Determination of bioavailability and bioactivity of enzymatically hydrolysed fish protein and phosphates in the African catfish *Clarias gariepinus*

by

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Supervisor

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Declaration

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Date: December 2017

List of Abbreviations

ADC	Apparent digestibility coefficient
ANOVA	Analysis of variance
AOAC	American Organization of Analytical Chemists
Ca	Calcium
Ca/P	Calcium to Phosphorous ratio
CF	Condition factor
DCP	di-calcium phosphate ($CaHPO_4$)
DE	Digestible energy ($kJ.g^{-1}$)
DOH	Degree of hydrolysis
EAA	Essential amino acid
EFA	Essential fatty acid
EM	Extruded maize
f	final
FA	Fatty acids
FCR	Feed conversion ratio
FB	Fish bones
FB-DCP	Fish bone di-calcium phosphate
FM	Fish meal
FPH	Fish protein hydrolysates
g	gram
i	initial
Ig	Immunoglobulin
ISO	International Organisation for Standardization
ICP	Inductively coupled plasma
kg	kilogram
L	Length (*mm*)
LSD	Least significant difference
l	Litre
M	Molarity (*mole/litre*)
m	mille
NFE	Nitrogen-free extractives
P	Phosphorus
PEG	Polyethylene glycol
PO_4^{-1}	Phosphate
p	Probability
P/E	Protein to energy ratio
RAS	Recirculating aquaculture system
R-DCP	Rock di-calcium phosphate
rpm	Revolutions per minute ($rev.min^{-1}$)
SE	Standard error
SGR	Specific growth rate ($\%.day^{-1}$)
SM	Soybean meal
T	Temperature (°*C*)
t	Time (*days or weeks*)
µ	micro
v	Volume
w	Weight (*g*)
WG	Weight gain (g/g)

Abstract

There is an ever-increasing demand for high-quality aquaculture feed ingredients by this growing food-production sector. Wild fish stocks are dwindling, and aquaculture is a potentially sustainable method for producing the food required to feed an increasing world population. The global population is increasing at 1% per annum, and current projections indicate that food production must be increased to sustain the global population. Fish plays an important role in the food sector globally, and fish consumption increased by over 100% from 1990 to 2014. During fish processing operations, by-products (wastes) are produced, which can be utilised to produce high-quality aquaculture feed ingredients. In this way, valuable natural resources are utilised optimally and nutrients are recycled back into the food-production sector, thereby decreasing pressure on natural resources.

The aim of this work was to conduct aquaculture feeding trials to evaluate two potential aquafeed ingredients derived from fish processing by-products. This evaluation indicated the suitability (bioactivity and bioavailability), performance and potential economic viability of the ingredients as aquaculture feed ingredients. The two feed ingredients were fish protein hydrolysates (FPH), and fish bone dicalcium phosphate (FB-DCP), both produced from monkfish (*Lophius vomerinus*) heads, which are by-products of the South African trawling industry.

The study utilised a standard aquaculture approach for the evaluation of the feed ingredients, by having a control feed formulation (recipe) and different experimental diets to incorporate (include) the experimental feed ingredients. The evaluation compared the performance of the experimental diets and the control diet. The formulation included seven experimental diets and one control diet. FPH replaced a portion of the fish meal (FM) in the diet, and FB-DCP replaced conventional rock dicalcium phosphate (R-DCP) as dietary phosphate source. The FPH replaced a fraction of the total FM (5%, 10%, 15% and 20% of FM) in diets named FPH-1, FPH-2, FPH-3, FPH-4 and distinguished as Trial 1. Two diets replaced R-DCP with either FB (untreated) or FB-DCP, and a final diet employed a 1:1 ratio of R-DCP and FB-DCP diets – named FB-5, DCP-6 and DCP-7 respectively and distinguished as Trial 2. Each diet was evaluated using six replicate tanks of 75 l each, which were initially

stocked with 30 fish of 1 g mean weight each, in a recirculating aquaculture system. A feeding trial of 10 weeks was performed and production performance parameters, such as growth, nutrient utilisation, non-specific immunity and production quality, were evaluated. In addition, a two-week digestibility trial was conducted on all FPH treatments to determine the influence of different levels of dietary FPH on the dry matter digestibility of the experimental diets.

In Trial 1, there were indications that FPH inclusion enhanced a component of the non-specific immune function of the experimental animals significantly, and that this action was dependent on the inclusion level of the FPH. Significantly higher immunoglobulin levels were found in diets (10% and 15% of FM replacement). There were no significant differences in production parameters (weight, length and protein utilisation), mortality or dry matter apparent digestibility between any treatments in Trial 1. FPH inclusion at levels 5% and 20% of FM replacement did, however, affect overall body composition of the experimental animals, as there were, compared to the control diet, significantly increased lipid deposits in diets FPH-1 and FPH-4.

In Trial 2 significant differences were measured for whole-body crude lipids when the phosphate diets were compared to the control, with increased lipids in diets FB-5, DCP-6 and DCP-7. In Trial 2 the production parameters, mortality, non-specific immunity and mineral composition of vertebrae illustrated no statistically significant diet-dependent differences.

The control diet's results were utilised to investigate the length-weight relationship of the African catfish (*Clarias gariepinus*), and a Condition Factor = Weight/Length^b relationship, b = 3.02 was found. This coincides with the b = 3 assumption during Trial 1 and Trial 2, as well as previous studies reported in open literature.

From the results, it can be concluded that both FPH and FB-DCP can be considered as high-quality feed ingredients for aquaculture, as their inclusion had no negative impacts on animal production performance, immune status or final body composition. However, the FPH influence has inclusion level dependencies – the product is optimally effective at the correct dietary inclusion level of FPH. The results indicate that FPH and FB-DCP can be successfully included into aquaculture feeds for African

catfish (*Clarias gariepinus)*, thereby reducing waste of high-value aquaculture feed ingredient products and promoting sustainable aquaculture.

Opsomming

Daar is ’n toenemende vraag na voerbestanddele van hoë gehalte vir akwakultuur, wat ’n vinnig groeiende sektor van voedselproduksie is. Voorrade van wilde vis is besig om te kwyn, en akwakultuur is geïdentifiseer as ’n potensieel volhoubare metode om voedsel vir ’n groeiende wêreldpopulasie te verskaf. Die wêreldpopulasie groei teen 1% per jaar, en huidige projeksiesyfers toon dat voedselproduksie moet toeneem om die populasie te onderhou. Vis speel ’n belangrike rol in voedselproduksie wêreldwyd, en verbruik het vanaf 1990 tot 2014 met 100% toegeneem. Verwerking van vis gee aanleiding tot neweprodukte (afval), wat gebruik kan word om akwakultuur voerbestanddele van hoë gehalte te produseer. So word waardevolle natuurlike hulpbronne optimaal gebruik en voedingstowwe word terug na die voedselproduksiesektor gesirkuleer, wat druk op natuurlike hulpbronne verlig.

Die doel van hierdie studie was om twee potensiële akwakultuur voerbestanddele wat van neweprodukte van visprosessering vervaardig word, deur middel van voerproewe te evalueer. Die evaluering het die geskiktheid (bioaktiwiteit en biobeskikbaarheid), prestasie en potensiële ekonomiese lewensvatbaarheid van die akwakultuur voerbestanddele bepaal. Hierdie twee voerbestanddele was visproteïen hidrolisaat (VPH) en visbeen dikalsium fosfaat (VB-DKF), wat albei van monnikvis- (*Lophius vomerinus*) koppe, wat neweprodukte van die Suid-Afrikaanse vissery is, vervaardig word.

Die studie het ’n standaard akwakultuur benadering gebruik om die voerbestanddele deur middel van ’n voerformulering (resep), as kontrole, en verskillende eksperimentele voere met eksperimentele voerbestanddele, te evalueer. Die prestasie van die eksperimentele voere is dus deur die evaluering met die prestasie van die kontrolevoer vergelyk. Die formulering is op sewe verskillende eksperimentele diëte en een kontroledieet toegepas. VPH het gedeeltes van vismeel in die dieet vervang, en VB-DKF het konvensionele klip di-kalsiumfosfaat (K-DKF) as fosfaatbron in die voer vervang. Die VPH vervang dele van die totale VM (5%, 10%, 15% en 20% van VM) in die voere, genoem FPH-1, FPH-2, FPH-3, FPH-4, en word onderskei as Proef 1. In twee ander voere is K-DKF met onbehandelde visbeen en VB-DKF onderskeidelik vervang, en in die laaste voer as ’n 1:1 verhouding van VB-

DKF en K-DKF; dié voername is FB-5, DCP-6 en DCP-7, en verteenwoordig Proef 2. Elke dieet is sesvoudig gedupliseer in tenks met ’n gesirkuleerde akwakultuur sisteem van 75 l, elk aanvanklik met 30 visse met ’n gemiddelde gewig van 1 g. Die voerproef was oor 10 weke, en het prestasieparameters, soos groei, benutting van voedingstowwe, niespesifieke immuniteit en produksiegehalte, gemeet. ‘n Verdere proef is oor twee weke op VPH voere uitgevoer om die invloed van verskillende vlakke van dieetkundige VPH op die verteerbaarheid van die droë middels op die eksperimentele diere te bepaal.

In Proef 1 was daar aanduidings dat VPH insluitingsvlakke ’n komponent van die niespesifieke immuniteit beduidend verbeter het, en dat hierdie verskynsel van die insluitingsvlak van die VPH afhanklik was. Beduidend hoër immunoglobulienvlakke is in voere (10% en 15% VM vervangingsvlakke) gemeet. Daar was geen beduidende verskille in prestasieparameters (gewig, lengte en proteïenverbruik), sterftesyfer of merkbare verteerbaarheid van droë middels tussen enige van die voere in Proef 1 nie. VPH insluitingsvlakke van 5% en 20% by VM vervanging het wel ‘n beduidende invloed op die heelliggaam-samestelling van die proefdiere gehad, met hoër vlakke van vetneerslag met FPH-1 en FPH-4 as met die kontroledieet.

In Proef 2, waar die fosfaatdiëte met die kontrole vergelyk is, is beduidende verskille ten opsigte van heelliggaam ruvet gemeet, met voere FB-5, DCP-6 en DCP-7 op hoër vlakke vet. In Proef 2, was daar geen beduidende dieetafhanklike verskille ten opsigte van prestasieparameters, sterftesyfer, niespesifieke immuniteit en mineraalinhoud van rugwerwels nie.

Die kontroledieet se resultate is aangewend om ’n gewig-lengte verhouding vir die Skerptandbaber (*Clarias gariepinus*) te bepaal. Deur die berekening van die verhouding van Kondisiefaktor = Gewig/Lengte^{b}, is b = 3.02 bevind, wat ooreenstem met die aanname wat in Proef 1 en Proef 2 gemaak is en wat ooreenstem met vorige ondersoeke in oop literatuur, naamlik, b = 3.

Uit die bevindinge kan afgelei word dat sowel VPH as VB-DKF as hoë gehalte voerbestanddele van akwakultuur beskou kan word, omdat die insluitingsvlakke nie enige nadelige invloed op die diere se produksieprestasie, immuniteitstatus of finale liggaamskomposisie gehad het nie. Daar is wel bepaal dat die invloed van VPH

insluitingsvlakafhanklik is – die produk is optimaal effektief teen die korrekte vlak van VPH insluiting. Die resultate dui aan dat VPH en VB-DKF suksesvol geïmplementeer kan word in voere vir Skerptandbabers (*Clarias gariepinus*). Hierdie proses verminder afval, deur hoëwaarde- akwakultuur voerbestanddele van neweprodukte te vervaardig, en bevorder volhoubare akwakultuur.

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Dedication

I dedicate this work to all the Swanepoels of Groenfontein, Ouma Marina and Inge.

The Swanepoels: Grandfather Hendri, my parents, Willie and Irene, sisters Janie and Sibella, brother Pieter.

List of Tables

List of Figures

Table of Contents

1. Introduction

The culture of aquatic species (referred to as aquaculture) is a method with the potential to supply food for a growing population in the future, and to ensure healthy, active and high-quality lives. However, the aquaculture industry requires high-quality feed ingredients if it is to produce the formulated feeds needed to grow the cultured animals to a harvestable size. The world population is increasing rapidly in size, at a rate of about 1% per annum (OECD/FAO, 2017). Aquaculture shows great promise for fuelling increasing demand for protein food sources, which is the fastest growing sector in food production (Ye et al., 2017). With the world facing dwindling wild fish stocks, sustainable aquaculture practices ensure that a more sustainable solution is available to meet the food-market demand for aquatic animal products, such as farmed finfish (FAO, 2016, Mathiesen, 2012). However, sustainable aquaculture industry growth requires high-quality feed ingredients.

The need for alternative aquaculture feed ingredients has increased as wild fisheries stock decreases and population increases. Currently fish meal (FM) and fish oils are major contributors to the aquaculture feed industry (Naylor et al., 2009). There is mounting pressure for research, and demands that the feed industry searches for alternative options for feed ingredients. However, fish-derived ingredients (e.g. FM, fish oil etc.) are preferred for aquaculture feeds, because these ingredients ensure the feed has a balanced essential amino acid (EAA) and fatty acid composition, and improves the palatability of feed. EAAs contain some of the building blocks of protein, which are required by the animal's metabolic system for growth – the animal cannot synthesise these amino acids (Nates, 2016). Essential fatty acids (EFA) are fatty acids that the animal cannot synthesise, and therefore they have to be included in diets for fish (NRC, 2011).

Regarding the utilisation and effective use of fish by-products, producing fish protein hydrolysates (FPH) from fish processing waste serve as a renewable technique to produce fish-derived feed ingredients. The production of FPH reduces the fish processing by-product to a liquid protein feed/food source with free amino acids and beneficial properties (Chalamaiah et al., 2012). Recycling the fish by-products by enzymolysis (the process of decomposing proteins in the presence of an enzyme

serving as as a catalyst) creates FPH, thereby utilising the processing by-products of wild caught and farmed fish effectively (Kristinsson and Rasco, 2000). FPH can be used as a substitute for FM as an aquaculture feed ingredient to utilise the protein from fish (Xu et al., 2016). FPH has the potential to be more profitable and more readily available to produce, e.g. it can be easier to manufacture than FM in certain instances, and it is less energy intensive to produce (Ishak and Sarbon, 2017). Simultaneously, enzymolysis creates fish bones as a secondary by-product. With further processing, using acid hydrolysis to leach out minerals, the fish bones can be used as a feed-grade phosphate additive. This phosphate additive can replace inorganic phosphate sources in aquafeed (Albrektsen et al., 2017).

Thus, by recycling fish processing by-products (waste), a *protein feed source* and a *phosphate feed additive* can be produced.

1.1 Motivation and background

Global aquaculture production needs to increase in order to supply the growing demand for fish products (FAO, 2016). Wild populations of most freshwater and marine fish are decreasing, yet the market demand for fish as a protein food source is increasing (OECD/FAO, 2017). A potential solution to this problem is offered by aquaculture, the artificial production of aquatic species in a controlled environment. Figure 1 shows global fish utilisation for human food and other uses, and global fish production (including wild caught fish and fish from aquaculture) between 1980 and 2014. The figure illustrates an increase in fish consumption by humans and a stagnant supply from wild caught fisheries. Figure 1 indicates that fish consumption by humans increased by over 100% from 1990 to 2014.

With the global human population increasing in size, the demand for farmed fish will increase, as people require protein (Lee et al., 2016). Aquaculture can supply in this increased demand, however, one of the current challenges is that formulated feed required to grow the fish to a sufficient size for harvesting is expensive, and in some cases, producing the feed is unsustainable. In unsustainable cases, feed production leads to overuse of decreasing wild fish populations, instead of minimising the impact of feed production on the environment and ecosystems. Further, feed ingredients that supply protein in aquafeeds are expensive and contribute a significant proportion

of the overall cost of aquafeeds (Nates, 2016). Expensive aquafeeds are creating high variable operating costs, of 30-40%, for aquaculture farms (FAO, 1978).

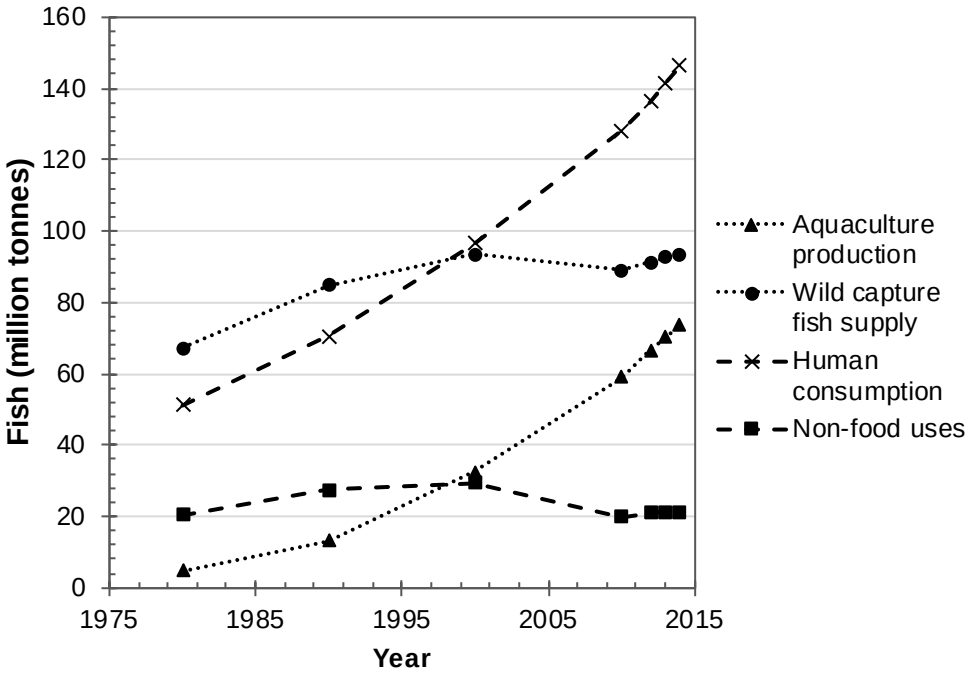

Figure 1 Global fish utilisation from 1980-2014 (FAO, 2016)

Despite the environmental disadvantages of utilising fish protein in aquafeeds, it is difficult for the aquafeed industry to replace/substitute all FM with plant proteins in balanced aquaculture feeds, because fish protein-derived products contain such good levels of EAA and EFA. Currently, FM and soybean meal provide the bulk of proteins used for aquafeed (Tacon and Metian, 2015). However, in most countries, soybean and FM exhibit fluctuating market prices, which are also heavily influenced by climate and currency instabilities (Asche et al., 2013, Peri, 2017). From an economic and environmental point of view, FM is not always a sustainable protein source for animal feeds. In some cases, edible and diminishing fish stocks are used for the production of animal feeds, and only about 25% of global FM production uses by-products (Tacon et al., 2011). Therefore, plant proteins are commonly used to substitute for fish-derived ingredients. The challenge posed by most plant proteins is that they contain insufficient levels of one or more EAAs required by aquatic animals, for which it is the only source of protein from aquafeed. In addition, plant proteins can contain antinutritional substances that hinder nutrient absorption and utilisation.

Therefore, plant proteins, such as soybean meal (SM) often exhibit low nutrient utilisations, leading to poor production performance in some fish species (Zhou et al., 2017) when they are incorporated as sole protein source in aquafeeds.

One potential solution is to decrease the use of FM in aquafeed, and to use FPH from fish processing by-products as a partial replacement for FM in aquaculture diets (Zheng et al., 2012). It is an accepted, standard aquaculture procedure to blend different protein sources, in this case a combination of FM and FPH, in such a way that the nutritional requirements are still achieved. FPH can act as a source of free EAA and enhance palatability of the feed. When the FPH is produced from by-products, it can give the aquaculture industry a means to optimise the utilisation of fish products to turn the 'waste' into a valuable high-quality feed ingredient in diets. This practice could decrease the reliance in wild caught fish and improve the sustainability of aquaculture.

Additionally, FPH, when compared to conventional FM, can exhibit specialist properties in diets of finfish, such as improved growth performance, palatability, survival and nutrient utilisation, and can have bioactive effects on fish health and immunity (Kotzamanis et al., 2007, Zheng et al., 2014). These additional functional and bioactive properties of FPH result in higher market prices as feed ingredients, which makes the production of FPH an attractive waste-processing option for fish processors.

Recently, two new products were developed to produce a) FPH under optimum conditions (Greyling, 2017); and b) a feed-grade phosphate supplement fish bone dicalcium phosphate (FB-DCP) (Swart, 2017) at the Department of Process Engineering at Stellenbosch University. The raw materials for both feed ingredients are monkfish (*Lophius vomerinus*) heads from local trawler fisheries. These newly developed feed ingredients were optimised in the laboratory, but have not been evaluated for their suitability as animal feed ingredients in actual feeding trials.

1.2 Objectives

The aim of the project was to evaluate two feed ingredients developed at the Department of Process Engineering, namely, FPH and FB-DCP produced from fish processing by-products, in an aquaculture feeding trial, using the African catfish

(*Clarias gariepinus*) as an experimental fish species. Both new ingredients were evaluated relative to ingredients that are currently deemed to be the industry standard: the FPH was compared with FM, while the FB-DCP was compared with rock phosphate (R-DCP).

The investigation was structured to the following approach: 1) Conducting a literature review of previous studies on how to use the feed ingredients (FPH and FB-DCP) in a standard aquafeed for African catfish (*Clarias gariepinus*), and to what suitable levels to include in diets according to dietary nutrient requirements. 2) Formulating aquafeed from the products into a suitable feed type for the African catfish (*Clarias gariepinus)*. 3) Implementing measures/parameters for determining the effects of ingesting the products on the animals. This step will illustrate some of the properties and consequences the product have on animals' growth, quality and health. This investigation (Steps 1-3) will evaluate the bioavailability and bioactivity of FPH and FB-DCP.

This document will present a literature survey in Section 2, which comprises the necessary background, terminology and standard procedures, and presents a critical review of similar and relevant previous studies. Section 3 presents the questions that were derived from the literature survey and which the study attempted to answer. Section 4 discusses the procedure and techniques applied to answer the questions; including all experimental design, procedures and analytical techniques for the analysis of samples. Section 5 presents the results gathered from the experimental work, explains the scientific phenomena illustrated by the results and how this compares to previous investigations, as well as presenting opinions relating to a critical evaluation of the findings. Section 6 provides the final conclusion regarding the evaluation of the feed ingredients, and suggests the impact of using these ingredients. Section 7 provides recommendations to industry and proposes further research on the topic. The appendices provide all additional information and requirements for the successful completion of the work.

2. Literature survey

This chapter is divided into five sections; Section 2.1 introduces the relevant terminology, background, and historical context of FPH production, its utilisation and advantages in aquaculture. Section 2.2 gives an overview of the standard procedure used for evaluating feed ingredients in aquaculture. Section 2.3 reviews the production and utilisation of FPH, and Section 2.4 discusses previous applications of some types of protein hydrolysates in aquaculture. Section 2.5 reviews the relatively new idea of using the secondary by-product, fish bones, to possibly produce a phosphate additive for use in animal feeds. Finally, Section 2.6 will provide the motivation for using African catfish (*Clarias gariepinus*) as experimental species to evaluate the feed ingredients, FPH and FB-DCP. In general, the chapter provides the necessary background regarding the use of fish processing by-products, FPH and FB-DCP, as aquafeed ingredients. In addition, the way this technique contributes to waste reduction and sustainable optimum utilisation of high-quality feed ingredients is discussed.

2.1 Background and historical context

The rapid growth of the aquaculture industry requires the development of cost effective, high-quality feed ingredients that can be used as replacements for FM and soybean, and new technology to improve current methods of production, and meet the demand of the growing industry (NRC, 2011). The world is heavily reliant on fish as a food source. This demand for fish can be met by either wild caught or farm raised fish, i.e. aquaculture (Tacon et al., 2009). The finfish (distinguished from shellfish) cultivation industry's most expensive running cost component is aquafeed (Tacon et al., 2011), which is required to grow the animals to harvestable size. The responsibility of the scientific community and feed industries is to convert the raw materials that are available into suitable feeds for aquaculture farmers. The total expenditure on feeds for these farmers can account to 50-60% of their total running costs (Nates, 2016), which means that, in many instances, the feed expenditure determines how profitable an aquaculture operation will be (Cai et al., 2017). Therefore, the aquaculture industry requires high-quality, cost effective and sustainable feed.

The aquafeed industry relies heavily on supply from wild caught fish to provide protein raw material for the production of aquafeeds (Glencross et al., 2007). The main function of FM is to provide protein for the diet (which consists of amino acids as building blocks; these amino acids have numerous metabolic and structural functions in the fish body), which is required to build muscle tissue in the production animals (Nates, 2016). Wild caught fish also supply fish oil and lipid supplements for diets, these oils and lipids can comprise a large range of components of which the primary roles are energy storage and fulfilling structural functions (NRC, 2011). To use ingredients harvested from wild caught fish as feed ingredients is, in some instances, an unsustainable practice (Cashion et al., 2017), as most wild fish stocks are depleted (Mathiesen, 2012).

Aquaculture requires high-quality feed ingredients from sustainable sources (Naylor et al., 2009). Several attempts have been made to replace the raw materials from wild caught fish in feeds by using alternative protein sources, e.g. plant protein, to replace FM (Hoffman et al., 1997, Hardy, 2010, Goda et al., 2007, Enyidi, 2012). Utilising the by-products of fish processing provides a new opportunity to develop fish-based feed ingredients as alternative sources of protein and feed additives, instead of plant-based products. Fish-based feeds are more sought after among nutritionists and farmers, as fish contain all the EAAs required (Naylor et al., 2009), while plant-based ingredients are often deficient in one or more EAA. The production of FM from fish processing by-products is common and about 25% of the FM produced globally is from by-products (Tacon et al., 2011). FM is of relatively low value compared to other fish-derived products, and the production process is energy intensive. Usually, when small amounts of by-products are produced at a particular facility, it is discarded as waste, as it is not economically viable to produce small volumes of FM. FM factories are only viable when large amounts of raw material are available, and it is rarely viable to transport low-value raw material over large distances to FM plants (Tacon et al., 2009).

Production of FPH from fish processing by-products provides the opportunity to produce another high-value aquafeed ingredient, in addition to FM. The enzymatic hydrolysis of the by-products has been proposed as a new way to produce high-value feed ingredients from the fish processing by-products (Ghaly et al., 2013, Dumay et

al., 2004, Kim and Mendis, 2006), as this process has the potential to produce a product that increases the digestibility and bioavailability of the protein in the feed (Refstie et al., 2004). The enzymatic protein hydrolysis process results in a product with shorter peptide chains and free amino acids, which can be utilised more effectively by animals (Harnedy and FitzGerald, 2012). Further, some protein hydrolysates can have beneficial bioactive properties, such as increased disease resistance and immune response (Xu et al., 2016), although there is limited information available on exactly how and why protein hydrolysates can be beneficial to animals.

During the enzymatic hydrolysis, fish bones are produced as secondary by-product, and these bones consist mainly of calcium phosphate, hydroxyapatite and structural proteins, such as collagen (Kim and Mendis, 2006, Nagai et al., 2004). Due to the relatively high (60-70%) mineral content of inorganic substances (Kim and Mendis, 2006) in fish bones, extraction of the minerals can yield a product that could be a potential phosphate source for aquafeed (Albrektsen et al., 2017, Ytteborg et al., 2016). A further benefit of this approach is optimal utilisation of the initial fish processing by-products.

This information confirms that there is a clear opportunity to produce two different products, FPH and FB-DCP, from fish processing by-products, with potential for application in aquafeeds. However, the suitability of these products as aquafeed ingredients needs to be established.

2.2 Aquaculture feed ingredient evaluation

The following section describes the standard procedure/practices employed to evaluate feed ingredients for aquaculture. Ingredient evaluation is a critical part of aquaculture development and research, with determining the following roles of components in the physiological development of animals being the most important: i) Ingredient bioavailability (digestibility), ii) practical implications of adding the ingredient to a feed (palatability), iii) the nutrient utilisation of the ingredient, and iv) potential positive/negative influence on health and wellbeing of the animal (Glencross et al., 2007). Production indicators, that is, growth rate and production per feed given, are important for determining the cost-effectiveness of the feed ingredients.

For evaluation of feed ingredients by research, the experimental design and methodology play an important role in determining the availability and digestibility of the feed ingredients. Although feed ingredient availability and digestibility are important, the evaluation is also specific to each situation as evaluation techniques are dependent on the type of ingredient and aquaculture species in which they are evaluated (Glencross et al., 2007).

