

# Acceptance of a Protein Concentrate from Alfalfa (*Medicago sativa*) by Yellow Perch (*Perca flavescens*) Fed a Formulated Diet

Jessica Coburn <sup>1</sup>, M. Scott Wells <sup>1</sup>, Nicholas B. D. Phelps <sup>2</sup>, T. Gibson Gaylord <sup>3</sup> and Deborah A. Samac <sup>4,\*</sup>

<sup>1</sup> Department of Agronomy and Plant Genetics, 1991 Upper Buford Circle, University of Minnesota, St. Paul, MN 55108, USA; jemcoburn@gmail.com (J.C.); mswells@umn.edu (M.S.W.)

<sup>2</sup> Department of Fisheries, Wildlife and Conservation Biology, 2003 Upper Buford Circle, University of Minnesota, St. Paul, MN 55108, USA; phelp083@umn.edu

<sup>3</sup> U.S. Department of the Interior, Fish and Wildlife Service, Bozeman Fish Technology Center, 4050 Bridger Canyon Road, Bozeman, MT 59715, USA; gibson\_gaylord@fws.gov

<sup>4</sup> U.S. Department of Agriculture, Agricultural Research Service, Plant Science Research Unit, 1991 Upper Buford Circle, St. Paul, MN 55108, USA

\* Correspondence: debby.samac@usda.gov

**Abstract:** The majority of plant proteins used in aquatic feeds are derived from seed meals, which may contain antinutritional factors. Protein concentrates from plant foliage have received less attention in fish feeding trials. Alfalfa protein concentrate (APC) is derived from fresh alfalfa foliage that contains approximately 52% protein and is low in fiber. A feeding trial was done to assess growth and feed efficiency responses of yellow perch (*Perca flavescens*) fed a formulated diet with 180 g/kg APC replacing all fishmeal compared to a control isonitrogenous diet with fishmeal. Yellow perch accepted the APC diet but gained weight at a lower specific growth rate (−0.07% per day) and had an elevated feed conversion ratio (+0.32 g feed/g growth) than fish on the control diet containing fishmeal. There was no impact on survivorship or condition nor differences in fillet yield or composition in fish on the diet with APC compared to the control fishmeal diet. These findings indicate that although replacing fishmeal with APC in a perch diet resulted in slower growth rates, the APC was accepted and has promise as a sustainable protein in aquatic feeds.

**Keywords:** alfalfa; feed efficiency; fishmeal; growth rate; sustainability; yellow perch



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## 1. Introduction

The global aquafeed market, worth USD 133.5 billion in 2020, is expected to reach USD 221.9 billion by 2025 [1]. Growing demand and environmental regulations on natural fisheries make it difficult to procure important raw materials for feed such as fishmeal. Inclusion of fishmeal in aquafeeds must be optimized and reduced as demand for aquaculture products grow [2]. There is increasing interest in abundant plant protein sources for replacing or supplementing fishmeal in feeds. A protein concentrate made from alfalfa (*Medicago sativa*) has been explored, to a limited extent, as a dietary protein for fish [3–6].

Alfalfa protein concentrate (APC) is derived by pressing fresh alfalfa foliage to make a protein-rich juice. In commercial production, the juice is heated to recover proteins, followed by centrifugation to separate proteins from the liquid fraction, and then drying to remove residual moisture from the protein fraction [7]. Refining of the juice and fiber-rich press residue can produce a range of products that include biofuels, animal feed, food grade protein, nutritional supplements, and industrial enzymes. Commercially produced APC has 520 g/kg crude protein with high amounts of lysine, threonine, and methionine; is high in vitamins and antioxidants such as carotenoids; contains approximately 100 g/kg fat, including 40 g/kg omega-3 fatty acids; and is low in fiber [7,8]. APC, marketed as VITALFA™ (Ingredients by Nature, Montclair, CA) is used in commercial production of

livestock feeds and pet foods and is readily available for aquaculture feed production. Additionally, because APC is made from the foliage of the plant (leaves and stems), it does not have the anti-nutritional concentrations of phytic acid or lectins that legume seed meals such as soybean meal often contain [9]. Feeding studies with poultry and swine indicate that APC may also have benefits as a dietary supplement that boosts immune function and helps combat diseases and stressors related to high densities and overcrowding [10]. Table 1 compares the composition of APC to soybean meal and Menhaden fishmeal. APC and soybean meal have a similar amino acid composition, although APC is higher in lysine and methionine.

