it would be wise to use such ingredient as protein source, especially when supplementation of crystalline AA is required. Thereby, optimum utilization will be achieved. However, using both fast digestible sources (i.e., hydrolysed fishmeal and starch) or both slowly digestible source (i.e., fishmeal and fat) interchangeably did not substantiate this hypothesis in African catfish (**chapter 4**). The reason might be related to the feeding habit of the fish (omnivorous) whereby different forms of ingredients have little effect on the overall utilization. Similarly, no improvement was seen in both performance and breast muscle yield when broilers were fed both fast digestible sources (i.e., rice starch and soy protein isolate) or both slowly digestible source (i.e., pea starch and SBM) simultaneously. In contrast, Rotger et al. (2006) used a combination of two sources of unstructured carbohydrates (barley and corn) and two sources of protein (soybean meal and sunflower meal) in their experiment on beef cattle. The fast-synchronous diet (barley and sunflower meal) and the slow synchronous diet (corn and soybean meal) produced more microbial N production in vitro. Similarly, in sheep, Witt et al. (1999b) found that synchronous diets resulted in greater efficiency of microbial protein synthesis (MPS). However, another study performed by the same group (Witt et al., 1999a) found no improvement in MPS. In another study, Herrera-Saldana et al. (1990) found that a quick synchronized diet (high digestible energy and protein) exhibited better microbial N flow and microbial protein synthesis efficiency compared to an asynchronous diet in lactating cows. Kim et al. (1999) injected maltodextrin directly into the cannula and demonstrated that synchronous treatments improved MPS in cattle. Although we could not substantiate this hypothesis in our study, it would be wise to validate this proposition in other species of fish (i.e., different trophic levels) in future studies.

6.8 Kinetics of protein digestion

In current fish feed evaluation system, the nutritional value of protein sources is based on the faecal digestibility of nutrients. In pigs and poultry nutrition, dietary protein sources are evaluated based on digestible amino acids at the end of the ileum, as it has been suggested that faecal and ileal protein digestibility differ substantially among commonly used feed ingredients. It is believed that microbial fermentation of food protein in the hindgut does not considerably contribute to animal AA supply (Lemme et al., 2004; Ravindran et al., 2005; NRC, 2012). The latter form of evaluation is not carried in fish due to the belief that the action of microbes in the hind gut are negligible in fish, consequently, there will be little differences between ileal and faecal digestibility (Halver and Hardy, 2002). This explains why faecal digestibility studies are still common in fish nutrition. The first step in faecal digestibility measurements is the collection of faecal samples. In contrast to terrestrial animals, total faeces collection is challenging due to the significant risk of nutrients leaching into the water before the faeces are collected (Smith et al., 1980). Different approaches have been applied in faeces collection for digestibility studies, each with its own set of benefits and drawbacks (Cho et al., 1982; NRC, 2011; Windell et al., 1978). Still, none could prevent the unavoidable problem associated with nutrient leaching, due to the contact of faeces with water. Furthermore, many researchers, notably those working on farm animals, have expressed doubts regarding the accuracy of evaluating AA digestibility using faecal samples. The latter issue stemmed mostly from the possibility of significant alteration of undigested AA by microflora present in the hindgut. These bacteria would break a considerable amount of undigested AA, resulting in lower AA excretion and consequently overestimated AA digestibility values. Owing to the modification of AA excretion by the hindgut

microflora, Parsons (2020) suggested that faeces collection may not be a reliable way to measure AA digestibility. This consequently led to the shift to using ileal digestibility assay in farm animals (e.g., pig and poultry), since the last 10 to 15 years. Diet formulation based on the faecal nutrient digestibility of ingredients (as used for fish), only accounts for the total amount of dietary nutrients that was apparently digested and assumed to be absorbed along the GIT (Chen, 2017; NRC, 2011). This does not take into consideration the kinetics of nutrients digestion along the GIT, which could significantly affect the post-absorption metabolism of AAs originating from dietary protein.

Generally, the kinetics of dietary protein digestion is determined by the rate at which digesta passes through the GIT, which is dependent on the physicochemical properties (e.g., solubility, viscosity, water binding capacity) of the digesta (Chen, 2017). Understanding how ingredients change nutrient digestion and absorption rates along the GIT is critical for understanding the differences between the digestion matrix in the GIT and faecal digestibility. In African catfish, protein digestion begins in the stomach and continues throughout the digestive tract with the foregut having the highest protease activity (Uys and Hecht, 1987). The release of pepsin and HCl in the stomach kicks off the digestive process, which is followed by the pancreas adding trypsin and chymotrypsin to the foregut. Crystalline amino acids do not require any of these proteases for digestion because they are already monopeptides, which are the simplest form of peptides. As such, monopeptides are readily available for absorption and are transported into the bloodstream via carriers in the basolateral membrane of the intestinal cells (Ballantyne, 2001). As the digestion process progresses, small peptides like di- and tripeptides are absorbed through active transcellular and paracellular transport (Verri et al., 2010). The final stage of dietary protein digestion occurs at the brush border membrane of the small intestinal mucosa (Erickson and Kim, 1990). Undigested dietary protein can be further fermented by the commensal microbiota in the hindgut. For instance, proteolytic fermentation mainly occurs in the colon of pigs and the caeca of poultry. The products of proteolytic fermentation include volatile fatty acids (VFAs) (an energy source for animals) and other metabolites.

