**Fortification of commercial catfish rations with superdoses of phytase enzymes increases hematological parameters and mineral stores in channel catfish (*Ictalurus punctatus*) and hybrid catfish (*Ictalurus punctatus x Ictalurus furcatus*)**

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A thesis submitted to the Graduate Faculty of

Auburn University

In partial fulfillment of the

Requirements for the degree of
Master of Science

Auburn, AL
May 07, 2017

Keywords: phytate, phytic acid, phytase, nutrition, catfish

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Thesis Abstract

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Master of Science, May 7, 2017
(B.S., Auburn University, May 9, 2015)

78 typed pages

Directed by Eric J. Peatman

Phytic acid is a well-known antinutritional factor and readily binds to di and trivalent ions reducing their bioavailability and thus absorption by the animal. Phytic acid levels in catfish diets have risen along with inclusion percentages of certain ingredients such as wheat middlings. While some of these ingredients contain phytase, the concentration of phytic acid may still inhibit mineral absorption. Accordingly, the impact of phytase superdosing (2500 FTU/kg) in a commercially available catfish diet was evaluated in channel catfish (*Ictalurus punctatus*) and hybrid catfish (channel x blue) fingerlings at the USDA-ARS in Stuttgart, AR and Auburn University. Catfish fed phytase-supplemented diets had higher mineral levels (including iron) in serum and liver along with improved hematocrit, hemoglobin, and red blood cell values. Weight gain and feed conversion ratios were also significantly improved in the 15-week replicated pond studies.

Acknowledgements

The author would like to thank Dr. Eric Peatman for his invaluable advice and guidance in all stages of this work. Furthermore, the author greatly appreciates the efforts of the staff of the USDA ARS of Stuttgart, AR and Auburn, AL as well as the staff at the EW Shell Fisheries Station for their support and assistance in completing this project, AB Vista, the Southfresh and Uniontown feed mills, and all directors, lab mates and technicians whose efforts greatly aided in the production of this work, namely: Dr. Benjamin Beck, Rashida Eljack, Lauren Davis, Taylor Brown, Dongdong Zhang, Wilson Holland and Karen Veverika. Further still, the author would like to thank Leigh Holland of Jubilee Farms and Craig Wyatt of AB Vista for their continued collaboration with projects of this lab.

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## Introduction

Aquaculture is one the fastest growing agriculture industries in the world, in fact, it has expanded faster in recent decades than any other livestock sector including poultry, pig, and cattle. Globally, in 2014, aquaculture was responsible for the production of 73.8 million metric tons of fish products, most of which was for human consumption (FAO, 2016). With the world's population projected to reach 9.7 billion people by 2050 and global capture fisheries unstable and steadily declining, the spotlight turns to aquaculture production to contribute significantly to global food security and adequate global nutrition and human health. In the United States, the commercial catfish industry is the predominant aquaculture sector. Responsible for over 50% of total aquaculture in the country, the sales value of the U.S commercial catfish industry was $386 million last year (NASS, 2016) with four states; Mississippi, Alabama, Arkansas, and Texas accounting for 96% of total sales, however, this industry is not without its barriers to growth. In the early 1990's, scarcity and rising price of fishmeal inputs forced industry feed producers to formulate a more sustainable, vegetable based diet, replacing fishmeal with meat and bone meal, cottonseed meal, and wheat middlings among other inputs. The industry peaked in 2002, and suffered greatly thereafter, decreasing in round-weight production by 49% as catfish farmers continued to go out of business and revert their ponds into corn and soybean acres (Hanson and Sites, 2011). Coincidentally, throughout this time period, catfish imports increased exponentially and now stand as the largest barrier to growth for US catfish producers. Additionally, prices for corn and soybeans rose steeply, increasing the price of feed per ton 19% from 2010 to 2011 (Hanson and Sites, 2011), and peaking at a staggering 562 USD/ton in 2012 (Hanson and Sites, 2015), and finally stabilizing around 350-400 USD/ton today (pers. comm., Alabama Catfish Feed Mill, Uniontown, AL). With high input costs and increased global competition, it becomes

imperative that fed fish are utilizing commercial catfish feeds as efficiently as possible; currently, this may not be the case. Studies have shown that phytic acid, a known antinutrient, in the diets of non-ruminants can significantly affect growth and overall health of livestock animals, through the formation of complexes with proteins and divalent cations (Dersjant-Li et al. 2014). Phytic acid is prevalent in plant matter, and especially abundant in ingredients such as cottonseed meal, wheat middlings, and soybean meal. The commercial poultry and swine industries have reported the use of phytases to aid in the destruction of phytic acid and increase bioavailability of minerals, protein, and the absorption of phosphorus (Selle and Ravindran, 2007). While these techniques have been used for decades in the latter industries, the use of phytases has been explored, or utilized relatively little in the diets of catfish. Our goal in this study is to assess the efficacy of exogenous application of a commercial phytase enzyme of bacterial origin to plant-based commercial catfish diets and observe physiological effects. The following is a review of the primary literature surrounding this topic.

## Status of Global Capture Fisheries and Aquaculture

There exist conflicting reports over the trends concerning global capture fisheries. The FAO claims in SOFIA (2016), that capture fishery production has been relatively static since the 1980's; however, the validity of this statement is in question. In a review by Pauly and Zeller (2016) commenting on the statements made by the FAO in their most recent SOFIA, he explains that catch reconstructions that have been conducted through *The Sea Around Us* over the last decade for all countries of the world, indicate that since 1996, total world catches are declining at a rate of 1.2 million metric tons per year (Pauly and Zeller, 2016). This declining trend is not reversed by the few years of reported data (2011-2014) that FAO has added compared to the *Sea Around Us* analysis covering 1950-2010. Overall, the state of the world's marine fish stocks has not improved. Based on FAO's analysis (SOFIA, 2016), the share of fish stocks within biologically sustainable levels decreased from 90% in 1974 to 68.6% in 2013. Thus, 31.4% of fish stocks were estimated as fished at a biologically unsustainable level and therefore overfished. Of the total number of stocks assessed by FAO in 2013, fully fished stocks accounted for 58.1% and under fished stocks 10.5%. The ten most productive species accounted for 27% of worlds capture fisheries production in 2013, however, most of their stocks are fully fished with no potential for increases in production and the remainders are overfished (SOFIA, 2016). This detrimental trend is supported by *The Sea Around Us* (Pauly and Zeller, 2016) and seems to validate a highly criticized publication on the disastrous effects of the loss of marine biodiversity as a result of overfishing (Worm et al. 2016).

Aquaculture has been responsible for the growth in the supply of fish for human consumption. Aquaculture provided only 7% of fish for human consumption in 1974; this share increased to 26% in 1994, and further to 39% in 2004 (SOFIA, 2016). China has played the

largest role in this impressive growth over the past several decades, as it represents more than 60% of world aquaculture production (SOFIA, 2016). It should be noted that these data are slightly misleading. While it is true that, globally, aquaculture has grown, this growth is dominated by China. Behind China, the other 'major' producers are India (6.6%), Indonesia (5.7%), Vietnam (4.6%), and Bangladesh (2.6%)(SOFIA, 2016). Thus, outside of China and the other mentioned producers (together accounting for 81.5% of total global aquaculture), aquaculture is much smaller and often not growing rapidly. In fact, many regions have declining or stagnating aquaculture over the last several years, e.g., North America (2010-2014: 1.2-0.8%), Western Europe (2010-2104: 0.6-0.4%), or the Caribbean (2010-2014: 0.06-0.05%) (Pauly and Zeller, 2016). The United States, number 17 of the top 25-aquaculture producers in 2014, produced 425,900 metric tons of total aquaculture products in 2014; 178,300 tonnes of which came from inland finfish (SOFIA, 2016). With the state of global capture fisheries a largely debated topic, but seemingly in decline, it becomes much more important for countries outside of China to expand their aquaculture production industries in order to bolster the supply of fish products around the world, thereby providing adequate food security and nutrition. According to the USDA ERS (2015), the United States imported over 40 billion USD worth of fish and shellfish products from both capture fisheries and aquaculture. While United States fish and seafood exports hit an all time high in 2015 as well; a total value of 5.3 billion USD (USDA FAS, 2015), the trade balance heavily favors imports. The catfish market in particular has suffered at the hands of foreign competition. More research must be done to develop more efficient methods of culturing catfish so that U.S. farmed products can become more competitive in both the local and global markets.

## The United States Catfish Industry

The catfish industry began in the late 1950's and since its establishment, has gone through four identifiable phases: the pioneering phase (1960-1970) characterized by rapid expansion and relatively high production costs that resulted in low yields and inefficiency (Swann, 1992). The period of 1971-1976 gave rise to major improvements in production and lower unit costs with average annual yields increasing from 1500-2000 lb / acre to 3000-4000 lb / acre (Swann, 1992). During this second phase, scarcity of fishmeal products forced marginal and unprofitable producers out of the market; a point that will be touched upon more later. The third phase (1977-1982) saw vastly improved productivity, greatly increased acreage, and lower production costs as producers shifted to wholesale processing as the major sales outlet (Swann, 1992). From 1982-1989, the industry experienced decreased rates of expansion, while production of other aquaculture species including salmon, striped bass, crawfish, and tilapia increased (Swann, 1992). Since its peak in 2003 when the industry processed 662 million pounds of round weight catfish, the United States catfish industry has drastically contracted (Hanson and Sites, 2015). In 2014, 301 million pounds were processed, down 32 million (-10%) from 2013; and a 54% decrease since the 2003 peak (Hanson and Sites, 2015). Production has risen slightly to 316 million pounds and 319 million pounds in 2015 and 2016, respectively (unpublished report from Dr. Terry Hanson). Some of this reduction in production can be explained by the loss of water acreage of catfish ponds in past and recent years as more farmers are leaving the industry and converting their land for cultivation of row crops as instability in the price of feed inhibited farmers from making expenses; there was simply more opportunity in row crops. Current production acreage for the top three catfish producing states, Alabama, Arkansas, and Mississippi was down 7,700 acres (-11%) to 62,100 acres from 2014 to 2015 (Hanson and Sites,

2015). Now, imports account for 80% of all U.S. sales of frozen catfish and catfish-like fillet products (Hanson and Sites, 2015). While imports did decrease by 42 million pounds from 2013 to 2014, in 2016, this number rose again to 299.026 million pounds of Siluriformes imported into the U.S. (unpublished report from Dr. Terry Hanson); this intense import competition has put significant strain on U.S. catfish farmers (Figure 1). In 2000, catfish was the fifth most consumed U.S. seafood product per capita, while in 2013, catfish dropped to eighth behind shrimp, salmon, canned tuna, *tilapia*, pollock, *Pangasius*, and cod (Hanson and Sites, 2015).

The price of catfish products per pound has increased since 2013. In 2014, the average price per pound received by producers was $1.189 compared to the 2013 average of $0.974; up $0.213 per pound (+22%) (Hanson and Sites, 2015). Price of catfish products fell to $1.138 per pound in 2015 and rose again last year in 2016 to $1.172 (unpublished report from Dr. Terry Hanson). The slight reduction in the amount of imported catfish products coupled with the continued decline of production by U.S. catfish farmers has seemingly led to a shortage of catfish products as explained by the significant increase in the value of product from 2013-2014. Feed prices remained relatively stable from 2013-2014, averaging about $480 USD per ton, but it should be noted that there are significant peaks and troughs throughout the production season as feed prices were $515 USD per ton in April, 2014 and $448 USD per ton in October (Hanson and Sites, 2015). In 2016, however, feed prices seem to have significantly dropped as the Alabama Catfish Feed Mill, one of the largest bulk catfish feed producers in the industry, reported an average price per ton of $381.54 USD. Today, the biggest challenges that producers face are imports, feed costs, and disease.

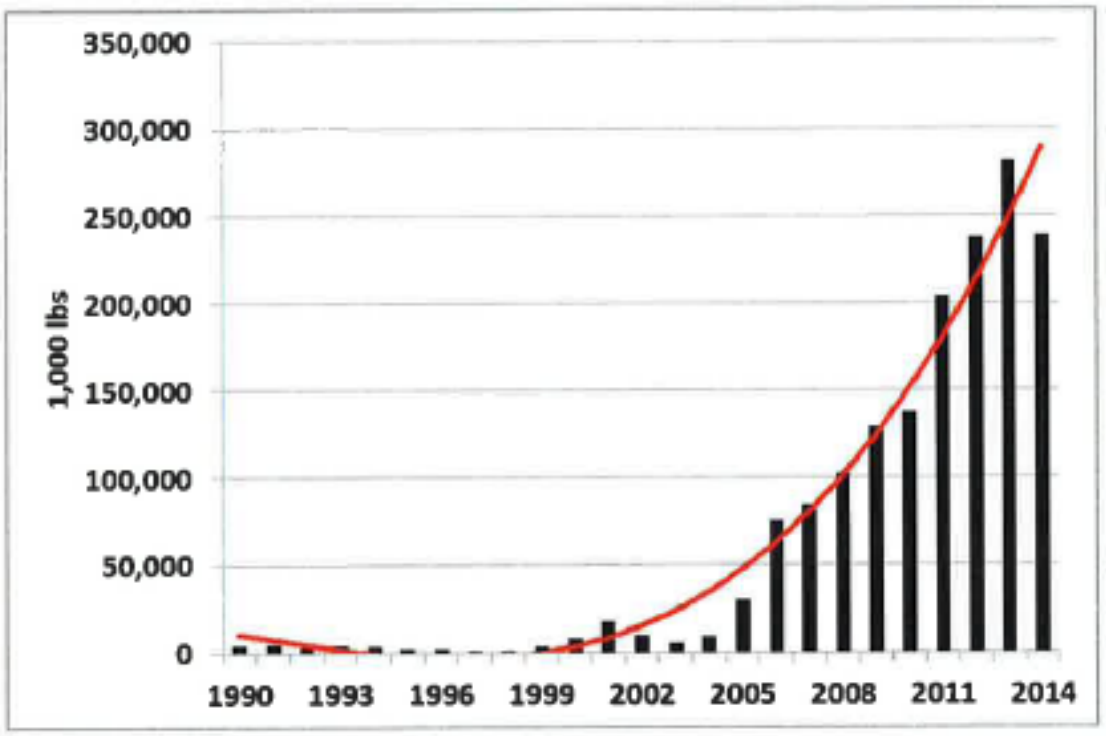


**Figure 1: Rise of imported catfish products since 2005 (Hanson and Sites, 2015).**

## Changes in the Composition of Catfish Diets

The main input since the beginning of the industry of feeds for aquaculture has been fishmeal sourced from pelagic fisheries, however, over a decades worth of maximum exploitation of this fishery has led reduced catch rates and gradual decline of the production of FM since 2005, leading to an increase in price of about 150% (IndexMundi, 2015; SOFIA, 2016). Given the instability of feed prices coupled with intense import competition from Pangasius, catfish farmers have been pushed to find ways of cutting their production costs; one way they cut costs was the use of more abundant and, therefore, more affordable feed ingredients.