**Table 1.** Composition (g/kg) of alfalfa protein concentrate (APC), soybean meal (SBM), and Menhaden fishmeal (MFM).

Component	APC	SBM	MFM
Crude protein	520	519	705
Crude lipid	104	17	104
Ash	140	ND <sup>§</sup>	ND
Carbohydrate	172	ND	ND
Crude fiber	27	ND	ND
Amino Acids			
Methionine	11.4	6.6	20.1
Lysine	33.8	27.2	52.8
Threonine	25.5	23.4	29.8
Histidine	12.5	13.1	18.7
Leucine	45.8	45.3	51.5
Phenylalanine	29.1	29.2	28.2
Valine	29.6	28.0	35.4
Arginine	31.2	44.3	51.3
Isoleucine	25.0	23.4	30.7
Tryptophan	10.4	ND	ND
Cysteine	4.7	ND	ND
Glutamic Acid	54.6	94.0	86.8
Proline	23.4	28.3	31.2
Tyrosine	23.4	21.9	23.3
Alanine	30.2	23.9	43.0
Aspartic Acid	50.4	61.0	61.1
Glycine	26.5	22.2	43.6
Serine	23.4	30.3	26.7

APC composition reproduced from [8]. MFM and SBM composition reproduced from [11]. <sup>§</sup> No data.

Alfalfa is a widely grown high-biomass perennial forage legume. The foliage is used primarily as a component for dairy and beef cattle feeds as dry hay or silage and in pastures for grazing. Cultivation of alfalfa requires fewer agronomic inputs such as fertilizers and pesticides than annual crops of maize (corn; *Zea mays*) and soybean (*Glycine max*). Alfalfa requires no nitrogen fertilizer and provides all of the nitrogen required for the subsequent grain crop [12]. Alfalfa is an important component of sustainable agricultural systems because of its ability to protect water quality, interrupt pest and pathogen cycles in crop rotations, improve soil carbon storage, and reduce the production of greenhouse gases in cropping systems [13]. Thus, alfalfa provides many environmental services that enhance sustainability of protein production, which is increasingly important to the public and consumers of aquaculture products, and for landscape conservation.

Past studies concluded that APC has benefits as a feed ingredient for several fish species including tilapia (*Oreochromis niloticus*) [4], carp (*Cyprinus carpio*) [5], and sea bream (*Sparus aurata*) [6]. This study was conducted to explore expanding use of APC with yellow perch, which is a species of increasing interest in aquaculture, shows tolerance to a range of dietary proteins, and is also considered to be carnivorous [14]. The growth and composition of yellow perch fed a diet in which fishmeal was replaced with APC were compared to fish fed an isonitrogenous control diet with fishmeal.

## 2. Results

Two experimental diets were formulated, the first was a fishmeal control diet formulated to mimic a commercial type formulation. The second diet was formulated to replace the protein from fishmeal with protein from APC (Table 2). Two runs of the feeding trial were done, one in 2017 and one in 2018. The results from both runs were similar and data were combined for the two years. No significant differences were detected in condition factor (K), survival, fillet yield (FY), or hepatosomatic index (HSI) for fish on the APC or fishmeal diets (Table 3). However, differences in final body weight (FBW) ( $p < 0.01$ ), weight gain (WG) ( $p = 0.04$ ), feed intake (FI) ( $p = 0.05$ ), feed conversion ratio (FCR) ( $p = 0.03$ ), and viscerosomatic index (VSI) ( $p = 0.01$ ) were observed with fish on the control fishmeal diet out-performing those on the APC diet. Growth was slower in the yellow perch fed the APC diet with a WG of 54.7% compared to yellow perch on the fishmeal diet with WG of 67.1%. Feed consumption was greater for yellow perch on the APC diet than yellow perch fed the fishmeal diet. Greater feed consumption combined with slower growth resulted in a higher FCR for yellow perch on the APC diet (FCR = 1.99) compared to yellow perch on the fishmeal diet (FCR = 1.67).