Although in this study, the digestibility of nutrients in the GIT did not reflect faecal nutrient digestibility, the importance of studying digestion kinetics cannot be overlooked. Previous studies conducted on digestion kinetics in fish have mainly focused on non-protein energy sources (e.g., Harter et al., 2013 and Harter et al., 2015). Yet, the kinetics of protein digestion of feedstuffs used in fish feed formulation remains largely unknown. Hydrolysed protein is now often being used as a protein source in aquafeeds. In humans, protein hydrolysates resulted in a faster postprandial increase of plasma AAs than their non-hydrolyzed equivalents, which suggests a quicker absorption of AA in the GIT (Morifuji et al., 2010). In this thesis, the passage rate of the digesta of fish fed a hydrolyzed fishmeal diet was faster than in those fed fishmeal (**chapter 5**). Hydrolysed protein contains short peptides, which are more easily dissolved in water (Ballantyne, 2001). In addition, and similar to the situation with crystalline amino acids, the peptidases released in the stomach are no longer required to first hydrolyse the longer peptides during digestion (Ballantyne, 2001; Uys and Hecht, 1987). Consequently, the fishmeal hydrolysate will display higher solubility, which aids quicker evacuation from the stomach (**chapter 5**). This implies that the dietary macronutrient composition can alter the postprandial protein digestion along the GIT (**chapter 5**) and as the passage rate is not equal among different AA sources and thus ingredients, it may affect the faecal digestibility data. Information on the digestion kinetics of new and emerging protein sources can be used to advance the concept of synchronizing the dietary supply of energy and protein (Chen, 2017), which could help improve protein retention and utilization efficiency in fish.

Analogue to pigs and poultry, where in the distal part of the GIT, microbial fermentation can lead to a misinterpretation of AA profile, there are no data of such occurrences in fish. Particularly, no information is available on whether fermentation influences the AA pattern in the hind gut of fish and whether this may hamper the proper estimation of AAs that are available for growth. Future research should focus on the location of protein digestion in the GIT and also elucidate the relevance of protein/amino acids fermentation in fish.

6.9 Implications of the research

With the fast-growing global population, providing a sustainable and steady supply of fish has become a huge challenge (World Bank, 2013). It is expected that the growing demand for fish will be primarily met through aquaculture. In 2016, aquaculture supplied approximately 47% of the global fish market, with Asia accounting for about 89% over the last 20 years. During this period, Africa made only a minor contribution of 2.5% in general and 0.9% for low-income food-deficient countries (FAO, 2018) despite its inherent potential. Yet, in the coming decades, the population growth in Africa will be the highest in the world (Asongu, 2013). As a result, there is a large imbalance between the demand and supply for fish, as local fish production remains marginal with low yields in most African countries. The two main identified factors are the scarcity of high-quality fish seeds and inadequate least-cost fish feeds (Adeleke et al., 2020; Changadeya et al., 2003; Gabriel et al., 2007). African catfish (*Clarias gariepinus*) is one of the most widely produced aquaculture species on the African continent, due to its productive ability and high market value. Nigeria is the African country with the second-highest aquaculture production, with African catfish being the most popular cultured species (Ekaitz Maguregui, 2021). The aquaculture production system in Africa has drastically shifted from extensive farming, where feed is less required, to a semi-intensive or intensive farming system, where feed is one of the most important productive components. In the intensive production of African catfish, the cost of feed is particularly relevant and often associated with the need to provide feed with high protein content for fish, in order to achieve high performance. This is because the majority of farmers in Africa heavily rely on imported fish feed from external markets (mostly European countries). In Nigeria, for example, an estimated 4,000 tons of high-quality fish feed are imported per year (AIFP, 2004). This is due to the low numbers of companies specifically dedicated to aquafeed production. This reliance results in huge price instability, which is difficult for producers to sustain, given that feed accounts for about 75 to 80% of the overall cost of production (Ekaitz Maguregui, 2021).