Animal proteins that have been used in catfish feeds include fishmeal, meat and bone meal, blood meal, meat and bone/blood meal blend, poultry by-product meal, and catfish offal

meal (Li and Robinson 2013; Miles and Chapman 2005). Today, the primary protein sources used in catfish feeds are oilseed meals, such as soybean meal and cottonseed meal; peanut meal and canola meal can also be used. When compared to animal proteins, most plant proteins, except for soybean meal, are deficient in lysine, the most limiting essential amino acid in catfish feeds (Li and Robinson, 2013).

Soybean meal is the predominant protein source in current catfish feeds. It has the best amino acid profile of all common plant protein sources and is highly palatable and digestible to catfish. Levels of soybean meal of up to 50 percent have been used in commercial catfish diets without detrimental effects, but the increasing cost has reduced its use in recent years (Li and Robinson, 2013). Cottonseed meal, which contains free gossypol and cyclopropenoic acids that can be toxic at high levels, is another common protein source in commercial catfish diets and has been used at anywhere from 10-30 percent (with lysine supplementation) to replace part of the soybean meal (Li and Robinson, 2013). Other components of typical commercial catfish diets include: Dried distillers grains with solubles (DDGS; protein source)(Li and Robinson, 2010), porcine meat and bone meal (protein source; usually not more than 10 percent of the total diet composition), corn grain (energy source), wheat middlings (energy source), corn gluten feed (energy source)(Li and Robinson, 2011), vitamin and mineral supplements including dicalcium phosphate and vitamin mixes, trace mineral mixes, and amino acid supplements typically in the form of Lysine HCl. Fishmeal contains 60-80 percent protein of excellent quality that is highly palatable to catfish when compared to the approximately 48 percent protein obtained from soybean meal, its chief replacement (Miles and Chapman, 2005). Porcine meat and bone meal contains roughly 52 percent crude protein and contains less lysine compared to fishmeal. While porcine meat and bone meal is a good source of minerals, its high ash content limits its use

because of the possibility of mineral imbalance, thus it is only added as 5-10 percent composition of the total diet if at all (Miles and Chapman, 2005). With virtually no fish meal, relatively little porcine meat and bone meal (blood meal blend), commercial catfish diets derive almost all protein from vegetable protein, mainly: soybean meal, wheat middlings, corn, and cottonseed meal.

Initially, this strategy to convert to a primarily soybean and corn based diet was successful in cutting the cost of feed as prices per ton were in the range of $250-300 USD in 1997 (Hanson and Sites, 2015). The instability of corn and soybean futures led to drastic increases in the price of soybean and thus, drastic increases in the price of fish feeds beginning in 2005. The high price mark of $562 USD per ton for 32% crude protein floating catfish feed was in August 2012 (Hanson and Sites, 2015), and throughout 2013 and 2014, the price of feed hovered around $450-500 USD per ton; providing little relief to farmers trying to cut costs by using plant-based feed ingredients. However, in 2015 and 2016, the price of soybean began to drop and the average price of feed in 2016 was $381.54 USD per ton (Alabama Catfish Feed Mill). While this switch to alternative feed may have reduced costs of production throughout the past decade (relative to continued fish meal/animal protein inclusion), the concentration of a known anti-nutritional factor, phytic acid (phytate or $IP_6$) is significantly higher in these alternatives compared to an animal protein based diet (Eeckhout and Paepe, 1994; Ravindran et al. 1993; Tahir et al. 2012).

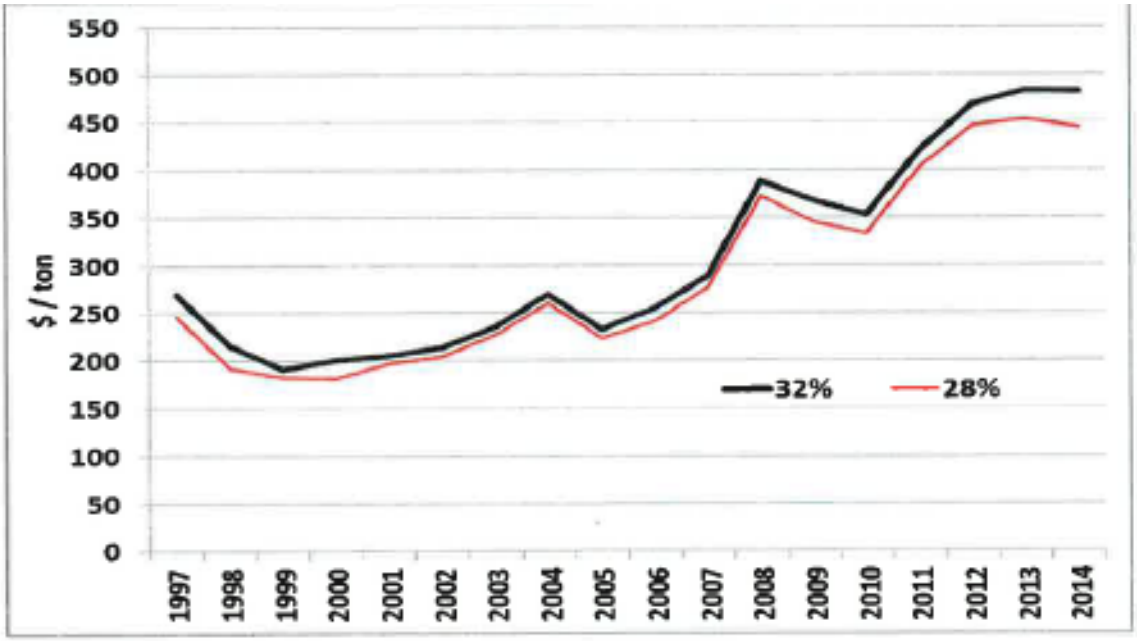


**Figure 2: Average feed costs for soybean-based floating catfish feeds of 28 and 32% crude protein respectively from 1997-2014 (Hanson and Sites, 2015).**

## Phytic Acid

Phytic Acid (*myo*-inositol hexaphosphoric acid, or, scientifically, 1,2,3,4,5,6-hexakis[di-hydrogen phosphate] *myo*-inositol) is of key importance when considering the composition of commercial catfish diets today, as it is widely known as an anti-nutritional factor in non-ruminants. In 1903, Posternak described phytic acid for the first time, but Pfeffer discovered it as early as 1872 (reviewed by Vohra and Satyanarayana, 2003). Phytates ($IP_6$'s) can be found in a variety of foods as demonstrated as early as Averill and King (1926), who reported a wide range of phytate levels as influenced by variety and byproduct of numerous cereals and nuts (Maga, 1982). Phytic acid occurs primarily as salts of mono- and divalent cations (Zhao et al. 2014) in discrete regions of cereal grains and legumes and accumulates during ripening. The structure of $IP_6$ makes it a very stable molecule and it confers a large negative charge over a wide range of pH. The six phosphate groups of the molecule can generate a total of 12 replaceable reactive

sites with negative charges in complete dissociation, where between 3 and 9 are dissociated under the usual range of pH in the digestive tract (Morales et al. 2016). Thus, $IP_6$ is able to bind di- and trivalent minerals and form very stable, insoluble complexes. Under normal physiological conditions, $IP_6$ is able to form complexes with zinc ($Zn^{2+}$), copper ($Cu^{2+}$), nickel ($Ni^{2+}$), cobalt ($Co^{2+}$), manganese ($Mn^{2+}$), calcium ($Ca^{2+}$), and iron ($Fe^{2+}$) in that order of stability (Cheryan, 1980). The formation of these insoluble complexes reduces the digestibility and thereby the absorption of these minerals, possibly leading to deficiencies and the physiological malfunctions associated.

Phytate is widely known to be indigestible by monogastrics, and the inability of catfish to utilize phytate was described by Andrews (1973) where reduced growth was observed when diets were formulated with 0.4% phytin phosphorus. Inclusion of phytate containing ingredients in fish feeds has been demonstrated to impair growth in a variety of cultured fish species, such as carp, tilapia, trout and salmon (reviewed in Francis et al. 2001). In catfish, supplemental dietary phytic acid at an inclusion rate of 2.2% significantly reduced weight gain and feed efficiency as compared to fish fed diets containing 0 or 1.1% supplemental phytic acid (Satoh et al. 1989). In these cases, these were probably deficient diets in terms of biological availability and the inclusion of phytic acid may have exacerbated the problem. The insoluble complex formed by phytic acid renders the phosphorus of the molecule unavailable to non-ruminants as well, limiting the absorption to non-phytate phosphorus in those ingredients with high phytate phosphorus like soybean meal (60% of total phosphorus) and cottonseed meal (69% of total phosphorus). The lack of availability of phosphorus from these ingredients leads producers to supplement their formulas with dicalcium phosphate at rates of about 1% inclusion, adding to their cost of production.

## Occurrence of phytate in the channel catfish diet

Phytic acid is abundant in aquaculture diets, especially those that are primarily plant-based; such is the case for many commercial catfish diets. Dominated by feedstuffs such as soybean meal, wheat middlings, corn grain, cottonseed meal, corn gluten feed, and DDGS, the occurrence of phytic acid in commercial catfish diets has increased as animal protein is replaced by soybean meal, corn products, and wheat products. As a proportion of dry matter weight, cottonseed meal contains 3.2-g/100 g phytic acid (Rodriguez et al. 2008), wheat middlings: 3-g/100g (Rodriguez et al. 2008, Tahir et al. 2012), soybean meal: 1.35-g/100 g DM (Ravindran, 1993), corn grain: 1-g/100 g (Rodriguez et al. 2008, Tahir et al. 2012), and DDGS: 0.5-g/100g DM (Batal and Dale, 2003a) [Calculations based on phytate P % of DM and assuming 20-28 % phosphorus by molecule].

It is common that all of the ingredients mentioned be present in significant amounts in a commercial channel catfish  diet. In one example provided by Li and Robinson (2013), a 32% protein food fish diet contained 30.6% soybean meal, 25% cottonseed meal, 15% corn grain, 10.82% wheat middlings, 15% DDGS, 1% dicalcium phosphate, 0.38% Lysine HCl, and 2% animal fat/oil. In a survey conducted by Beck and Peatman (2016), it was determined that participating feed mill formulations contained between 1.54 – 2.22% phytate (average 1.92%), consistent with the hypothetical example given.

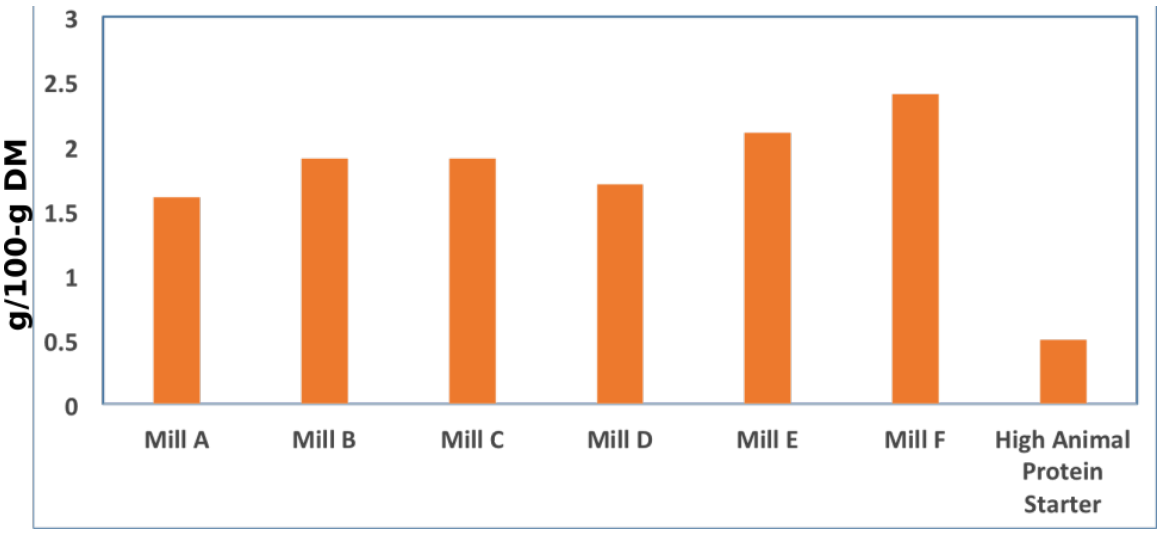


**Figure 3: Unpublished survey conducted by Beck and Peatman (2016) of phytic acid concentration of catfish feed produced by major feed mills in AL and MS**

## Effects of phytic acid on protein availability

Increasing evidence shows that $IP_6$ may affect the digestibility of protein and amino acids in pigs and even fish (Cowieson et al. 2006; Kumar et al. 2012). Similar to its action on minerals, phytic acid may interact with proteins to form insoluble $IP_6$ protein complexes over a wide range of pH. Phytic acid can create binary (protein-$IP_6$) or ternary (protein-mineral-$IP_6$) complexes (Kies et al. 2006). Selle et al. (2000) proposed that de novo formation of binary protein $IP_6$ complexes in the animal gastrointestinal tract is a key mechanism determining the digestibility of protein. The main modes of $IP_6$-protein complex formation that have been suggested are related to the presence of these complexes in feed ingredients, the formation of binary and ternary complexes in the gut, and the inhibition of fish proteolytic enzymes (Dvorakova, 1998; Kies et al. 2006; Selle et al. 2006).

Given the high values of pKa (dissociation constant) of amino acids, they are highly susceptible to chelation at low pHs such as those seen in the gastrointestinal tract. At pH lower than the isoelectric point of proteins and amino acids, cationic proteins have a high affinity for

negatively charged $IP_6$ molecules and readily form binary complexes of macromolecular aggregations or insoluble coacervates. As pH decreases, the binding potential of $IP_6$ to protein increases until it reaches its maximum at a pH of 2.5 (Adeola and Sands, 2003). Dietary calcium has a significant affinity for phytic acid as well, thus, sufficient dietary calcium may dissociate binary $IP_6$ complexes through competitive inhibition. Selle et al. (2012) also discussed the possibility of phytic acids role as a 'Hofmeister anion,' which categorizes cations and anions based on their ability to stabilize or destabilize proteins (Baldwin, 1996). In the case of phytic acid, it can be suggested that phytic acid has strong kosmotropic properties under the Hofmeister series, that is, it has an innate ability to stabilize bound protein and reduce solubility.

Furthermore, it was hypothesized by Anderson (1985) and evaluated by Morales et al. (2013) that the content of basic amino acids (arginine, lysine, histidine) in proteins can also have an effect on the interactions between $IP_6$ and proteins. Under the acidic conditions of the fish stomach, such proteins would be particularly prone to binary complex formation with $IP_6$. Using SDS- PAGE electrophoresis, it was shown that, among protein fractions, legumin and vicilin (proteins found in broad beans; 17.4 and 19.9% basic AA) showed higher increase in their solubalization in response to dephosphorylation by phytase enzymes when compared to gliadins and glutenins (wheat protein; 4.1% basic AA). These results support the hypothesis that the proportion of basic AA's plays a significant role in the formation of binary phytic acid complexes. Therefore, soybean meal, which is in abundance in commercial catfish diets, may be inefficiently utilized as a protein source due to the affinity of its components to bind to $IP_6$.