In evaluating ingredients, cost/price is considered, as aquafeed is usually the biggest expense for farmers (Nates, 2016). In most cases the cost relates to production -- which is therefore the *specific growth rate (SGR)* of the animals -- and the amount of weight gain per feed given: *the feed conversion ratio (FCR)* (NRC, 2011). The SGR and FCR are common measurement parameters during aquaculture feeding trials. In order to ensure that the measured results are reliable, some important interfering aspects need to be controlled, including running trials for a sufficient length of time, ensuring appropriate general husbandry of fish (water conditions, stocking density etc.), and applying correct feeding and sampling methods (length and weight measurements) (Glencross et al., 2007).

To ensure a consistent and high-quality product for the market it is important for aquaculture farmers to attend to production or nutrient quality. To determine the nutrient composition of the product, protein, lipid and ash analyses are used to quantify the nutrient composition; this is known as *whole-body proximate composition*. Additional body composition analyses can include mineral analyses of both whole-body and bone (vertebrae) composition. If phosphate feed additives are evaluated it is important to also analyse for Ca and P in bones, in order to evaluate the feed ingredients appropriately (Ytteborg et al., 2016, Albrektsen et al., 2009).

The general wellbeing and health of experimental animals are important during the evaluation of feed ingredients. This also applies to production in aquaculture, as increased health and wellbeing can relate to increased production, survival rate, and profit. Mortality is a common parameter used as an indicator of animal health in production experiments, however, Kiron (2012) argues that, although growth and mortality are important indicators for evaluation of feed ingredients, it is important to determine any influence of feed ingredients on other health parameters. Positive health influences (by the feed ingredients) on the animal include increased disease

resistance, survival rate and immunity, while negative influences will have the opposite effect, which could lead to negative/positive production. Therefore, potential effects on animal health should also be taken into consideration during the formulation of aquafeeds. In specific cases, certain high-value feed ingredients can improve immune response for disease resistance, this is known as the immunostimulant effect of the feed ingredient (Dawood et al., 2017). The Food and Agricultural Organization (FAO) declared that the scientific community should investigate, specifically, the role of nutritional compounds such as immunostimulants and immune enhancers (Subasinghe, 1997). To evaluate immunity during feeding trials, blood serum samples can be analysed for non-specific immunity parameters, to evaluate the influence of feed ingredients on immune response (Ardó et al., 2008).

Apparent digestibility coefficients (ADC) determine the digestibility of a feed ingredient relative to other ingredients (Glencross et al., 2007). Digestibility indicates how readily the experimental animals absorb nutrients during feeding trials. This process involves adding a known amount of an inert marker in the diet and measuring the concentration of this marker in the faeces. The digestibility of various nutrients (e.g. protein or lipid) can be determined, or that of total dry matter, and digestible energy. This process is influenced by the method of faeces collection, which could involve stripping the faeces from the animal's anus, surgically removing the faeces from the gut and using automatic collectors, such as tank funnels with an area with low velocity flow, to ensure that the faecal matter is not swept away (Tacon and Rodrigues, 1984). Each of these methods has drawbacks. So-called stripping and surgical methods do not necessarily mean complete reabsorption in the gut or that is the digestion is complete. The challenge posed by automatic collectors/funnels is that the faeces becomes unstable and leaches some components, thereby changing its composition, during the release of faeces in the water by the animal. In faeces collection there will be a degree of compromise, which will influence the results, and repeatability of experiments.

In evaluating FPH the important factors are growth performance, bioavailability, feed utilisation, digestibility and non-specific immunity (Bui et al., 2014). Disease resistance is also important, although there are some practical and ethical challenges

related to testing disease resistance which show variable results (Murray et al., 2003).

In conclusion, there are various standard procedures for feed ingredient evaluation for different studies, applications and species. The most important parameters that should be evaluated to determine the quality/value of a feed ingredient can be categorised as bioavailability (growth, nutrient utilisation, and digestibility), production quality and bioactivity (health).

2.3 Enzymatic hydrolysis of fish processing by-products

There are various methods that can be applied to extract protein from fish by-products with enzymes, and each process has unique applications and/or techniques. The specific process employed will depend on the raw material and the desired type of product (Chalamaiah et al., 2012). The hydrolysis of proteins has a long history with vegetables and milk, but the hydrolysis of fish only started in the 1960s, when FPH was used as an additive in food formulations, although the bitter taste of FPH made it unsuitable for food applications (Kristinsson and Rasco, 2000).

The type of enzymes and substrates used in the process of protein hydrolysis distinguish some of the techniques used to produce FPH with enzymes. The two most common biochemical methods employed are either to add proteolytic enzymes from an external source to the substrate under controlled conditions, or making use of the endogenous enzymes already present in the raw material (Klompong et al., 2009). When using autolytic enzymes, it is important to prevent the highly perishable fish product from spoiling microbially by controlling the hydrolysis with pH; if acid is added, it is known as fish silage (Kristinsson and Rasco, 2000, Greyling, 2017). However, using endogenous enzymes does not ensure consistent quality; alternatively, using proteolytic enzymes produces a consistent product with known qualities and composition.

The literature reviewed includes aquaculture trials, which employed all the different types of FPH, i.e. hydrolysates produced through controlled hydrolysis, and both chemical and fermented fish silage. Fish silage results when the substrate (fish raw material) is fermented with bacteria and/or enzymes, a carbohydrate source (e.g.

molasses) is added, and acidification (e.g. formic or sulphuric acid) is performed to preserve it. The result can be stored as wet or dry product (Fagbenro, 1996).

The most basic fish silage is chemical silage, which is produced by using only the endogenous enzymes present in the fish (normally in the digestive system), to break down the substrate to a liquid protein in the presence of an acid to prevent microbial spoilage. Fermented silage results when the added or present lactic acid bacteria are utilised together with a carbohydrate source (sugar, such as molasses) to break down the substrate (Raa et al., 1982).

FPH is produced under controlled hydrolysis by adding an enzyme or combination of enzymes to the raw material, and then controlling the conditions of the hydrolysis, such as temperature, pH and enzyme concentration (Chotikachinda et al., 2013). By adding the enzymes to the fish by-product (raw material) under controlled conditions, the resulting product will contain proteins (a mixture of intact protein, peptides and free amino acids), fats (oils) and bones from the fish (Kristinsson and Rasco, 2000). The amounts/levels of these different components will reflect the composition of the starting raw material (Goosen et al., 2014a). Each component (protein, fats, bones) can be liberated, and the protein components will contain soluble and non-soluble peptides and amino acids to be used as protein feed ingredients (Guerard et al., 2001) – they are known as FPH in this study. The fish bones remaining after protein hydrolysis are a secondary by-product and are normally not included in the final FPH product.

As mentioned, the advantage of using controlled hydrolysis is that the composition of the final product can be controlled more accurately, thus the product quality will be more consistent with a known degree of hydrolysis (DOH) (Šližytė et al., 2005). The optimised process for producing the most efficient FPH can be dependent on the raw material used and thus be unique for each process (Šližytė et al., 2005). For this project, the optimised process was developed and designed by Greyling (2017), and the methodology will be used to produce the feed ingredient that is to be evaluated.

The composition of the hydrolysates can vary, depending on the raw material (substrate), enzymes (type and concentration) and conditions (pH, temperature, time) of the hydrolysis process (Klompong et al., 2009). Although the beneficial properties

of the FPH are dependent on the process parameter factors, it is essential to know how well the product will perform as a feed ingredient. Therefore, it is critical to investigate the bioavailability and bioactivity via feeding trials, i.e. *in vivo*.

2.4 Application of fish protein hydrolysates in aquafeed

The FPH produced by controlled hydrolysis using added enzymes can be used in various applications in the animal feed and pharmaceutical industries, because of its well balanced amino acid composition (Kristinsson and Rasco, 2000). Several articles detail functional and other properties of FPH (e.g. antioxidant capacity and properties in food systems) (Chalamaiah et al., 2012, Pires et al., 2013); however, the scope of this study is limited to the application of FPH as an aquaculture feed ingredient.

The main advantages of FPH as a feed ingredient are the replacement of FM in the formulation, and the immunostimulant properties of the product (Hardy, 2010, Refstie et al., 2004, Murray et al., 2003, Johannsdottir et al., 2014). FPH also contains more free amino acids and shorter peptide chains, which give the ingredient its bioactive properties (Harnedy and FitzGerald, 2012), and more levels of EAA than plant proteins, and it is therefore a good candidate as a feed ingredient to replace FM and plant protein sources (e.g. SM).

Results of previous studies that used FPH as an ingredient in aquafeed vary, which might be due to the fact that each hydrolysis method (technique, enzymes) and raw material will produce a product with unique nutrient and amino acid composition, and bioactive compounds (Klompong et al., 2009). From literature, it is apparent that the most common advantage of using FPH in aquafeed is by replacing FM, and beneficial effect on growth, feed intake, protein utilisation and, in certain cases, an immunostimulating effect, which can translate into improved disease resistance. These beneficial properties of FPH are attributed to the characteristic shorter peptide chains and free amino acids that are found in these products (Refstie et al., 2004, Kotzamanis et al., 2007, Zheng et al., 2014, Liang et al., 2006). In several feeding trials, successful growth enhancement and proof of beneficial health properties have been found after FPH inclusion in aquafeed. These effects were, further, found for a wide variety of finfish and other aquatic species.

Successful feeding trials report using FPH in aquafeed with various species, for example, Atlantic salmon with up to 15% inclusion level (Refstie et al., 2004); studies by Hevrøy et al. (2005); Berge and Storebakken (1996) and Espe et al. (2012).

Other common aquaculture species include red sea bream and olive flounder (Khosravi et al., 2015a), rainbow trout (Aksnes et al., 2006c) and Nile tilapia (Borghesi et al., 2008). Some of the feeding trials illustrate improved production (Chotikachinda et al., 2013, Khosravi et al., 2015a, Khosravi et al., 2015b) and immune response from using FPH as a feed ingredient instead of FM (Bui et al., 2014) and administering a high plant protein diet (Xu et al., 2016, Aksnes et al., 2006a, Zheng et al., 2014).

The summary of FPH feeding trials is divided into separate tables for marine fish (Table 1), shellfish (Table 2) and freshwater species (Table 3). These summaries include protein hydrolysates produced through controlled hydrolysis, and chemical and fermented fish silage as feed ingredients in feeding trials. For the purpose of this study, the review only includes literature that investigated FPH-type ingredients as a feed ingredient for aquatic animals. Other applications, e.g. as food ingredient for humans, as feed ingredient for other animals, or as fertilizer, are all excluded. In addition, the raw materials for producing the feed ingredients are fish/marine processing by-products, such as tuna viscera from the canning industry (Ovissipour et al., 2014, Khosravi et al., 2015a).

The summary (Table 1, Table 2 and Table 3) illustrates that the influence of FPH-type products are not consistent over all trials, species, development stages and inclusion levels. It is clear, however, that production techniques, conditions and raw materials were different, and that each species and development stage ingested the ingredient differently. The diet within which the ingredient was included also had an influence on the performance of the experimental ingredient, that is, the level and type of other ingredients in the diet, for example, the silage only performed efficiently if a diet's FM levels were high enough (Soltan et al., 2008).

Over different species, and using the identical product, the influences are inconsistent. A study by Cahu et al. (1998) illustrates increased survival and growth with two different species, European sea bass (*Dicentrarchus labrax*) and common carp (*Cyprinus carpio*) – carnivore and omnivorous species respectively. Kvåle et al. (2009) found that, in the same study, Atlantic cod (*Gadus morhua*) had an increased survival rate, while Atlantic halibut (*Hippoglossus hippoglossus*) had a decreased survival rate.

The inclusion level of the FPH products is important: for each species and product the optimal level is unique. By including three different levels of tuna hydrolysate (44.9 $g.kg^{-1}$, 187.3 $g.kg^{-1}$ and 374.7 $g.kg^{-1}$) and replacing FM for Persian sturgeon (*Acipenser persicus*) larvae, Ovissipour et al. (2014) found that the intermediate level was the most effective to achieve good growth and feed utilisation. Studies investigating low and high FPH inclusion levels with Turbot (*Scophthalmus maximus*) (Xu et al., 2016) and Japanese sea bass (*Lateolabrax japonicus*) (Liang et al., 2006) illustrate similar results. Thus, the influence of FPH is inclusion level dependent, however, it is a function of species, development stage and product type, and high inclusion levels can have a negative effect on fish development (Johannsdottir et al., 2014).

The type of FPH is important, especially its molecular weight, composition of di- and tripeptides and raw material, which will influence the growth and feed utilisation on animals. Investigations with Japanese flounder (*Paralichthys olivaceus*) found increased performance results with low molecular weight products, and high-level molecular weight products having no effect (Zheng et al., 2012, Zheng et al., 2014). Delcroix et al. (2015) illustrate that both raw material and composition will have an influence on the growth of European sea bass (*Dicentrarchus labrax*). Kotzamanis et al. (2007) suggest that the molecular weight of the ingredient can influence the immune status of animals.

The influence of FPH products on health factors, such as disease resistance, and haematology, clinical and immune parameters, vary among investigations, however, from the summary (Table 1, Table 2 and Table 3), the general trend is that investigations found an increase in or no influence on health. An investigation with Coho salmon (*Oncorhynchus kisutch*) found no influence on disease resistance by the particular pathogen investigated (Murray et al., 2003), while Red sea bream (*Pagrus major*) had good disease resistance when FPH was included in the diet (Bui et al., 2014, Khosravi et al., 2015a, Khosravi et al., 2015b).

Table 1, Table 2 and Table 3 illustrate variable results, as discussed, in relation to the inclusion of FPH in diets. The investigations confirm that the influence is not always positive, but is dependent on species, inclusion level, development stage (larva,

juvenile etc.), the product's raw material, production techniques (enzyme, OH) and composition (molecular weight).

Table 1 Summary of FPH feeding trials using marine fish

Species	Comment on experimental finding	Reference
Atlantic salmon (*Salmo salar*)	Proved to be an efficient feed stimulant and increased apparent digestibility.	Refstie et al. (2004)
	Increased SGR, FCR and no impact on haematology and clinical parameters. High growth rates and feed intake using soluble FM proteins.	Hevrøy et al. (2005)
	Challenged by pathogen, reduced mortality with lactic acid bacteria.	Kousoulaki et al. (2012)
	Increased growth rate, no difference in mortality or condition factor.	Gildberg et al. (1995)
	Increased visceral mass and growth in low FM diets.	Berge and Storebakken (1996)
	No difference for dogfish vs herring silage.	Espe et al. (2012)
		Heras et al. (1994)
Coho salmon (*Oncorhynchus kisutch*)	No influence on disease resistance when animals were infected with pathogen.	Murray et al. (2003)
Red sea bream (*Pagrus major*)	Increased SGR, FCR, digestibility, no difference in haematological parameters and whole-body composition, increased non-specific immune response and disease resistance.	Khosravi et al. (2015a)
	Improve SGR, FCR, digestibility, innate immunity and disease resistance in low FM diets.	Khosravi et al. (2015b)
	Increase SGR, FCR, digestibility, innate immunity and disease resistance.	Bui et al. (2014)
European sea bass (*Dicentrarchus labrax*)	Inclusion level and molecular weight influence growth and immune status.	Kotzamanis et al. (2007)
	Illustrates the successful use of raw materials other than fish (yeast, pig blood).	Skalli et al. (2014)
	Different raw materials, composition of di- and tripeptides illustrates variable results in growth.	Delcroix et al. (2015)
	Increased survival and growth with larvae (Compared to *Cyprinus carpio*)	Cahu et al. (1998)
Japanese flounder (*Paralichthys olivaceus*)	Small molecular weight FPH showed increased FPH, FCR, digestibility.	Zheng et al. (2012)
	Moderate low molecular weight increased growth and protein retention; high-level molecular weight had no influence.	Zheng et al. (2014)

Species (cont'd)	Comment on experimental finding	Reference
Atlantic cod (*Gadus morhua*)	No difference in growth or feed intake with high plant protein diet. Reduced performance and promoted development in early stages of larvae. Increased survival rate, no influence on growth (compared to Atlantic halibut) Increased protein absorption in digestive tract.	Aksnes et al. (2006b) Johannsdottir et al. (2014) Kvåle et al. (2009) Bakke et al. (2010)
Atlantic halibut (*Hippoglossus hippoglossus*)	Survival rate decreased and no influence on growth.	Kvåle et al. (2009)
Large yellow croaker (*Larimichthys crocea*)	Growth, survival, absorption, digestion influenced by filtration of FPH.	Cai et al. (2015)
Persian sturgeon (*Acipenser persicus*)	Intermediate inclusion levels influence SGR, FCR and intestinal performance in larvae.	Ovissipour et al. (2014)
Asian seabass (*Lates calcarifer*)	Decreased growth and feed intake to FM; illustrate the influence of FPH production conditions (DOH).	Chotikachinda et al. (2013)
Japanese sea bass (*Lateolabrax japonicus*)	Intermediate inclusion levels supported highest growth, and all levels supported increased phagocytic activity.	Liang et al. (2006)
Japanese eel (*Anguilla japonica*)	Promotes larval growth and survival.	Masuda et al. (2013)
Turbot (*Scophthalmus maximus*)	No difference in SGR, FCR and protein retention with intermediate inclusion levels of graded levels of FPH; dose-dependent body composition (lipids). Low-level ultra-filtered FPH has positive influence on growth and feed utilisation.	Xu et al. (2016) Wei et al. (2016)
Dover sole (*Solea solea*)	Positive correlation of FPH in weaning diet, no influence in diets after weaning.	Day et al. (1997)

Table 2 Summary of feeding trials with FPH using shellfish

Species	Comment on experimental finding	Reference
Molluscs		
Abalone (*Haloitis fulgens*)	Successful replacement of all FM with abalone viscera silage and soybean; reduced water stability.	Guzman and Viana (1998)
	Mackerel and abalone viscera silage in diet increased growth compared to kelp, no influence of DOH.	Viana et al. (1996)
Abalone (*Haloitis midae*)	Intermediate inclusion levels increase cellular immunity and growth.	Goosen et al. (2014a)
Crustaceans		
Giant river prawn (*Macrobrachium rosenbergii*)	Insignificant influence on growth, utilisation and protein retention.	Ali and Sahu (2002)
Whiteleg shrimp (*Litopenaeus vannamei*)	Increased hydrolysis time (2 hours) allowed for increased FM substitution and promotes growth.	Quinto et al. (2017)
	Fish silage processed with formic acid proved beneficial in diets, compared to fish waste meal.	Gallardo et al. (2012)
	Increased SGR, FCR and weight gain, and provided significant positive nutritional influence.	Hernández et al. (2011)

Table 3 Summary of feeding trials with FPH using freshwater fish

Species	Comment on experimental finding	Reference
Rainbow trout (*Oncorhynchus mykiss*)	Removing small molecular weight fractions from FPH reduces growth and feed efficiency.	Aksnes et al. (2006c)
	Does not influence the metabolism of taurine, anserine, and eating quality.	Aksnes et al. (2006c)
	No influence on SGR, FCR, ADC and performs similarly to FM.	Barrias and Oliva-Teles (2000)
	With the correct inclusion levels, no influence on growth and possible increased influence on lysozyme activity.	Jo et al. (2017)
Mozambique Tilapia (*Oreochromis mossambicus*)	No negative influence on production, health and intestinal morphology, influence can be diet inclusion level dependent	Goosen et al. (2014a)
Nile Tilapia (*Oreochromis niloticus*)	No influence on ADC for dry matter, crude protein and energy using fermented fish silage in diets.	Fagbenro and Jauncey (1998)
	Determined good (similar to FM) ADC coefficients for protein and amino acids using fermented, chemical and enzymatic silages.	Borghesi et al. (2008)
Common carp (*Cyprinus carpio*)	Larvae fed with FPH as the only protein source did not perform as well as those receiving a mixed protein source from intact and hydrolysed protein.	Carvalho et al. (1997)
	Increased survival and growth compared to FM.	Cahu et al. (1998)
	Low formic acid concentrations in silage performed more effectively to higher concentration for silage in diets.	Ramasubburayan et al. (2013)
African catfish (*Clarias gariepinus*)	Fish silage influence is dependent on the other protein component in the diet; although effective for growth and feed efficiency.	Fagbenro and Jauncey (1995)
	Intermediate inclusion levels show increased growth compared to high inclusion levels of fermented shrimp head. No influence on ADC and FCR.	Nwanna (2003)
	High replacement levels of FM with silage reduced growth and influenced body composition. Intermediate replacements had no influence.	Soltan et al. (2008)
Rohu or Indian Major carp (*Labeo rohita*)	Fermented silage in diets influenced lipid and protein in carcass and increased feed intake, no influence on ADC.	Mondal et al. (2007)

Species (cont'd)	Comment on experimental finding	Reference
Pacú (*Piaractus mesopotamicus*)	Reduced final weight compared to FM for larvae.	Macedo-Viegas et al. (2004)

2.5 Phosphates from fish bone by-product

During the process of enzymatic hydrolysis described in Section 2.3, one of the secondary by-products is fish bones. This product can be utilised to create a phosphate (PO_4^{3-} or Phosphorous, P) feed additive (Ytteborg et al., 2016), which is an important feed additive to all formulated aquaculture diets. Phosphate is important for animal growth (Nwanna et al., 2009), bone mineralisation, reproduction and energy metabolism (Albrektsen et al., 2009) and it plays an important role as a buffer system in blood (Nwanna et al., 2009). In most common commercial feeds sufficient P is available, and if a deficiency of P is signalled by poor growth and skeletal abnormalities (Nwanna et al., 2009). Supplemental P-source can be included as an additive in aquafeed (Ahmad, 2008), and is mostly provided in the form of phosphate obtained from mining of rock phosphate (Van Vuuren et al., 2010).

Inorganic rock phosphate is a common feed additive for providing P in aquaculture diets. Phosphorous can be added in different forms to animal feeds, e.g. as inorganic phosphates: monocalcium phosphate, monodicalcium phosphate, dicalcium phosphate (DCP), tricalcium phosphate, and natural tricalcium phosphate (Swart, 2017), to be included in diets to prevent P deficiency (Nwanna et al., 2009).

The requirements of P by African catfish (*Clarias gariepinus*) are given by Nwanna et al. (2009) as 6.7-8.2 g kg^{-1}. Considering the increasing trend of replacing FM (containing relatively high levels of P) in aquaculture diets, more P additives need to be added to the feeds to satisfy the animals' requirements. The animal (poultry, pig, cattle etc.) feed and fertilizer industry competes for the same P source, which means high volumes of P sources are required (Van Vuuren et al., 2010). However, rock phosphate reserves are limited and mining these reserves can have high production cost (Van Vuuren et al., 2010), meaning that alternative forms of P feed additives need to be developed for use in aquaculture.

There is very limited open literature on the subject, specifically on alternative phosphate additives from fish bone extraction. Adebayo and Akinwumi (2015) investigated the utilisation of various phosphate-rich raw materials (fish and chicken bones) for African catfish (*Clarias gariepinus*), and provide a good indication on how important the utilisation of phosphates is for growth and bone mineralisation; as is the

utilisation of various raw materials normally seen as waste. Investigations into P from fish bone hydrolysis in diets of Atlantic salmon (Albrektsen et al., 2009, Albrektsen et al., 2017), illustrate the successful use as a P additive in feed. The replacement/addition of other P sources include monocalcium phosphate for Rainbow trout (Hernández et al., 2005, Hua and Bureau, 2006); these studies investigated including the P source at an optimum level, as high P loading in the diet can cause polluted water in aquaculture systems. Hua and Bureau (2006) did modelling with a variety of P sources, and verified with a feeding trial to ensure optimum P inclusion and minimum waste output.

FB-DCP could be a more suitable product than other common aquafeed phosphate additives, as it contains common phosphate-type molecules required by fish for pH control, and is a component of various important structures and functions (Nates, 2016). Because PO_4^{3-} levels in most natural waters are low, phosphate is a dietary requirement and is often the main cost related to mineral supplementation. P and Ca concentrations are deemed to be the most important values that must be determined, as deficiencies in these elements would cause abnormalities in the bone development of experimental animals (Ytteborg et al., 2016). Because the phosphorous source is fish bones, the optimum Ca/P ratio will be 1:1 or 1:2 for dietary supplements for fish (Nates, 2016).

Plant protein ingredients, such as soybean, have low levels of available P (Schäfer et al., 1995) and, with the trend towards aquafeeds that include more plant proteins, the development of new P additive ingredients was initiated, including the recycling of fish offal bones (Albrektsen et al., 2017). The utilisation of fish bones increases the sustainability of wild fisheries and aquaculture farms, and the effective utilisation of by-products (fish bones) from fish processing (Kim and Mendis, 2006). The P available in fish bones occurs in the form of insoluble minerals (Albrektsen et al., 2009) and, therefore, the bones are extracted by hydrolysis with a strong acid before use. Treated fish bone minerals have been shown to be more digestible and available for intake by Atlantic salmon (*Salmo salar*) than the P available in pure, untreated fish bone, as illustrated by a study with fry weighing at least 15 g, suggesting greater utilisation in later stage development (Ytteborg et al., 2016).

However, Albrektsen et al. (2017) illustrate successful utilisation with postsmolt Atlantic salmon (*Salmo salar*); they utilised P from hydrolysed herring bone.

In conclusion, acid hydrolysed fish bones can be utilised as P inclusion in aquafeeds, as shown by previous studies (Ytteborg et al., 2016, Albrektsen et al., 2017). It is, however, not known if the feed ingredient will be utilised by various species and on different diets, and if the feed ingredient is required to be treated with acid hydrolysis instead of being used as pure, untreated fish bone.

2.6 African catfish (Clarias gariepinus) as experimental species

The African catfish (*Clarias gariepinus*) is considered to be an important species for aquaculture, with global production at an estimated 33 924 tonne per annum; with an additional 135 507 tonne of hybrid species (*Clarias gariepinus x Clarias macrocephalus*) being produced per annum (Tacon et al., 2011). The African catfish (*Clarias gariepinus*) species is also known to be relatively easy to culture, as it is hardy, it can breathe air (and therefore doesn't always require the addition of oxygen to the culturing system) and it has rapid growth rates (Enyidi, 2012). In addition, African catfish (*Clarias gariepinus*) have a very good FCR, and the species is therefore an attractive option for commercial production (Hecht et al., 1988). The African catfish (*Clarias gariepinus*) is a suitable candidate for the evaluation of feed ingredients, as brood stock is locally available, the animals tolerate high stocking density (which reduces variation in results), and it is an species indigenous to South Africa (Ali, 2001).

In South Africa, the African catfish (*Clarias gariepinus*) has shown substantial aquaculture potential, and the species is currently used in various semi-commercial and research projects, e.g. the Camdeboo Satellite Aquaculture Project, Blue Karoo Trust, China-South Africa Agricultural Technology Demonstration Centre at Gariep Dam, and Operation Phakisa, which is supported by the South African government. The projects rely on African catfish (*Clarias gariepinus*) for food production, education/training, and developing the economy of South Africa (Straton, 2016, Rogers, 2016).

There are reports in open literature on previous feeding trials that used African catfish (*Clarias gariepinus*) to evaluate various products, focusing particularly on alternative

sources of protein (Enyidi, 2012). In most cases, the evaluation of feed ingredients (Table 4) using the African catfish (*Clarias gariepinus*) as a trial species was successful. Previous studies on the African catfish (*Clarias gariepinus*) have evaluated products similar to FPH as feed ingredients, such as fermented shrimp heads (Nwanna, 2003) and fish silage (Fagbenro and Jauncey, 1995, Fagbenro et al., 1997, Balogun et al., 1997, Soltan et al., 2008, Mondal et al., 2007). In some cases, it was reported that total replacement of FM could be a likelihood applied successfully for African catfish (*Clarias gariepinus*). Thus, investigations reveal an optimum inclusion level of certain feed ingredients, i.e. the optimum inclusion of 300 $g.kg^{-1}$ SM in diets (Imorou Toko et al., 2008).

However, no open literature was found with reference to the evaluation of FPH for African catfish (*Clarias gariepinus*). In similar feed ingredient studies, fermented shrimp heads (Nwanna, 2003) and fish silage performed effectively as protein replacements for FM. The FPH has a similar composition as the fermented shrimp heads and fish silage, with short peptide chains and free amino acids, and therefore the expectation is that the produced FPH (from the optimised, controlled enzymatic hydrolysis) will produce a similarly effective performance as a feed ingredient. Fagbenro et al. (1997) and Soltan et al. (2008) provides evidence that African catfish (*Clarias gariepinus*) does not tolerate high inclusion levels of fish silage.

