Improving High Soy Feed Formulations for Florida Pompano Trachinotus carolinus through Phytase Supplementation.

by

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Abstract

The economically sound and sustainable aquaculture of many marine species in the coming years is dependent on the reduction of fish meal and the increased inclusion of plant protein sources. Florida Pompano perform well on soy-based diets. However, these diets may be improved through the use of certain enzymes to improve digestibility coefficients. Research in other marine fish has shown the potential for phytase supplementation to improve the digestibility of phosphorus, protein, and possibly other minerals. Three growth trials were conducted in recirculating systems at the Claude Peteet Mariculture Center, Gulf Shores, Alabama, USA, which both consisted of 12 tanks stocked with twenty pompano juveniles in each. Fish were fed four times daily one of the four, randomly assigned diets in each trial. The first trial consisted of both low and high levels of phytase (Basal+CaP, Basal, 500 FTU, 4000 FTU, trial 1). The following two trials consisted of a greater spectrum of phytase supplementation levels in both the low (200 FTU, 400 FTU, 600 FTU; Trial 2) and high (1000 FTU, 2000 FTU, 4000 FTU; Trial 3) ranges to further elucidate the response to this enzyme. Digestibility trials were carried out for trial 1 and trail 2. Growth, retention, and digestibility results did not indicate a significant difference in growth or performance with enzyme supplementation. It is possible that the phosphorus requirement for Florida pompano was met or that the enzyme was damaged during storage; resulting in the lack of the observable benefit of phytase supplementation.

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ADMD	Apparent dry matter digestibility
APD	Apparent protein digestibility
AED	Apparent energy digestibility
APHD	Apparent phosphorus digestibility
FCR	Feed conversion ratio
TGC	Thermal growth coefficient
CP	Crude protein
CL	Crude lipid
FW	Final weight
CaP	Calcium phosphate
Progain	Protein gained
Prooffered	Protein offered
ANPR	Apparent net protein retention
Phsfed	Phosphorus fed
Phsgain	Phosphorus gained
Phsret	Phosphorus retention
Egain	Energy gained
Eoffered	Energy offered
ANER	Apparent net energy retention

Introduction

Biology of Pompano

The Florida pompano, *Trachinotus carolinus*, is a member of the jack family, *Carangidae*, and is endemic to the western Atlantic Ocean and Gulf of Mexico. The species is migratory, following warmer water seasonally, from Cape Cod, Massachusetts to south-eastern Brazil. It is especially common along the Florida coast and hence its common name. Colloquially, Florida pompano is simply referred to as pompano. Florida pompano reach a maxium length of about 63.5 cm at a weight of approximately 7.5 lbs. Individuals over 4 lbs are uncommon although, on rare occasions pompano have been caught weighting 10+ lbs (Gilbert 1986).

Florida pompano typically have 23 to 25 dorsal fin rays and 21 to 22 anal fin rays. No teeth are present on the tongue during any stage, no bars or patterning along the body side, and no enlargement of ribs or elongated fins. The body is compressed with both dorsal and ventral sides similar. The eyes are small and the head slopes into a blunt snout. The anal-fin base is shorter than the second dorsal-fin base and the pectoral fins are small. The scales are small and cycloid. Florida pompano are blue-green on the dorsal that shades into a silver color on the sides. The stomach area, head, and fins are often yellow and the intensity of color varies between individuals. The tips of the dorsal fins may also appear darker or near black at times and the pelvic fins are white (Gilbert, 1986).

There are twenty recognized species within the genus Trachinotus, with two other species in the western Atlantic and Gulf of Mexico aside from *T. carolinus*. Permit, *Trachinotus falcatus*,

have fewer dorsal soft rays, fewer anal soft rays, enlarged 2-4 ribs, teeth on the tongue of juvenilles that are absent in adults, juvenilles have a bright orange anal fin in contrast to the yellow found on *T. carolinus*, and the maximum size is much larger at approximately 20-30 lbs. The other species is *Trachinotus goodie*, the palometa, also has fewer dosal soft rays and fewer anal soft rays, four narrow bars on the upper part of the body, the anterior dorsal and anal soft rays are elongate (Gilbert, 1986).

Florida pompano generally feed upon many invertebrates including, polychaetes, mollusks, crustaceans, invertebrate eggs and larvae throughout the day and have been described as "grazers". Their pharyngeal plates are well developed as would be expected of a species that routinely feeds on organisms with a hard exoskeleton or shell. Pompano are generally less selective with their feeding choices when young and become more selective as they age (Finucane 1969; Gilbert 1986). In 1969 19 adult pompano were sampled and all 19 had fed exclusively on *Brachidontes exustus*, the scorched mussel (Finucane 1969). Pompano collected in the vicinity of oil rigs have been found to feed on penaeid shrimp.

Florida pompano larvae spend most of their early larval stage in offshore waters, moving towards shore at approximately 10-30mm. low turbidity surf along beaches is the preferred nursery habitat for juveniles (Fields 1962; Gilbert 1986). Estuaries, shallow bays, piers become more common as juveniles grow. Pompano remain near shore until anywhere from 60-120mm in length at which point they depart for deeper waters. This migration seems to be modulated by

temperature. When Florida near-shore waters drop below 19° C, the vast majority of juveniles have abandoned beaches (Fields 1962; Gilbert 1986).

Pompano prefer warm water ranging from 28 to 32° C with critical temperatures being 10 and 38° C. (Gilbert 1986). Pompano have been recorded in waters with salinity as low as 9 g/L though this appear to be quite the exception (Gunter and Hall, 1963). Pompano are typically found in waters with a salinity of 28-37 g/L (Gilbert 1986).

Aquaculture and Wild Fishery

Florida pompano, *Trachinotus carolinus*, is a prized species among both recreational and commercial fishermen. Pompano command high prices, selling for $10.12 per pound in 2014 when supply was at its lowest (NOAA, statistics 2014). Due to the 1995 ban of entanglement gear and regulations to the offshore fisheries the commercial catch of Florida pompano account for only a small portion of pompano harvested in the United States. Recreational anglers continue to be the primary harvesters of Florida pompano (FWC 2014). As there is limited commercial catch but certainly market demand, the species has a promising outlook for aquaculture. Indeed, the trends in supply and price have gone in opposite directions since roughly 1998; commercial catch continues to decrease while price increases (Figure 1.). This is indicative of a resource where there is considerable demand yet low supply.

As Florida pompano are a highly prized marine species with a high market value and no substantial commercial fishery; aquaculture of the species by private corporations has expanded significantly over the past several years. AquaGreen, Pompano Farms, Troutlodge Marine Farms,

Proaquatix, and Mariculture Technologies International are all producing Florida pompano within the United States. However, production of the species within the US remains relatively small compared with other aquaculture species. In 2014 a total of 436 tons of Florida pompano were produced along with 110,258 tons of Asian pompano globally. All pompano species produced globally constituted 119,450 tons in 2014 (FAO, 2016). Within Asia, *Trachinotus blochii*, is the leading species of pompano being cultured and the market for Asian pompano, within Asia, is far more established relative to the market for Florida pompano within the United States.

Pompano have many characteristics that make them an ideal aquaculture species outside of their high market value. Firstly, broodstock and larval rearing techniques are established for the species (Hoff, 1972, 1978a, 1978b; Main et al., 2007) and continue to be improved upon (Weirich and Riley, 2007). Pompano possess a wide salinity tolerance. Research from Mote Marine Laboratory has shown no significant difference in growth or mortality for pompano grown in as low as 10 ppt salinity (Main et al., 2010). However, lower salinity does come with some caveats, that is, pompano are more sensitive to ammonia and nitrite at lower salinites, requiring more upkeep (Weirich and Riche, 2006). Pompano grow relatively quickly and may reach market size within roughly one year and possibly as short as nine months under optimal conditions (McMaster, 2006). Market size for the species is roughly 1-1.5 pounds (Main et al., 2007). Florida pompano will readily accept a variety of pelleted feeds that are lower or completely void of fishmeal; instead incorporating other animal protein sources in conjunction with plant protein sources (Waldemar and Davis, 2012). This has the potential to significantly lower the costs of production for the producer as well as sway public opinion favorably as production of the species could have a low

"fish in : fish out" ratio. Indeed, Florida pompano produced in recirculating systems have received a final rank of "Best choice" from the Monterary Bay Seafood Watch which may influence public perception and market appeal for the species (Welch, 2013).

As with many species, a great deal of research has been carried out in an effort to optimize feed formulations for Florida pompano. Feeds may account for 50-70% of the cost of cultured fish. Dietary protein being the most expensive component with marine species typically needed between 40-50% protein for optimal growth and performance (Riche, 2015). Alternatives feeds, particularly plant based feeds, have potential to reduce costs of production for various species and past research has shown that Florida pompano perform well on such diets as long as supplementation of limiting nutrients is met (Waldemar and Davis, 2012). As the cost of fish meal continues to rise it is increasingly more important to seek out methods for improving alternative feed sources. In addition, the improvement of plant based feed sources can help to reduce the dependence on fishmeal in aquaculture, potentially enhancing public perception. The use of fish meal and fish oil as feed constituents is suspected to decline over the long term. The reasons for this speculative decline include: static and potential diminishing supply of wild forage fish, increased demand (static/decreasing supply combined with greater aquaculture globally), increased costs driving aquaculture producers to use other ingredients, and public pressure for sustainability (Tacon and Metian, 2008).

Alternative Feeds

Before alternative feeds can be explored, dietary requirements need to be established. Fortunately for Florida pompano, this is already largely the case. The dietary requirement of protein and lipid in pompano feeds has been investigated (Lazo et al., 1998; Riche, 2009; Williams, 1985). Williams et al. (1985) reported that the ideal level of fish oil in a 42% protein fish and soybean meal diet was between 4 and 8%. At 12% percent body fat increased and protein gain was significantly lower. Like other marine species, Florida pompano likely have an omega-3 fatty acid requirement. As oils other than fish oil become more common as alternative lipid sources more research will be needed to determine specific fatty acid requirements in Florida pompano. Lazo et al. (1998) determined that the ideal protein content for Florida pompano fed a fishmeal based diet was 45%. This 45% protein inclusion increased final weight gain, protein efficiency ratio, feed conversion efficiency, and feed conversion efficiency; whilst simultaneously reducing percent daily feed consumption. In 2009, Riche reinvestigated the dietary protein needs of Florida pompano to achieve maximal growth. In his experiment the dietary requirement of Florida pompano for protein was between 35.6 and 36.6% of the diet on a dry matter basis.