**Table 2.** Composition (g/kg) of diets to evaluate alfalfa protein concentrate (APC) replacing fishmeal in a diet for yellow perch (*Perca flavescens*).

Ingredient	Fishmeal Control	APC
Menhaden Fishmeal, Omega Protein, Special Select	150.0	0.0
Alfalfa Nutrient Concentrate	0.0	184.0
Soybean Meal 48% crude protein	150.0	150.0
Poultry Meal <sup>1</sup>	150.0	150.0
Corn Protein Concentrate, Emphyreal 75 <sup>2</sup>	50.0	50.0
Wheat Gluten Meal	3.2	5.6
Pastry Flour	342.6	300.6
Lecithin	10.0	10.0
Stay-C	1.5	1.5
Vitamin premix ARS 702 <sup>3</sup>	10.0	10.0
Trace Mineral Mix ARS 640 <sup>4</sup>	1.0	1.0
Sodium Chloride	2.8	2.8
Magnesium Oxide	0.6	0.6
Potassium Chloride	5.6	5.6
Monocalcium Phosphate	18.0	32.0
Choline Chloride 50%	10.0	10.0
DL-Methionine	4.2	5.0
Lysine Hydrochloride	18.1	18.8
Threonine	3.2	3.2
Taurine	5.0	5.0
Yttrium Oxide	1.0	1.0
Menhaden Fish Oil	63.2	53.3
Calculated Composition		
Crude Protein	404	401
Crude Lipid	120	120
Digestible Energy (Kcal/g)	4.08	4.02
Proximate Chemical Composition		
Crude Protein	411	399
Crude Lipid	97.0	93.7
Ash	72.9	70.6

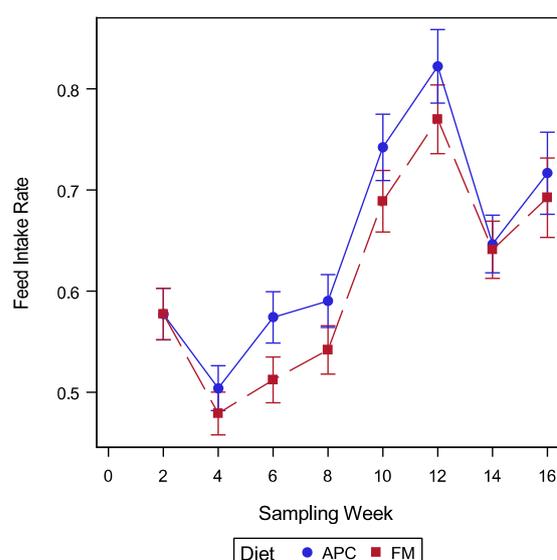
<sup>1</sup> American Dehydrated Foods Inc. <sup>2</sup> Cargill Inc. <sup>3</sup> Contributed per kg diet: 9650 IU vitamin A; 6600 IU vitamin D; 132 IU vitamin E; 1.1 mg vitamin K3; 9.1 mg thiamin mononitrate; 9.6 mg riboflavin; 13.7 mg pyridoxine hydrochloride; 46.5 mg calcium DL-pantothenate; 0.03 mg cyanocobalamin; 21.8 mg nicotinic acid; 0.34 mg biotin; 2.5 mg folic acid; 600 mg inositol. <sup>4</sup> Contributed per kg diet: 40 mg zinc; 17 mg manganese; 80 mg iron; 6 mg iodine; 15 mg copper; 0.4 mg selenium.

**Table 3.** Effects of replacing fishmeal (FM) with alfalfa protein concentrate (APC) in a diet for yellow perch on growth performance and fillet composition.