Previous studies, listed in Table 4, illustrate that FM can be successfully replaced by various plant proteins, such as SM, cotton seed (Imorou Toko et al., 2008), sesame seed (Enyidi, 2012), oilcake (Nyina-Wamwiza et al., 2010) and by-products, such as animal protein meal (Goda et al., 2007). Investigations into replacing FM found that, in general, high inclusion levels of plant proteins, such total replacement of FM by SM, can influence growth negatively (Goda et al., 2007). In addition, African catfish (*Clarias gariepinus*) utilise and digest plant proteins well (Enyidi, 2012), which supports the successful use of a high plant protein diet without decreased growth.

The nutrient requirements of African catfish (*Clarias gariepinus*) is 300-400 $g.kg^{-1}$ (Ahmad, 2008, Ali and Jauncey, 2005) of crude protein in diets and 100-120 $g.kg^{-1}$ crude lipid (Ahmad, 2008, Ali, 2001, Machiels and Henken, 1985). The amino acid requirements for African catfish (*Clarias gariepinus*) is not fully established (Pantazis,

1999), however, various studies suggest that it is comparable to the needs of Channel catfish (*Ictalurus punctatus*) (Goda et al., 2007, Ayoola, 2016).

In conclusion, the African catfish (*Clarias gariepinus*) is a species that is suitable for evaluation of fish-derived products, and it is a suitable species for investigating the influence of new feed ingredients in laboratory conditions. In addition, the African catfish (*Clarias gariepinus*) is an important aquaculture species, and research via a feeding trial will provide useful data for the industry.

Table 4 Summary of important feeding trials with African catfish (*Clarias gariepinus*)

Ingredient	Comment	Reference
Soybean meal	Efficient growth and feed utilisation of 750 g.kg^{-1} and 1000 g.kg^{-1} replacement of FM; diet-dependent differences in whole-body composition.	Goda et al. (2007)
	Can utilise effectively to up to 500 g.kg^{-1}, and to 750 g.kg^{-1} if supplemented with methionine; also illustrated decreased ADC.	Fagbenro and Davies (2001)
	No influence on survival, FCR and hepatosomatic index, and can replace FM to a maximum of 300 g.kg^{-1}.	Imorou Toko et al. (2008)
	Different treatments of the SBM before inclusion in diet influence growth, and diets with phytase discard less P in environment.	Nwanna et al. (2005)
	Silage inclusion in diets found to influence growth negatively.	Balogun et al. (1997)
Sesame seed	Increased lipid deposits and can successfully replace up to 600 g.kg^{-1} of FM.	Enyidi (2012)
Bambaranut seed	Produced good growth and can replace up to 250 g.kg^{-1} of FM or SM, should, however, not be used without any FM in diet.	Enyidi (2012)
Corn meal	Poorly utilised ingredient, could serve as basal ingredient, and does not serve as protein supplement.	Enyidi (2012)
Cotton seed	Illustrates good growth as protein supplement, can replace FM up to 300 g.kg^{-1}, and influence body mineral composition.	Imorou Toko et al. (2008)
Pea seed	Feasible dietary inclusion of 300 g.kg^{-1}, also, different treatments of products influence results, and increased utilisation compared to SM.	Davies and Gouveia (2008)
Sunflower oilcake	No influence on growth in low levels with maximum replacement of FM by 250 g.kg^{-1}, increased digestibility with deshelled ingredient.	Nyina-Wamwiza et al. (2010)
Groundnut oilcake	Decreased growth and utilisation in diets.	Nyina-Wamwiza et al. (2010)
Brewer's yeast	Decreased growth compared to FM and in performance comparable to SM.	Hoffman et al. (1997)

Ingredient (cont'd)	Comment	Reference
Tomato	Decreased growth compared to FM and SM.	Hoffman et al. (1997)
Different sources of lipids	Palm oil was found to be the most effective lipid source due to its cost, availability and composition, compared to sunflower and cod oil.	Ng et al. (2003)
Animal protein meal	Satisfactory growth and feed utilisation to replace FM with poultry by-products, meat and bone meal.	Goda et al. (2007)
Fish silage	Animal does not tolerate total FM replacement with dried fish silage and SM. Replacing FM with fish silage, SM, poultry by-products and hydrolysed feather meal has no influence on growth and development. Reduces growth and utilisation if diets contain fish silage and SM. High replacement levels of FM with silage reduced growth and influenced body composition. Intermediate replacements had no influence.	Fagbenro et al. (1997) Fagbenro and Jauncey (1995) Balogun et al. (1997) Soltan et al. (2008)
Fermented shrimp head	No influence on ADC and FCR. Intermediate inclusion levels show increased growth compared to high inclusion levels of fermented shrimp head.	Nwanna (2003)

3. Problem Statement

This section will formulate the main problem statement, why the problem needs to be addressed, what gaps there are in the field and the main questions to answer during the project.

3.1 Key questions

The Department of Process Engineering at Stellenbosch University developed two processes to produce feed ingredients from fish processing by-products (FPH and FB-DCP). The key question that the project addresses is whether these two feed ingredients will be good feed ingredients in aquafeed for African catfish (*Clarias gariepinus*), and whether there are any additional benefits in terms of bioactivity or bioavailability. Both the FPH and the FB-DCP were produced under optimised conditions from monkfish heads, which is a fish processing by-product. The FPH is produced through enzyme hydrolysis of the proteins in the heads, while the FB-DCP is produced from the bones that remain after hydrolysis. Even though production of both FPH (Greyling, 2017) and FB-DCP (Swart, 2017) has been optimised in the laboratory, the actual nutritional value as determined by animal trials still needs to be determined.

A further important question to answer is how each of these ingredients affects the chosen experimental species, the African catfish (*Clarias gariepinus*). It is known that each aquaculture species has specific nutrient requirements, and dietary ingredients may affect species in different ways (NRC, 2011). For this reason, the effects on production performance of the feed ingredients need to be determined and optimised for a particular species.

In order to answer these questions, the project will be divided into two sections. Each ingredient (FPH and FB-DCP) will be investigated separately in order to answer the following key questions:

I. Will the FPH produced from local fish processing by-product, and produced under the optimum controlled enzymatic hydrolysis conditions, be a successful aquafeed protein ingredient for African catfish, *Clarias gariepinus*?

II. Will the FB-DCP product produced from the secondary by-products of enzymatic hydrolysis (fish bones), treated with acid hydrolysis to separate the minerals and crystallised, be a successful phosphate aquafeed additive for African catfish, *Clarias gariepinus*?

3.2 Identification of research area gaps

No previous research has evaluated FPH and FB-DCP with reference to African catfish (*Clarias gariepinus*) from available open literature. FPH and FB-DCP are both recently developed ingredients, and were required to be evaluated in a feeding trial to determine their bioavailability and bioactivity. Similar feeding trials have been conducted for using FPH for other species (See Table 1, Table 2 and Table 3); there has, however been no trial illustrating the influence of FPH on African catfish (*Clarias gariepinus*); there have only been investigations into the influence of chemical and fermented silage (Fagbenro and Jauncey, 1995, Nwanna, 2003, Soltan et al., 2008). Utilising hydrolysed fish bones as a feed ingredient (FB-DCP) is a relatively new concept and, as far as this researcher is ware, its use has only been evaluated for Atlantic salmon (*Salmo salar*) (Albrektsen et al., 2017, Ytteborg et al., 2016).

FPH and FB-DCP are both new feed ingredients with unique properties and composition, and have not been evaluated in any animal feeding trial. Thus, the research gap is the evaluation of two feed ingredients FPH and FB-DCP for African catfish (*Clarias gariepinus*).

3.3 Conclusion of problem statement

In conclusion, two new, high-value feed ingredients produced from fish processing by-products, FPH and FB-DCP, are available. Valorising the feed ingredients via a feeding trial would illustrate the value of the ingredients, thereby determining the viability of producing the ingredients on a larger scale. Thus, illustrating the value of the feed ingredients in aquafeed for African catfish (*Clarias gariepinus*) will determine the suitability of the ingredients for aquafeed and whether the feed ingredients can meet industry standards. This investigation as a whole will reduce waste, relieve pressure on wild fish stocks and promote sustainable fishing and aquaculture.

4. Materials and Methods

The materials and method section includes information on the production of feed ingredients and feed and an evaluation of feed ingredients in diets for African catfish (*Clarias gariepinus*). Valorising these ingredients includes evaluating production performance parameters, dry matter digestibility, whole animal body composition (as an indication of final product quality) and non-specific immunity. This section will explain the overall project approach and experiment design, and list all equipment, materials and experimental procedures. Appendix E contains information on the detailed analytical methodologies that were used throughout the trials.

4.1 Methodology approach

From the problem statement (Section 3), the investigation required the valorisation of FPH, a product developed by Greyling (2017), and FB-DCP, a product developed by Swart (2017) as aquafeed for African catfish (*Clarias gariepinus*). The valorisation of these ingredients was done by formulating a standard aquafeed suitable for the species by including similar ingredients in the experimental feed ingredients. For this purpose, FM was chosen to be compared with the FPH, and R-DCP was chosen to be compared with the FB-DCP. Therefore, the original feed formulation was deemed a standard, the control treatment, and where the standard ingredients were replaced with FPH and FB-DCP, the experimental treatments. Therefore, the experiment involved feeding different experimental diets to the experimental animals over a period, and evaluating the performance of the animals over time. Figure 2 and Figure 3 provides a schematic diagram of the investigation, the origin of FPH and FB-DCP in fish processing by-products, its inclusion in aquafeed and the evaluation of the feed ingredients in an aquafeed.

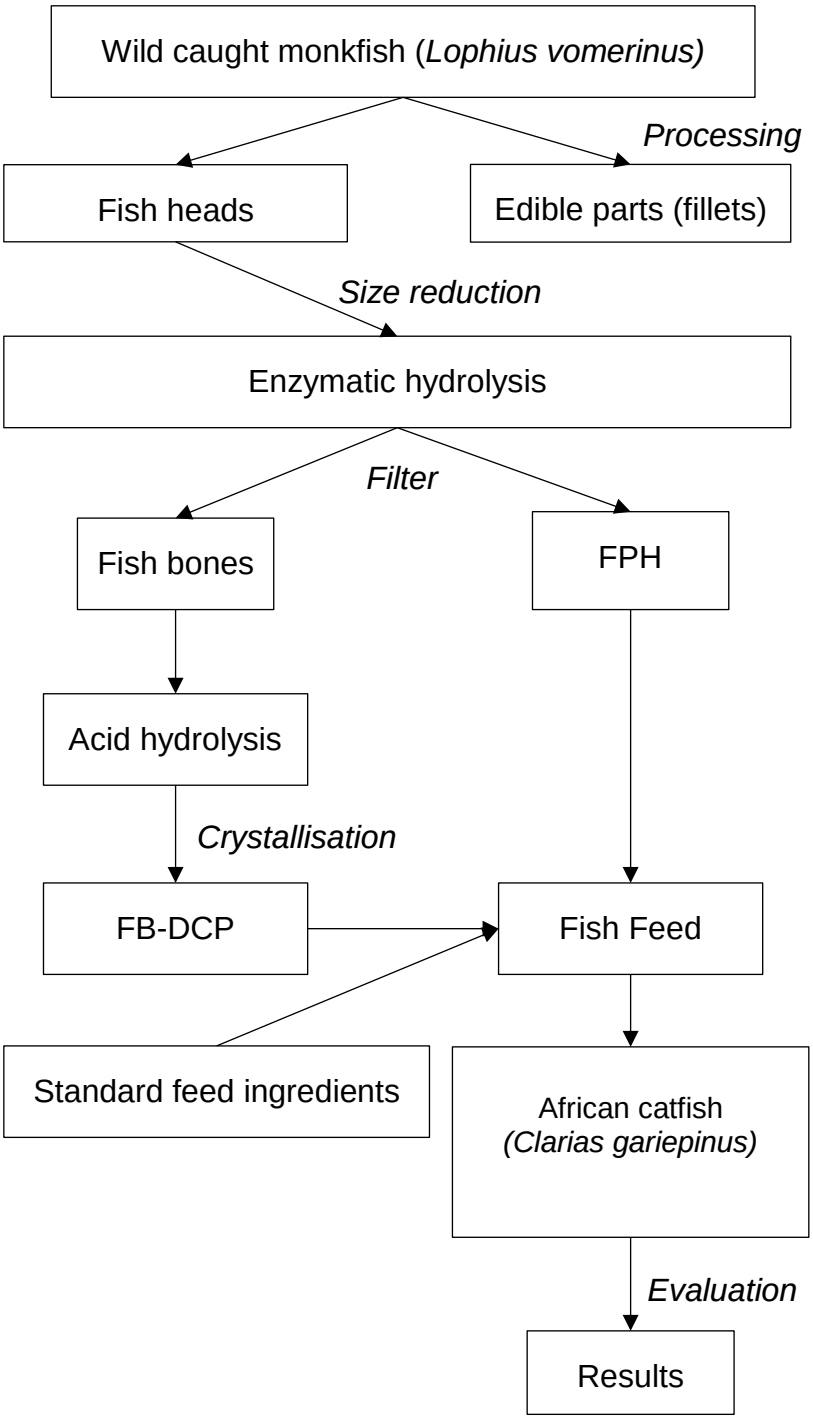

Figure 2 Schematic diagram of investigation, showing origin of feed ingredients

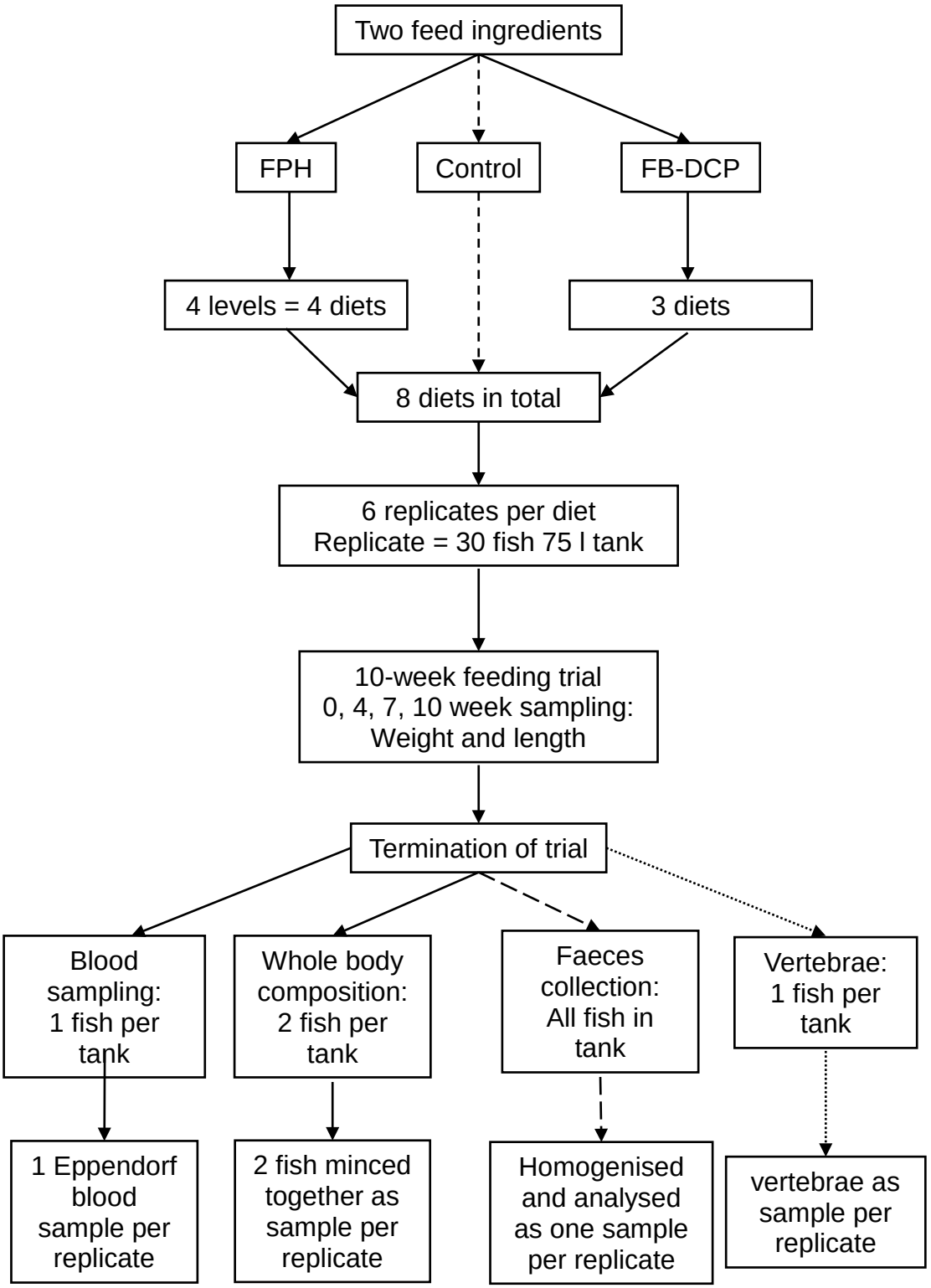

Figure 3 Schematic methodology of experimental design, sampling and sample analysis, where faeces collection only in Trial 1 and vertebrae collection only in Trial 2

4.2 Experimental design, animals and system

The main objective of the experiment was to conduct a feeding trial with *Clarias gariepinus*, to evaluate the fish processing waste-derived products FPH and FB-DCP. These potentially high-value feed ingredients were produced from fish processing by-products, namely, monkfish (*Lophius vomerinus*) heads from local commercial fisheries.

For the feeding trials, eight different diets were formulated. There was one control diet, four diets in Trial 1 for the FPH evaluation, and in Trial 2 for the FB-DCP evaluation; a mutual control diet was used. For Trial 1, FM was replaced by FPH at increasing levels of replacement, at 5%, 10%, 15%, and 20% replacement levels. For Trial 2, R-DCP was replaced fully or partially in the diets in three different ways: one diet replaced R-DCP with untreated fish bones (diet FB), another diet replaced the R-DCP using FB-DCP, and a third diet employed a 1:1 ratio of R-DCP and FB-DCP as a phosphate additive (further information provided in Table 8).

For both Trial 1 and Trial 2, the following applied: Each diet was replicated six times by feeding six tanks of fish a different diet each for 10 weeks. Each tank was initially stocked with 30 fish of 1 g initial mean weight (W_i); the volume of a tank was 75 l. The experimental tanks formed part of a recirculating aquaculture system (RAS), in which water was continuously supplied to the tanks and recirculated. The RAS was in a closed greenhouse with a particle and bio-filter, heating elements and air-conditioning, at Welgevallen Experimental Farm, Stellenbosch University. During the trial, the water temperature was maintained within a range (23 - 29°C, Hoffman et al. (1991)) suitable for African catfish (*Clarias gariepinus*); a thermostat (*Danfoss EKC 102*) was used to control the heating elements, in conjunction of the air-conditioning within the greenhouse. Furthermore, an air blower through air-stones in each tank supplied the RAS tanks with continuous aeration.

For Trial 1, an additional two-week feeding was allocated to include chromium oxide (Cr_2O_3) in the feed as an inert marker to determine the ADC for the FPH diets.

The Agricultural and Technology Demonstration Centre at Gariep Dam, Free State, supplied all experimental fish. The animals were maintained in the experimental

system for two weeks, to enable the experimental animals to acclimatise within the system after transport, and were fed the control diet until the trials commenced.

The trial was performed by feeding the fish in each tank a specified diet, by randomly allocating diets to tanks in the RAS. The diet involved 50 g feed per kg of body weight per day; divided into proximately three equal parts, so fish were fed three times a day, at 8:00, 12:00 and 16:00. For both trials, the performance measures were determined by periodically sampling the fish over the 10-week trial period: at the start of the trial (Sampling 0), after 4 weeks (Sampling 1) and at 7 and 10 weeks (Sampling 2 and 3 respectively) of the trial. Sampling included performance parameters (length and weight), blood, whole animal body composition, faeces (only Trial 1, two week trial) and vertebrae (only Trial 2). Details of the sampling and analytical procedures are presented in Sections 4.5 and 4.6.

4.3 Feed ingredient preparation (FPH and FB-DCP)

FPH production method

The FPH was produced from monkfish (*Lophius vomerinus)* heads by the enzymatic protein hydrolysis methodology optimised and described by Greyling (2017). Fresh monkfish heads were sourced from the local fish processing industry and transported to the laboratory on ice. The raw material was prepared for enzymatic hydrolysis by removal of any viscera from the heads, and then reducing the size by homogenising it in a commercial bowl cutter (*Dampa CT55*, Universal Butchery Equipment, South Africa) for 10 minutes, thereby converting the raw material into a mince that contained some solids (fish bones).

Prior to hydrolysis, the raw material was heated to 90°C for 15 minutes in hot water in a glass beaker, in order to deactivate any endogenous proteolytic enzymes. This step was necessitated by Greyling (2017) finding significant enzyme activity when she investigated endogenous enzymes in the raw material. After endogenous enzyme deactivation, the beaker was placed in a hot water bath (*Labotec 132*, South Africa) at 61°C and the solution was stirred continuously (overhead, *DragonLab OS40-Pro*, China) until the mixture reached the bath temperature. Temperature and pH were continuously measured using a combined temperature and pH meter (*MI150*, Milwaukee, US). The pH of the mixture was adjusted by adding 1M NaOH

(Sigma) until the mixture reached a pH of 8.2. When both the pH and temperature were at the required values, *Alcalase® 2.4L FG* enzyme (*Novozyme*, Denmark) was added at 3.33 µl/g of raw material (enzyme/substrate ratio). The hydrolysis conditions were controlled by adding 1M NaOH with a burette to maintain the pH at 8.2, while the temperature was maintained at 61°C by the water bath. Base addition was continuously monitored and the hydrolysis was stopped when 0.047 ml of NaOH/g per raw material had been added (8-10 minutes). Hydrolysis was stopped by heating the mixture to 90°C for 15 minutes, to deactivate the added enzyme and to stop any further hydrolysis. The degree of hydrolysis (DOH) of the product, using the conditions and techniques, was between 15% and 20% (Greyling, 2017). The product was then cooled at room temperature, and filtered with a 1 mm sieve to remove all solids (fish bones), and stored in food-grade bottles at -20 °C.

FB-DCP production method

The production of FB-DCP was done according to the methodology described by Swart (2017). The filtered fish bones from the hydrolysis mixture were washed and rinsed with tap water to remove any FPH or other foreign material (manually removing anything that were not fish bones). The fish bones were prepared in a food processor (*Barbanita® BBEK-1051*) with 1:1 w/v ratio water for 10 minutes to reduce particle size and increase surface area.

Minerals were leached from fish bones by using a 1:5 w/v ratio of the fine fish bones and 1M H_3PO_4, under continuous mixing for 24 hours, at room temperature. After the first extraction, the mixture was filtered and re-extracted for another 24 hours using fresh volume of H_3PO_4, filtered again, and the process was repeated a third time. Three consecutive H_3PO_4 filtrate batches were collected and mixed, with the minerals of the fish bones extracted in the filtrate solution. The DCP was prepared by precipitating the filtrate at 75°C with a 0.952:1 ratio of 1M $Ca(OH)_2$/mineral filtrate, in a water bath with continuous mixing. At this temperature, the DCP crystalised immediately and was removed from the water bath, filtered with a Buchner funnel and dried at 100°C for 5 hours to have a final product as anhydrous fish bone dicalcium phosphate (FB-DCP). Analysis confirmed that the phosphate phase was indeed

DCP, resulting in a major element composition of 215.2 $g.kg^{-1}$ P and 323.9 $g.kg^{-1}$ Ca as dry matter (Swart, 2017).

4.4 Diet preparation

To design feeds that included FPH and FB-DCP, the first step was to formulate a diet according to the requirements of African catfish (*Clarias gariepinus),* using available local feed ingredients. It was decided to employ two protein ingredients, SM and FM, as protein sources, maize as a source of carbohydrates, and vegetable and fish oil as sources of energy. The additional additives and ingredients required to formulate a complete feed that satisfied all the requirements of the experimental species included vitamin/mineral premix and R-DCP as a phosphate source. Pre-gelatinised starch was used as a feed binder, and cellulose as a formulation variable to ensure that the diets were iso-nitrogenous and isocaloric. The proximate compositions of the major ingredients (FM, FPH, SM and EM) are shown in Table 5, standard feed analytical methods are given in Section 4.6, and carbohydrates were determined as the remaining fraction from moisture, crude protein, crude fat and ash. From Table 5, the FM and FPH had approximately equal amounts of protein as dry matter, but the FPH contained less fat and ash.

Table 5 Proximate composition of major ingredients locally available and the FPH (g.kg^{-1} dry matter)

	Ingredients			
	FM	FPH[c]	SM	EM
Moisture*	85	945	98	106
Crude protein	751	763	519	83
Crude fat	59	14	9	36
Ash	188	132	72	15
Carbohydrates[a]	2	90	317	866
Gross energy (kJ g^{-1})[b]	20.0	20.0	21.3	18.3

*Value as the wet weight (g kg^{-1})

[a]Determined by difference 1 000 - Moisture - Crude Protein - Crude Lipids - Ash

[b]GE = 23.4 Protein + 39.8 Fat +17.2 Carbohydrates

[c]Produced with methodology explained in Section 4.3 (Greyling, 2017)

Although FM and FPH are ingredients derived from similar raw material, the FPH raw material consisted only of monkfish heads (a low-fat fish), whereas FM was produced from a combination of species and a combination of raw materials, including viscera, frames and heads. In addition, during the production of FPH, the fish bones were removed through filtration, which decreased the ash content of FPH compared to FM. The nutrient content of SM and extruded maize (EM) showed similar values to those

obtained for these ingredients by literature (Nates, 2016, NRC, 2011) and provide sufficient protein (from SM) and carbohydrates (from EM) to the diet formulation.

Table 6 and Table 7 include the amino acid composition analysis of the major ingredients available locally. As expected, EM had very low amounts of amino acids, because of EM's low protein content, and, being a plant ingredient, it showed an unbalanced amino acid profile with low EAA content. Although the SM showed high amounts of total amino acids, it had low levels of EAA such as methionine and lysine. The FM and FPH had similar amino acid compositions, as both ingredients were produced from fish raw material. When expressing each amino acid as the percentage of total amino acids (Table 6), it is clear that some of the EAA (in bold) is lower in FPH than in FM. A possible explanation for the shortage of EAA could be the raw material used: FM was made from all fish by-products, including viscera, while the FPH comprised monkfish heads only.

Table 6 Amino acid composition of major ingredients as dry matter ($g.kg^{-1}$)

Amino acid	Ingredient ($g.kg^{-1}$ dry matter)			
	FM	FPH	SM	EM
Histidine*	14.8	13.4	17	3.9
Serine	36.8	40.5	26.5	5.4
Arginine*	49.6	45.9	41.1	6.5
Glycine	50.1	89.9	29.2	6.4
Aspartic acid	78.8	73.4	55.4	7
Glutamic acid	117.1	111.6	92.2	19.8
Threonine*	33.7	34.5	20.4	4.3
Alanine	49.5	52.8	22	7.4
Proline	38.3	52.3	32.3	10.9
Lysine*	77.2	67.6	15.4	2.2
Tyrosine	28.5	25.8	28.8	6
Methionine*	33.9	27	4.3	1.7
Valine*	35.4	30.5	22	5
Isoleucine*	26.3	23.1	20.1	2.9
Leucine*	58.9	48.2	38.3	11.7
Phenylalanine*	30.2	29.4	40	7.8
ΣEAA	360	319.6	218.6	46

*Essential amino acids (EAA)

Table 7 Amino acid composition of major ingredients as a percentage of measured total amino acids

Amino acid	Ingredient ($g.kg^{-1}$ of total measured amount of amino acids)			
	FM	FPH	SM	EM
Histidine*	19.5	17.5	33.7	35.5
Serine	48.5	52.9	52.4	49.6
Arginine*	65.4	59.9	81.3	59.7
Glycine	66	117.4	57.9	58.5
Aspartic acid	103.9	95.8	109.7	64.2
Glutamic acid	154.2	145.7	182.5	182
Threonine*	44.4	45	40.4	39.5
Alanine	65.2	68.9	43.6	68
Proline	50.4	68.3	64	100.2
Lysine*	101.7	88.3	30.5	20.7
Tyrosine	37.6	33.7	57	54.7
Methionine*	**44.6**	**35.2**	8.6	15.9
Valine*	46.6	39.9	43.6	45.8
Isoleucine*	34.6	30.2	39.7	26.5
Leucine*	77.7	63	75.9	107.9
Phenylalanine*	39.8	38.3	79.2	71.3

*Essential amino acids (EAA)

The formulation of the treatments of Trial 1 required that the energy and protein levels were maintained at constant levels over all diets, to enable comparison of the different treatment results and to reach conclusions about the influence of ingredient level. Table 8 illustrates the diet formulation formulated with *Winfeed 2.8* (Winfeed Ltd, Cambridge, UK), and the proximate composition analysed for each diet. The software utilises linear programming to do a mass balance to formulate the feed, with inputs comprising the feed ingredients with the composition and the constraints according to the dietary requirements (Section 2.6) of African catfish (*Clarias gariepinus*). The results of the proximate composition analysis in Table 8 illustrate isonitrogenous (constant protein) and isocaloric (constant energy) diets. The diets correspond with the minimum and maximum requirements for effective growth (Ali and Jauncey, 2005, Ahmad, 2008). The diets of Trial 1 represent the variation of different inclusion levels of FPH, and the diets of Trial 2 represent the different sources of P (FB and FB-DCP).