One of the underdeveloped aquaculture sectors is fish feed technology, especially in Africa and other developing countries around the world (FAO, 2003). Hecht (2000) pointed out that research on the use of least-cost ingredients for aquafeed formulation has not significantly contributed to aquaculture development in Africa. He proposed that more research be carried out in this area, such as the use of plant protein in fish diet. Siluriformes, like other aquaculture species, require a high protein content in their diet. For African catfish, the protein requirement is around 37%. Initially, a high percentage of fishmeal was included in the feed to cover this high dietary demand for protein. Currently, there is a growing trend towards substituting fishmeal with plant-based protein sources (Ekaitz Maguregui, 2021). Amino acids profile in ingredients are well documented for commonly used ingredients in fish feeds but for many fish species, information on the apparent digestibility coefficient (ADC) of AA are still lacking at the ingredient level. Currently, no reliable information is available on the amino acid digestibility of ingredients in African catfish, so also the amino acids requirement has not been extensively researched. One of the main aims of this thesis was to investigate the AA profile of common

ingredients used in aquafeed production and specifically in the diet of African catfish (**chapter 2**). It is critical to ensure that animal feed is properly digested and absorbed for farms to remain viable (Ekaitz Maguregui, 2021). This thesis highlighted the digestibility of these ingredients in African catfish in line with the goals of replacing conventional feedstuffs with novel ingredients. Furthermore, novel ingredient such as insect meal was shown to display similar performance as fishmeal in this study (**chapter 2**). Overall, the current research exposes how well African catfish can utilize locally available ingredients without reducing the quality. In addition, the methionine requirement of African catfish was investigated (**chapter 3**). Knowledge of this will enable local nutritionists to formulate diets based on nutritional requirements. Such information is crucial to the success of aquaculture development, growth and expansion in Africa.

Discovering feeding strategies that might help to decrease the loss of nutrients from feeds to waste is a strategic way of providing solutions that can reduce the environmental footprint left by the aquaculture sector. Therefore, there is a need to establish the effect of feeding times on feed management, nutrient utilization and growth rate of fish (Aderolu et al., 2010). Several authors have suggested that feeding twice or three times per day is sufficient for the optimum growth of African catfish (Aderolu et al., 2010; Eyo and Ekanem, 2011; Marimuthu and Muralikrishnan S, 2010). In this study, African catfish fed two times per day displayed the lowest nutrient digestibility (**chapter 5**). This implies that feeding more frequently than twice per day is optimal for good digestibility. This information is useful biologically and economically for the aquaculture industry by improving our understanding of the right husbandry methods for optimum utilization of nutrients in fish.

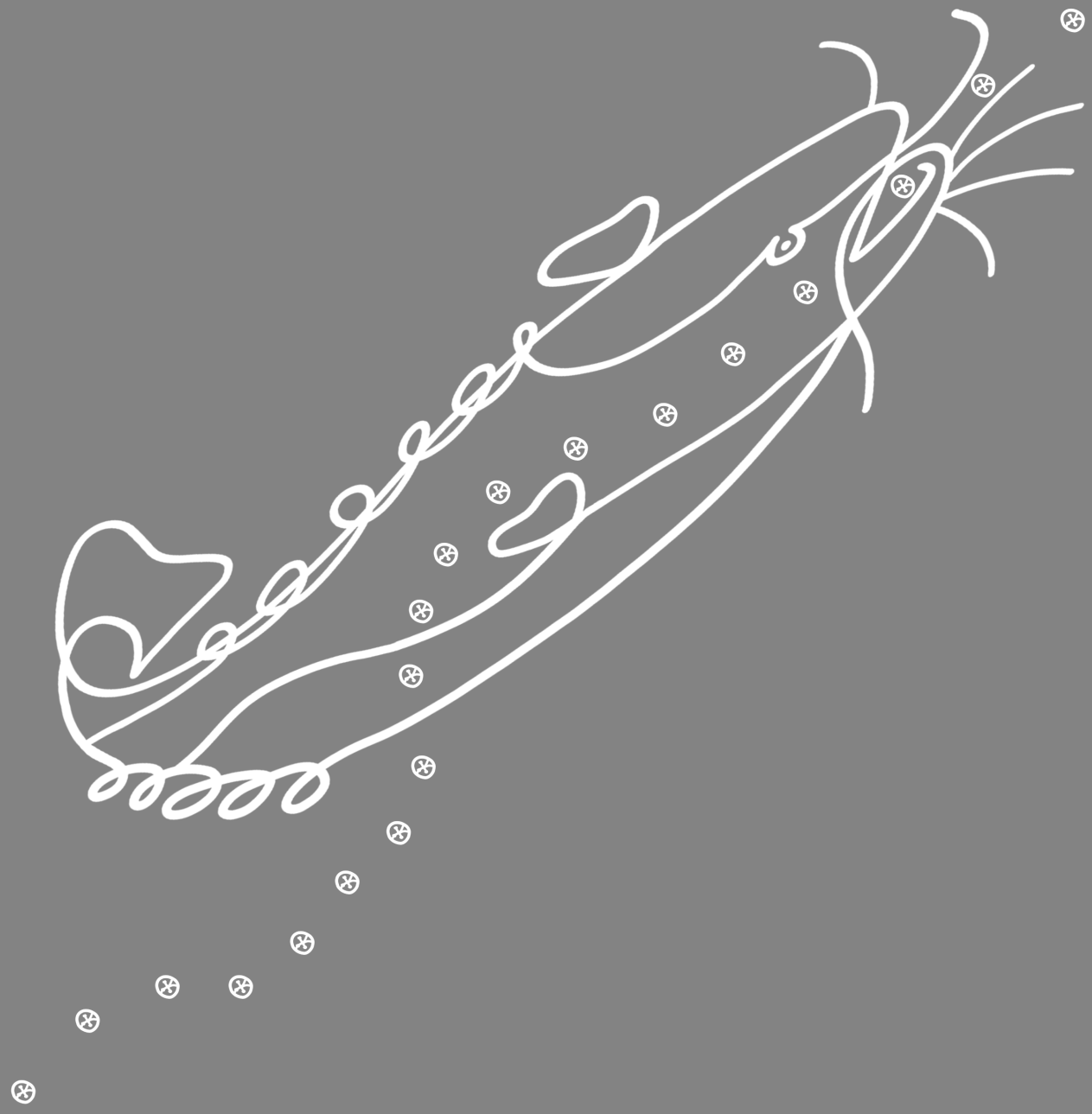
6.10 Recommendations for science and practice