In addition to amino acids, evidence exists that suggest that phytic acid may also have significant impacts on function of several enzymes. Polyphenols and phytic acid may affect starch digestibility through interaction with amylase enzymes (Thompson and Yoon, 1984). The

actions of other enzymes including trypsin, acid phosphatase, and tyrosinase have been shown to be inhibited by phytic acid and also by inositol pentaphosphate ($IP_5$) (Harland and Morris, 1995).

## Reduction of mineral bioavailability by phytic acid

There have been numerous studies overall the past several decades that conclude phytic acid can bind essential dietary minerals, most notably, zinc, calcium, and iron. However, disparities in the literature remain due to differences in diets used throughout these studies; other components of the feed, especially fiber, can play significant roles in the utilization of minerals (Erdman, 1979). Additionally, studies involving "pure" phytates are also open to question since chemical binding is not considered (Davies and Nightengale, 1975) and phytic acid localization differs with product (Tombs, 1967). Regardless, the plethora of studies conducted that have shown phytic acid to have significant affects on mineral utilization cannot be ignored.

### *Calcium and phosphorus*

Calcium deficiency is not common in fish. Most freshwater and saltwater environments contain enough Ca to meet the requirements of fish via absorption through the gills, fins, and oral epithelia, therefore, unlike terrestrial animals; bone is not the main regulatory site for calcium regulation in fish. However, absorbed calcium is deposited into bone, scales, and skin. Scales are also the sites of Ca storage in fish. The importance of Ca in fish is mainly through its interactions and regulation of phosphorus. Many studies with monogastrics have shown that an optimum Ca to P ratio is important, and increasing the Ca to P ratio interferes with the absorption of P and, conversely, a high P to Ca ratio may restrict the Ca absorption. While these ratios are not as important in fish when compared to other monogastrics, optimum dietary Ca to P ratio has been

reported in red sea bream and Japanese eel to be 1:2 and 1:1 respectively (Lall and Lewis-McCrea, 2007).

Plant-based diets typical of the commercial catfish industry are generally low in both calcium and phosphorus with the main ingredients: soybean meal, cottonseed meal, DDGS, corn grain, wheat middlings, and corn gluten feed; all below 0.25% Ca and available P (except cottonseed meal comprising of 0.32% available P) (Batal and Dale, 2011). These feeds are supplemented with dicalcium phosphate at an inclusion rate up to 1% total composition of the diet (Li and Robinson, 2013).

Phosphorus deficiency is characterized by reduced growth and feed efficiency, and reduced bone mineralization and skeletal abnormalities including curved spine and soft bones, cephalic deformities in the frontal bones, and compressed vertical bodies resulting in scoliosis. Robinson et al. (2002) recommend 3.5g of phosphorus /kg diet supplementation to satisfy the requirement for channel catfish. Composed of 18.5% phosphorus (Batal and Dale, 2011), a 1% inclusion of dicalcium phosphate accounts for 1.85g of that supplement. However, dicalcium phosphate is 22% composed of calcium, contributing 2g of calcium /kg of feed. In an alkaline environment, the addition of dicalcium phosphate could lean the calcium to phosphorus ratio toward calcium, and reduce absorption of phosphorus.

### *Zinc*

Zinc is involved in various metabolic pathways and serves as a specific cofactor of several enzymes. Zinc is also an integral part of roughly 20 metalloenzymes such as alkaline phosphatase, alcohol dehydrogenase, and carbonic anhydrase. It is connected with prostaglandin metabolism and also may have a structural role in nucleoproteins (Watanabe et al. 1997). Zinc

deficiency leads to growth retardation (Ogino and Yang, 1978), lower digestibility of protein and carbohydrate, most likely due to reduced carboxypeptidase activity (Ogino and Yang, 1978), eye lens cataracts and fin erosion (Ogino and Yang, 1978; Hughes, 1985), and reduced appetite in catfish (Gatlin and Wilson, 1983).

Gatlin and Phillips (1989) reported a tight relationship between calcium, phytate, and zinc. Interactions among the three were investigated and in diets containing 20 mg Zn/kg, 2% Ca, and 2% phytate, catfish bone zinc was significantly decreased. Gatlin and Wilson (1984), determined that channel catfish fed a predominantly soybean based diet require approximately 150 mg Zn/kg to maintain optimum zinc status. A supplemental zinc level of 200 mg Zn/kg diet provides sufficient available zinc to channel catfish even with high concentrations of calcium and phytate.

### *Iron*

Iron is important to oxidation/reduction reactions and electron transport associated with cellular respiration. It can be found in complexes bound to proteins such as heme, in enzymes such as microsomal cytochromes, catalases, etc., and in non-heme compounds such as transferrin, ferritin and flavin iron enzymes. Hemoglobin occurs in erythrocytes while transferrin is found in the blood plasma; the latter is the principal carrier of iron in the blood (Watanabe et al. 1997). Iron is also one of the primary metals involved in lipid oxidation. Ferrous iron catalyzes the formation of hydroperoxides and free radical peroxides by providing a free radical initiator in the presence of unsaturated fatty acids and oxygen (Chvapil et al. 1974; Lee et al. 1981; Fujimoto et al. 1982). Both an inadequate supply of iron and an excess of iron in the body can lead to significant morbidity.

Catfish fed a basal diet of purified egg whites containing 9.6 mg Fe/kg diet exhibited suppressed growth and feed efficiency, as well as reduced hemoglobin, hematocrit, plasma iron, transferrin saturation and erythrocyte count values. Normal growth and feed efficiency were observed for catfish fed an additional 10 mg Fe/kg supplemental iron; however, 20 mg Fe/kg supplemental iron was required to maintain hematological values (Gatlin and Wilson, 1986).

The major factors influencing the absorption of iron are the proportion of organic and inorganic components of the diet, the amount ingested, and the conditions of the digestive tract. When comparing the effectiveness in preventing anemia, it was shown that ferrous and ferric chloride was more highly available than ferric citrate (Sakamoto and Yone, 1979). Feeds of animal origin, such as fishmeal, are rich in iron compared to oil seeds (fish meal: 880 ppm, soybean meal: 171ppm). Additionally, iron may be complexed with phytic acid, reducing its solubility at a wide range of pH. The availability of iron, therefore, depends heavily upon its form.

Iron behaves as though it is derived from one of two common iron pools in the meal (Bjorn-Rasmussen, 1974). The larger pool comprises iron in vegetables, any soluble inorganic iron present, and iron in meat that is not in the physical form of heme (non-heme iron pool). Heme compounds are comprised mainly of hemoglobin and myoglobin from meat and blood.

Iron storage status is the most important determinant of the rate of non-heme iron absorption, however, factors present in the intestinal lumen exert a powerful influence over the body's ability to extract iron from the luminal non-heme iron pool (Lynch, 1997). Several dietary factors may act as either inhibitors or enhancers of non-heme iron absorption and the balance between these factors determines the relative bioavailability of non-heme iron.

The presence of ascorbic acid, organic acids such as citric, malic, and lactic acids, and animal tissues tend to enhance the absorption of non-heme iron. Above a pH of 4, almost all iron is precipitated from a solution of ferric chloride. However, if ascorbic acid is added to soluble ferric chloride in acid solution, a complex of iron and ascorbic acid is formed that remains soluble (Conrad and Schade, 1968). Factors in animal tissues that attribute to increased iron status remain poorly characterized, however, it has been suggested that peptides released during proteolytic digestion by pepsin in the stomach may increase the solubility of inorganic iron (Kane and Miller, 1984; Slatkavitz and Clydesdale, 1988; Hurrel et al. 1988).

Far outnumbering the enhancers of non-heme iron absorption are inhibitors of non-heme iron absorption. Several factors including phytic acid, polyphenols, protein digestive products, calcium (in tandem with phytic acid in a similar action as inhibition of zinc absorption), and fiber (Lynch, 1997), inhibit the absorption of non-heme iron.

### *Anemia in channel catfish*

Anemia has been a persistent, unresolved problem in the United States catfish industry for several decades. Severe anemia, first referred to as "no-blood disease" by Lovell (1983), is characterized by packed cell volumes (PCV) or hematocrits around 5-10%. Severe anemia can often be detected through noticeably reduced feeding or decreased tolerance of low oxygen. However, less is known regarding milder (hematocrit in the 20% range), chronic cases of anemia, which may be widespread and largely undiagnosed while still having detrimental impacts on fish growth and disease susceptibility. Numerous factors can depress hematocrit levels; and with respect to severe anemia, a range of etiologies have been ruled out including pathogenic insult, heavy metals, pesticides, and mycotoxins. Instead, a number of studies to date

have pointed to feed-related origins for anemia in catfish. Indeed, the incidence of anemia is heightened during late spring and early summer as water temperatures approach 21 ºC and fish are actively consuming feed (Lovell 1983; Klar et al. 1986). More convincing is a series of studies, which demonstrated that the occurrence of anemia could be corrected by changing the feed (i.e., the brand of feed) or through a complete cessation of feeding (Plumb et al. 1986, Klar et al. 1986; Butterworth et al. 1986). Following these reports, folic acid levels—and those of its antagonist pteroic acid—were implicated in the pathogenesis of anemia, which necessitated a determination of dietary folate requirements for channel catfish (Butterworth et al. 1986; Duncan et al. 1993; Plumb et al. 1994).

Despite these findings, anemia remains prevalent in the industry with an estimated 4.7% of annual cases submitted to the National Warmwater Aquaculture Center in Stoneville, Mississippi from 1994-2012 being anemia-related (Camus et al. 2014). While the precise mechanisms governing feed-related anemia are not known, recent findings by Camus et al. (2014) indicated sufficient dietary iron levels in examined commercial catfish diets but a "failure of iron uptake, either as result of poor dietary bioavailability or from an unknown malabsorptive process." Intriguingly, administration of parenteral iron to anemic catfish brought about a recovery from anemia documented by marked increases in PCV and iron stores in the serum and liver (Camus et al. 2014).

According to Allen et al. (2014), metabolic patterns in catfish tissue extracts indicated anemia was primarily having effects via reduction in available oxygen, reduced aerobic capacity resulting in an increase in anaerobic and alternative energy pathways. Decreases in erythrocyte concentrations confirmed the loss of aerobic capacity. This study, using 1-D and 2-D metabolomics, highlights the possibility that mild, chronic anemia may be caused by the

inhibition of iron absorption and is responsible for decreased growth and feed efficiency in channel catfish by forcing their metabolism into less efficient, anaerobic activity.

### *Inhibition of iron absorption by phytic acid and tannic acid*

A large number of subsequent studies suggest that phytic acid is a major inhibitor of iron absorption, and its occurrence in commercial catfish diets is evident. Studies using both wheat and soybeans show that even small concentrations of phytic acid can have a significant effect on iron absorption (Hurrel, Juillerat, and Reddy et al. 1992). Although it has been extensively investigated, numerous opposing data have appeared. For example, McCance and Widdowson (1943), Nakamura and Mitchell (1943), Sathe and Krishnamurthy (1953), and Davis and Nightingale (1975) have shown phytic acid to be inhibitory of iron absorption, while others (Cowan et al. 1966; Callendar and Warner, 1970; Ranhotra et al. 1974b; Welch and Van Campen, 1975), have shown no effect.

Perhaps one of the more convincing, more recent studies, Glahn and Wortley (2002) demonstrated the effects of phytic acid, tannic acid, and zinc on iron uptake in an in vitro digestion/ Caco-2 cell culture model. The results from the study indicated that molar ratios of Fe to phytic acid of 1:10 or greater result in maximum inhibition of iron uptake. Inhibition was less than maximal at some point between 1:3 and 1:10 Fe to phytic acid ratio, but still significantly different when compared to the absence of phytic acid (28 vs. 6 mg ferritin/ mg cell protein). Interestingly, heme iron was less affected by phytic acid compared to non-heme iron. It is thought that heme iron absorbed by the enterocyte as an intact metalloporphyrin (a cyclic molecule consisting of four pyrolic groups joined by methane bridges with a ferrous iron ion in the center) following liberation from globin protein by proteolytic enzymes (Conrad et al. 1966).

Maximal inhibition of Fe uptake in vivo was 88% by tannic acid and occurred at or near a 1:1 molar ratio of Fe to tannic acid, suggesting that tannic acid concentration in the diet is also relevant when considering the availability of iron. Similar to phytic acid, the inhibition of Fe by tannic acid is likely due to binding of the two to form insoluble complexes (South and Miller, 1998).

## Phytic acid destruction

### *Phytase*

Phytases have been employed in animal feeds for several years and have been used primarily to reduce the environmental impact and phosphorus loads from farm effluents. Numerous studies have shown the positive effect of phytase supplementation on the availability of dietary phosphorus and trace minerals within plant-based diets for monogastric farm animals, including farmed fish and shrimp (Lemos and Tacon, 2015).

Phytase (myo-inositol hexakisphosphate phosphohydrolase) is a phosphatase enzyme that catalyzes the hydrolysis of phytic acid to myo-inositol and inorganic phosphorus in a stepwise manner forming myo-inositol intermediates. Phytase has been reported in rice, wheat, maize, soybeans, corn seeds, lettuces, dwarf beans, mung beans, fababean, rye, and other legumes and oil seeds (Chang, 1967; Eskin and Wiebe, 1983; Gibson and Ullah, 1990). The first report on animal phytase in calf liver and blood was that by McCollum and Hart (1908). A further search for mammalian blood phytase was unsuccessful; phytase was detected in the blood and liver of lower vertebrates such as birds, reptiles, fishes, and sea turtle (Rapoport et al. 1941). Patwardhan (1937) first noted phytate hydrolysis in the rat intestine. Phytase activity was also observed in the intestine of pig, sheep, and cow (Spitzer and Phillips, 1972). The ruminants probably digest

phytate through the action of phytase produced by microbial flora in the rumen (Vohra and Satyanarayana, 2003).

Several microorganisms possess the ability to produce phytases with a wide range of characteristics; having different temperature and pH optima most notably. Bacterial phytases are mostly cell associated, with the exception of *Bacillus subtilus*, *Lactobacillus amylovorus*, and *Enterobacter* sp. 4 (Vohra and Satyanarayana, 2003). Over 200 fungal isolates belonging to the genera *Aspergillus, Penicillium, Mucor,* and *Rhizopus* have been tested for phytase production (Liu et al. 1998). Of these fungal species, *Aspergillus niger* was identified as the most active fungal phytase producer and has been used in many fish trials between 1990 and 2010. Production of phytase can be either constitutive (produced all the time) or inducible (expressed only under conditions in which it is of clear adaptive value) depending upon the organism (Table 1).

Commercial phytases have been used in animal feeds for more than twenty years, but primarily for swine and poultry; only recently have these enzymes begun to be used in the aquaculture industry. The positive effects of using dietary phytase supplementation have been related to the limited ability of monogastrics to hydrolyze dietary phytate into absorbable forms of phosphorus. Amendment of feeds with phytase has been reported to increase the bioavailability of amino acids, trace minerals, and dietary energy (Ravindran et al. 2001; Selle et al. 2010). Anderson (1985) proposed that the hypothetical beneficial effects of phytase on P and protein availability are through disruption of the binary protein $–IP_6$ complexes. The result of the disruption breaks down phytic acid into its components: myo-inositol, bioavailable phosphorus, and associated protein that can be broken down by fish gastric proteases upon release from the complex.