The soybean and EM required milling (*Drotsky S8,* hammer mill) before mixing, to a fine particle size (<1 mm), to ensure homogenous particle size for extrusion. The diets were prepared by measuring the required amounts of each ingredient (wet weight), as the formulation shows in Table 8, adding water (35% of the total batch weight) and oils to the dry ingredients, and mixing in a commercial dough mixer (*MACADAMS SM 401*) for 10 minutes. A single-screw extruder (custom model, *Reomach Engineering,* South Africa) with a 5 mm die was used to form pellets from the extruded mixture. The resulting pellets were dried overnight in a convection drying oven at 55°C.

Table 8 Formulation and proximate composition of the experimental diets of Trial 1 and 2 (g.kg^{-1} as mixed, wet)

Trial #	Diet							
	Trial 1 & 2	Trial 1	Trial1	Trial 1	Trial 1	Trial 2	Trial 2	Trial 2
	Control	FPH-1	FPH-2	FPH-3	FPH-4	FB-5	DCP-6	DCP-7
Ingredients (as mixed)								
Fishmeal	200	190	180	170	160	200	200	200
Soya meal	483	483	483	483	483	483	483	483
Extruded maize	100	100	100	100	100	100	100	100
Starch	80	80	80	80	80	80	80	80
Vegetable oil	50	50	50	50	50	50	50	50
Fish oil	50	50	50	50	50	50	50	50
R-DCP	20	20	20	20	20	-	-	10
Mineral/Vitamin Premix	7	7	7	7	7	7	7	7
Cellulose	5	5	6	7	9	-	14	12
Inert Marker (Cr_2O_3)[c]	5	5	5	5	5	-	-	-
FPH(dry weight g.kg^{-1})*	-	**10**	**19**	**28**	**38**	-	-	-
FB-DCP	-	-	-	-	-	-	**16**	**8**
FB	-	-	-	-	-	**30**	-	-
Proximate composition								
Moisture	29	26	27	27	25	29	29	29
Crude protein	383	384	369	375	375	383	383	383
Crude lipid	121	123	116	119	124	121	121	121
Ash	96	97	94	93	91	96	96	96
Carbohydrates[a]	371	370	394	386	385	371	371	371
Gross energy (kJ g^{-1})[b]	20.2	20.2	20.0	20.2	20.3	20.2	20.2	20.2

*Formulated as dry weight, and the moisture (945 g.kg^{-1}) accounted for in the mixing

[a]Determined by difference 1 000 - Moisture - Crude Protein - Crude Lipids - Ash

[b]GE = 23.4 Protein + 39.8 Fat +17.2 Carbohydrates

[c] Only included in the two-week digestibility trial, filled by cellulose when not included.

The 5 mm pellets were not suitable for the small animals, and pellet size reduction was done with a hopper-mill (*ROFF® maize roller mill*), which resulted in four different size fractions: fines (not used), 1-2 mm, 2-3 mm and >3 mm. These small pellets were suitable for feeding the differently sized fish as the feeding trial progressed. The feed was stored in cool, dark room in airtight polyethylene bags in sealed food-grade buckets. The feedings trials started immediately upon completion of the feed production, to prevent any possible expiration of feed. Table 9 shows the amino acid analysis of the FPH-containing diets from Trial 1; amino acid profiles of the FB and DCP diets were not determined, as these diets contained the same ingredients as the control diet, except for the source of phosphorous. From Table 9, it can be seen that the specific amino acids that are present in lower levels in FPH than in FM, are mostly also reflected in the overall levels of these amino acids in the formulated feeds. This is only a concern in the case of limiting amino acids, especially for methionine, which is a common limiting EAA in aquafeed for carnivores.

Table 9 Amino acid composition of the FPH diets from Trial 1 (g.kg^{-1} as fed)

Amino acid	Diet				
	Control	FPH-1	FPH-2	FPH-3	FPH-4
Histidine*	13.8	12.4	13	12.1	11.4
Serine	23.9	21.7	21.7	21.1	21.4
Arginine*	37.1	29.9	33.5	27.6	29.5
Glycine	32.9	27.8	28.1	24.6	27.9
Aspartic acid	47.6	45.7	44.3	45.7	46
Glutamic acid	78.4	75	75.1	72.1	76.9
Threonine*	20.3	19.8	18.2	18.6	20.3
Alanine	26.3	23.4	22	21.9	22.2
Proline	31.5	27.2	25.4	20.6	28.5
Lysine*	28.7	31.6	23.2	34	29.5
Tyrosine	25.9	19	22.7	17.3	19.8
Methionine*	15	10.8	9.8	8.3	8.7
Valine*	24.2	20.6	20.3	18.5	20.1
Isoleucine*	23	18.7	17.8	17.8	18.2
Leucine*	41	32.7	31.8	29.5	32
Phenylalanine*	34.7	25.2	29.1	20	24.3
Σ EAA	237.8	201.7	196.7	186.4	194

*Essential amino acids (EAA)

4.5 Experimental and sampling procedures

Stellenbosch University Research Ethics Committee: Animal Care and Use approved all procedures and protocols of this project in relation to animal care, usage and handling (Protocol #: SU-ACUD16-00062 in Appendix A). The project minimised stress to the experimental animals, and environmental impacts.

The experiments consisted of eight dietary treatments, containing 30 fish per 75 l tank, with six replicates per diet, for a total of 48 tanks. The treatments were randomly allocated to tanks in the RAS, and the feeding trial was run for a total of 10 weeks. The fish received approximately 50 g feed per kg of their body weight per day; divided into approximately three equal parts, fed three times a day at 8:00, 12:00 and 16:00. During the feeding it was observed that the fish ate all the feed, and the assumption of total consumption throughout the trial was concluded. African catfish (*Clarias gariepinus*) are enthusiastic feeders and all feed was consumed within a couple of minutes after feeding. During the trial, the most important requirements were to feed the fish the correct amount of feed and to ensure the application of good general husbandry procedures. The feeding was controlled by allocating a small food grade bucket (2 l) with a lid to each tank containing a known weight of the allocated feed. The feed was replenished bi-weekly; the initial and final weight of the bucket, and the amount of feed that was added to each tank over two weeks, was known. The feeding was also controlled with a monitoring sheet – every feeding time was ticked off and the temperature noted.

Before sampling, the animals were not fed for 48 hours, to minimise stress. The sampling of the fish (at 0, 4, 7, 10 weeks) involved removing the fish from the tanks with minimal stress and placing the fish in a tank containing anaesthetics (100 mg/L MS-222, Sigma; 100 mg/L $NaHCO_3$, food grade) to ensure minimal handling stress during weighing and measuring procedures. For each animal, the total animal length was taken on a measuring board (mm) and the total weight was recorded with 0.01 g accuracy on a balance (*Kern® PLE-N*, Germany). At termination of Sampling 4 (10 weeks), blood was taken from one randomly selected fish in each tank with a 27-gauge needle and 1 ml syringe. The blood was stored in Eppendorf tubes to clot overnight at 4°C, centrifuged at 500 xg for 10 minutes after clotting, and the blood serum was collected as the supernatant (at least 300 µL), using a pipette and stored

in Eppendorf tubes at -20°C until further analysis. The whole-body samples were taken by euthanising the fish with an overdosage of anaesthetic (500 mg/L MS-222) followed by percussion to the brain. For this purpose three fish were removed from each tank for Trial 2 and two fish for Trial 1.

One fish from Trial 2 was utilised by surgically removing the vertebrae for bone mineral analysis. The vertebrae were treated with enzymes (*Alcalase® 2.4L FG, Novozyme,* Denmark) at 60°C for 1 hour, to remove any protein and cleaned with distilled water and then dried at 100°C for 4 hours.

For Trial 1, a two-week digestibility trial was conducted to determine the ADC of the diets with FPH inclusion levels. The concentration of an inert marker (Chromium (III) oxide, Cr_2O_3) was measured in the feed and in the faeces, and ADC of a nutrient was calculated with Equation 5. During the trial, the faeces was collected with the 'stripping' method for all fish at one and two weeks. One hour after the morning feeding session (9:00), the animals were placed in an anaesthetic tank (100 mg/L MS-222, Sigma; 100 mg/L $NaHCO_3$, food grade) and faeces was collected by gently pressing the area above the anus until the faeces could be collected with a sampling bottle. The sample was stored at -20°C until further analysis was done. For this two-week trial, only 20 animals (mean initial weight of 60 g) per tank were used, with four replicates.

The production performance parameters of the animals were determined using data on the weight, length and feed consumed throughout the trial. For calculation of the feed parameters, it was assumed that all feed fed to each tank was completely consumed by the animals. The weight gain (WG) by the experimental animals, as the fraction of the initial weight (Jo et al., 2017), is given as:

$$WG = \frac{W_f - W_i}{W_i} \quad (1)$$

where:
W_f = the mean final weight (*g*),
W_i = the mean initial weight (*g*).

The SGR shows the growth of the animals as an exponential growth model (Nates, 2016),

$$SGR\% = \frac{(\ln W_f - \ln W_i)}{t} \times 100 \quad (2)$$

where:
$\ln W_f$ = the natural logarithm of the mean final weight,
$\ln W_i$ = the natural logarithm of the mean initial weight,
t = the time (days) between $\ln W_f$ and $\ln W_i$.

Calculating the amount of feed the fish used to grow to a certain size, using a known amount of feed, is described by the FCR, which is the ratio between the change in size (weight) of the fish and the amount of feed given over the time of the growth. The parameter is commonly known as the nutrient utilisation by the animal. The FCR is determined as,

$$FCR = \frac{\text{Feed consumed}}{(W_{f,total} - W_{i,total})} \quad (3)$$

where:
$W_{f,total}$ = the total mean final weight of animals in the tank (*g*),
$W_{i,total}$ = the total initial weight of animals in the tank (*g*),
Feed = the amount of feed the fish received over the period of growth (*g*).

With the SGR and the FCR, the production performance parameters illustrate the growth of the fish for amount of feed given over a time. These parameters are of particular concern for an aquaculture farmer, as they will determine how profitable the feed and production is.

The condition factor (CF) of the animals, as an indication of an animal's body condition relative to others, is the ratio of weight and length, described as (Froese, 2006),

$$CF = \frac{W}{L^3} \quad (4)$$

where:
W = the weight of the animal (*g*),
L = length of animal (*mm*).

In some cases, the height of the fish is included to provide a more accurate description of the animal's condition, as in the model described by Jones et al. (1999). It is unpractical and inaccurate to measure the height of African catfish

(*Clarias gariepinus)* due to its flat body shape, and therefore the weight/length ratio should be sufficient to describe body condition differences over the treatments.

The ratio of the chromium oxide concentration in the faeces and the feed expresses the ADC. By measuring the concentration of an inert marker (Chromium (III) oxide, Cr_2O_3) in the feed, and again in the faeces, and calculating ADC of a nutrient is described by (Zheng et al., 2012),

$$ADC = \left[1 - \frac{C_{diet}}{C_{faeces}} \times \frac{N_{faeces}}{N_{diet}}\right] \times 100\% \quad (5)$$

where:
ADC = the apparent digestibility coefficient (%)
C = the chromium oxide concentration in the diet and faeces (*assayed/measured*)
N = nutrient concentration in diet and faeces ($g.kg^{-1}$)

This calculation will give a good indication of the digestibility and bioavailability of the different diets, and therefore the digestibility of FPH in Trial 1. In Trial 1 the only variable ingredient among the treatments are the FPH inclusion level, therefore, any differences in digestibility can be attributed to the FPH component in the feeds.

4.6 Analytical procedure

This section describes the methodology and techniques implemented to analyse feeds, feed ingredients and the various animal tissue samples collected throughout the project. The analytical procedures includes approximate analysis, amino acid analysis, blood serum analysis, chromium oxide analysis and mineral analysis of fish bones.

Approximate composition

The approximate composition analysis involves analysing the crude protein, crude fat, moisture and ash. This is only a ap*proximate* analysis of the nutrients available for consumption and is therefore used as a general guide.

The crude protein was analysed by the Kjeldahl method (AOAC 979.09) by measuring total nitrogen, where the sample is digested at 420°C, with sulphuric acid (H_2SO_4) in the presence of a catalyst ($CaSO_4 \cdot 5H_2O$ and K_2SO_4). To liberate any NH_3 present, the digestion mixture was diluted, and mixed with NaOH. The distillation of the solution evaporates the NH_3, which is then trapped using a receiving boric acid (H_3BO_3) solution. With HCl endpoint titration, the percentage nitrogen was determined in the receiving boric acid, and the protein was calculated from the nitrogen using a conversion factor of 6.25.

To determine crude lipid (fat) in approximate analysis, solvent extraction with ether (AOAC 920.39) was used. Moisture was determined as the weight difference before and after drying (above 105°C), and the ash percentage was determined after placing the sample in a muffle furnace at 600°C for 3 hours (AOAC 942.05). Carbohydrate level is determined by the remaining fraction, that is, the difference of 1 000 $g.kg^{-1}$ - Moisture - Crude Protein - Crude Lipids – Ash ($g.kg^{-1}$).

Amino acid composition

Amino acid analysis determines the potential nutritive value of feedstuff, where the amount of amino acids provides an indication of the nutrients available for growth of the experimental animals. Amino acid profiles were determined using HPLC according to a standard method (AOAC 985.28). This method determines total amounts of individual amino acids and does not indicate the form of the amino acids

(i.e. free or bound amino acids). Therefore, it does indicate the level of amino acids, but not the necessarily the amino acids available for digestion.

Blood serum analysis

Section 4.5 describes the serum sampling method, which analysed for protein, lysozyme activity and immunoglobulin as an indication of non-specific immunity in the experimental animals.

The serum lysozyme concentrations were determined spectrophotometrically according to the method of Sankaran and Gurnani (1972). To determine the serum lysozyme, 50 µl of blood serum was added to 250 µl suspension solution in a 96-well microplate, and absorbance was read using a microplate reader (*BioTek ELx 800*™) after 5 and 20 minutes of incubation (using a microplate shaker) at 530 nm. The suspension solution contains 0.075% (w/v) *Micrococcus lysodeiticus* (Sigma, M3770) and a phosphate buffer of 0.5 M and 6.2 pH. The standard curve is prepared similarly, with the known serum lysozyme (Sigma, L6876) concentration instead of a blood serum sample. The lysozyme activity of the serum is determined by the standard curve (Appendix E, Table 20) and the decrease in absorbance of the blood serum.

The total serum protein was also determined spectrophotometrically using the linearised Bradford assay (Zor and Selinger, 1996). The protein standard used was lysozyme (Sigma, L6876) and a standard curve (Appendix E, Table 21) was set up for ranging concentrations between 0 and 1420 µg/ml. The standard concentrations were prepared with 0.85% NaCl solution as a dilution agent. Samples were measured in triplicate by adding 50 µl of serum/standard to a 96-well flat-bottomed microplate, with 200 µl of Bradford reagent. The absorbance of samples was measured at 450 nm and 630 nm and using the ratio of absorbances (630 nm/450 nm) to set up the standard curve (Appendix E, Table 21) and calculate the total protein levels of the serum.

The immunoglobulin (Ig) concentration of serum samples was measured with the method of Siwicki et al. (1994). Equal volumes (100 µl each) of blood serum and 12% (v/w) polyethylene glycol (PEG, Sigma, with 10 000 Dalton average molecular weight) were added to Eppendorf tubes and left to incubate for 2 hours at ambient

temperature. After incubation, the Eppendorf tubes were centrifuged at 14 000 rpm for 5 minutes, which resulted in a white pellet forming at the bottom of the Eppendorf tube. The protein concentration of the remaining supernatant was then measured using the linearised Bradford method as described above. The immunoglobulin concentration of the blood serum was then calculated as the difference in the total protein concentration in the serum before and after PEG incubation. Each blood serum sample was analysed in triplicate.

Chromium oxide analysis

Chromium oxide (Cr_2O_3) concentration in feed and faeces samples was analysed spectrophotometrically by oxidation of the sample with a molybdate reagent, as described by Divakaran et al. (2002), Furukawa (1966) and Kimura and Miller (1957). The samples (feed/faeces) were dried (see Section 4.5) and then crushed with a porcelain mortar and pestle in preparation for the oxidation. The molybdate reagent was prepared by dissolving 5 g of $Na_2MoO_4 \cdot 2H_2O$ (*Sigma*) in 75 ml of distilled water and 75 ml of concentrated H_2SO_4; it was allowed to cool to room temperature, and then 100 ml of $HClO_4$ was added. The oxidation was done by adding 200 mg of finely crushed sample to a 100 ml volumetric flask with 3 ml of the molybdate reagent (as prepared), and the flask was then heated with a hotplate until boiling and allowed to boil for a minimum of 5 minutes, until fumes subsided. The procedure was carried out in a fume hood.

After boiling, the solution was allowed to cool at ambient temperature, and another aliquot of 3 ml 70% $HClO_4$ was added to the solution, then the boiling step was repeated for an additional 10 minutes. After the second boiling step, the solution was allowed to cool at ambient temperature, the solution was washed out of the flask using distilled water, into a 25 ml volumetric flask, and the volume adjusted to exactly 25 ml. The chromic oxide concentration of each sample was determined from this final solution by transferring triplicate samples of 250 µl to a 96-well flat-bottomed microplate, and measuring absorbance at 340 nm. The calculation of the concentration of chromic oxide in the feed and faeces was done using a standard curve (Appendix E, Table 19 and Figure 9), which was set up with a known concentration of oxidised chromic oxide solution.

Mineral analysis of vertebrae

P, Ca, Mg, Al, Mn and Si in the vertebrae of the animal were analysed with inductively coupled plasma mass spectrometry (ICP-MS, *Agilent 7700*). The major element components were analysed by digesting samples of known weight with nitric acid and hydrogen peroxide in a microwave digester, and using a standard curve, the element concentrations were calculated.

4.7 Statistical analysis

Data were analysed with a one-way analysis of variance (ANOVA), using *Statistica 13* (Dell, Inc.) software. In this study, differences were viewed to be significant between means for p-values <0.05. If ANOVA analysis indicated significant differences between means, the means that were different were identified by performing Fischer's LSD post-hoc test. All data in this work are presented as the mean with the standard error (±SE) between the ranges of means.

Statistical regression analysis was done by fitting Length-Weight data in order to determine b in $CF = W/L^b$, where W is the weight and L the length. This was done with the *Statistica 13* (Dell, Inc) software with a 95% prediction interval, calculated from the known mean square error and the sum of squares.

5. Results and Discussions

The results of the study are given in this section, and include a summary of analysed data from feeding trials that used two different feed ingredients: FPH (Trial 1 in Section 5.1) and FB-DCP (Trial 2 in Section 5.2). These results illustrate how the experimental feed ingredients influenced the production performance, body composition and non-specific immune status of the African catfish (*Clarias gariepinus*) during the in vivo feeding trial conducted.

During the feeding trials, the animals adapted well to the RAS system as the animals were enthusiastic feeders that consumed the feed well within a few minutes, and the animals did not exhibit any obvious signs of disease. The temperature in the system ranged between 23 and 29°C, with a mean of 26.1±2°C. There was however, some cannibalistic behaviour throughout the trial, as some remains of fish were found – this accounted for the majority of mortalities. Consequently, it was not always clear if the experimental animals succumbed from a cause other than the cannibalistic behaviour. Overall, visual examination revealed no obvious distress among the fish, and the fish seemed to adapt well within the RAS.

5.1 Trial 1: Feeding trial with fish protein hydrolysate diets

This section reports how the FPH, compared to a control diet, influenced the production of African catfish (*Clarias gariepinus*); treatments replaced FM with FPH in increasing levels in iso-nutrient diets. Table 8 provides detailed information about the diets in Trial 1.

Production parameters

The following production parameters were determined for growth: initial (W_i) and final animal weights (W_f), weight gain (WG) fraction of initial weight to weight gain, SGR, FCR and CF. The growth and development of the animal are proportional to the bioavailability of the feed, in the case of the protein feedstuff, it will be proportional to the supply of EAA; for example, an increase in SGR and decrease of the FCR could indicate that the protein is more digestible to the animal. The production parameters of the FPH-1, FPH-2, FPH-3 and FPH-4 treatments, with the control diet, over the 10-week feeding trial, are shown in Table 10.

Table 10 Summary of production performance parameters with FPH treatments in Trial 1

	Diet				
	Control	FPH-1	FPH-2	FPH-3	FPH-4
W_i (g)	0.96 ± 0.08	0.98 ± 0.07	1.00 ± 0.09	1.08 ± 0.17	0.91 ± 0.05
W_f (g)	31.2 ± 0.95	32.3 ± 1.61	29.0 ± 1.23	28.2 ± 0.97	32.0± 0.36
WG (g/g)	32.6 ± 3.04	32.5 ± 1.50	29.1 ± 2.95	27.5 ± 3.35	34.5 ± 1.81
SGR (%.day^{-1})	4.99 ± 0.13	5.01 ± 0.07	4.82 ± 0.16	4.72 ± 0.20	5.09 ± 0.08
FCR	0.82 ± 0.01	0.82 ± 0.01	0.83 ± 0.01	0.86 ± 0.03	0.86 ± 0.02
CF	0.70 ± 0.01	0.70 ± 0.01	0.68 ± 0.01	0.71 ± 0.01	0.70 ± 0.01

Data represented as mean ± SE
p>0.05 for all parameters

It is clear from Table 10 that replacement of FM with FPH at increased levels did not have a statistically significant effect (p>0.05) on the production performance of the experimental animals. Neither SGR, FCR nor CF changed significantly over the increased inclusion of FPH (from FPH-1 to FPH-4). SGR ranged from 4.72 to 5.09 %.day^{-1}, which was a very good growth rate compared to previous studies with similar protein and a lipid content (Al-Dohail et al., 2009, Ahmad, 2008, Fagbenro and Davies, 2001). The FCR ranged from 0.82 to 0.86, illustrating very good feed utilisation of FCR<1, due to the small initial weight and the wet versus dry weight of the FCR. The ranges of the SGR and FCR show that all the diets succeeded in meeting the requirements for sufficient growth and feed utilisation of African catfish (*Clarias gariepinus*).

The only difference in the composition of the diets compared to the control, is the replacement of FM by FPH. Because the replacement is a protein source, the phenomenon of no significant differences over the treatments indicates that all diets satisfied the minimum required EAA of the animals, even though Table 9 illustrates slightly lower values for methionine in the FPH diets compared to the control (see Section 4.4).

The FM and FPH possibly have similar bioavailability to African catfish (*Clarias gariepinus*) of this age over the trial period. The results illustrate that the FPH, compared to the industry standard of FM, does not have any negative effects on the production parameters and can, therefore, be considered to be a good feed ingredient for African catfish (*Clarias gariepinus*). The growth and development of the animal is proportional to the bioavailability of the feed; in the case of the protein feedstuff, it will be proportional to the supply of EAA, for example, an increase in

SGR and decrease in the FCR could indicate that the protein is more digestible for the animal.

The protein requirements of African catfish (*Clarias gariepinus*) are reported to be around 300-400 g.kg^{-1} (as fed) for a size range of 10.7-67 g (Ali and Jauncey, 2005, Nates, 2016, Ahmad, 2008). The diets designed for the current study have a protein content of 380 g.kg^{-1} (as fed) with fish size 1-30 g, which was slightly is less than the maximum requirements. Excessive dietary protein content can disguise the influence of relatively low FPH inclusion levels in aquaculture growth trials. Dietary protein levels influence protein retention; this is the utilisation of protein as growth source versus protein as energy source (Ali and Jauncey, 2005). In this study, a low dietary protein level was required to ensure all protein was retained for growth and not used to meet energy requirements. However, in aquaculture, optimum dietary protein levels are the minimum required for optimal growth, because of the high cost of protein ingredients (NRC, 2011).

The purpose for the designed diet (FM of 200 g.kg^{-1}) was to ensure that the influence of the test FPH ingredients with controlled feeding was not disguised; therefore, the SGR and FCR should be sensitive to any influence of the FPH. Table 6 shows the amino acid composition of FPH compared to FM, and Table 9 the amino acid composition of the diets. The methionine level in FPH was slightly lower than in the control diet (which contained FM only); the slightly lower methionine levels in FPH diets reflect that FPH composition. All diets preformed equally, and there was no evidence of any nutrient limitations in any of the diets. The diets with the lowest levels of EAA had the same production performance as the diet with the highest EAA levels (the control diet), thereby, suggesting no nutrient limitations. The rapid SGR also illustrates that the experimental animals developed well with FPH, receiving all the required nutrients for absorption.

Previous studies that used FPH found possible beneficial growth properties, because of the shorter peptide chains and free amino acids (Khosravi et al., 2015a, Khosravi et al., 2015b, Bui et al., 2014), although this was not visible in the current trial results. The behaviour of the experimental animals (e.g. cannibalism), husbandry practices, and the genetics of the experimental animals could also have had an influence on the trial results (Glencross et al., 2007), however, attempts were made to reduce these

influences to a minimum through good experimental practice. Further, six replicates per treatment were employed in an attempt to provide sufficient statistical power to detect any differences between treatments.

There were no statistically beneficial advantages to including FPH in diets in the current trial. The results indicate no differences between any of the FPH treatments and the FM control diet. Although the beneficial production advantages described by literature (Refstie et al., 2004, Kotzamanis et al., 2007, Zheng et al., 2014, Liang et al., 2006, Espe et al., 1999, Murray et al., 2003) were not seen in the results in this study, there is a possibility of other advantages of FPH inclusion. The inclusion of FPH in aquaculture diets does not always show improvements in production performance. Other researchers also found that FPH inclusion had no significant beneficial effects on various species, animal development stages, animal behaviour (cannibalism), FPH inclusion levels, trial length, feed ingredients, feed formulation and conditions (Fagbenro et al., 1997, Cahu et al., 1998, Murray et al., 2003, Aksnes et al., 2006c).

Cumulative mortality

Figure 4 illustrates the mortality of the animals as a fraction of the total initial animals. The cannibalistic nature of the African catfish (*Clarias gariepinus*) created a challenge regarding determining the mortality of the animals over the trial period, as the majority of the mortalities were due to cannibalistic behaviour. Mortalities related to cannibalism were therefore also counted as part of the mortalities during the trial, and therefore the cumulative mortality is presented on sampled days (0, 28, 49, 70 days).