Provided that a diet is formulated based on the digestible AA, then accurate values for all digestible AA requirements are needed. Beyond methionine, we recommend that more digestible AA requirement data for African catfish and other commonly cultured species are investigated. Especially data on digestible lysine, sulfur AAs, threonine, and other essential amino acids are needed. Additional data on the requirements for all developmental stages of fish should also be assessed since studies on other animals (e.g., birds) have revealed that age can influence the AA digestibility of ingredients (Parsons, 2020). In both broiler chickens and turkey poults, AA digestibility was seen to be lower at very young ages and increases with increasing age. These improvements in protein evaluation could be an important step towards a more precise practical feeding of fish according to their actual requirements for digestible amino acids. However, nutrient evaluation goes beyond assessing the digestibility of ingredients but also utilization efficiency. In the context of the method of protein evaluation proposed in this thesis, we recommend further investigations to validate the concept of nutrient synchrony in other fish species. Furthermore, in case this concept would prove to be invalid for African catfish (omnivorous fish), it still could be of importance to fish in other trophic levels (e.g., carnivorous fish). Furthermore, since low-quality ingredients like plant proteins are increasingly used, this hypothesized concept might still be relevant to fish that naturally eat diets of lower quality (i.e., herbivorous fish). Finally, nutrient digestibility studies in fish should go beyond only faecal measurement and include digesta examination.

6.11 General conclusion

The main conclusions drawn from the different studies carried out in this thesis, are:

- Within ingredients, the digestibility coefficients are not equal for the different amino acids.
- This variability in digestibility between amino acids is larger in low-quality ingredients compared to high-quality ingredients when using crude protein digestibility as a quality indicator.
- Crude protein digestibility values of ingredients can be used as a predictor for the digestibility of their total sum of amino acids.
- Individual amino acids digestibility coefficients can be predicted from the sum of amino acids digestibility in ingredients.
- Across fish species, the digestibility of the amino acids, arginine, methionine, glutamic acid and phenylalanine decreases less strongly compared to the digestibility of SAA when the protein quality of an ingredient declines. In contrast, the digestibility of valine, glycine, aspartic acid, proline and cysteine have a stronger decline compared to the digestibility of SAA when the protein quality declines.
- The impact of feeding level on macronutrient digestibility in African catfish is ingredient-dependent.
- The choice of the mathematical model used in studies to estimate nutrient requirement can impact requirement estimates, for instance, quadratic regression can lead to an overestimation of nutrient requirements.
- Based on the linear plateau model, the digestible methionine requirement of juvenile African catfish ranges between 18.7 and 21.4 g/kg per unit of digestible protein, depending on the response criterion.
- In African catfish, the protein utilization efficiency of digested protein is not influenced by feeding frequency.
- Ingredient macronutrient composition can alter the kinetics of nutrient digestion. Changing dietary fat by starch alters the intestinal location of protein digestion in African catfish.
- Hydrolysation of protein (e.g., fishmeal) increases the stomach evacuation rate of protein.
- In this thesis, no proof was found for the existence of nutrient asynchrony in African catfish.



