MANUFACTURE AND USE OF ALFALFA (*Medicago sativa* L.) LEAF PROTEIN CONCENTRATE AS A PROTEIN SUPPLEMENT IN FISH CULTURE DIETS

A Thesis
SUBMITTED TO THE FACULTY OF THE
UNIVERSITY OF MINNESOTA
BY

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IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
MASTER OF SCIENCE

Dr. Deborah A. Samac & Dr. M. Scott Wells

May 2019

Acknowledgements

This research was possible due to the incredible support of many different individuals and organizations. I am very grateful to have worked with my advisers, Dr. Deborah Samac of the USDA Agricultural Research Service and Dr. M. Scott Wells of University of Minnesota Agronomy and Plant Genetics Department. The research herein was conceived by my advisers to protect soil and water by finding additional markets for alfalfa and increasing the incentive to grow alfalfa in fields and buffers around the Midwest. I was attracted to the project because I re-entered school with a passion to affect improvement in water quality by working in food and agriculture. With appreciation, I thank Drs. Samac and Wells for giving me the space and time to learn new fields of research.

Dr. Roger Ruan of UMN BBE assisted my understanding of industrial processing techniques for alfalfa. Guidance on fish and animal science was patiently provided by Dr. Nicholas Phelps, University of Minnesota, and Dr. Gibson Gaylord, US Fish and Wildlife Service, as they assisted the development of the feeding trials. Paula Phelps, Derrick Casper, and the other staff members of the MN DNR Lanesboro Hatchery provided time and resources to running feed trials. Jay Maher, Marie Abbey and Michelle Sherman of the University of Minnesota provided assistance in establishing and maintaining the aquaponic tanks for feeding trials. Ted Jeo, Peter Lenz, Riley Ellarson, Rebekah Carlson, Alex Hard, Eric Ristau, Artur Marcondes, John Harris, and many others gave time, ideas, and humor to the work. Thank you.

Thank you to the Minnesota Department of Agriculture and the USDA that funded this work. This research was supported by a grant from the Minnesota Department of Agriculture and USDA-ARS project 5026-12210-002-00D. *Mention of any trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.*

Dedication

To faith, family and future.

To my family, that has supported me through all of this, you are my strength and my reason. My parents, partner, and sister each gave me great support in times of need. With much love and appreciation to my kids, who patiently waited for mom to come home and play and sometimes let me tell you about alfalfa.

Thesis Abstract

Alfalfa protein concentrate (APC) is a high-protein, low-fiber, refined product from the legume *Medicago sativa* L. intended for feeding monogastric animals. The research herein extends understanding of the potential for APC use in aquaculture by conducting feeding trials with yellow perch (*Perca flavescens*) and rainbow trout (*Oncorhynchus mykiss*) and evaluating APC yield and content by producing APC with alternative methods, from reduced-lignin varieties, and from stripped alfalfa leaves. Both fish species accepted APC feeds. Growth was slowed in the perch trial where APC was included at 18% of diet, while the trout did not show significant differences among feeds when APC was included at low levels to enhance growth. APC produced with acid contained less fiber while heating produced the highest protein concentration. APC produced from stripped leaves contained less protein than APC from whole forage. APC produced from reduced-lignin varieties did not differ from APC produced from conventional varieties.

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1.1 Protein Consumption around the World and in Aquaculture

Providing a sustainable source of protein for the growing global human population is a major challenge for the 21st century. Animal proteins provide a high density of protein and essential nutrients. Fish and shellfish are the most highly consumed animal proteins in the world at 19.5 kg capita^{-1} annual consumption (FAO, 2017). This figure has doubled since the 1960s primarily due to the increase in global incomes (FAO, 2016). The next three most consumed meats, which have been traditionally provided by agriculture, are pork, poultry, and beef at 16.0, 15.0, and 9.3 kg capita^{-1} annual consumption, respectively (FAO, 2017). While global populations and per capita fish consumption have grown over the last 60 years, wild fisheries have not been able to meet demand and aquatic agriculture, termed aquaculture, has become the fastest-growing food sector in the world (FAO, 2016). Similar challenges exist between both terrestrial agriculture and aquaculture, that is, supplying feed in a sustainable economic manner, competition for habitat and interactions with wildlife, and crowding and disease prevention (Hixson, 2014; Subasinghe, Soto, & Jia, 2009). Production and utilization of aquatic feeds affects each of these challenges (Mente, Pierce, Santos, & Neofitou, 2006).

Feed ingredients must provide necessary nutrients but need to be economical for the production systems, feed waste must be minimized to reduce waste levels discharged into the surrounding environment, and feed ingredients influence the health and stress tolerance of the animals.

Aquafeeds, a $99 billion industry in 2016, are expected to rise to the value of $163 billion by 2021 (Market Data Forecast, 2016). Feeds for aquatic animals often have a higher protein content than feeds for terrestrial animals; in the case of carnivorous fish, protein can be 50% of the diet. Protein in aquatic feeds has traditionally been supplied by fishmeal. However, fishmeal supplies are currently limited with static or declining wild fisheries and their inclusion in aquatic feeds must be optimized and reduced as demand for aquatic feed grows (Rust et al., 2011). Therefore, due to increased demand for high-protein aquafeeds, abundant plant-based feed sources are often used in replacement of fishmeal. Over the last 30 years the aquatic feeds industry reduced the amount of fishmeal within aquatic feeds due to limited supplies, rising costs, increased understanding of nutritional needs of aquatic species, and increased options for feed components to meet those needs. The use of fishmeal decreased from 24% of the overall aquatic diet in 2000 down to 16% in 2008 (Fry et al., 2016). Because of price and competition, fishmeal use has also declined in terrestrial animal feeds for swine and poultry, now making aquaculture the largest user of fishmeal.

Research over the past 100 years has identified the protein requirements (e.g. 25-50%) for many aquatic species grown in confinement (Pillay, 1990). Finfish, crustaceans, and mollusks all require 10 essential amino acids, though in differing amounts (Pillay,

1990). Improved aquatic feeds have developed rapidly in conjunction with the rise in aquaculture. One method of measuring feed efficiency is the feed conversion ratio (FCR) calculated as g feed intake per g weight gain in the animal. Salmon and trout are very efficient with an FCR close to 1.0 (Okumus & Mazlum, 2002). Other aquaculture species also have efficient feed conversion with an FCR ranging from 1.3-3.0 (Naylor et al., 2009). Optimizing the balance of amino acids in the diet is of critical importance to an efficient feed, particularly when the protein requirements are half the feed. The most common alternate feed ingredients used to date in aquaculture include soybean meal, bone and feather meals, corn gluten, wheat gluten, and a few other plant products and plant residues (Table 1-1). The three main limiting amino acids of terrestrial mammals are similarly limiting in the diets of the main aquaculture species of salmon, trout, and other predatory species when including plant-based ingredients (Gaylord, Sealey, Barrows, Myrick, & Fornshell, 2017). Terrestrial agriculture by-products such as blood meal and bone meal cannot fully replace fishmeal because they are poorly digested and their amino acid profiles differ greatly from those needed by aquatic species (Lovell, 1998; Webster & Lim, 2002). However, the use of plant-based ingredients not only reduces demands on the limited aquatic resources of the oceans, but also allows increased control of the amount of mercury and other toxins that build up in fish and seafood products.

Soybean meal is the most widely used fishmeal replacement (Lovell, 1998). The use of defatted soybean meal (DFSM) has increased in use due to its high protein content, acceptance by fish, availability, and low cost (FAO, 2009). For rainbow trout (*Oncorhynchus mykiss*), the typical inclusion rate for soybean meal in the diet is close to

15% (Collins et al., 2012). However, growing soybean (*Glycine max*), an annual crop, has environmental costs, upper limits for inclusion in feed, and important health deterrents (Fry et al., 2016). Phytoestrogens, allergens, lectins, phytic acid, and other anti-nutrients within DFSM can impede growth or feed acceptance, and research is ongoing to reduce or remove these compounds from DFSM (Alexis, Papaparaskeva-Papoutsoglou, & Theochari, 1985; Allan & Booth, 2004; Kasper, Watkins, & Brown, 2007). Plant geneticists are also working to breed soybean varieties that have a more complete amino acid balance in seeds than is currently available (Park et al., 2017). Rainbow trout (*Oncorhynchus mykiss*) have also been bred to respond more positively to DFSM (Overturf, Barrows, & Hardy, 2013).

A protein concentrate made from the foliage of alfalfa (lucerne; *Medicago sativa* L.), has many of the same qualities in macronutrients as DFSM: long chain polyunsaturated fatty acids, high quality amino acids, but lower anti-nutritional components. From component analysis, alfalfa protein concentrate (APC) has a slightly higher quality for aquaculture feeds than DFSM with more crude protein and 27% more of the limiting amino acids lysine, methionine, and threonine (Table 1-1). Since APC is manufactured from the leaves and stems of alfalfa plants, the anti-nutrients differ from those of DFSM and other common plant protein sources, which are produced from plant seeds (Francis, Makkar, & Becker, 2001). More omnivorous and lower-trophic level aquatic species such as catfish and shrimp may easily accept APC as a replacement or in addition to DFSM while more carnivorous fish such as salmon and walleye would likely have a defined upper limit of tolerable amounts of APC. Carp and tilapia have already

been shown to digest and utilize alfalfa meal or APC in their diets (Gaweł & Grzelak, 2012; Kim et al., 2019; Lara-Flores et al., 2007).

Alfalfa, the "Queen of Forages," is widely used as a terrestrial animal forage due to its high protein content in conjunction with good fiber content and other nutritive qualities (Barnes, Goplen, Baylor, Hanson, & Hill Jr, 1988). The impacts of livestock and row crop agriculture on soil, water, and the environment have gained wider study and recognition. Alfalfa provides a range of positive environmental impacts through soil and carbon retention, increasing N fertility, and improving water and nutrient infiltration (King & Hofmockel, 2017; Russell, Laird, & Mallarino, 2006; Russelle, 2001; Sheaffer & Seguin, 2003) and is often used in rotation with annual row crops. Alfalfa also provides wildlife habitat, and serves as a nectar source for pollinators (Jung & Lamb, 2011). Refining alfalfa into a digestible ingredient for fish feeds with additional co-products has ecological and economic advantages (Bals, Dale, & Balan, 2012).

1.2 Refined Alfalfa Protein Concentrate

The fractionation and refinement processes for production of APC consists of first dewatering the fresh alfalfa using various types of presses (Edwards, de Fremery, Mackey, & Kohler, 1977). The dewatering results in a press residue (PR) that can be used fresh, for haylage, dried and pelleted for terrestrial animal feeds, or used for biomass-derived energy. Nutrients available in the PR depend on both the quality of the starting material as well as the dewatering process (Digman, Runge, Shinners, & Hatfield, 2013).

The press filtrate, or juice, can be further processed to extract an insoluble green chloroplastic protein concentrate and a soluble white edible leaf protein concentrate. Separation of the two fractions can be done by differential heating (Edwards et al., 1975; Koegel & Straub, 1996; Lamsal, Koegel, & Boettcher, 2003; Miller, de Fremery, Bickoff, & Kohler, 1975), pH shift (Merodio, Martin, & Sabater, 1983), or ultrafiltration (Eakin, Singh, Kohler, & Knuckles, 1978). From 30-70% of the soluble fraction is composed of ribulosebisphosphate carboxylase (Hood, Cheng, Koch, & Brunner, 1981; Lamsal, Koegel, & Gunasekaran, 2007), which catalyzes carbon dioxide fixation in photosynthesis.

Research, although limited, has demonstrated the successful use of APC in aquaculture diets. The first published study was in 1990 reporting the results of a Mexican team of scientists who used two different types of locally produced APC in diets of tilapia (*Oreochromis mossambicus*) (Olvera-Novoa, Campos, Sabido, & Martínez Palacios, 1990). Olvera-Novoa et al. (1990) found that the fish accepted a high inclusion rate, and it improved their growth, feed efficiency, and other health markers. The research showed that APC can, not only be a possible protein for tilapia and possibly other fish, but it also improved the growth and feed efficiency when used at the right level. The fish responded positively to the APC that was more refined (Olvera-Novoa et al., 1990).

Since the publication by Olvera-Novoa et al. (1990), there have been eight additional published feeding trials that vary by aquatic species, alfalfa products used, and results (Ali, Al-Asgah, Al-Ogaily, & Ali, 2003; Chatzifotis, Esteban, & Divanach, 2006;

Harpaz, Rise, Arad, & Gur, 1998; Jia, He, & Yang, 1991; Rechulicz, Ognik, & Grela, 2014; Robert, Coulmier, Divanach, & Cuzon, 2004; Yanar, Erçen, Özlüer Hunt, & Büyükçapar, 2008; Yousif, Alhadrami, & Pessarakli, 1994). Feeding trials with tilapia fed dehydrated alfalfa leaves in pelleted feeds found negative effects on growth largely related to the fiber content in the unprocessed alfalfa (Yousif et al., 1994). Conversely, another study on pigmentation in freshwater crayfish found that ground dried alfalfa leaves did not significantly affect growth or survival when compared to the control, they did however, significantly increase pigmentation (Harpaz et al., 1998). Also, a blunt snout bream (*Megalobrama amblycephala*) trial with alfalfa meal found tolerance levels as high as 40% of the diet (Jia et al., 1991). Lovell states in his book, *The Nutrition and Feeding of Fish* (1998), that alfalfa meal is a standard feed ingredient high in vitamin E and K. These studies and statements from the last decade of the 20th century indicate that alfalfa was being evaluated for the purposes of fish feeds, but the fiber in the unrefined meal was leading to mixed results. A 2008 study included alfalfa meal in the diets of goldfish (*Carassius auratus*) in order to improve coloring (Yanar et al., 2008). The researchers tested six different levels from 0 to 40% and found that 25% inclusion resulted in optimal pigmentation. However, detrimental effects in growth and feed conversion efficiency were observed with ≥25% inclusion of alfalfa protein. The authors recommended 15% alfalfa meal inclusion for pigmentation in goldfish (Yanar et al., 2008).