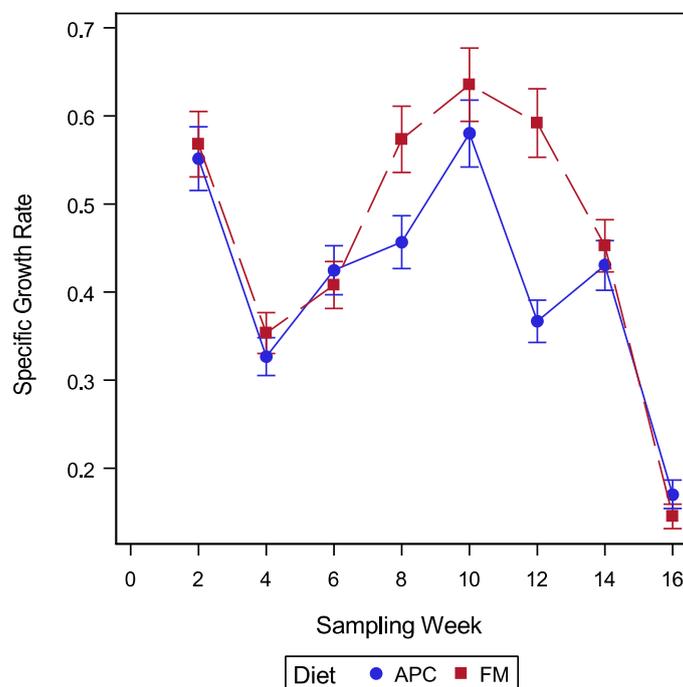
Parameter	Diets		SE	p-Value
	FM	APC		
Growth Performance Indexes				
IBW (g)	23.4	21.7	–	–
FBW (g)	40.4 <sup>a</sup>	33.3 <sup>b</sup>	3.13	<0.01
WG (%)	67.14 <sup>a</sup>	54.67 <sup>b</sup>	3.80	0.04
SGR (%/day)	0.46 <sup>a</sup>	0.39 <sup>b</sup>	0.02	0.04
K	1.10	1.08	0.02	0.35
Survival (%)	87	83	4	0.26
FI (% BW/day)	0.59 <sup>b</sup>	0.65 <sup>a</sup>	0.02	0.05 <sup>†</sup>
FCR	1.67 <sup>b</sup>	1.99 <sup>a</sup>	0.23	0.03
FY (%)	39.82	39.81	0.77	0.97
VSI	7.58 <sup>b</sup>	8.13 <sup>a</sup>	0.78	0.01
HSI	2.30	2.40	0.10	0.24
Fillet Composition				
Moisture (g/kg)	788.6 <sup>b</sup>	792.6 <sup>a</sup>	4.2	0.05
Crude Protein (g/kg)	196.3	193.8	5.3	0.20
Crude Fat (g/kg)	1.6	1.4	0.2	0.18
Ash (g/kg)	12.8	12.7	0.5	0.72
Gross Energy (kcal/g)	1.04	1.03	0.01	0.15

Parameters measured are initial body weight (IBW), final body weight (FBW), weight gain (WG), specific growth rate (SGR), condition factor (K), feed intake (FI), feed conversion ratio (FCR), fillet yield (FY), viscerosomatic index (VSI), hepatosomatic index (HSI), and composition of the carcass fillets. Yellow perch diets were used to test replacement of fishmeal protein with APC and included the control diet with 150 g/kg fishmeal and 0 g/kg APC (FM) along with the experimental diet including 0 g/kg fishmeal and 180 g/kg APC (APC). Within a row, treatment means with differing letters are significantly different ( $p \leq 0.05$ ). SE = standard error. <sup>†</sup> FI calculated by two-week growth periods.

Daily feed intake rate ranged from 0.48 to 0.82% of mean body weight (Figure 1). The FI patterns of the APC and fishmeal diets were similar during the course of the study with no significant differences between means at each sampling week ( $\alpha = 0.05$ ). Specific growth rate (SGR) patterns for fish on the two feeds were similar with no significant differences between means at each sampling week ( $\alpha = 0.05$ ). Growth of fish decreased on both feeds after week 12 when air and water temperature in the greenhouse in which fish were housed increased with outside summer temperatures (Figure 2).