Several different plant based products have been investigated as a replacement protein source. Soybean meal possesses many of the qualities required to be a viable alternative to fish meal and has been previously shown to support Florida pompano growth (Lech 2012). Soybean meal is widely available, has a favorable amino acid profile, is easily shipped and stored, and is priced competitively with other plant based food sources (Gatlin et al., 2007). Despite these qualities there are still limitations to the use of soybean meal and other plant based protein sources. Among these are low levels of certain amino acids, anti-nutritional factors, and adverse effects on

the intestine such as enteritis (Gatlin et al., 2007). In comparison with fish meal, soybean meal is relatively low it total crude protein. This in turn makes diets completely devoid of animal protein increasingly difficult for species that require higher levels of dietary protein, such as marine carnivores. Advanced processing methods have the potential to concentrate traditionally lower-protein sources resulting in products such as soy protein concentrate (Salze et al., 2010). However, this increased inclusion and concentration of plant based alternatives carries with it the potential to amplify the deficiencies of these alternatives. That is, more highly concentrated anti-nutritional factors, decreased palatability, and potentially difficulty extruding the product to make a pellet. Soybean meal is low in methionine and lysine, two of the ten essential amino acids in fish diets. Soy protein concentrate and soy protein isolate approach or exceed the levels of lysine and methionine that are limiting in soybean meal. However, due to processing costs these products are not yet economical (Gatlin et al. 2007). Therefore, it is prudent to investigate the dietary requirement of these amino acids in practical soy-based diets for Florida pompano. Gatlin et al. recommends >3.5% lysine and >1.5% methionine be included in alternative diets. Indeed this is close to the values reported by others for these two amino acids. Patro et al. (2011) presented data demonstrating that the dietary requirement for maximal growth in Florida pompano was between 1.17% and 1.60%. Below 1.17%, pompano had a decreased growth rate. Nunes (2014) reports the dietary requirement of lysine as a percent of diet in many species around 1.5-2.2% with most species being around 2%.

Another concern is taurine. Taurine biosynthesis varies among fish species (Goto et al., 2003). This variability amongst fish species to synthesize taurine coupled with the fact that plant

based ingredients and poultry by-product contain low levels of taurine, it is entirely possible that taurine may be limiting in diets of some fish species that are low or devoid of fishmeal. It has been shown that taurine supplementation can enhance pompano growth and survival (Rossi and Davis, 2012, 2014). In a dose-response study it was purposed that Florida pompano have a dietary taurine requirement of 0.54-0.65% of the diet (Salze and Davis, 2015).

One of the primary problems with plant based alternatives is that many contain anti-nutritional factors that may limit their inclusion potential in some species. There are a variety of different anti-nutritional factors depending on the plant from which the protein source is derived. Among these are protease, amylase, and lipase inhibitors, lectins, saponins, oligosaccharides and non-starch polysaccharides, glucosinolates, tannins, lectins, phytoestrogens, alkaloids, antigenic compounds, gossypols, and phytic acid. These anti-nutritional factors have been shown to influence a variety of physiological processes from digestion to endocrine function to organ dysfunction (Francis et al. 2001). Generally, these negative qualities are of less concern as plant based proteins are used only partially in conjunction with fish meal. However, as the inclusion of plant based proteins continues to increase and levels of fishmeal decrease, these negative attributes possess the potential to impact growth and performance to an extent that could hinder aquaculture endeavors. There are a variety of negative consequences that follow higher inclusion of anti-nutritional factors. Firstly, reduced palatability. A given species may simply be less likely to consume a feed high in plant products as opposed to one high in fish meal or other animal protein. This gives rise to another potential area of research; attractants to be used in plant based feeds. There is also evidence that anti-nutrients may alter the intestinal microflora in ways that are not

fully understood as of yet (Krogdahl et al., 2010). There is an interplay between various minerals in their bioavailability; plant based diets that are low in certain minerals and thus supplemented may have nutrient imbalances that need to be further understood and overcome. Certain anti-nutrients, e.g. phytate, may completely inhibit the bioavailability of certain nutrients unless deliberate action is taken to confront this problem. Additional concerns are intestinal dysfunction, immune modulation, pancreatic hypertrophy, hypoglycemia, and liver damage all of which could lead to problems affecting cost in an aquaculture setting (Krogdahl et al., 2010).

Phosphorus deficiency

One of the anti-nutritional factors found within soybeans is phytate. This is the primary storage form of phosphorous in plant seeds, accounting for approximately two-thirds of the total phosphorous bound as phytate (Cao et al., 2008). As phytate is indigestible by fish, or any monogastric in significant amounts, inorganic phosphorous must be supplemented to meet dietary requirements. The dietary requirement of phosphorus ranges from 0.3 to 1.5% of the diet depending on species and the phosphorus sources within the diet (NRC, 2011). Phosphorus is currently the most expensive mineral supplement in aquatic feeds. Additionally, as supplementation of phosphorus increases, less is utilized as a percent included in the diet (Fox et al., 2006). Therefore, increasing the bioavailability of phytate bound phosphorus already present in soybean meal has the potential to reduce cost.

Phosphorus plays a variety of roles in fish nutrition. Most obvious is the role in calcification and hard tissue formation. This not only includes bones but scales and teeth as well. In addition to

the role of phosphorus in hard tissue formation, phosphorus has roles in intermediary metabolism as well and is required for nucleotides, phospholipids, coenzymes, deoxyribonucleic acid (DNA), ribonucleic acid (RNA), adenosine triphosphate (ATP), and adenosine diphosphate (ADP) (NRC, 2011). Thus, the potential to affect growth and survivability does exist. A deficiency in phosphorus will not only impair the formation of hard tissues in the short term but lead to impairments in metabolism in the long term (Sugiura et al., 2004). This impaired metabolism may ultimately reduce growth and feed conversion. Furthermore, phosphorus deficiencies have been indicated in decreased immune function and disease resistance (NRC, 2011).

Day and McCollum demonstrated the outcome of phosphorus deficiency in rats in 1939. Rats grew slowly for the first 5 to 6 weeks. In the following 2 to 3 weeks, these rats collapsed and died. Rarefaction of bone had occurred as phosphorus was mobilized from hard tissues to permit the growth of soft tissue. In addition, phosphorus deficiency was characterized by increased excretion of calcium. As calcium and phosphorus are both integral to bone mineralization, phosphorus deficiency resulted in calcium being excreted as the optimal level of bone mineralization is impeded by the lack of phosphorus. However, phosphorus will not greatly increase in excretion as it is being utilized by soft tissue for growth. Baeverfjord, Åsgård, and Shearer (1998) demonstrated in Atlantic salmon, *Salmo salar*, fed phosphorus deficient diets; that a deficiency was first observed by reduced whole-body calcium and phosphorus levels along with the development of softer and more frequently malformed bones. In later stages of growth salmon were severely growth impaired and suffered increased mortality compared with salmon fed a diet replete in phosphorus. Fjelldal, 2012 demonstrated that phosphorus deficient diets during juvenile

salmon rearing predisposes Atlantic salmon to vertebral deformities later in life. Presumably, due to sub-optimal bone mineralization during early developmental stages. It has also been shown in rainbow trout, *Oncorhynchus mykiss*, that phosphorus deficiency leads to decreased bone density and an increased incidence of skeletal malformation (Fontagné et al., 2009).

Furthermore, excessive phosphorus levels have also been shown to lead to reduced growth and in some cases reduced survivability (Satoh et al., 1993). A possibility is that excessive phosphorus inclusion leads to the reduced absorption of trace elements such as zinc. Decreased intestinal absorption of zinc has been observed in rats fed excess calcium (Heth et al., 1965). In addition this effect seems to be increased with higher levels of phosphorus alongside excessive calcium levels (Heth et al., 1966).

In addition to possibly interfering with optimal growth and development, excess dietary phosphorus can contribute to water quality problems as well as eutrophication through effluents to connecting bodies of water. Diets consisting of supplemental phosphorus need to reach a middle ground where the dietary requirement for the given species is met and a minimum amount of excess phosphorus is released into the culture water or connecting bodies of water (Sugiura et al., 2004). Excessive phosphorus load can lead to the fouling of water quality in both recirculating systems as well as eutrophication in the environment by providing a nutrient needed for algal growth. As aquaculture increases in the coming years and more aquaculture effluent is released into the environment, it is necessary to find methods to reduce the amount of phosphorus loading (Herath and Satoh, 2015).

Phytate

The dietary requirement for phosphorus in fish is clearly influenced by a multitude of factors. Notably, the inter-relationship with calcium. However, with the increased inclusion of plant based protein sources, phytate has become a major concern. Phytic acid, Myo-Inositol hexakisphosphate, and its salts, phytate, are the primary storage form of phosphorus in plant seeds. Both phytic acid and phytate are found simultaneously in plant seeds and consequently it is common to find them used almost interchangeably (Reddy and Sathe, 2002). Phytate is indigestible for monogastrics and consequently feeds high in phytate are often over supplemented with inorganic phosphorus to account for this difference. As mentioned earlier, undigested inorganic phosphorus as well as phytate bound phosphours then enters the culture system or connecting bodies of water; leading to fouling of the culture system or connecting bodies of water by providing a substrate for undesirable algal blooms(Bohn, 2008).

Phytate strongly binds with various minerals, particularly divalent cations such as copper, calcium, magnesium, zinc, and iron (Cheryan, 1980). Once these phytate-mineral chelates are formed, the minerals are not available for digestion by monogastrics, thus phytate may reduce the digestibility of other minerals aside from phosphorus. Phytates may also react with proteins or with minerals and proteins forming phytate-protein complexes or phytate-mineral-protein complexes. This may also reduce protein bioavailability (Knuckles, 1985). Additionally, it has been shown that phytate may bind with starch, potentially impeding carbohydrate digestibility (Yoon, 1983).

