ABSTRACT

Title of Document:	TAURINE: AN INDISPENSABLE INGREDIENT IN THE DEVELOPMENT OF SUSTAINABLE AQUAFEEDS
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Aquaculture as a global industry is at a crossroad; increased production cannot rely on the unsustainable harvest of forage fish for feed production. The use of fishmeal and fish oil as components in feeds for aquaculture, most notably for high value marine carnivores must be reduced or eliminated. The most promising and sustainable sources of replacement feed must be plant derived, such as soybean meal, wheat flour, and corn gluten along with dozens of other plant derived sources. Likewise for fish oil the most promising sources are plant oils such as soybean and canola oil supplemented with necessary omega-3 fatty acids.

This work was undertaken to examine the effects of switching marine carnivores from fishmeal-based feeds to fishmeal-free, plant-based diets. The majority of this research has been conducted with cobia, *Rachycentron canadum*, a promising species for intensive aquaculture due to its rapid growth rates, high disease resistance, and lack of a major

commercial fishery as competition. A variety of plant proteins, plant protein blends and alternative lipid sources were examined for digestibility and efficacy as fishmeal replacement sources in regards to their effects on growth rates, feed conversion, and a range of physiological characteristics.

This work has explored the hypothesis that marine carnivores have lost the ability to synthesize taurine, a non-protein amino acid, in sufficient quantities and must therefore be supplied through the diet, and should be considered essential for all marine carnivores. By measurement of gene expression of the genes in taurine biosynthesis, this work shows that cobia do not possess the ability to regulate taurine biosynthesis confirming taurine must be supplied through the diet.

Overall, this work has developed multiple plant protein-based feeds that perform equivalently or better than commercial and commercial-like diets. Taurine has been shown to be an essential ingredient when seeking to reduce or preferably, eliminate fishmeal and thereby making aquaculture sustainable in providing protein to meet the world's growing population.

TAURINE: AN INDISPENSABLE INGREDIENT IN THE DEVELOPMENT OF SUSTAINABLE AQUAFEEDS

By

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Preface

I want to preface this dissertation with a brief explanation of its structure. Following the general introduction and up to the concluding chapter, the chapters and sub-chapters are written in manuscript format for the various journals they have been submitted to for publication where possible. Where a section represents a published manuscript, the citation follows the title of that section. Although references, subsections, tables, and figures have been reformatted to be consistent within this dissertation, there are acknowledgements at the end of the majority of sections. These are specific to each section as they would be for individual manuscripts and denote the specific people whose assistance was vital for that study as well as the funding sources for each, as appropriate. Contribution numbers have been removed from these acknowledgement sections to be consistent within the dissertation format.

Dedication

This work is dedicated to my family, for all of their love and support in my twenty-four years of school. To my mother, Sharon, for her unwavering support and encouragement to continually achieve no matter the endeavor. To the memories of my father, Jesse David Watson III, and grandfather, Jesse David Watson Jr., for being the two most powerful role models a son could ask for; for setting me on the right track through their work ethics and love. To my grandmother, Rachel Watson, for always reminding me of the important things. To my brother, Evan, for being one of the strongest people I've ever known. And finally to my wife, Jessica, for, everything, I wouldn't have made it this far without you.

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There are entirely too many people to thank for all the assistance I have received during my duration as a graduate student in completing this work and many individuals are thanked at the end of individual sections of this dissertation but there are a few who warrant special thanks. My advisor, Dr. Allen Place, for letting me run with ideas to fruition or (often) frustration, and for begging and borrowing, sometimes “with no intent to return” to keep me funded during this work. Dr. Place has allowed me to pursue collaborations with a variety of people across the country, all of which have strengthened this work. In addition to this I have been encouraged to pursue roles with the United States Aquaculture Society and the World Aquaculture Society which have greatly enhanced my graduate education. Dr. Place has encouraged me as a mentor of five LMRCSC summer interns (Michelle Thompson, Gordon Taylor, Vanessa Richards, Travonya Kenly, and Jonathon Peake), which have not only aided in this research, but has been an important component of my training. Dr. Rosemary Jagus, as project director for the LMRCSC at IMET for coordinating the summer intern program as well as for the stipend and travel support I have received from her and the LMRCSC. Dr. Frederick (Rick) Barrows of the USDA-ARS for formulating many and manufacturing most of the experimental feeds used throughout these projects and for allowing me to visit his facility in Bozeman, MT to learn about the feed manufacturing processes. Ernest Williams for constant assistance in lab work, analysis, editing, and errands no matter how important or insignificant. Steve Rodgers and Chris Tollini for all of their assistance with animals and equipment and for letting me work by the motto “it is easier to ask forgiveness than permission”.

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List of Abbreviations

AA	Atomic absorption
ADC	Apparent digestibility coefficient
ADO	Cysteamine dioxygenase
ALA	α-Linolenic acid
ANF	Anti-nutritional factor(s)
ARA	Arachidonic acid
ARS	Agricultural research service (control diet)
CDO	Cysteine dioxygenase
CF	Condition factor
CL	Crude lipid
CAN+EFA	Canola oil + essential fatty acids diet
CP	Crude protein
CSD/CSAD	Cysteinesulfinate decarboxylase/Cysteine sulfinic acid decarboxylase
DCP	Digestible crude protein
DE	Digestible energy
DHA	Docosahexaenoic acid
DL	Digestible lipid
DM	Dry matter
DNA	Deoxyribose nucleic acid
DPA	Docosapentaenoic acid
EAA	Essential amino acid(s)
EFA	Essential fatty acid(s)
EPA	Eicosapentaenoic acid
EPP1	Experimental plant protein diet #1
EPP2	Experimental plant protein diet #2
FAME	Fatty acid methyl ester(s)
FAO	Food and agriculture organization of the United Nations
FCR	Feed conversion ratio

FE	Feed efficiency
GC	Gas chromatography
GMO	Genetically modified organism
HSI	Hepatosomatic index
IACUC	International animal care and use committee
IMET	Institute for Marine and Environmental Technology
LA	Linolenic acid
LC	Long chain
LCMS	Liquid chromatography mass spectrophotometer
LMRCSC	Living Marine Resources Cooperative Science Center (NOAA-EPP)
LNA	Linoleic acid
MUFA	Monounsaturated fatty acid(s)
NOAA	National Oceanographic and Atmospheric Administration
NWFSC	Northwest Fisheries Science Center (NOAA)
PCB	Polychlorinated biphenyl(s)
PCR	Polymerase chain reaction
PER	Protein efficiency ratio
PUFA	Polyunsaturated fatty acid(s)
RAS	Recirculating aquaculture system(s)
RNA	Ribose nucleic acid
RT-PCR	Reverse transcriptase – PCR
RT-qPCR	Reverse transcriptase – quantitative PCR
SFA	Saturated fatty acid(s)
SGR	Specific growth rate
TAUT	Taurine transporter
TGC	Thermal growth coefficient
TM+SOY	Thraustochytrid meal + soybean oil diet
USDA	United States department of agriculture

Chapter 1: General Introduction

World population and capture fisheries

The greatest problem facing the world over the next century will be how humanity adapts to global population increase. Everything from energy, water, and food shortages to environmental degradation and climate change are all directly rooted simply to the number of people inhabiting the planet. One of the most challenging aspects of this concerns the worlds growing population and that the majority of the increase is going to come from developing nations (Figure 1.1.1; United Nations, 2009), nations with poor infrastructures and poor technologies to cope with increasing demands. Food concerns will highlight the problems in many of these countries, where much of the current population still relies on capture fisheries for the bulk of their animal protein. Fish accounted for 16.6 % of the world population's intake of animal protein and 6.5 % of all protein consumed with roughly 4.3 billion of the world's people relying on fish for at least 15 % of their protein intake (FAO, 2012).

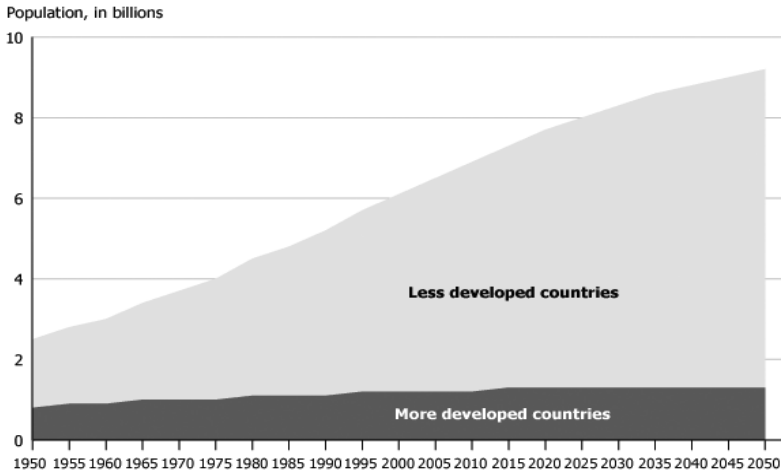

Figure 1.1.1. Expected global population increase through 2050, divided by developed (dark gray) or less developed (light gray) nations; from United Nations (2009).

However, worldwide landings from commercial fisheries have either declined or leveled off for virtually every species in every region of the world. Total global landings have leveled off between 90-95 million tons per year and have been at this level for close to two decades (Figure 1.1.2.; FAO, 2012, 2010). Even at these consistent levels, many of the commercial fisheries still in operation are not doing so under sustainable practices, with many regularly undergoing periodic closures or continuing restraints. Public concern has only continued to increase over issues such as habitat degradation, over-fishing dwindling stocks, and special concern has been paid to increasing contamination levels in many wild caught species (Brar *et al*., 2010; Burger and Gochfeld, 2013; Du *et al.*, 2012; Hayward *et al*., 2007; Kim *et al*., 2012; Mita *et al*., 2011; Stewart *et al*., 2011).

Overfishing of stocks has always been the biggest factor in declining wild stocks, with habitat degradation due to poor fishing practices being another strong factor. Since none of the increase in supply can be expected to come from wild fish populations that are already beyond their sustainability levels, aquaculture must step in to help increase global seafood production and act as an aid in easing over-fishing and habitat degradation.

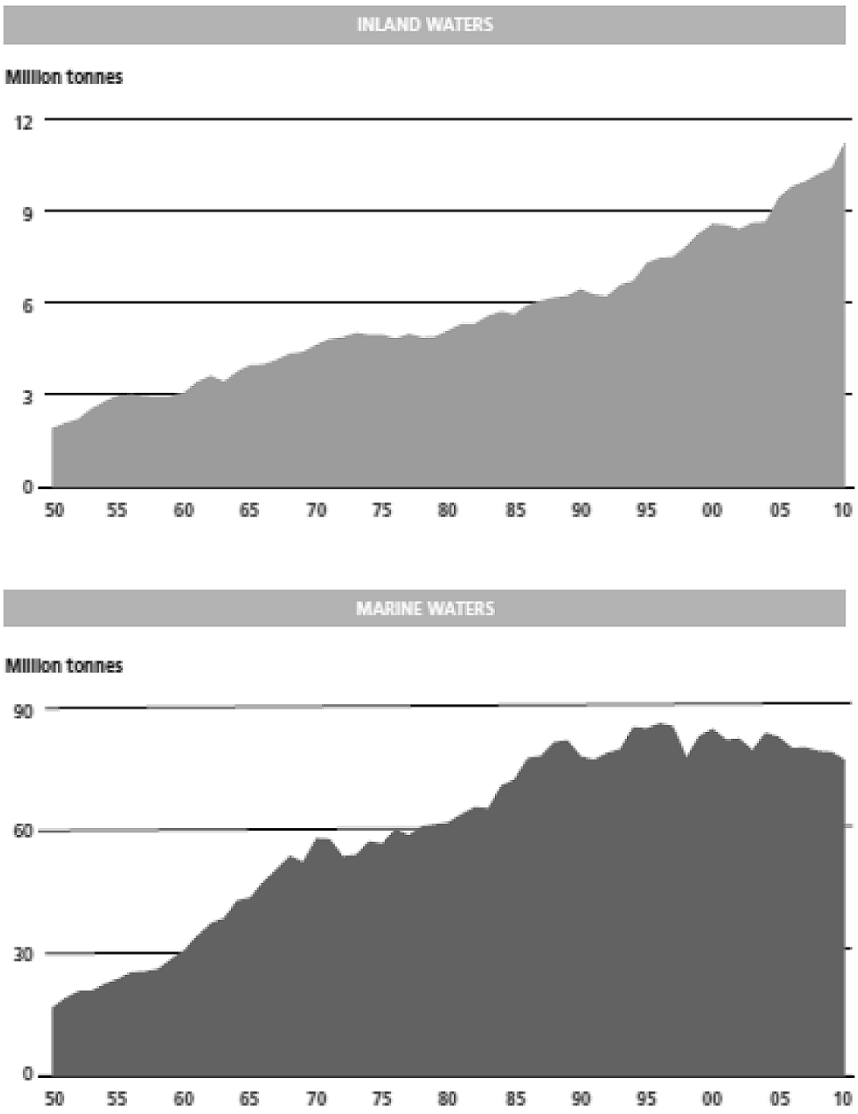

Figure 1.1.2. World capture fisheries production from inland waters (top) and marine waters (bottom), from FAO (2012).