**Figure 1.** Feed intake rate for yellow perch averaged over the 16- and 14-week trials in 2017 and 2018, respectively. Values are means for each treatment ( $n = 8$  for weeks 2–14 and  $n = 4$  for week 16)  $\pm$  standard error. No significant differences were measured between means at each week ( $\alpha = 0.05$ ).

The treatments were isonitrogenous balanced diets with either 180 g/kg alfalfa protein concentrate (APC; solid blue line) or 150 g/kg fishmeal (FM; dashed red line). Feed intake rate =  $100 \times \text{weight of feed consumed} / (\text{mean fish weight} \times \text{population}) / \text{number of days}$ .



**Figure 2.** Specific growth rate for yellow perch averaged over the 16- and 14-week trials in 2017 and 2018, respectively. Values are means for each treatment ( $n = 8$  for weeks 2–14 and  $n = 4$  for week 16)  $\pm$  standard error. No significant differences were measured between means at each week ( $\alpha = 0.05$ ). The treatments were isonitrogenous balanced diets with either 180 g/kg alfalfa protein concentrate (APC; solid blue line) or 150 g/kg fishmeal (FM; dashed red line).

Diet affected moisture content of the yellow perch fillets in a small but measurable amount. Fish on the APC diet had 79.3% moisture content in the fillet as opposed to 78.9% moisture in fillets from fish on the fishmeal diet (Table 3;  $p = 0.05$ ). Protein, lipid, ash, and energy content of fillets did not change significantly with diet, and values ranged from 19.4 to 19.6%, 0.14 to 0.16%, 1.27 to 1.28%, and 1.03 to 1.04 kcal/g, respectively (Table 3).

### 3. Discussion

Yellow perch accepted APC in the diet resulting in survivorship, condition factor, and an HSI similar to fish on the fishmeal diet. Although yellow perch on the APC diet had a slightly higher VSI ( $8.13 \pm 0.78$ ) than those on the fishmeal diet ( $7.58 \pm 0.78$ ), the VSI for fish on the APC diet was lower than those found in studies testing combinations of dietary distillers dried grain and soybean meal with yellow perch of a similar size as those in our study [15]. However, growth rate and FCR were lower for fish on the APC diet. Our results differ from a previous study in which tilapia fed similar levels of APC had a growth rate and FCR that did not differ from tilapia on a control diet with fishmeal [4]. In that study not all fishmeal was replaced by APC, which may have improved growth rate. Additionally, omnivorous species such as tilapia may have greater tolerance for plant-based proteins than carnivorous species. Additional studies on APC digestibility and testing a range of inclusion rates should clarify the optimal amount of APC in yellow perch diets.

The SGR for yellow perch on APC and fishmeal diets was on the lower end of what is typically reported, which range from 0.33 to 1.33 [15–19]. Though the yellow perch were offered feed to satiation, both treatment populations consumed feed at a low level. This may have been due to low densities leading to less aggressive feeding behavior, avoidance

due to movements around the tanks from researchers [20], or possible other feed sources from the aquaponics tanks such as algae [21,22]. Although algal strands were removed by hand, no other control of algae was attempted. The repeat of the experiment in 2018 was conducted to test if a longer period of feed training and greater fish density would increase feed consumption and SGR. However, the increase in population density and more gradual feed training in 2018 did not increase feed intake.

Some of the yellow perch on the APC diet had pellets remaining in their stomachs at the time of necropsy, which indicates that the pellets were slower to digest than those of the fishmeal diet. Fish on the APC diet had slightly higher moisture content in the fillet compared to fish on the fishmeal diet. Often higher moisture content is observed when difficulty in absorbing and utilizing fats occurs [23]. Saponins, triterpenoids, or steroidal aglycones that are substituted with varying numbers of sugar sidechains have been found to be an antinutritional component for fish and other animals that interfere with fat digestion and absorption when present at high levels [24]. If present in high enough concentrations in APC, they could affect digestion and growth. Alfalfa varieties vary in their production of saponins, and saponin concentration may be affected by environmental conditions and maturity of the crop at harvest [25]. Further research is needed on the concentrations of saponins in APC depending on alfalfa variety used, condition of the crop at harvest, and methods of producing APC, which can alter the final composition of the protein concentrate [26].