Phytase

The addition of the enzyme phytase has been shown to increase phosphorous digestibility and retention and therefore lessens the need for supplementing with inorganic phosphorous, assisting in the formulation of cost-effective feeds (Sajjadi 2004). Phytases have been used in agriculture for over twenty years, mostly in swine and poultry feeds (Lemos, 2016). Recently, their use has expanded into aquaculture as plant based alternative feeds have become more common. Currently, the bulk of phytase products are histidine acid phytases isolated from recombinant strains of fungi and yeasts (Greiner and Konietzny, 2010). Most of these phytases are coming from *Aspergillus* species (Lemos, 2016). Phytases are commonly utilized in a dried powder form or liquid suspension; occasionally encapsulated phytases have been used (Kumar et al. 2012; Vandenberg et al. 2011).

Phytases may be included within complete feeds with expected activity within the low pH of the gut. However, phytases may also be used to treat feeds before mixing, known as pre-condition or dephytinization, this form of treatment may be most useful when the feed is to be subjected to high temperatures that could destroy the enzyme, species that have a less acidic pH within the gut, or species that are going to be reared at a low temperature (Mwachireya et al., 1999; Denstadli et al. 2007) as activity of the enzyme is pH and temperature dependent. The optimal pH for phytase activity is 4.5-6.0 with a temperature of 45-60°C (Cao et al., 2007).

Some pre-treatment options involve soaking, cooking, germination of seeds, and fermentation. These methods work by relying on endogenous phytases. However, phytate must be

reduced to a very low level to increase mineral digestibility and thus exogenous phytases are warranted (Kumar, 2010). Pretreatment options may also involve exogenous phytases. For instance, Fortes-Silva et al. (2011) top coated feeds that had already been extruded in a spray form. In this case, increased phosphorus retention was seen in bone while no differences were seen in growth in European sea bass (Fortes-Silva et al., 2011). In 1995, Rodehutscord and Pfeffer showed that phosphorus digestibility increased from 25 to 57% in trout reared at 15°C fed a plant based diet supplemented with phytase. However, when reared at 10°C phosphorus digestibility only increased from 6 to 25% (Rodehutscord and Pfeffer, 1995). In such cases it may be most effective to pretreat the feed before processing. The intent would be to optomize pH and temperature while limiting the amount of water used to avoid problems with subsequent extrusion. During this process other enzymes, such as carbohydrases, may also be utilized to reduce non-starch polysaccharide content (Denstadli et al., 2007).

Sajjadi and Carter (2004) demonstrated the use of phytase with a canola-meal based diet in Atlantic salmon, *Salmo salar*. A 12 week growth trial was carried out that resulted in no difference in weight gain, feed intake, or survivability. However, the addition of either phytase or inorganic phosphorus resulted in significantly higher bone and whole body phosphorus when compared to phosphorus deficient diets. Additionally, phosphorus digestibility and retention were significantly higher while phosphorus load was significantly lower compared to diets supplemented with inorganic phosphorus. Similar results were found by Forster et al. (1999) in canola-meal based diets for rainbow trout. Phytase supplementation in these diets resulted in greater phosphorus availability from phytate bound phosphorus. The digestibility coefficients for

phytate bound phosphorus were directly related to the dosing of the enzyme. Diets not supplemented with phytase had 0% phytate bound phosphorus digestibility however, with 4500 FTU/kg 45.4% of the phytate bound phosphorus was available.

Vandenberg et al. (2011) investigated the use of encapsulated phytase in rainbow trout, *Oncorhynchus mykiss*. Supplementation with phytase improved apparent digestibility coefficients for energy, protein, ash, P. Phosphorus retention, final growth, and feed efficiency were also enhanced by phytase supplementation. Encapsulated phytase did not perform as well when compared to non-encapsulated phytase, presumably due to reduced contact of the enzyme with the phytate.

One of the minerals that has received attention aside from phosphorus in plant based diets is zinc. Fish meal contains adequate levels of zinc and supplementation in fish meal based diets is likely unnecessary. However, plant based protein sources are lower in zinc and high in phytate that further reduces bioavailability (Welker et al. 2016). Zinc is required for normal growth and development in aquatic organisms and it may be absorbed from the water making the establishment of a dietary requirement complicated. Furthermore, there is an interplay between calcium, phosphorus, and zinc in that calcium and phosphorus levels may influence zinc digestibility (Welker et al. 2016).

Zinc's largest impact on growth and performance is due to the high number of zinc dependent enzymes. Zinc deficiency may result in reduced growth, cataracts, dwarfism due to impaired bone metabolism, fin and skin erosion, skeletal abnormalities, reduced enzyme activity,

endocrine disturbances, and increased mortality (Welker et al., 2016). Thus, it is prudent to prevent zinc deficiencies and plant based diets are where these are most likely to occur.

Much like phosphorus, zinc is often over supplemented in plant based diets as it is both naturally low in these feeds and less bioavailable due to phytate-mineral interactions (Satoh et al., 1993). A study by Sebastian et al. in 1996 revealed that phytase increased the retention of zinc in low phosphorus plant based diets by 62.3%. In addition, the retention of P, Ca, and Cu was also improved (Sebastian et al., 1996). Morales et al. demonstrated that phytase supplementation improved digestibility of P, Ca, Mg and Zn and retention of P, Ca, and Mg in plant based diets for juvenile rainbow trout (Morales et al., 2015). Cheng et al. (2003) also found that phytase supplementation significantly increased the digestibility of zinc with 7.2% digestibility with no phytase in a plant based diet but 85.1% digestibility with 400 FTU/kg (Cheng et al. 2003).

Phytase has also been reported to improve protein digestibility by breaking down insoluble phytate-protein complexes. However, the studies on this are quite mixed. Several studies have reported improved protein digestibility with phytase supplementation in plant based diets for Rainbow trout (Vielma et al., 2004; Vandenberg et al., 2012), Atlantic salmon (Storebakken et al., 1998), catfish spp. (Zhu et al., 2014). Others have not detected such an effect (Morales et al., 2015; Riche et al., 2001).

The primary objective of this study is to establish the efficacy of phytase supplementation to improve nutrient digestibility and retention, particularly of phosphorus, in soy based diets for Florida pompano. In addition, these studies may help bolster the current research demonstrating

that soy based diets for Florida pompano are a viable alternative. The first experiment was designed to compare low and high phytase supplementation to two different phytase free soy diets one of these diets contained supplemental inorganic phosphorus while the other did not. This would not only give a measure of phytase efficacy in both the high and low dosing ranges, but also allow for comparison with calcium phosphate supplementation. The second and third experiments were designed to further elucidate the optimal dosing regimen of phytase in soy based diets for Florida pompano in the low and high ranges, respectively.

Materials and Methods

Trial design

Three growth trials were carried out. Each trial consisted of four treatments with varying levels of phytase supplementation. Phytase is measured in terms of phytase units (FTU) and one phytase unit is defined as the amount of the enzyme needed to liberate 1 µmol of phosphorus/min from phytate at a pH of 4.5 and temperature of 60ºC (Wilkinson et al. 2013). The phytase used in each trail was Phytase Quantum Blue from AB Vista, Marlborough, Wiltshire, UK. Each diet was formulated to contain approximately 400 g/kg^{-1} protein and 80 g/kg^{-1} lipid. All diets contained 15% poultry meal and ~47% soybean meal (Tables 1, 2, 3) and were based on previous work developing low animal meal feed formulations for this species. Proximate analysis of each of the diets was carried out at University of Missouri, Agricultural Experiment Station Chemical Laboratories, Columbia, Missouri, USA (ESCL) and may be found in Tables 1, 2, 3 for the respective trials.

For the first trial, treatment 1 (Basal P replete) contained 400 $g\ kg^{-1}$protein, 79.7 $g\ kg^{-1}$ lipid, with supplemental calcium phosphate (1.13% total phosphorus). Treatment 2 was the basal diet without supplemental inorganic phosphate (0.65% total phosphorus). Treatments three and four were the same as treatment 2 with the exception of the inclusion of phytase at 500 and 4000 active phytase units (FTU) per 100g of feed for treatments 3 and 4 respectively. The next two trials were then designed to refine and confirm dose responses, in terms of growth, nutrient retention, and nutrient digestibility, of phytase supplementation by looking at traditional low levels of

supplementation (Trial 2; 0, 200, 400 and 600 FTU) and high levels (Trial 3; 0, 1000, 2000, 4000 FTU).

Diet preparation

Experimental diets were produced at Auburn University, School of Fisheries, Aquaculture and Aquatic Sciences, Auburn AL, USA. The diets were prepared through the mixing of pre-ground dry ingredients along with menhaden fish oil in a food mixer (Hobart A200FT, Troy OH, USA) for 15 minutes. Boiling water was added to the mixture to help ensure the appropriate consistency of the mix for pelleting. The mash from each diet was passed through a 3.0 mm die in the grinder. Wet diets were then placed in a forced air drying oven (<45 °C) until a moisture content of less than 12% and crumbled to achieve a range of sizes for feeding. Dried diets were stored at -20 °C until use.

Culture Conditions

Two groups of Juvenile Florida pompano (~1g mean weight) obtained from Proaquatix in Vero Beach, FL. One group was acquired in May of 2014 and the other in May of 2015. The husbandry procedures for each group were the same until the initiation of growth trials. These pompano were transported via a hauling tank equipped with supplemental oxygenation to the Alabama Department of Conservation and Natural Resources Marine Resource Division, Claude Peteet Mariculture Center (CPMC), in Gulf Shores, Alabama. After arriving at the mariculture center, pompano were acclimated to natural seawater sourced from the Gulf of Mexico. Pompano

were then transferred to a series of nursery tanks equipped with a biological filter, air lift pumps and aeration provided by a regenerative blower and air diffusers. The pompano remained in these tanks until an adequate size for the initiation of growth trials was reached; that is, large enough to consume the experimental feed. During this period pompano were fed to satiation with a 40% crude protein and 12% crude fat commercial diet (EXTR 400, Rangen Inc., Angelton, TX).