Studies including refined, low fiber, APC are also variable in findings. While including APC at low levels (3.6 and 7.2%) in the diet of gilthead seabream (*Sparus aurata*), feed conversion ratios and coloring were improved (Robert et al., 2004).

However, a publication on sharp snout seabream (*Diplodus puntazzo*) showed that including APC at three levels (7, 14, and 21%) was detrimental at each level for growth, efficiency, and mortality (Chatzifotis et al., 2006). They hypothesized that the differing results may have to do with the processing techniques used in producing the APC as well as the pelleted feed. Compound pelleted feeds are often used in aquaculture. Pelleted feeds can be made with standard equipment and are commonly made by farmers. However, professionally and consistently made extruded feeds further process and heat the ingredients of the feeds to reduce the amount of heat-liable anti-nutrient factors within the feeds and enhance digestion (Drew, Borgeson, & Thiessen, 2007). A study with carp (*Cyprinus carpio*) was conducted with a 5% supplementary addition of APC that resulted in a higher specific growth rate, greater length and weight, and overall condition (Rechulicz et al., 2014). This study did not specify what type of pelleting technology was used; however, they hypothesized that the oxidative stress responses in association with anti-nutrients in alfalfa would vary greatly not only with inclusion level but also by species. Feeding studies using monogastric terrestrial animals show that the protein in APC is digestible, and that APC can also be an immunostimulant when used in small doses (Gaweł & Grzelak, 2012). Thus, previous studies show that APC is a promising new protein source for use in aquaculture diets that may also improve health of the animals produced in confined spaces.

Nonetheless, previous research has left knowledge gaps in important areas. While some feeding trials were completed, research is warranted on palatability, inclusion rates, and feed conversion of APC by additional commercial fish species, the effect of processing technologies, and the interaction of ingredient combination including the

interaction of anti-nutrients in plant products in the diets. Research into these topics is limited by lack of APC production in the US and communication between nutritionists and producers of APC. The bioprocessing industry also requires additional research in producing APC. The industry would benefit from knowing the possibilities for optimizing their product for different uses, and updated economic data on establishing and conducting APC production. Farmers and APC producers are also likely to benefit from an increased understanding of what varieties and agronomic practices will optimize the quality of the end product.

A commercial APC process was developed in the 1960s and many aspects patented in the 1970s (Bickoff et al., 1976; Bickoff, Spencer, & Kohler, 1972; Bickoff & Kohler, 1974). The promise of the technology led the USDA to run an economic analysis on converting alfalfa dehydration plants to APC production facilities (Enochian, Köhler, Edwards, Kuzmlcky, & Vosloh, Carl J., 1980). However, the technology was not adopted and there is little information on the economics of APC production since that time.

Alfalfa is the fourth most widely harvested crop of Minnesota with 1,285,000 acres harvested in 2016 (National Agricultural Statistics Services, 2017). New waterway perennial buffer laws in Minnesota that went into place in 2016, accompanied by increased awareness and concern for sustainable cropping techniques, are inspiring more producers to think about markets for perennial crops. APC provides a unique opportunity for Minnesota grain producers and feed suppliers to reduce the environmental footprint of feeding the world while adopting waterway buffers. For example, Cargill, a Minnesota based company, has been heavily investing in developing aquafeeds since 2015

(Anonymous, 2017). The research described in this thesis investigates questions of how APC affects the growth of commercially important rainbow trout and yellow perch, and how agronomic practices effect the product quality of APC for use in aquaculture feeds.

Researchers are searching for solutions that can be implemented quickly that fulfill human needs for food, fiber, and fuel with fewer strains on the environment. Alfalfa cultivation is already well established and the aquaculture industry is rapidly expanding, providing economic opportunities for the aquaculture industry and forage producers alike. For the aquaculture industry, alfalfa offers a renewable plant-based protein source that may have fewer anti-nutrients and better amino acid balance than soybean and other proteins in current use. For crop producers, a new high value product would offer increased financial incentive to grow this environmentally beneficial plant. The challenge is to provide sufficient research data for investment in commercial APC production and adoption of APC in aquaculture feeds.

1.3 Tables

Table 1-1 Composition of common aquatic feed ingredients and alfalfa protein concentrate (APC)

Ingredient[‡]	Protein %	Fat %	Energy kcal kg^{-1}	Met %	Lys %	Thr %	Total AA %
Menhaden Fishmeal	69.2	7.8	5679	1.8	4.7	3.1	64.8
Poultry Byproducts	66.4	UL[ϕ]	5622	1.4	3.9	2.6	60.8
Feather Meal	87.4	UL	6422	0.5	1.3	4.0	80.6
Soybean Meal (SBM)	53.9	1.8	4791	0.6	2.9	2.4	52.9
Solvent extracted dehulled SBM	53.9	UL	4981	1.0	4.3	3.1	71.2
Corn Protein Conc.	82.0	2.9	5867	2.1	1.2	3.3	95.5
Wheat Pastry Flour	13.2	UL	5501	0.2	0.2	0.3	10.0
APC[§]	55.1	12.4	5645	1.2	3.5	2.8	40.1

[‡] Source of first seven ingredients and their compositions are from a USDA and US Fish and Wildlife Service joint database on common and novel ingredients (Barrows, Gaylord, Sealey, & Rawles, 2015).

[§] Source of APC and its composition is Alfalfa Nutrient Concentrate crumble from Ingredients by Nature (Diversified Ingredients, Ballwin, MO) (Ingredients By Nature, 2010)

[ϕ] UL stands for unlisted as these ingredients did not have ether extract data in the source file.

Chapter 2: Alfalfa Protein Concentrate in the Diets of Yellow Perch and Rainbow Trout

2.1 Chapter Summary

Due to a rising demand in aquaculture and limited supply of fishmeal for aquatic feeds, research into plant-derived proteins for aquaculture species is a key factor in continued growth of the industry. Alfalfa protein concentrate (APC) is a high-protein product of refining alfalfa (*Medicago sativa*) foliage for fractionation into multiple products. The objectives of the experiments described in this chapter were to determine the growth and feed efficiency response of yellow perch (*Perca flavescens*) fed a diet with APC replacing 100% of the fishmeal, and to evaluate the use of APC as an additive to support efficient growth of rainbow trout (*Oncorhynchus mykiss*) with APC replacing 3% and 6% of the fishmeal. Results showed that yellow perch reared for 14-16 weeks accepted the APC diet but gained weight at a slightly lower specific growth rate (-0.07% day^{-1}) and had an elevated feed conversion ratio (+ 0.32 g feed for every g of growth) than fish on the control diet. The trout diets supplemented with APC were also accepted well and no growth or efficiency differences were detected. This research indicates that although APC at 18% inclusion resulted in slower growth rates, the feed protein was accepted and did not impact survivorship or condition in either yellow perch or rainbow trout. Further evaluation is needed to find the appropriate levels to optimize benefits and determine limits to growth rates.

2.2 Introduction

Alfalfa protein concentrate (APC) has been explored, to a limited extent, as a dietary protein for fish (Chatzifotis et al., 2006; Olvera-Novoa et al., 1990; Rechulicz et al., 2014; Robert et al., 2004). APC is a high-protein co-product of refining alfalfa for fractionated products that range from biofuels, high quality foods, and isolated enzymes (Koegel & Straub, 1996). APC is commercially produced with 52% crude protein, has a well-balanced amino acid profile, is high in vitamins and antioxidants such as carotenoids, contains approximately 10% fat including 4% omega-3 fatty acids, and is very low in fiber (Ingredients By Nature, 2010; Koegel & Straub, 1996). In contrast to corn and soybean meals used in fish diets, APC has a higher proportion of key amino acids (lysine, methionine, cysteine) and because APC is made from leaves rather than the seeds, it has low fiber and fewer anti-nutritional factors that may allow for increased digestibility in aquafeeds. Cultivation of alfalfa requires fewer agronomic inputs than annual crops and provides many environmental services enhancing sustainability of protein production. In addition, APC may have benefits as a dietary supplement. A small portion of APC included in livestock rations, such as for poultry and swine, may boost the immune system and help fight diseases related to high densities and overcrowding (Gaweł & Grzelak, 2012).

Past studies concluded that APC has benefits as a feed ingredient for diverse fish species. In diets for tilapia (*Oreochromis niloticus*), APC can replace up to 35% of fishmeal protein with no effect on growth (Olvera-Novoa et al., 1990). Inclusion of 5% APC in carp (*Cyprinus carpio*) diets led to increased growth rates (Rechulicz et al.,

2014). Inclusion of 7.2% APC in an extruded diet for sea bream (*Sparus aurata*) was found to increased growth and feed efficiency compared to the control diet (Robert et al., 2004) but that growth and feed efficiency decreased when feeds were not extruded (Chatzifotis et al., 2006). These studies indicate that feed preferences and performance to alternative plant proteins differ by fish species and need to be tested empirically.

This study investigated APC as a component of diets for rainbow trout (*Oncorhynchus mykiss*) and yellow perch (*Perca flavescens*). Yellow perch were selected due to their growing use in aquaculture, their midrange carnivorous diet requiring 30-40% protein, and their tolerance to a range of temperatures, dietary proteins, and seasonality (Hart, Garling, & Malison, 2006). In this study the experimental feed for yellow perch had APC included at 18% of the diet, completely replacing fishmeal in order to study the effects on growth and body composition. Rainbow trout were selected due to their importance in North American fisheries, their carnivorous diets that require 40% protein or more, and their close relationship to other prominent and important aquaculture species in the salmonid family (Webster & Lim, 2002). Inclusion of some plant-based proteins, particularly soybean meal, causes hindgut inflammatory response and enteritis in fish in this family (Drew et al., 2007; Krogdahl, Penn, Thorsen, Refstie, & Bakke, 2010). In this study, rainbow trout were fed small amounts of APC, at 3 and 6% of their diet, to study the effects of APC as a dietary supplement rather than a main component on growth and body composition.

The objectives and hypotheses of this study were to: (1) measure the growth and composition of yellow perch where 100% fishmeal is replaced with APC,

inisonitrogenous and isocaloric diets; and (2) measure the growth and composition of rainbow trout when diets included 3% or 6% APC. We expect that growth rates for perch will not differ between diets and that trout growth will be increased.

2.3 Materials and Methods

2.3.1 Alfalfa Protein Concentrate as Fishmeal Replacement in the Diets of Yellow Perch

Diet formulation. Diets were formulated to be isonitrogenous based on a digestible nutrient basis (Barrows et al., 2015) and an estimation of the digestibility of APC. Floating 3 mm pellets were formulated with 40% crude protein and 12% crude lipids (Table 2-1). The control diet contained 15% fishmeal while APC replaced all the fishmeal in the experimental diet. The APC used was produced by Désialis (Paris, France) and obtained in crumbled form as Alfalfa Nutrient Concentrate from Ingredients by Nature (Diversified Ingredients, Ballwin, MO). The two diets were manufactured in October 2016 at the U.S. Fish and Wildlife Service Fish Technology Center in Bozeman, MT using steam extrusion and oil coating as described by Gaylord et al. (2017). Briefly, the ingredients were mixed and finely milled, extruded through a 3 mm die at approximately 122^{o}C, dried at approximately 102^{o}C, and cooled on an air table. Both diets were stored under sealed conditions at -18°C until needed for feeding.

Feeding trials. Eight 600 L aluminum recirculation aquaponic tanks, maintained with 480-560 L of water were utilized for the feeding trials. 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 700 GPH magnetic drive pumps (Danner Manufacturing Inc., Islandia, NY). Floating insulation rafts contained lettuce in 2017 and basil in 2018 to filter the nitrates from the water. Tanks and biological filters had been started in 2015 for previous yellow perch populations and maintained in between fish populations with ammonium chloride (Hawkins Chemical Co., Roseville, MN). Nitrite, pH, and ammonia levels were monitored three times per week and adjusted to maintain optimal levels for the fish (0-0.5 ppm nitrite and ammonia and pH 7.2-7.8). Greenhouse air temperature was set to maintain between 20.0^{o}C and 23.5^{o}C for the rearing and experimental period. Water temperature was controlled through acclimation to room temperature. Dissolved oxygen was maintained with bubblers at 7-8 mg L^{-1}. Lighting within the greenhouse was supplemented with overhead halogen lights to maintain a 16-hour 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 in 2016 and Minnesota Muskie Farm, Alexandria, MN in 2017). The perch were feed-trained and reared on site until the start of the experiment in February 2017 and March 2018. Feed training was done in large groups to benefit from schooling behavior (Hart et al., 2006) by mixing various fishmeal based 3 mm pellets with a mix of freeze dried feeds including tubifex worms, shrimp, and krill. The ratio of freeze dried feeds to pelleted feeds

decreased as increasing numbers of fish learned to eat the pellets. Once feed training was completed, fish were randomly assigned to tanks and four tanks per diet were randomly assigned within blocks based on greenhouse layout. Approximately 35% and 45% of the original perch survived transportation, acclimation to tanks, and feed training to begin the trials 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. The eight tanks were randomly stocked with 14 and 22 fish $tank^{-1}$ in 2017 and 2018, respectively. Initial mean weights and standard deviations of mean weights of the fish in each tank were 24.7 g ± 3.2 g and 20.4 g ± 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 windows in 2017 and 2018, respectively. Feed was offered at approximately 0800 and 1630 over a 20-minute period in small doses. As the floating pellets were eaten, additional pellets were added. If more than 20 pellets (0.3 g dry basis) remained at the end of the feeding period, the pellets were skimmed off and discarded. Feed buckets were weighed daily to track consumption.