Our results indicate that yellow perch are able to use relatively high levels of APC without major impacts on growth or fillet yield and composition. Additional studies should clarify the optimal amount of APC and appropriate supplements in yellow perch diets for maximum performance. Sustainable plant-based sources of protein are needed for continued expansion of aquaculture production. Utilization of alfalfa protein in aquaculture feeds has environmental and social benefits important to consumers. APC use in aquaculture feeds should not negatively impact supply of alfalfa for ruminant animals. Processing of alfalfa foliage to produce APC results in a fibrous press residue that can be used for ruminant animal feeds [26]. Presently, the cost of APC is similar to that of fishmeal; however, decreases in cost should occur with increased demand and competition in APC production. This study demonstrates that APC is an acceptable protein source for aquaculture species such as yellow perch with a midrange carnivorous diet.

## 4. Materials and Methods

### 4.1. Experimental Diets

Diets were formulated to contain 400 g/kg digestible protein supplemented with lysine, methionine, and threonine to provide a digestible amino acid target of 34, 12, and 19 g/kg, respectively. The APC used was produced by Désialis (Paris, France) and obtained in crumbled form as VITALFA Alfalfa Nutrient Concentrate (ANC) from Ingredients by Nature (Montclair, CA, USA). Since the digestibility coefficients of nutrients in ANC were not known for yellow perch at the time of formulation, surrogate apparent digestibility coefficients were utilized from soybean meal as needed for presumably conservative estimates due to the similarity in amino acid content. Diet crude lipid and digestible energy formulation targets were 120 g/kg and 17 kJ/kg, respectively (Table 2). The two diets were manufactured in October 2016 at the U.S. Fish and Wildlife Service Fish Technology Center in Bozeman, MT using cooking extrusion as described by Gaylord et al. [27]. Briefly, the ingredients were mixed and finely milled, extruded through a 3 mm die at approximately 122 °C, dried at approximately 102 °C, and cooled on an air table. Fish oil was added post-cooling by vacuum infusion top coating (AJ Mixing, Ontario, CA, USA). Both diets were stored under sealed conditions at −18 °C until use in the feeding trial.

### 4.2. Feeding Trial

Eight 600 L aluminum recirculation aquaponic tanks, maintained with 480 to 560 L of water, were utilized for the feeding trial. Tanks were located within a greenhouse at

the University of Minnesota-Twin Cities. Two 118 L gravel biological filter beds were attached to each tank with recirculation provided by 2650 L/hour magnetic drive pumps (Danner Manufacturing Inc., Islandia, NY, USA). Floating insulation rafts contained lettuce in 2017 and basil in 2018 to filter nitrates from the water. Tanks and biological filters were started in 2015 for previous yellow perch populations and maintained in between fish populations with ammonium chloride (Hawkins Chemical Co., Roseville, MN, USA). Nitrite, pH, and ammonia levels were monitored three times per week and adjusted to maintain optimal levels for the fish (0 to 0.5 ppm nitrite and ammonia and pH 7.2 to 7.8). Greenhouse air temperature was set to remain between 20.0 and 23.5 °C. Water temperature was controlled through acclimation to room temperature. Dissolved oxygen was maintained with airstones at 7 to 8 mg/L. Lighting within the greenhouse was supplemented with overhead halogen lights to maintain a 16 h photoperiod.

Two runs of this feeding trial were conducted, one in early 2017 and a second in 2018. Young-of-the-year yellow perch were obtained in November of 2016 and 2017 from unfed outdoor rearing ponds (Oswald Fisheries, Ellendale, MN, USA in 2016 and Minnesota Muskie Farm, Alexandria, MN, USA in 2017). The yellow perch were feed-trained and reared on site until the start of the experiment in February 2017 and March 2018. Once feed training was completed, fish were randomly assigned to tanks with four tanks per diet. Approximately 35% and 45% of the original yellow perch survived transportation, acclimation to tanks, and feed training to begin the trials in 2017 and 2018, respectively. The tanks were stocked with 14 and 22 fish/tank in 2017 and 2018, respectively. Fish were acclimated to assigned tanks and the two diets for two weeks prior to the start of the trial. Initial mean weights and standard deviations of mean weights of the fish in each tank were  $24.7 \pm 3.2$  g and  $20.4 \pm 3.4$  g in 2017 and 2018, respectively. Fish were fed to apparent satiation twice daily five times per week and once daily on weekends over the 16- and 14-week trial periods in 2017 and 2018, respectively. Throughout the study, growth was tracked every two weeks by netting and weighing fish from each tank in batches. Individual and batch weights were taken  $\pm 0.1$  g. Total length from anterior-most part of the fish to the end of the caudal fin rays was taken by hand  $\pm 0.5$  cm.