Aquaculture

Aquaculture is one of the oldest forms of agriculture, with roots dating back as far as 5,000 years (Nash, 2011). Aquaculture production has increased from roughly 1 million tons per year in the early 1950's to 63.6 million tons per year as of 2011, increasing roughly

7 % annually and representing a global value of approximately $119.4 billion in 2010 (FAO, 2012). However, in terms of being developed into a worldwide high intensity practice, aquaculture is significantly behind its terrestrial counterparts of animal husbandry and crop farming. With the attempt to rapidly develop aquaculture and aquaculture stocks to have a similar status with terrestrial livestock production densities and technologies, many issues are being encountered. Fishing is the oldest form of hunting/gathering that is still being practiced on a large scale. The belief that the world's oceans can never be "out-fished" has only fairly recently been tossed aside. The technical issues encountered, coupled with the reluctance to accept that our current rate of capture fishing is unsustainable, has produced difficulties in developing aquaculture into a sustainable, high intensity practice that can meet global seafood demands. One of these major stumbling blocks for the advancement of aquaculture, both politically and socially, are the industry's real and perceived negative environmental impacts.

Eutrophication, the increased concentrations of chemicals, most notably nitrogen and phosphorous, has been an issue associated with agriculture and urbanization for decades. Runoff from agricultural land where excess fertilizers are used and runoff from cities and industrialized areas are often rich in nutrients. These nutrients can lead to the rapid degradation of local water quality, as well as increased algal production, which turns into bacterial blooms that can consume virtually all usable oxygen, driving these local systems into periods of hypoxia or anoxia. Even shortened periods of anoxia or hypoxia can have negative health effects such as lowered growth, delayed or stunted sexual development, or can become fatal to the majority of prey species and higher life such as fish and other vertebrates, with sessile organisms being the most at risk. Every summer

there is an anoxic zone, roughly the size of New Jersey, that grows and shrinks in the Gulf of Mexico in correspondence to the outflow rate of the Mississippi River, a direct result of the increased nutrients flowing down the river (Dagg and Breed, 2003). There have been dozens of documented water bodies such as this around the world that undergo either seasonal anoxia, or have become permanently anoxic due to eutrophication and other anthropogenic causes (Zhao *et al*., 2012). Although this problem is exacerbated in fresh water systems that tend to be more closed than ocean systems, coastlines and estuaries are not immune to these effects and there are fears that large scale open ocean aquaculture pens could easily lead to similar problems in a variety of ecosystems worldwide, or fall victim to seasonal episodes.

Outside of possible eutrophication there are other issues that have plagued traditional aquaculture in terms of its public perception and application. Escapes from aquaculture pens, although rare, have made national and world headlines in the past (Elvira and Almodovar, 2001; Jensen *et al*., 2010; Volpe *et al*., 2013). Invasive species have the potential to be spread rapidly from large or small-scale escapes from aquaculture operations. A good example is in the salmon industry, where genetic populations from the two sides of the Atlantic basin and the Pacific are very distinct. Escapes of Atlantic salmon in the natural environments of the other have taken place to the extent there is fear of feral populations of Atlantic salmon developing on the Pacific coast (Volpe *et al*., 2013). Even in the ornamental culture industry, which is often thought of on a miniscule scale in comparison to food-grade fish culture, populations of invasive lionfish have been documented and monitored in the Caribbean and Atlantic (Johnston and Purkis, 2011). The northern snakehead, *Channa argus*, is an invasive species in North America that is

decimating local fish populations and is believed to have been released from pet owners and small-scale aquaculture operations (Lapointe *et al*., 2010) The negative potentials for this type of problem are felt and understood by a wide range of communities throughout the world due to the fact that invasive species have been a major problem in terrestrial ecosystems for much longer than aquatic ecosystems and the large majority of communities are very familiar with their implications. Although to date this has been much more of a potential and publically feared problem than an actual one, with a few major exceptions (e.g. Asian carp in the Mississippi river (Sampson *et al*., 2008)), it has greatly hindered and prevented the expansion of intensive aquaculture for certain species and in certain regions.

An emerging issue with aquaculture and one that will only continue to undergo heated debate is the use of genetically modified organisms (GMO's). Historically this has only been thought of in terms of the organism that is raised or released and genetic modification has been thought of as the genes that have been manipulated, inserted, or selected for in selective breeding programs. The definition of genetic modification is widening to include things such as DNA vaccines and the genetically modified feedstuffs that go into the diet formulation of farmed fish. The answers to questions, such as whether or not horizontal gene transfer can or will occur from genetically modified feedstuffs or DNA vaccines, have not been answered so the moral and ethical stumbling blocks of allowing these practices in intensive aquaculture have also not been explored fully. As one of the major challenges in aquaculture, the reduction in the use of fishmeal and fish oil in diets is being explored, to increase the variety of alternate feedstuffs for use in diets. Many issues will arise in the future in relation to GMO's and their use in aquaculture, especially

their use in traditional cage and pen culture operations where excess feed and or escapes are issues (Myhr and Dalmo, 2005).

One of the most promising alternatives to traditional aquaculture practices that can alleviate many of the current and future concerns within aquaculture is the use of recirculating aquaculture systems (RAS). Recirculating systems are self-contained systems that include tanks for the target species, biological and mechanical filtration, as well as all necessary monitoring apparatus (Figure 1.1.3) such as temperature, salinity, and pH control as well as ozone or UV irradiation to control bacterial levels.

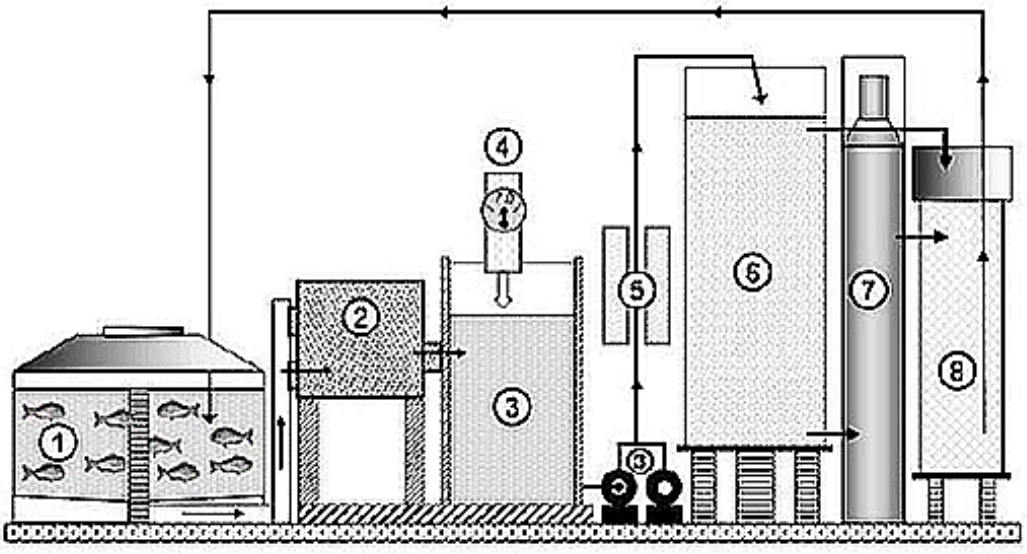

Figure 1.1.3. A schematic drawing of the experimental Baltimore Recirculating Mariculture System. Numbered system components are: (1) fish tank, (2) particle removal, (3) sump [left] and pump [right], (4) pH doser, (5) temperature control; (6) biofiltration, (7) protein skimmer, (8) oxygen delivery. From (Zohar *et al*., 2005).

Most recirculating systems do just what their name suggests, recirculate the water, relying on the biological and mechanical filtration units to maintain high water quality. This greatly reduces waste and can disconnect the system from possible impacts to the local

environment, providing them with the capability of raising non-native species and GMOs, as well as the ability to be placed virtually anywhere having the required infrastructure. This benefit of RASs also gives them the ability to maintain proper spawning conditions for broodstock such as temperature and photoperiod, as well as conditions for maximal growth throughout the year, regardless of the external environment they are situated in. RASs also have the potential for year-round production of eggs, currently one of the significant bottlenecks for successful aquaculture, and final products that traditional aquaculture simply cannot match.

There is a high cost associated with recirculating systems that has greatly limited their use and expansion on a large scale, both freshwater and marine, in the past decades (Zohar *et al*., 2005). However, with decreasing fish stocks and increasing demand, alternatives to traditional aquaculture, such as RASs need to expand to fill the void. On-going research has the potential to continually improve systems, increase fish densities and increase our ability to raise high-value species anywhere.

Aquafeeds

Besides the decimation of natural populations and ecosystems, another major issue with aquaculture is its feed source. Aquaculture, unlike its terrestrial counterparts, relies heavily on species that are carnivorous or omnivorous in the wild. Cows, pigs, sheep, and chickens, some of the highest production terrestrial animals can all subsist on grain crops or wastes from human food production without much modification to their diets. Many fish species on the other hand, especially high-value marine species, rely heavily upon meat sources for nutrition, particularly for protein. This can result in feed costs comprising more

than 50 % of the total budget for raising fish to market size, which means even small fluctuations in ingredient costs can make the difference between profit and failure. Fish are much better converters of food into mass than any terrestrial species, reflecting their high protein diets. Most fish species can convert 1-2 kilograms of dry food into one kilogram of wet flesh while terrestrial animals have much higher conversion ratios with poultry in the range of 3-5:1, pork at about 8:1 and beef ranging from 5-20:1 (NOAA/USDA 2011). This significant difference results from the fact that fish are cold-blooded, which reduces the amount of calories required for respiration and total metabolism. Also, by living in water, fish do not require energy to support their body weight, which also frees up more consumed energy to be utilized for growth as opposed to maintenance.

Currently and historically, a major source of the protein and lipid used as feed components in aquaculture comes from marine derived fishmeal and fish oil. This is mainly due to the high digestibility of these ingredients along with their appropriate amino and fatty acid compositions for most fish species, especially marine species. Until recently, with the increasing production of aquaculture, these ingredients were in ready supply at manageable prices. Fishmeal production, both from the reduction of wild-caught stocks and offal from the processing of fish for human consumption, peaked in 1994 at roughly 30.2 million tonnes, and has dropped dramatically to 15.0 million tonnes in 2010 (FAO, 2012), with 36 % of 2010 production coming from offal. The price of fishmeal over that period increased by approximately 400 % from ~$300 per tonne to ~$1,200 per tonne in early 2012. Of this global fishmeal production, aquaculture consumes roughly 60.8 %, with the remainder going to non-human consumption industries such as the pet-food industry.