Appendices

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A

Appendices

Summary

Acknowledgements

About the author

List of scientific publications

WIAS Training and Supervision Plan (TSP)

Summary

The increasing usage of alternative and novel ingredients for fishmeal replacement in aquafeed makes it necessary to strengthen our basic knowledge on their nutritional content, digestibility and utilization, especially in relation to the requirement for specific nutrients, such as amino acids in commonly cultured species. Since the larger part of aquafeed formulation is based on the protein content, reliable data on the digestible AA content of these different ingredients for each species is considered a necessary prerequisite. This is because the biological value of protein is determined by the optimal composition of amino acids that are digested and absorbed. Currently, the protein values of aquafeeds are evaluated based on the concentration and digestibility of protein in the selected ingredients. In the same light, amino acid requirements are estimated on the crude protein value of ingredients with the assumption that individual AAs within protein react in the same way. However, evidence suggests that this evaluation system might not be the accurate representation of fish needs based on the differences in crude protein and also individual amino acids digestibility. The benefits of using digestible AA data for protein evaluation have been demonstrated in pigs and poultry for decades but are yet to be adopted in fish. The goal of this thesis was to assess the digestible amino acids of commonly used ingredients in order to improve protein evaluation of fish feeds/ingredients. An additional aim was to estimate AA requirements based on digestible AA in protein and compare this with the currently used approach (i.e., AA requirement based on crude protein).

However, the optimization of feed formulation does only depend on the accurate characterisation of feed but also on its utilization efficiency. This implies that despite supplying a balanced AA profile, which meets the daily requirement of fish, the release of nutrients and absorption might be affected by some intrinsic factors. For instance, crystalline AA that is usually supplemented to the diet in order to overcome the AA deficiency problem caused by low quality (e.g., plant) ingredients are said to be less utilised in fish, compared to AA in intact protein. Apparently, the former, which are already in the free form are quickly catabolised and lost rather than used for protein synthesis leading to asynchronous absorption of dietary AA. The concept of 'nutrient synchronization' has been applied in other farms animals (e.g., pigs and poultry) to improve protein utilization efficiency but yet to be explored in the field of fish nutrition. Therefore, this thesis further explored some factors that are affecting the optimal utilization of available AA to check whether nutrient asynchrony does occur in fish.

The first two chapters (Chapter 2 and 3) focused on the digestibility and requirement of AA in fish with a view of improving protein evaluation of fish feeds. African catfish (*Clarias gariepinus*) was used as a case study. **Chapter 2** of this thesis investigated the apparent digestibility coefficients (ADC) of amino acids in 13 feed ingredients of both plant and animal origin (including single-cell protein) in African catfish. We checked if the AA ADC values are equal among different AAs, as well as to the overall crude protein. Furthermore, the effect of feeding level on nutrient ADC was determined by feeding fish restrictively and subsequently to apparent satiation. This chapter revealed that ADCs of nutrients were significantly affected by feeding level but this effect was dependent on ingredient type. For instance, the digestibility of ingredients with high carbohydrate content declined with feeding level. Furthermore, this chapter demonstrated a variation in the digestibility of amino acids and therefore, emphasized the need to quantify individual digestible AA based on digestible protein, especially when low-quality ingredients are used. Due to these observed differences, **Chapter 3** estimated the AA requirements of African catfish on the basis of digestible AA per digestible crude protein. Compared to other animals, AA requirements of fish are usually expressed per kg of feed or kg of crude protein. However, it was shown in Chapter 2 that the ADC of individual AA can differ between ingredients and

among other AAs constituents of protein. Since methionine is one of the first limiting AA in plant ingredients and no reliable information is available for African catfish, methionine was used as a case study. A plant-based diet deficient in methionine was formulated and supplemented with graded levels of crystalline methionine and then fed at 90% satiation to ascertain the requirement. Three (the linear plateau model (LP), broken line model (BL), or quadratic regression model (QR)) out of the common models used in the field of fish nutrition were selected to check if a mathematical model can impact requirement estimates. It was shown that LP and BL had similar values for requirement estimates while QR recorded a 57% higher value. The digestible methionine requirement of African catfish for growth (using LP) ranges between 18.7 and 21.4 g/kg per unit of digestible protein. This equates to a minimum dietary methionine level of 6.3 g/kg diet (19.2 g/kg Crude protein), which is lower than what has been previously reported for this species. Comparing values of methionine requirement when expressed on a digestible methionine or crude protein basis only displayed a slight difference.