The result in Figure 6 illustrates that, over the 70 days, at least 64% of all fish survived the feeding trial. In general, this is not a good survival rate. During the trial, there were no obvious signs of diseases, and the high mortality rate was mostly caused by cannibalism. At small sizes, differential growth of African catfish (*Clarias gariepinus*) tends to result in outliers (fish that are significantly larger than the mean in the tank); the larger fish can then turn cannibalistic. Considering the small initial size/age (±1 g) of the fish in this trial, the cannibalism noted is not surprising (Enyidi, 2012, Hecht and Appelbaum, 1988, Sallehudin and Mukai, 2014). Despite the

cannibalism, it was decided against removing outliers from tanks, as this might have interfered with trial results. For these reasons, no definite conclusions can be made from the mortality rate; nonetheless, from Figure 4, FPH-1 (0.35) and FPH-4 (0.36) had the worst survival, and FPH-2 (0.26) and FPH-3 (0.25) had the best survival, and the control (0.30) an average survival. To assign these differences in mortality to

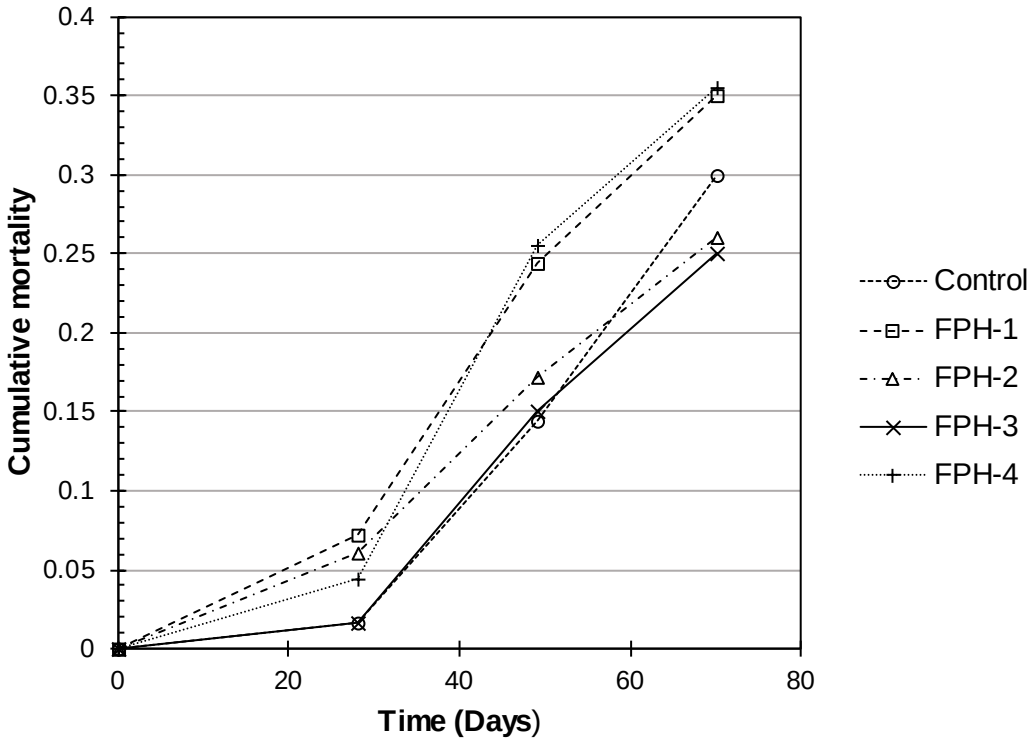

Figure 4 Cumulative mortality of the animals as a fraction of initial total animals for FHP diets in Trial 1

differences in the dietary treatments would be invalid; these differences have no general trend and the diets do not cause the cannibalism observed.

Non-specific innate immunity

Non-specific immune parameters were measured during the evaluation of the feed ingredients in order to determine whether the feed ingredients had any effects (negative or positive) on the health on the experimental animals. Non-specific immunity was characterised using non-cellular mechanisms in blood serum: lysozyme activity, immunoglobulin, and total serum protein. In many cases, immune function is proportional to survival rate and disease resistance of fish in aquaculture systems and the immune, in turn, affects the production of fish by an aquaculture farmer (NRC, 2011).

It is important to take the health impacts of feed ingredients into account during aquafeed formulation, and to evaluate the effect of novel feed ingredients on animal immune function (Kiron, 2012). The non-specific parameters of the diets are included in Table 11, and analytical methodologies are described in Section 4.6.

There are statistically significant differences ($p=0.02$) for the immunoglobulin (Ig) concentrations between treatments, but no statistically significant differences ($p>0.05$) for lysozyme activity and total serum protein. The diets of FPH-2 and FPH-3 show statistically more significant different values than that of the Control, FPH-1 and FPH-4 in measured Ig levels. The increased level of measured Ig is an indication of improved immune response, which may translate into increased disease resistance in production systems. Ig acts as an antibody in the non-specific immune system, neutralising harmful bacteria and viruses that cause disease (Cuesta et al., 2004, Pilström and Bengtén, 1996). Khosravi et al. (2015b) and Bui et al. (2014) found increased Ig levels for red sea bream (*Pagrus major*) on a low FM diet, compared to krill, shrimp and tilapia hydrolysates.

The possibility is that FPH increased the Ig level because of the increase in the measured Ig levels in diets FPH-2, and FPH-3. There was, however, a significant decrease in the measured Ig levels in FPH-4. This phenomenon could possibly be attributed the over-stimulation of the non-specific immune systems of the experimental animals by the FPH ingredient. From these current data, the Ig stimulation was therefore dose-dependent on the FPH, where too low or too high levels did not contribute to improvement of innate immunity. There are previous reports that high levels of FPH can decrease the immune response in juvenile fish by excessive diet inclusion levels (Cahu et al., 1998). This dose-dependency highlights why it is necessary to determine the optimal inclusion level for FPH. These results further indicate that FPH inclusion had no negative impacts on the other components of the non-specific immune function, as shown by lack of differences in lysozyme activity and total serum protein between treatments.

The potentially beneficial health properties of the higher Ig measured in diets FPH-2 and FPH-3 agree with studies, such as Refstie et al. (2004), Kotzamanis et al. (2007), Bui et al. (2014), Khosravi et al. (2015b) and Liang et al. (2006). In some trials the animals were also challenged by being infected with pathogenic bacteria, to

determine disease resistance, although FPH diets showed no significant beneficial health properties (Gildberg et al., 1995). No artificial bacterial challenges were introduced in this study, because of ethical complications and the requirement to keep the recirculating aquaculture system pathogen-free. The results of FPH studies are challenging to analyse and compare over different studies, because each FPH results in a unique composition of peptides, which are a function of the raw material, hydrolysis enzyme, DOH, and hydrolysis conditions. The FPH inclusion had a positive influence on the Ig levels, indicating that one component of the non-specific immunity was improved; it is however, unclear if the FPH will result in actual disease resistance during an outbreak of disease. FPH could, however, still have had an influence on other components of the immune response (e.g. the cellular immune function), however, these effects were not measured by the current study. Further investigations with increased trial length, consistent genetics, different growth periods (age) and diet formulations could find additional beneficial immune responses.

Table 11 Summary of non-specific immunity indicators on FPH diets in Trial 1

	Diet				
	Control	FPH-1	FPH-2	FPH-3	FPH-4
Lysozyme (µg.ml^{-1})	7.4 ± 0.19	7.3 ± 0.14	7.4 ± 0.13	7.5 ± 0.18	7.0 ± 0.10
Immunoglobulin (mg.ml^{-1})	4.94 ± 2.68^{ab}	2.17 ± 0.82^{a}	16.37 ± 4.92^{c}	15.01 ± 3.91^{bc}	2.50 ± 1.15^{a}
Total protein (mg.ml^{-1})	27.0 ± 1.53	28.7 ± 0.78	29.8 ± 4.16	33.2 ± 4.24	31.5 ± 1.81

Data represented as mean ± SE

$p>0.05$ for all treatments for lysozyme and total protein

$p<0.05$ for all treatments for immunoglobulin ($p=0.02$)

Different superscripts (e.g. ^{a}, ^{b}) in the same row indicate which means are significantly different ($p<0.05$)

Whole-body approximate composition

Investigating whole-body approximate composition enables research to evaluate the effects diets have on the production quality (nutrient content) of the animal, as if the animal would be used for human or animal consumption. In addition, whole-body composition provides an indication whether the experimental ingredient improved/disrupted any normal digestive and physiological processes (Shearer, 1994). Therefore, the final body composition was evaluated as an indication of the product quality, and the FPH diets were compared to a normal ingredient (FM). Whole-body approximate composition is shown in Table 12, as moisture content, crude protein, crude lipid and ash.

In Table 12 the whole-body lipid concentration indicates a statistically significant difference ($p=0.002$) between diets, while there were no differences for moisture, crude protein and ash ($p>0.05$). The lipid concentration in the whole bodies increased in diets FPH-1 and FPH-4, compared to the control, FPH-2, FPH-3 diets.

Table 12 Summary of whole-body approximate composition on FPH diets in Trial 1

	Diet				
	Control	FPH-1	FPH-2	FPH-3	FPH-4
Moisture ($g.kg^{-1}$)	713 ± 2.13^{a}	700 ± 2.08^{b}	704 ± 1.49^{b}	704 ± 2.84^{b}	700 ± 3.49^{b}
Crude protein ($g.kg^{-1}$)	220 ± 3.3	210 ± 6.03	214 ± 2.11	214 ± 1.04	213 ± 5.13
Crude lipid ($g.kg^{-1}$)	249 ± 1.55^{a}	276 ± 6.02^{b}	257 ± 4.14^{a}	255 ± 6.76^{a}	272 ± 4.98^{b}
Ash ($g.kg^{-1}$)	113 ± 2.60	113 ± 3.67	113 ± 3.10	112 ± 4.03	118 ± 2.81

Data represented as mean ± SE
$p>0.05$ for crude protein and ash
$p<0.05$ for moisture and crude lipids
Different superscripts in the same row indicate which means are significantly different ($p<0.05$).

The phenomenon of increased whole-body crude lipids can be attributed to the diets fed to the animals and external/condition factors (NRC, 2011, Nates, 2016). Some of the major contributors to an increased lipid deposit content can be minerals available in diets (metabolic functions for lipid storage), as well as low energy utilisation. Lipid deposits are most notable in adipose tissue, and with whole-body composition, analysis is evident in liver, muscle, skin and flesh (Nates, 2016). In the current trial, the only difference between treatments was the diets fed to the experimental animals, therefore, the levels of FM and FPH. The FPH possibly contributed to a higher lipid concentration in the body at specific dietary inclusion levels, and thus suggests that

the FPH ingredients can cause the animal's metabolic function to increase lipid deposits in the body. However, the inconsistent results provide inconclusive evidence of the true effect of FPH on the metabolic lipid deposit of the animal.

The increase of whole-body lipids is of critical importance, as it can influence product quality (Lee et al., 2002). It is common for fish fed on higher lipid diets to exhibit increased lipid deposits (McGoogan and Gatlin, 1999). In this case, all diets have equal lipid, protein and energy content. In addition, because of the nature of the analysis, it is unclear in which specific tissues the increased lipid deposits are found. Increased lipid deposits can be from the liver, internal organs, muscle tissue or skin (Chatzifotis et al., 2010). Lipid deposits are also dependent on species, as the deposit type is different for each species (Nates, 2016). It is therefore challenging to conclude why the whole-body composition lipid increased in the FPH diets. Therefore, with the information currently available, no conclusive evidence can be provided that FPH increased lipid deposits.

Apparent dry matter digestibility of FPH diets

A two-week digestibility trial was conducted in addition to the 10-week feeding trial to determine the apparent digestibility coefficients (ADC) of the FPH diets. Chromium oxide (Cr_2O_3) was added to the diets as an inert marker to determine ADC, and the coefficient was calculated by measuring the concentration of chromium oxide in the faeces (see Equation 5). Therefore, the ADC is the change in ratio of the concentration of chromium oxide in the feed and faeces. The ADC can be helpful to determine how well animals digest certain feedstuff, in this case FPH. The sampling (by stripping faeces) of the animals took place once a week, with weight and length measurements taken at the start and end of the trial. As the digestibility trial was completed only over a short growth period, which is insufficient to generate reliable production data, the data shown in Table 13 only serve as a crosscheck to verify that growth did occur during the trial, to ensure that husbandry and growth conditions were favourable.

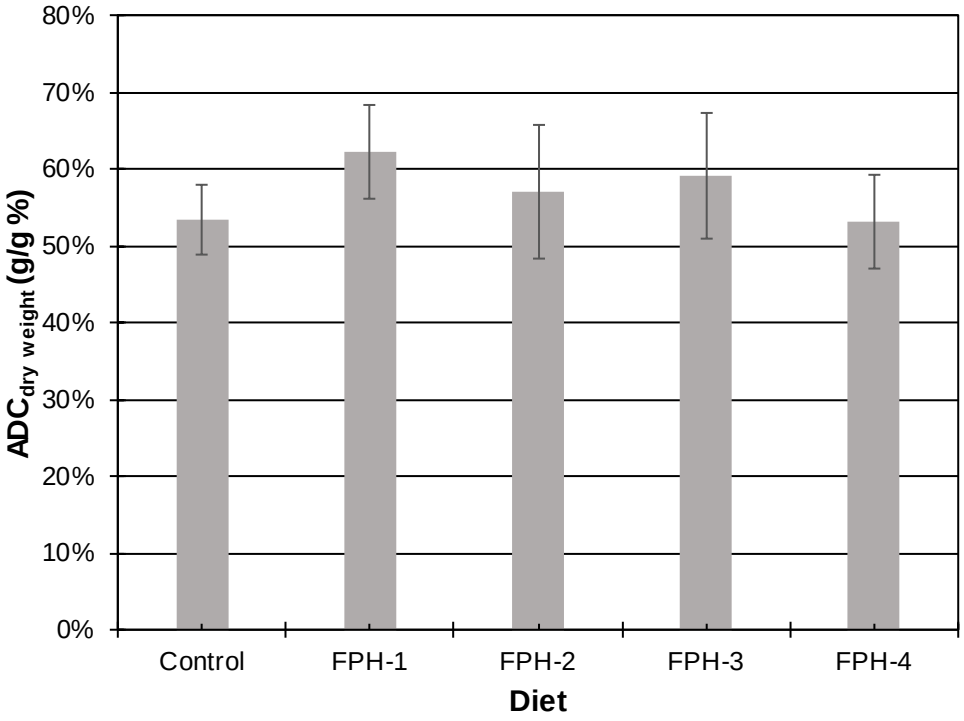

Figure 5 Apparent digestibility coefficient (ADC) with dry matter of FPH diets, for all diets p>0.05, insignificant statistical difference in Trial 1

Figure 5 illustrates the ADC for dry matter during the growth period in Table 13. It is clear from Figure 5 that there were no statistically significant differences (p>0.05) for the $ADC_{dry\ matter}$ during the two-week trial, with values ranging from 53±4% to 62±6%, which is within the ranges of previous studies (Leenhouwers et al., 2006, Nwanna, 2003, Fagbenro and Bello-Olusoji, 1997). These data suggest that FM and increased levels of FPH have similar digestibility for African catfish (*Clarias gariepinus*) of the particular size and age of the experimental animals.

The high variance in the results might be attributable to the nature of the experimental species' digestive system. It is a challenge to determine the ADC for African catfish (*Clarias gariepinus*), compared to other fish species (Slawski, 2016). The faeces of the catfish is not solid, and forms a slurry with a high moisture content, even when using the stripping method of collection (Choubert et al., 1982, Leenhouwers et al., 2006), as was done in this trial. Other methods of faeces collection, such as funnel tanks, result in the faeces mixing with tank water, which can lead to high rates of nutrient leaching and low faeces recovery, giving variable results.

Previous studies with African catfish (*Clarias gariepinus*) have found a significant difference in dry matter digestibility for different diets, which also translates into significant differences in digestibility of protein, energy and phosphorus in the diets. It is difficult to compare the results of these studies, as they each employed different faeces collection methods and analytical techniques; they used different sizes and ages of fish; and trial lengths were not standardised (Nwanna, 2003, Fagbenro and Davies, 2001, Fagbenro and Bello-Olusoji, 1997). In addition, in most studies the ADC was determined as protein or digestible energy, in contrast to this work, which determined it as dry matter. For the purpose of this study, the only requirement is comparing the ADC of each diet investigated, which was successful. The ADC of the diets shows that both FM and the FPH exhibited similar digestibility, and statistical analysis did not show any significant differences between treatments.

The $ADC_{dry\ matter}$ does not show a significant statistical difference ($p>0.05$), however, the results show similar digestibility for FM, and high and low levels of FPH. This leads to the conclusion that the FPH does not have any influence on digestibility for the African catfish (*Clarias gariepinus*), and the absorption of nutrients by the experimental animals.

The results in Table 13 show a good growth rate range of SGR 3.5-4 $\%.day^{-1}$ and an average weight gain of ± 30 g per fish during the two-week trial. Statistically significant differences ($p<0.05$) can be observed in Table 13 for final mean animal weight (W_f)and condition factor (CF), where the FPH-2 diet outperformed the control and all other diets.

However, the growth and production performance data for the digestibility trial alone should not be used to reach conclusions regarding which treatments provided optimal production performance, because of the short trial period (2 weeks) and the lower number of replicates used than in the growth trial (4). The production performance data of this phase of the trial can be viewed only as supporting evidence when viewed alongside the data from the 10-week growth trial. The conclusion can, however, be reached that the animals showed sufficient growth, suggesting that the diets were well utilised during the trials, which is the requirement for the digestibility trial.

Table 13 Summary of production parameters for the 2-week apparent digestibility trial

	Diet				
	Control	FPH-1	FPH-2	FPH-3	FPH-4
W_i (g)	61.8 ± 2.76	58.6 ± 2.05	63.0 ± 2.38	57.7 ± 3.05	60.9 ± 1.64
W_f (g)	97.1 ± 3.41^{a}	92.7 ± 2.80^{a}	106 ± 3.50^{b}	92.5 ± 2.91^{a}	98.0 ± 0.81^{ab}
WG (g/g)	0.58 ± 0.08	0.58 ± 0.01	0.69 ± 0.07	0.61 ± 0.04	0.61 ± 0.05
SGR (%.day^{-1})	3.49 ± 0.41	3.53 ± 0.04	4.00 ± 0.32	3.65 ± 0.20	3.67 ± 0.24
FCR	1.03 ± 0.13	1.05 ± 0.25	2.59 ± 1.67	1.08 ± 0.13	0.96 ± 0.08
CF	0.76 ± 0.01^{a}	0.74 ± 0.02^{a}	0.79 ± 0.01^{b}	0.75 ± 0.01^{a}	0.76 ± 0.00^{a}

Data represented as mean ± SE

$p>0.05$ for W_i, WG, SGR, FCR

$p<0.05$ for W_f and CF

Different superscripts (e.g. ^{a}, ^{b}) in the same row indicate which means are significantly different ($p<0.05$)

5.2 Trial 2: Feeding trial with different phosphate supplements

This section reports how the FB-DCP in an aquafeed influenced growth performance of the African catfish (*Clarias gariepinus*), compared to R-DCP and untreated, pure fish bones. Four different diets were formulated, each with a different phosphate additive: **Control** (R-DCP), **FB-6** (raw fish bones), **DCP-7** (FB-DCP) and **DCP-7** (1:1 R-DCP/FB-DCP), Table 8 provides detailed feed formulation.

Production parameters

The production performance parameters indicate whether the phosphate additives influenced the growth and development of the experimental animals. The availability of the dietary phosphates is proportional to the skeletal development of an animal (Nwanna et al., 2009), therefore, sufficient available P in the diet will promote growth and development, as well as supplying the animal with sufficient P required to perform metabolic functions (Ytteborg et al., 2016). The production parameters of the phosphate treatments are given in Table 14.

Table 14 Summary of production parameters for diets containing different forms of DCP in Trial 2

	Diet			
	Control	FB-5	DCP-6	DCP-7
W_i (g)	0.96 ± 0.08	1.04 ± 0.12	0.96 ± 0.06	0.95 ± 0.03
W_f (g)	31.2 ± 0.95	29.9 ± 1.13	31.4 ± 2.24	30.5 ± 0.60
WG (g/g)	32.6 ± 3.04	28.8 ± 1.77	32.4 ± 3.17	31.4 ± 1.00
SGR (%.day^{-1})	4.99 ± 0.13	4.83 ± 0.09	4.98 ± 0.15	4.96 ± 0.05
FCR	0.82 ± 0.01^{a}	0.82 ± 0.01^{a}	0.89 ± 0.01^{b}	0.89 ± 0.02^{b}
CF	0.70 ± 0.01	0.69 ± 0.01	0.70 ± 0.01	0.70 ± 0.01

Data represented as mean ± SE
p>0.05 for all diets for parameters: SGR and CF
p<0.05 for FCR (p=0.008)
Different superscripts in the same row indicate the means are significantly different (p<0.05)

It is clear from Table 14 that the phosphate supplements exhibit a diet-dependent statistical difference for the FCR; diets DCP-6 and DCP-7 had a negative effect on the nutrient utilisation (FCR). All other parameters indicate a statistically insignificant (p>0.05) influence on the production of the African catfish (*Clarias gariepinus*). The initial and final weight illustrate an increase of ± 30 g; high growth rates were seen during the trial, with all growth rates ranging between SGR 4.8 and 5 %.day^{-1}, and a consistent CF over all diets, indicating that P deficiency in the diets is unlikely.

Although FCR<1 can be considered a good value, DCP-6 and DCP-7 is significantly worse than in the Control and FB-5. A possible explanation for the phenomenon is that the FB-DCP reduced nutrient utilisation by African catfish (*Clarias gariepinus*). Because of the high plant (SM and EM) content of the diets, the utilisation of available P is source dependent, and thus nutrient utilisation dependent, because of low P values in water (NRC, 2011). The explanation for the difference between R-DCP and FB can be the amount of P available for uptake by the experimental animals, rather than the total amount of P present in the feed, that is, the P digestible by the experimental animals. Therefore, there could be less available P in the FB-DCP than in the R-DCP.

However, according to Albrektsen et al. (2017), the pure form of the P in untreated fish bone is mostly inorganic hydroxyapatite, which is poorly digestible because of its stable, insoluble crystalline structure. Accordingly, FB-5 P-utilisation should be lower than that of DCP-6 and DCP-7. The results indicate otherwise, suggesting that the negative impact on FCR seen in diets DCP-6 and DCP-7 is probably not due to the P source, although the current data available cannot confirm this conclusion.

The available P is also influenced by the FM (200 $g.kg^{-1}$) in the diets. The P in the FM can disguise the effects of the phosphate additive during the feeding trial. In addition, for each diet the P/Ca ratio and available P have an influence on animal development. The good SGR>4.5 $\%.day^{-1}$, and a consistent CF for all diets is an indication that P deficiency in the treatments was unlikely. Similar experiments on Atlantic salmon (*Salmo salar)* used acid hydrolysed fish bone successfully as an efficient P source in aquafeed for fry and postsmolt (Albrektsen et al., 2017, Ytteborg et al., 2016).

The FCR<1 can be attributed to the small size of the fish (mean growth period 1-30 g over 10 weeks) and the manner in which FCR is calculated as the amount of dry feed given versus wet weight gain of the fish. These factors influence the value of FCR, although these factors were constant over all treatments. If the treatments had had any significant effect on the experimental animals, it should be visible in the production parameters statistical analysis.

Cumulative mortality

Figure 6 illustrates the mortality of the animals as a fraction of the total initial animals. The cannibalistic nature of the African catfish (*Clarias gariepinus*) created a challenge in relation to determining the true mortality of the animals over the trial period, as the majority of the mortalities were due to cannibalistic behaviour. Mortalities due to cannibalistic behaviour were therefore counted as mortalities as part of the trial – the cumulative mortality presented at sampled days (0, 28, 49, 70 days).

The result in Figure 6 illustrates that, over the 70 days; at least 65% of all fish survived the feeding trial. In general, this is not a good survival rate. During the trial, there were no obvious signs of diseases, and the high mortality rate was mostly caused by cannibalism. At small initial sizes, differential growth of African catfish (*Clarias gariepinus*) tend to result in outliers (fish significantly larger than the mean); these larger fish turn cannibalistic (Enyidi, 2012, Hecht and Appelbaum, 1988, Sallehudin and Mukai, 2014). Considering the small initial size/age (±1 g) of the fish, it was not surprising to find cannibalism among the experimental animals. Despite the cannibalistic behaviour it was decided not to remove outliers from the tank, as doing so might have interfered with the results.

One replicate was, however, removed from the data set after extreme cannibalism, in this case only six fish survived (of 30), with one large fish as an extreme outlier. For these reasons, no definitive conclusions can be made from the mortality rate. Nonetheless, from Figure 6, DCP-6 (0.35) and DCP-7 (0.34) had the worst survival, where FB-5 (0.28) had the best survival and the Control (0.30) an average survival. To assign these differences in mortality to variations in dietary treatments would be invalid; these differences have no general trend and it is unlikely that the different diets caused the observed cannibalism.

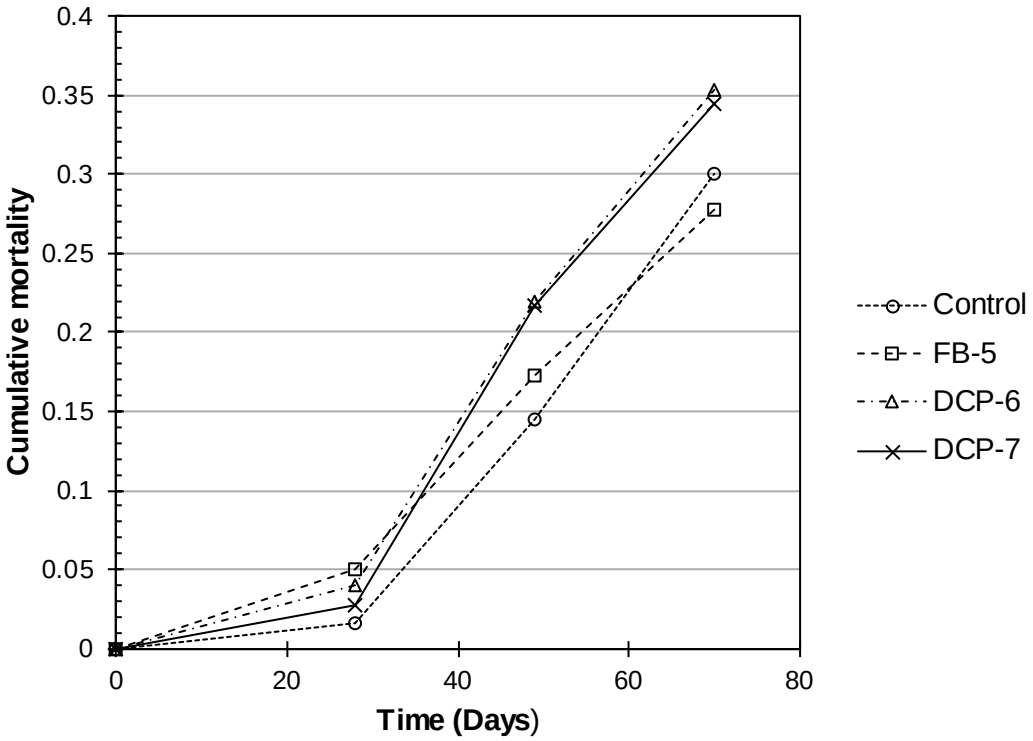

Figure 6 Cumulative mortality of the animals as a fraction of initial total animals for DCP diets in Trial 2

Non-specific innate immunity

The non-specific immunity parameters were measured by evaluating the dietary phosphate ingredients, in order to determine whether the feed ingredients showed any effects (negative or positive) on the health of the experimental animals (Kiron, 2012).

Non-specific immunity was characterised using none-cellular mechanisms in blood serum: lysozyme activity, immunoglobulin, and total serum protein. In many cases, immune function is proportional to survival rate and disease resistance of fish in aquaculture systems, and immune function, in turn, affects the production of the fish by an aquaculture farmer (NRC, 2011). It is important to take the health impacts of feed ingredients into account during aquafeed formulation, and to evaluate the effect of novel feed ingredients on animal immune function (Kiron, 2012).

Non-specific immunity parameters are used to evaluate the phosphate feed ingredients with potential beneficial effects, such as micro minerals and high

availability of P (Swart, 2017). The non-specific parameters of the diets are shown in Table 15, and analytical methodologies are described in Section 4.6.

Table 15 Summary of non-specific immunity indicators on DCP diets in Trial 2

	Diet			
	Control	FB-5	DCP-6	DCP-7
Lysozyme (µg.ml^{-1})	7.4 ± 0.19	6.8 ± 0.062	7.1 ± 0.27	7.1 ± 0.10
Immunoglobulin (mg.ml^{-1})	4.94 ± 2.68	9.88 ± 2.50	13.6 ± 3.59	7.61 ± 1.77
Total Protein (mg.l^{-1})	27.0 ± 1.53	27.8 ± 0.78	30.5 ± 1.71	25.8 ± 1.88

Data represented as mean ± SE
p>0.05 for all diets for parameters

Table 15 illustrates that there were insignificant statistical differences (p>0.05) between the treatments regarding non-specific immunity parameters. These data indicate that the FB-DCP product did not influence the immunity of the animals negatively compared to standard R-DCP (Control), providing further evidence that FB-DCP is an acceptable feed ingredient and P source for African catfish (*Clarias gariepinus*) diets. In addition, the FB and FB-DCP did not contain known harmful substances or bacteria that influenced the animals' non-specific immunity negatively at the inclusion levels of the ingredients used in this trial.

Measuring non-specific immunity parameters during phosphate trials is not common in open literature. Commonly measured parameters include welfare indicators and bone development (Ytteborg et al., 2016, Albrektsen et al., 2009). The non-specific immunity parameters only illustrate whether the micro and macro minerals had an influence on the animal's non-specific immunity. The results of the current trial show that the FB and FB-DCP did not influence the non-specific immunity significantly, and that there are no dietary dependent non-specific immunity differences (P source of FB-DCP/FB).