Fish oil resources have seen similar declines in production coupled with increasing costs. Global fish oil production peaked in 1986 at 1.67 million tonnes, but has declined at a steady rate of 2.6 % annually since, with global production in 2009 at 1.07 million tonnes. This decrease in availability has seen prices rise to roughly $1,500 per tonne in early 2012 (FAO, 2012). Aquaculture is the largest consumer of global fish oil resources, utilizing 73.8 % of global fish oil produced annually.

Not only are these trends alarming from economic and ecological perspectives, they become a major problem when considering that in order for aquaculture to continue to expand, fishmeal and fish oil production would also need to increase. As previously discussed however, wild fisheries production has been at a consistent or declining level for years. Fishmeal and fish oil production are also at the mercy of weather patterns such as El Nino that can greatly affect the quantity of fish produced for reduction. The time of year that fish are harvested can also affect the amino and fatty acid profiles of the meal and oil, leading to inconsistent quality.

An underlying issue with the whole concept of fishmeal and fish oil use in aquaculture is the concept of using fish to feed fish, which in no way increases the sustainability or productivity of the industry. All this practice is doing is using wild fish that are not suitable for human consumption as a resource in producing species that are viable for sale or consumption. The species caught for reduction to fishmeal and fish oil are often critical components or keystone species in their natural environments. Significantly reducing their numbers has negative impacts on local ecosystems such as reduced water quality when removing filter feeders as well as reducing the natural prey populations of species that are fished for human consumption. This causes further dietary

shifts in other species resulting in cascading negative effects throughout local and large-scale ecosystems. This results in unfavorable ratios of the amount of fish products used to create new fish products, or a “fish in: fish out” ratio. Currently, trends for these ratios for production of high-value marine fish can be as high as 4.5-6, meaning it takes 4.5-6 kg of wild caught fish to produce 1 kg of aquacultured fish. This is not a sustainable or beneficial type of production, and industry goals are to dramatically reduce this ratio by replacing fishmeal and fish oil with alternative, more sustainable sources. For aquaculture to successfully expand, these ratios need to be reduced to at least 1:1, with the preference to reach below 1, a situation which would actually result in a net production of fish protein from non-fish sources. Reaching these goals would allow the aquaculture industry to expand at much more sustainable levels, which would not only aid in meeting global protein demands, but could potentially aid in reducing the anthropogenic impacts on the environment due to over-fishing and reduction fisheries. Reduction fisheries also rely on the feed sources of many species fished for human consumption. Lessening the impact on these forage fish species would also further enable recovery of these populations.

Besides the ecological and economic challenges the industry faces, another important issues involved with aquaculture and fisheries recognized by the public and consumers is contamination. Polychlorinated biphenyls (PCB's) and mercury concentrations receive ever increasing scrutiny and publicity. There are already limits to the amounts of wild-caught fish humans should consume from the high trophic level, apex predators such as tuna and swordfish, as these compounds are known to undergo biomagnification, increasing in concentration in tissues up the food-chain. It has been shown that many environmental contaminants, especially those that accumulate in lipids,

are found in higher concentrations in farm-raised animals than in wild animals (Hites *et al*., 2004). This is due to the fact that these contaminants are greatly concentrated and increased relative to ration size during the production and inclusion of fishmeal and fish oil into diets used for aquaculture. Taken as a whole, not only are fishmeal and fish oil unsustainable, they should also be considered unsafe.

Food sources for fish grown in aquaculture is not a new or recently emerged problem and a great deal of research over the past few decades has been conducted on alternative protein and lipid sources in a variety of species with a wide range of success and failure. The greatest success seen in fishmeal replacement has been with animal source proteins, such as chicken, pork, and beef by-product meals, or with fish processing waste such as carcass and visceral meal for shrimp. Many of these meals naturally meet requirements in terms of protein and lipid and have appropriate amino acid profiles to serve as excellent replacement sources for most species. Besides meat meals, many other protein sources have been utilized as potential fishmeal replacements including plants, algal meals, yeast meals, and microbial products.

Generally speaking, freshwater omnivorous species, such as tilapia and catfish, have been the easiest to convert to low or no fishmeal diets and marine carnivorous species have been the most challenging. This may be due in part to the omnivorous nature of many freshwater species that are cultured, generally giving them the ability to digest a wider range of protein sources. Omnivores potentially maintain the ability to synthesize more of the needed essential and semi-essential components than their marine counterparts since their diets can be varied and unpredictable, reducing their dietary demands.

Fishmeal and fish oil replacement

A great deal of research has concentrated on the topics of replacing fishmeal and fish oil with a variety of alternative sources. Although animal by-product meals are readily available in consistent quantities in many parts of the world and tend to be highly digestible, public concern and outcry over their use has led them to being banned in European and other markets, and lessened their use in much of the rest of the world (Tomas *et al*., 2005). Plants, on the other hand, are a readily available source of proteins and lipids and already represent 94 % of global protein production and 86 % of the total edible oil production (NOAA/USDA 2011). Proteins such as wheat flour, wheat gluten, soy protein concentrate, soybean meal, barley meal, corn gluten, flax, pea protein concentrate, castor bean meal, and even yeast extracts, have all been studied as possible fishmeal replacements (Gaylord *et al*., 2007; Gomes *et al*., 1995; Lunger *et al*., 2007; Luo *et al*., 2006; Tomas *et al*., 2005; Xie *et al*., 1998; Zhou *et al*., 2005; Zhou *et al*., 2004).

Plant proteins have their own range of advantages and disadvantages just as any other feedstuff. Advantages to the use of plant proteins include the reduced reliance on a limited resource that itself is reliant upon environmental conditions, reduced fluctuations in quality and quantity produced annually, lowered mineral content giving the user more control over environmental enrichment, and lowered fears of disease transfer via animal based ingredients such as that seen with "mad-cow" disease. The biggest advantage though is the increased degree of sustainability that plants provide when compared to animal based products, especially fishmeal and fish oil. Along with sustainability comes the possibility of having aquaculture products deemed "organic" or “green”, which alone would allow these products into a niche market that already demands higher prices from informed and

demanding consumers, greatly offsetting the current increased production costs of both RAS's and the use of some alternative feedstuffs. However, the use of plant proteins has several disadvantages, including high fiber and carbohydrate levels, unfamiliar to many marine species that can cause significant health problems such as intestinal enteritis. Anti-nutritional factors (ANFs) such as the toxic chemicals gossypol, ricin, and erucic acid, as well as hormonal and protein breakdown inhibitors such as trypsinase are common in plants. Some of the negative characteristics of plant proteins are alleviated and some are exacerbated by the industrial processes required for their preparation and inclusion in diets for aquaculture. Taken as a whole, these factors have historically led to low overall digestibility and availability of proteins and other nutrients for fish from plant sources.

Plants such as soybeans, corn, rapeseed, and wheat are renewable, sustainable sources of terrestrial protein that have been cultivated and bred for many reasons that increase their nutritive value to humans. Size, protein quantity, amino acid profiles, annual yield, and reduced anti-nutritional components are all characters that have been selected for (both in GM and non-GM strains) in almost all species and strains of domesticated crops. Many of these beneficial traits extend to the use of these products as feed ingredients for aquaculture. Although there are many alternative protein sources available besides crop plants such as animal by-product meals, algal biomass as a by-product of biofuels production, algal meals, insect products, and aquaculture and fisheries by-products; terrestrial plant proteins provide one of the most readily available and applicable protein sources for use in the United States and globally. Plants are however, naturally low in some of the beneficial long chain polyunsaturated fatty acids (n3 LC-PUFAs). In addition, processing to remove some forms of anti-nutritional factors is expensive and can strip the

proteins of some beneficial vitamins and minerals (NOAA/USDA 2011). Plants also exhibit different amino acid profiles than the natural diet and requirements of most fish, especially marine carnivores. Supplementing dietary formulations with vitamins, minerals, amino acids, and other components known to be lacking in plant protein sources is a solution to this problem but requires a species by species knowledge of minimal and optimal requirement levels for the optimization of dietary formulations and productivity. Amino acid profiles that are unbalanced can be detrimental in terms of optimizing growth potential, and these issues can very challenging to address when using alternative protein sources, especially plants (Van Nguyen *et al*., 2013).

Partial and complete replacement of fishmeal in diets for rainbow trout, *Oncorhynchus mykiss*, have been successful with plant protein sources such as soybean meal, lupin flour, corn gluten meal, rapeseed meal, and cottonseed meal (Maria N. Alexis *et al*., 1985; Burel *et al*., 1998; Gomes *et al*., 1995; Hughes, 1991; Luo *et al*., 2006; Refstie *et al*., 1998; Vielma *et al*., 2002). Tomas *et al*. (2005) found increased FCR and lowered growth rates with inclusion of soybean meal above 30% in diets for Mediterranean yellowtail, *Seriola dumerili*, and cited varying levels of success other researchers had had with soybean meal inclusion with other species of yellowtail, most notably Japanese yellowtail. China, which houses one of the largest and most rapidly growing sectors of the aquaculture industry, has been very involved in alternate feedstuffs for fish culture. Cai *et al*. (2005) explored the effects of detoxified castor bean meal as a possible fishmeal replacement for juvenile grass carp, *Ctenopharyngodon idellus*. Castor beans contain high concentrations of the anti-nutritional factors and toxins ricin, lectin, and ricinine, which can be highly toxic to fish and higher vertebrates. However, upon detoxification processes,

caster bean meal was shown to be an effective fishmeal replacement with no detrimental effects on growth and feed utilization up to about a 40% replacement level. Xie *et al*. (1998) found that increasing levels of soybean cake as a fishmeal replacement led to decreased growth rate and feed utilization and deemed soybean cake an unsuitable replacement candidate for Chinese longsnout catfish, *Leiocassis longirostris*. Borgeson *et al*. (2006) were successful in replacing up to 67% of fishmeal in Nile tilapia, *Oreochromis niloticus*. This was a significant study, although not the first, because it focused on comparing diets that utilized a variety of plant proteins as replacements as opposed to simply replacing fishmeal with one plant protein ingredient. Comparisons were made between simple replacement diets, diets using only one replacement source, and complex diets, diets using blends of replacement sources. The results showed that fish fed the complex diets had higher growth rates, protein efficiency ratios, and feed utilization than fish fed the simple replacement diets. However growth rates and feed efficiencies dropped when 100% of fishmeal was replaced either in the simple or complex diets.

These studies have encountered varying degrees of success, rarely matching growth rates, feed conversion ratios and survival rates when compared to typical fishmeal-based diets. Several of the possible explanations for this have been uncovered and corrected. It is known that many plant sources lack several essential amino acids such as lysine and methionine as well as many vitamins and minerals that are needed in micro quantities. It has become common practice to supplement alternate protein source diets as well as even fishmeal-based diets with these types of additives to ensure that amino acid, vitamin, and mineral requirements are met. Barrows *et al*. (2010) reported positive effects on weight gain, FCR, feed intake, hepatosomatic index (HSI), and nutrient retention when

supplementing plant-based diets for rainbow trout with macro-minerals sodium chloride, potassium chloride, and magnesium oxide as well as inositol. It is important to note that very few species specific requirement levels have been identified for more than a handful of individual ingredients and many researchers and diet manufacturers work from the same nutritional guidelines regardless of the species for which a particular diet is being formulated (NRC, 1993).