At the beginning and end of the trial fish were netted, counted, weighed individually, and their lengths measured. 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 ± 0.1 g by allowing excess water to drain from the individuals and placing fish in a tared plastic tray. Total length from anterior-most part of the fish to the end of the caudal fin rays was taken by hand ± 0.5 cm.

Upon the termination of the study, fish were netted by tank and euthanized in a solution of buffered tricaine methanesulfonate (MS 222) at 250 mg L^{-1}. Necropsy was completed by hand to remove fillets and skin, as well as to remove the internal organs from the esophagus to the anus. The following measurements were made: total mass, length, mass of both skinless fillets, liver mass, and viscera mass from esophagus to anus including liver. Fillets from each fish were preserved for compositional analysis by freezing at -20ºC in polyethylene bags.

All experiments were conducted at the University of Minnesota Twin Cities campus and adhered to methods approved by the University of Minnesota Institutional Animal Care and Use Committee (Protocol No. 1611-34299A).

Analytical procedures. 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 (Latimer, 2012). Moisture, ash, crude fat, and crude protein were determined with AOAC methods 930.15, 942.05, 2003.05, 978.10, 990.03, respectively.

Statistical Analysis. 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 below calculated based on weight of individual fish averaged by tank:

$$\text{Percent weight gain (WG)} = 100\% * (Wt_{final} - Wt_{initial}) / Wt_{initial}$$

$$\text{Specific Growth Rate (SGR)} = (\ln Wt_{final} - \ln Wt_{initial}) / Time_{days} * 100\%$$

$$\text{Condition factor (K)} = 100\% * Wt / Length^3$$

Feed intake (FI) = 100% * Wt of feed consumed / (mean fish Wt * population) / # of days

Feed Conversion Ratio (FCR) = Dry Wt of Feed Consumed / Wet Wt gain

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

Fillet Yield (FY) = 100 * mass of both skinless fillets / whole body mass

Viscerosomatic Index (VSI) = 100 * mass of viscera contents / whole body mass

Hepatosomatic Index (HSI) = 100 *mass of liver / whole body mass

Differences among 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) ($\alpha = 0.05$). Where significant differences were detected, mean separations were evaluated with Tukey's HSD.

2.3.2 Alfalfa Protein Concentrate as a Supplement in the Diets of Rainbow Trout

Diet formulation. Diets were formulated for 45% crude protein and 19% crude fat with ingredients in the control diet to match as closely as possible to the standard Minnesota Department of Natural Resources (MN DRN) hatchery feed and two experimental diets with 3% and 6% APC (Table 2-3). Extruded 4 mm sinking pellets were manufactured at Prairie Aquatech (Brookings, SD). Feed was stored in original packaging on shelves in the MN DNR storage shed and used completely before it reached six months post manufacture.

Feeding trials. The rainbow trout feeding trial was completed in partnership with the MN DNR at the Lanesboro Hatchery. Rainbow trout hatched and raised on site were used for this trial. On October 30, 2017, roughly 20,000 fingerling trout with an approximate mean individual weight of 45 g were removed from the main population and evenly distributed into eight raceways. The fish acclimated to the new environment until the beginning of the trial on November 23, 2017. Raceways were flow-through and were fed by the Duschee Creek spring at a consistent 9^{o}C year-round. Low density of fish was maintained with a density index in the raceways between 0.11-0.20 for the duration of the study.

Trout were fed twice daily by MN DNR staff. Feeding rate was calculated based on mean length and historic growth in order to meet a length target set by MN DNR. To estimate the average initial weight of the population, 50 individuals were randomly selected from each raceway, anesthetized with clove oil, and individual weights and

lengths were recorded. The remainder of the population was netted and batch weighed in tared buckets of water to obtain a weight for the entire raceway population. Samples (n = 100-300) of trout were also weighed and enumerated twice a month throughout the study to calculate average individual fish weight and growth. For these measurements, three to five nets of 20-60 trout per net were weighed in a tared bucket of water and individuals counted as returned to the population. These intermediate measurements were used for measuring SGR.

At the end of the study, three fish from each raceway were euthanized and the fillets (skin intact), viscera, and liver were weighed. Fillets were frozen at -20°C until sent for compositional analysis at MVTL as described above.

Protocols and all activities related to the experiments through euthanasia were conducted at the MN DNR Lanesboro Hatchery and adhered to methods approved by the University of Minnesota Institutional Animal Care and Use Committee (Protocol No. 1708-35020A) as well as MN DNR management.

Statistical Analysis. Response variables were the same as in the yellow perch trial above with the exception that survival was not consistently tracked by raceway and therefore was not listed by diet, and feed offered was set equally across all tanks and therefore feed intake was not statistically tested. Differences among response variables were evaluated with a randomized complete design with diet and sampling period as fixed effects using PROC MIXED in SAS software (version 9.4; SAS Institute Inc., Cary, NC), ($\alpha = 0.05$).

Where significant differences were discovered, mean separations were evaluated with Tukey's HSD.

2.4 Results and Discussion

2.4.1 Alfalfa Protein Concentrate as Fishmeal Replacement in the Diets of Yellow Perch

No significant differences were detected in K, survival, FY, and HSI indicating that neither diet was detrimental to the growth and health of the perch. The study found significant differences in FBW ($p < 0.01$), FI ($p = 0.05$), WG ($p = 0.04$), FCR ($p = 0.03$), and VSI ($p = 0.01$) at trial completion with fish on the fishmeal diet out-preforming those on the APC diet. Growth was slower in the perch fed the APC test diet with 54.7% (± 3.8 SE) WG compared to perch on the control fishmeal diet at 67.1% (± 3.8 SE) WG. At the end of the trial, the difference in body weights had grown to nearly 20% with mean weights of 40.4 g (± 3.1 SE) and 33.3 g (± 3.1 SE) for fish on the fishmeal and APC diets, respectively. Dietary consumption was greater with perch on the APC diet than the perch fed the fishmeal diet and this combined with growth resulted in a larger FCR for perch on the APC diet (FCR = 1.99 (± 0.23 SE)) compared to perch on the control diet (FCR = 1.67 (± 0.23 SE)).

In an attempt to classify essential amino acid (EAA) needs of yellow perch, Hart et al. (2010) found a similar growth difference between feeds that contained a margin of safety of all EAA at 20 and 40% above predicted needs versus feeds that only boosted

levels of threonine, isoleucine, and tryptophan by a margin of safety of 20 and 40%. Hart et al. (2010) concluded that threonine, isoleucine, and tryptophan were not the limiting amino acids and therefore the difference in growth was due to other EAA provided at the base level without a margin of safety. This may indicate that, similarly, the APC feed is lower in a particular EAA or the fish are having trouble utilizing it. It was noted that some of the perch on the experimental diet had pellets remaining in their stomachs at the time of necropsy. Though pellets were removed prior to weighing the viscera, this observation indicates that the pellets were slower to digest than those of the fishmeal diet.

Perch on the APC diet also had a larger VSI (8.13 ± 0.78SE) than those on the fishmeal diet (7.58 ± 0.78SE). The VSI found in this study were lower than those found in studies testing dietary distillers grain and soybean meal with yellow perch of a similar size (FBW was between 43-53 g, VSI ranged from 9.9 to 10.6) where the diet contained approximately 30% crude protein (Schaeffer, Hennen, Brown, & Rosentrater, 2012). The VSI is tracked in order to assess the level of fat deposits around organs and can indicate an inefficient use of carbohydrates when the protein level is too low (Ighwela, Ahmad, & Abol-Munafi, 2014). Although the low VSI in this study is a positive indicator, the difference between diets may indicate that the carbohydrates within the APC deserve further investigation.

The combination of increased appetite, and increased FCR point out that the perch were not able to digest or absorb the APC diet as easily as the fishmeal diet. Saponin, an antinutritional components which may be present in APC, has been shown to interfere with fat digestion and absorption when given at high percentages (Sen, Makkar, &

Becker, 1998). Alfalfa varieties vary in their production of saponins and production may be affected by environmental conditions (Tava, Odoardi, & Oleszek, 1999). Saponins are not heat labile (Savage, 2003), and if present in feed may have led to a decrease in digestible energy for the fish on the experimental feed. Fish on the APC diet had an increased feed intake by week 10, before feed intake increased for the fish on the control diet in week 12 (Figure 2-1). However, fish on the APC diet had a decrease in growth at week 12 (Figure 2-2) after consuming a greater amount of the experimental diet, indicating that there may have been a problem in absorption or utilization of the nutrients.

The two treatments had similar FI patterns during the course of the study, and no significant differences were noted by measurement period. Feed intake ranged from 0.48-0.82% mean body weight (Figure 2-1). Gauthier et al. (2008) noted that 0.5% BW feed intake is near a basic rate to maintain health while 3% BW feed intake is closer to maximum satiation level. Though the perch were offered feed to satiation, both treatment populations consumed feed at a lower level to maintain minimal growth rather than a more aggressive growth pattern. This may have been due to low densities leading to less aggressive feeding behavior, avoidance due to movements around the tanks from researchers (Malison & Held, 1992), or possible other feed sources from the aquaponics tanks (Andriani et al., 2018; Pinho, Molinari, de Mello, Fitzsimmons, & Coelho Emerenciano, 2017). Malison and Held (1992) reported that yellow perch feeding patterns were significantly affected by both density as well as by the presence of shadows from researchers when lighting is external to the tank, as was the case in this study. The aquaponic tanks supported large populations of algae, and likely at least some of the plankton species listed by Andriani et al. (2018) and Pinho et al. (2017), though presence

of these organisms was untested. While the feeds were consumed at lower rates than seen in other trials, perch on the APC diet consumed more in relation to their body weight than perch on the fishmeal diet on a consistent basis ($p = 0.05$, Table 2-4) showing feed acceptance that indicates palatability.

Diet affected moisture content of the 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 fillet from fish on the fishmeal diet (Table 2-4; $p = 0.05$). Higher moisture content is associated with a difficulty in absorbing and utilizing fats (Tidwell et al., 1999). Protein, lipid, ash, and energy content of fillets did not change significantly with diet and values ranged from 19.4-19.6%, 0.14-0.16%, 1.27-1.28%, and 1.03-1.04 kcal g^{-1}, respectively (Table 2-4). The moisture, protein, and ash values are similar to those reported by Mjoun, Rosentrater, & Brown (2012) when testing plant based lipids in the diet. However, the lipid content of the perch fillets was much higher, at 0.90-1.20% in the Mjoun et al. (2012) report, most likely a difference of including the skins on the fillets. Perhaps focusing future trials on the bile, fat, and saponin interaction would help understand the mechanisms of growth difference. Measurement of saponins in APC would facilitate diet formulations.

Replacing fishmeal in the diets of young-of-the-year yellow perch with APC for 14-16 weeks had a significant negative affect on growth (Table 2-4). Figure 2-2 displays the progression of SGR between the populations on the two diets. No significant differences were detected between the two diets by measurement period. However, for the length of the study, perch on the fishmeal diet had a higher SGR than those on the

experimental diet at 0.46 and 0.39 % BW day^{-1}, respectively ($p < 0.05$). Both diets had SGR on the lower end of what is typically reported in the literature which range from 0.33 to 1.33 (Brown, Dabrowski, & Garling, 1996; Hart et al., 2010; Kasper et al., 2007; Schaeffer, Brown, & Rosentrater, 2011; Schaeffer et al., 2012). Since the FI was low and the SGR are within 0.07%, it is difficult to determine the cause for the growth difference. Additional studies measuring APC digestibility and determining optimal levels of APC in yellow perch diets are needed. Fecal content can be considered for digestion and absorption, as well as studied for extra bile and lipid content that may indicate a reaction to the possible saponins.

2.4.2 Alfalfa Protein Concentrate as a Supplement in the Diet of Rainbow Trout

Alfalfa protein concentrate was supplemented in the diet of hatchery-raised rainbow trout at 3% and 6% of the standard diet for 93 days to observe differences in growth, feed efficiency, and fillet composition. No differences in mean final body weights ($p = 0.48$) were observed among diets over the 13-week trial. The mean weight of rainbow trout in the raceways doubled in size during the trial, from 54 ± 3 g at the start of the trial to 115 ± 2 g at the conclusion, a 0.8% day^{-1} SGR ($p = 0.48$). Average results of growth parameters by diet are reported in Table 2-5. The growth seen in the study is consistent with past performance of trout at the hatchery (P. Schmidt, personal communication, June 21, 2017) but lower than often seen in other rainbow trout feeding trials due to the reduced feed rate applied in order to keep the trout at target sizes for the hatchery and MN DNR programs. In a 72 day trial (Zhang et al., 2012), rainbow trout at a

similar initial size gained 251 ± 9% of their IBW with a SGR of 1.74 ± 0.04% day^{-1} when fed 10% excess of satiation, which was roughly 1.25% BW day^{-1}. Even greater growth was seen in trout fed approximately 2% BW day^{-1} over a 13-week period where the fish gained approximately 500% of the IBW (Gaylord et al., 2017). Higher feeding rates and faster growth rates may reveal differences in performance of rainbow trout on APC feeds in the future. There was also no difference detected in FCR among diets (p = 0.50). The FCR in the present trial, at 0.88 ± 0.06 is in line with the results in prior studies, demonstrating a similar efficiency with the feeds. Zhang et al. (2012) found an FCR of 0.81 ± 0.05 while Gaylord et al. (2017) found an FCR of 0.92 ± 0.11. While the APC diet did not show the increase in growth or efficiency hypothesized, the study shows rainbow trout can grow efficiently and at the same rate as standard diets when APC is included in up to 6% of the diet. This differs from the results seen in a trial with another carnivorous species, sharp snout sea bream (*Diplodus puntazzo*), where APC was included at 7% of the diet and the bream experienced negative effects on WG, FCR, and SGR (Chatzifotis et al., 2006). There are many differences between these two trials, including species, feed pellet production methods, feed rates, and more. Increased study of APC with carnivorous species would help differentiate which variables matter most in the success of APC in fish diets.