The final part of the thesis (Chapter 3 and 4) explored the factors that could hamper optimal protein digestion and utilization in fish. It was hypothesized that the forms of AA and the timing of digestion can affect the optimal utilization of total protein in fish. Other factors such as asynchronous availability of energy at the protein synthesis site could also cause sub-optimal utilization of dietary protein, since optimal protein synthesis is highly dependent on energy availability. Therefore, the question of whether asynchronous nutrient digestion occurs in fish was studied. Moreover, bridging the gap in knowledge on the difference in digestion and absorption rates of nutrients as affected by ingredients characteristics is needed in fish nutrition. In monogastric animals, it has been reported that the physical state of the dietary proteins and carbohydrates can influence the digestion and passage rate of the nutrients before they are absorbed. Studies on kinetics of digestion are numerous in pigs and poultry but the limited studies performed on fish have mainly focused on non-protein energy (NPE) and not protein. In **Chapter 4**, the kinetics of nutrient digestion and development of chyme characteristics in African catfish were assessed in response to fishmeal hydrolysation and non-protein energy sources. This was to ascertain whether the kinetics of protein digestion can be altered by dietary composition. This was assessed by feeding African catfish four diets, which were formulated to contain starch or fat as NPE source, and fishmeal or hydrolysed fishmeal as protein source. Four hours after the consumption of a single meal, chyme was collected from the stomach, proximal-and distal intestine and analysed for dry matter content, crude protein and marker concentration. Postprandial water fluxes to the GIT and stomach evacuation were also assessed. We observed that fishmeal hydrolysation can alter the digestion kinetics of digesta along the GIT. Furthermore, dietary macronutrient composition can alter the postprandial digestion of nutrients in the GIT without being reflected in the faecal digestibility.

The observed differences in the kinetics of nutrient digestion in chapter 4 indicated that there may be asynchronous availability of nutrients during absorption. Therefore, it was assumed that among other factors, feeding frequency can affect the utilization efficiency of AA. In order words, low feeding frequency may affect the optimum utilization of amino acids, especially in a diet that is supplemented with crystalline amino acids. In **Chapter 5,** we investigated the effect of feeding frequency and its interaction with crystalline methionine supplementation level on nutrient digestion and AA requirement. The goal was to investigate if the timing of nutrient availability (nutrient synchronization) can be used as a means of improving AA utilization. Thus, we hypothesized that increasing feeding frequency will aid the utilization of CAA supplementation in diets deficient in AA, thereby leading to an increased protein deposition in fish. To test this hypothesis, two diets that contained methionine

either just fulfilling or exceeding the methionine requirement of African catfish were formulated. These diets were fed to African catfish at four feeding frequencies (six, two, one time (s) per day and two times out of three days). Feeding frequency affected feed intake and the apparent digestibility coefficient (ADC) of nutrients. Feeding at a low frequency hampered daily feed intake while higher frequencies improved nutrient digestibility in African catfish. However, none of the tested parameters was affected by the interaction between dietary methionine levels and feeding frequencies, except for digestible nitrogen intake and dry matter body content. Thus, it was concluded that the asynchronous availability of AA for protein synthesis, which is often suggested to cause a sub-optimal utilization of crystalline AA, was not influenced by feeding frequency.

In **Chapter 6**, the main outcomes of the different studies of this thesis were summarized and discussed in the context of protein evaluation for fish. This chapter further tackled the unresolved question in the field of fish nutrition about the use of protein ADC to predict AA digestibility, since AAs are the building blocks of protein. Feedstuffs are acquired in batches and it is crucial to obtain the AA digestibility data of each batch for proper feed formulation. However, the analysis of the ADC of AA is expensive, therefore, cheaper alternative methods are required. The prediction of AA digestibility from the ADC of crude protein or the sum of total AA (SAA) has been suggested in literature but has been accompanied by different submissions. A meta-analysis was conducted in this chapter to further check the relationship between the ADCs of AAs and the SAA in protein across different ingredient/fish species. It was shown that there is a possibility of using protein ADC as a predictor for the ADC of SAA. However, the relationships of the ADC of individual AA with the ADC of SAA revealed that these relationships strongly varied between the individual AA. For some AAs, like arginine, methionine, glutamic acid and phenylalanine, the ADC declined less strongly compared to the decline in SAA ADC for ingredients with a lower protein quality (i.e., the slope of the linear regression being smaller than 1). While for some other AA like valine, glycine, aspartic acid, proline and cysteine, the decline in ADC with declining SAA ADC was stronger (slope being larger than 1). Moreover, for various AA, the relationship between their ADCs with the ADC of SAA was different between different types of ingredients (animal versus plant sources).

Overall, the following conclusions can be drawn from this thesis.

- Within ingredients, the digestibility coefficients are not equal for the different amino acids.
- This variability in digestibility between amino acids is larger in low-quality ingredients compared to high-quality ingredients when using crude protein digestibility as a quality indicator.
- Crude protein digestibility values of ingredients can be used as a predictor for the digestibility of their total sum of amino acids.
- Individual amino acids digestibility coefficients can be predicted from the sum of amino acids digestibility in ingredients.
- Across fish species, the digestibility of the amino acids, arginine, methionine, glutamic acid and phenylalanine decreases less strongly compared to the digestibility of SAA when the protein quality of an ingredient declines. In contrast, the digestibility of valine, glycine, aspartic acid, proline and cysteine have a stronger decline compared to the digestibility of SAA when the protein quality declines.
- The impact of feeding level on macronutrient digestibility in African catfish is ingredient-dependent.