Taurine

Taurine is a sulfur containing beta-amino acid found in high concentrations in a variety of tissues in vertebrates. Taurine is not incorporated into any known proteins and is therefore only considered semi-essential in most species and essential in a few strict carnivores, most notably felines. The various roles of taurine in the eye, heart, liver, kidneys, and leukocytes have received the most attention (Schaffer *et al*., 2010). These include conjugation to bile salts, cell differentiation, photoreceptor protection, regulation of neural transmission in the retina, hemolytic suppression, osmoregulation, and as a powerful anti-oxidant (Schuller-Levis and Park, 2003). Growing evidence also supports beneficial effects on improved paternal reproductive quality in the form of improved spermatogenesis (Higuchi et al., 2012b). Taurine is considered to be a conditionally indispensable amino acid for humans and non-human primates (Schuller-Levis and Park, 2003), however little attention has been paid to requirement levels of this amino acid and its roles in fish. The primary roles of taurine in teleosts are shown in Figure 1.1.4. Taurine is found in appreciable concentrations in the natural diet of marine carnivores and the fishmeal component of traditional feeds (Satake *et al*., 1988). All terrestrial plant protein

sources are devoid of taurine however, yet it is rarely supplemented in aquaculture diets, and may be the biggest contributing factor to the drop-off in production characteristics described previously when fishmeal levels in feeds are reduced.

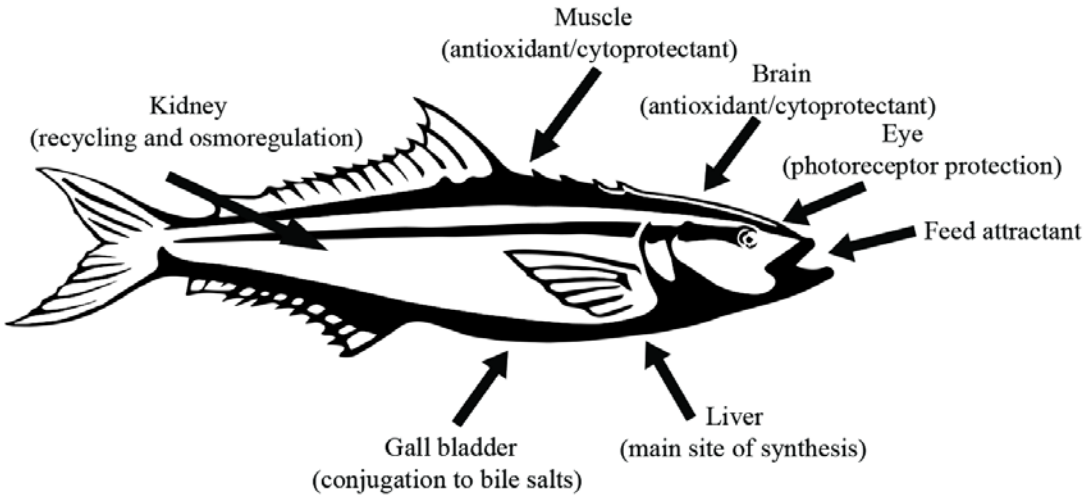

Figure 1.1.4. Primary functions and organs with high concentrations of taurine in a marine teleost. Cobia, *Rachycentron canadum*, figure modified by Alexandra Casmer.

Taurine deficiencies in some species have been described and include symptoms such as green liver syndrome and blindness (Goto *et al*., 2001b; Maita *et al*., 1997; Takagi *et al*., 2011, 2006, 2005). The positive effects of taurine supplementation to fish diets (Gaylord *et al*., 2007; Lunger *et al*., 2007b; Pinto *et al*., 2010; Takagi *et al*., 2008) has led to the hypothesis that marine carnivores have a dietary requirement for taurine. This hypothesis is supported by the high levels of taurine observed in the natural diet of marine carnivores and thus the ability to synthesize sufficient quantities of taurine has been lost or down regulated. Taurine must therefore be considered an essential amino acid and its dietary requirement for each species must be identified and met either through inclusion of meat meals containing appreciable quantities of taurine or through direct supplementation of taurine to the feeds. Examining the species-specific capacity for taurine biosynthesis

will not only help establish minimal and optimal taurine requirement levels, but will also enable the feeds industry to replace higher and higher quantities of fishmeal with plant-based proteins without losing production efficiencies or using ingredients such as by-product meals from animal processing that are banned in some countries (Tomas *et al*., 2005) and carry significant public concern (e.g. prions, heavy metal accumulation).

Synthesis of taurine can occur in two ways (Figure 1.1.5, Stipanuk *et al*., 2009, 2011) with both pathways relying on cysteine, a semi-essential amino acid biosynthesized from the essential amino acid methionine. Synthesis occurs mainly in the liver (Tappaz, 2004), after which taurine is taken up by other tissues through plasma circulation and a taurine transporter (TauT).

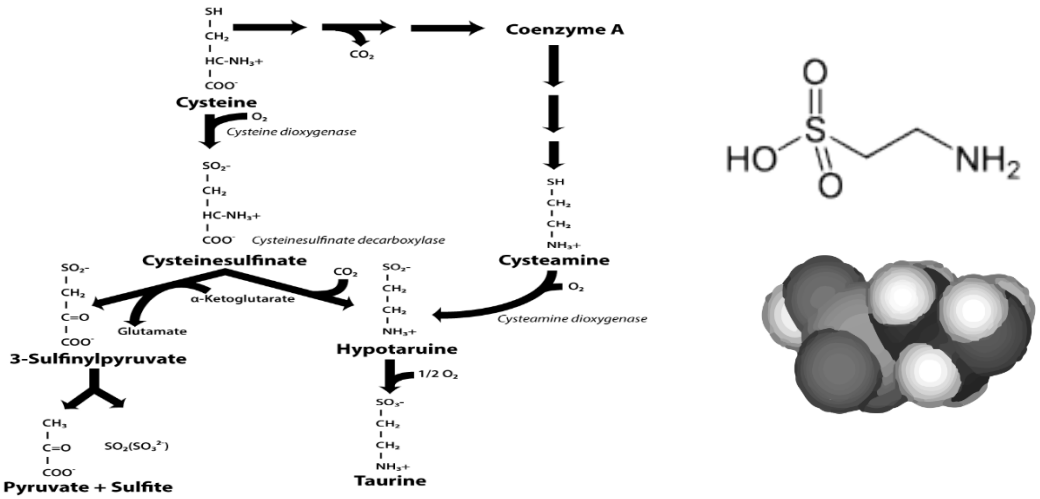

Figure 1.1.5. Taurine biosynthesis pathway (left) modified from Stipanuk *et al*. (2011) with the chemical structure of taurine.

Cysteine dioxygenase (CDO, EC 1.13.11.20)

Much of the work that has been performed on the individual enzymes of the taurine synthesis pathway have been conducted *in vivo* with mice or rats or *in vitro* with cell lines derived from mice, rats or humans. Very little work has been conducted in teleost species, however, many parallels can be drawn between the mammalian work that has been conducted and teleost systems, as both groups of vertebrates rely on many of the same cellular mechanisms and transcription/translational control machinery.

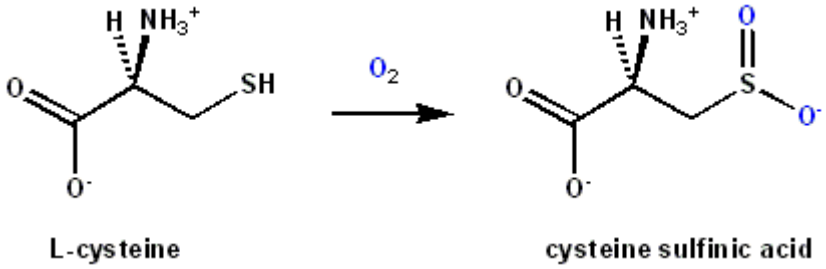

Figure 1.1.6. Cysteine dioxygenase converts cysteine to cysteinesulfinic acid.

Cysteine dioxygenase (CDO) is a non-heme containing, mononuclear iron enzyme (Simmons *et al*., 2006) that catalyzes the first of two reactions (Figures 1.1.6 and 1.1.7) required to produce taurine from cysteine (Figure 1.1.5). In this reaction molecular oxygen is added to the sulfur of cysteine, creating cysteinesulfinic acid (McCoy *et al*., 2006). CDO activity reflects the balance between cellular cysteine levels and the presence of a thioether bond between a cysteine (Cys93) and a tyrosine (Tyr157) within the protein that acts as a cofactor. Although basal catalytic activity is present without the thioether bond, as evidenced by mutated CDO expressed in cell lines, the presence or absence of the thioether bond is responsible for a 10-fold potential difference in activity (Dominy *et al*., 2008). The mature, thioether bond-containing, isoform of CDO is dependent on the presence of cellular cysteine, a metal cofactor (Fe^{2+}) and oxygen (Stipanuk *et al*., 2009).

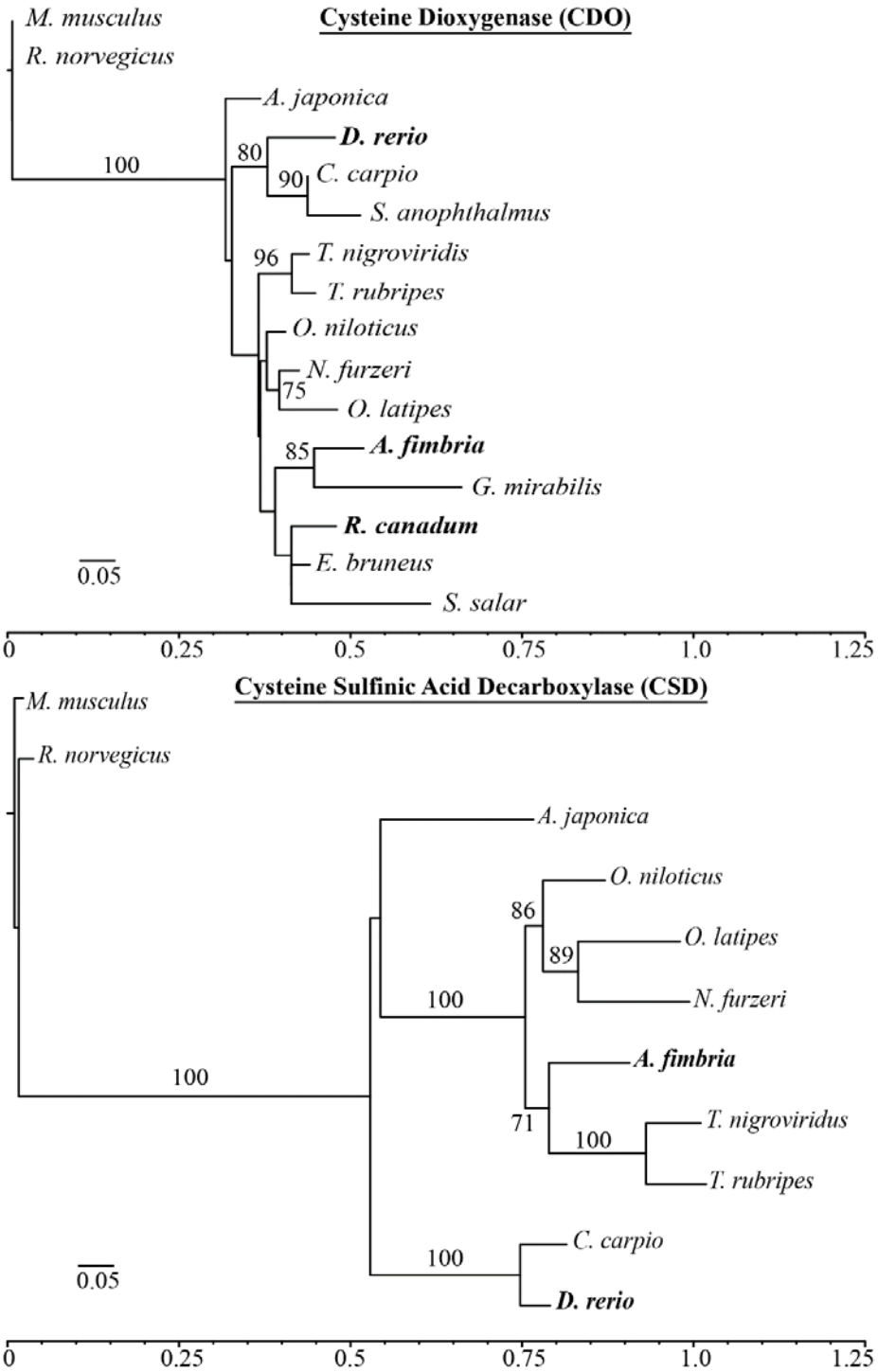

Figure 1.1.7. Phylogenetic relationships for cysteine dioxygenase (CDO) and cysteine sulfinic acid decarboxylase (CSD). Protein sequences, all except for *R. canadum* obtained from published sequences in NCBI database. *R. canadum* sequence translated from sequenced cDNA. Bold species represent those discussed in detail in subsequent sections. Numbers are bootstrap values (<70 considered not significant and are not shown).