Studies conducted on juvenile yellow catfish fingerlings demonstrated phytase inclusion at a rate of 1000 FTU/kg increased phosphorus digestibility by 71%, protein digestibility by 15%, weight gain by 74%, and feed efficiency by 32% (Zhu et al. 2014). Additional studies done by Zhu et al. (2015) and Cheng et al. (2015) show similar increases in digestibility of phosphorus and protein and in weight gain and feed efficiency. While these are some of the more astounding results, the effect of phytase application is highly dependent upon temperature and pH as the enzyme has optimum activities for both of those parameters. Therefore, the efficacy of application may vary between species as a result of differences in gut pH, the temperature at which those species are cultured, and the source of the enzyme (Table 2). In addition, phytase enzymes cannot remain stable under conditions of pellet extrusion, limiting the use of phytase to aquafeeds that do not undergo pellet extrusion, or the application of the enzyme may require ancillary equipment.

Still, the possibilities of a potential increase in the availability of phosphorus, amino acids, and trace minerals may outweigh the cost of additional equipment for coating pelleted feed with enzyme or the cost of research and development of new strains of bacteria that can produce highly heat stable phytases. Other nutritional benefits beyond phosphorus release and the destruction of anti-nutritional factors include the release of inositol (myo-inositol) from the dephosphorylation of phytic acid. Inositol is required at elevated doses for several fish species and inositol deficiency has been linked to symptoms including increased lipid liver, hematological changes, pathological organ change, lethargy, and poor appetite (NRC, 2011).

While the potential of phytase to break down phytic acid and free bound minerals, amino acids, phosphorus, and inositol is apparent, there is evidence that the reduction of $IP_6$ to $IP_4$, $IP_3$, and perhaps even lower does not remove the antinutritive properties of phytic acid. Bedford and

Walk (2016), report that the presence of $IP_4$ and $IP_3$ may still possess similar antinutritive properties as $IP_6$. In order to produce what is referred to as an 'extraphosphoric effect' (superdosing) with a phytase, the goal should be removal of all IP esters in the intestinal contents to produce the most beneficial effects. This brings about the emphasis on the differences in kinetics amongst different phytases and their effects on $IP_6$ and its associated breakdown esters (Bedford and Walk, 2016). It is paramount that the source of the phytase be understood if successful superdosing is to be achieved.

A study conducted by Jackson et al. (1996) demonstrated the practical implications of phytase amendment in channel catfish feeds. Fish fed diets containing 0, 500, 1000, 2000, or 4000 units of microbial phytase/kg of diet were subjected to a 10-week feeding trial in which all fish fed phytase diets benefited from increased consumption, weight gain, and feed efficiency as well as bone ash and bone phosphorus. In a follow-up study, Li et al. (2004) demonstrated that dietary phytase supplementation at a rate of 250 FTU/kg applied post-pelleting can effectively replace the dicalcium phosphate supplement in catfish diets without affecting growth, feed efficiency, or bone phosphorus deposition. This experiment holds both economical and environmental implications in that the reduced use of dicalcium phosphate may allow mill owners and farmers to benefit from lower production costs and the use of phytase improves the utilization of phosphorus, thereby reducing phosphorus loading in ponds and pond effluent. The reduction in phosphorus loading to the culture environment may control harmful algal blooms and bacterial populations, however, Li et al. (2004) did not report significant differences in P levels between treatment ponds.

**Table 1: Abbreviated summary of production of phytases by microorganisms (excluding yeasts) as summarized by Vohra and Satyanarayana (2003).**

| Bacteria | T opt (°C) | pH opt[1] | Inducible/ constitutive | Location of enzyme | Reference |
|---|---|---|---|---|---|
| *Aerobacter aerogenes* | 27 | 6.8 | Constitutive | Cell bound | Greaves et al. (1967) |
| *Bacillus sp. DS11* | 37 | 6.5 | Constitutive | Extracellular | Kim et al. (1998) |
| *Bacillus subtilis* | 30 | 6.5 | Inducible | Extracellular | Powar and Jagannathan (1982) |
| *Enterobacter sp.4* | 39 | 5.5 | Inducible | Extracellular | Yoon et al. (1996) |
| *Escherichia coli* | 37 | 7.5 | Constitutive | Cell bound | Greiner et al. (1993) |
| *Klebsiella aerogenes* | 30 | NA | Inducible | Cell bound | Tambe et al. (1994) |
| *Lactobacillus amylovorus* | 37 | 6 | Inducible | Extracellular | Sreeramulu et al. (1996) |
| *Pseudomonas sp.* | 25 | NA | Constitutive | Cell bound | Irving and Cosgrove (1971) |
| *Selenomonas ruminantium* | 39 | NA | Constitutive | Cell bound | Yanke et al (1999) |
| **Fungi** | | | | | |
| *Aspergillus carneus* | 30 | 6 | Constitutive | Extracellular | Ghareib (1990) |
| *A. carbonarius (SSF)* | 30 | NA | Constitutive | Extracellular | Al Asheh and Duvnjak (1995) |
| *A. ficuum NRRL 3135* | 27 | 5 | Constitutive | Extracellular* | Shieh and Ware (1968) |
| *A. niger* | 30 | 5 | Constitutive | Extracellular* | Shieh and Ware (1968) |
| *Rhizopus oligosporus* | 25 | 5.5 | Constitutive | Cell bound | Howson and Davis (1983) |

[1] *: May vary depending upon species strain

**Table 2: Reported beneficial effects of direct dietary phytase supplementation on nutrient digestibility and performance in warm water catfish species (Reviewed by Lemos and Tacon, 2016).**

| Species | Diet Composition | Phytase Source/ Application Method | Dietary Inclusion Level (FTU/kg) | Improvement (nutrient digestibility, performance) | Reference |
|---|---|---|---|---|---|
| Channel Catfish *Ictalurus punctatus* (Juvenile) | Soybean (46%), cottonseed meals (14%), corn (32%) | *A. niger*/ added to ingredient mix | 1000, 3000 | Positive dose-response: phosphorus (up to 100%) | Eya and Lovell (1997) |
| | Soybean meal (53%), corn (33%) | *A. niger*/ sprayed onto pellets | 250 to 750 | At 250 FTU/kg: weight gain (41%) and feed efficiency (22%) | Li and Robinson (1997) |
| African Catfish *Clarius gariepinus* (Juvenile) | Soybean meal (68%) | *A. niger*/Not Stated | 15 to 1000 | Positive dose response: phosphorus (>100%) | Van Weerd et al. (1999) |
| Pangas Catfish *Pangasius pangasius* (Fingerlings) | Soybean meal (45%), wheat (15%), corn (15%) | *A. niger*/ sprayed onto pellets | 150 to 2000 | Positive dose-responses, up to 250: zinc (6%), potassium (1%), copper (10%). Up to 500: calcium (>100%), phosphorus (30%) Up to 1000: magnesium (31%), manganese (75%), iron (81%) | Debnath et al. (2005) |
| Striped Catfish *Pangasianodon hypophthalmus* (Juvenile) | Soybean meal (50%), rice bran (11%), cassava (11%), canola meal (8%) | Not Stated | 750 and 1500 | At 750 FTU/kg: phosphorus (31%), protein (6%) At 750 FTU/kg: weight gain (46%) and feed efficiency (20%) | Hung et al. (2014) |

## Xylanase

Xylanase is a class of enzyme that degrades linear polysaccharides, and breaks down hemicelluloses that are a major component of plant cell walls (Ganguly et al. 2013). Xylan, the substrate for xylanase, is found in large quantities in hardwoods from angiosperms (15-30% of the cell wall content) and softwoods from gymnosperms (7-10%), as well as in annuals (<30%)(Singh et al. 2003). Many microorganisms produce multiple xylanases (Gilbert and Hazelwood, 1993; Gilbert et al. 1988; Yang et al. 1989). Much like phytases, these may have diverse physiochemical properties, structures, specific activities and yields, as well as overlapping, but dissimilar specificities, thereby increasing the efficiency and extent of hydrolysis, but also the diversity and complexity of the enzymes (Collins et al. 2005). The vast majority of xylanases are excreted into the extracellular environment as the large size of xylan prevents its penetration into the cell (Collins et al. 2005). The heterogeneity and complexity of xylan has led to immense diversification of xylanases with different specificities, primary sequences and folds, and hence has led to limitations regarding the classification of the enzymes by substrate alone (Collins et al. 2005). Xylanase enzymes have a potential application in animal feeds, particularly in monogastrics such as swine and poultry with the goal of decreasing the content of non-starch polysaccharides, thereby reducing the intestinal viscosity and improving the utilization of proteins and starch, improving performance, and increasing the digestibility and nutritive value of poorly degradable feeds such as wheat and barley (Bhat, 2000; Mathlouthi et al. 2002, 2003a,b) In broilers, this enzyme has proven to increase the digestion and absorption of starches and protein by disrupting the cell wall and allowing water hydration and entrance of endogenous enzyme, phytase (Sinha et al. 2011). Increased protein digestibility as an effect of

xylanase supplementation could produce a sparing affect that could be utilized in the feed formulation of the diet (Bogevik, 2015).

The results of several experiments with the use xylanase in fish are highly variable and most beneficial effects, it would seem, occur in diets that are not extruded, the enzyme is applied to a wet feed mass, and are associated in a multi enzyme complex amongst other unnamed, nutritionally beneficial enzymes (likely phytase included). There were no significant effects on protein digestibility and growth in rainbow trout fed a soybean meal based diet supplemented with 67-mg/kg-xylanase enzyme added by coating after extrusion (Dalsgaard et al. 2012). It is possible that the activity of the enzyme is rendered ineffective due to the high heat and pressure of the extrusion process; the cell wall of the plant material may have already been disrupted due to the process, rendering the treatment ineffective. Considering that commercial catfish feeds are extruded, the use of this enzyme would have smaller implications, however, the inconsistency in the literature surrounding the use of xylanase enzymes leave too large of a question mark to ignore, as more studies need to be done that utilize different application methods, for example, pre-treatment of feedstuffs prior to pellet extrusion.

## Justification

The exodus from fishmeal diets in the commercial catfish industry has presented many challenges for the formulation of effective, plant-based diets. The attractiveness of fishmeal, considering its amino acid profile, mineral profile, and lack of antinutrients, has left much to be desired from the current roster of plant based ingredients including soybean meal, wheat middlings, corn grain, cottonseed meal, DDGS, corn gluten feed, etc. While these ingredients are indeed high in protein and do satisfy some of the basal amino acid requirements of channel catfish (hybrid catfish), the abundance of phytate in these ingredients may curb the performance of farmed fishes. Indeed, the first steps toward an economical, sustainable, and nutritionally complete commercial feed have been taken, but more research pertaining to the complete destruction of phytate, phytase superdosing, and the full extent of the complicated interactions of phytic acid in the gastrointestinal tract must be conducted to more fully unlock the potential of plant-based commercial catfish diets.

We conducted the following study in order to add to the growing list of phytase dose-response analyses so that we may fill in some of the gaps missing from the primary literature and continue to take strides in the direction of nutritional, economical, and sustainable excellence.

## Materials and Methods

The study consisted of four trials in which commercially available, closed formula, 32% and 28% protein catfish diets were top-coated with super-doses of enzymes from AB Vista (Plantation, FL). Two studies were conducted with hybrid catfish; our Auburn preliminary tank trial was conducted with on-site, residual Jubilee x D&B hybrids and our pond trial was conducted with hybrids donated from Leigh Holland at Jubilee Farms. In Stuttgart, AR, both trials were conducted with channel catfish.

### *Tank Trials*

Our preliminary tank trial in Auburn ranged from late June 2015 to mid-November and was conducted with Jubilee x D&B hybrids. Eight total tanks were stocked with 575 ± 50 fish with an average total biomass of 45 ± 2.5 kg; 4 control tanks and 4 phytase tanks. The feed used was a commercially available, closed formula, 32% protein diet and determined to contain 2.2% phytic acid from Southfresh feed mill located in Demopolis, AL. Prior to the trial, all fish were fed once daily with Southfresh control feed. The mill had the ancillary equipment necessary to provide a control diet and a diet with superdose (2500 FTU/ kg) topcoat of AB Vista Quantum Blue phytase enzymes; so no coating by our lab was necessary in this trial. The fish were fed twice daily to apparent satiation for the first 9 weeks, and as water temperatures cooled below 25° C, we fed only once daily to apparent satiation. The culture period lasted 140 days to final harvest.

At weeks 9 and 12, 10 fish per tank were sampled and harvested for hematology analysis. On November 15, all tanks were harvested, weighed, counted, and 10 fish from each tank were sampled and harvested for hematology analysis.

An initial tank trial at the USDA ARS of Stuttgart, AR was conducted to assess the impact of dietary phytase inclusion on erythrocyte packed cell volume, a rapid assessment of hematologic health. Two fiberglass tanks (675L) were stocked with 250 channel catfish fingerlings each averaging 28.2±2.4 g, which were spawned at the Harry K. Dupree Stuttgart National Aquaculture Research Center (HKDSNARC). Animal care and experimental protocols were approved by the HKDSNARC Institutional Animal Care and Use Committee and conformed to ARS Policies and Procedures 130.4 and 635.1. Prior to stocking fish had been reared on a standard commercial diet for catfish fingerlings. Tanks received flow-through well water (24ºC ± 2) with forced air aeration. Beginning at day 0, fish were fed at a rate of 3% body weight per day with a commercially available closed-formula diet with a label indication of 28% protein, 2.5% lipid, and 7.5% fiber which was top-dressed with 0 or 2500 FTU/kg of Quantum Blue phytase (AB Vista, Plantation, Florida). One FTU is defined as the phytase activity that liberates 1 micromole of orthophosphate per minute from 5.1 mM sodium phytate at 37°C and at pH 5.5 (Engelen et al., 1994). The diet was determined to contain 1.9% phytic acid as determined with the Phytic Acid Assay Kit (Megazyme, Wicklow, Ireland). At the beginning of the trial and 6, 9, and 12 weeks after initiation a random subset of fish was collected (n = 24) from each treatment and blood was collected from the caudal vein for packed cell volume determination

*Pond Trials*

The following spring and summer, we conducted a larger scale pond study to evaluate the commercial application of phytase fortification using a model that more closely resembles conditions that can be found in the commercial catfish industry. For this trial, 12, 1/10- ha ponds were stocked with 860 fish each. Due to the lack of uniformity in fingerlings that we had available for the study, the fish were graded and split into two classes: small and large consisting of 6 ponds of each class, 2 ponds per treatment, per class. The average weight of the smaller class was 57 ± 4 grams and the larger class was 97 ± 9 grams.

For this trial, 32% protein commercial feed was used from the Alabama Catfish Feed Mill in Uniontown, AL. This mill had also recently been outfitted with the ancillary equipment necessary to topcoat feed with enzyme. The target dose for phytase enzyme was 2500 FTU/ kg, and in this trial, we also looked to evaluate the dose-response of the application of the enzyme xylanase as well, as such, there were three treatments in this trial: 4 ponds of control, 4 ponds of phytase superdose (2500 FTU/kg), and 4 ponds of phytase superdose (2500 FTU/kg) and Xylanase (16,000 units/kg).