- The choice of the mathematical model used in studies to estimate nutrient requirement can impact requirement estimates, for instance, quadratic regression can lead to an overestimation of nutrient requirements.
- Based on the linear plateau model, the digestible methionine requirement of juvenile African catfish ranges between 18.7 and 21.4 g/kg per unit of digestible protein, depending on the response criterion.
- In African catfish, the protein utilization efficiency of digested protein is not influenced by feeding frequency.
- Ingredient macronutrient composition can alter the kinetics of nutrient digestion. Changing dietary fat by starch alters the intestinal location of protein digestion in African catfish.
- Hydrolysation of protein (e.g., fishmeal) increases the stomach evacuation rate of protein.
- In this thesis, no proof was found for the existence of nutrient asynchrony in African catfish.

A

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About the author

Folasade Esther Elesho (Nee Oyawale) was born on the 22nd of April 1987 in Ileoluji, Ondo state, Nigeria. After completing her secondary school education, she proceeded to Adekunle Ajasin University Akungba-Akoko, Nigeria for her B.Sc. degree in 2004. Having grown up in a riverine environment, her passion for Fisheries & Aquaculture necessitated her choice of Environmental Biology & Fisheries as a course of study for her undergraduate program. Her B.Sc. thesis was based on the proximate composition of four important fish species in Nigeria. In 2010, she later proceeded to study Environmental Control & Management at master's level, with a research that focused on the impact of cassava effluent on the physico-chemical properties of a river water. After, the completion of her M.Sc. program in 2012, she immediately began a career as an academic lecturer at the Federal University, Oye-Ekiti, Nigeria and remained active in service till date. Her background in educational and professional career made her to be aware of the inter-connection of aquaculture and environmental management. Therefore, she pursued a PhD in the field of fish nutrition within the Aquaculture and Fisheries group (AFI) at Wageningen University, the Netherlands. For her PhD research, she received a scholarship from Tertiary Education Trust Fund, TETfund Nigeria. In addition, the project was partially funded by Skretting Aquaculture Research Centre, Norway and Wageningen University. The research focused on protein evaluation in fish, using African catfish as a case study, which resulted in this thesis.

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List of scientific publications

Elesho, F.E., Sutter, D.A.H., Swinkels, M.A.C., Verreth, J.A.J., Kröckel, S., Schrama, J.W., 2021. Quantifying methionine requirement of juvenile African catfish (*Clarias gariepinus*). Aquaculture. 532, 736020.