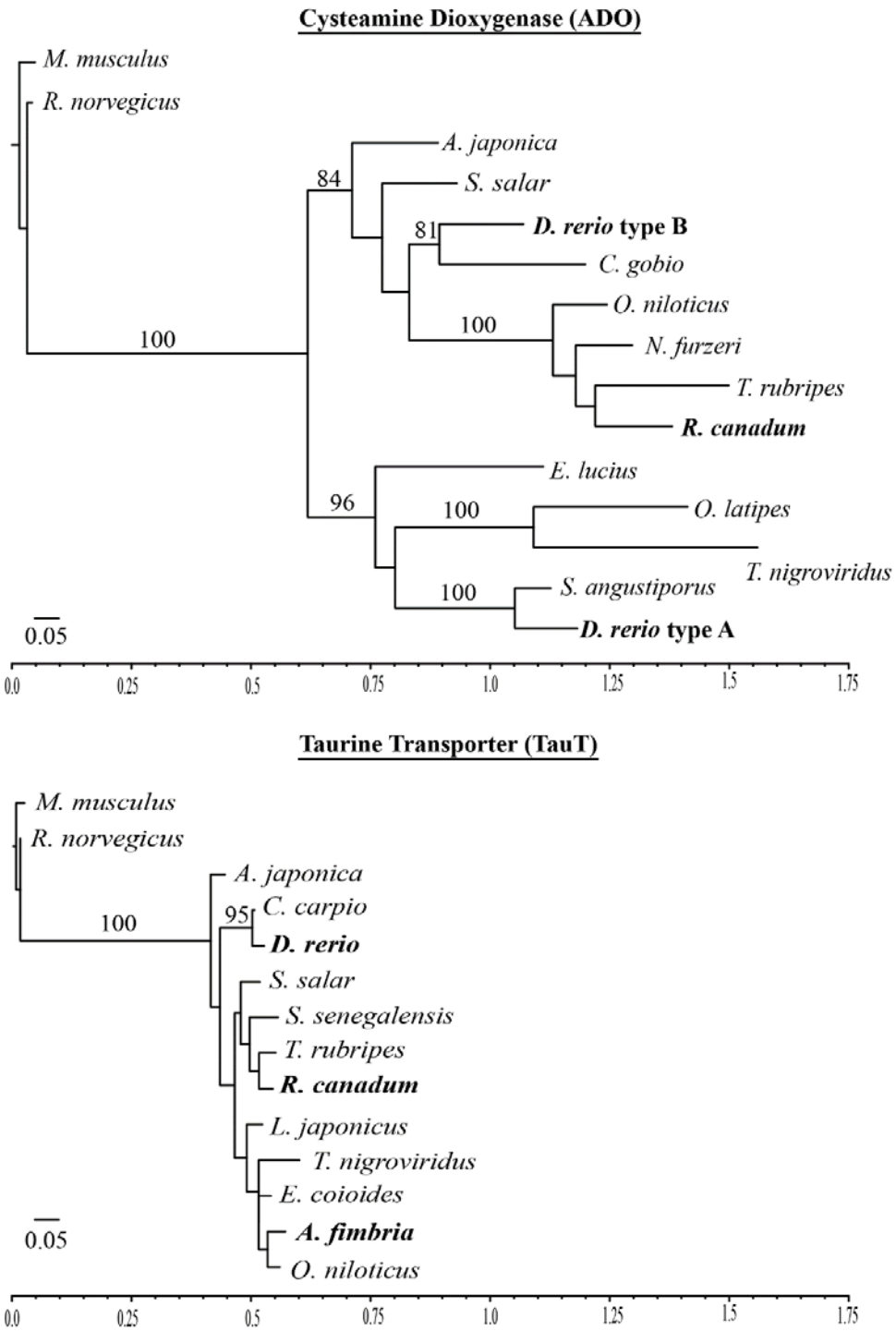

Figure 1.1.8. Phylogenetic relationships for cysteamine dioxygenase (ADO) and the taurine transporter (TauT). Protein sequences, all except for *R. canadum* obtained from published sequences in NCBI database. *R. canadum* sequence translated from sequenced cDNA. Bold species represent those discussed in detail in subsequent sections. Numbers are bootstrap values (<70 considered not significant and are not shown).

Cellular CDO concentrations are tightly regulated by the rate of proteasomal degradation as controlled by polyubiquitination (Stipanuk *et al*., 2004). CDO concentrations can change up to 45-fold (Lee *et al*., 2004), multiplied by the potential 10-fold difference in catalytic efficiency (Dominy *et al*., 2008), for a total potential change in CDO activity of up to 450-fold. This change in activity occurs within minutes and stabilizes within hours after the introduction of either high protein or high sulfur amino acid diets or cell media. This is one of the fastest and broadest ranges of activity of any metabolic enzyme that responds to dietary input (Stipanuk *et al*., 2009). Interestingly, the rapid increase in cellular concentration of CDO does not appear to be the result of increased transcript levels. Instead, the prevention of proteasomal degradation results in more active CDO remaining in cells. Low cellular cysteine concentrations result in the polyubiquitination of both the immature and mature isoforms of CDO, which are then rapidly degraded. The introduction of higher cellular cysteine concentrations either through high protein or high sulfur amino acid containing diets results not only in an increase in the Cys-Tyr thioether bond formation, but also in a decrease in the ubiquitination of CDO and its subsequent proteasomal degradation, allowing for the rapid increases in CDO activity (Dominy *et al*., 2006; Stipanuk *et al*., 2004). It has been hypothesized that cysteine itself, when bound by CDO, initiates a conformational change that prevents subsequent polyubiquitination and degradation (Lee *et al*., 2004; Stipanuk *et al*., 2004).

The tight regulation of this step of the pathway is not to ensure the synthesis of sufficient taurine, instead is a mechanism to reduce cellular cysteine which can rapidly become toxic at high concentrations (Stipanuk et al., 2006). Although cysteine toxicity can occur in any cell type, it is a serious concern in brain and neuronal tissues. The mechanisms

of cysteine toxicity result from the L-cysteine enantiomer, which can form a variety of excitotoxins, which can lead to cell death at high concentrations due to over-excitation. Cysteine α-carbamate (a toxic analog of N-methyl-D-aspartate, NMDA), S-nitrosocysteine (a potential nitric oxide surrogate), catecholamine derivatives, and other toxic oxidized cysteine derivatives can be formed from excess cysteine. Cysteine can also interrupt the NMDA receptor redox sites and chelate Zn^{2+} and other metals which can result in blocking of receptor sites (Janáky *et al*., 2000). S-nitrosocysteine other S-conjugates of cysteine, and the closely related 4-thiaalkanoates are all nephrotoxic and hepatotoxic in rats. Their main effects are in the mitochondria and result in decreased cellular respiration, decreased ATP, depletion of glutathione, and damage to the mitochondrial genome (Anders, 1995), each which can lead to reduced performance of the mitochondria and may contribute to several known mitochondrial disorders.

Loss of, or insufficient, CDO activity can lead to the same negative effects as exhibited by high cellular cysteine levels. Ueki *et al*. (2011) developed a *CDO*$^{-/-}$ knockout mice to explore the effects of a reduced capacity for regulating cysteine metabolism and taurine synthesis. The authors found that the knockout mice had extremely low taurine levels and elevated cysteine levels along with postnatal mortality, growth impairment, and connective tissue pathology. Supplementation of these knockout mice with taurine improved survival, but did not otherwise affect the knockout phenotype (Ueki *et al*., 2011). This is consistent with the hypothesis that CDO is critical in both the synthesis of taurine and the reduction of cellular cysteine to prevent toxicity. The mouse *CDO* gene contains multiple 5′ upstream promoter elements (HNF-3β, HFH-1, HFH-2, HFH-3, C/EBP, C/EBPβ) that are consistent with tissue specific expression. Both mRNA and protein levels

are consistent with activity measurements in varying tissues, with the liver having the highest, but with detectible expression in kidney, lung, brain, adipose tissue and small intestine (Hirschberger *et al*., 2001). These other tissues have been shown to compensate for the loss of liver CDO activity in liver-specific knockout mice (Ueki *et al*., 2012). This compensation not only results in lowered concentrations of cysteine in these tissues, but also an increase in the concentrations of hypotaurine and taurine.

Cysteine sulfinic acid decarboxylase (CSD, EC 4.1.1.29)

Cysteinesulfinic acid decarboxylase (CSD) converts cysteinesulfinate, the product of CDO, to hypotaurine (Figures 1.1.7 and 1.1.9). CSD is also referred to as cysteine sulfinic acid decarboxylase (CSAD), especially in the zebrafish literature, but for simplicity and continuity will only be referred to as CSD throughout this dissertation. CSD is a pyridoxal phosphate requiring enzyme (Kaisaki *et al*., 1995) that decarboxylates cysteinesulfinate to yield hypotaurine, which spontaneously devolves into taurine (De La Rosa and Stipanuk, 1985). This is the rate-limiting step in the synthesis of taurine from cysteine, and appears to be the reason that many species, such as cats, do not appear to have the ability to synthesize enough taurine *in vivo* (Worden and Stipanuk, 1985). Cysteinesulfinate can also be transaminated to form pyruvate and sulfate as part of the shunt to relieve excess cellular cysteine levels.

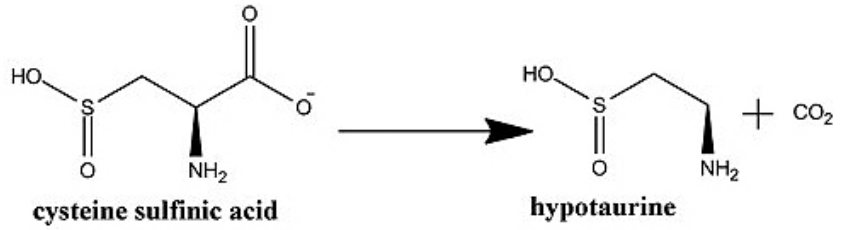

Figure 1.1.9. Cysteinesulfinic acid decarboxylase (CDO) converts cysteinesulfinic acid to hypotaurine.