2.5 Conclusions

Both yellow perch and rainbow trout accepted APC in their diets and the diets promoted survivorship, condition, and growth. The acceptance of the diets indicates that

APC was palatable to both rainbow trout and yellow perch. Additional replacement values should be tested in combination with variable amounts of soybean meal to evaluate effects on gut inflammation. Replacing 3% and 6% of fishmeal with APC did not impact condition or growth rate of rainbow trout. However, APC at 18% inclusion slowed yellow perch growth while increasing feed intake. The reduced efficiency seen with 18% APC inclusion indicates that the perch had difficulty digesting, absorbing, or utilizing the feed. Future studies into feed digestibility that examine the nutrients remaining in feces, the health of the intestinal lining, and whole body incorporation of nutrients would provide essential information to accurately formulate feeds with APC for efficiency. Efficient feeds allow producers to maximize the economic benefit of APC, and minimize feed waste products, therefore maintaining higher water quality. Future studies should also conduct feeding studies with APC at different inclusion levels in order to establish an optimal APC-based feed formulation for species of interest. Feed studies that focus on current commercial APC products, produced by steam injection heating, will allow the market to grow as suitable levels are identified. Experimental APC products may also identify advantages in digestion and utilization if feeding studies are conducted. The acceptance and maintenance of condition in the yellow perch trial are positive indicators that currently available APC can be optimized in yellow perch diets. The lack of differences seen in the rainbow trout trial suggests that higher amounts of APC can be used to replace fishmeal in the diet. Alternately, vitamin C and astaxanthin in experimental trout diets could be reduced or eliminated to challenge whether low amounts of APC would boost immunity. Studies with smaller numbers of rainbow trout would allow researchers to have more replicates and allow fish feed to satiation without

interfering with the needs of a full hatchery. Small confined studies would also provide the opportunity to test immunological response of fish fed APC by challenging the trout with higher population densities or small doses of pathogens. While feeding trials with perch and trout conducted in this research advance the understanding of carnivorous response to currently available APC, further determination of health effects and efficiency in trout and perch diets will require examination of fecal output, changes in intestinal cell walls, or perhaps simply a longer test period.

2.6 Tables and Figures

Table 2-1 Formulation of diets for trials with yellow perch and rainbow trout.

Diet	CP	CL	FM	APC	FO	SBM	DE
	---------- % w w^{-1} ----------						Kcal g^{-1}
Yellow Perch Control	40.4	12.0	15.0	0.0	6.3	15.0	4.08
Yellow Perch APC	40.1	12.0	0.0	18.4	5.3	15.0	4.02
Rainbow Trout Control	39.0	19.0	15.0	0.0	8.0	16.0	4.15
Rainbow Trout 3% APC	39.2	19.0	12.9	3.0	8.0	16.0	4.15
Rainbow Trout 6% APC	39.4	18.9	10.9	6.0	8.0	16.0	4.15

CP: crude protein, CL: crude lipids, FM: fishmeal content, APC: alfalfa protein concentrate content, FO: fish oil content, SBM: soybean meal content, DE: digestible energy.

Table 2-2 Ingredients (g kg-1) of two diets to evaluate alfalfa protein concentrate replacing fishmeal in the diets of yellow perch (*Perca flavescens*).

Ingredient	Control	APC	Difference
Menhaden Fishmeal Special Select	150.0	0.0	-150.0
Desialis Alfalfa Nutrient Concentrate	0.0	184.0	184.0
Soybean Meal 48% CP	150.0	150.0	0.0
Poultry Meal	150.0	150.0	0.0
Corn Protein Concentrate	50.0	50.0	0.0
Wheat Gluten Meal	3.2	5.6	2.4
Mayflower Pastry Flour	342.6	300.6	-42.0
Lecithin	10.0	10.0	0.0
Stay-C	1.5	1.5	0.0
Vitamin premix ARS 702[‡]	10.0	10.0	0.0
Trace Mineral Mix ARS 640[§]	1.0	1.0	0.0
Sodium Chloride	2.8	2.8	0.0
Magnesium Oxide	0.6	0.6	0.0
Potassium Chloride	5.6	5.6	0.0
Monocalcium Phosphate	18.0	32.0	14.0
Choline Chloride 50%	10.0	10.0	0.0
DL-Methionine	4.2	5.0	0.8
Lysine Hydrochloride	18.1	18.8	0.7
Threonine	3.2	3.2	0.0
Taurine	5.0	5.0	0.0
Yttrium Oxide	1.0	1.0	0.0
Menhaden Fish Oil	63.2	53.3	-9.9

[‡] Contributed, per kg diet; vitamin A, 9650 IU; vitamin D, 6600 IU; vitamin E, 132 IU; vitamin K3, 1.1 mg; thiamin mononitrate, 9.1 mg; riboflavin 9.6 mg; pyridoxine hydrochloride, 13.7 mg; pantothenate, DL- calcium, 46.5 mg; cyanocobalamin, 0.03 mg; nicotinic acid, 21.8 mg; biotin, 0.34 mg; folic acid, 2.5 mg; inositol, 600 mg.
[§] Contributed mg per kg of diet: zinc, 40; manganese, 17; iron, 80; iodine, 6; copper, 15; selenium, 0.4.

Table 2-3 Ingredients (g kg^{-1}) of three diets to evaluate alfalfa protein concentrate as a supplement to rainbow trout feed (*Oncorhynchus mykiss*).

Ingredient	Control	APC3	APC6
Desialis Alfalfa Nutrient Concentrate	0	30	60
Wheat Flour	168.8	161.5	154.2
Soy Oil	68	65.8	63.5
Fish Meal	150	129.5	109.1
Sodium Chloride	2.8	2.8	2.8
Magnesium Oxide	0.6	0.6	0.6
Mold Zap	5	5	5
Potassium Chloride	5.6	5.6	5.6
Blood Meal	40	40	40
Poultry Meal	120	120	120
Feather Meal	40	40	40
Empyreal	65	65	65
Vitamin Premix - open blue	10	10	10
Lysine Hydrochloride	23	23	23
DL-Methionine	6.2	6.2	6.2
Choline Chloride	10	10	10
Mineral Premix - open blue	1	1	1
Stay-C	1.5	1.5	1.5
Fish Oil	80	80	80
Astaxanthin	0.8	0.8	0.8
Monocalcium Phosphate	15	15	15
Taurine	10	10	10
Threonine	6.7	6.7	6.7
Soy Bean Meal	160	160	160
Lecithin	10	10	10

Table 2-4 Effects of replacing fishmeal with alfalfa protein concentrate in the diets of yellow perch for 98-112 days on growth and fillet composition.

Parameter	Diets		SE	*p*-value
	FM	APC		
Growth Performance Indexes				
IBW (g)	23.4	21.7	--	--
FBW (g)	40.4 a	33.3 b	3.13	< 0.01
WG (%)	67.14 a	54.67 b	3.80	0.04
SGR (% day^{-1})	0.46 a	0.39 b	0.02	0.04
K	1.10	1.08	0.02	0.35
Survival (%)	87	83	4	0.26
FI (% BW day^{-1})	0.59 b	0.65 a	0.02	0.05 †
FCR	1.67 b	1.99 a	0.23	0.03
FY (%)	39.82	39.81	0.77	0.97
VSI	7.58 b	8.13 a	0.78	0.01
HSI	2.30	2.40	0.10	0.24
Fillet Proximate Composition				
Moisture (%)	78.86 b	79.26 a	0.42	0.05
Crude Protein (%)	19.63	19.38	0.53	0.20
Crude Fat (%)	0.16	0.14	0.02	0.18
Ash (%)	1.28	1.27	0.05	0.72
Gross Energy (kcal g^{-1})	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 15% fishmeal and 0% APC (FM) along with the experimental diet including 0% fishmeal and 18% APC (APC). Within a row, treatment means with differing letters are significantly different ($p \leq 0.05$).

† FI calculated by two-week growth periods.

Table 2-5 Effects of supplementing rainbow trout diets with 3 and 6% alfalfa protein concentrate for 93 days on growth and fillet composition.

Parameter	Diets			SE	*p*-value
	Control	APC3	APC6		
Growth Performance Indexes					
IBW (g)	56	51	55	--	--
FBW (g)	116	113	116	4	0.48
WG (%)	108.50	122.33	112.67	9.93	0.49
SGR (% day^{-1})	0.79	0.86	0.81	0.05	0.48
K	1.21	1.22	1.18	0.02	0.20
FI (% BW day^{-1})	0.69	0.69	0.69	--	NT†
FCR	0.82	0.91	0.89	0.02	0.50
FY (%)	49.48	47.00	48.27	1.42	0.51
VSI	10.88	11.85	11.10	0.56	0.31
HSI	1.53	1.63	1.57	0.11	0.65
Fillet Proximate Composition					
Moisture (%)	75.23	74.16	75.37	0.57	0.12
Crude Protein (%)	17.55	17.60	18.02	0.24	0.19
Crude Fat (%)	4.94	5.89	4.96	0.55	0.21
Ash (%)	1.43	1.53	1.58	0.06	0.53
Gross Energy (kcal g^{-1})	1.42	1.55	1.46	0.05	0.20

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. Rainbow trout diets were used to test alfalfa protein concentrate (APC) as a supplement and included the control diet and two diets replacing small portions of fishmeal, soy oil, and wheat flour with 3 and 6% APC (APC3 and APC6 respectively). No statistically significant differences were found ($p \leq 0.05$).

† Feed rates were set by DNR staff and not determined individually by diet, therefore, FI was not statistically tested but was averaged across all raceways.

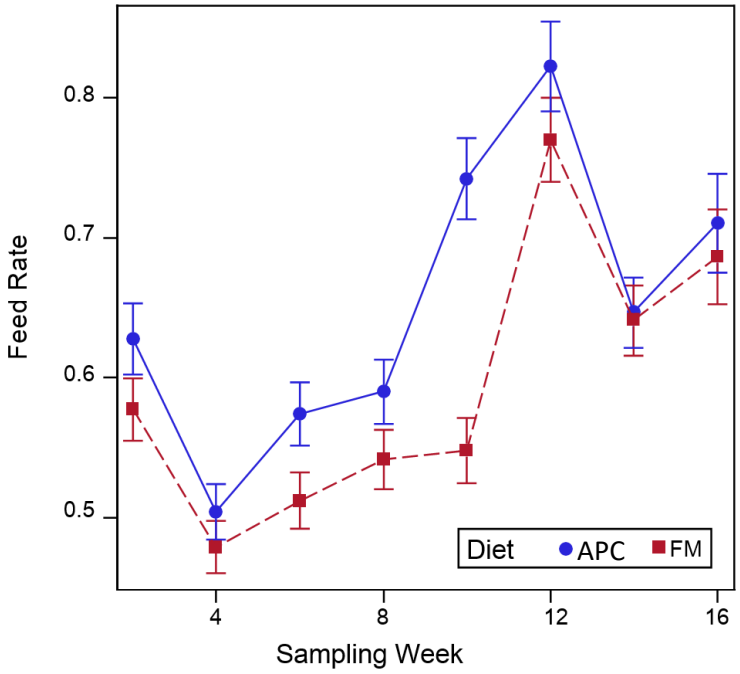

Figure 2-1. Feed intake rate for yellow perch over the 14- and 16-week alfalfa protein concentrate trials in 2017 and 2018. Values are means for each treatment (n=8 for weeks 2-14 and n=4 for week 16) ± standard error. The treatments were isonitrogenous balanced diets with either 18% alfalfa nutrient concentrate (APC; solid blue line), or 15% fishmeal (FM; dashed red line).

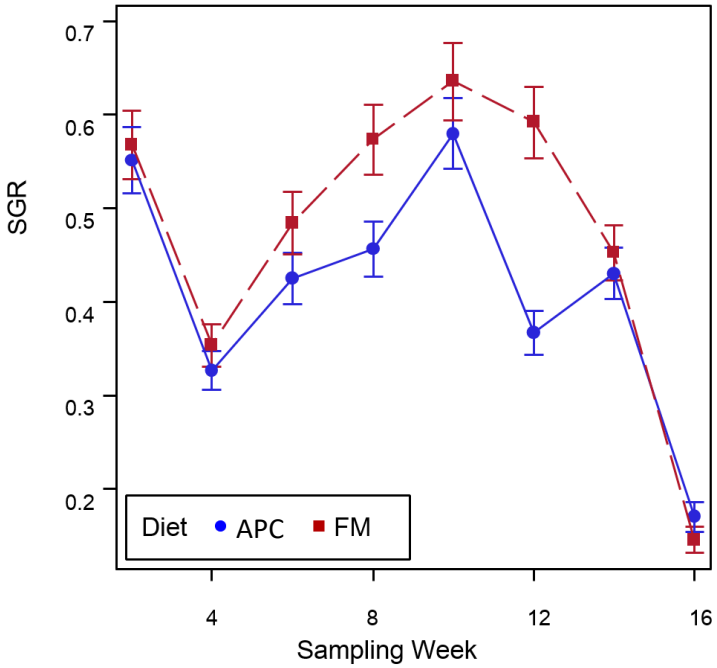

Figure 2-2 Specific growth rate for yellow perch over the 14- and 16-week trials in 2017 and 2018. Values are means for each treatment (n=8 for weeks 2-14 and n=4 for week 16) ± standard error. The treatments were isonitrogenous balanced diets with either 18% alfalfa nutrient concentrate (APC; solid blue line), or 15% fishmeal (FM; dashed red line).