The feed was top coated using a garden 1.5 L weed sprayer and a cement mixer. Dry feed from the Uniontown Feed Mill was loaded into the cement mixer 50 kg batches at a time. The enzymes were diluted in the garden weed sprayer in 500 ml of Klaufmann's fish oil, 500 ml of Klaufmann's corn oil, and 500 ml of water. Solution was mixed thoroughly before application. Feed was added to the mixer about 3 kg at a time while feed was tumbling and enzyme solution was applied as feed was added to ensure and even coating.

The fish were fed to apparent satiation once daily at least five days per week. Feed consumption, water temperature, dissolved oxygen was recorded daily. Water quality was

conducted bi-weekly, and water was collected for phosphorus analysis monthly. Ten fish from each pond were harvested for sampling at week 9 for individual weight and hematology analysis using hook and line fishing. At the end of the study, ponds were seined and harvested; fish were weighed and counted to determine yield and mortality and donated to the EW Shell Fisheries Station live fish market. Twenty fish from each pond were set aside for individual weight, hematology analysis, liver collection for mineral analysis and RNA sequencing (place in RNA later solution), feces collection for phytic acid assay, and a portion of the distal intestine was also collected for RNA sequencing and placed in RNA later solution. The total culture period was 14 weeks.

The pond trial in Stuttgart, AR featured a single commercially available closed-formula diet labeled to contain 32% protein, which was top-dressed with 0 or 2500 FTUs of Quantum Blue phytase (AB Vista, Plantation, Florida). The diet was found to contain 2.2% phytic acid as determined with the Phytic Acid Assay Kit (Megazyme, Wicklow, Ireland). Six, 0.1-ha earthen ponds located at the USDA Agricultural Research Service (ARS) Harry K. Dupree Stuttgart National Aquaculture Research Center (HKDSNARC), Stuttgart, Arkansas USA, were used for this completely randomized feeding study. Ponds were filled with well water in late-May 2015. Rice bran was added to ponds to stimulate algal production one week prior to stocking. Well water was added to ponds as needed to replace losses to evaporation and seepage. Ponds were stocked with 2014-year class fingerling channel catfish, which were spawned at the HKDSNARC. Ponds were stocked with 600 fish each with a mean individual stocking weight of 22.4 ± 0.6 g/fish and acclimated for three weeks (while being fed the control diet) until the initiation of the feeding trial beginning in July when all ponds were switched over to their respective study diets. Fish were fed a minimum of 5 days per week. Feeding occurred each day

around 1300 h. Each pond was fed to apparent satiation, meaning if feed was not completely eaten within 15 minutes from the last addition of feed, that pond was not offered more feed that day. The quantity of feed offered each day was recorded. Individual weights were tracked monthly throughout the study (25 fish collected per pond; 75 per treatment) and final individual weights were obtained from 120 fish per treatment (40 per pond) at the termination of the study in early November.

### *Fish and feed composition*

In Stuttgart, eight fish collected at the end of the trial were randomly sampled, pooled, and stored at -20 ºC for determination of whole-body proximate composition. Each sample was analyzed in duplicate following the standard methods (AOAC 1990). Moisture content was determined by drying fish samples in an oven at 105 ºC until constant weight was reached. Samples used for dry matter were digested with nitric acid and incineration in a muffle furnace at 600 ºC for overnight ash content. Protein (N x 6.25) was determined by the Dumas method using a LECO nitrogen analyzer (LECO Corporation, St. Joseph, MI). The lipid content of samples was determined by petroleum ether extraction using a Soxtec System (2055 Soxtec Avanti; Foss Tecator, Höganäs, Sweden). Mineral content was measured (Arkansas Analytical, Inc., Little Rock, Arkansas) using AOAC 2011.14 - Microwave digestion and inductively coupled plasma-optical emission spectrometry (AOAC 1990). In the tank and pond trials, blood was collected and pooled from 3 fish per pool, so as to obtain the amount required for mineral analysis; with 3 pools for each treatment in the tank trial and 6 pools per treatment in the pond study. Due to adequate sample size liver samples were analyzed individually from 9 fish per treatment in the tank trial and 45 fish per treatment in the pond study. At the termination of the

study the inositol phosphate ester content within feces was measured using high-performance liquid chromatography (University of East Anglia, Norwich, Norfolk, United Kingdom).

### *Hematology Analysis*

Hematology was performed as described by Brown (1988). On all blood samples collected, we performed total cell count, total blood cell count, hemoglobin assay, hematocrit, and calculated red blood cell indices that included mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), and mean corpuscular hemoglobin (MCH).

Along with the hematocrit, the measurement of hemoglobin is used to follow the treatment and occurrence of anemias. Hemoglobin was measured using the Cyanmethemoglobin Method (Sigma Chemical Co., St. Louis, MO, USA). Whole blood was added to cyanmethemoglobin (HiCN) reagent that contains potassium cyanide and potassium ferricyanide. The ferricyanide converts the hemoglobin iron from the ferrous state ($Fe^{++}$) to the ferric state ($Fe^{+++}$) to form methemoglobin (Hi), which then combines with potassium cyanide to form the stable pigment, cyanmethemoglobin. The color intensity of this mixture is measured in a spectrophotometer at a wavelength of 540 nm. The optical density of the solution is proportional to the concentration of hemoglobin. Values were adjusted using a correction factor for channel catfish described by Larsen (1964).

When anticoagulated whole blood is centrifuged, the space occupied by the packed red blood cells is termed the hematocrit reading and is expressed as the percent of red blood cells in a volume of whole blood. It is also less commonly known as the PCV (packed red blood cell volume). The values for the hematocrit closely parallel the values for the hemoglobin and red blood cell count. Whole blood was centrifuged for maximum red blood cell packing. The space

occupied by the red blood cells was measured and expressed as a percentage of the whole blood volume.

The red blood cell count (RBC) is the number of red blood cells in 1 liter of whole blood. Whole blood was kept from coagulating using heparinized syringes, centrifuge and capillary tubes during storage and analysis. To facilitate counting and prevent lysis of the red blood cells, whole blood was diluted with an isotonic diluting fluid, in this case, phosphate buffer saline to 1:10000 and was then counted using a Spencer BrightLine hemacytometer.

The red blood cell indices were used to determine the size and hemoglobin content of the red blood cells. They consist of MCV, MCH, and MCHC and described earlier. The MCV indicates the average volume of the blood cell in femtoliters (fL) and was calculated using the following formula:

$$MCV = Hct \times 10^3 \text{ fL} / (RBC/L)$$

The MCV indicates whether the red blood cells appear normocytic, microcytic, or macrocytic. The MCHC is an expression of the average concentration of hemoglobin in the red blood cells and gives the ratio weight of hemoglobin to the volume of the blood cell. MCHC was calculated using the following formula:

$$MCHC = Hgb \times 100\% / Hct$$

The MCHC indicates whether the red blood cells are normochromic, hypochromic, or hyperchromic. MCH indicates the average weight of hemoglobin in each red blood cell and was calculated using the following formula:

$$MCH = Hgb\ (g/L) / RBC\ (/L)$$

In pg's

The MCH should always correlate with MCV and MCHC. A Pearson Correlation was performed to ensure data were accurately obtained and calculated.

### *Mineral Analysis: Liver / Blood*

Liver samples were collected from 10 fish per pond (40 per treatment) in the pond study conducted at the EW Shell Fisheries Station in Auburn. After hematology analysis, the blood collected from these fish pooled by pond into 5 replicates of 3 ml each. Both types of specimen, liver and blood, were freeze dried to remove all moisture, sealed in whirl pack bags, and outsourced to the University of Arkansas Poultry Science Center where they conducted a mineral panel using ICP (Inductively Coupled Plasma Mass Spectrometry).

### *Phytic Acid Assay*

We conducted a phytic acid assay on the feces that were collected from the pond trial using a Megazyme Phytic Acid (Phytate)/ Total Phosphorus kit to determine the extent to which phytic acid was broken down as a result of enzyme fortification. A stepwise reaction results in the hydrolysis of phytic acid into *myo*-inositol $(phosphate)_n$ and inorganic phosphate by phytase, further breakdown by alkaline phosphatase, and finally conversion to molybdenum blue which can be measured by spectrophotometer as absorption increases proportionally with the amount of molybdenum blue present at 655 nm. $P_i$ was quantified as phosphorus from a calibration curve generated using standards of known phosphorus concentrations.

### *Pond Phosphorus Analysis*

Three times throughout our pond study conducted at the EW Shell Fisheries Station in Auburn, AL, we collected water samples for free and reactive phosphorus analysis. Samples were collected in untreated, unpainted, non-reactive plastic bottles and chilled upon collection. Samples were analyzed the samples in the lab of Dr. Claude Boyd using the ascorbic acid method following digestion in potassium solution as described by Eaton et al. (2005).

### *Statistics*

Probabilities of $< 0.05$ were considered statistically significant. All statistics and figures were performed and produced using Graphpad Prism 6.0 or R Studio version 1.0.136. Differences between performance, hematological parameters, mineral content, and phytic acid content of feces were detected using Welch Two-Sample T-tests and/ or ANOVA with Sidak's multiple comparison test (nested analysis).

## Results and Analysis

### *Tank Trial Stuttgart*

Initial proof-of-concept trials were performed in Auburn and Stuttgart. In Stuttgart, the trial was conducted in 675 L tanks, in order to assess the effect of a 2500 FTU/kg phytase inclusion rate on erythrocyte packed cell volume (hematocrit). As shown in Figure 4, fingerlings fed a phytase-amended diet had significantly higher hematocrit values ($p<0.02$) beginning as early as six weeks, with differences increasing in magnitude at 9 and 12 weeks ($p<0.001$).

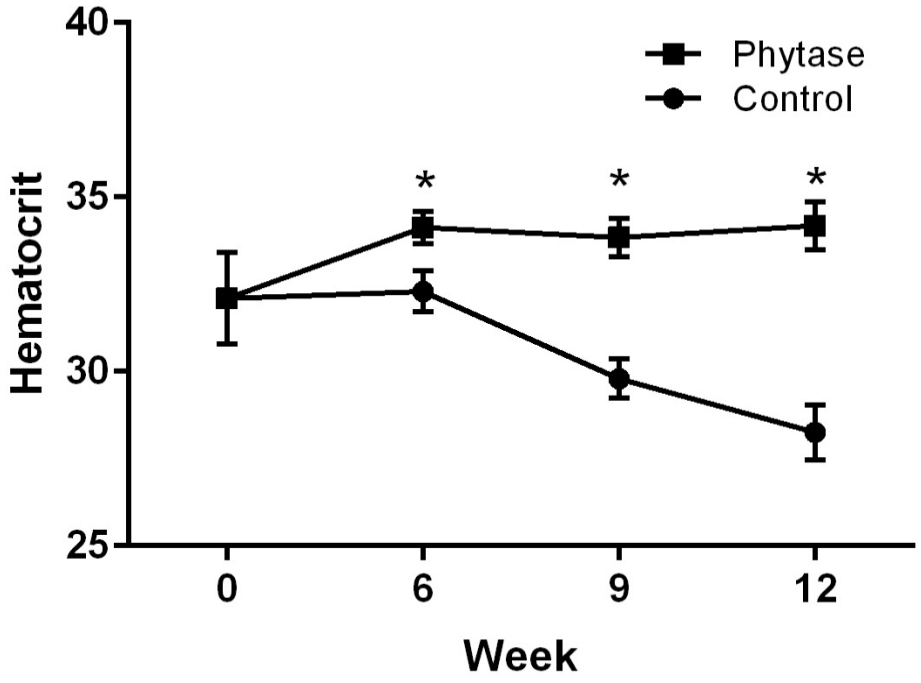


**Figure 4: Hematocrit values of channel catfish fed a ration containing 0 FTU/kg (Control) or 2500 FTU/kg (Phytase) of Quantum Blue Phytase in an indoor tank trial (error bars are ± SEM). Asterisks denote statistically significant differences at P<0.05. Data was taken from individual fish from each of the two tanks.**

Analysis of liver and blood showed marked differences in the uptake of minerals in fish fed the phytase-supplemented diet (Table 3). In particular, elevated levels of iron were present in both the blood and liver of fish fed the diet superdosed with phytase. In the liver, phytase-fed animals showed a nearly 50% increase in iron stores in the liver. At 24 h post-feeding, feces were analyzed for phytic acid content and fish fed the phytase-supplemented diet showed a 70% reduction in phytic acid content in the feces of the distal intestine.

**Table 3: Channel catfish mineral content in blood and liver (ppm) at the conclusion of a 12-week indoor tank study (ppm ± SEM). Asterisks denote statistically significant differences at P<0.05.**

| Mineral | Control Blood | Phytase Blood | Control Liver | Phytase Liver |
|---|---|---|---|---|
| Calcium | 547.2±55.4 | 604.7±5.8 | 96.1±13.3 | 96.7±6.2 |
| Phosphorus | 5699.7±121.0 | 6541.7±140.4* | 7409.6±311.5 | 8869.8±136.4* |
| Sodium | 10963.3±721.3 | 12125.0±89.0 | 2059.0±230.5 | 2283.5±108.9 |
| Aluminum | ND | ND | ND | ND |
| Copper | 1.1±0.3 | 1.0±0.2 | 6.6±0.85 | 8.5±0.39 |
| Iron | 1072.7±66.8 | 1312.0±53.2* | 38.7±3.53 | 73.1±14.5* |
| Magnesium | 427.1±10.0 | 526.7±13.9* | 487.6±23.7 | 562.2±16.8* |
| Manganese | ND | ND | 5.0±0.47 | 3.6±0.26* |
| Potassium | 7945.7±171.7 | 9159.7±215.2* | 11771.6±362.3 | 12591.5±164.8 |
| Sulfur | 2414.3±30.2 | 2563.7±62.4 | 3803.3±304.7 | 4170.4±136.4 |
| Zinc | 100.2±8.0 | 156.8±34.7 | 62.5±5.3 | 73.3±3.1 |

*Tank Trial Auburn*

A similar proof-of-concept trial was conducted in Auburn in 8-trough style, 1250- liter fiberglass tanks; 4 controls and 4-phytase superdosed stocked with 575 ± 50 Jubilee x D&B hybrid catfish. At the end of the 140-day culture period, there were significant differences in total growth, feed consumed, and FCR between the two treatments. Upon hematological analysis, we observed elevated concentrations of total cells per volume microliter of blood (TCC, $p<0.05$), red blood cell per microliter (RBC, $p<0.05$, Figure 5a), and packed cell volumes (Ht, $p<0.05$, Figure 5b), however, we did not observe a significant difference in hemoglobin concentration although fish fed the phytase-fortified diet were slightly elevated (Table 4).