In rats, two distinct mRNAs have been identified encoding identical CSD proteins. These transcripts differ only in their 5´-untranslated region. This difference is most likely due to alternative splicing, but may be a regulatory element for the expression of CSD in different tissues (Tappaz *et al*., 1999). Phosphorylation of the α-subunit of eukaryotic initiation factor 2, and the subsequent downstream effects, is a common response to stress in many animals, with several eIF2α kinases responding specifically to nutritional stress. The main kinase that responds to amino acid nutritional stress is GCN2, which responds to unbound tRNAs involved in protein synthesis, resulting from a deficiency in either essential amino acids in the diet or amino acid metabolism from supplied precursors. Activation of GCN2 has the general effect of down-regulating protein synthesis and can be involved in the downstream pathway of protein catabolism during periods of prolonged amino acid starvation (Sikalidis and Stipanuk, 2010). Since taurine is derived from the sulfur amino acids methionine and cysteine, it is important to understand whether or not there is a different stress response induced from sulfur amino acid deficient diets compared to diets deficient in non-sulfur amino acids. Sikalidis and Stipanuk (2010) fed rats diets deficient in methionine and observed prolonged phosphorylation of eIF2α and an increased translation of activating transcription factor 4 (ATF4) that stimulates the integrated stress response. However, since taurine does not have a corresponding tRNA, it seems unlikely

that taurine deficiency would activate GCN2 directly. However, it is possible that ATF4 could increase transcription of CSD or that eIF2α phosphorylation could increase CSD translation. However, it is equally likely that taurine synthesis is regulated by a different pathway(s). Similar to other stress responses, CSD activity is regulated by phosphorylation. CSD is active when phosphorylated by protein kinase C and inhibited when dephosphorylated by protein phosphatase 2C (Tang *et al*., 1997).

The activity of CSD has been assayed in an array of mammals and some teleost species and a variety of tissue types. Postnatal male and female rats displayed high levels of hepatic CSD activity (3.4-5.6 mmol CO_2 min^{-1} mg $protein^{-1}$), which declined in females during development to adulthood, to the point where adult male rats had 16-fold higher activity than did adult females. Mice, on the other hand maintained fairly consistent levels of hepatic CSD activity through 16 weeks of age (10.7-23.2 mmol CO_2 min^{-1} mg $protein^{-1}$). Cats and kittens had the lowest levels of CSD activity compared to all other animals in the study, with hepatic CSD activity at 0.61-0.63 mmol CO_2 min^{-1} mg $protein^{-1}$ in five-six week-old kittens which declined rapidly, with 15 month-old cats having activity 73 times lower than kittens (0.008 mmol CO_2 min^{-1} mg $protein^{-1}$). CSD activity in guinea pig liver was significantly lower than rats or mice in the same study (Worden and Stipanuk, 1985). Similar trends were observed in brain CSD activity for all species examined, although at much lower rates than those observed in the liver. Overall taurine concentration in these tissues mimicked CSD activity, decreasing with age and activity level. Eppler and Dawson (1999) observed similar reductions in CSD and CDO activity in aged rats resulting in reduced tissue taurine concentrations, however the decline is strain-specific, with Fischer

344 rats showing the decline with age, but not Sprague-Dawley or F344/Brown-Norway hybrids.

CSD has also been shown to be highly expressed, at both the protein and transcript level, in the outer medulla of rat kidneys, supporting a role for taurine as an osmolyte (Reymond *et al.*, 2000). CSD activity has been confirmed in the astrocytes of the cerebellum and hippocampus of adult rats, supporting the importance of taurine in the brain, although which of its myriad functions is a priority in the brain has yet to be confirmed (Reymond *et al*., 1996). Hepatic CSD activity can be inhibited in rats by the administration of thyroid hormone, however the same administration results in an increase in renal CSD activity (Jerkins and Steele, 1991). Adrenalectomy, also results in reduced CSD activity (Jerkins and Steele, 1992).

Although there have been fewer studies on hepatic CSD, this also declines in activity in animals fed high protein or high sulfur amino acid diets (Jerkins and Steele, 1992; Tappaz, 2004). Goto *et al*. (2001) measured hepatic CSD activity in livers of several teleost species, although source and diet of fish prior to sampling was not specified. Three freshwater species had hepatic CSD activity of 0.3 (carp), 2.33 (rainbow trout) and 15.8 (bluegill) nmol taurine produced mg protein^{-1} min^{-1}. Five marine species measured ranged from 0.00 to 0.24 nmol taurine produced mg protein^{-1} min^{-1}. Although this study was measuring CSD activity through the measurement of synthesized taurine, rates for CSD activity were much lower in these teleost species than the previously discussed CSD activities described for mammalian species.

Taurine is such an important contributor to osmoregulation, lipid metabolism and oxidative stress reduction that maternally deposited mRNA transcripts for CSD have been

detected in the embryos and developing stages of zebrafish, alongside embryonic derived transcripts. Knockdown of CSD in zebrafish embryos significantly reduces taurine level and results in increased mortality and cardiac abnormalities. Injection of mRNA and supplementation with taurine can rescue the abnormal cardiac phenotypes (Chang *et al*., 2013) indicating not only how critical taurine is to embryonic development, but how critical the CSD pathway is in the synthesis of taurine in zebrafish.

Cysteamine dioxygenase (ADO, EC 1.13.11.19)

In comparison to CDO and CSD, much less is known about cysteamine dioxygenase (ADO), the enzyme responsible for the reaction that produces hypotaurine from cysteamine (Figures 1.1.8 and 1.1.10). Although the existence of the enzyme has been known for some time, it was only recently identified and characterized at the gene level through bio-informatic genome analyses (Buongiorno and Straganz, 2013). Similar to CDO, ADO is in the cupin superfamily of enzymes and also requires a metal cofactor, almost exclusively iron. CDO and ADO however, do not show any cross-reactivity of substrates, as CDO will not use cysteamine and ADO will not use cysteine (Dominy *et al*., 2007). Although cysteine and cysteamine are very similar thiol compounds, the fact that there is no cross-reactivity between the two enzymes indicates that, at least in mammals, these two pathways are very tightly and independently regulated. Cysteamine is produced in cells as a by-product of the degradation of coenzyme A (Besouw *et al*., 2013). Due to the low activities of CSD observed in many mammalian and teleost species, there has been a renewed interest in the potential of cysteamine and ADO in the synthesis of taurine (Coloso *et al*., 2006).

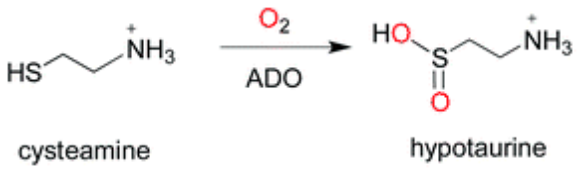

Figure 1.1.10. Cysteamine dioxygenase (ADO) converts cysteamine to hypotaurine.

Rats have been shown to have a high capacity for taurine synthesis in adipose tissues through both the CDO/CSD and ADO pathways (Ueki and Stipanuk, 2009) resulting from an increase in both mRNA and protein abundances for the enzymes of each pathway.

Goto *et al*. (2001a) measured the ADO activity in the livers of several species of fish. Source and dietary input of the fish was not specified, but the activity of ADO ranged from 0.09-3.06 nmol hypotaurine + taurine produced min^{-1} mg $protein^{-1}$ with no significant difference between marine and freshwater species. Combined with the studies of the Goto laboratory on CSD, the authors concluded that the low activity levels were not consistent with with the overall levels of taurine detected in the fish. This led to the conclusion that most of the species assayed, most notably the marine species, although possessing the ability to synthesize taurine to some degree do not synthesize sufficient taurine to meet their needs.

Taurine transporter (TauT)

In addition to the enzymes responsible for the synthesis of taurine, a highly conserved membrane bound transporter is critical in the transport and recycling of taurine (Figure 1.1.8). The taurine transporter (TauT) is a sodium chloride dependent transporter which exchanges two Na^+ ions and one Cl^- ion for each molecule of taurine transported across the membrane. The transporter is highly expressed in the apical membrane of

intestinal cells as well as kidney cells (Bröer 2008). The transporter contains at least two intracellular phosphorylation sites for some degree of regulation. The taurine transporter is a substrate for both PKC and PKA and there is evidence that they are responsible for inhibiting or increasing the rate of taurine transfer, respectively. However, there appear to be differences between tissue types and species as to the specific effects of each kinase (Tappaz, 2004).

In general, an animal's body taurine levels are regulated by the circulating plasma taurine levels and an increase or decrease in renal TauT expression and activity. TauT activity is up-regulated when circulating taurine levels are low to increase the reabsorption and recycling of existing taurine levels, and down-regulated when plasma taurine levels are high to allow for excretion and maintenance of appropriate concentrations. The majority of circulating taurine is derived from dietary intake since, as previously discussed, many animals do not have the ability to synthesize sufficient taurine. This has been confirmed through TauT activity of animals fed high protein or high taurine diets relative to those fed low protein or low taurine diets (Tappaz, 2004). Animals that are capable of regulating TauT expression and activity do so rapidly after dietary intake or changes in dietary protein or taurine content.

Besides dietary protein and taurine input, there are many factors that can affect TauT expression and activity. Hypertonicity can induce an increase in TauT expression in many cell types, and the 5´-flanking region of the gene upstream of the transcription start site contains a sequence that is consistent with osmotic response elements (Tappaz, 2004). Rat TauT expression has also been shown to increase *in vivo* during induced antidiureses in the renal papilla, in the outer renal medulla under high salt conditions, and in the brain

and retina under hypertonic conditions (Bitoun and Tappaz, 2000; Bitoun *et al*., 2001). Similar results were obtained from a carp cell line (Takeuchi *et al*., 2000) and up and down-regulation of the taurine transporter in canine kidney cells also responded to the taurine concentration of the media (Han *et al*., 1997). Human intestinal epithelial cells, Caco-2, also respond to hypertonicity by increasing TauT expression and increased taurine uptake (Satsu *et al*., 1999). Caco-2 cells also respond to specific cytokines, such as TNF-α, with an increase in TauT expression and activity (Mochizuki *et al*., 2002). This serves to mediate the typical inflammatory response of cytokines in intestinal cells. TNF-α did not have similar effects in kidney, liver, or macrophage cells however, and the uptake of other osmolytes such as glycine, L-leucine, and L-glutamic acid were not affected by TNF-α (Mochizuki *et al*., 2002). This response could explain why fish fed diets high in plant protein ingredients not supplemented with taurine can develop intestinal enteritis. TauT expression in human embryonic kidney 293 cells can be significantly up-regulated by the WT1 gene (Han and Chesney, 2003), and TauT is a target of the tumor suppressor protein p53, which results in a down-regulation of TauT expression in the developing kidney (Han *et al*., 2000). These studies have led to the conclusion that TauT is critical in the proper development of the kidneys in mammals. TauT is also present in murine neuronal precursor cells and addition of taurine to cell media increases cell proliferation (Hernandez-Benitez *et al*., 2010).

TauT knockouts have been developed for the mouse model. Mice with no taurine transporter have significantly reduced taurine concentrations in skeletal and cardiac muscles (Warskulat *et al*., 2004) as well as changes in $GABA_{A,}$ kainite, and AMPA receptor densities in the brain (Oermann *et al*., 2005). Exercise capacity was also

significantly reduced in TauT knockout mice which accompanied a 28% reduction in conduction velocity in muscle tissue. Interestingly cardiac function was not affected by knocking out the taurine transporter. Instead there was an up-regulation of the cytosolic concentrations of several other organic solutes in heart tissues to compensate for the loss of taurine availability (Warskulat *et al*., 2004). This compensation was not observed in the muscle cells however. Contrasting results were found by Ito *et al.* (2010, 2008) who observed significant negative effects in cardiac structure and function in TauT knockout mice. The differences between the Warskulat and Ito studies have been attributed to the genetic background of the strains of mice used. TauT knockout mice showed retinal degeneration at a significantly younger age than control mice. Retinal cells in mice have been shown to lack CSD activity and are therefore reliant upon the taurine transporter to maintain high taurine concentrations needed for its anti-oxidant properties to protect photoreceptors and aid in their recovery from bright light. Even mice kept in total darkness suffer this rapid degeneration when compared to control mice, and the use of standard day/night light cycles accelerates this process. Since cell differentiation is not affected by TauT knockout, this is likely to reflect the inability of mature photoreceptor cells to survive without a functional taurine transporter, (Rascher *et al*., 2004). TauT knockout mice also suffer from hepatitis and liver fibrosis with over 80% of mice over age 1 developing these symptoms compared to only ~20% in wild type mice (Warskulat *et al*., 2006). Interestingly, many of the pathologies observed in homozygous knockout mice are also observed in the heterozygous knockouts, although at intermediate levels between the homozygous knockouts and wild type mice (Warskulat *et al*., 2007).