Whole-body approximate Composition

Whole-body approximate composition enables research to evaluate the influence of diet on the production quality (nutrient content) of the animal. The nutrient composition of the complete animal carcass is expressed as moisture, crude protein, crude lipid and ash, as if the animal would be used for human or animal consumption. This enables research to illustrate the influence of the phosphate feed ingredients (FB and FB-DCP) if the quality of the animals is disrupted/improved compared to the

control (Shearer, 1994). Therefore, whole-body approximate composition measurements compare the effects of the different phosphate supplements (R-DCP, FB, and FB-DCP) in the feed on the nutrient composition production (quality) of the animals. Table 16 presents the whole-body approximate composition analysis for the phosphate treatments in Trial 2.

Table 16 Summary of whole body approximate composition on DCP diets in Trial 2

	Diet			
	Control	FB-5	DCP-6	DCP-7
Moisture ($g.kg^{-1}$)	713 ± 231	699 ± 245	688 ± 1.11	706 ± 2.55
Crude protein ($g.kg^{-1}$)	220 ± 3.30	218 ± 5.00	226 ± 4.93	218 ± 5.96
Crude lipid ($g.kg^{-1}$)	249 ± 1.55^{a}	270 ± 6.21^{bc}	275 ± 2.29^{b}	261 ± 3.63^{c}
Ash ($g.kg^{-1}$)	113 ± 2.60	115 ± 5.71	116 ± 4.28	116 ± 43.77

Data represented as mean ± SE
$p>0.05$ for all diets for moisture, crude protein and ash
$p<0.05$ for crude lipid ($p=0.0008$)
Different superscripts (e.g. ^{a}, ^{b}) in the same row indicate what means are significantly different ($p<0.05$)

From Table 16 it is clear that there were statistically significant differences in crude lipid approximate composition, though there were no differences for moisture, crude protein and as ($p>0.05$). Diet-dependent differences for approximate crude lipid were found, where FB-5, DCP-6 and DCP-7 all showed significantly higher whole-body lipid content than the control diet. In animal diets, the composition of dietary lipids can increase lipid storage in the body of experimental animals, which might be the reason for the differences seen in the current trial, as temperature, genetics and age (all common factors that can influence lipid storage) were kept constant over all diets (Nates, 2016).

High lipid levels in whole-body composition can indicate phosphorous deficiency in general aquaculture (Ye et al., 2006); however, in this trial there is insufficient evidence to correlate the slightly higher lipid levels in experimental animals with P deficiency. To the contrary, all other growth and immune-function data suggest that all diets provided sufficient dietary nutrients; this, coupled with the absence of any skeletal deformities in the experimental animals used in the trial, indicate that the varying P treatments all provided sufficient levels of P.

However, there is a general shortage of research detailing dietary mineral requirements of African catfish (*Clarias gariepinus*) (NRC, 2011), and the reason for the increased lipid levels in the current trial could therefore not be determined. In some species, increased lipid deposits do not mean a phosphorous deficiency, and the influence of sub-optimal dietary P levels is not always known (Tacon, 1992).

Mineral composition of vertebrae

The mineral composition of the vertebrae was analysed to evaluate any diet-dependent differences in bone development in the experimental animals. Table 17 shows the results of the mineral analysis of vertebrae for Trial 2.

It is clear from Table 17 that there are no diet-dependent statistically significant differences ($p>0.05$) between any of the diets. Elemental analysis of P, Ca, Mg, Al, Mn, and Si illustrate no differences. The levels of P and Ca were the highest, as was expected, because these elements result from the hydroxyapatite content of fish bones (Kim and Mendis, 2006). P and Ca concentration are deemed to be the most important values, as deficiencies in these elements would indicate any abnormalities in the bone development of the experimental animals (Ytteborg et al., 2016).

Table 17 Mineral analysis of vertebrae of animals ($mg.kg^{-1}$ and $g.kg^{-1}$) in Trial 2

	Diet			
	Control	FB-5	DCP-6	DCP-7
P ($g.kg^{-1}$)	137 ± 6.26	141 ± 17.0	117 ± 1.94	127 ± 6.51
Ca ($g.kg^{-1}$)	281 ± 11.9	291 ± 40.7	241 ± 4.65	263 ± 13.5
Mg ($mg.kg^{-1}$)	5847 ± 347.9	6090 ± 789.8	4798 ± 101.4	5164 ± 235.3
Al ($mg.kg^{-1}$)	81 ± 6.6	88 ± 13	74 ± 8.3	73 ± 4.1
Mn ($mg.kg^{-1}$)	117 ± 14.6	143 ± 11.7	147 ± 5.4	145 ± 8.2
Si ($mg.kg^{-1}$)	12 ± 2.3	15 ± 3.6	13 ± 2.0	10 ± 1.0

Data represented as mean ± SE
$p>0.05$ for all diets for all minerals

The Mg, Al, Mn and Si content also showed no diet-dependent differences ($p>0.05$). With no comparable open literature data available on African catfish (*Clarias gariepinus*) bones, no conclusive evidence is available to illustrate the influence of the P sources on the experimental animals.

The Ca/P ratios in the bones were calculated and are shown in Figure 7. In general, the values for Ca/P varied within a very narrow band of 2.056 to 2.070 (as the concentration ratio $mg.kg^{-1}$: $mg.kg^{-1}$) for all diets, with no diet-dependent statistically

significant differences (p>0.05). It could, however, be noted that DCP-7 showed the highest numerical Ca/P ratio, with a value of 2.070±0.010.

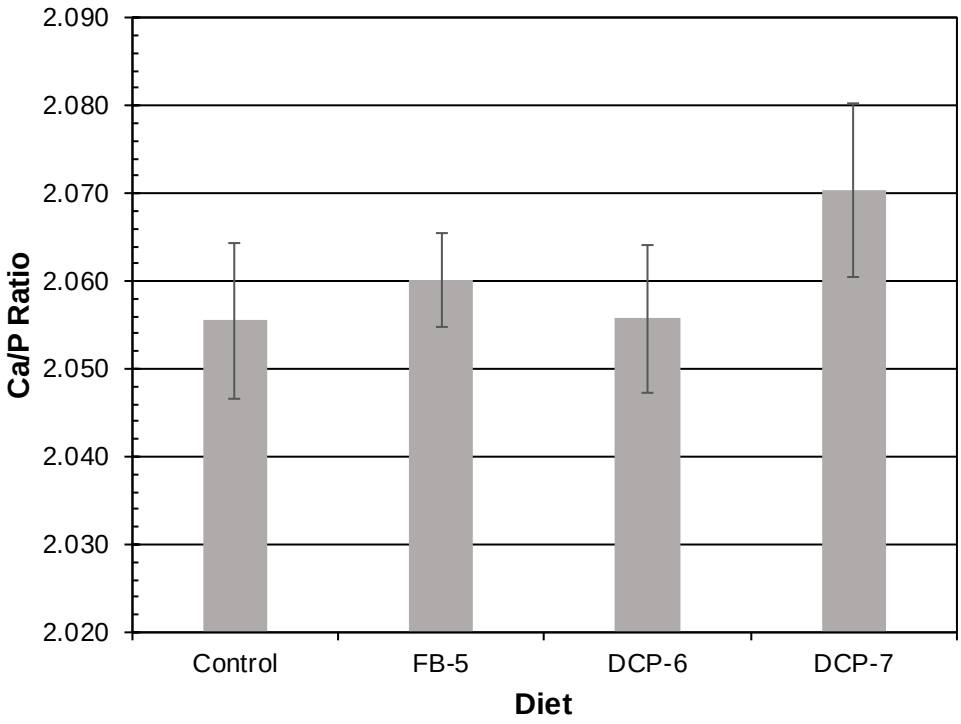

Figure 7 Ca/P ratio of composition of vertebrae of Trial 2

5.3 Relationship between length and weight

In research the weight length relationship is important for determining the body condition of animals, which may provide an indication of whether animals are experiencing some form of undetected physiological stress and/or whether the animals are in 'good' condition. In essence, the relationship calculates the 'density' of each experimental animal, by assuming the volume can be determined from only one dimension (in this case, the length). In Section 5.1 and 5.2 the relationship described by Fulton in 1904 (Froese, 2006), Equation 3 (See Section 4.5), was used. This relationship assumes b = 3 with a $W=aL^b$ relationship as biological variable described by Fulton. However, the assumption of b = 3 does not always work for all species, and vary from 2.5 to 3.5 (Froese, 2006). When b < 3, it represents fish that tend be less rotund when length increases, and when b > 3 the fish tend to be more rotund when length increases (Jones et al., 1999). In general, the shape of fish determines the value of b, and fish such as halibut, flounder and tilapia, which have a much flatter body shape, will give significantly different values than African catfish (*Clarias gariepinus*) would. In addition, it is generally accepted that a heavier fish for a given length is in better condition, that is, a higher value of b (Jones et al., 1999). Figure 8 illustrates the plots of the normal weight-length and the natural logarithmic

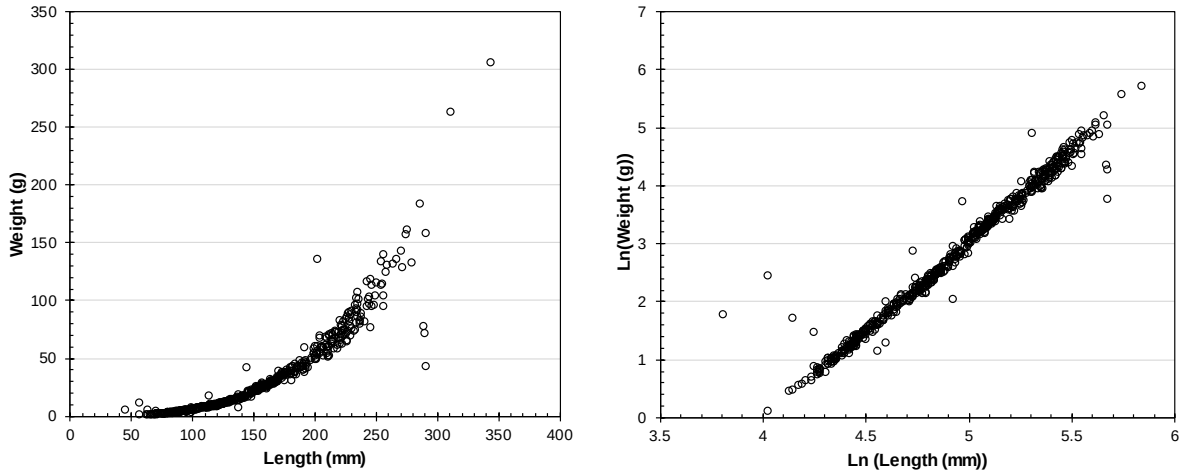

Figure 8 Weight-length relationship of all data points for the control diet

values of all the Control diet data points for both the 10-week and 2-week trials.

Statistical regression analysis with a 95% prediction interval (Appendix F, Figure 10), with a linear fit of the natural logarithm in Figure 8, resulted in values for a = -12.0761, b = 3.0236 and R^2 = 0.9725. This confirms that the assumption of b = 3 in the Fulton condition factor calculation provides a reasonably good fit to actual data, and the CF parameter will give a good indication of the condition of the experimental animal. Using the parameters obtained from the statistical analysis, the following equation will be true:

$$CF = \frac{W}{L^{3.02}} \qquad (6)$$

The current result corresponds with data obtained for a range of different species, such as channel catfish (*Ictalurus punctatus*) (Carlander, 1969), Silver catfish (*Schilbe intermedius*) (Swanepoel, 1988), various salmonids, native North American fish and marine fish, such as sardine (Froese, 2006). FishBase (2017), which provide mean b = 3.00 for 21 studies of African catfish (*Clarias gariepinus*), from various natural and artificial populations, with a range of 2.699<b<3.254.

6. Conclusion

FPH, FB-DCP and FB derived from fish processing by-products have the potential to be successful aquafeed ingredients. The results show that both the FPH and FB-DCP can be included successfully in aquafeed for African catfish (*Clarias gariepinus)*, and that both are good feed ingredients comparable to products that are deemed the current industry standards. In the case of FPH, it was shown that up to 250 $g.kg^{-1}$ (dry matter) of FM could be replaced by FPH, while all R-DCP could be replaced entirely by FB-DCP. The production of both these feed ingredients from fish processing by-products has the potential to reduce fish processing by-product, by utilising the waste to produce high-value products, thereby increasing sustainability of wild fisheries and aquaculture. The results, however, did not find significant improvements in production performance when either FPH or FB-DCP were incorporated as aquafeed ingredients in diets for African catfish (*Clarias gariepinus)*.

Further, the findings clearly show that FPH exerted a specialist bioactivity effect in the experimental diets, as evidenced by a significant influence on the immunoglobulin concentration in the blood serum for diets FPH-2 and FPH-3. It was further found that these effects on this component of the non-specific immune function were dependent on the level of FPH inclusion, meaning that, if the correct inclusion levels of FPH are utilised in aquafeed, there could be some beneficial effects for African catfish (*Clarias gariepinus*). In the case of the Trial 2, P-source inclusion, there were no effects on non-specific innate immunity, thereby emphasising the fact that FB and FB-DCP did not influence the non-specific immunity in any way and that the inclusion of these ingredients had no detectable negative health impacts on the experimental animals.

There were no statistically significant differences in production parameters and mortality for either the FPH or FB-DCP diets, except in nutrient utilisation (FCR) in Trial 2, where diets with the FB-DCP and 1:1 ratio of FB-DCP and R-DCP showed significantly decreased nutrient utilisation. It is challenging to confirm an explanation for this finding, as no other important parameters, such as SGR, showed any significant difference, and the exact reason for the decreased FCR could not be established.

Both feed ingredients affected the whole-body composition of the experimental animals significantly. All diets in which conventional rock phosphate (R-DCP) was replaced showed significant increases in whole-body lipid content (diets FB-5, DCP-6 and DCP-7). In the FPH diets of Trial 1, FPH-1 and FPH-4 also illustrated significantly higher crude lipid composition in the carcass. Lipid deposits in experimental animals can be attributed to various non-dietary factors, and to dietary lipid content; however, in the current trial all experimental conditions and lipids added to the diets were standardised. Therefore, in the case of constant lipid (isocaloric) diets, such as the current trial, the increased lipid deposits can only be attributed to some dietary influence related to the phosphate source (FB and FB-DCP) and the protein source and inclusion level (FPH). The exact reason for increased lipid deposits could not be determined with the available data, and warrants further investigation.

FPH inclusion did not have any effect on total dry matter digestibility of the diets. There were no significant differences in apparent digestibility of the dry matter between treatments, and all diets and control had similar $ACD_{dry\ matter}$ coefficients. This confirms that FPH had similar digestibility to the ingredient that it replaced in the diets (in this case, FM).

The FB and FB-DCP did not show influence on bone mineralisation. The mineral content in the vertebrae for the phosphate supplements did not indicate any significant dietary dependent differences. Therefore, it do however indicate that the FB (untreated fish bone) and the FB-DCP (acid hydrolysis fish bone) have equal influence on the experimental animals for the specific diet and species.

7. Recommendations

It is recommended that further in vivo trials be preformed to investigate some of the effects observed by the current trials further. It is possible that further investigation will reveal further potential and beneficial properties of the aquafeed ingredients FPH and FB-DCP. Additional research could be done on different fish species, trial length can be increased, genetics improved, and more measurements taken, which have the potential to outline/emphasise the beneficial influences of high-value feed ingredients. It is known from literature that not all feeding trials determine all the beneficial properties of FPH, as happened in the current work.

The cannibalistic nature of the African catfish (*Clarias gariepinus*) caused variation in the results and mortality of the animals in the experiment, and therefore it is recommended that a higher stocking density is applied and size/age of the animals is increased, to decrease cannibalism and the effect of outliers on feeding trials. A further recommendation is to use larger fish for the feeding trial (<10 g) to decrease variation in the results; this adaptation would also possibly decrease mortality. In addition, during the juvenile stages of the fish development, it is difficult to detect significant differences, causing the statistical analysis to show insignificant statistical differences that may not exist.

In general, it is recommended to investigate the influence of African catfish (*Clarias gariepinus*) size, stocking density, trial length, genetic strains, feeding (controlled vs ad libitum), sampling methods/intervals, and to determine if removing the outliers from the feeding trial influences the results. To use a phosphate free protein source (i.e. not FM) to investigate phosphate additives can also be useful in the future to ensure no other available phosphate in the feed. These data will be extremely useful for aquaculture research, nutritionists and farmers, especially if further research on this species is conducted in future.

For disease resistance research, the potential of doing a disease challenge trial (artificially infecting animals with a pathogen) and applying different feed ingredients can be useful to determine if specific diets have any influence disease resistance, instead of merely measuring immunity parameters. This type of research, however, does have some practical and ethical challenges.

During the 2-week digestibility trial, the amount of faeces collected was only sufficient to determine dry matter digestibility, but not to apparent digestibility for individual nutrients (e.g. proteins). The recommendation is that, either the trial length, the number of replicates, or the size of fish should be increased to ensure enough faeces is collected. Doing so would ensure sufficient amounts of faeces samples with sufficient replicates to do chromium oxide, protein and energy analysis on faeces. Another option would be to use facilities such as funnel tanks or automatic collectors to collect faeces throughout the trial, and to avoid using the stripping method, (Choubert et al., 1982).

Regarding blood sampling, future studies should ensure that the animals reach sufficient size, so that enough blood can be collected to do the non-specific innate immunity analysis. From experience of this trial, fish with a length of >180 mm and >40 g will provide enough (>1 500 µl) blood for total serum protein, immunoglobulin and lysozyme activity analysis.

7.1 Economic viability of feed ingredients

One important area for future investigation is determining the economic viability of using the two feed ingredients, which would include an evaluation of production costs and potential market prices of the ingredients. For FPH to compete realistically against FM, and to compare FB-DCP with R-DCP, the feed ingredients must be economically viable, which means that the ingredients should have competitive market prices and should be readily available if they are to compete as major feed ingredients in the aquafeed ingredient market. Regarding the cost of FB-DCP, according to the results of this study there are no advantages to using FB-DCP compared to untreated fish bone, therefore, from an ingredient production cost point of view, it would not be necessary to employ any additional treatment to produce a feed ingredient from fish bones, thereby lowering overall production cost.

In order to make the results generated from this study relevant from an engineering perspective, it is recommended that a complete desktop economic viability study is performed to determine the economic viability of the two feed ingredients. If these results are favourable, further steps towards commercialisation can be taken, e.g. by designing a pilot plant. Even though the current study clearly demonstrates that the

two feed ingredients that were evaluated performed to current market standards, and that present some additional beneficial properties (e.g. improving non-specific immune function), these results do not indicate the economic feasibility of producing these ingredients. For these reasons, it is recommended that particular attention be given to this aspect in future.

8. References

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Appendices

A. Ethics Protocol Approval

UNIVERSITEIT·STELLENBOSCH·UNIVERSITY
jou kennisvennoot • your knowledge partner

Protocol Approval

Date: 20-Feb-2017

PI Name: Swanepoel, Johannes JC

Protocol #: SU-ACUD16-00062

Title: Valorisation of low-value fish processing waste with African catfish Clarias gariepinus

Dear Johannes Swanepoel, the Response to Modifications, was reviewed on 09-Feb-2017 by the Research Ethics Committee: Animal Care and Use via committee review procedures and was approved. Please note that this clearance is only valid for a period of twelve months. Ethics clearance of protocols spanning more than one year must be renewed annually through submission of a progress report, up to a maximum of three years.

Applicants are reminded that they are expected to comply with accepted standards for the use of animals in research and teaching as reflected in the South African National Standards 10386: 2008. The SANS 10386: 2008 document is available on the Division for Research Developments website www.sun.ac.za/research.

As provided for in the Veterinary and Para-Veterinary Professions Act, 1982. It is the principal investigator's responsibility to ensure that all study participants are registered with or have been authorised by the South African Veterinary Council (SAVC) to perform the procedures on animals, or will be performing the procedures under the direct and continuous supervision of a SAVC-registered veterinary professional or SAVC-registered para-veterinary professional, who are acting within the scope of practice for their profession.

Please remember to use your protocol number, SU-ACUD16-00062 on any documents or correspondence with the REC: ACU concerning your research protocol.

Please note that the REC: ACU has the prerogative and authority to ask further questions, seek additional information, require further modifications or monitor the conduct of your research.

Any event not consistent with routine expected outcomes that results in any unexpected animal welfare issue (death, disease, or prolonged distress) or human health risks (zoonotic disease or exposure, injuries) must be reported to the committee, by creating an Adverse Event submission within the system.

We wish you the best as you conduct your research.

If you have any questions or need further help, please contact the REC: ACU secretariat at WABEUKES@SUN.AC.ZA or 218089003.

Sincerely,

Winston Beukes

B. Research Permit

CapeNature

REGION/ OFFICE/ DIRECTORATE

postal Private Bag X29 Gatesville 7766
physical PGWC Shared Services Center cnr Bosduif & Volstruis Streets Bridgetown 7764
website www.capenature.co.za
enquiries Zimkitha Dwangu
telephone +27 21 483 0154 **fax** 086 576 7404
email zdwangu@capenature.co.za
reference 1/2/1/6/5/F8
date 05 December 2016

Mr C Swanepoel
University of Stellenbosch
Department Process Engineering
P O Box 1
MATIELAND
7602

Dear Mr Swanepoel

APPLICATION TO COLLECT FAUNA SPECIMENS FOR SCIENTIFIC RESEARCH PURPOSES

I refer to your application to collect fauna and flora specimens in the Western Cape Province.

Attached is permit No. **0056-AAA043-00025** dated **05 December 2016** to collect specimens in the Western Cape Province. Please take special note of the standard conditions attached to the permits. I specifically draw your attention to permit condition (i). **It is imperative that you make contact with the Reserve Manager BEFORE you intend collecting on any nature reserve, conservation area, wilderness area and / or state forest**. No deviation is allowed from the fore-mentioned conditions without the prior written approval of the Chief Executive Officer: Western Cape Nature Conservation Board.

Please also take note of the *pro forma* (copy attached), which must please be used when submitting your collection / distribution records to CapeNature as per the conditions to your permit. Please feel free to add columns for extra data to the *pro forma* but no columns should be deleted. This pro forma is also available electronically from CapeNature.

Should you have any queries please do not hesitate to contact this office.

Yours faithfully,

CHIEF EXECUTIVE OFFICER

Western Cape Province

Telephone No: (027) 021 483 0000
EMail: permits.fax@capenature.co.z
PGWC Shared Services Centre
cnr Bosduif and Volstruis Streets
Bridgetown
7764

Facsimile No: (027)0865567734
Internet: www.capenature.co.za
Private Bag X29
Gatesville
7766

PERMIT TO HUNT WITH
PROHIBITED HUNTING METHOD OF WILD ANIMALS - RESEARCH PURPOSES

(Issued in terms of the provisions of the Nature Conservation Ordinance 1974, (Ord 19 of 1974)Section29&33)
Not Transferable

Holder

Full Name	Mr C Swanepoel	**Identity No.**	9310315110084
Trade Name	NA	**Registration No.**	AAA043-01183
Postal Address	University of Stellenbosch Department Process Engineering Private Bag X1	**Physical Address**	30 Simonsrust, Cluver Street
Suburb\Town	Matieland	**Suburb\Town**	Stellenbosch
Province\State	Western Cape	**Province\State**	Western Cape
Country	South Africa	**Country**	South Africa
Postal\Zip Code	7602	**Longitude**	.0000
		Latitude	.0000

In terms of and to the provisions of the abovementioned Ordinance and the Regulations framed thereunder, the holder of this permit is hereby authorised to Hunt (capture/disturb/stampede/kill) the protected wild animal(s) specified below on the property mentioned on this permit. See conditions on last page:

Details

Permit/Licence No	**0056-AAA043-00025**
Expiry Date	05/04/2017
Date Issued	05/12/2016
Amount Paid	R 0.00
Reference	NO CHARGE
File Code	1/2/1/6/5/F8

Stamp: CapeNature FAUNA • FLORA • HUNTING • CITES

Description	Property
Organization	NA
Person	Swanepoel C Mr
ID	9310315110084
Properties	Within the Western Cape Province only.
Physical Address	NA
District	NA
Province/State	Western Cape
Country	South Africa
Longitude	.0000
Latitude	.0000

Species(Scientific Name)	Qty	Note
Barbel(Clarias gariepinus)	1 600	Conditions apply, note special conditions.

Issued by:	Approved on Behalf CEO	Effective Date	Signature of Holder
Zimkitha Dwangu	Western Cape Nature Conservation Board	05/12/2016	I acknowledge, accept and understand fully the permit conditions as described

07/12/2016 08:20:14

software by: www.u-rad.com

Standard Conditions

1. When the holder of this permit *kills/captures/collect any wild animal in terms thereof, he shall, before leaving the above-mentioned property, or if he does not leave it, after each day's *hunt/capture/collection, record the particulars regarding the date, species and number of each sex of each species, or if it is impossible to distinguish the sex, the total number of each species of such wild animals which he had *killed/capture/collected.

2. The holder of this permit shall return it to the Chief Executive Officer: Western Cape Nature Conservation Board, Private Bag X29, Gatesville, 7766, within 14 days of the date of expiry thereof.

3. THIS PERMIT IS SUBJECT TO THE ADDITIONAL CONDITIONS AS SET OUT IN THE ADDENDUM HERETO.

Special Conditions

Title of this project:-Determination of bioavailability and bioactivity of enzymatically hydrolysed fish protein, and phosphates with African catfish Clarias gariepinus

Number of persons engaged in the research project:-
1.Dr NJ Goosen, Supervisor (8303065019085)

Special conditions:-
1. Fish are for research purposes only and no live fish may be moved from Welgevallen Experimental farm.
2. All fish must be euthanized upon completion of the experimental work.

CONDITIONS APPLICABLE TO RESEARCHERS UNDERTAKING RESEARCH OR OTHER COLLECTING WORKS ON PROVINCIAL CONSERVATION AREAS AND / OR PRIVATELY OWNED LAND IN THE PROVINCE OF WESTERN CAPE:

1. THE MANAGER OF THE RELEVANT CONSERVATION AREA(S) (IF ANY) MUST BE INFORMED TIMEOUSLY BEFORE ANY CONSERVATION AREA IS ENTERED FOR COLLECTING OR RESEARCH PURPOSES AND THE MANAGER'S WRITTEN PERMISSION TO ENTER SUCH RESERVE MUST BE ACQUIRED BEFOREHAND. THIS PERMIT DOES NOT GRANT THE PERMIT HOLDER AUTOMATIC ACCESS TO ANY NATURE RESERVE, CONSERVATION AREA, WILDERNESS AREA AND / OR STATE FOREST. ANY OTHER / FURTHER CONDITIONS OR RESTRICTIONS THAT THE MANAGER MAY STIPULATE AT HIS / HER DISCRETION MUST ALSO BE ADHERED TO. THIS PERMIT MUST BE AVAILABLE TO BE SHOWN ON DEMAND.
2. The owner of any other land concerned (be it privately or publicly owned land) must give WRITTEN consent allowing the permit holder to enter said property to collect flora / fauna. This written permission must reflect the full name and address of the property owner (or of the person authorised to grant such permission), the full name and address of the person to whom the permission is granted and the number and species of the flora / fauna, the date or dates on which such flora / fauna may be picked / collected and the land in respect of which permission is granted. Copies of this written permission must be made available to The Western Cape Nature Conservation Board upon request.
3. Type-specimens of any newly described / discovered species or other taxon collected must be lodged with a recognised South African scientific institution / museum / herbarium (preferably within the Province of Western Cape) where such material will be available to other researchers. For every flora specimen collected on a Western Cape Nature Conservation Board nature reserve, one additional (extra) herbarium specimen must be forwarded to the Western Cape Nature Conservation Board Herbarium at Jonkershoek (c/o MJ Simpson, Private Bag X5014, Stellenbosch 7599).
4. A list of all collected specimens / material including the; species name, the number collected, the collection date and the precise locality of the collection must be submitted within 14 days from the date of expiry of your permit to The Chief Executive Officer: CapeNature, Private Bag X29, Rondebosch, 7701
5. The maximum number of specimens per species specified in the permit (if at all) may not be exceeded without the prior permission of The Chief Executive Officer: Western Cape Nature Conservation Board.
6. For projects of more than one year's duration a progress report must be submitted to The Chief Executive Officer: Western Cape Nature Conservation Board before 31 December of each year.
7. One copy of all completed reports, publications, or articles (including books, videos, CDs, DVDs etc.) resulting from the project/collection must be submitted to The Chief Executive Officer: Western Cape Nature Conservation Board free of charge.
8. Should a report, publication, article or thesis arise from this project/collection, an acknowledgement to Western Cape Nature Conservation Board must be included.
9. The Forest Act 1984 (Act 122 of 1984) and regulations, the Nature Conservation Ordinance, 1974 (Ordinance 19 of 1974) and all regulations in terms of the Ordinance must be adhered to.
10. Should it be envisaged to export any material / specimens across the boundaries of the Western Cape Province, an export permit will be required in respect of certain species and a further application form will have to be completed. The permit holder must confirm with the Western Cape Nature Conservation Board whether an export permit is required BEFORE exporting any material / specimens from the Western Cape Province.
11. No species that appear on the Red Data List or species listed as endangered in terms of the Nature Conservation Ordinance, 1974 (Ordinance 19 of 1974) may be collected, except for those mentioned on the permit.
12. Unless otherwise specifically indicated in writing, no material or specimens collected with this permit or material or specimens bred or propagated, from material or specimens collected with this permit, may be donated, sold or used for any commercial purpose by any party.
13. IF APPLICABLE, ETHICS CLEARANCE MUST BE ACQUIRED FROM YOUR RESEARCH INSTITUTE PRIOR TO COLLECTION.