Chapter 3: Evaluation of Alfalfa (*Medicago sativa* L.) Protein Extracts and Press Residue from Whole Forage and Stripped Leaves

3.1 Chapter Summary

As interest grows in refining plants to make protein concentrates as well as extract enzymes and other products, it is important to examine the agronomic practices that affect the processes. The proteins, fatty acids, and other cellular components of alfalfa protein concentrate (APC) derived from processing alfalfa foliage could be a viable component for aquaculture feeds, which will increase the value of harvested alfalfa. Although a number of methods for protein purification have been reported, they have not been compared using the same plant material. The objectives of the experiments described in this chapter were to evaluate the effects of protein extraction methods, use of reduced-lignin alfalfa varieties, and field separation of leaves on the quantity and composition of APC. Five methods of precipitating APC from press filtrate of alfalfa foliage were tested. Acid based precipitation methods resulted in the largest recovery of APC, while heating the foliage extract produced the highest concentration of protein and limiting amino acids in the product. Extraction of APC from reduced-lignin varieties did not result in higher protein extraction compared to conventional varieties. Yields of APC on a dry matter basis were similar for total forage and stripped leaves only. The implication is that APC does not need to be produced from lower yielding immature foliage, but can be produced from standard varieties harvested at later maturities to maximize yields.

3.2 Introduction

Feed-grade alfalfa protein concentrate (APC) is composed of both insoluble and soluble protein fractions and is a low-fiber product high in protein and xanthophylls (Edwards et al., 1977). Use of APC in the diets of poultry, swine, and other animals can increase feed efficiency, enhance the quality of meat and eggs, and contribute to increased animal health parameters (Gaweł & Grzelak, 2012). Poultry fed with APC were able to utilize the xanthophyll 1.7-times more efficiently than xanthophylls in dehydrated alfalfa to gain skin and yolk pigmentation equal to that of pure lutein extract (Kuzmicky et al., 1977). These brief examples illustrate that APC can be used to replace other proteins or enhance the growth of the animal.

Refining APC, and other leaf protein concentrates, has been under study since at least the 1950s (Raymond & Harris, 1957). APC production was found to be a cost-effective way to provide protein to world populations, produce a biomass feed stock for fuels, and as a way to more carefully control the nutrients animals receive in their rations. Patents were filed in the U.S. in the 1970s to define the "Pro-Xan" process (Bickoff et al., 1976, 1972; Bickoff & Kohler, 1974). The name Pro-Xan was used to describe the feed-grade APC due to the high content of both protein and xanthophylls. In 1980, the USDA released a financial report outlining the production methods and costs to produce Pro-Xan for feeding poultry and other non-ruminants (Enochian et al., 1980). However, after 1980, little is documented about Pro-Xan production in the U.S. Currently, commercial APC is available from one company in France, Désialis (Bals et al., 2012).

The fractionation and refinement processes for production of APC consist of first dewatering the fresh alfalfa using various types of presses (Edwards et al., 1977). The dewatering results in a press residue (PR) that can be used fresh, for haylage, dried and pelleted for animal feed, or used for biomass derived energy. The press filtrate, or juice, can be further processed to extract an insoluble green chloroplastic protein concentrate (Pro-Xan) and a soluble white edible leaf protein concentrate ("Welpro"). Separation of the two fractions can be done by differential heating (Edwards et al., 1975; Koegel & Straub, 1996; Lamsal et al., 2003; Miller et al., 1975), pH shift (Merodio et al., 1983), or ultrafiltration (Eakin et al., 1978). Ribulosebisphosphate carboxylase composes 30-70% of the soluble fraction (Hood et al., 1981; Lamsal et al., 2007). The differing fractionation methods result in differences in composition of the resultant APC, with differential heating generally recovering the highest measures of crude protein (Zhang, Grimi, Jaffrin, Ding, & Tang, 2017).

Changing economies of energy, crops, and feeds has contributed to the difficulties of stabilizing a production and marketing system for APC (Bals et al., 2012). Transportation of fresh cut alfalfa is more costly than transporting hay due to the extra water weight, which usually makes up 80% of a fresh crop weight (Koegel & Bruhn, 1978; Shinners, Herzmann, Binversie, & Digman, 2007). Thus, extracting the most APC from each fresh ton of alfalfa transported is important for recovering transportation costs. One solution to minimize transportation costs is to juice the alfalfa directly on site with a mobile extractor (Marcotte, Savoie, Hamel, & Vezina, 2002). Another means of reducing transportation costs is field separation of alfalfa leaves and stems (Digman et al., 2013). Separating leaves and stems in the field can be done with a harvester with rotating tines

that are able to pull off the apical, most tender portion of the plant and the majority of the leaves (Shinners et al., 2007). Results showed that the pressed leaves could be refined into three or more products: field biomass residue for hay, press residue for haylage, and APC. Field separating the leaves of a non-lodging biomass type of alfalfa (Lamb, Jung, Sheaffer, & Samac, 2007) would allow the plants to grow to a later stage of maturity, accumulate more biomass, and reduce the number of harvests to maximize the value of the stand and reduce harvest costs. However, fractionation of biomass-type alfalfa for APC production has not been tested previously. Koegel and Straub (1996) noted that more mature alfalfa plants produced less APC than less mature plants, possibly due to the larger amount of fiber in the press residue blocking protein extraction. They also reported that the ratio of soluble to insoluble proteins did not change with crop maturity indicating that quality of the proteins was consistent in young and mature leaves. Amino acid content of APC does not significantly differ at different plant maturities (Balde, Vandersall, Erdman, Reeves, & Glenn, 1993). New alfalfa varieties have been developed that maintain lower levels of acid detergent lignin and higher digestibility than conventional varieties of alfalfa (Grev, Wells, Samac, Martinson, & Sheaffer, 2017). Leaf stripping mature alfalfa and reduced lignin varieties of alfalfa may offer a chance to produce higher yields of APC without affecting the ratio of soluble proteins due to a possible effect of lignin on the ability to extract protein rich juice.

The increasing need for a protein replacement in aquaculture diets (Rust et al., 2011) and expanding markets for specialized feeds and sustainable products (Subasinghe et al., 2009) warrants reconsideration of APC production. In aquafeeds, fishmeal, a limited resource, is being replaced by alternate plant-based proteins (FAO, 2016; Rust et

al., 2011). Soybean meal is often utilized to replace fishmeal in aquafeeds (Fry et al., 2016) due to its availability, low price, and ability to maintain reasonable growth in many fish species (Naylor et al., 2009). However, due to a differing amino acid balance, as well as anti-nutritional components, soybean meal is not able to substitute for all the protein in an aquafeed diet (Burrells, Williams, Southgate, & Crampton, 1999; Francis et al., 2001).

APC has been used successfully in feeds for tilapia (Olvera-Novoa et al., 1990), sea bream (Chatzifotis et al., 2006), shrimp (Harpaz et al., 1998; Robert et al., 2004), and carp (Rechulicz et al., 2014) as both a protein source as well as a source of vitamins and carotenoids (natural color enhancers). Tilapia fed a diet with 11% APC replacing fishmeal maintained growth and feed use efficiency comparable to the control, while tilapia fed a diet with 20% Welpro showed improvements in growth and feed use efficiency significantly over the control (Olvera-Novoa et al., 1990). With the varied uses of APC as a protein supplement as well as a source of dietary micronutrients, a comprehensive nutritional profile is needed for APC produced by various production methods. Therefore, to develop further use of APC in aquaculture, an investigation is needed to compare APC composition from differing starting materials and from different juice processing methods.

Carnivorous fish require feeds with 45-50% protein, and therefore require a highly digestible protein in their rations. When replacing fishmeal with plant-based proteins, methionine, lysine, and threonine are often the amino acids in most limiting supply (Bendiksen, Johnsen, Olsen, & Jobling, 2011; Naylor et al., 2009). Crystalline amino acid supplementation can add great cost to aquaculture feeds. Therefore, protein

products that maximize these limiting amino acids may assist in creating economical and balanced diets. Changes in pH, additives, and temperature of the press filtrate have all been explored for APC production (Edwards et al., 1977; Lu, Jorgensen, Straub, & Koegel, 1981; Merodio et al., 1983; Miller et al., 1975). However, a comparison of the composition of APC resulting from each production method, which may affect the available nutrients and digestibility by fish, has not been investigated previously.

The objectives of the study were to: (1) measure the yields and composition of APC from a biomass type alfalfa when using differing APC extraction methods; (2) determine whether using reduced lignin alfalfa varieties results in more efficient protein extraction; and (3) compare yields and composition of APC and PR produced from stripped leaves and total foliage.

3.3 Materials and Methods

3.3.1 Testing Five APC Extraction Methods

Forage Production and Processing. A biomass type experimental alfalfa germplasm (UMN 4624) was planted at the University of Minnesota Southern Research and Outreach Center in Waseca, MN at a rate of 13.45 kg ha^{-1} with a target density of 180 plants $meter^{-2}$ in 12.2 x 4.6 m plots. UMN 4624 was developed for large, non-lodging, woody stems at the late-flower maturity stage (Lamb et al., 2007). The plants were part of a rotational study using alfalfa, soybean (*Glycine max*), and corn (*Zea mays*). The alfalfa plots used were established in spring of 2015 and harvested for this study on September

13, 2016. The field for this experiment was a split plot design with four blocks and three plots in each block with differing potassium treatments: 0, 224, and 448 K_2O kg ha^{-1}. Total foliar biomass was harvested for this study from a mix of field plots with 224 and 448 K_2O kg ha^{-1}, when plants were at 50% flowering stage, using a forage flail harvester. Fresh plant matter was stored in coolers with ice for transport to the laboratory and placed in a cold room for one to six days before macerating with deionized water in a CB15 3800 ml stainless steel commercial blender (Waring Commercial Products, Torrington, CT). After the plant material had been macerated, the pulp was hand pressed through four layers of cheesecloth to separate the juice from the PR. Once all material had been macerated and pressed, all storage containers of juice were combined and homogenized before splitting samples for protein precipitation.

Concentrate Preparation and Characterization. Five methods of whole juice APC coagulation were evaluated for yield and nutritional content. The methods were: (1) heating the juice in an 80°C water bath for 30 minutes, (2) freezing the juice at -15°C, (3) lowering the pH of the juice to 4.0 with 6 N hydrochloric acid, (4) lowering the pH to 4.0 of juice chilled under refrigeration with chilled 6 N hydrochloric acid, and (5) raising the pH of the juice to 10.0 with 6 N sodium hydroxide, letting it set for 15 minutes at room temperature, then lowering the pH to 4.0 with 6 N hydrochloric acid. These methods are hereafter referred to as “heat”, “freeze”, “acid”, “cold acid”, and “base + acid,” respectively. Freezing was chosen as a control, heat to compare to prior studies, and pH changes to test for simplified methods of in-field processing. In each replication, all processes were started simultaneously, including overnight refrigeration of the chilled juice for cold acid. Precipitation methods with pH change were done using a magnetic

stir plate to mix the solution. Frozen juice samples from freeze were thawed for approximately an hour at room temperature before centrifugation. Three replicate samples were produced using each coagulation method.

The protein precipitate was obtained by centrifugation in a model J-6B with a swinging bucket rotor at 4,000 rpm for 12 min, RCF_{max} = 4,050 x g (Beckman Coulter, Indianapolis, IN). The supernatants were discarded and pellets were dried on parchment paper at 43°C until a stable weight was obtained. Samples were then ground in a 8000M Mixer Mill (SPEX SamplePrep, Metuchen, NJ) to a fine powder before being sent for compositional analysis and amino acid profiling at Minnesota Valley Testing Laboratories (MVTL), New Ulm, MN using the Association of Official Analytical Chemists (AOAC) Official Methods of Analysis (Latimer, 2012): Moisture (AOAC 930.15), ash (AOAC 942.05), crude fat (AOAC 2003.05), crude fiber (AOAC 978.10), and crude protein (AOAC 990.03). In addition, sugar profiles were obtained using the American Association of Cereal Chemists Approved Methods of Analysis 80-04 modified method (AACC International, 1999) and fatty acid profiles obtained using the AOAC 996.06 method. Specifically, values for 18:2 linoleic (omega-6), 18:3 linolenic (omega-3), and 20:5 eicosapentaenoic (EPA) were compared. Starch and tryptophan content were obtained using proprietary methods by MVTL Research and Development.

Statistical Analyses. Analyses were completed using a randomized complete block design. ANOVA was completed in SAS software (version 9.4; SAS Institute Inc., Cary, NC). The number of days after homogenizing the juice before processing for APC was treated as the block and the treatments were the coagulation methods. Statistical

significance was set at $p < 0.05$. Mean separations were tested for significant effects using Tukey's HSD. Response variables included APC yield; and component content on a dry basis including protein; sum of the three amino acids methionine, lysine, and threonine; fat; sugar; and fiber.