Upon the termination of the study, fish were euthanized in a solution of buffered tricaine methanesulfonate (MS 222) at 250 mg/L. Necropsy was completed by hand to remove fillets and skin, as well as to remove the internal organs from the esophagus to the anus. Fillets from each fish were preserved for compositional analysis by freezing at  $-20$  °C in polyethylene bags.

#### 4.3. Analytical Procedures

The two diets and frozen fillets were sent to Minnesota Valley Testing Laboratories (MVTL), New Ulm, MN for composition analysis using the Association of Official Analytical Chemists (AOAC) Official Methods of Analysis [28]. Moisture, ash, crude fat, and crude protein were determined with AOAC methods 930.15, 942.05, 2003.05, 978.10, and 990.03, respectively.

#### 4.4. Statistical Analysis and Calculations

Response variables include percent weight gain (WG), specific growth rate (SGR), condition factor (K), survival, feed intake (FI), feed conversion ratio (FCR), fillet yield (FY), viscerosomatic index (VSI), hepatosomatic index (HSI), and composition components.

Responses calculated based on weight of individual fish averaged by tank:

$$\text{Percent weight gain (WG)} = 100 \times (\text{Weight}_{\text{final}} - \text{Weight}_{\text{initial}}) / \text{Weight}_{\text{initial}}$$

$$\text{Specific Growth Rate (SGR)} = (\ln \text{Weight}_{\text{final}} - \ln \text{Weight}_{\text{initial}}) / \text{Time}_{\text{days}} \times 100$$

$$\text{Condition factor (K)} = 100 \times \text{Weight} / \text{Length}^3$$

$$\text{Feed intake (FI)} = 100 \times \text{Weight of feed consumed} / (\text{Mean fish weight} \times \text{population}) / \text{number of days}$$

Feed Conversion Ratio (FCR) = Dry weight of feed consumed/Wet weight gain.

Responses calculated based on individuals then averaged and statistically tested by tank (replication):

Fillet Yield (FY) =  $100 \times \text{Mass of both skinless fillets} / \text{Whole body mass}$ .

Viscerosomatic Index (VSI) =  $100 \times \text{Mass of viscera contents} / \text{Whole body mass}$ .

Hepatosomatic Index (HSI) =  $100 \times \text{Mass of liver} / \text{Whole body mass}$ .

Differences between response variables were evaluated with randomized complete blocking with diet and sampling period (in the case of FI and SGR) modeled as fixed-effects and year as random using PROC MIXED in SAS software (version 9.4; SAS Institute Inc., Cary, NC, USA) ( $\alpha = 0.05$ ). Where significant differences were detected, mean separations were evaluated with Tukey's HSD.

**Author Contributions:** Conceptualization, J.C., M.S.W., N.B.D.P., and D.A.S.; Methodology, J.C., M.S.W., N.B.D.P., and T.G.G.; Formal Analysis, J.C., and M.S.W.; Investigation, J.C., N.B.D.P., and D.A.S.; Resources, T.G.G.; Data Curation, J.C.; Writing—Original Draft Preparation, J.C. and D.A.S.; Writing—Review and Editing, J.C., M.S.W., N.B.D.P., T.G.G., and D.A.S.; Project Administration, D.A.S.; Funding Acquisition, M.S.W., N.B.D.P., and D.A.S. All authors have read and agreed to the published version of the manuscript.

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**Conflicts of Interest:** The authors declare that they have no conflict of interest.

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