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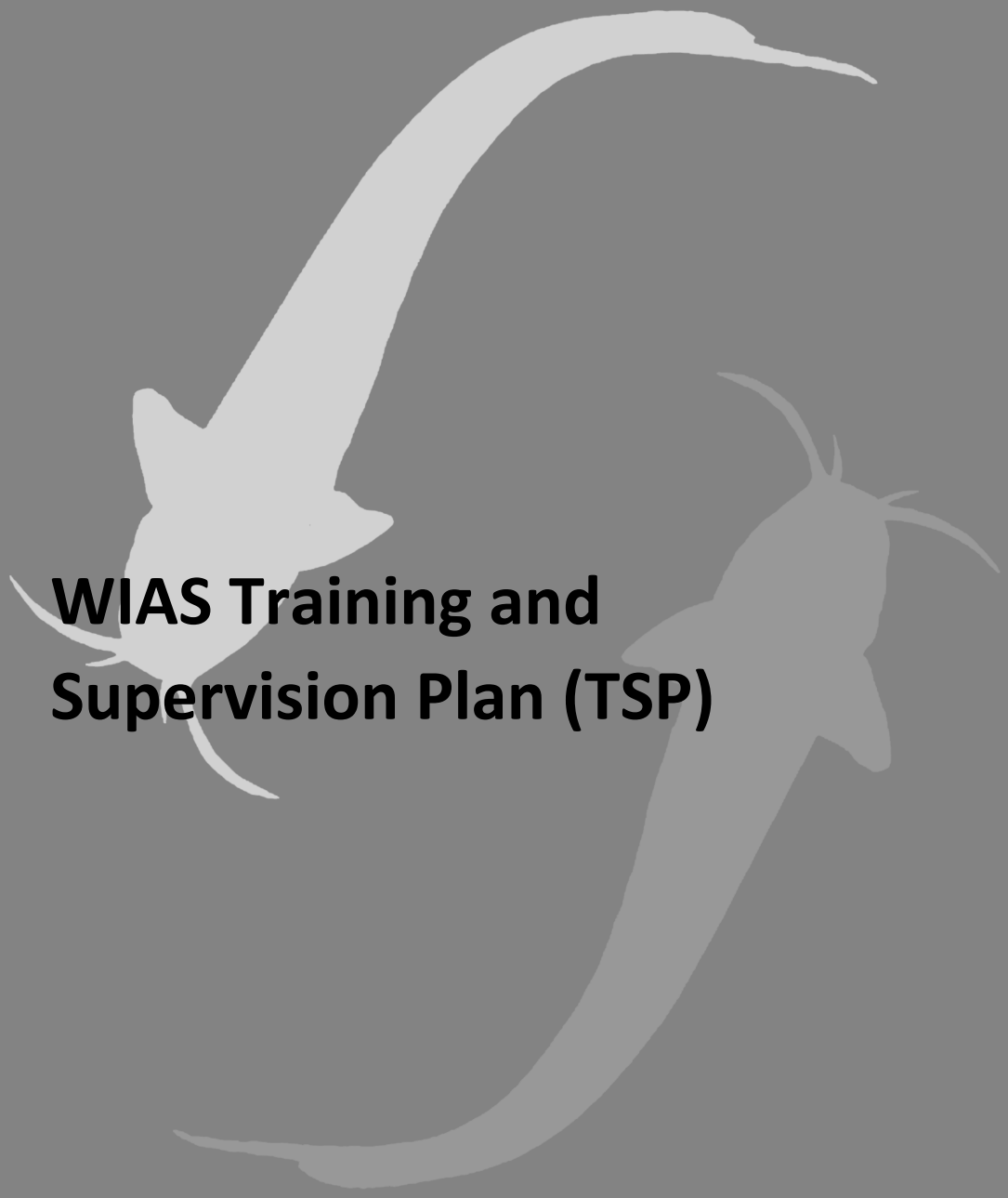
Babalola T.O., **Oyawale F.E.,** Adejumo I.O., and Bolu S.A (2016): Effects of Dietary Fish Oil Replacement by Vegetable Oil on the Serum Biochemical and Haematological Parameters of African Catfish (Heterobranchus longifilis) Fingerlings, Iranian Journal of Fisheries Science., 15(2): 775-788.

Okoya, A.A., **Oyawale F.E.,** Ofoezie E.E., and Akinyele A.B (2016): Impact of Industrial Cassava Effluent Discharge on the Water Quality of Ogbese River, Ayede Ogbese, Ondo State, Nigeria, Ethiopian Journal of Environmental Studies and Management, 9(3): 339 – 353.



WIAS Training and Supervision Plan (TSP)

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The Basic Package	***3 ECTS***
WIAS Introduction Day	2016
Course on philosophy of science and/or ethics	2017
Course on essential skills	2017

Disciplinary Competences	***15.6 ECTS***
Writing a research proposal and developing a TSP	2017
WIAS Advance Statistics Course design of experiment	2017
Laboratory Animal Science: Design and Ethics in Animal Experimentation	2017
Laboratory Animal Science: Species Specific Course Fish	2017
WIAS course statistic for the life science	2018
AFI knowledge exchange programme (China)	2019
Workshop: Fish nutrition: Interaction with Aquaculture system and Water Quality	2021

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Information Literacy including Endnote Introduction	2017
Project and Time Management (PTM)	2019
AFI knowledge exchange programme organizing committee member	2019
WIAS science day organizing committee member	2019
Reviewing a scientific manuscript	2020
Brain-friendly working and writing	2020

Presentation Skills	***4 ECTS***
WIAS Science Day, Wageningen, The Netherlands (Poster presentation)	2018
WIAS Science Day, Lunteren, The Netherlands (Oral presentation)	2019
European Aquaculture Society (EAS) Conference, Berlin, Germany (Poster presentation)	2019
International Symposium of PhD candidate in Chinese and European Aquaculture and Fisheries (Oral presentation)	2019
International Symposium of PhD candidate in Chinese and European Aquaculture and Fisheries (Poster presentation)	2019
WIAS Science Day, Lunteren, The Netherlands (Poster presentation)	2020

Teaching competences	***6 ECTS***
Supervising major MSc thesis: Retno Nuraini	2017
Supervising major MSc thesis: Chen JueQi, Ian	2018
Supervising major MSc thesis: Marieke Swinkels	2018-2019
Supervising major MSc thesis: Roel Frenken	2019-2020
Supervising practicals and excursions	2016-2020

Education and Training Total	***36 ECTS***

Completion of the training activities in in fulfilment of the requirements for the education certificate of the Graduate School of the Wageningen Institute of Animal Sciences (WIAS). One ECTS equals a study load of 28 hours.

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Colophon

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