3.3.2 Investigating the Impact of Reduced-lignin Alfalfa Varieties on APC Yield and Crude Protein Concentration

Forage Production and Harvest. Three varieties of alfalfa were grown at the University of Minnesota UMore Park, Rosemount, MN. The varieties were selected to compare two reduced-lignin types with a standard variety for yield and quality of both APC and the PR. Hi-Gest 360 (Alforex Seeds) is a reduced lignin variety with high forage quality developed by conventional selection and was planted at 24.7 kg seed ha^{-1}, referred to here as "HiGest". HX-14376 (Forage Genetics International) is a genetically modified reduced lignin variety with high forage quality planted at 18.5 kg seed ha^{-1}, referred to here as "HarvX". Pioneer 54VR08 (Pioneer Hi-Bred International, Inc.) is a standard high-quality alfalfa often used in the northern U.S. planted at 24.7 kg seed ha^{-1}, referred to here as "Control". Field plots included four replication blocks with four varieties of alfalfa planted in a quadrant of each block. The plots were planted May 18, 2016 and harvested for this study in 2017. Total above ground foliage was harvested using a flail forage harvester (Carter Manufacturing Company) when the overall field was at flowering stage. Plant maturity was assessed using the mean stage by count method to determine the average maturity for each variety (Kalu & Fick, 1983). Three harvests were done on June

9, July 18 and September 13, 2017. Each harvest was planned for when the field was at roughly at the early flowering stage, but individual plots were at differing stages of growth and ranged, for this trial, between early bud stage to early flowering stage. Hand-cut samples were taken for dry matter assessment as well as for forage quality assessment as described below. Machine-cut sample material was brought to the laboratory for processing and refinement to APC.

Processing of Plant Material. Total proteins were extracted by combining 1,000 g of fresh material from each of the three replicate plots, making a 3,000 g experimental unit. Material was homogenized in a CB15 3,800 ml stainless steel commercial blender (Waring Commercial Products) in ~500 g portions mixed with 1,000 g deionized water. Once a portion was homogenized, the liquid was collected and used for homogenizing the next portion of plant material. This practice was repeated until all 3,000 g of plant material from a variety had been processed. Once all material was blended, pressing of the total homogenate was done using a stainless-steel cider press lined with a 5-gallon mesh paint strainer bag (Reaves & Co., Inc., Durham, NC). Two liquid samples were processed from each variety in a shaking water bath at 69-72°C, cooled to room temperature, transferred to 1 L centrifuge bottles, and stored overnight at 4°C. The protein precipitate was obtained by centrifugation, dried, and ground to a powder as described above. The PR was dried at 60°C until a stable weight was obtained, ground to pass a 1 mm sieve, and stored at room temperature until analyzed.

Sample Analyses. PR samples were scanned using near infrared reflectance spectroscopy (NIRS) using a Perten NIRS (Model DA 7200; Perten Instruments, Springfield, IL) with calibration equations developed at the University of Minnesota to estimate forage nutritive value for crude protein (CP), neutral detergent fiber (NDF), acid detergent lignin (ADL), and neutral detergent fiber digestibility (NDFD). The standard error of cross validation was 0.98, 1.98, 1.52, and 2.64, respectively, for CP, ADL, NDF, and NDFD, while the R^2 was 0.98, 0.80, 0.86, and 0.87, respectively. Relative forage quality (RFQ) was calculated from the NIRS results and hay equation as described previously (Undersander et al., 2011). At MVTL, wet chemistry procedures for compositional analysis were completed on dried and milled PR and powdered APC as described above. Moisture, ash, amino acid profiles, crude fat, crude fiber, and crude protein were assessed as before with AOAC standard methods. For the APC only, the total amino acid profile was determined (AOAC 994.12) and the percentage of lysine, methionine, and threonine were calculated for these amino acids.

3.3.3 APC Production from Field Separated Leaves

Plant Material Production and Harvest. For this trial, the same biomass type alfalfa (UMN 4624) planted at the University of Minnesota Southern Research and Outreach Center in Waseca, MN was used as described above. The alfalfa plots used were established in spring of 2016 and harvested for this study in 2017. For APC production, second, third, and fourth harvests were done on July 6, August 8, and October 16, 2017, respectively, when plants were at approximately 50% flowering stage. Total foliar

biomass was harvested using a forage flail harvester in a 3 m strip. Foliage was separated into a leaf fraction using a custom-built leaf stripper in the remainder of the plot as described previously (Shinners et al., 2007). Leaf fractions consisted of the upper portion of the plant with tender stems. After leaf stripping, the stems were harvested, from the same strip as the leaves, using the flail forage harvester.

Concentrate Preparation and Characterization. A single-screw press (S-Press model RSP-6-H2, Rietz Manufacturing Company, West Chester, PA) was used to produce a protein rich "juice" fraction and a fibrous PR from total foliage and the leaf fraction in the same manner as previously described (Digman et al., 2013). Material from each plot was run through the press twice to allow for additional juice extraction and the screw press was paused between each plot to collect and weigh material. Run number was recorded to account for changes from the first plot to the end plot run. The plant material was loaded into the hopper one plant fraction at a time with random order of plots. Each plot was pressed until juice expression had ceased, then the fiber fraction was processed a second time to obtain any remaining juice. Fiber and juice fractions were weighed and sub-sampled for each plot. Whole foliage was processed first followed by the leaf fraction. The juicing process was delayed for the second harvest and material was bagged and stored in coolers overnight. For the third and fourth harvests, material was juiced on the same day.

Sub-samples of the PR were weighed and dried in ovens at 60°C until stable dry matter was achieved. The dry weights were recorded and used to calculate PR moisture.

Dried PR was ground to pass a 1 mm sieve, scanned with NIRS and submitted to MVTL for compositional analysis as described above. Sub-samples of approximately 700 ml of the juice portion were heated in a shaking water bath until they reached 74°C and were held at that temperature for 15 minutes, cooled to room temperature, then stored overnight at 4°C. Centrifugation, drying, and grinding of APC were completed as described above. APC samples were combined by block and harvest date before being sent for compositional analysis and amino acid profiling at MVTL. This resulted in mixing APC from the 224 and 448 kg ha^{-1} potassium treatments for the compositional and amino acid analysis.

Statistical Analyses. Statistical analyses were performed using the MIXED procedure in SAS software (version 9.4; SAS Institute Inc., Cary, NC) where fixed effects included alfalfa portion (i.e., alfalfa leaves vs. whole foliage), alfalfa harvest date (July 6, August 8, and October 16, 2017), and potassium fertilization rate (224 vs. 448). Potassium fertilization did not impact ($\alpha = 0.05$) or interact with alfalfa portion and harvest date; therefore, a parsimonious model including alfalfa portion and harvest date was selected for further analysis. Due to the screw press uptime limitations, and potential carryover between samples, 'press run' was considered a random effect. Response variables included APC and PR yield; protein, fat, and fiber APC content; content of three limiting amino acids of APC, methionine, lysine, and threonine; and protein content and RFQ of PR. Random effects (with appropriate nesting) included block, run, as well as all

interactions between these effects. Mean separations were performed using Tukey's HSD at $p \leq 0.05$ for all response variables.

3.4 Results and Discussion

3.4.1 Effect of Coagulation Method on Concentration of Crude Protein and Nutritional Quality of APC

Five coagulation methods were tested for whole APC coagulation from homogenized total foliage. Each method significantly impacted total product yield ($p < 0.001$), protein concentration in the product ($p < 0.001$), and concentration of limiting amino acids ($p < 0.001$), fiber ($p < 0.01$), fats ($p < 0.01$), and sugars ($p < 0.001$) in the products. Yield (g APC kg^{-1} of starting material (foliage)) on a dry matter (DM) basis was highest in the three acid coagulation methods at 141.7, 137.5, and 134.2 g kg^{-1} for the base followed by acid, acid alone, and cold acid method, respectively (Table 3-1). The freezing and heating coagulation techniques yielded significantly less APC at 108.8 and 105.0 g kg^{-1}, respectively. These values are similar to those reported by Bruhn and Koegel (1977) for yield of APC from immature alfalfa after the juice was heated to 80°C to coagulate proteins.

Heating resulted in the highest CP concentration at 50.9%, followed by freezing at 48.9%, then the acidification methods at 45.3%-45.8% (Table 3-1). This pattern is consistent with past results that showed acid precipitates additional cellular components in addition to proteins (Hernandez, Martinez, & Alzueta, 1989). However, because the total APC yield was highest with acidification methods, these methods yielded the

highest total amount of CP kg^{-1} starting dry matter. The CP yields are lower than those previously reported. Using steam to heat the press filtrate to 85°C, Edwards et al. (1977) obtained coagulums with 49.6-65.8% CP and Lu et al. (1981) reported obtaining 59-61% CP heating the press filtrate to 80°C using steam. Results from this experiment may be lower due to proteolysis occurring during storage of the filtrate before processing and the relatively long period needed to heat the press filtrate in a water bath.

The total content of the three limiting amino acids (i.e. 3 Lim AA) lysine, methionine, and threonine was the highest in APC made with heating the filtrate, at 5.8% of content, and significantly different from the other four methods at 5.2-5.3% of content (Table 3-1). Ameenuddin et al. (1983) also found small changes in amino acid composition between APC produced by heating and by acid precipitation. However, all methods tested resulted in a product that is high in lysine (2.35-2.53%), methionine (0.70-0.88%), and threonine (1.78-2.13%) (

Table 3-2).

All coagulation methods resulted in low fiber content in the APC products, making the APC products potentially more easily digested by monogastric species. Fiber content significantly differed between the heat coagulation method and the acid and base followed by acid method, with the lowest fiber content present in the APC product from the acid treatment methods at 0.6-0.7% (Table 3-1). The heating method, in contrast, resulted in the highest fiber content, 1.2%, similar to that reported by Edwards et al. (1977) for concentrates produced by heating. When making APC for use in diets of monogastric species, controlling the fiber content is critical in the efficiency of feeds and reducing wastes (Lovell, 1998).

Fat content was significantly different in APC products produced by heating, acid, and cold acid methods, with the lowest fat content present in the APC produced using the acid coagulation method at 9.0% (Table 3-1). The heating method, in contrast, produced 11.4% fat content. Crude fat in APC products from our study was similar to published product standards from commercially produced heat coagulated APC at 10.4% fat (Ingredients By Nature, 2010). Edwards et al. (1975), however, reported 13.7% crude fat from green protein fractionated with heat. Whereas Hanczakowski, Szymczyk, & Skraba (1991) reported 5.2% crude fat when green proteins were produced by heating juice to 80^{o}C and the green APC made for feeding trials with tilapia contained 6.77% crude fat (Olvera-Novoa et al., 1990). This wide range of differences could be related to differences in lipid oxidation, alfalfa varieties, or seasonal changes and may be worth further investigation due to the high energy effect of fats in the diet. No differences in

essential fatty acids were detected among coagulation methods. The three primary fatty acids within the APC were omega-3, palmitic, and omega-6 comprising roughly 42-44%, 24-27%, and 16-18% of total fatty acids, respectively. The high presence of omega-3 fatty acids could be a benefit for aquatic and terrestrial species alike. However, digestibility is affected by the fat content and source (Hanczakowski et al., 1991; Mjoun et al., 2012) and separation of protein and fat into different feed ingredients allows further control in feed formulations.

Total sugar content was lowest in APC products resulting from the heating method as well as the base + acid method at 1.1% and 1.3%, respectively (Table 3-1). Sugar content was highest in the APC from the freezing method at 3.8%. Sugar content within APC is not well reported, but could potentially affect protein digestibility through glycation in a Maillard reaction during APC production as well as during pellet formation when feeds are cooked or extruded (Fremery, Bickoff, & Kohler, 1971; Gaylord, Barrows, & Rawles, 2010).

Although several methods for coagulating total proteins from alfalfa press filtrate have been published, this is the first study to compare yield and composition of APC produced by five different methods. Acidification, freezing, or heating the press filtrate resulted in products with different compositions. Thus, it is possible to somewhat tailor the APC composition by the coagulation method. Due to the high demand for protein and limiting amino acids (Kim et al., 2019) as well as the need for omega-3 fatty acids in carnivorous fish diets (Lovell, 1998), further experiments within this research used the heating method for APC preparation. Heating is also the industry standard and the most

economical for large scale APC production (Bals et al., 2012; Edwards et al., 1975; Enochian et al., 1980).

3.4.2 Effect of Reduced Lignin Varieties on Yield and Quality of APC and the PR

Alfalfa varieties with reduced lignin composition were tested for their effects on protein coagulation. The first harvest of alfalfa foliage was made on June 9, 2017. The second harvest was taken 39 days later on July 18, and the final harvest was taken on September 13, 57 days after the second harvest. Total dry matter yields of unprocessed alfalfa were lower for HarvX plots compared to the HiGest and Control varieties with an overall season average of 9.77, 12.50, and 11.81 t ha^{-1} for HarvX, HiGest, and Control, respectively (

Table 3-3). However, HarvX had higher forage quality at each harvest based on NIRS estimation of RFQ (

Table 3-4). The difference in forage quality between HarvX and the other two varieties was particularly striking for the second harvest in which all varieties were harvested at early flowering stage Table 3-4. The RFQ for HarvX was above 151 at each harvest date, the forage quality recommended for high producing dairy cows (Undersander et al., 2011).The preservation of forage quality in HarvX foliage with increasing plant maturity is consistent with past research on yield and quality of HarvX alfalfa varieties (Grev et al., 2017).

The alfalfa varieties did not differ in APC yield (p = 0.45). The amount of APC produced ranged from 58.0 to 72.1 and was 66.9 g APC kg^{-1} foliage dry matter averaged over the three varieties and three harvests (Table 3-5). This is an indication that lignification due to forage maturity may not be a determining factor for APC yields as was suggested by Koegel and Straub (1996). The percentage of CP content in the APC ranged from 48.3% to 50.1% (Table 3-5). There was no difference by alfalfa variety (p = 0.39). The limiting amino acids for aquaculture feeds are methionine, lysine and threonine. The concentration of these amino acids did not vary by variety. The lack of significant difference among varieties is consistent with previous findings from Balde et al. (1993) as well as Koegel and Straub (1996) who determined the ratio of amino acids and the ratio of soluble to insoluble proteins in APC produced using alfalfa of differing maturities. Without differences between varieties in yield and CP content of APC, this information gives producers increased options to make decisions based on co-product outputs or site and producer specific management plans.