The growth trails were conducted separately in their own independent systems. Each system consisted of twelve culture tanks of 800-1000 L capacity each, sump tanks, bead filter, biological trickle filter, circulation pumps, and aeration through diffuser stones. The systems were located in greenhouses and therefore subjected to a natural lighting of approximately 14 hour light / 10 hour dark. Each trial consisted of four treatments each with three replicates.

Trial 1 was conducted over a 9 week period using a 1000 L culture tanks stocked with 20 fish with an average weight of 7.95g. Trial 2 was carried out in the same recirculating system as trial one and conducted over 8 weeks. Tanks were stocked with 20 fish per tank with an average of 22.5 grams initial weight. Trial 3 was carried out in a recirculating system containing 800L culture tanks and conducted over an 8 week period. Due to the smaller tank size as well limitations on fish, 10 fish of an average initial weight of 23.8 grams were stocked in each tank. A sample of fish was frozen at the start of the trials for later whole body analysis. Upon termination of the growth trials, four fish from each tank were frozen for whole body analysis.

Temperature, dissolved oxygen, salinity and pH were monitored and recorded twice daily using an YSI 556 multi probe meter (Yellow Spring Instruments Co., Yellow Springs, OH, USA). total ammonia nitrogen was determined twice a week using an ion selective electrode (Orion EA 940, Thermo Electron Corporation, Beverly, MA, USA). Nitrite and Nitrate were tested weekly with a LaMotte test kit (3354-01, 3352-01, LaMotte Company, Chestertown, MD).

Feed management

The amount of food offered was based on a percentage of bodyweight adjusted after sampling the fish every two weeks as well as observations of feeding behavior. Four feedings were spaced throughout the day occurring at 700, 1100, 1500, 1900 hours. Every two weeks fish were weighed and counted. After sampling fish were placed in chloroquine dip at a concentration of 63 mg/L (MP Biomedicals, Solon OH) followed by a freshwater dip for approximately one minute to help reduce the possibility of parasitic infection. Feed conversion survival and growth rate were calculated at each sampling. One week preceding each sampling the fish were treated with 20% copper sulfate (EarthTec, Earth Science Laboratories, Bentonville, AR, USA) at a dosage of 0.18ppm to prophylactically treat for parasites.

The formula used for determining growth and feed calculations are listed below:

Survivability = (remaining individuals/total initial individuals) x 100

Biomass = total weight of all individuals in a tank

Mean weight = total weight of all individuals/number of fish

Weight gain (%) = ((final body weight – initial body weight)/(initial body weight) x 100)

Feed conversion ratio = (total amount fed/weight gain)

Thermal growth coefficient = 100 x (final mean weight^0.33 – initial mean weight^0.33)/(average temperature x days)

Digestibility, Retention, and Biochemical Analysis

Apparent digestibility coefficients were determined for dry matter (ADMD), protein (APD), energy (AED), and phosphorus (APHD). Fish were fed the same diets from the growth trial with an inert marker added (chromic oxide at 10 g kg^{-1}). Fish were acclimated to the diet three days prior to manual stripping, in which pressure is applied to the abdomen to force fecal extrusion, and fed on the same schedule as the growth trials at 0700, 1100, 1500, and 1900 hours based on a fixed percentage of body weight. On the day of stripping, fish were fed on a staggered feeding schedule. Four tanks were fed per feeding block and stripping occurred three hours after feeding to allow appropriate time for digestion. Each feeding block contained one tank from each treatment to minimize any influence of time of day on sampling. Feeding occurred at 0600, 0800, and 1000 hours, with stripping at 0900, 1100, and 1300. Fish were anesthetized using MS-222 and then manually stripped by applying pressure to the gut to express and collect feces. Each fecal sample was dried in an oven at 105 °C until a constant weight was obtained.

Protein, used to determine apparent digestible protein (APD), was determined using the standard micro-Kjeldahl method (Ma and Zuazaga, 1942). Phosphorus (APHD) was analyzed by a modification of Fiske and Subbarow (1925). Gross energy content (AED) was determined using a semimicro-bomb calorimeter (Model 1425, Parr Instrument Co. Moline, IL, USA), and chromic oxide content using McGinnis and Kasting (1964) method.

Digestibility coefficients were calculated according to Cho et al. (1982) as is depicted in the following formulae:

$$\text{ADMD}(\%) = 100 - \left[100 \times \left(\frac{\%\ \text{Cr2O3 in feed}}{\%\ \text{Cr2O3 in feces}}\right)\right]$$

$$\text{APHD, APD, and AED}\ (\%) = 100 - \left[100 \times \left(\frac{\%\ \text{Cr2O3 in feed}}{\%\ \text{Cr2O3 in feces}} \times \frac{\%\ \text{nutrient feces}}{\%\ \text{nutrient feed}}\right)\right]$$

Whole body analysis was also carried out through independent lab analysis at the University of Missouri, Agricultural Experiment Station Chemical Laboratories, Columbia, Missouri, USA (ESCL) for crude protein, crude fat, crude fiber, moisture, ash, and phosphorus For trials 2 and 3, whole body mineral analysis was also conducted for: phosphorus, sulfur, potassium, magnesium, calcium, sodium, iron, manganese, copper, and zinc. Nutrient retention was determined based on differences in whole body nutrient content of the initial and final samples.

Statistical Analysis

Statistical analysis were conducted using SAS (V9.1 SAS Institute, Cary, NC, USA). Initial weight, biomass, number, final weight, TGC, percent weight gain, FCR, and survival were analyzed using a one-way analysis of variance to determine statistical significance amongst the treatments in each growth trial. For the digestibility coefficients a one-way analysis of variance was also used to determine statistical significance amongst treatments. Apparent digestibility of dry matter (ADMD), apparent energy digestibility (AED), apparent protein digestibility (APD), and apparent phosphorus digestibility (APHD) were all analyzed for statistical significance. Whole body retention data was also analyzed for protein gained, protein offered, protein retention, phosphorus fed, phosphorus offered, phosphorus retention, energy gained, energy offered, and energy retention. Student-Neuman Keuls multiple comparison test was used to define significant differences amongst the treatment means.

RESULTS

Water quality

In trial 1, the water quality parameters (mean ± standard deviation) for dissolved oxygen were maintained at 5.84 ± 0.4 mg L^{-1}, temperature at 27.4 ± 1.5 °C, salinity at 25.5 ± 3.6 g L^{-1}, pH at 7.4 ± 0.7, total ammonia nitrogen at 0.01 ± 0.02 mg L^{-1}, nitrite at 0.42 ± 0.32, and nitrate at 9.75 ± 3.78.

In trial 2, water quality parameters for dissolved oxygen were maintained at 5.1 ± 0.6 mg L^{-1}, temperature at 28.9 ± 1.6 °C, salinity at 36.1 ± 3.4 g L^{-1}, pH at 7.8 ± 0.2, total ammonia nitrogen at 0.02 ± 0.04 mg L^{-1}, nitrite at 4.11 ± 4.9, and nitrate at 7.37 ± 6.23.

In trial 3, water quality parameters for dissolved oxygen were maintained at 5.0 ± 0.7 mg L^{-1}, temperature at 29.03 ± 1.8 °C, salinity at 35.95 ± 3.95 g L^{-1}, pH at 7.9 ± 0.3, total ammonia nitrogen at 0.04 ± 0.06 mg L^{-1}, nitrite at 1.72 ± 2.6, and nitrate at 8.12 ± 5.63.

Growth trials

Trial 1 was conducted over a 9 week period with the biological response of the fish to the various diets presented in Table 4. Initial weight ranged from 7.6g to 8.2g and was not significantly different. There were no significant differences in initial weight ($p = 0.9901$); survival ($p=0.5957$); FCR ($p=0.2493$); percent weight gain ($p=0.1365$); or TGC ($p=0.0559$). Statistically significant differences were found for final weight ($p=0.0294$) and biomass ($p=0.0204$) among treatments. Final weight was highest for fish offered the diet supplemented with 500 FTU. Final weight for the 4000 phytase unit treatment was similar to the basal

treatment with supplemental calcium phosphate. Treatment 2 had the lowest final weight; this was the basal diet with no supplemental phosphorus or phytase. Biomass differences reflected final weight with the 500 FTU treatment having the highest biomass while the unsupplemented basal diet was the lowest and the 4000 FTU treatment was similar to the basal diet supplemented with CaP.

For trial 2, initial weight ranged from 20.40g to 23.50g and was not significantly different. Statistical analysis revealed no statistical differences on growth parameters for this trial (Table 5).

Trial 3, initial weight ranged from 22.05g to 25.30g and was not significant. Statistical analysis revealed significant differences in FCR and biomass between treatments. The basal diet and the 4000 FTU treatment were similar to each other with the basal diet having the lowest FCR. The 1000 FTU and 2000 FTU treatments were similar with the highest FCRs. Biomass was highest for the 4000 FTU treatment and similar to the basal diet. The 1000 FTU treatment was statistically less than the basal and 4000 FTU treatments in biomass but greater than the 2000 FTU treatment which had the lowest biomass. Growth data for trial 3 may be found in table 5.

Whole body retentions

Based on nutrient intake and whole body gains in nutrients levels whole body retention were determined. Phosphorus fed was significantly higher for the basal diet supplemented with inorganic phosphorus and fish fed the basal diet with CaP had significantly lower phosphorus retention than the other three treatment groups. However, phosphorus fed and phosphorus

retention were not significantly different among the basal or phytase supplemented diets. Whole body proximate analysis data for trial 1 fish may be found in table 6 and retention data in table 8.

No statistically significant differences were found for retention in trial 2 (Table 9).

For trial 3, protein retention was significantly different. The basal and 4000 FTU treatments were similar while the 1000 and 2000 FTU treatments were significantly lower. Whole body crude lipid was also found to be significantly different. Fish fed the basal diet and 4000 FTU diet had similar whole body lipid retention while fish fed the 1000 FTU diet had significantly less lipid retention. Fish fed the 2000 FTU diet were significantly lower than all other three treatments in terms of whole body crude lipid. No significant differences were found for mineral retentions. Whole body proximate analysis data are displayed in table 7 and retention data is presented in table 9.