**Table 4: Effects of dietary phytase superdosing (2500 FTU/kg) on hematological parameters during tank trial conducted in Auburn, AL (n = 40 fish per treatment, 4 tanks per treatment) ± SEM. An asterisk denotes statistically significant differences in values.**

| Diet | TCC/µL ($10^6$) | RBC/µL ($10^6$) | WBC/µL ($10^5$) | Hemoglobin (g/dL) | Hematocrit (%) | MCV (fl) | MCH (pg) | MCHC (%) |
|---|---|---|---|---|---|---|---|---|
| Control | 2.12±0.07 | 2.03±0.06 | 1.28±0.06 | 8.56±0.13 | 31.85±0.5 | 162.28±1.15 | 43.38±1.23 | 27.04±0.49 |
| Phytase | 2.32±0.05* | 2.21±0.05* | 1.16±0.05 | 8.80±0.15 | 34.23±0.63* | 157.08±3.46 | 40.47±1.05 | 25.96±0.65 |

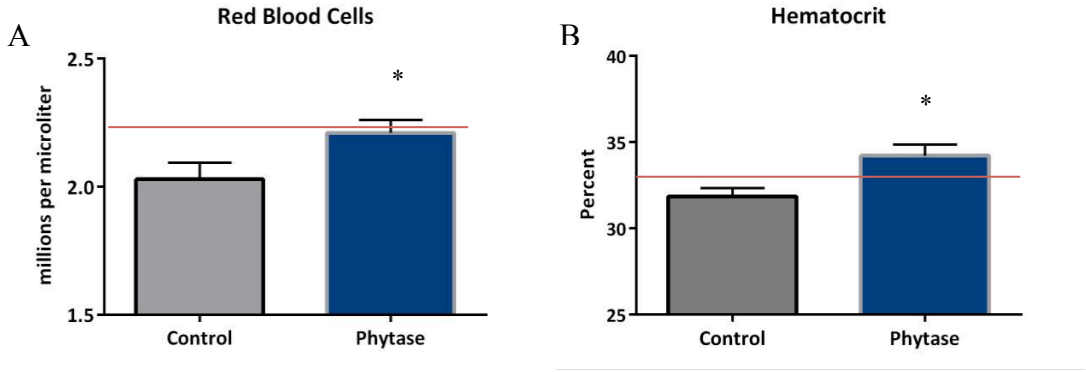


**Figure 5a,b: RBC concentration and hematocrit of fish fed phytase fortified feed compared to control in Auburn, AL tank study. Asterisk denotes significant differences ($p<0.05$); red lines represent historical averages.**

We observed a 17% difference in FCR in favor of phytase superdosing ($p < 0.02$), a 14% difference in feed consumption (Figure 7) in favor of phytase superdosing ($p < 0.02$), and a 29% difference in total growth per tank in favor of phytase superdosing ($p < 0.008$, Figure 6). In general, hematological parameters in this study were lower compared to the trial conducted in Stuttgart. The intensity of the trial and a system effect could have contributed significantly to the difference in results. This observation is paralleled by the FCR's during the culture period (we achieved FCR's of 1.59 and 1.92); the higher FCR's suggest that stocking densities may have become too intense and performance suffered. Typically, an FCR of 1.2 to 1.4 is not uncommon for hybrid catfish in a similar system.

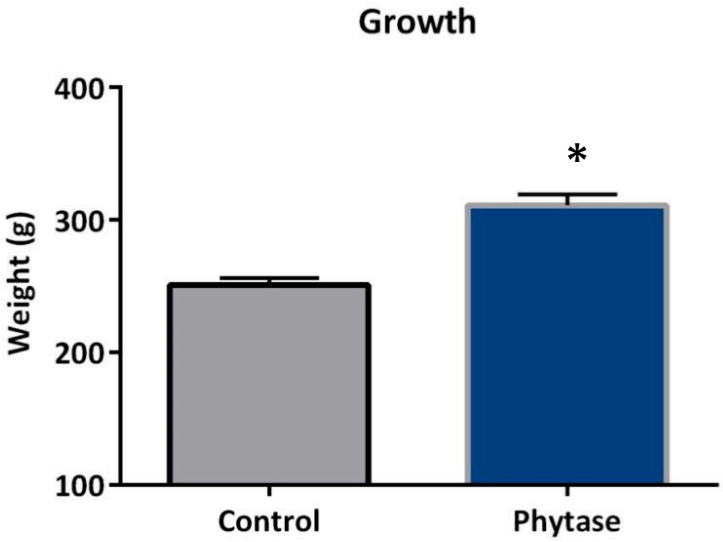


**Figure 6: Total growth per fish between the control and phytase treatments at the end of the culture period of the tank trial conducted in Auburn, AL. The asterisk denotes a statistically significant difference in the values $p < 0.05$**

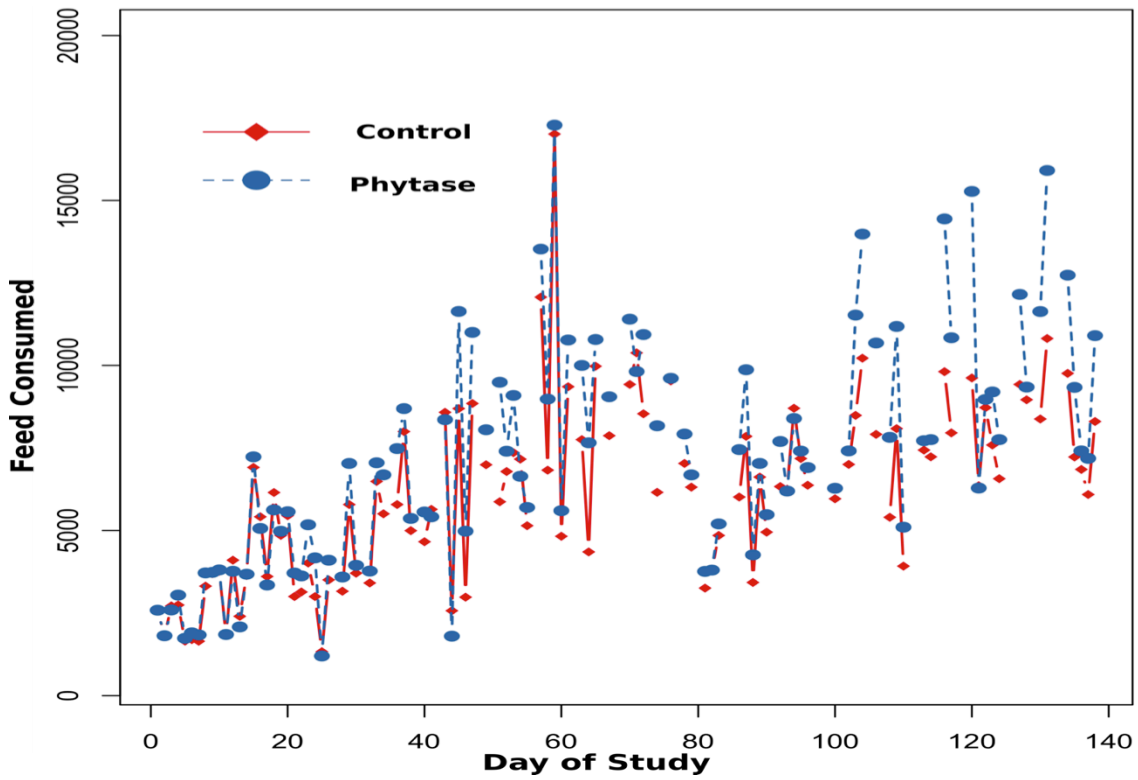


**Figure 7: Daily feed consumption (g) throughout the 140-day culture period of the tank trial conducted with hybrid catfish in Auburn, AL.**

*Stuttgart Pond Trial*

A larger, replicated pond study utilizing a commercial diet from the same supplier, but with a higher protein level (32% versus 28% that was utilized in the tank trial) and the same inclusion rate of Quantum Blue phytase (2500 FTU/kg) was conducted in Stuttgart, AR. Channel catfish fed diets containing phytase consumed more feed and gained more weight than fish fed the control ration without supplemental phytase (Figure 8).

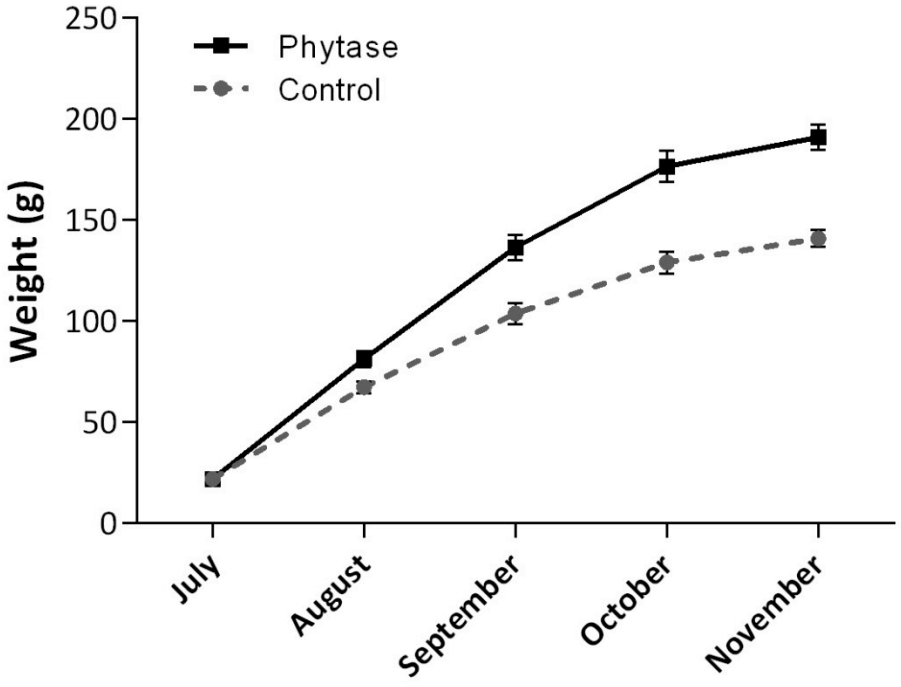


**Figure 8: Weight gain of channel catfish over the duration of a pond study where fish were fed a ration containing 0 FTU/kg (Control) or 2500 FTU/kg (Phytase) of Quantum Blue Phytase (error bars are ± SEM).**

Beginning in August, approximately four weeks after initiation of the study, fish fed the phytase-supplemented diet were significantly ($P<0.001$) larger in mass, and were statistically larger at each sampling time point thereafter with final individual weights of control fish averaging 140.8±8.1g and phytase-fed fish averaging 191.3±9.4g. Feed conversion ratio (FCR) was significantly different ($P<0.05$) for the two groups and was determined to be 1.76 for the control treatment compared to 1.43 for fish fed supplemental phytase. At the termination of the study, examination of feces collected from the distal intestine showed that phytase fed fish had significantly lower levels of inositol hexaphosphate ($P<0.05$; Figure 9). Control fish showed slightly lower levels of the phosphate esters inositol pentaphosphate (IP5), inositol tetraphosphate (IP4), inositol triphosphate (IP3), and inositol diphosphate (IP2), but the

differences were not significant (P>0.05). At the time of feces collection in the pond study, the water temperature of the culture ponds averaged 19±0.9 ºC (mean±SD).

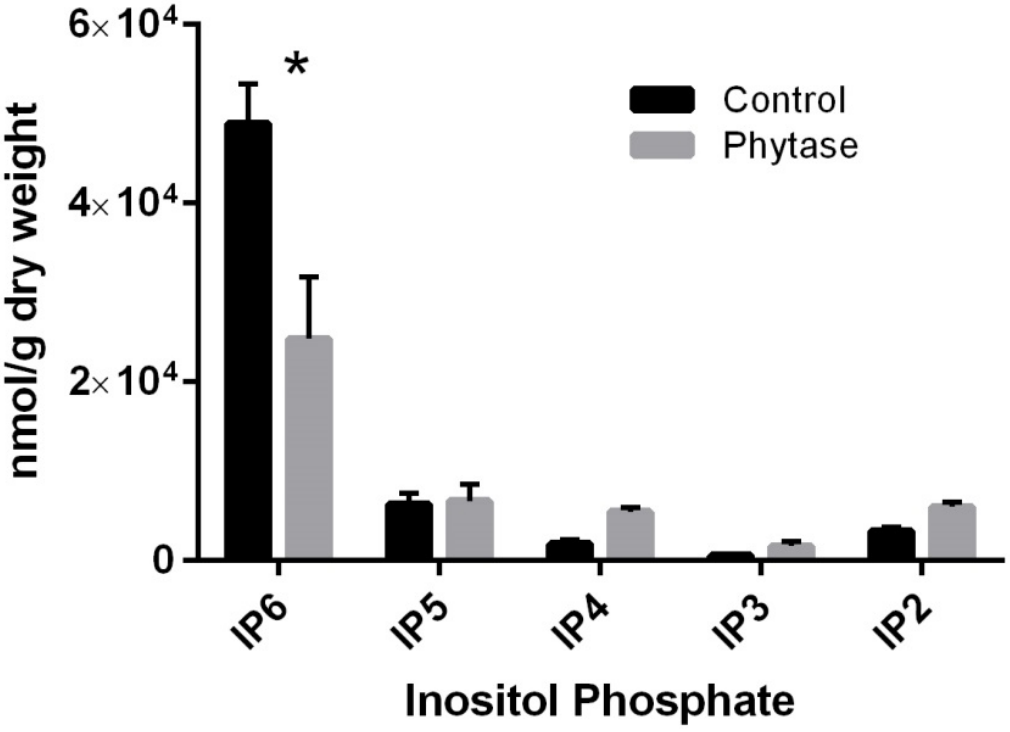


**Figure 9: Inositol phosphate ester content of feces collected at the termination of the pond study. Samples were collected 24 hours after feeding. Water temperature at time of collection was 19 C.**

Fish fed the diet containing supplemental phytase showed significant (P<0.001) increases in hematocrit values (Figure 10), beginning at 9 weeks post-initiation and continuing until the completion of the trial at 15 weeks.