The PR co-product retained 14.68 to 16.01% CP with no differences among varieties (p = 0.12). The PR from HarvX had the highest RFQ, but with a relatively low RFQ of

97, it is most suited for feeding beef cattle, older heifers, or for biomass energy (Undersander et al., 2011). The low RFQ values of the PR here are most likely associated with the blender maceration method as previous reports on maceration technique highly influence the products (Carroad, Anaya-Settano, Edwards, & Kohler, 1981). The yields of PR obtained were significantly different by variety ($p < 0.01$). The material harvested from the Control resulted in the highest amount of PR at each harvest followed by HarvX and HiGest with averages across the three harvests of 776.8, 690.5, and 667.2 g kg^{-1}, respectively (

Table 3-6) suggesting that the conventional variety retains more fibrous material than the reduced lignin varieties after processing. This result was consistent with higher percent fiber in the PR from the Control and HiGest compared to HarvX.

3.4.3 Effects of Field Separation of Leaves and Stems on APC and PR Yield and Composition

Dry matter yields of unprocessed total alfalfa foliage from the first, second, third, and fourth harvests were 4.1, 4.1, 2.8, and 1.5 t DM ha^{-1}, respectively. The total yield of 12.5 t DM ha^{-1} for the season harvested at full flower is similar to yields achieved with the same planting density harvested at green pod in two MN locations in a previous report (Lamb, Sheaffer, & Samac, 2003). These yields are higher than the national average of 7.8 t ha^{-1} which is usually an earlier growth stage and higher density planting (Samac, Jung, & Lamb, 2006). It is also a higher yield than the 10.1 to 10.6 t ha^{-1} achieved when harvested at late flower at a medium density planting of 270 seeds m^{-2} in Minnesota (Sheaffer et al., 2000). The reduced density of planting allows for increased stem yield as well as decreased leaf drop (Samac et al., 2006). Yield of leaves plus immature stems using the leaf stripper from the second and third harvests averaged 2.1 and 1.3 t ha^{-1}, respectively, which was 51% and 46% of total herbage yield. Though APC yield per hectare is lower when the biomass variety is harvested by stripping the apical potion, harvesting and drying the residual stems could be used as a biofuel product (Bals et al., 2012; Lamb et al., 2007, 2003; Samac et al., 2006) offering producers another use for their crop.

APC yields from leaves and total foliage differed ($p < 0.05$) by harvest (Table 3-7). Yields of APC were 102.3 and 97.2 g kg^{-1} for second harvest, 105.3 and 130.6 g kg^{-1} for the third harvest, 107.5 and 108.9 g kg^{-1} for the fourth harvest for total foliage and stripped leaves, respectively (Table 3-8). The yield that differs from the others is the stripped leaf yield in the third harvest at 130.6 g kg^{-1}. It is not clear what the difference was in this harvest material that led to this 25% increase in DM yield and may warrant further study to determine if it was related to changes in soil moisture availability (Zhang & Shi, 2018) or other physiological changes through the season. The yield per hectare results are as expected because of the biomass difference in the harvests between the total foliage from the second and third harvest and that of the stripped leaves. The yield of APC per hectare produced from total foliage differed from the yield of APC per hectare produced from stripped leaves ($p < 0.001$) for the second and third harvest (Table 3-8). The fourth harvest had similar yields of APC from stripped leaves and total foliage on a hectare basis.

Protein content within the APC across the two material types differed by harvest timing ($p <0.001$, Table 3-7) at 42.0, 38.0, and 36.1% for the second, third, and fourth harvest, respectively (

Table 3-9). This result was not unexpected, since Sheaffer et al. (2000) observed variation in CP content in total alfalfa herbage during the season. However, these findings are unique, as studies in the past have focused on differing maturities rather than the change of APC quality through the season with sequential harvests. APC CP concentration across the three harvests also differed by material type ($p < 0.05$) at 39.5 and 37.9% for total forage and stripped leaves, respectively (

Table 3-9). This is also a unique finding, as CP in APC produced from stripped leaves and total foliage has not been compared previously. It was a surprising result, because the majority of protein in total foliage is found in leaves (Sheaffer et al., 2000). The difference may be due to the difference in amount of maceration of the two materials with harvest, rather than the differences in plant fractions. The total foliage material harvested with the flail forage harvester was cut roughly into ~75 mm sections, while the stripped leaves were pulled from the stems without further chopping. Digman et al. (2013), found a 5% increase in recoverable protein from macerated material over unmacerated alfalfa. Similarly, Edwards et al. (1977) found an increase in both yield and CP extraction from grinding chopped alfalfa before pressing to extract juice. Neither of the materials used in this experiment went through a maceration step, but the difference in chop length is likely to have contributed to the difference found. Further studies may be warranted on the amount of CP in APC made from stripped leaves and the total foliage with different amounts of maceration. If maceration is the cause of the difference between the two materials for CP content in APC, the results showing differences as the season progressed may suggest that seasonality has more effect on protein content than maturity. The lack of difference in CP content between different varieties from the reduced-lignin variety trial also suggests that protein is not being bound by lignin content as previously suggested.

Overall, the protein concentrations in APCs for this trial are lower than those reported in the literature, which range from 45-60% CP in APC made with heat coagulation (Edwards et al., 1977; Lu et al., 1981; Zhang et al., 2017). The lower values obtained here may be due to the use of the biomass cultivar compared to forage varieties

used in other studies. However, in the experiments on methods for APC coagulation, the biomass alfalfa foliage yielded APC with a CP content from 45-51%, which is within range of values reported in the literature. Because of the differences in the year, harvest timing, and juicing methods, a direct comparison of APC CP concentration between the two experiments cannot be made. Further investigation is warranted to compare CP yields in APC from year to year and with differing maceration techniques.

There were no differences in yields of PR between stripped leaves and total foliage when measured on a g output kg^{-1} input basis ($p = 0.105$, Table 3-7). Yields of PR were 912.1 and 798.7 g kg^{-1} for harvest 2 (July 6, 207), 863.0 and 791.5 g kg^{-1} for harvest 3 (August 8, 2017), and 693.7 and 702.2 g kg^{-1} for harvest 4 (October 16, 2017) for the total foliage and the stripped leaves, respectively (Table 3-10). However, once again, total foliage yielded more than double the amount of PR ha^{-1} than stripped leaves in the July and August harvests (Table 3-10). This was expected because only the apical portion of the plant was stripped resulting in lower yield ha^{-1} prior to juicing. While the yield of PR ha^{-1} was lower for stripped leaves than total foliage, RFQ and protein content of PR from stripped leaves was significantly greater than the PR from total foliage at each harvest. The stripped leaves consistently resulted in a PR with RFQ suitable for lactating dairy cows (139-194 RFQ) while the PR from total foliage was more suited for beef cows, heifers, or for biomass energy production at the lower values of 66-100 RFQ (Undersander et al., 2011). These results are similar to previous results that found that PR from stripped leaves produces a high-quality silage chemically similar to high-quality whole plant alfalfa silage (Digman et al., 2013). The amount of CP retained in the PR

indicates that higher yields of APC may be possible if maceration methods for increasing protein extraction from total foliage and stripped leaves can be optimized.

3.5 Conclusions

The results of this study show that lignin content has less impact on yield of APC while harvest timing (early vs. later in the summer) and the production methods for APC and PR have a much greater effect on yield and quality of the two products. This information may help producers decide on when to harvest their alfalfa when processing it into APC and associated products. The differences seen in PR protein content and RFQ values also supports the notion that the alfalfa variety and harvest timing can more directly relate to co-product choices and APC remains a viable product, whether the PR is used as feed or as biomass fuel stock.

Pressed total foliage resulted in a greater amount of APC on a per hectare basis than separated leaves, but the associated PR had a lower feed quality. An economic and market analysis would be useful of the APC and biofuel markets in order to inform producers and processors of the economic balance of investing in new leaf stripping equipment. At this time, with whole foliage producing a higher protein content APC and higher yields, standard equipment, such as a flail forage harvester, is likely the better choice for harvesting the alfalfa.

This is the first study to document the range of compositional differences in APC produced by different pH and heat coagulation methods. Acidification methods resulted in the highest yield of APC and total protein extracted, but do not result in the highest

concentration of protein within the product. This makes sense because the CP is there, but is diluted by other constituencies that the pH change also coagulated. The percentage of limiting amino acids and concentration of fatty acids did not vary by production method. Compositional analysis of APC produced by the methods tested indicated that APC is suitable for incorporation into aquaculture feeds. Further studies should investigate the combined effects of alfalfa variety, plant fractionation, harvest timing and multistep refining on yields of insoluble (chloroplastic) and soluble (cytoplasmic) protein products. While past studies have focused on the food-grade products, feed-grade APC could possibly contribute to more sustainable protein supply chains. The significant differences observed in protein and amino acid content, as well as fat, sugars, and fiber in the APC resulting from different production methods will help inform future studies to match nutritional needs of different species with feed-grade APC production methods.

Reduced lignin content in alfalfa foliage did not have a significant effect on APC yields or CP content, but follow up studies are needed to determine if the observation are stable with additional varieties across environments and years. Furthermore, stripping biomass-type alfalfa for higher quality apical materials did not have a significant effect in increasing either yield or quality of the APC. In fact, the stripped leaf material resulted in lower protein concentrations in the end APC product. Yield results varied by harvest and protein content of the APC declined as the season progressed. The implication is that because variety or plant fraction did not have a significant impact on APC quality, producers can grow the variety of alfalfa that is best suited for their location and quality goals for hay or PR and APC. The non-lodging biomass type alfalfa, which allows crop

producers more time between harvests, and in turn, reduces labor, fuel, and equipment costs, could be a reasonable choice for APC production along with goals for hay or PR.

3.6 Tables

Table 3-1 Alfalfa protein concentrate (APC) yields and contents from five coagulation methods.

	Yield	Protein	Fat	Sugar	Fiber	3 Lim AA[†]
	g kg^{-1}	% content dry basis				
Acid	137.5 a[ǂ]	45.8 c	9.0 c	2.0 bc	0.6 bc	5.3 b
Base + Acid	141.7 a	45.3 c	10.1 abc	1.3 cd	0.6 c	5.2 b
Cold Acid	134.2 a	45.5 c	9.6 bc	2.1 b	0.7 abc	5.2 b
Freeze	108.8 b	48.9 b	11.1 ab	3.8 a	1.1 ab	5.3 b
Heat	105.0 b	50.9 a	11.4 a	1.1 d	1.2 a	5.8 a

[†]3 Lim AA is the sum of the contents of methionine, lysine, and threonine.
[ǂ]Significant differences ($p \leq 0.05$; Tukey's HSD) are indicated by different letters within columns.

Table 3-2 Alfalfa protein concentrate (APC) amino acid contents from five coagulation methods averaged over triplicate samples (% w/w).

Amino Acid	Heat	Freeze	Cold Acid	Acid	Base + Acid
Cysteine	0.43	0.38	0.39	0.39	0.40
Methionine	0.88	0.83	0.72	0.72	0.70
Lysine	2.53	2.35	2.35	2.37	2.36
Alanine	2.75	2.84	2.45	2.42	2.37
Arginine	2.71	2.29	2.37	2.42	2.38
Aspartic Acid	4.59	3.68	4.00	4.12	4.13
Glutamic Acid	4.74	4.40	4.23	4.28	4.21
Glycine	2.51	2.35	2.19	2.20	2.18
Isoleucine	2.34	2.16	2.03	2.09	2.02
Leucine	4.30	3.99	3.69	3.73	3.68
Serine	1.93	1.56	1.65	1.67	1.67
Threonine	2.13	1.78	1.92	1.92	1.91
Valine	2.77	2.54	2.41	2.47	2.40
Histidine	1.16	1.09	1.02	1.03	1.02
Phenylalanine	2.91	2.64	2.48	2.51	2.48
Tyrosine	1.89	1.53	1.66	1.68	1.64
Taurine	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
Tryptophan	1.04	0.89	0.85	0.88	0.89

Table 3-3 Foliage dry matter yield of two reduced lignin alfalfa varieties and one standard variety from Rosemount, MN harvested in 2017 and used for alfalfa protein concentrate and press residue preparation.

	1st Harvest 6/9/2017		2nd Harvest 7/18/2017		3rd Harvest 9/13/2017		Total Yield
Variety[†]	Maturity	t ha^{-1}	Maturity	t ha^{-1}	Maturity	t ha^{-1}	t ha^{-1}
HiGest	Late Bud	4.51	Early Flower	4.21	Early Bud	3.77	12.50
HarvX	Late Bud	3.45	Early Flower	3.27	Early Bud	3.07	9.77
Control	Early Bud	3.90	Early Flower	3.97	Late Bud	3.92	11.81

[†] The varieties were selected to compare alfalfa protein concentrate (APC) production from two reduced-lignin types with a standard variety for yield and quality of both the APC and the press residue (PR). Hi-Gest 360 (Alforex Seeds) is a reduced lignin variety with high forage quality developed by conventional selection, referred to here as "HiGest". HX-14376 (Forage Genetics International) is a genetically modified reduced lignin variety with high forage quality, referred to here as "HarvX". Pioneer 54VR08 (Pioneer Hi-Bred International Inc.) is a standard high-quality alfalfa often used in the northern USA, referred to here as "Control".