Digestibility

Digestibility analysis was carried out for dry matter, protein, energy and phosphorus. For trial 1, significant differences were found for ADMD, APD, and APHD. Fish fed 4000 FTU had the highest ADMD while those fed the basal diet had the lowest. Fish fed the basal+CaP diet and the 500 FTU diet were similar with an intermediate value. APD was lowest for those fed the 500 FTU diet but was similar for all other treatments. APHD was highest for fish fed 4000 FTU and similar for the other three treatments. All digestibility results for trial 1 are presented in table 10. No significant differences were found for digestibility in trial 2 (table 11).

Discussion and Conclusion

The use of phytases in plant based feeds has the potential to reduce the influence of one of the most commonly discussed antinutritional factors in soybean meal, phytate. Currently, phosphorus is the most expensive mineral supplement and is often supplemented in excess when plant based ingredients dominate the feed formulation. Phytases possess the potential to liberate phytate-bound phosphorus, and potentially other nutrients, thereby improving growth and performance, cost effective feed formulations, and lowering phosphorus waste coming from aquaculture facilities. Given the high market value of pompano, their tolerance of soybean based diets, and the trends in the cost of fishmeal, this research seeks to improve soybean based feed formulations for the Florida pompano; through the use of an enzyme that has over twenty years of support through its use in terrestrial agriculture. Additionally, this research may aid in the formulation of plant based diets for other marine fish species. The potential of phytase to impact growth in a positive manner certainly exists through the improvement of nutrient digestibility. Improvements in protein, energy, and mineral digestibility have all been observed and indeed in several studies these have correlated with significant improvements in growth and feed efficiency (Lemos and Tacon, 2016).

There was only slight indication that phytase supplementation had a significant impact on growth, retention, or digestibility and these indications came entirely from trial 1. The 500 FTU treatment in trial one significantly outperformed other treatments in this trial in both biomass and final mean weight. However, as stated previously, retention and digestibility are far better

measures of phytase efficacy. The phytase supplemented treatments in trial one did not significantly differ from the unsupplemented basal diet in terms of phosphorus, energy, or protein retention. Interesting results were found for phosphorus digestibility in trial one; in which the 4000 FTU treatment had a significantly higher phosphorus digestibility while other treatments were similar to one another. However, contrary to what would be expected, this increase in phosphorus digestibility did not translate into an increase in phosphorus retention and potential reasons for this will be explored herein. Indications of phytase supplementation effectiveness were not seen in the subsequent second and third trials for potential reasons that will also be explored in the following paragraphs.

A simple but possible explanation as to why indications of phytase activity were observed in the first trail, but not in the second or third thrial, is that the percent weight gain was much lower in the second and third trials (404.28% ± 25.74%; 355.07% ± 39.13%) compared to the first trial (762.48% ± 49.05%). There are a couple of reasons for this difference in percent weight gain. Firstly, the initial weight of the fish in the second and third trials (22.57g ± 0.85g; 23.8g ± 1.0g) was greater than in the first trial (7.95g ± 0.19g). Additionally, the first trial was run for one week longer than the second and third trials. These two factors created a situation in which the percent weight gained would be greater in the first trial. Smaller fish grow more rapidly and the additional week added time for growth. As the percent weight gain was greater, the tissue replacement was also presumably greater, and therefore an observable impact of phytase may have been more likely seen in the first trial due to this reason.

A second possible explanation for the lack of measures that would indicate phytase efficacy is that the phosphorus requirement for Florida pompano was met by the ingredients alone. This would significantly if not completely reduce the differences in phosphorus retention that may be indicative of phytase activity. In a study with rainbow trout, Riche and Brown (1996) found that the digestibility of phosphorus is maximal at the dietary requirement and then declines when the phosphorus content exceeds the requirement. Regulation of phosphorus absorption occurs in the blood (Sajjadi and Carter, 2004). When the blood phosphorus level is saturated, absorption decreases and therefore digestibility coefficients decrease. Therefore, one would expect fish fed a diet meeting the requirement of phosphorus to have similar digestibility values and no significant differences in phosphorus retention; whether the diets were supplemented with phytase or not.

Phosphorus requirements are based upon bioavailable phosphorus. In order to determine whether the phosphorus requirement may have been met, it is first important to attempt to estimate the bioavailable phosphorus within these experimental diets. Across each of the experiments phosphorus content as a percentage of the diet ranged from .65 to .77% based on proximate analysis; with the exception of the supplemental calcium phosphate treatment in experiment one at 1.13%. Riche and Brown (1996) reported phosphorus bioavailability in solvent extracted soybean meal to be 22.5% while Sugiura et al. (1998) reported 22%. Some reports of phosphorus availability in soybean meal have been as high as 29% (Wilson et al. 1982). These numbers are reasonable as 50% to 80% of the total phosphorus in soybean meal is

in phytate form (Kumar et al., 2012). Presumably, the remaining phosphorus would be available for digestion.

Nearly half of the phosphorus in all diets, aside from treatment one, was from poultry by-product meal (0.33%) while the other half was from soybean meal (0.32%) in the formulation. As some diets reached as high as 0.77% phosphorus, there was clearly some variability when diets were produced. This variation could be from when the diet was actually produced, that is, it was not done with the highest accuracy, or the phosphorus sources themselves varied from their normal levels. Phosphorus availability from poultry by product meal will depend on the solubility of the salts within the meal and thus it is not likely that it will be entirely available; though it would be expected to be far more available than soybean meal. Variability in the total phosphorus content of animal protein sources is not uncommon and literature reviews have shown that anywhere from 16-42 g/kg of phosphorus is in fish meal, 25-56 g/kg in meat and bone meal, and 17-35 g/kg in poultry by product meal (Hua et al., 2005). Various processing methods, source materials, and equipment differences, all contribute to this variability.

To further compound the issue of estimating bioavailable phosphorus, not all of the phosphorus in animal protein sources is bioavailable. For example, the digestibility of phosphorus in a fish meal diet for rainbow trout ranged from 21.5 to 55.4% (Riche & Brown, 1996). Sugiura et al. (1998) found the digestibility of phosphorus in poultry by product meal for Coho salmon to be 68% and for Rainbow trout to be 64%. Essentially, there are two broad categories of phosphorus that one would expect to find in an animal protein source. These are

"bone phosphorus" and "organic phosphorus". Bone phosphorus is that bound to calcium while organic phosphorus is found in molecules such as nucleic acids, amino acids, and phospholipids (Hua et al., 2005). When high variability of phosphorus digestibility is seen with animal based protein sources, it is likely differences in the salt forms between diets that contributes substantially to such a difference.

There is little information of the specific forms of phosphorus that compose various animal protein sources. Approximately 85-88% of phosphorus in vertebrates exists as bone phosphorus while the remaining 12-15% exists as organic phosphorus (Hua et al., 2005). However, this does not translate directly to processed animal meal products. Hua and Bureau found that bone phosphorus accounted for 53-95% of the total phosphorus in various animal protein meal. Specifically, Hua et al. (2005) found that 60-91% of the total phosphorus in poultry by product meal was bone phosphorus. Bone phosphorus is poorly bioavailable to fish. Nordrum, Åsgård, and Shearer investigated the availability of different inorganic phosphate salts and fish bone meal in Atlantic salmon (*Salmo salar*) and found that fish bone meal had the lowest digestibility. Additionally, phosphorus digestibility from multiple sources may not be additive (Nordrum et al., 1997; Hua et al., 2005).

Estimating phosphorus bioavailability based on Riche and Brown's (1996), Sugiura's (2004), and Wilson's numbers for phosphorus digestibility in soybean meal and Sugiura's 1998 numbers for the digestibility of phosphorus in poultry by product meal; the bioavailability of

phosphorus in these diets would range from 0.33% to 0.37% based on an average phosphorus formulation of 0.77% from proximate analysis.

The dietary requirement of phosphorus ranges from 0.3 to 1.5% of the diet depending on species and the phosphorus sources within the diet (NRC, 2011). Such variability in the dietary requirement is not likely solely species dependent; but also dependent upon the phosphorus content of the ingredients, the distribution of salt forms within ingredients, and potentially the interaction with other ingredients such as calcium. Phosphorus dietary requirements as low as 0.3 and 0.34% have been reported for subadult and fingerling channel catfish (*Ictalurus punctatus*) by Eya and Lovell in 1997 and Wilson et al., 1982, respectively. A requirement of 0.44% for yellowtail amberjack (*Seriola quinqueradiata*) was reported by Sarker et al. in 2009. While on the lower end of the dietary requirement spectrum at a speculated 0.33 to 0.37% bioavailable phosphorus, it is possible that sufficient phosphorus existed in these experimental diets for Florida pompano. Additionally, it is possible that more than 29% of the phosphorus found in the soybean meal, and more than 68% of the phosphorus in the poultry by product meal, of these experimental diets was available to the fish which would further elevate the speculated bioavailable phosphorus.

The fish used in these experiments were young, roughly 8 grams in the first trial and 22-24 grams in the second and third trials at the beginning of the experiments. If, like fingerling catfish, young Florida pompano have a dietary requirement for phosphorus that is low; it is unlikely differences would have been seen in phosphorus retention or growth parameters with

phytase supplementation in these experimental diets. Therefore, it would be prudent to investigate the dietary phosphorus requirement in Florida pompano before attempting to elucidate ideal phytase supplementation levels in soy based diets. Another method would be to remove poultry meal entirely from the diets. This may lead to other complications such as palatability, extrusion capability, and other nutrient deficiencies. However, this would likely create a situation in which phosphorus is more likely to be limited and therefore the beneficial effects of phytase could be observed.