It is thought that the taurine transporter may also play a role in sulfide detoxification in organisms adapted to deep-sea hydrothermal vents. The deep-sea mussel *Bathymodiolus septemdierum* has a high concentration of TauT mRNA in its gills and gonads, and the transporter has been shown to function under a wide range of salinities with affinity for thiotaurine and hypotaurine. Similar results were found in another deep-sea mussel, *Bathymodiolus platifrons* (Koito *et al*., 2010), where long-term exposure to sulfides also resulted in a maintenance of high TauT mRNA in gill tissue. Thiotaurine is a derivative of taurine with two sulfurs instead one just one, and has been found in high concentrations in the tissues of many vent and seep invertebrates, and is believed to be involved in sulfide detoxification (Inoue *et al*., 2008). Two other bivalves, although shallow water molluscs as opposed to deep-sea molluscs, have also been shown to maintain high transcript levels and activity of TauT in gill tissues. Both the Mediterranean blue mussel, *Mytilus galloprovincialis*, and the giant Pacific oyster, *Crassostrea gigas*, rely heavily upon the taurine transporter to aid in maintaining osmotic balance in the face of changing salinities that can be experienced in shallow coastal areas (Hosoi *et al*., 2007; Toyohara *et al*., 2005).

In fish, TauT is only known for its role in osmoreguletion. The starry flounder, *Platichthys stellatus*, Japanese eel, *Anguilla japonica*, Senegalese sole, *Solea senegalensis*, and tilapia, *Oreochromis mossambicus*, have all been shown to up-regulate TauT expression or activity in response to high-salinity in a variety of tissues (Fincham *et al*., 1987; Pinto *et al*., 2012; Takeuchi *et al*., 2001). Dogfish, *Squalus acanthais*, and the little skate, *Raja erinacea*, both excrete taurine through the kidneys when adjusted to lower than full strength seawater (Schrock *et al*., 1982). This is the opposite effect observed of previously discussed species being acclimated to higher salt concentrations, but fits with

the hypothesis that lower salt concentrations would lead to lower TauT expression and activity resulting in increased excretion of plasma taurine, further evidence of the role of taurine as an osmolyte. Expression of TauT in the Senegalese sole also indicated a potential mechanism for taurine uptake in the stomach for use in bile salt conjugation, and taurine recycling with high levels of TauT expression in the hindgut to prevent loss (Pinto *et al*., 2012). Mechanisms such as this may be critical not only in maintaining the existing taurine pool within the animal, but also in the dietary importance, of taurine, since a recycling loop would not be necessary if the species had the capacity for sufficient synthesis. Zebrafish embryos in early cleavage stages contain taurine (~192 pmol embryo^{-1}) and TauT mRNA, indicating as with CSD that these are maternally derived transcripts and critical to normal development (Kozlowski *et al*., 2008). During later stages of development, zygotic expression of TauT is found in the retina, brain, heart, kidney, and blood vessels. Not only does the zebrafish TauT expressed in mammalian cells transport taurine with similar kinetics, but knockdown of TauT in developing zebrafish embryos by antisense morpholino oligonucleotides results in CNS cell death and significantly increased mortality, indicating the importance of taurine in development (Kozlowski *et al*., 2008).

Overall, a great deal of work has been done on the individual components of the taurine synthesis and transport pathways in a variety of species, with relatively little work done encompassing the whole pathway at once. Also, little work has been done on the ability of teleosts to synthesize taurine, although its physiological importance in development, osmoregulation, and digestion has been well established for an array of species. Assessing the ability of any species to synthesize taurine, and other critical dietary components, will be paramount in designing species specific diets that can maximize

growth while reducing redundant or excessive input that could result in undue metabolic stress or excretion of expensive or eutrophying compounds.

Hypotheses

This work sets out to test several hypotheses. The primary hypothesis is that taurine in an essential, rather than semi-essential amino acid for marine carnivores. The premise for this hypothesis comes from the finding of high taurine levels in the natural prey of these species. This is similar to the need for omega-3 fatty acids in marine carnivores where high natural abundance in their prey has resulted in down regulation of biosynthesis. This taurine requirement has only recently been recognized when fishmeal began being replaced with plant proteins, as plants are naturally devoid of taurine. In order to test this hypothesis we had to first develop an LC-MS method to rapidly and efficiently measure taurine in a variety of sample types (Chapter 2). Chapter 3 discusses the taurine biosynthesis potential of several teleosts using RT-qPCR methods to quantify transcript abundance for genes required for taurine synthesis. Chapter 4 examines the utilization of taurine as a dietary supplement in efforts to reduce and eliminate fishmeal and the effects of no and low taurine feeds to illustrate the essential nature of taurine. Chapter 5 examines the effects of total fish product (fishmeal and fish oil) replacement with taurine supplemented feeds for two marine carnivores with two alternative lipid sources to replace fish oil. Finally, Chapter 6 addresses potential consumer concerns that exist for aquaculture and how the replacement of fishmeal with plant proteins. PCB and mercury concentrations were measured in fillets of fish raised on plant based feeds compared to commercial feeds and taste-testing panels analyzed the same fillets to determine potential differences consumers may expect in the marketplace.

Chapter 2: Measuring Taurine

Chapter 2 begins with a description of a newly developed LC-MS method for rapidly quantifying taurine that subsequent work was reliant upon. The second section of this chapter discusses the utilization of this method to determine taurine content in striped bass, *Morone saxatilis*, eggs and common prey items of marine carnivores. Overall, this chapter describes the method used to measure taurine throughout this dissertation and the establishment of the argument that marine carnivores consume substantial quantities of taurine in their natural diets.

Leaching of taurine from commercial type aquaculture feeds

Abstract

Leaching of soluble compounds from pelleted feeds is an issue for the aquaculture industry which can increase environmental impact and reduce the ingested quantity of essential components. This study was undertaken to examine the leaching rates of taurine, a non-protein amino acid with critical physiological roles in teleosts. To this end we adapted a new liquid chromatography mass spectrometry (LC-MS) method for quantifying taurine. Twelve different feeds (3-4 mm dia.), varying in protein source and taurine supplementation, were examined. Fishmeal ranged from 0.0% to 45.5% with taurine supplementation ranging from 0.0% to 5.0%. Taurine was extracted and quantified from individual pellets in triplicate at 6 time points (0, 1, 5, 10, 20, 40 min). Leaching rates ranged from 0.026 ± 0.005 to 0.826 ± 0.121 mg min^{-1} over 40 min at 27°C and were strongly correlated to initial taurine content of the feeds (for distilled water n=12, $p<0.001$, $R^2=0.91$ for artificial seawater, 25 ppt, n=4, $p=0.020$, $R^2=0.96$). Loss of taurine from feeds was 59.48 ± 16.49% after 40 min. This study shows that taurine supplementation should exceed requirement level for slow consumers or feed being delivered in multiple additions, as a significant amount of taurine is lost over time.

Introduction

Leaching of essential nutrients such as vitamins, minerals and amino acids can lead to inadequate nutrition and further environmental pollution for intensive aquaculture operations (Gadient *et al*., 1992; Goldblatt *et al*., 1979; Marchetti *et al*., 1999). This issue is especially problematic with microdiets for larval fish (Kvåle *et al*., 2006; Yufera *et al*.,

2002) and with feeds for species such as shrimp and crabs who macerate their diet prior to ingestion often after extended immersion times (Obaldo *et al*., 2002).

Taurine, a non-protein amino acid, found in the natural prey for many species of commercial interest in aquaculture (Satake *et al*., 1988), is a recent addition to pelleted diets for marine species (Gaylord *et al*., 2007; Lunger *et al*., 2007; Matsunari *et al*., 2008; Qi *et al*., 2012; Takagi *et al*., 2008). Plant protein sources such as soybeans, wheat, and corn are devoid of taurine. Although taurine requirements have not been explicitly identified for many species, it is considered a semi-essential amino acid in mammals since it can be synthesized from the essential amino acid, methionine (Pinto *et al*., 2013). In marine fish however, the synthesis of taurine has been shown to be highly variable between species, and in many cases fish fed diets devoid of taurine have low feed consumption, growth and survival (Kim *et al*., 2007, 2005b; Watson *et al*., 2012). However, the FDA has yet to approve taurine as an additive for fish feeds in the United States, and it does not appear on the FDA's Generally Recognized as Safe (GRAS) database.

The aim of the current study was to determine the leaching rates of taurine from several different experimental feeds used in trials with juvenile cobia *Rachycentron canadum* using a simple extraction protocol and novel LC-MS detection and quantification method. Taurine supplementation to low fishmeal and fishmeal-free feeds has been the key to the palatability, consumption, survival, and growth of juvenile cobia (Watson *et al.*, 2012). Due to the important physiological roles that taurine plays in marine carnivores (Kader *et al*., 2012; Schuller-Levis and Park, 2003), it is critical to ensure adequate quantities are being delivered with the feed and not leached too rapidly. Accordingly, we measured taurine pellet levels after immersion in distilled water and tank water for various

aquaculture feeds to obtain the first reported leaching rates for this highly water soluble dietary supplement.

Methods and Materials

Diet Preparation

All diets tested in the leaching study were used in two separate trials involving juvenile cobia, *Rachycentron canadum,* to be reported elsewhere. Dietary formulations and proximate compositions are presented in Table 2.1.1. For all diets, dry ingredients were ground using an air-swept pulverizer (Model 18H, Jacobsen, Minneapolis, MN) to a particle size of <200 μm. For four of the diets tested, all ingredients including oil were mixed prior to extrusion. Pellets were prepared with a twin-screw cooking extruder (DNDL-44, Buhler AG, Uzwil, Switzerland). The pellets were dried using a pulse bed drier (Buhler AG, Uzwil, Switzerland). Eight of the diets tested were top coated with the oil ingredient after extrusion. Final moisture levels were less than 10% for each diet. Diets were stored in plastic lined paper bags at room temperature, and were fed within six months of manufacture. Pellet sizes for all twelve diets tested in the leaching trial were 3-4 mm in diameter and proximate compositions of each diet were determined by New Jersey Feed Labs, Inc. (Trenton, NJ, USA).

Leaching Trials

Five grams of feed pellets (approximately 50 pellets) were placed into 200 ml of water in 250 ml beakers. Water was circulated by elevated magnetic stir bars at approximately 200 rpm (BellStir Multistir 4, Bellco Biotechnology, NJ, USA) to simulate

water movement within a tank environment and avoid grinding of the pellets. Three feed pellets were individually removed with a large bore transfer pipette from each beaker immediately upon starting each trial (0 min) and placed individually into pre-weighed test tubes. Three additional pellets were individually removed at each subsequent time point (1, 5, 10, 20, and 40 min). Excess water was removed from all pellet samples in test tubes; pellets were frozen and lyophilized prior to taurine extraction.

A total of 12 different diets were tested throughout the study. Eight extruded and top coated feeds with varying taurine levels based on dietary fishmeal inclusion level in D.I. water, and four extruded not top coated feeds with different taurine supplementation levels in both D.I. water and the artificial seawater (25 ppt) utilized at the Institute of Marine and Environmental Technology's Aquaculture Research Center (ARC), for a total of 16 leaching trials.