Table 3-4 Forage quality values of two reduced lignin alfalfa varieties and one standard variety harvested from Rosemount, MN in 2017 and used for alfalfa protein concentrate and press residue preparation.

	1st Harvest 6/9/2017		2nd Harvest 7/18/2017		3rd Harvest 9/13/2017	
Variety[†]	Maturity	RFQ[§]	Maturity	RFQ	Maturity	RFQ
HiGest	Late Bud	172	Early Flower	140	Early Bud	134
HarvX	Late Bud	175	Early Flower	170	Early Bud	158
Control	Early Bud	150	Early Flower	149	Late Bud	99

[†] The varieties were selected to compare alfalfa protein concentrate (APC) production from two reduced-lignin types with a standard variety for yield and quality of both the APC and the press residue (PR). Hi-Gest 360 (Alforex Seeds) is a reduced lignin variety with high forage quality developed by conventional selection, referred to here as "HiGest". HX-14376 (Forage Genetics International) is a genetically modified reduced lignin variety with high forage quality, referred to here as "HarvX". Pioneer 54VR08 (Pioneer Hi-Bred International Inc.) is a standard high-quality alfalfa often used in the northern USA, referred to here as "Control".

[§]RFQ = Relative Forage Quality (Undersander et al., 2011).

Table 3-5 Yield and composition of alfalfa protein concentrate extracted from two reduced lignin varieties and one standard variety from Rosemount, MN in 2017.

Variety[†]	Yield g kg^{-1}	Protein %	3 Lim AA[§]	Fat %	Fiber %	Ash %
HiGest	72.1	49.9	5.4	5.1	0.0	10.4
HarvX	58.0	48.3	5.1	4.8	0.0	10.6
Control	70.5	50.1	5.3	5.2	0.0	11.4

No statistically significant differences (Tukey's HSD; $p < 0.05$) were found in APC yields or composition from the different varieties.

[†] The varieties were selected to compare alfalfa protein concentrate (APC) production from two reduced-lignin types with a standard variety for yield and quality of both the APC and the press residue (PR). Hi-Gest 360 (Alforex Seeds) is a reduced lignin variety with high forage quality developed by conventional selection, referred to here as "HiGest". HX-14376 (Forage Genetics International) is a genetically modified reduced lignin variety with high forage quality, referred to here as "HarvX". Pioneer 54VR08 (Pioneer Hi-Bred International Inc.) is a standard high-quality alfalfa often used in the northern USA, referred to here as "Control".

[§]Sum of the percentage of three limiting amino acids that are frequently added to fish feeds: methionine, lysine, and threonine.

Table 3-6 Alfalfa press residue yield and composition produced from two reduced lignin varieties and one standard variety from Rosemount, MN in 2017.

Variety[†]	Yield g kg^{-1}	Protein %	Fat %	Fiber %	Ash %	RFQ[§]
HiGest	667.2 b[‡]	15.43	1.65	40.55 a	7.13 b	83 b
HarvX	690.5 b	16.01	1.80	33.07 b	8.02 a	97 a
Control	776.8 a	14.68	1.57	40.66 a	7.13 b	80 b

[†] The varieties were selected to compare alfalfa protein concentrate (APC) production from two reduced-lignin types with a standard variety for yield and quality of both the APC and the press residue (PR). Hi-Gest 360 (Alforex Seeds) is a reduced lignin variety with high forage quality developed by conventional selection, referred to here as "HiGest". HX-14376 (Forage Genetics International) is a genetically modified reduced lignin variety with high forage quality, referred to here as "HarvX". Pioneer 54VR08 (Pioneer Hi-Bred International Inc.) is a standard high quality alfalfa often used in the northern USA, referred to here as "Control".

[§]RFQ=relative feed quality calculated from NDF, NDFD, lipid and ash content (Undersander et al., 2011).

[‡]Different letters within a column denote a significant difference (Tukey's HSD; $p < 0.05$).

Table 3-7 Test of fixed effects for material type and harvest period in alfalfa protein concentrate (APC) production from biomass type 1st production year alfalfa grown in Waseca, MN (2017).

	APC yield (g kg^{-1} DM) [1]	APC yld (kg ha^{-1})[2]	APC CP[3]	APC 3 Lim AA[4]	APC Fat[5]	APC Fiber[6]	PR Yield (g kg^{-1} DM) [7]	PR yield (kg ha^{-1})[8]	PR CP[9]	PR RFQ[10]
Fixed Effect	---------- P > F ----------									
Material (M)	0.112	<0.001	0.024	0.016	0.652	0.687	0.105	<0.001	<0.001	<0.001
Cut Time (C)	0.006	<0.001	<0.001	<0.001	<0.001	0.500	0.002	<0.001	<0.001	<0.001
M x C	0.019	<0.001	0.834	0.462	0.095	0.263	0.382	<0.001	<0.001	<0.001

1. APC yield tested based on g product kg^{-1} alfalfa input (on a DM basis).
2. APC yield tested based on kg product ha^{-1} of growing space.
3. Crude protein (CP) content within the APC product tested based on percentage of dry matter content.
4. Content of three limiting amino acids within the APC product, methionine, lysine, and threonine tested based on percentage of dry matter content.
5. Crude fat content within the APC product tested based on percentage of dry matter content.
6. Fiber content within the APC product tested based on percentage of dry matter content.
7. Press residue (PR) yield tested based on g product kg^{-1} alfalfa input (on a DM basis).
8. Press residue (PR) yield tested based on kg product ha^{-1} of growing space.
9. Crude protein (CP) content within the PR tested based on percentage of dry matter content.
10. Relative feed quality (RFQ) of the PR tested based on score from NIRS scans and RFQ equation (Undersander et al., 2011).

Table 3-8 Comparison of alfalfa protein concentrate (APC) yield and composition produced from field separated materials of a biomass type alfalfa grown in Waseca, MN (2017).

Harvest Period	APC Yield[1]		APC Components			
	g kg^{-1}	kg ha^{-1}	Protein %	3 Lim AA %[2]	Fat %	Fiber %
Harvest 2 (Jul 6 2017)						
Total Foliage	102.3 a	388.3 a	42.87	4.61	6.15	1.33
Stripped Leaves	97.2 a	198.4 b	41.13	4.40	6.76	0.77
Harvest 3 (Aug 8 2017)						
Total Foliage	105.3 b	300.9 a	38.92	4.14	7.47	0.84
Stripped Leaves	130.6 a	176.1 b	37.03	3.76	6.30	0.86
Harvest 4 (Oct 16 2017)						
Total Foliage	107.5 a	164.5 a	36.64	3.70	8.94	0.64
Stripped Leaves	108.9 a	149.5 a	35.58	3.59	9.05	0.92

Different letters within column and harvest denote a significant difference (Tukey's HSD; $p < 0.05$).

[1] Yield of g APC kg^{-1} of starting material on a dry matter basis and calculation of kg APC $hectare^{-1}$.

[2] Content of three limiting amino acids within the APC product, methionine, lysine, and threonine reported as percentage of dry matter.

Table 3-9 Composition of alfalfa protein concentrate (APC) produced from field separated materials of a biomass type alfalfa grown in Waseca, MN (2017).

	Protein	3 Lim AA[1]	Fat	Fiber
	---------- [% w/w] ----------			
Material Type				
Total Foliage	39.47 a	4.15 a	7.52	0.93
Stripped Leaves	37.92 b	3.92 b	7.37	0.85
Harvest Period				
Harvest 2 (Jul 6, 2017)	42.00 a	4.50 a	6.46 b	1.05
Harvest 3 (Aug 8, 2017)	37.97 b	3.95 b	6.88 b	0.85
Harvest 4 (Oct 16, 2017)	36.11 c	3.65 c	9.00 a	0.78

Different letters within a column denote a significant difference (Tukey's HSD; $p < 0.05$) between either the material type or the harvest period.

[1] Content of three limiting amino acids within the APC product, methionine, lysine, and threonine reported as percentage of dry matter content.

Table 3-10 Press residue (PR) yield and feed quality from field separated materials from a biomass type alfalfa grown in Waseca, MN (2017).

Harvest Period	PR Yield[1]		PR Components	
	g kg^{-1}	kg ha^{-1}	% Protein	RFQ[2]
Harvest 2 (Jul 6 2017)				
Total Foliage	912.1	3595.4 a	14.85 b	66 b
Stripped Leaves	798.7	1635.9 b	19.47 a	177 a
Harvest 3 (Aug 8 2017)				
Total Foliage	863.0	2460.5 a	14.91 b	100 b
Stripped Leaves	791.5	1039.7 b	18.03 a	194 a
Harvest 4 (Oct 16 2017)				
Total Foliage	693.7	1052.7	17.67 b	94 b
Stripped Leaves	702.2	959.9	20.85 a	139 a

Different letters within a column and harvest denote a significant difference (Tukey's HSD; $p < 0.05$).

[1] Yield determined in g PR kg^{-1} of starting material on a dry matter basis and calculated kg PR^{-1} hectare.

[2] Relative feed quality (RFQ) of the PR based on NIRS (Undersander et al., 2011).

Chapter 4: APC Use and Manufacture for Aquaculture Conclusions

This collaborative research investigated two portions of sustainable protein supply with the use of alfalfa protein concentrate (APC) from *Medicago sativa* L. foliage. In contrast to plant proteins from annual crops, alfalfa can increase sustainability of animal feeds while protecting soil and water resources in cropping systems. Currently, alfalfa's use is mainly driven by the dairy and cattle industries. The hope is that this research will stimulate interest in further studies to develop APC for monogastric use such as commercial aquaculture and to establish a robust supply chain for APC to stimulate rural economies, and provide protein for a growing global population. The first portion of the research tested a commercially available APC in the feed of two carnivorous fish species, yellow perch (*Perca flavescens*) and rainbow trout (*Oncorhynchus mykiss*). The second portion of the research compared yield and composition of APC manufactured by different methods and from different feedstocks. This research advances the understanding of how carnivorous finfish respond to APC in the diet, and identified compositional differences between APC products manufactured with different methodologies and plant variations that inform producers on alterations that affect products available.

The fish feeding trials showed acceptance and growth when the two species were fed diets including APC. However, 18% inclusion in the yellow perch diet slowed the

growth and lowered the efficiency of fish on the experimental diet. Lower inclusion rates at 3 and 6% in rainbow trout showed no difference in growth and efficiency when compared to the fish on the control diet. Further studies on these two species, as well as others, are warranted to find an optimal level for this protein source that has been shown to be digestible and utilizable. Further research should consist of not only additional levels of APC inclusion, but examination of the intestinal health as well as fecal remains of the fish on APC diets in order to establish what nutrients are hindering digestion or not being absorbed. Finding optimal levels of inclusion in aquaculture feeds would allow for reduced reliance on fishmeal, additional growth of alfalfa, and additional protection of soils and water.

A novel finding of the APC production research was the compositional differences between APC products beyond crude protein, which included fat, fiber, and sugar content as well as detailed amino acid profiles. These composition data in conjunction with yield data will help inform future research into development of commercial APC production. Fish feeding studies with APC produced using different methods may identify a process that provides nutrients and digestibility best suited to the intended fish species. Building upon this research, more information is needed on the concentrations of saponins in APC produced by different methods, and from different plant fractions, varieties, maturities, and harvests as well as feed processing methods to reduce saponins in the final product.

In contrast to production methods, the research on differing starting alfalfa material from reduced lignin alfalfas as well as stripped leaves and whole foliage indicates that these differences have little to no effect on the composition of resulting APC product. Surprisingly, APC produced from stripped leaves had lower concentrations of crude protein and the limiting essential amino acids methionine, lysine, and threonine than APC from whole herbage. This difference may have been due to the difference in maceration of the two feedstocks. Although some research has been done on efficiency of protein extraction with different amounts of maceration, more research is needed on the effect of maceration on APC composition. The current commercial production of APC appears to be driven more by production of alfalfa pellets from press residue (PR) than production of APC. Here, PR from reduced lignin alfalfa varieties and stripped leaves was shown to have high feed value, which could influence facilities to produce PR from these materials to increase the value of this product. Stripping leaves from mature alfalfa for use in APC and PR manufacture provides alfalfa producers the opportunity to obtain multiple valuable products while providing more flexibility in harvest schedules from alfalfa plants that would otherwise produce a low value hay.

To move forward in sustainably feeding the growing populations over the next few decades, research must continue on both APC production and aquaculture diets. Aquaculture needs nutritional studies including plant proteins into diets for different fish species in order to reduce production costs and maintain the growth that has been experienced over the last 25 years. A bottleneck to adoption of APC in aquaculture feeds

is the lack of domestic production. APC will need to compete economically with soybean meal as a component of the feed and/or by providing economic benefits in fish health. Information on the economics of APC production with modern equipment and alfalfa production costs at varying farm scales would help promote a domestic APC industry. Such an industry will benefit alfalfa producers and the alfalfa industry. Including APC at a rate of 1% in 100 million MT of aquatic feeds will require approximately 10 million MT of alfalfa (dry weight basis, assuming 10% APC yield). This would require a 20% increase in U.S. alfalfa production area with an average alfalfa yield of 10 MT per hectare. However, because press residue from the production of APC can be utilized in haylage and pelleted, new acreage would also require increased use of these products in feed for ruminants or a use of press residue for biomass energy. A highly sustainable system can be envisioned in which APC extraction is optimized with little protein remaining in the press residue, which is used to power APC and co-product production. Excess energy could be sold to the power grid or used by associated aquaculture and/or aquaponics production facilities. Taking these steps towards a sustainable future will help human populations meet the future of increased protein demand and limited resources.

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