The biggest concern raised when working with phytase is whether or not the enzyme could have been active in dephytinization of the feeds before extrusion or the possible destruction of the enzyme at extrusion temperatures or boiling water prior to extrusion. Additionally, the phytase used in these experiments was granulated and stored in a freezer. Many enzymes cannot withstand temperatures below freezing; however, this is somewhat dependent upon the preparation of the enzyme product. For instance, the liquid preparation of Phytase Quantum Blue from AB Vista (Marlborough, Wiltshire, UK) is reported by the manufacture to be more sensitive to freezing than the granulated product used in this experiment.

Phytase is predominately active at a low pH and higher temperature. The optimal pH for phytase activity is 4.5-6.0 with a temperature of 45-60°C (Cao et al., 2007). The phytase used in these experiments was a third-generation modified E coli phytase from AB vista, Marlborough, Wiltshire, UK known as phytase Quantum Blue 5g with an analyzed activity of 7269 FTU/g (AB vista, Marlborough, Wiltshire, UK). Based on characteristics of the individual enzyme optimal

activity conditions may vary. The optimal range of activity for Phytase Quantum Blue has been found at 3.5-5.0 pH and is nearly inactive at a pH of 7.0 (Menezes-Blackburn et al., 2015). The pH of fish stomachs ranges from 1 to 6 depending on the species, time after feeding, diet composition, and feeding frequency (NRC, 2011). Tap water was used to achieve the proper consistency of the feed prior to extrusion. The average pH of tap water in Auburn, Alabama is 7.28 according to the Water Works Board of Auburn Consume Confidence report in 2012. Therefore, it is highly unlikely that the enzyme would have been active at the pH of the mixing feed.

It is also possible that the phytase could have been degraded or inactivated by high temperatures during processing; in commercial phytase applications, this is certainly something to take note of and apply the best application of the enzyme given the production method. However, it is unlikely that the temperature was sufficiently high enough to negatively affect the enzyme with the moderate temperatures used to produce the feeds in these experiments. Phytase Quantum Blue 5g is a heat stabilized phytase. Improving heat stability of enzymes can be achieved in several ways; coating with fats or waxes, granulation, genetic engineering, or simply application of the enzyme post pelleting (Wilkinson et al. 2013). Phytase Quantum Blue appears to withstand conditioning temperatures up to approximately 92.5°C; after which the enzyme is increasingly less recoverable from the diet (AB Vista, 2015). Under true extrusion temperatures, 100+°C, it is entirely possible that the enzyme would have be destroyed during processing if the procedure used in this experiment were utilized for producing the diets. However, true extrusion was not utilized in this experiment and what was done to produce the diets is more readily

compared to cold pelleting. While no temperature measurements were taken of the feed while in the barrel of the extruder or directly after exiting the apparatus; the feed was easy to handle and nowhere near a temperature that would scald the hand of the feed producer (~100+°C). A reasonable estimate, based upon feel alone would be 40-50°C; well below the temperature at which Phytase Quantum Blue is destroyed. Additionally, while it is true that boiling or near boiling water was used to achieve a consistency of extrusion, water was added slowly and rapidly mixed into the total feed. Throughout the process of adding water, the feed was handled to test for the appropriate consistency. At no point was the feed harmful or overly hot to the touch and a temperature of 40-50°C again seems to be a likely temperature during this stage of preparation.

Additionally, some phytases have been shown to be susceptible to proteolytic cleavage. Fortunately, bacterial phytases appear to be pepsin tolerant while fungal phytases seem to show reduced activity after exposure to pepsin preparations (Menezes-Blackburn et al., 2015). The phytase used in this experiment, Phytase Quantum Blue, is an *E. coli* derived phytase and thus reduced activity due to hydrolysis by proteolytic enzymes is not suspected.

Yet another concern is the even distribution of the enzyme throughout the feed. Phytase Quantum Blue 5G is a granulated phytase. During preparation of the feed, the phytase granules were mixed with other dry ingredients in a Hobart A200ft mixer, Troy, OH, USA for 15 minutes. During this time, boiling or near-boiling water was slowly added to achieve the proper consistency. The concern here would be that complete solubilization of the phytase granules did

not occur. If the granules were not completely solubilized, the enzyme may not have been evenly distributed throughout the feed. Without testing, it cannot be known if the enzyme was well distributed. Phytase activity, phytate content (g/100g), and phytate bound phosphorus (g/100g) were all tested and results are presented in table 12 (Enzyme Services & Consultancy, Unit 6, Innovation & Technology Centre, Tredomen Park, Ystrad Mynach, UK). Based on these tests, the small amount of variability is not expected to be a concern between diets. Phytate content ranged from 0.72-0.86 g/100g feed and phytate bound phosphorus ranged from 0.274-0.384 g/100g feed. However, without several replicates the question of even distribution remains. Had the enzyme not been evenly mixed this would likely interfere with the aim of this study. That is, rather than consuming the enzyme with each feeding, fish would have consumed various doses of the enzyme over the course of the experiment. For example, fish may have consumed a portion of feed with an incompletely solubilized phytase granule and thus received a large dose of phytase during that singular feeding event. Later, those same fish may have received a portion of the feed containing very little, if any, of the enzyme. Such a scenario may help to explain why such a large spike in phosphorus digestibility was seen in experiment 1 treatment 4 yet no corresponding increase in phosphorus retention was observed. Perhaps these fish had received a large dose prior to stripping to collect feces.

In conclusion, there are several sources of error that may have interfered with the aims of this study. That is, to evaluate the efficacy of phytase to improve nutrient digestibility and retention, particularly of phosphorus, in soy based diets for Florida pompano as well as determine the ideal dosing regimen for the enzyme. Firstly, the dietary requirement for

phosphorus absolutely may have been met with these experimental feed formulations. If this did occur, the beneficial influence of phytase likely would have gone undetected. Additionally, it is unlikely, given the temperature and pH of the feed during processing, that the enzyme was destroyed or active at this time. However, complete solubilization and even mixing is a concern. This may help to explain the spikes in digestibility that were seen yet no corresponding increase in phosphorus retention.

In future studies there are several potential means for improvement. First of all, the diets need to be limited in phosphorus as this will allow for differential effects of phytase dosing to be observed; if there are any. There are a couple of ways to accomplish this. The easiest way may be to create a diet that is simply very low in phosphorus; potentially by removing poultry by product meal entirely. This may lead to other problems mentioned earlier such as palatability or extrusion capability and other nutrient deficiencies. If only a small inclusion of animal protein source (~15%) is needed to meet the phosphorus requirement in Florida pompano; phytase supplementation may not be practical until greater percentages of the diet are composed of plant based ingredients. This may give credence to experimenting with diets that have a greater inclusion of soybean meal and how phytase supplementation may affect those diets.

Given the demand for pompano, its high market value, lack of a commercial fishery, and overall suitability for aquaculture; it seems more prudent to investigate the phosphorus requirement for the species. If Florida pompano aquaculture endeavors are to expand in the

coming years, and phosphorus remains the most expensive mineral supplement, knowledge of the dietary requirement for phosphorus is valuable information.

An even mixing of the enzyme in the feed is also of paramount importance. Without testing it is not possible to know with certainty if this was a factor in the results of these trails. However, as described above, this can lead to an improper dosing of the enzyme and consequently poor ability to achieve the targeted goal of freeing phytate bound phosphorus. Liquid preparations are available and may be more evenly distributed with the feed preparation methods used in these experiments. Perhaps grinding the granules into smaller pieces would allow for a more even distribution. Another method is to approach phytase application in these diets as it would be undertaken in a commercial operation. That is, top coating of the diet with a liquid preparation. This would bring other factors into consideration such as porosity of the feed and mechanical stress post spray-application. However, this method would be the most practical and valuable information for those seeking to use phytase supplementation in commercial Florida pompano aquaculture operations employing soy based feed formulations.

As Florida pompano are a commercially valuable species; interest in the species for aquaculture has increased and likely will continue to do so. Going forward, there are many ways this study could be improved upon. Given the market interest in the species and past research that has shown that Florida pompano perform well on soy based diets, seeking means to improve soy based feed formulations for the species is a prudent endeavor (Waldemar and Davis, 2012). Phosphorus remains the most expensive mineral supplement in aquatic feeds and phytase has the

potential to free phosphorus that is already contained in soy based formulations; thereby reducing the need to supplement. This in turn may reduce costs, increase the viability of plant based feeds, and reduce phosphorus waste is aquaculture effluents.

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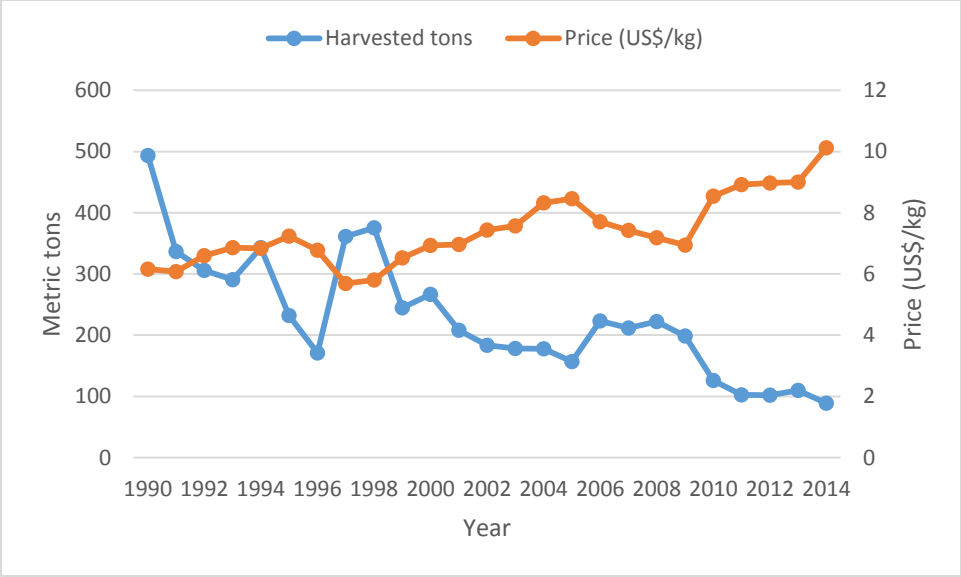

Figure 1. Pompano price over the years in comparison with the number of tons commercially harvested from 1990 to 2014 (NOAA, Fisheries and Statistics Division, www.st.nmfs.noaa.gov).