CHIEF EXECUTIVE OFFICER

CapeNature
FAUNA • FLORA • HUNTING • CITES

C. *Transport Permit*

Western Cape Province

Telephone No: (027) 021 483 0000
EMail: permits.fax@capenature.co.za
PGWC Shared Services Centre
cnr Bosduif and Volstruis Streets
Bridgetown
7734

Facsimile No: (027)0865567734
Internet: www.capenature.co.za
Private Bag X29
Gatesville
7766

CapeNature

PERMIT TO
IMPORT AND STOCK LIVE FISHES

(Issued in terms of the provisions of the Nature Cons. Ordinance, 1974 (Ord 19 of 1974) (Section 57 & 58))

Not Transferable

Holder			
Full Name	Mr C Swanepoel	**Identity No.**	9310315110084
Trade Name	NA	**Registration No.**	AAA043-01183
Postal Address	University of Stellenbosch Department Process Engineering Private Bag X1	**Physical Address**	30 Simonsrust, Cluver Street
Suburb\Town	Matieland	**Suburb\Town**	Stellenbosch
Province\State	Western Cape	**Province\State**	Western Cape
Country	South Africa	**Country**	South Africa
Postal\Zip Code	7602	**Longitude**	.0000
		Latitude	.0000

In terms of sections 57 and 58 of the Nature Conservation Ordinance, 1974 (Ordinance 19 of 1974), a permit is hereby issued to import, transport and stock in the Province of the Western Cape for the under-mentioned live fishes:

Details		
Permit/Licence No	**0060-AAA043-00056**	*Stamp:* CapeNature FAUNA • FLORA • HUNTING • CITES
Expiry Date	09/09/2017	
Date Issued	09/09/2016	
Amount Paid	R 150.00	
Reference	C Swanepoel	
File Code	1/2/3/6	

Description	Supplier	Destination
Organization	NA	NA
Person	Swanepoel Pieter	Swanepoel C Mr
ID	NA	9310315110084
Properties	Gariepdam Fish Hatchery	US Aquaculture, Welgevallen
Physical Address	NA	Experimental Farm
District	Gariep dam	Stellenbosch
Province/State	Free State	Western Cape
Country	South Africa	South Africa
Longitude	.0000	.0000
Latitude	.0000	.0000

Species(Scientific Name)	Qty	Note
Barbel(Clarias gariepinus)	1 600	Note special conditions, conditions apply.

Issued by:	Approved on Behalf CEO	Effective Date	Signature of Holder
Zimkitha Dwangu	Western Cape Nature Conservation Board	09/09/2016	I acknowledge, accept and understand fully the permit conditions as described

09/09/2016 08:33:25 software by: www.u-rad.com

Standard Conditions

1.This permit is valid for one year only.
2.This permit is not transferable.
3.This permit is only valid if the fish are accompanied by a valid health certificate indicating that they are disease-free.
4.This permit is only valid for the import of species listed on the Permitted List / White List.
5.This permit is not valid for the import of any freshwater invertebrates.

Special Conditions

Special conditions:-

Fish are for research purposes only and no live fish may be moved from Welgevallen Experimental farm.
All fish must be euthanized upon completion of the experimental work.
A CapeNature research permit must be applied for within 30 days of the date of issue of this import permit.

GPS coordinates - S: 33° 56' 36. 0"
E: 18° 51' 58. 62"

CHIEF EXECUTIVE OFFICER

CapeNature
FAUNA • FLORA • HUNTING • CITES

D. Feed ingredient information

Table 18 South African feed ingredient suppliers

Feed ingredient	Supplier	Details				
		Contact Person	**Phone**	**E-mail**	**Price**	**Location**
Fish meal	I&J	Stephanie J	0214407220	StephanieJ@ij.co.za	R22/kg	Cape Town (WC)
Extruded Maize	Equifeeds	Cornell L	0219751910	cornell@afreshbrands.co.za	R5.96/kg	Fisantekraal (WC)
Soya oilcake	FeedPharm	Esterlyne P	0218530393	feedpharmproduction2@xsinet.co.za	R9.73/kg	Strand (WC)
Fish oil	Energy Oil	Aldo	0219751910	N/A	Donation	Blackheath (WC)
Soya oil	Rein Oil	Melani S	0218454418	info@reinoil.co.za	R19/L	Strand (WC)
MCDP	SAFP	Pieter l R	0760202086	Pieterlr@safp.co.za	Donation	Christiana (NW)
DCP	Biominerale	Paul B	0132467451	paul.britz@biominerale.co.za	Donation	Wonderfontein (MP)
Min/Vit Premix	Advit	Frances K	0764124876	frances@advit.co.za	R49.51/kg	N/A
Cellulose	Carbocraft	Suzaan v N	0112340675	suzaanv@carbocraft.co.za	Donation	Johannesburg (GP)
Inert marker	Sigma	N/A	0119791188	rsa@sial.com	N/A	N/A
Raw materials						
Monkfish heads	I&J	Pheobius M	0781321386	PheobiusM@ij.co.za	Donation	Cape Town

E. Detailed Methodologies and protocols

Protocol for crude protein determination in feed and body tissue: Total Kjeldahl Nitrogen (TKN) method

References: VELP Distillation Unit User Manual, AOAC 979.09

Requirements:

- VELP Digester, Distillation Unit, SMS scrubber
- Catalyst Tabs ($CaSO_4 \cdot 5H_2O$ and K_2SO_4)
- Concentrated Sulphuric acid 96-98% (H_2SO_4), Sodium hydroxide (NaOH), Hydrochloric acid (HCL), Boric acid (H_3BO_3), 95% ethanol (C_2H_6O), methyl red, bromocresol green

Solution preparation:

Sodium hydroxide: 35% w/v preparation by dissolving 350g NaOH pellets (analytical grade) in 1000ml distilled water (MilliQ water can also be used). The solution is a stock solution with the VELP Distillation Unit, with a tube connecting the solution.

Boric acid solution: 4% w/v preparation by dissolving 20g boric acid powder in 500 ml distilled water (MilliQ water can also be used). Use automatic stirrer, as boric acid powder dose not dissolve easily. Note: Solubility limit in room temperature water is 4.7% w/v.

Titrant solution: 0.2 N HCL preparation by adding 10 ml of concentrated hydrochloric acid in a 250 ml volumetric flask and fill up to the 250 ml mark with distilled water. Note: The make-up accuracy of this solution will have a significant influence on the assay accuracy and repeatability.

Indicator solution: Dissolve 0.2 g methyl red in 100 ml 95% ethanol. Dissolve 1g bromocresol green in 500 ml 95% ethanol. Combine both solutions (1:5 make-up) as the working solution.

Note: Always add one blank, without any sample. Caution with sulphuric acid, use appropriate procedures and PPE.

Procedure: Prepare sample by grinding the sample to a powder to ensure homogeneity. Weight off 1.00 g (±0.1mg accuracy and note value) of sample

(tissue/feed ingredient etc.) in a digestion tube, add two catalyst tubes and 12 ml of concentrated sulphuric acid and shake gently. Digest tubes in VELP Digester for 60 minutes at 420 °C. After digestion, remove tubes from heating block at let the solution cool to 50-60°C. Place tube in VELP Distillation unit, with settings: time = 5 minutes and NaOH = 50 ml. Ensure sufficient water and NaOH stock for distillation unit. Before starting distillation, place a 250ml Erlenmeyer flask with 30ml boric acid solution as trapping solution. After distillation, add indicator titrate the solution with 0.2N HCL solution until a clear endpoint (first trace of pink in solution). Calculate the approximate protein content using the 6.25 conversion factor and percentage nitrogen,

$$\% \text{ Nitrogen} = \frac{1.4007 \times (V_{HCL,\ sample} - V_{HCL,blank})}{W_{sample}} \times C_{HCL} \qquad (7)$$

where:
$V_{HCL,\ sample}$ = the titrant volume of the sample (ml)
$V_{HCL,blank}$ = the titrant volume of the blank (ml)
W_{sample} = the total weight of the sample (g)
C_{HCL} = concentration of the titrant HCL (mole.l^{-1})

Protocol for chromium oxide analysis in African catfish (Clarias gariepinus) feed and faeces analysis

References: Divakaran et al. (2002), Furukawa (1966), Kimura and Miller (1957)

Requirements:

- *BioTek ELx800* (Microplate reader) to measure at 340 nm with 96-well microplates (flat bottomed)
- Sodium Molybdate ($Na_2MoO_4 \cdot 2H_2O$), Concentrated Sulphuric acid (H_2SO_4), 70% Perchloric acid ($HCLO_4$)
- 250 ml, 100 ml, 25 ml volumetric flasks
- Sample preparation: Dried at 66°C overnight, ground and sieved to pass through 0.42 mm mesh

Reagent Preparation:

Sodium molybdate reagent:

Dissolve 5 g of sodium molybdate ($Na_2MoO_4 \cdot 2H_2O$) in 75 ml of distilled water, and add 75 ml of concentrated sulphuric acid (H_2SO_4). Cool to room temperature and add 100 ml of 70% perchloric acid ($HCLO_4$). Can be stored to up to two weeks in glass stopper flask (250 ml).

Sample oxidation:

Add a known amount of feed/faeces (200-220 mg) in a 100 ml volumetric flask with 3 ml of molybdate reagent. Heat the tube until boiling, and fumes subsidised for a minimum of 3 minutes. The solution will become yellow due to the oxidation of chromic oxide to monochromate. Take care for charred particles not to stick to the side of the tube. Let the solution cool to room temperature and add 3 ml of 70% perchloric acid to repeat the process for 10 minutes. The flask is rinsed repeatedly with distilled water to up to 25 ml into a volumetric flask.

Note: Caution with perchloric acid, use appropriate methods and PPE.

A known weight of chromic oxide (3 mg or 1 ml of 3 mg/ml solution) treated to a similar manner to create a standard with a final concentration of 120 µg/ml. Also, add blank feed/faeces without any chromic oxide. Prepare standards in Eppendorf tubes.

Table 19 Preparation of solutions for standard curve during chromium oxide assay

Sample	Concentration (µg/ml)	Dilution factor	Standard volume (µl)	Dilution volume (µl)	Total volume (µl)
1	0	N/A	N/A	1500	1500
2	2	60	25	1475	1500
3	4	30	50	1450	1500
4	8	15	100	1400	1500
5	12	10	150	1350	1500
6	16	7.5	200	1300	1500
7	20	6	250	1250	1500
8	40	3	500	1000	1500
9	60	2	750	750	1500
10	120	N/A	1500	N/A	1500

Protocol to determine lysozyme activity in blood serum

References: Goosen et al. (2014b), Sankaran and Gurnani (1972)

Requirements:

- *BioTek ELx800* (Microplate reader) to measure at 490 nm with 96-well microplates (flat bottomed) and microplate shaker (DLAB-MXM)
- Potassium phosphate monobasic (KH_2PO_4), Sodium phosphate dibasic (Na_2HPO_4), Sodium chloride (NaCl), *Micrococcus lysodeiticus*, Lysozyme from chicken egg white

Reagent/solution preparation:

0.05 M Buffer solution: Dissolve 6.8045g KH_2PO_4 and 7.098g Na_2HPO_4 in 1l distilled water, and adjust the pH to 6.2

Bacteria solution (new preparation for each assay): Add 0.75mg of Micrococcus lysodeiticus to 100ml of buffer solution. Keep stirred on an automatic stirrer and hotplate at 25°C.

Lysozyme standard: Prepare 0.85 %w/v NaCl solution and add 200 mg lysozyme to 100 ml in a volumetric flask, this will result in a 2000 µg/ml solution. Also, prepare a 20 µg/ml lysozyme solution by adding 100 µl (2000 µg/ml solution) to 9900 µl 0.85 %w/v NaCl solution, which will be used to create the standard curve.

Table 20 Standard curve solution preparation during lysozyme activity assay

Sample	Concentration (µg/ml)	Standard volume (µl)	Dilution volume (µl)	Total volume (µl)
1	0	N/A	1000	1000
2	20	1000	N/A	1000
3	10	500	500	1000
4	5	250	750	1000
5	2.5	125	875	1000
6	1	50	950	1000
7	0.5	25	975	1000

Standard: 20 µg/ml lysozyme
Dilution: 0.85 %w/v NaCl

Procedure: Use 50 µl of each standard and 1:1 dilution (0.85 %w/v NaCl) of the blood serum, 250 µl of the stirred (25°C) bacteria solution in the 96-well plates. Use a

repeater pipette to add the bacteria solution, for least possible amount of time, shake the plate for 2 min and measure the absorbance at 490nm. Shake for 20 minutes and measure the absorbance again. The standard curve can be prepared as the change (Δ) in absorbance, and the results calculated with the standard curve using the change of absorbance at t = 2 min and t = 20 minutes, taking into account the 1:1 dilution.

Protocol to determine total serum protein and Immunoglobulin in blood serum with the Bradford protein assay

References: Zor and Selinger (1996), Ardó et al. (2008), Goosen et al. (2014b)

Requirements:

- *BioTek ELx800* (Microplate reader) to measure at 490 nm with 96-well microplates (flat bottomed) and microplate shaker (*DLAB-MXM*)
- Potassium phosphate monobasic (KH_2PO_4), Sodium phosphate dibasic (Na_2HPO_4), Sodium chloride (NaCl), Lysozyme from chicken egg white, Coomassie brilliant blue G-250, 95% ethanol (C_2H_6O), 85% w/v phosphoric acid (H_3PO_4), of polyethylene glycol, PEG (average molecular weight 10,000 Dalton)

Solution preparation:

Saline dilution agent: Prepare 0.85 %w/v NaCl solution with MilliQ water.

Bradford dye reagent: Dissolve 100 mg Coomassie brilliant blue G-250 in 50 ml of 95% ethanol, with a 100ml of 85% w/v phosphoric acid in a 1000ml volumetric flask. Dilute the mixture up to the 1000ml mark with MilliQ water and mix well. Filter the mixture immediately, twice through filter paper and a Buchner funnel (glass filter). The mixture will be a light brown colour after filtering, where it can then be stored at 4°C away from light (cover with aluminium foil).

12% w/v PEG solution: Add 6 g of polyethylene glycol, PEG (average molecular weight 10,000 Dalton) to 50 ml of MilliQ water.

Protein standard: Prepare 1420 µg/ml by adding 710 mg Lysozyme to 500 ml dilution agent (0.85 %w/v NaCl) as prepared.

Table 21 Standard curve solution preparation during total protein assay

Sample	Concentration (µg/ml)	Standard volume (µl)	Dilution volume (µl)	Total volume (µl)
1	0	0	1000	1000
2	21.5	20	1300	1320
3	38.4	25	900	925
4	49.0	25	700	725
5	74.7	50	900	950
6	113.3	75	865	940
7	188.8	125	815	940
8	473.3	250	500	750
9	946.7	500	250	750
10	1420	1000	0	1000

Standard: 1420 µg/ml lysozyme
Dilution: 0.85% w/v NaCl

Procedure for total protein: Dilute blood serum in Eppendorf tubes, by adding 20 µl to 1500 µl dilution agent; invert the tubes twice to ensure mixing. Using 96-well microplates add 50 µl of diluted serum/standard in triplicates to the plate and 200 µl Bradford dye reagent. Measure the absorbance at 450nm and 630nm. The ratio 630nm/450nm in absorbance used to plot a standard curve and calculate the total serum protein values.

Procedure for Immunoglobulin assay: Add 100 µl of blood serum with 100 µl of 12% w/v PEG solution in an Eppendorf tube, incubate at room temperature for 2 hours and then centrifuge for 10 minutes at 14,000 rpm. Use the supernatant content of the tube to measure the total protein taking into account the 1:1 dilution of the PEG solution.

F. Raw Data and Statistical Analysis

Mean weight per tank between samplings

Table 22 Mean weight of animals in each tank (0-4 weeks)

Weight (g)	Sampling 0 (0 weeks)						Sampling 1 (4 weeks)					
Replicate	1	2	3	4	5	6	1	2	3	4	5	6
Control	1.19	1.22	0.89	0.89	0.79	0.78	4.91	4.46	3.56	4.24	4.24	4.08
FPH-1	1.29	1.05	0.88	0.83	0.86	0.95	4.17	5.16	4.07	3.77	3.87	3.79
FPH-2	1.41	1.05	0.87	0.91	0.90	0.88	4.13	3.91	3.74	4.45	3.67	4.44
FPH-3	1.94	0.92	0.90	1.01	0.90	0.83	4.08	4.26	3.72	4.06	3.89	2.85
FPH-4	1.13	0.95	0.89	0.87	0.82	0.82	5.02	4.41	3.86	3.65	3.11	2.69
FB-6	1.61	0.95	1.01	0.95	0.87	0.82	5.05	4.40	3.88	3.34	3.38	3.56
DCP-7	1.16	0.91	1.02	-	0.84	0.84	3.92	3.96	4.13	-	2.20	3.55
DCP-8	1.00	0.92	0.98	0.84	0.93	1.00	3.79	5.16	4.08	3.06	3.36	4.12

Table 23 Mean weight of animals in each tank (7-10 weeks)

Weight (g)	Sampling 2 (7 weeks)						Sampling 3 (10 weeks)					
Replicate	1	2	3	4	5	6	1	2	3	4	5	6
Control	13.29	11.68	9.89	11.69	12.23	12.65	29.35	31.78	27.38	33.67	32.56	32.23
FPH-1	13.33	15.87	11.56	11.56	12.67	11.56	35.15	36.36	31.75	25.40	31.15	34.20
FPH-2	11.55	11.31	12.85	12.59	11.23	11.67	26.33	25.65	33.18	31.74	27.57	29.47
FPH-3	11.20	11.38	11.60	11.11	12.01	10.65	27.46	26.36	28.16	25.37	31.03	31.09
FPH-4	14.41	12.90	11.74	13.43	11.29	10.89	31.80	31.66	32.19	31.74	33.55	30.89
FB-6	14.39	11.47	10.95	10.27	9.86	12.17	34.89	28.89	30.65	29.39	26.74	28.56
DCP-7	10.32	10.96	12.90	-	11.80	11.21	26.85	26.36	38.51	-	32.26	32.98
DCP-8	11.96	15.37	12.29	10.40	11.13	11.21	31.16	31.47	32.09	29.27	30.97	28.19

Means length per tank between samplings

Table 24 Mean length of animals in each tank (4-10 weeks)

Length (mm)	Sampling 1 (3 weeks)						Sampling 2 (7 weeks)						Sampling 3 (10 weeks)					
Replicate	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6
Control	88	90	82	84	86	83	130	124	118	123	124	128	164	168	158	167	166	166
FPH-1	86	91	85	84	82	83	129	134	123	119	126	123	173	173	163	156	165	169
FPH-2	86	84	82	87	83	89	123	124	126	126	121	124	157	157	169	166	162	160
FPH-3	85	87	85	84	83	76	118	124	125	120	124	119	158	156	161	152	164	160
FPH-4	91	86	84	82	78	74	133	128	122	132	123	118	168	167	165	167	169	164
FB-6	91	88	84	81	81	81	131	123	122	117	117	124	171	162	166	160	157	161
DCP-7	85	84	86	84	-	82	118	122	128	-	123	126	157	157	174	-	165	168
DCP-8	84	89	85	76	80	86	126	133	126	117	122	121	167	165	167	158	164	161

Total feed fed over trial between samplings

Table 25 Total amount of feed fed over 0-4 weeks in each tank

Feed (g)	Sampling 0-1 (0-4 weeks)					
Replicate	1	2	3	4	5	6
Control	81.24	80.68	80.31	82.62	81.50	80.80
FPH-1	81.56	81.02	80.64	80.20	80.19	81.04
FPH-2	81.62	79.75	78.12	80.65	80.44	81.69
FPH-3	82.49	82.21	79.87	80.47	82.12	79.75
FPH-4	80.43	81.53	81.56	80.80	81.28	80.49
FB-6	82.36	80.90	79.99	82.89	83.18	79.69
DCP-7	80.17	80.97	80.51	81.72	81.00	80.66
DCP-8	79.99	82.49	81.00	79.60	80.08	82.05

Table 26 Total amount of feed fed over 4-7 weeks in each tank

Feed (g)	Sampling 1-2 (4-7 weeks)					
Replicate	1	2	3	4	5	6
Control	138.30	138.03	138.04	137.94	138.19	138.10
FPH-1	138.09	138.27	138.27	138.25	138.19	138.18
FPH-2	138.19	138.12	138.24	138.39	138.24	138.87
FPH-3	138.35	138.24	138.08	138.21	138.11	138.20
FPH-4	138.94	138.17	138.46	138.12	138.04	138.08
FB-6	138.49	138.31	138.29	138.23	138.10	138.32
DCP-7	138.29	138.57	138.28	138.64	138.38	138.44
DCP-8	138.48	138.18	138.94	138.39	138.30	138.13

Table 27 Total amount of feed fed over 7-10 weeks in each tank

Feed (g)	Sampling 2-3 (7-10 weeks)					
Replicate	1	2	3	4	5	6
Control	287.90	292.63	290.08	290.87	289.19	288.25
FPH-1	291.19	281.44	279.77	276.82	281.69	275.63
FPH-2	286.27	289.41	286.96	284.92	285.07	283.38
FPH-3	291.87	287.37	292.00	290.46	290.24	288.51
FPH-4	288.89	288.94	286.93	285.64	290.75	290.92
FB-6	284.83	281.16	283.74	283.43	288.52	284.23
DCP-7	287.42	283.84	290.80	285.96	290.38	283.84
DCP-8	285.21	286.58	285.32	283.91	285.19	282.89

Total weight of biomass in tanks over trial

Table 28 Total weight of all animals in each tank (0-4weeks)

Weight (g)	Sampling 0 (0 weeks)						Sampling 1 (4 weeks)					
Replicate	1	2	3	4	5	6	1	2	3	4	5	6
Control	35.65	36.65	26.69	26.55	23.84	23.48	142.33	133.82	106.77	127.25	123.06	118.25
FPH-1	38.67	31.38	26.51	24.80	25.94	28.36	121.05	113.49	122.24	109.22	112.26	106.18
FPH-2	42.33	31.41	26.17	27.20	27.07	26.28	119.85	117.31	100.89	115.79	110.04	119.97
FPH-3	58.07	27.59	26.93	30.19	27.06	24.96	122.54	127.69	111.46	121.82	109.03	82.58
FPH-4	34.03	28.48	26.64	26.20	24.48	24.54	135.57	123.53	108.02	109.37	90.10	80.58
FB-6	48.38	28.48	30.28	28.64	26.02	24.67	126.21	123.06	116.46	96.74	101.41	103.12
DCP-7	34.83	27.43	30.70	30.27	25.21	25.34	117.68	114.90	119.76	111.68	63.82	95.85
DCP-8	30.14	27.62	29.41	25.13	27.98	29.99	109.79	139.33	122.43	91.92	97.38	123.62

Table 29 Total amount of feed fed over 7-10 weeks in each tank

Weight (g)	Sampling 2 (7 weeks)						Sampling 3 (10 weeks)					
Replicate	1	2	3	4	5	6	1	2	3	4	5	6
Control	318.9	315.2	276.9	303.82	318.04	291.02	675.1	667.3	629.9	639.8	683.9	612.3
FPH-1	293.2	285.7	289.1	292.71	266.08	289.08	632.7	654.4	603.2	635	623	581.4
FPH-2	300.3	282.7	282.6	302.13	303.24	291.63	658.3	666.9	630.3	634.8	634	589.4
FPH-3	302.4	307.3	290.1	311.01	288.15	234.30	658.9	685.4	647.6	634.3	620.6	528.6
FPH-4	316.9	283.8	270	281.93	259.74	250.48	667.8	633.2	643.7	603.1	570.4	586.8
FB-6	302.2	286.7	284.7	267.11	276.01	279.89	697.7	635.6	613.1	617.1	641.7	656.9
DCP-7	288.9	296	283.7	270.91	224.29	235.37	617.5	606.2	616.1	513.4	580.6	560.6
DCP-8	275	307.4	295	259.89	255.97	291.46	592.1	566.5	641.8	585.4	557.5	648.4

Total count of animals over 10-week trial

Table 30 Animal count in each tank (0-4 weeks)

Count	Sampling 0 (0 weeks)						Sampling 1 (4 weeks)					
Replicate	1	2	3	4	5	6	1	2	3	4	5	6
Control	30	30	30	30	30	30	29	30	30	30	29	29
FPH-1	30	30	30	30	30	30	29	22	30	29	29	28
FPH-2	30	30	30	30	30	30	29	30	27	26	30	27
FPH-3	30	30	30	30	30	30	30	30	30	30	28	29
FPH-4	30	30	30	30	30	30	27	28	28	30	29	30
FB-6	30	30	30	30	30	30	25	28	30	29	30	29
DCP-7	30	30	30		30	30	30	29	29		29	27
DCP-8	30	30	30	30	30	30	29	27	30	30	29	30

Table 31 Animal count in each tank (7-10 weeks)

Count	Sampling 2 (7 weeks)						Sampling 3 (10 weeks)					
Replicate	1	2	3	4	5	6	1	2	3	4	5	6
Control	24	27	28	26	26	23	23	21	23	19	21	19
FPH-1	22	18	25	25	21	25	18	18	19	25	20	17
FPH-2	26	25	22	24	27	25	25	26	19	20	23	20
FPH-3	27	27	25	28	24	22	24	26	23	25	20	17
FPH-4	22	22	23	21	23	23	21	20	20	19	17	19
FB-6	21	25	26	26	28	23	20	22	20	21	24	23
DCP-7	28	27	22		19	21	23	23	16		18	17
DCP-8	23	20	24	25	23	26	19	18	20	20	18	23

Production performance parameters of FPH diets

Table 32 Initial mean weight for FPH diets with statistical analysis

Mean Wi (g)	Mean initial weight for FPH treatments							
Replicate	1	2	3	4	5	6	Mean	±SE
Control	1.19	1.22	0.89	0.89	0.79	0.78	0.96	0.08
FPH-1	1.29	1.05	0.88	0.83	0.86	0.95	0.98	0.07
FPH-2	1.41	1.05	0.87	0.91	0.90	0.88	1.00	0.09
FPH-3	1.94	0.92	0.90	1.01	0.90	0.83	1.08	0.17
FPH-4	1.13	0.95	0.89	0.87	0.82	0.82	0.91	0.05

F(4, 25) = 0.38509, p = 0.81719

Table 33 Final mean weight for FPH diets with statistical analysis

Mean Wf (g)	Mean final weight for FPH treatments after 10-weeks							
Replicate	1	2	3	4	5	6	Mean	±SE
Control	29.35	31.78	27.38	33.67	32.56	32.23	31.16	0.95
FPH-1	35.15	36.36	31.75	25.40	31.15	34.20	32.33	1.61
FPH-2	26.33	25.65	33.18	31.74	27.57	29.47	28.99	1.23
FPH-3	27.46	26.36	28.16	25.37	31.03	31.09	28.24	0.97
FPH-4	31.80	31.66	32.19	31.74	33.55	30.89	31.97	0.36

F(4, 25) = 2.7382, p = 0.05124

Table 34 Weight gain for FPH diets with statistical analysis

WG	Weight gain for FPH treatments after 10-weeks							
Replicate	1	2	3	4	5	6	Mean	±SE
Control	23.70	25.01	29.78	37.05	39.98	40.17	32.62	3.04
FPH-1	26.27	33.76	34.93	29.73	35.03	35.18	32.48	1.50
FPH-2	17.66	23.50	37.03	34.01	29.55	32.64	29.07	2.95
FPH-3	13.18	27.66	30.37	24.21	33.40	36.37	27.53	3.35
FPH-4	27.03	32.35	35.25	35.34	40.12	36.76	34.47	1.81