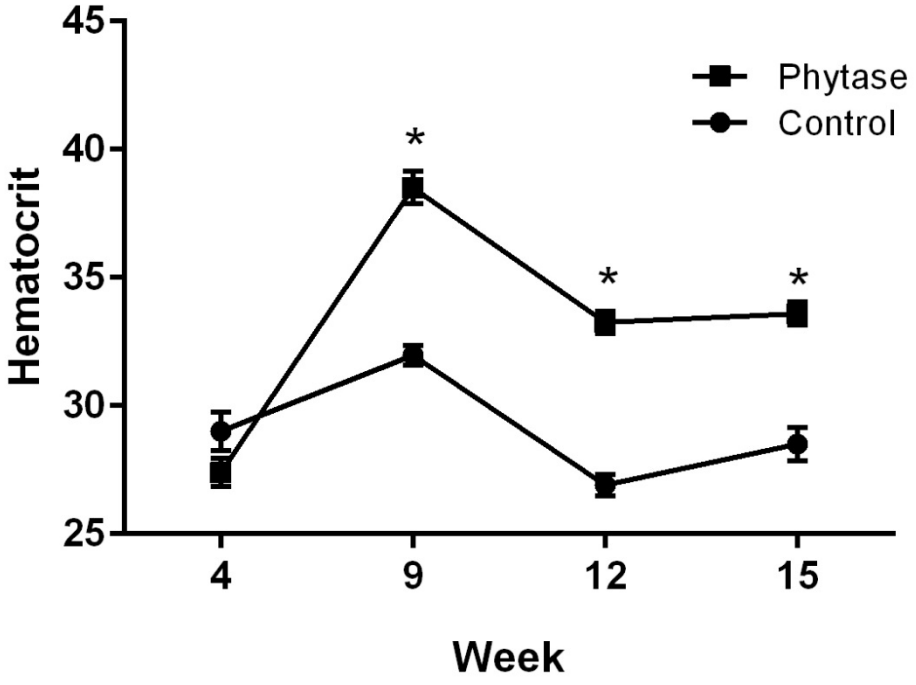


**Figure 10: Hematocrit values of channel catfish (n=24 per treatment, 8 per pond) fed a ration containing 0 FTU/kg (Control) or 2500 FTU/kg (Phytase) of Quantum Blue Phytase in an earthen pond trial (error bars are ± SEM). Asterisks denote statistically significant differences at P<0.05**

However, to rule out differences in red blood cell size/volume, we performed more comprehensive analyses of the blood including absolute red blood cell counts, which revealed a greater red blood cell content in the phytase treatment (P<0.001). Strikingly, fish fed the phytase superdosed feed had over 0.5 x $10^6$ more red blood cells/μL (an approximate 20% increase) than fish fed the control ration. Hemoglobin values were also significantly higher (p<0.001) in the

phytase treatment (>20% increase). Mean corpuscular volume was significantly higher ($p<0.05$) in fish fed the control diet indicating larger erythrocyte size (Table 5).

**Table 5: Effects of dietary phytase superdosing (2500 FTU/kg) on hematological parameters in channel catfish at the conclusion of the pond trial in Stuttgart, AR (n = 45 fish per treatment). Asterisks denote statistically significant differences at p<0.05.**

| Diet | TCC/μL ($10^6$) | RBC/μL ($10^6$) | WBC/μL ($10^5$) | Hemoglobin (g/dL) | Hematocrit (%) | MCV (fl) | MCH (pg) | MCHC (%) |
|---|---|---|---|---|---|---|---|---|
| Control | 2.21±0.06 | 2.01±0.06 | 1.87±0.09 | 7.92±0.18 | 28.5±0.65 | 145.6±4.0 | 40.9±1.7 | 28.2±0.8 |
| Phytase | 2.70±0.06* | 2.52±0.05* | 1.85±0.09 | 9.97±0.17* | 33.6±0.46* | 135.4±2.8* | 40.1±0.8 | 29.9±0.6 |

While the red cell compartment was augmented by dietary inclusion of phytase, there were no significant differences detected in the absolute number of white blood cells between either treatments. Microscopic examination of blood smears did not reveal any differences in the incidence of malformed red cells or alterations in nuclei as previously observed under conditions of anemia (Duncan and Lovell, 1994).

Fish fed the diet containing supplemental phytase showed significant increases of iron within the blood and numerous significant ($P<0.05$) differences in mineral content within the liver including over 40% more iron and substantial increases in phosphorus, sodium, copper, magnesium, potassium, sulfur, and zinc (Table 6).

**Table 6: Channel catfish mineral content in blood and liver (ppm) at the conclusion of a 15-week pond study (ppm ± SEM). Asterisks denote statistically significant differences at P<0.05**

| Mineral | Control Blood | Phytase Blood | Control Liver | Phytase Liver |
|---|---|---|---|---|
| Calcium | 586.3±21.5 | 609.5±27.1 | 100.14±3.9 | 105.9±5.8 |
| Phosphorus | 6783.5±60.4 | 6909.4±83.0 | 7809.1±185.2 | 8925.5±149.1* |
| Sodium | 14420.6±210.3 | 14186.9±356.3 | 2319.2±83.8 | 2675.9±85.8* |
| Aluminum | 8.2±2.4 | 4.8±1.6 | 20.1±3.6 | 26.3±6.7 |
| Copper | 1.2±0.2 | 1.4±0.3 | 8.21±0.4 | 9.6±0.4* |
| Iron | 1567.9±21.8 | 1680.3±28.2* | 87.6±6.9 | 146.6±8.8* |
| Magnesium | 727.8±12.5 | 723.0±7.8 | 574.7±17.7 | 650.2±12.3* |
| Manganese | 1.3±0.1 | 0.9±0.0* | 4.0±0.3 | 4.6±0.6 |
| Potassium | 7571.1±197.6 | 7608.0±198.8 | 9924.4±218.5 | 10943.3±202.3* |
| Sulfur | 4118.1±57.5 | 3958.3±46.6 | 6061.6±165.2 | 6633.3±197.3* |
| Zinc | 140.0±2.8 | 138.6±2.7 | 73.6±2.5 | 87.0±1.9* |

Accordingly, whole body proximate analysis showed that fish fed the phytase-supplemented diet had significantly higher ash 3.2% versus 2.5% in control fish (P<0.01). Fish did not differ in dry matter, lipid, or crude protein content. Proximate analyses and whole body mineral levels are shown in Tables 7 and 8 respectively.

**Table 7: Channel catfish whole body proximate analysis (%). Asterisk denotes statistically significant difference at P<0.05.**

| Diet | Dry matter | Ash | Lipid | Protein |
|---|---|---|---|---|
| Control | 27.4±0.23 | 2.5±0.23 | 7.4±0.17 | 16.7±0.08 |
| Phytase | 27.8±0.11 | 3.2±0.11* | 7.4±0.12 | 16.3±0.17 |

**Table 8: Whole body mineral composition (ppm) after 15-week pond trial ± STDEV. Asterisks denote statistically significant differences at P<0.05, n=20.**

| Mineral | Control Body (ppm) | Phytase Body (ppm) |
|---|---|---|
| Calcium | 17521.5±1342.4 | 25345.8±936.3* |
| Phosphorus | 14743.1±719.4 | 18837.9±387.5* |
| Sodium | 3399.9±73.7 | 3604.7±80.6 |
| Aluminum | 6.0±1.8 | 6.7±0.7 |
| Copper | 1.6±0.0 | 1.4±0.0* |
| Iron | 50.4±0.9 | 50.1±2.0 |
| Magnesium | 958.0±18.2 | 1136.8±63.3 |
| Manganese | 3.8±0.2 | 7.6±0.1* |
| Potassium | 10445.4±51.3 | 10150.0±162.3 |
| Sulfur | 7567.4±59.8 | 6922.9±47.3* |
| Zinc | 63.4±1.7 | 80.9±1.3* |

*Auburn Pond Trial*

Another larger scale study was performed at the EW Shell Fisheries Station in Auburn University, AL, this time utilizing a commercial diet sourced from the Alabama Catfish Feed Mill, Uniontown, AL. The feed is a bulk produced, 32% protein, extruded, floating catfish pellet determined to contain 1.9% phytic acid by DM and was top-coated at the same inclusion rate of 2500 FTU/kg AB Vista Quantum Blue Phytase (Plantation, FL). An additional treatment included the fortification of feed with AB Vista Econase XT xylanase enzymes at an inclusion rate of 16,000 U/kg in addition to the previously stated inclusion rate of phytase. None of the physical results that we calculated (yield, FCR, percent gain, or survival) were significantly

influenced by any treatment, however, it should be noted that during the first half of the trial, from April until June, we discovered that our control feed was consistently contaminated with phytase enzymes with concentrations upwards of 1500 FTU/kg. The Alabama Catfish Feed Mill is designed to produce a bulk product; therefore, during their peak of production, it became increasingly more difficult for them to produce feed without phytase enzymes. As a result, we began purchasing feed directly from their driers, as we, with help from the mill managers and technicians, were able to determine that their fat/ oil coating equipment was the source of the contamination. Despite remedying the situation, we fear our results were significantly impacted.

While physical data may have been affected, we still observed significant differences in the hematological parameters between treatments. Reminiscent of the results we observed in both Stuttgart studies, fish fed phytase-fortified feed benefitted from increased packed-cell volumes or hematocrits ($p < 0.002$), hemoglobin ($p < 0.05$), MCHC ($p < 0.05$), and MCV ($p < 0.05$). Unlike the latter mentioned studies, however, we did not observe significant differences in total cell counts or red blood cell counts although fish fed phytase-fortified feed were slightly higher (TCC 2.72* $10^6$/μL vs. 2.57* $10^6$/μL and RBC 2.59* $10^6$/μL vs. 2.44* $10^6$/μL). Interestingly, addition of the xylanase enzymes in addition to phytase enzymes seemed to be inhibit the effects of phytase fortification as hematological parameters resembled the control in fish fed this diet (Table 9).

**Table 9: Effects of dietary super dosing of phytase (2500 FTU/kg) and Xylanase (16,000 U/kg) on hematological parameters during pond trial conducted at the EW Shell Fisheries Station (n=40 per treatment, 10 per pond). Asterisks denote significantly different values compared to the control treatment ($p < 0.05$).**

| Diet | TCC/µL ($10^6$) | RBC/µL ($10^6$) | WBC/µL ($10^5$) | Hemoglobin (g/dL) | Hematocrit (%) | MCV (fl) | MCH (pg) | MCHC (%) |
|---|---|---|---|---|---|---|---|---|
| Control | 2.57±.32 | 2.44±.30 | 1.28±.31 | 8.53±1.1 | 31.32±4.7 | 128.99±16 | 35.17±4.1 | 27.40±2.3 |
| Phytase | 2.72±.33 | 2.59±.32 | 1.24±.35 | 9.23±.72* | 36.25±4.3* | 141.41±20* | 35.94±3.4 | 25.69±2.5* |
| Phytase + Xylanase | 2.56±.35 | 2.43±.34 | 1.33±.44 | 8.96±1.1 | 31.43±3.2 | 125.67±34 | 37.45±6.5 | 28.62±3.6 |

Analysis of blood and liver mineral showed significant increases in blood Potassium, and Magnesium of fish fed phytase fortified diets ($p < 0.05$). There were no significant differences in blood minerals between the control and fish that were fed feeds fortified with both phytase and xylanase enzymes (Table 10). In liver tissue, fish fed phytase fortified feeds benefitted from increase stores of calcium, copper, iron, magnesium, manganese, and phosphorus ($p < 0.05$). Fish being fed feeds fortified with both phytase and xylanase enzymes benefitted from increased stores of aluminum, calcium, iron, magnesium, and manganese ($p < 0.05$)[Table 11].

**Table 4: Hybrid catfish blood mineral analysis collected from fish reared in ponds at the EW Shell Fisheries station in Auburn, AL (n = 20 per treatment, 5 per pond) ± STDEV. Asterisks denote a statistically different means compared to the control ($p < 0.05$).**

| Mineral | Control Blood | Phytase Blood | Phytase+Xylanase Blood |
|---|---|---|---|
| Calcium | 358.7±62 | 338.9±58 | 421.1±109 |
| Phosphorus | 5814.3±150 | 5868.9±115 | 5624.2±346 |
| Sodium | 8967.21±1865 | 9482.6±700 | 7743.9±2309 |
| Aluminum | 2.04±.45 | 3.41±1.1 | 2.0±.35 |
| Copper | 1.1±.35 | 1.0±.23 | 1.4±.41 |
| Iron | 1780.8±168 | 1835.95±74 | 1739.8±128 |
| Magnesium | 449.7±29 | 493.26±23 | 423.1±63 |
| Potassium | 10520.4±314 | 10841.4±422 | 11333.0±849 |
| Sulfur | 2627.27±145 | 2451.0±168 | 2605.1±254 |
| Zinc | 104.9±9.3 | 105.9±7.6 | 112.3±16 |

**Table 5: Hybrid catfish liver mineral analysis collected from fish reared in ponds at the EW Shell Fisheries Station in Auburn, AL (n = 40 per treatment, 10 per pond) ± STDEV. Asterisks denote significantly different means compared to the control treatment ($p < 0.05$).**

| Mineral | Control Liver | Phytase Liver | Phytase+Xylanase Liver |
|---|---|---|---|
| Calcium | 49.50±40.9 | 95.44±44* | 92.8±54* |
| Phosphorus | 8062±1195 | 8642.13±1446* | 8399.08±859* |
| Sodium | 1638±181 | 1603.45±271 | 1625±215 |
| Aluminum | 2.31±3.1 | 2.05±1.2 | 3.6±2.7* |
| Copper | 9.9±2.8 | 11.52±4.4* | 9.87±2.2 |
| Iron | 458±252 | 684.73±260* | 633.86±218* |
| Magnesium | 559±103 | 601.08±92* | 602.05±71* |
| Manganese | 2±.92 | 2.56±1.1* | 2.51±.97* |
| Potassium | 9896±783 | 9679.77±1078 | 9665.33±477 |
| Sulfur | 4285±761 | 4555.72±903 | 4420±587 |
| Zinc | 78±14 | 82.48±18 | 79.05±11 |

Upon analysis of water samples collected for phosphorus determination in the lab of Dr. Claude Boyd, we found no significant differences in either soluble reactive phosphorus (Figure 11) or total phosphorus (Figure 12). It should be noted, however, that water exchanges within all ponds occurred as needed and was frequent throughout the study to replace water lost through evapotranspiration. Replenishing water in this manner may have affected our results.