Table 1. Practical diet formulated to 40% protein and 8% lipid to initially evaluate the response of juvenile Florida pompano to phytase supplement. Formulation and proximate composition of trial 1 diets g/100g as is.

Ingredient	Basal + CaP	Basal	500	4000
Poultry by product meal[1]	15.00	15.00	15.00	15.00
Soybean meal solvent extracted[2]	47.70	47.70	47.70	47.70
Menhaden Fish Oil[3]	4.78	4.78	4.78	4.78
Corn Starch[4]	4.57	6.67	6.66	6.61
Whole Wheat[4]	17.5	17.5	17.5	17.5
Corn protein concentrate[5]	6.30	6.30	6.30	6.30
ASA Trace Mineral premix[6]	0.25	0.25	0.25	0.25
ASA Vitamin premix w/o choline[7]	0.50	0.50	0.50	0.50
Choline chloride[4]	0.20	0.20	0.20	0.20
Stay C 250 mg/kg using 35%[8]	0.10	0.10	0.10	0.10
CaP-Dibasic[4]	2.10	0.00	0.00	0.00
Lecithin[4]	0.50	0.50	0.50	0.50
Taurine[4]	0.50	0.50	0.50	0.50
Phytase[9]	0.00	0.00	0.007	0.055
Proximate analyses (%, as-is)				
Crude Protein (%)[10]	41.64	40.71	41.03	41.56
Crude Fat (%)[10]	8.77	8.51	8.60	8.74
Crude Fiber (%)[10]	3.38	2.99	3.05	2.86
Moisture (%)[10]	5.46	7.55	6.89	6.46
Ash (%)[10]	7.52	5.76	5.58	5.64
Phosphorus (%)[11]	1.13	0.65	0.65	0.65

[1]Griffin Industries, Inc., Mobile, Alabama, USA.
[2]De-hulled, solvent-extracted soybean meal, Bunge, Alabama, USA.
[3]Omega Protein Inc., Reedville, Virginia, USA.
[4]MP Biochemicals Inc., Solon, Ohio, USA.
[5]Empyreal75™ Cargill, Blair, NE, USA.
[6]ASA Premix (g $100g^{-1}$ premix): cobalt chloride, 0.004; cupric sulphate pentahydrate, 0.250, ferrous sulfate heptahydrate, 4.0, manganous sulfate anhydrous, 0.650; potassium iodide, 0.067; sodium selenite, 0.010; zinc sulfate heptahydrate, 13.193, and α cellulose 81.826.
[7]ASA Premix (g/kg Premix): thiamin HCl, 0.5; riboflavin, 8.0; pyridoxine HCl, 5.0; Ca-pantothenate, 20.0; niacin, 40.0; biotin, 0.040; folic acid, 1.80; cyanocobalamin, 0.002; vitamin A acetate (500,000 IU g^{-1}), 2.40; vitamin D_3 (400,000 IU g^{-1}), 0.50; DL-α-tocopheryl acetate, 80.0; and α cellulose, 834.258.
[8]Stay C®, (L-ascorbyl-2-polyphosphate 35% Active C), Roche Vitamins Inc., Parsippany, NJ, USA.
[9]Phytase Quantum Blue 5G, AB Vista, Marlborough, Wiltshire, UK.
[10]Analyzed.
[11]Calculated.

Table 2. Practical diet formulated to 40% protein and 8% lipid to examine different doses of phytase response in juvenile Florida pompano diets. Formulation and proximate composition of trial 2 diets g/100g as is.

	0	200	400	600
Poultry by product meal[1]	15.00	15.00	15.00	15.00
Soybean meal solvent extracted[2]	47.21	47.21	47.21	47.21
Menhaden Fish Oil[3]	4.81	4.81	4.81	4.81
Corn Starch[4]	2.63	2.63	2.62	2.62
Whole Wheat[4]	22.00	22.00	22.00	22.00
Corn protein concentrate[5]	6.30	6.30	6.30	6.30
ASA Trace Mineral premix[6]	0.25	0.25	0.25	0.25
ASA Vitamin premix w/o choline[7]	0.50	0.50	0.50	0.50
Choline chloride[4]	0.20	0.20	0.20	0.20
Stay C 250 mg/kg using 35%[8]	0.10	0.10	0.10	0.10
Lecithin[4]	0.50	0.50	0.50	0.50
Taurine[4]	0.50	0.50	0.50	0.50
Phytase[9]	0.00	0.003	0.006	0.008
Proximate analyses (%, as-is)				
Crude Protein (%)[10]	41.74	41.85	42.16	42.33
Crude Fat (%)[10]	7.29	8.34	9.67	9.49
Crude Fiber (%)[10]	3.11	3.07	3.59	2.95
Moisture (%)[10]	8.32	7.13	6.84	6.26
Ash (%)[10]	5.96	6.00	5.89	5.99
Phosphorus (%)[10]	0.77	0.73	0.77	0.77

[1]Griffin Industries, Inc., Mobile, Alabama, USA.
[2]De-hulled, solvent-extracted soybean meal, Bunge, Alabama, USA.
[3]Omega Protein Inc., Reedville, Virginia, USA.
[4]MP Biochemicals Inc., Solon, Ohio, USA.
[5]Empyreal75^{TM} Cargill, Blair, NE, USA.
[6]ASA Premix (g $100g^{-1}$ premix): cobalt chloride, 0.004; cupric sulphate pentahydrate, 0.250, ferrous sulfate heptahydrate, 4.0, manganous sulfate anhydrous, 0.650; potassium iodide, 0.067; sodium selenite, 0.010; zinc sulfate heptahydrate, 13.193, and α cellulose 81.826.
[7]ASA Premix (g/kg Premix): thiamin HCl, 0.5; riboflavin, 8.0; pyridoxine HCl, 5.0; Ca-pantothenate, 20.0; niacin, 40.0; biotin, 0.040; folic acid, 1.80; cyanocobalamin, 0.002; vitamin A acetate (500,000 IU g^{-1}), 2.40; vitamin D_3 (400,000 IU g^{-1}), 0.50; DL-α-tocopheryl acetate, 80.0; and α cellulose, 834.258.
[8]Stay C®, (L-ascorbyl-2-polyphosphate 35% Active C), Roche Vitamins Inc., Parsippany, NJ, USA.
[9]Phytase Quantum Blue 5G, AB Vista, Marlborough, Wiltshire, UK.
[10]Analyzed.

Table 3. Practial diet formulated to 40% protein and 8% lipid to examine different doses of phytase response in juvenile Florida pompano diets. Formulation and proximate composition of trial 3 diets g/100g as is.

	0	1000	2000	4000
Poultry by product meal[1]	15.00	15.00	15.00	15.00
Soybean meal solvent extracted[2]	47.21	47.21	47.21	47.21
Menhaden Fish Oil[3]	4.81	4.81	4.81	4.81
Corn Starch[4]	2.63	2.62	2.60	2.57
Whole Wheat[4]	22.00	22.00	22.00	22.00
Corn protein concentrate[5]	6.30	6.30	6.30	6.30
ASA Trace Mineral premix[6]	0.25	0.25	0.25	0.25
ASA Vitamin premix w/o choline[7]	0.50	0.50	0.50	0.50
Choline chloride[4]	0.20	0.20	0.20	0.20
Stay C 250 mg/kg using 35%[8]	0.10	0.10	0.10	0.10
Lecithin[4]	0.50	0.50	0.50	0.50
Taurine[4]	0.50	0.50	0.50	0.50
Phytase[9]	0.00	0.014	0.028	0.055
Proximate analyses (%, as-is)				
Crude Protein (%)[10]	42.31	42.07	42.81	42.38
Crude Fat (%)[10]	9.28	9.96	10.19	10.35
Crude Fiber (%)[10]	3.38	3.23	3.06	3.18
Moisture (%)[10]	5.12	6.13	5.14	5.61
Ash (%)[10]	6.18	6.04	6.07	5.98
Phosphorus (%)[10]	0.77	0.75	0.76	0.76

[1]Griffin Industries, Inc., Mobile, Alabama, USA.
[2]De-hulled, solvent-extracted soybean meal, Bunge, Alabama, USA.
[3]Omega Protein Inc., Reedville, Virginia, USA.
[4]MP Biochemicals Inc., Solon, Ohio, USA.
[5]Empyreal75™ Cargill, Blair, NE, USA.
[6]ASA Premix (g $100g^{-1}$ premix): cobalt chloride, 0.004; cupric sulphate pentahydrate, 0.250, ferrous sulfate heptahydrate, 4.0, manganous sulfate anhydrous, 0.650; potassium iodide, 0.067; sodium selenite, 0.010; zinc sulfate heptahydrate, 13.193, and α cellulose 81.826.
[7]ASA Premix (g/kg Premix): thiamin HCl, 0.5; riboflavin, 8.0; pyridoxine HCl, 5.0; Ca-pantothenate, 20.0; niacin, 40.0; biotin, 0.040; folic acid, 1.80; cyanocobalamin, 0.002; vitamin A acetate (500,000 IU g^{-1}), 2.40; vitamin D_3 (400,000 IU g^{-1}), 0.50; DL-α-tocopheryl acetate, 80.0; and α cellulose, 834.258.
[8]Stay C®, (L-ascorbyl-2-polyphosphate 35% Active C), Roche Vitamins Inc., Parsippany, NJ, USA.
[9]Phytase Quantum Blue 5G, AB Vista, Marlborough, Wiltshire, UK.
[10]Analyzed.

Table 4. Growth response of juvenile Florida pompano of initial weight 7.95g ± 0.19 offered test diets over a 9 week period. Results for trial 1. Significance (P<0.05) determined by SNK grouping.