F(4, 25) = 1.1651, p = 0.34990

Table 35 Specific growth rate for FPH diets with statistical analysis

SGR (%.day^{-1})	Specific growth rate over 10 weeks for FPH treatments							
Replicate	1	2	3	4	5	6	Mean	±SE
Control	4.58	4.66	4.90	5.20	5.30	5.31	4.99	0.13
FPH-1	4.72	5.07	5.12	4.89	5.12	5.13	5.01	0.07
FPH-2	4.18	4.57	5.20	5.08	4.88	5.02	4.82	0.16
FPH-3	3.79	4.79	4.92	4.61	5.05	5.17	4.72	0.20
FPH-4	4.76	5.01	5.13	5.13	5.31	5.19	5.09	0.08

F(4, 25) = 1.1850, p = 0.34158

Table 36 Feed conversion ratio for FPH diets with statistical analysis

FCR	Feed conversion ratio over 10 weeks for FPH treatments							
Replicate	1	2	3	4	5	6	Mean	±SE
Control	0.79	0.81	0.84	0.83	0.77	0.86	0.82	0.01
FPH-1	0.86	0.80	0.86	0.81	0.84	0.89	0.85	0.01
FPH-2	0.82	0.80	0.83	0.83	0.83	0.89	0.83	0.01
FPH-3	0.85	0.77	0.82	0.84	0.86	1.01	0.86	0.03
FPH-4	0.80	0.84	0.82	0.87	0.93	0.91	0.86	0.02

F(4, 25) = 0.73741, p = 0.57528

Table 37 Condition factor for FPH diets with statistical analysis

CF	Condition Factor over 10 weeks for FPH treatments							
Replicate	1	2	3	4	5	6	Mean	±SE
Control	0.67	0.67	0.70	0.72	0.71	0.71	0.70	0.01
FPH-1	0.68	0.71	0.73	0.67	0.69	0.71	0.70	0.01
FPH-2	0.68	0.66	0.69	0.70	0.65	0.71	0.68	0.01
FPH-3	0.69	0.69	0.68	0.73	0.70	0.76	0.71	0.01
FPH-4	0.67	0.69	0.72	0.69	0.70	0.71	0.70	0.01

F(4, 25) = 0.93570, p = 0.45942

Non-specific immunity parameters for FPH diets

Table 38 Lysozyme activity for FPH diets with statistical analysis

Lysozyme activity ($\mu g.ml^{-1}$)	Lysozyme activity after 10 weeks for FPH treatments							
Replicate	1	2	3	4	5	6	Mean	±SE
Control	6.8	7.8	7.3	7.9	7.7	6.8	7.4	0.19
FPH-1	-	7.7	7.0	-	7.0	7.3	7.3	0.14
FPH-2	-	7.4	6.9	7.7	7.7	7.2	7.4	0.13
FPH-3	-	7.2	-	7.5	7.3	8.1	7.5	0.18
FPH-4	-	6.8	-	6.9	7.3	7.2	7.0	0.10

F(4, 18)=0.98039, p = 0.44284

Table 39 Immunoglobulin in blood serum for FPH diets with statistical analysis

Immunoglobulin ($mg.ml^{-1}$)	Immunoglobulin after 10 weeks for FPH treatments							
Replicate	1	2	3	4	5	6	Mean	±SE
Control	13.16	6.24	0.44	0.08	-	-	4.94^{ab}	2.68
FPH-1	3.57	0.09	0.90	1.31	5.00	-	2.17^{a}	0.82
FPH-2	-	20.72	27.19	-	17.03	0.55	16.37^{c}	4.92
FPH-3	-	14.04	14.20	-	26.89	4.90	15.01^{bc}	3.91
FPH-4	-	0.18	2.26	-	5.05	-	2.50^{a}	1.15

F(4, 15)=3.7457, p = 0.02634

Different superscripts (e.g. ^{a}, ^{b}) in the same column indicate what means are significant different ($p<0.05$)

Table 40 Total serum protein levels for FPH diets with statistical analysis

Total Protein ($mg.ml^{-1}$)	Total serum protein after 10 weeks for FPH treatments							
Replicate	1	2	3	4	5	6	Mean	±SE
Control	31.3	28.8	31.3	25.6	21.7	23.3	27.0	1.53
FPH-1	30.8	30.7	30.1	27.5	26.5	26.4	28.7	0.78
FPH-2	-	32.6	42.0	15.2	35.2	23.9	29.8	4.16
FPH-3	26.4	37.9	28.4	24.7	54.5	27.4	33.2	4.24
FPH-4	37.6	30.9	31.8	24.9	32.1	-	31.5	1.81

F(4, 23)=0.62363, p = 0.65033

Whole body composition analysis of FPH diets

Table 41 Approximate moisture composition for FPH diets with statistical analysis

Moisture ($g.kg^{-1}$)	Moisture content 10 weeks for FPH treatments							
Replicate	1	2	3	4	5	6	Mean	±SE
Control	708	710	710	711	714	724	713^{a}	2.31
FPH-1	691	702	700	704	705	698	700^{b}	2.08
FPH-2	698	709	703	704	706	706	704^{b}	1.49
FPH-3	701	694	707	714	702	708	704^{b}	2.84
FPH-4	709	701	701	684	700	706	700^{b}	3.49

F(4, 25)=4.1659, p = 0.01013

Different superscripts (e.g. ^{a}, ^{b}) in the same column indicate what means are significant different (p<0.05)

Table 42 Approximate crude protein composition for FPH diets with statistical analysis

Crude Protein ($g.kg^{-1}$)	Crude protein after 10 weeks for FPH treatments							
Replicate	1	2	3	4	5	6	Mean	±SE
Control	219	223	218	207	231	223	220	3.30
FPH-1	186	221	222	207	200	222	210	6.03
FPH-2	213	214	206	219	212	221	214	2.11
FPH-3	212	215	216	211	216	216	214	1.04
FPH-4	217	199	206	227	204	228	213	5.13

F(4, 25)=0.90673, p = 0.47513

Table 43 Approximate crude lipid composition for FPH diets with statistical analysis

Crude Lipid ($g.kg^{-1}$)	Crude lipid after 10 weeks for FPH treatments							
Replicate	1	2	3	4	5	6	Mean	±SE
Control	247	252	243	253	250	247	249^{a}	1.55
FPH-1	300	272	271	275	255	283	276^{b}	6.02
FPH-2	257	274	255	259	242	254	257^{ac}	4.14
FPH-3	261	251	258	271	224	266	255^{ac}	6.76
FPH-4	266	271	274	261	284	286	272^{b}	4.98

F(4, 25)=5.4525, p = 0.00269

Different superscripts (e.g. ^{a}, ^{b}) in the same column indicate what means are significant different (p<0.05)

Table 44 Approximate ash composition for FPH diets with statistical analysis

Ash ($g.kg^{-1}$)	Ash content after 10 weeks for FPH treatments							
Replicate	1	2	3	4	5	6	Mean	±SE
Control	102	114	119	112	120	111	113	2.60
FPH-1	101	121	106	109	121	122	113	3.67
FPH-2	106	115	105	120	122	108	113	3.10
FPH-3	115	106	115	124	132	129	120	4.03
FPH-4	127	122	107	119	113	121	118	2.81

$F(4, 25)=1.0712$, $p = 0.39158$

Production performance parameters of FPH diets during 2-week digestibility trial

Table 45 Mean initial weight of FPH diets during 2-week digestibility trial

Mean Wi (g)	Mean initial weight for FPH treatments					
Replicate	1	2	3	4	Mean	±SE
Control	56.99	59.95	69.72	60.34	61.75	2.76
FPH-1	60.62	-	-	56.51	58.56	2.05
FPH-2	64.78	58.58	59.81	68.94	63.03	2.38
FPH-3	63.65	50.38	61.74	55.12	57.72	3.05
FPH-4	63.64	56.33	62.89	60.59	60.86	1.64

F(4, 13) = 0.73535, p = 0.58412

Table 46 Mean final weight of FPH diets during 2-week digestibility trial

Mean Wf (g)	Mean final weight for FPH treatments after 2-weeks					
Replicate	1	2	3	4	Mean	±SE
Control	88.50	102.12	94.69	102.97	97.07[a]	3.41
FPH-1	95.46	-	-	89.85	92.65[a]	2.80
FPH-2	112.71	97.02	109.92	104.31	105.99[b]	3.46
FPH-3	97.78	87.33	97.38	87.67	92.54[a]	2.91
FPH-4	96.96	99.31	99.45	96.26	98.00[ab]	0.81

F(4, 13) = 3.4533, p = 0.03933
Different superscripts (e.g. [a], [b]) in the same column indicate what means are significant different (p<0.05)

Table 47 Weight gain of FPH diets during 2-week digestibility trial

WG	Weight gain for FPH treatments after 2-weeks					
Replicate	1	2	3	4	Mean	±SE
Control	0.55	0.70	0.36	0.71	0.58	0.08
FPH-1	0.57	-	-	0.59	0.58	0.01
FPH-2	0.74	0.66	0.84	0.51	0.69	0.07
FPH-3	0.54	0.73	0.58	0.59	0.61	0.04
FPH-4	0.52	0.76	0.58	0.59	0.61	0.05

F(4, 13) = 0.46523, p = 0.76026

Table 48 Specific growth rate of FPH diets during 2-week digestibility trial

SGR (%.day^{-1})	Specific growth rate over 2 weeks for FPH treatments					
Replicate	1	2	3	4	Mean	±SE
Control	3.39	4.10	2.36	4.11	3.49	0.41
FPH-1	3.49	-	-	3.57	3.53	0.04
FPH-2	4.26	3.88	4.68	3.19	4.00	0.32
FPH-3	3.30	4.23	3.51	3.57	3.65	0.20
FPH-4	3.24	4.36	3.53	3.56	3.67	0.24

F(4, 13) = 0.44709, p = 0.77276

Table 49 Feed conversion ratio of FPH diets during 2-week digestibility trial

FCR	Feed conversion ratio over 2 weeks for FPH treatments					
Replicate	1	2	3	4	Mean	±SE
Control	1.18	0.64	1.10	1.21	1.03	0.13
FPH-1	1.30	-	-	0.81	1.05	0.25
FPH-2	0.71	0.78	1.27	7.58	2.59	1.67
FPH-3	1.41	0.96	0.83	1.14	1.08	0.13
FPH-4	1.19	0.98	0.87	0.80	0.96	0.08

F(4, 13) = 0.72318, p = 0.59145

Table 50 Condition Factor of FPH diets during 2-week digestibility trial

CF	Condition Factor over 2 weeks for FPH treatments					
Replicate	1	2	3	4	Mean	±SE
Control	0.78	0.74	0.77	0.76	0.76^{a}	0.01
FPH-1	0.76	-	-	0.73	0.74^{a}	0.02
FPH-2	0.80	0.80	0.81	0.76	0.79^{b}	0.01
FPH-3	0.78	0.75	0.73	0.75	0.75^{a}	0.01
FPH-4	0.77	0.76	0.77	0.76	0.76^{a}	0.00

F(4, 13) = 3.8195, p = 0.02888

Digestibility coefficients of 2-week digestibility trial for FPH diets

Table 51 Dry matter digestibility coefficients of FPH diets during 2-week digestibility trial

		Weight (mg)	Absorbance 340 nm	Concentration (ug/ml)	Cr2O3 (ug/mg)	ADC	Mean	SE
Feeds	Control	195.6	0.909	58.67	7.499			
	FPH-1	198.3	0.868	55.47	6.993			
	FPH-2	227.2	0.932	60.45	6.652			
	FPH-3	200.0	0.779	48.46	6.057			
	FPH-4	187.5	0.906	58.43	7.791			
Faces								
1	Control	139.0	1.100	73.71	13.257	43.4%		
2	Control	71.7	0.928	60.17	20.978	64.3%		
3	Control	132.6	1.150	77.67	14.644	48.8%		
4	Control	136.1	1.371	95.07	17.464	57.1%	53.4%	4.58%
1	FPH-1	98.4	1.112	74.65	18.967	63.1%		
2	FPH-1	210.4	1.575	111.08	13.199	47.0%		
3	FPH-1	96.5	1.073	71.56	18.538	62.3%		
4	FPH-1	40.1	0.773	47.93	29.884	76.6%	62.3%	6.05%
1	FPH-2	188.1	1.116	74.94	9.960	33.2%		
2	FPH-2	103.6	0.960	62.69	15.127	56.0%		
3	FPH-2	56.9	0.878	56.20	24.693	73.1%		
4	FPH-2	56.2	0.726	44.23	19.677	66.2%	57.1%	8.70%
1	FPH-3	73.9	0.806	50.56	17.104	64.6%		
2	FPH-3	163.4	0.944	61.43	9.398	35.5%		
3	FPH-3	51.3	0.752	46.28	22.554	73.1%		
4	FPH-3	113.0	1.109	74.44	16.470	63.2%	59.1%	8.16%
1	FPH-4	109.0	0.971	63.55	14.576	46.5%		
2	FPH-4	138.5	1.067	71.14	12.841	39.3%		
3	FPH-4	88.5	1.094	73.24	20.688	62.3%		
4	FPH-4	76.7	1.021	67.49	21.997	64.6%	53.2%	6.12%

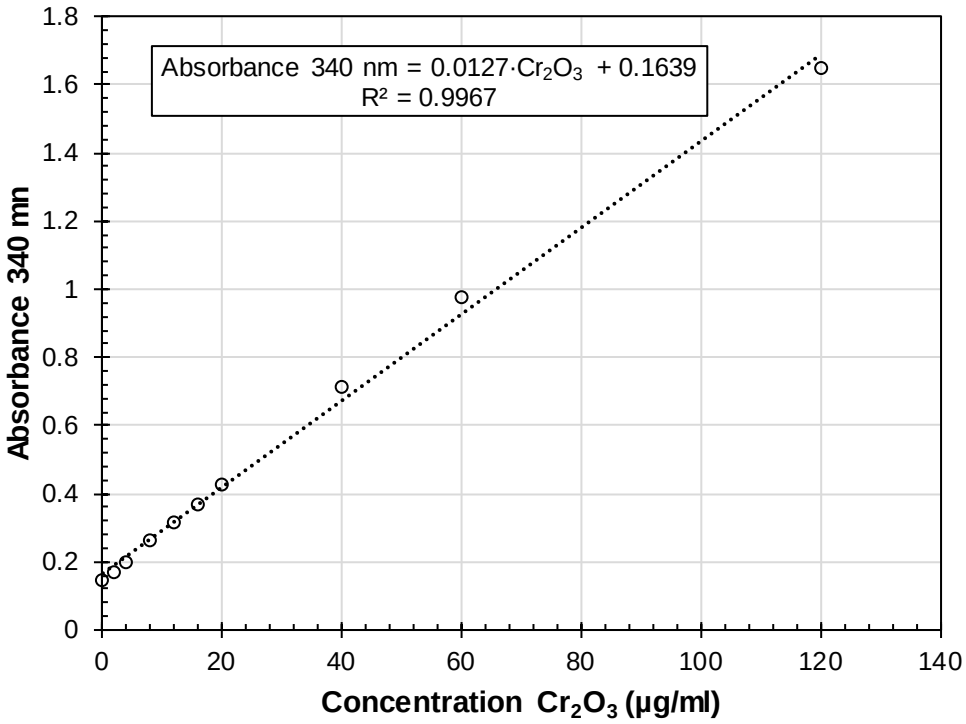

Figure 9 Standard curve for chromium oxide assay

Production performance parameters of DCP diets

Table 52 Mean initial weight for DCP diets with statistical analysis

Mean Wi (g)	Mean initial weight for DCP treatments							
Replicate	1	2	3	4	5	6	Mean	±SE
Control	1.19	1.22	0.89	0.89	0.79	0.78	0.96	0.08
FB-5	1.61	0.95	1.01	0.95	0.87	0.82	1.04	0.12
DCP-6	1.16	0.91	1.02	-	0.84	0.84	0.96	0.06
DCP-7	1.00	0.92	0.98	0.84	0.93	1.00	0.95	0.03

F(3, 19) = 0.27201, p = 0.8448

Table 53 Mean final weight for DCP diets with statistical analysis

Mean Wf (g)	Mean final weight for DCP treatments after 10-weeks							
Replicate	1	2	3	4	5	6	Mean	±SE
Control	29.35	31.78	27.38	33.67	32.56	32.23	31.16	0.95
FB-5	34.89	28.89	30.65	29.39	26.74	28.56	29.85	1.13
DCP-6	26.85	26.36	38.51	-	32.26	32.98	31.39	2.24
DCP-7	31.16	31.47	32.09	29.27	30.97	28.19	30.53	0.60

F(3,19) = 0.229234, p = 0.8304

Table 54 Weight gain for DCP diets with statistical analysis

WG	Weight gain for DCP treatments after 10-weeks							
Replicate	1	2	3	4	5	6	Mean	±SE
Control	23.70	25.01	29.78	37.05	39.98	40.17	32.62	3.04
FB-5	20.63	29.43	29.37	29.78	29.83	33.73	28.80	1.77
DCP-6	22.12	27.83	36.63	-	37.38	38.04	32.40	3.17
DCP-7	30.02	33.18	31.73	33.94	32.21	27.20	31.38	1.00

F(3,19) = 0.56974, p = 0.64172

Table 55 Specific growth rate for DCP diets with statistical analysis

SGR (%.day^{-1})	Specific growth rate over 10 weeks for DCP treatments							
Replicate	1	2	3	4	5	6	Mean	±SE
Control	4.58	4.66	4.90	5.20	5.30	5.31	4.99	0.13
FB-5	4.39	4.88	4.88	4.90	4.90	5.07	4.83	0.09
DCP-6	4.49	4.80	5.18	-	5.21	5.24	4.98	0.15
DCP-7	4.91	5.05	4.98	5.08	5.00	4.77	4.96	0.05

F(3, 19) = 0.46567, p = 0.70963

Table 56 Feed conversion ratio for DCP diets with statistical analysis

FCR	Feed conversion ratio over 10 weeks for DCP treatments							
Replicate	1	2	3	4	5	6	Mean	±SE
Control	0.79	0.81	0.84	0.83	0.77	0.86	0.82[a]	0.01
FB-5	0.78	0.82	0.86	0.86	0.83	0.79	0.82[a]	0.01
DCP-6	0.87	0.87	0.87	-	0.92	0.94	0.89[b]	0.01
DCP-7	0.90	0.94	0.83	0.90	0.95	0.81	0.89[b]	0.02

F(3, 19) = 5.3551, p = 0.00764
Different superscripts (e.g. [a], [b]) in the same column indicate what means are significant different (p<0.05)

Table 57 Condition factor for DCP diets with statistical analysis

CF	Condition Factor over 10 weeks for DCP treatments							
Replicate	1	2	3	4	5	6	Mean	±SE
Control	0.67	0.67	0.70	0.72	0.71	0.71	0.70	0.01
FB-5	0.70	0.69	0.67	0.71	0.69	0.68	0.69	0.01
DCP-6	0.70	0.69	0.73	-	0.72	0.70	0.70	0.01
DCP-7	0.67	0.70	0.69	0.74	0.70	0.67	0.70	0.01

F(3, 19) = 0.35069, p = 0.78910

Non-specific immunity parameters for DCP diets

Table 58 Lysozyme activity for DCP diets with statistical analysis

Lysozyme activity ($\mu g.ml^{-1}$)	Lysozyme activity after 10 weeks for DCP treatments							
Replicate	1	2	3	4	5	6	Mean	±SE
Control	6.8	7.8	7.3	7.9	7.7	6.8	7.4	0.19
FB-5	6.7	6.9	6.8	6.8	7.0	6.5	6.8	0.06
DCP-6	6.6	6.6	8.2	-	7.4	7.0	7.1	0.27
DCP-7	7.3	7.4	6.9	7.1	6.7	7.3	7.10	0.10

F(3, 18)=2.0310, p = 0.14557

Table 59 Immunoglobulin concentration for DCP diets with statistical analysis

Immunoglobulin ($mg.ml^{-1}$)	Immunoglobulin after 10 weeks for DCP treatments							
Replicate	1	2	3	4	5	6	Mean	±SE
Control	13.16	6.24	0.44	0.08	-	-	4.94	2.68
FB-5	18.83	13.47	7.22	6.81	3.07	-	9.88	2.50
DCP-6	-	8.97	8.97	-	10.49	26.01	13.61	3.59
DCP-7	1.88	-	6.95	8.73	14.10	6.40	7.61	1.77

F(3, 14)=1.3815, p = 0.28935

Table 60 Total serum protein for DCP diets with statistical analysis

Total Protein ($mg.ml^{-1}$)	Total serum protein after 10 weeks for DCP treatments							
Replicate	1	2	3	4	5	6	Mean	±SE
Control	31.3	28.8	31.3	25.6	21.7	23.3	27.0	1.53
FB-5	30.7	28.3	27.0	25.0	26.4	29.4	27.8	0.78
DCP-6	-	30.2	30.2	-	26.0	35.6	30.5	1.71
DCP-7	21.7	-	21.6	28.4	32.6	24.6	25.8	1.88

F(3, 17)=1.2407, p = 0.32589

Whole body composition analysis of DCP diets

Table 61 Approximate moisture composition for DCP diets with statistical analysis

Moisture ($g.kg^{-1}$)	Moisture content 10 weeks for DCP treatments							
Replicate	1	2	3	4	5	6	Mean	±SE
Control	708	710	710	711	714	724	713^{a}	2.31
FB-5	700	704	707	693	700	691	699^{ab}	2.45
DCP-6	710	694	694	-	645	696	688^{b}	1.11
DCP-7	701	697	712	708	712	704	706^{a}	2.55

F(3, 19)=3.8962, p = 0.02516
Different superscripts (e.g. ^{a}, ^{b}) in the same column indicate what means are significant different ($p<0.05$)

Table 62 Approximate crude protein composition for DCP diets with statistical analysis

Crude Protein ($g.kg^{-1}$)	Crude protein after 10 weeks for DCP treatments							
Replicate	1	2	3	4	5	6	Mean	±SE
Control	219	223	218	207	231	223	220	3.30
FB-5	210	227	203	216	218	237	218	5.00
DCP-6	211	225	242	-	223	229	226	4.93
DCP-7	201	212	223	212	219	244	218	5.96

F(3, 19)=0.58237, p = 0.6338

Table 63 Approximate crude lipid for DCP diets with statistical analysis

Crude Lipid ($g.kg^{-1}$)	Crude lipid after 10 weeks for DCP treatments							
Replicate	1	2	3	4	5	6	Mean	±SE
Control	247	252	243	253	250	247	249	1.55
FB-5	272	269	254	265	264	299	270	6.21
DCP-6	266	279	279	-	277	277	275	2.29
DCP-7	261	269	261	274	251	252	261	3.63

F(3, 19)=8.6763, p = 0.00078

Table 64 Approximate ash composition for DCP diets with statistical analysis

Ash ($g.kg^{-1}$)	Ash content after 10 weeks for DCP treatments							
Replicate	1	2	3	4	5	6	Mean	±SE
Control	102	114	119	112	120	111	113	2.60
FB-5	111	98.0	114	120	140	110	115	5.71
DCP-6	130	111	111	-	122	107	116	4.28
DCP-7	120	110	102	118	117	129	116	3.77

F(3, 19)=0.11468, p = 0.395042

Mineral analysis of vertebrae

Table 65 ICP-MS results from vertebrae analysis for DCP diets

	Diet	Mineral analysis in mg.kg-1						
		Al	Ca	Mg	Mn	P	Si	Ca/P
1	**Control**	96	343271	7667	117	170062	19.6	2.019
2	**Control**	69	255736	5259	91	123995	11.5	2.062
3	**Control**	67	284142	5880	187	136063	<5	2.088
4	**Control**	87	262028	5130	82	126573	8.7	2.070
5	**Control**	62	272027	5637	133	132855	8.4	2.048
6	**Control**	106	271054	5511	92	132466	<5	2.046
1	**FB-5**	66	243276	5066	123	117676	8.7	2.067
2	**FB-5**	99	273506	5903	138	134156	16.6	2.039
3	**FB-5**	153	511900	10342	203	247798	25.6	2.066
4	**FB-5**	77	238467	5259	113	116537	<10	2.046
5	**FB-5**	61	246855	5264	141	118893	13.0	2.076
6	**FB-5**	73	230722	4704	137	111663	12.0	2.066
1	**DCP-6**	104	221933	4529	136	109369	18.2	2.029
2	**DCP-6**	62	246361	4912	143	120458	<10	2.045
3	**DCP-6**	85	249711	5073	167	119702	<10	2.086
4	**DCP-6**	-	-	-	-	-	-	-
5	**DCP-6**	51	248458	4949	154	120609	9.8	2.060
6	**DCP-6**	70	236935	4528	136	115120	12.6	2.058
1	**DCP-7**	65	249424	5146	139	123289	<5	2.023
2	**DCP-7**	71	242724	4779	132	117934	9.5	2.058
3	**DCP-7**	85	251393	4903	131	120640	13.5	2.084
4	**DCP-7**	61	249586	4806	144	119285	8.1	2.092
5	**DCP-7**	70	249456	4925	135	119271	8.06	2.091
6	**DCP-7**	88	337077	6426	189	162615	12.67	2.073

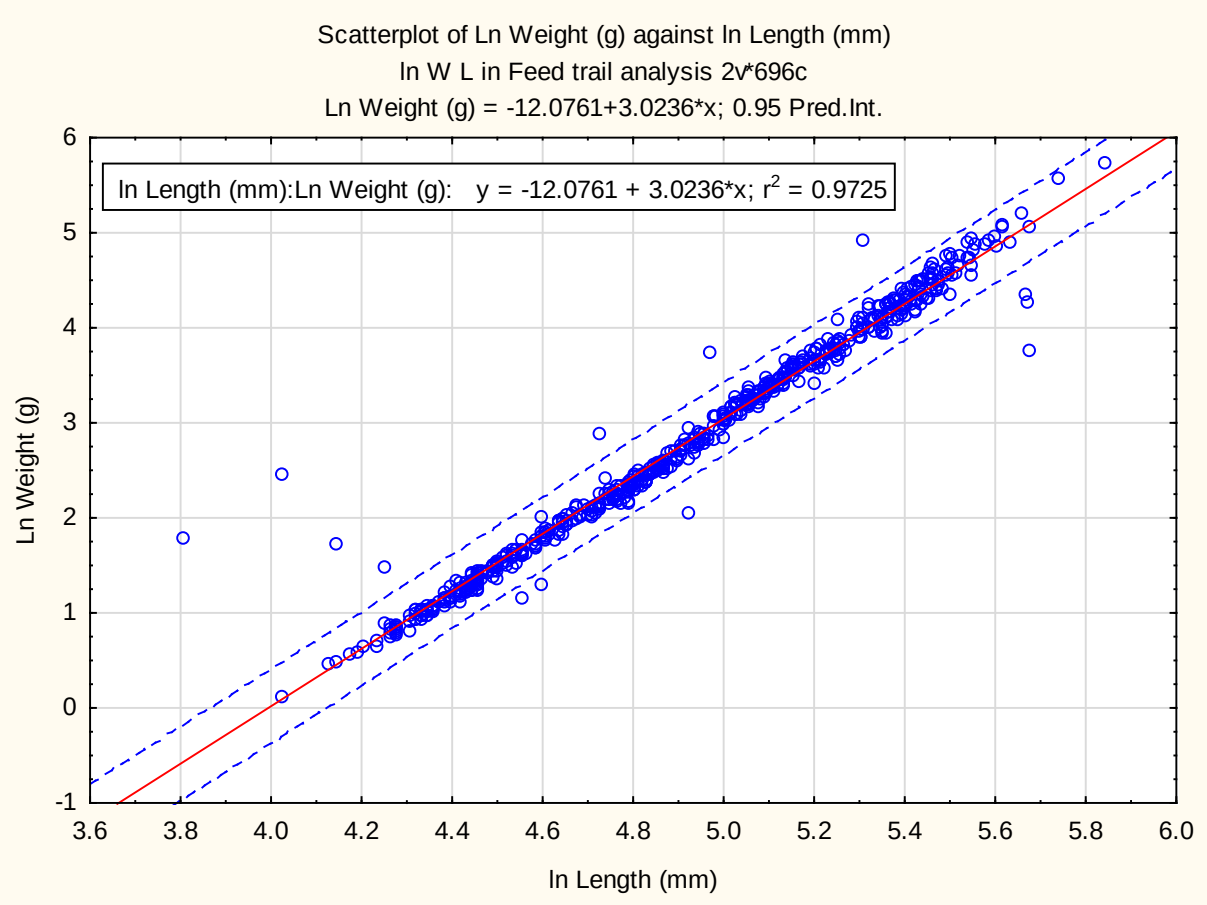

Figure 10 Statistical analysis for length-weight relationship with a 95% prediction interval