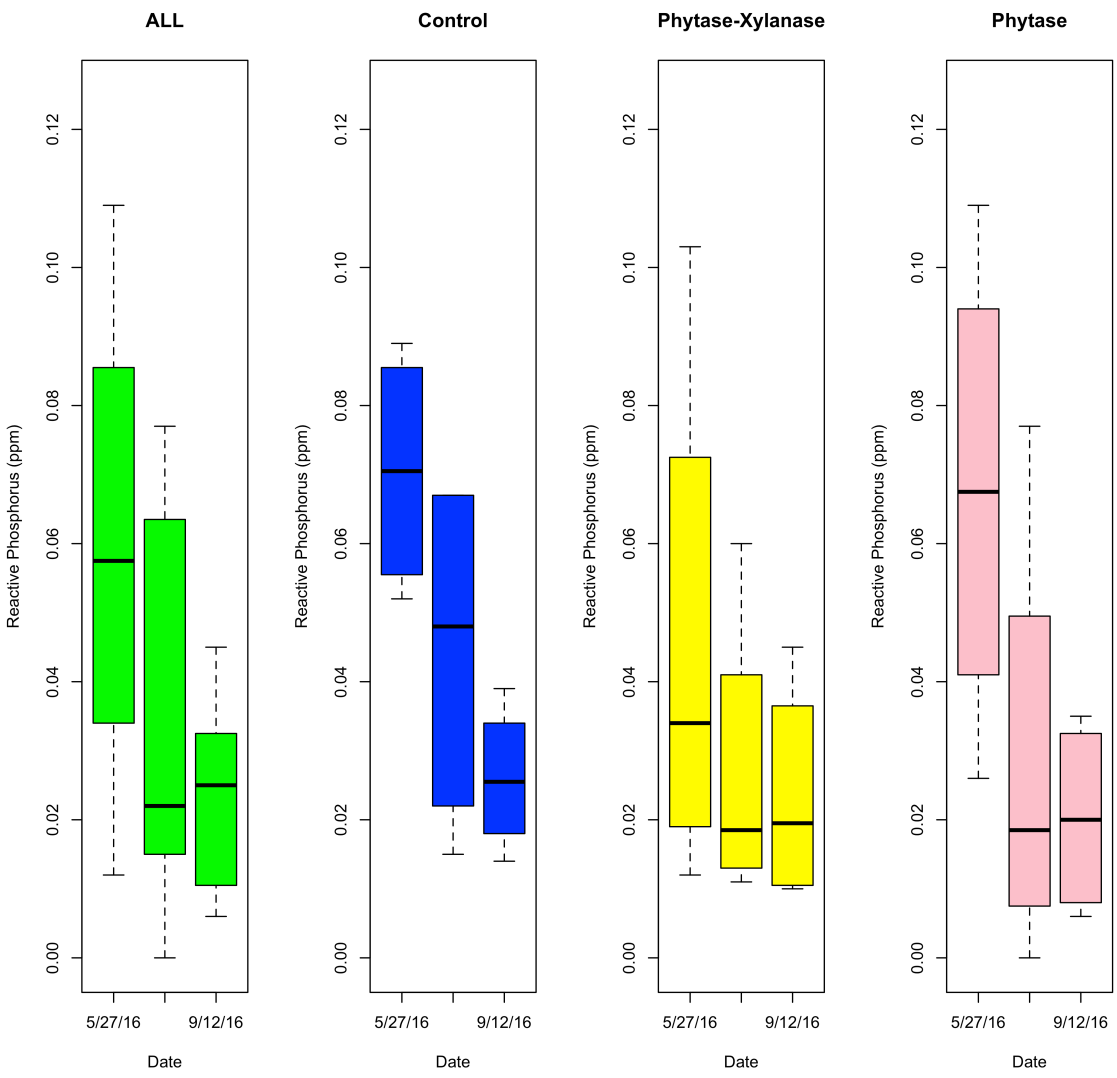


**Figure 11: Soluble reactive phosphorus in hybrid catfish ponds fed diets fortified with phytase or phytase + xylanase diets versus a control diet**

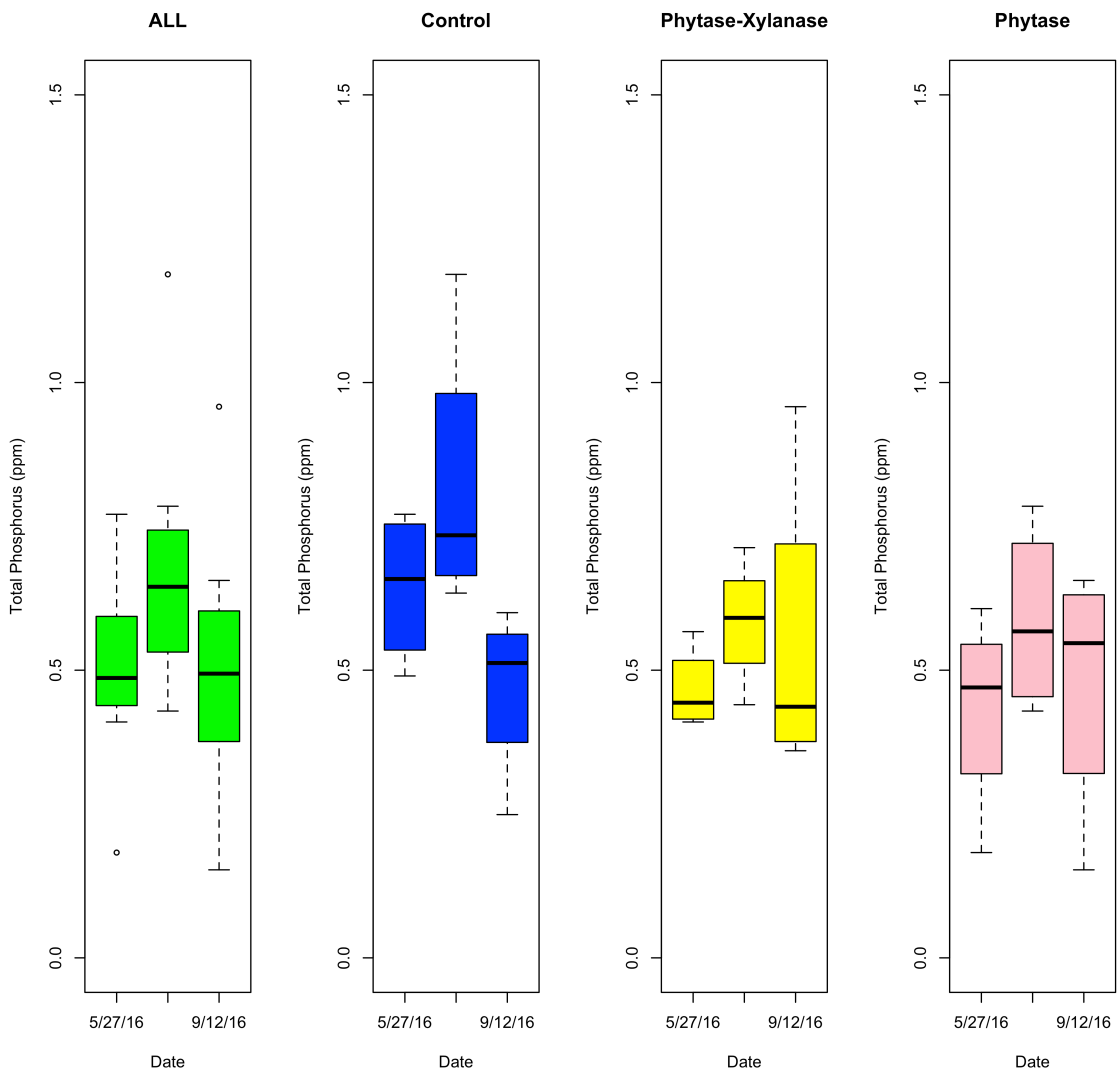


**Figure 12: Total phosphorus in hybrid catfish ponds fed diets fortified with phytase or phytase + xylanase diets versus the control.**

## Discussion

Overall, the present study indicates that fortification of commercial catfish diets with high "superdose" levels of phytase enzymes could significantly improve mineral absorption, hematological parameters, growth, and feed conversion. Specifically, and critical to our research question of combating cases of mild, chronic, anemia, we observed significantly higher levels of iron retention, higher concentration of RBC's, and elevated hematocrit and hemoglobin concentration as a result of phytase superdose fortification to a commercial catfish ration. Our results indicate that high phytic acid content of some plant-based diets may be contributing to the incidence of feed-related anemia on commercial catfish farms in the United States (Peatman and Beck, 2016).

Shifts in dietary content from fishmeal towards certain plant-based materials such as soybean meal, cottonseed meal, and wheat middlings, have been associated with reduced hematological parameters in some species of fish (Imanpoor et al. 2012; Barros et al. 2002). Furthermore, balance of dietary calcium and phosphorus can also impact hematocrit levels in catfish (Andrews et al. 1973) and vegetable based diets typical of commercial catfish industries are generally low in both calcium and phosphorus with the main ingredients all below 0.25% Ca and available P (except cottonseed meal comprising of 0.32% available P) (Batal and Dale, 2011). It is likely that these observations are a result of the phytate ($IP_6$) content of these diets. Due to the rise in price, fishmeal has largely been eliminated from commercial catfish diets and replaced with less expensive, but higher phytate sources of protein (Li and Robinson, 2013). Price constraints on lower phytate vegetable matter such as soybean meal and corn grain have pushed the inclusion of wheat middlings to inclusions of 25% or more of commercial diets (pers. comm., Southfresh Feeds). In depth analyses demonstrate that wheat middlings have the highest

concentration of phytic acid (0.8% phytate-P, 234% of the NRC [1993]) amongst feedstuffs (Rodriguez et al. 2008; Tahir et al. 2012; Batal and Dale, 2011). Tahir et al. (2012) also found increasing levels of phytate-P in soybean meal over the last two decades, likely due to genetic selection for improved nitrogen levels, inadvertently increasing phytate concentration as well.

The cumulative effects of shifts towards higher-phytate plant-based ingredients coupled with temporally rising phytate content in those ingredients has translated into commercial catfish diets often with greater than 2% phytic acid inclusion as seen in our survey and in the diets used in our studies, however, these concentrations vary; the same ingredients have shown to have differing levels of phytic acid based on region (Rodriguez et al. 2008; Tahir et al. 2012; Batal and Dale, 2011). Comparisons of results among phytase studies, without accounting for differing phytic acid levels in base diets are, therefore, often difficult. However, the studies outlined in Table 2 and described in previous sections support our findings in the present studies (Peatman and Beck, 2016).

Currently available phytases are unable to withstand the high temperatures and pressures of extrusion processing common to the manufacture of floating feeds for catfish and other fish species, necessitating the addition of ancillary equipment and change in mill logistics to allow spraying of phytase on finished pellets (Kumar et al. 2012; Lemos and Tacon, 2015). When only considering the gain of phosphorus and calcium as a result of phytase fortification and the elimination of the need for dicalcium phosphate supplementation, feed mills often outweigh the potential of phytase fortification with the capital costs associated with installing this equipment. Now, our current research accompanied with the latter mentioned studies that have increased our understanding of extra-phosphoric of phytase superdosing in addressing the anti-nutritional aspects of phytate may provide momentum for commercial feed mills to incorporate phytase in

commercial catfish diets. In fact, in 2016 Alabama-based catfish mills added post-extrusion spray equipment to their feed mills, opening the doors to industry-wide research and utilization of not only phytase, but also other nutritional enzymes. While our results in the Auburn studies were variable in terms of growth, the results we found in the hematology and mineral data were consistent throughout each trial in Auburn and Stuttgart. During our Auburn pond trial, we picked up a ton and a half of feed at a time from the Alabama Catfish Feed Mill and we tested aliquots of feed using an AB Vista dipstick test designed to non-quantifiably detect the presence of Quantum Blue phytase. We discovered that several batches of our control feed were contaminated with phytase. These results were furthermore confirmed by ELISA-based test, and measured levels of Quantum Blue phytases as high as 1500 FTU/kg in control batches of feed. Upon realization that the source of the contamination was the oil/fat coating, we began taking food directly from the driers and coating the feed with oil and enzymes ourselves, but the lack of control feed for an extended period of time likely affected the resulting data.

More trials in the future are required to explore whether or not phytase superdosing can correct anemia and to the extent to which it can do so if it can. These trials would involve using phytase to amend feeds that are observed to cause anemias of varying severities and tracking the potential hematologic recovery of the animals. These findings extend beyond the scope of anemia and may be of more broad, practical importance. Farmers devote tremendous effort and resources into the management of nighttime dissolved oxygen concentrations as both channel catfish and hybrid catfish (channel x blue) feed intake, growth, and overall yields are dramatically affected by chronic diurnal hypoxia (Green et al. 2012). It stands to reason that the gains in blood parameters that we have observed would allow fish to better cope with diurnal hypoxia common in intensive production settings. Earlier reports of blood cell counts in channel

catfish across four studies reported erythrocyte values of 2.44, 2.23, 2.16, and 2.19*$10^6$ cells/µL respectively (Grizzle and Rogers; Dogden and Sullivan; Haws and Goodnight; Scott and Rogers, 1981). In comparison, our present studies showed slightly higher (2.21*$10^6$, 2.44*$10^6$, 2.59*$10^6$) red cell counts while fish fed control diets were lower in the Stuttgart studies and Auburn tank study (2.01*$10^6$, 2.03*$10^6$). In the Auburn pond trial, RBC counts of the control fish (2.44*$10^6$) resembled those of fish fed phytase diets in the Stuttgart's studies; however, this could have been a lingering result of phytase contamination earlier in the study as they were lower, but not significantly, to the phytase treatment (2.59*$10^6$) and about equal to the phytase and xylanase treatment (2.43*$10^6$). The compositions of the diets from the historical data were not known and further, fish reared in Stuttgart were not subject to high stocking densities and therefore large, diurnal swings in oxygen as they would in a commercial setting. However, in Auburn, stocking densities were much higher and reached 10,000 lbs/ha at harvest.

Contrary to expectation, we did not observe the same hematological responses in the fish being fed feed fortified with phytase and xylanase enzyme as we did in those supplemented with only phytase. The concentrations of phytase were the same (2500 FTU/kg), and the xylanase dose (16,000 U/kg) was added in the same manner. We could come up with no clear explanation as to why we did not at least see the same effects as the phytase only feed. One potential explanation may be that potentially these enzymes mixed together before coating led to reduced efficacy of phytase or that xylanase activity in the gut led to phytase inhibitory effects. These fish (phytase + xylanase) still benefitted from increased hematological parameters such as hematocrit and hemoglobin. Still, more research is necessary to develop a comprehensive understanding of the optimal values or blood work signatures needed beforehand for catfish to tolerate, or be unaffected by hypoxic insult.

Further study is also needed to better understand the impact of shifting pond water temperatures on phytase performance (Morales et al. 2011). Catfish in the United States are actively feeding at water temperatures ranging from 15 C to 35 C (Tucker and Robinson 1990). As ectothermic animals, the body temperature of catfish approximates the water temperature. While phytase efficiency is known to decrease at lower temperatures (Dersjant-Li et al. 2015) necessitating higher amounts of phytase, systematic studies examining the optimal dose rates at varying temperatures are lacking for fish species. The majority of reviewed phytase studies in fish were conducted for a short duration in constant or near-constant temperature conditions (e.g. aquaria studies or mid-summer pond studies). Here, we observed superior phytate destruction and mineral absorption at 24 C (preliminary tank study) when compared to 19 C (temperature at November harvest for pond study). Follow-up studies will track phytate destruction and attendant effects across temperatures encountered over the catfish-growing season. In the future, commercial feed mills may optimize phytase dosing depending on pond water temperature, with the highest doses reserved for the early spring and late fall when temperatures are the lowest.

Finally, further study is needed to achieve optimum calcium to phosphorus ratios (Andrews et al. 1973) and, more broadly, dietary electrolyte balance (Dersjant-Li et al. 1999; Ravindran et al. 2008; Saravan et al. 2013), in light of gains in mineral absorption achieved through phytase superdosing. In commercial diets, such as used here, this work is critical, and formulation-specific, as sources and levels of phosphorus and other elements can differ substantially, even among diets for the same fish species. Achieving optimum nutrient balance is critical to avoid antagonistic mineral effects (Gatlin and Phillips 1989), achieve acid-base balance (Dersjant-Li et al. 1999), and reduce maintenance costs for homeostasis. In addition, exploration of the occurrence and effects of other antinutritional factors, such a tannic acid, may

be beneficial if found to be relevant in the aquaculture industry (Peatman and Beck, 2016). Further study into the molecular changes that occur within the animal as a result of benefitting from phytase superdosing may help us understand and target the underlying mechanisms that allow fish species to utilize phytase enzyme supplements most efficiently. The present study provides a solid foundation on which to build and continue the conversation of enzyme-mediated breakdown of catfish feedstuffs to improve physiological and hematological parameters of these fish for the benefit of the commercial industries. Continued work in this discipline could play a significant role in further growth and competiveness of United States farmed raised catfish for years to come.

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