Phytase	Biomass (g)	Final mean weight (g)	Thermal growth coefficient	Weight gain (%)	Feed conversion ratio	Survival (%)
Basal+CaP	1351.56[ab]	68.69[ab]	0.1234	764.68	1.96	98.33
Basal	1270.63[b]	64.65[b]	0.1187	734.48	1.93	100
500	1450.46[a]	72.52[a]	0.1276	807.07	1.83	100
4000	1368.23[ab]	68.41[ab]	0.1230	785.91	1.86	100
P-value	0.0204	0.0294	0.0559	0.1365	0.2493	0.5957
PSE[1]	30.47	1.425	0.0018	23.934	0.0536	1.1785

[1]PSE=Pooled Standard Error, n=3

Table 5. Growth response of juvenile pompano initial weight 22.57g ± 0.85 for trial 2 and 23.8g ± 1.0 for trial 3 fed test diets for an 8 week period. Results for both low (trail 2) and high (trial 3) phytase trials. Significance (P<0.05) determined by SNK grouping.

Phytase	Biomass (g)	Final mean weight (g)	Thermal growth coefficient	Weight gain (%)	Feed conversion ratio	Survival (%)
0	1733.16	91.38	0.10347	406.90	2.25	95
200	1704.16	92.88	0105687	418.65	2.25	91.6
400	1766.66	92.96	0.10333	401.31	2.36	95
600	1716.16	87.31	0.099547	390.27	2.28	98.33
P-value	0.9059	0.4527	0.599	0.6596	0.5204	0.4532
PSE[1]	63.73	2.689	0.0031	15.862	0.0579	2.7638
0	1107[a]	114.62	0.1212	375.65	2.27[b]	96.66
1000	996.66[b]	99.66	0.1089	323.81	2.61[a]	100
2000	876.66[c]	101.37	0.1109	333.87	2.60[a]	86.66
4000	1136.5[a]	118.22	0.1236	386.97	2.28[b]	96.66
p-value	0.0022	0.0971	0.0821	0.1099	0.0135	0.0519
PSE[1]	33.55	5.411	0.0040	18.53	0.0733	2.886

[1]PSE=Pooled Standard Error, n=3

Table 6. Whole body proximate analysis for trial 1 Florida pompano. Significance (P<0.05) determined by SNK grouping.

Phytase	Crude protein (%)	Moisture (%)	Crude lipid (%)	Fiber (%)	Ash (%)	Phosphorus (%)
Basal+CaP	17.48	68.70	12.22	0.40	3.30	0.48
Basal	17.32	68.21	12.29	0.37	2.66	0.46
500	17.02	67.30	13.18	0.44	2.88	0.48
4000	16.63	67.04	14.08	0.40	2.64	0.44
P-value	0.3764	0.5373	0.1277	0.7971	0.3189	0.3656
PSE[1]	0.3463	0.5792	0.5463	0.0516	0.2623	0.0201

[1]PSE=Pooled Standard Error, n=3

Table 7. Whole body proximate analysis and minerals for both low (trial 2) and high (trial 3) phytase trials. Significance (P<0.05) determined by SNK grouping.

Phytase	Crude protein (%)	Moisture (%)	Crude lipid (%)	Ash (%)	Phosphorus (%)	Sulfur (%)
0	16.6	68.57[b]	10.19	3.57	0.59	0.25
200	16.77	70.87[ab]	8.39	3.36	0.51	0.25
400	16.63	72.8[a]	7.38	3.03	0.67	0.25
600	16.47	69.23[ab]	10.33	3.47	0.6	0.25
P-value	0.9732	0.0454	0.2098	0.6846	0.5899	0.9106
PSE[1]	0.458	0.917	1.041	0.328	0.076	0.007
0	16.93	68.37	11.32[a]	2.56	0.62	0.23
1000	16.13	71.17	10.5[ab]	2.8	0.61	0.23
2000	16.1	71.43	8.46[b]	3.32	0.62	0.23
4000	17.13	68.5	11.57[a]	2.53	0.61	0.24
P-value	0.1187	0.3794	0.0411	0.6165	0.9918	0.7278
PSE[1]	0.4582	0.9169	1.0414	0.3282	0.0762	0.0076

[1]PSE=Pooled Standard Error, n=3

Phytase	K (%)	Mg (%)	Ca (%)	Na (%)	Fe (%)	Mn (%)
0	0.3	0.05	0.86	0.17	14.2	3.17
200	0.3	0.04	0.69	0.14	13.63	1.77
400	0.3	0.04	1.01	0.15	12.4	2.53
600	0.3	0.04	0.86	0.15	12.33	3.23
P-value	0.9804	0.4872	0.6057	0.557	0.6975	0.0645
PSE[1]	0.008	0.0029	0.1607	0.015	1.318	0.3586
0	0.3	0.04	0.91	0.12	11.5	2.73
1000	0.29	0.04	0.91	0.12	11.9	2.73
2000	0.28	0.04	0.93	0.12	10.6	2.63
4000	0.31	0.04	0.87	0.11	13.43	2.83
P-value	0.3723	?	0.9544	0.7867	0.4971	0.9727
PSE[1]	0.0079	?	0.1607	0.015	1.3180	0.3586

[1]PSE=Pooled Standard Error, n=3

Phytase	Cu (%)	Zn (%)
0	1.7	17.7
200	Nd	16.07
400	Nd	17.07
600	1.6	17.27
P-value	?	0.8539
PSE[1]	?	1.362
0	Nd	18.33
1000	Nd	18.1
2000	Nd	16.57
4000	1.1	16.87
P-value	?	0.8448
PSE[1]	?	1.3619

[1]PSE=Pooled Standard Error, n=3

Table 8. Whole body retentions for protein, phosphorus, and energy for trial 1 fish. Significance (P<0.05) determined by SNK grouping.

Phytase	Protein gained (g)	Protein offered (g)	ANPR (%)	Phosphorus fed (g)	Phosphorus gained (g)	Phosphorus retention (%)
Basal+CaP	10.81	49.61	21.80	1.43[a]	0.28	19.46[b]
Basal	9.97	45.11	22.12	0.88[b]	0.25	28.10[a]
500	11.12	48.02	23.19	0.92[b]	0.30	32.10[a]
4000	10.15	48.40	21.06	0.92[b]	0.24	26.49[a]
P-value	0.0908	0.1774	0.3992	<.0001	0.0536	0.0023
PSE[1]	0.3093	1.315	0.8413	0.02868	0.0127	1.5051

[1]PSE=Pooled Standard Error, n=3

Phytase	Energy gained (kcal)	Energy offered (kcal)	ANER (%)
Basal+CaP	1384.77	5708.42	24.21
Basal	1261.92	5902.26	21.39
500	1521.25	6064.50	25.16
4000	1468.92	5220.91	28.33
P-value	0.9636	0.8529	0.9809
PSE[1]	100.93	258.16	2.417

[1]PSE=Pooled Standard Error, n=3

Table 9. Whole body retention data for protein, phosphorus, and energy in both low (trial 2) and high (trial 3) phytase trials. Significance (P<0.05) determined by SNK grouping.

Phytase	Protein gained (g)	Protein offered (g)	ANPR (%)	Phosphorus fed (g)	Phosphorus gained (g)	Phosphorus retention (%)
0	12.4	82.93	14.95	1.53	0.56	36.66
200	12.63	85.26	14.86	1.49	0.48	32.17
400	12.45	88.12	14.12	1.55	0.62	39.99
600	11.87	80.77	14.7	1.47	0.54	37.28
P-value	0.933	0.1328	0.9369	0.4323	0.5155	0.6862
PSE[1]	0.8839	1.9914	1.0166	0.0357	0.0639	4.5403
0	15.54	87.07	17.86[a]	1.58	0.7	44.38
1000	12.31	83.77	14.7[b]	1.49	0.6	40.4
2000	12.59	86.72	14.5[b]	1.54	0.62	40.49
4000	16.45	90.24	18.13[a]	1.62	0.72	44.14
P-value	0.0563	0.5749	0.0098	0.4705	0.378	0.577
PSE[1]	0.8839	1.9913	1.0166	0.0357	0.0639	4.5403

[1]PSE=Pooled Standard Error, n=3

Phytase	Energy gained (kcal)	Energy offered (kcal)	ANER (%)
0	1456.72	9327.46	15.72
200	1332.96	9854.71	13.58
400	1241.36	10404.29	11.97
600	1423.63	9640.06	14.79
P-value	0.67	0.0564	0.43
PSE[1]	132.20	230.99	1.59
0	1866.54	9963.25	18.84
1000	1506.53	9799.34	15.39
2000	1386.15	9760.54	14.17
4000	2022.44	10819.54	18.48
P-value	0.1564	0.2225	0.1754
PSE[1]	197.80	369.01	1.576

[1]PSE=Pooled Standard Error, n=3

Table 10. Digestibility data from trial 1 for ADMD, AED, APD, and APHD. Significance (P<0.05) determined by SNK grouping.

Phytase	ADMD (%)	AED (%)	APD (%)	APHD (%)
Basal+CaP	46.10[b]	63.06	81.90[a]	25.67[b]
Basal	49.33[ab]	67.87	82.65[a]	35.61[b]
500	52.10[ab]	64.90	75.97[b]	32.94[b]
4000	54.87[a]	66.19	83.78[a]	51.36[a]
P-value	0.047	0.2845	0.0369	0.015
PSE[1]	1.837	1.652	1.621	4.226

[1]PSE=Pooled Standard Error, n=3

Table 11. Digestibility data from trail 2 digestibility trial for ADMD, AED, APD. Significance (P<0.05) determined by SNK grouping.

Phytase	ADMD (%)	AED (%)	APD (%)
0	25.67	45.95	77.43
200	25.67	48.45	75.72
400	25.67	50.19	76.87
600	24.19	47.73	80.42
P-value	0.9866	0.6801	0.7715
PSE[1]	3.493	2.435	3.255

[1]PSE=Pooled Standard Error, n=3

Table 12. Phytase activity, phytate content (g/100g), and Phytate bound phosphorus (g/100g) for trial 1 diets.

Phytase	Phytase activity	Phytate (g/100g)	Phytate-P (g/100g)
Basal+CaP	<50	0.82	0.384
Basal	<50	0.76	0.274
500	432	0.86	0.301
4000	3510	0.72	0.306