Taurine Extraction and Quantification

Following removal of bound water by lyophilization, taurine was extracted from individual pellets in 2 ml cold 70% ethanol using 20 min sonication in a 4°C cold-room. Extracts were then dried completely overnight at 70°C, re-suspended in 1 ml HPLC grade water, and filtered (GF/F) prior to LC-MS analysis (modified from Chaimbault *et al*. 2004). LC–MS was performed using an Agilent 1100 Series LCMSD system, comprising binary pump system, autosampler and diode array detector (DAD) with a micro high-pressure flow cell (6 mm pathlength, 1.7 ml volume), fraction collector and quadrupole mass spectrometer (G1956A SL) equipped with an electrospray ionization (ESI) interface. Extracted samples in H_2O were injected onto a PGC column, Hypercarb (100 X 3.0mm

i.d., particle size 5 µm) from Thermo Scientific subjected to a 0.2 ml min^{-1} isocratic elution for 12 min at 30°C using 10 mM ammonium acetate buffer adjusted to pH 9.3. The eluate from the column was subjected to MS analysis under the following spray chamber conditions: drying gas (N2) flow rate 12 L min^{-1}, pressure 35 psi, temperature 250 °C, fragmentor voltage 70 V, capillary voltage 4000 V. Isopropanol (0.01 ml min^{-1}) was added post-column via a T-connector to provide enhanced sensitivity for negative mode ionization for taurine (Chaimbault *et al*., 2004). Selected ion monitoring (SIM) was used for quantification (124 for taurine at RT = 3.64 ± 0.2 min. and 109 for hypotaurine at RT = 4.12 ± 0.1 min). By using a single MS detection mode, the limits of detection (signal-to-noise (S/N) ratio 3) were 10 µg L^{-1} for hypotaurine (Sigma-Aldrich Co., St. Louis, MO, USA) and taurine (Sigma-Aldrich Co., St. Louis, MO, USA). Calibration curves were performed between 0.1 and 10 mg L^{-1} (using fourteen standard calibration levels in triplicate: 0.1, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, and 10 mg L^{-1}). This concentration range was selected according to the sulfur amino acid content of the diets. Coefficients of correlation were found to be greater than 0.998 for hypotaurine and taurine using a second order polynomial model.

In order to the verify the LC-MS method, eight diet samples (including the EPP and REF 2 diets used in this leaching study) from a previous growth study were ground up (approximately 2 g), and three replicates of ~50 mg were extracted as described previously, with results being compared to taurine content determined by AOAC method 994.12 (New Jersey Feed Labs, Inc. Trenton, NJ, USA) on the same eight dietary samples. Further validation for the method was performed using a NIST standard reference material (SRM)

1849a (Infant/Adult Nutritional Formula) at three different quantities (50, 100, and 250 mg).

Statistics

All statistics were run using Aabel 3.0.6 (Gigawiz, OK, USA). Linear regression analysis was used to determine leaching rate for each trial and ANCOVA was used to assess the relationship between leaching rates between the two water types and between top coated or not top coated feeds.

Table 2.1.1. Simplified dietary component formulations and proximate compositions of the twelve diets used for leaching trials.

Ingredient (g Kg^{-1})	FM 1 (NONE)[3]	FM 1 (HIGH)[3]	FM 2 (MED)[3]	PP 1 (LOW)[3]	PP 1 (HIGH)[3]	PP 2 (MED)[3]	PP 3 (MED)[3]	PP 4 (MED)[3]	PP 5 (MED)[3]	PP 6 (MED)[3]	PP 7 (MED)[3]	PP 8 (MED)[3]
Menhaden Meal	345	345	455	0.0	0.0	229	180	134	274	235	184	0.0
Poultry Meal	118	118	75	0.0	0.0	38	30	23	45	38	30	0.0
Total Plant Ingredients	377	327	405	745	700	634	681	712	586	639	695	806
Taurine	0.0	50	15	5	50	15	15	15	15	15	15	15
Proximate Composition (%)												
Water[1]	2.15	2.43	4.41	1.73	2.13	4.87	4.77	4.53	4.74	4.78	5.19	6.56
Protein[1]	49.2	51.4	47.7	48.5	50.3	45.6	44.5	45.5	46.1	45.4	44.9	46.4
Fat[1]	12.28	12.66	8.02	11.73	11.91	9.92	10.12	9.99	9.54	9.85	9.90	7.92
Fiber[1]	0.54	0.53	1.64	1.09	1.21	1.19	1.32	1.30	1.42	1.27	1.48	2.39
Ash[1]	10.62	10.51	9.51	7.84	7.77	9.41	8.20	7.49	7.29	8.33	7.72	8.13
Carbohydrate[2]	27.36	24.9	28.72	30.84	28.81	29.01	31.09	31.19	30.91	30.37	30.81	28.6
Energy (MJ Kg^{-1})	19.58	19.64	18.65	20.23	19.91	18.97	19.22	19.29	19.19	19.19	19.29	19.04
Top-coated (Y or N)	N	N	Y	N	N	Y	Y	Y	Y	Y	Y	Y

[1] New Jersey Feeds Labs analysis.
[2] Calculated by difference (100-Water-Protein-Ash-Fat-Fiber).
[3] Value in parentheses is supplemental taurine level.

Results

Comparison of the LC-MS extraction and taurine quantification method for the pelleted diets to the AOAC method 994.12 (New Jersey Feed Labs, Inc. Trenton, NJ, USA) on the samples is presented in Figure 2.1.1. Essentially identical taurine levels are obtained (R^2 = 0.98) for both methods. Moreover, the taurine level using the LC-MS method for NIST SRM 1849a found 0.033 ± 0.007 percent (n=12), well within the certificate of analysis (certificate issue date: 8/7/2012) of 0.0366 ± 0.0018 percent by mass of taurine. It is important to note that new taurine and hypotaurine standards must be made fresh and standard curves run with each set of samples, as the standards gradually lose accuracy, presumably due to the loss of the sulfonate group over time.

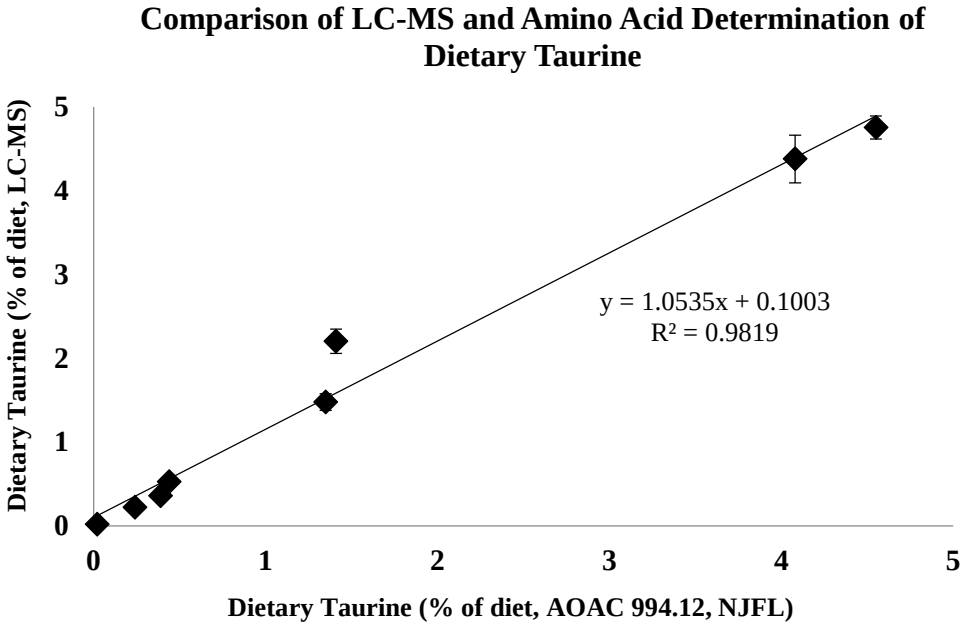

Figure 2.1.1. Linear regression comparing LC-MS method (% of diet) of taurine quantification to amino acid determination method AOAC 994.12 (% of diet, New Jersey Feed Labs, Inc.).

The results of the eight leaching trials conducted on the four non top coated feeds are shown in Table 2.1.2. Rates of taurine loss due to leaching ranged from 0.026 ± 0.005 to 0.051 ± 0.008 mg min^{-1} for the two diets with low or no taurine supplementation, PP 1 (LOW) and FM 1 (NONE). The diets with higher taurine supplementation, PP 1 (HIGH) and FM 1 (HIGH) had higher leaching rates ranging from 0.747 ± 0.072 to 0.826 ± 0.121 mg min^{-1}. For these eight trials, pellets placed into D.I. water resulted in a percent loss of taurine of 63.97 ± 23.7 compared to a percent loss in artificial seawater of 55.69 ± 5.6, with all but PP 1 (LOW) diet resulting in higher rate of leaching and total percent loss of taurine in D.I. water compared to artificial seawater.

Results of the eight leaching trials with top coated feeds are shown in Table 2.1.3. Although all diets were supplemented with 1.5 % taurine, varying levels of fishmeal and poultry meal in seven of the eight feeds resulted in different initial concentrations of taurine. Rates of taurine loss ranged from 0.111 ± 0.012 to 0.576 ± 0.090 mg min^{-1} with an average percent taurine loss of 59.13 ± 17.54 over 40 min. One of the diets, PP 4 (MED), completely dissolved by the 40 min time point, so all analyses were conducted from the 20 min time point. All other diets and trials maintained enough pellet integrity to remove whole, individual pellets at each time point.

Table 2.1.2. Taurine concentrations and leaching data for the four non top coated feeds.

Diet[1]	Water Type	Initial Taurine Content (mg g^{-1})	Rate of Taurine Loss (mg min^{-1})	$R^2(n)^2$
PP1 (LOW)	D.I.	2.38 ± 0.15	0.026 ± 0.005	0.82 (18)
PP1 (LOW)	ARC	3.01 ± 0.10	0.039 ± 0.003	0.90 (18)
FM 1 (NONE)	D.I.	2.33 ± 0.64	0.051 ± 0.008	0.73 (18)
FM 1 (NONE)	ARC	3.07 ± 0.66	0.039 ± 0.006	0.72 (18)
PP 1 (HIGH)	D.I.	46.50 ± 6.28	0.766 ± 0.081	0.85 (18)
PP 1 (HIGH)	ARC	47.69 ± 11.22	0.826 ± 0.121	0.74 (18)
FM 1 (HIGH)	D.I.	45.16 ± 6.89	0.747 ± 0.072	0.87 (18)
FM 1 (HIGH)	ARC	56.93 ± 3.75	0.751 ± 0.075	0.86 (18)

[1] Value in parentheses represents supplemental taurine level.
[2] R^2 value for linear regression of taurine content by immersion time, n (number of data points for each regression).

Table 2.1.3. Taurine concentrations and leaching data for the eight top coated feeds.

Diet[1]	Water Type	Initial Taurine Content (mg g^{-1})	Rate of Taurine Loss (mg min^{-1})	$R^2(n)^2$
FM 2 (MED)	D.I.	26.30 ± 1.98	0.576 ± 0.090	0.76 (18)
PP 2 (MED)	D.I.	20.77 ± 0.78	0.304 ± 0.034	0.83 (18)
PP 3 (MED)	D.I.	23.70 ± 3.07	0.231 ± 0.045	0.78 (18)
PP 4 (MED)[3]	D.I.	14.29 ± 0.91	0.221 ± 0.029	0.78 (15)
PP 5 (MED)	D.I.	20.38 ± 2.12	0.367 ± 0.059	0.83 (18)
PP 6 (MED)	D.I.	22.41 ± 2.12	0.474 ± 0.049	0.85 (18)
PP 7 (MED)	D.I.	10.99 ± 1.19	0.111 ± 0.012	0.85 (18)
PP 8 (MED)	D.I.	10.57 ± 0.65	0.167 ± 0.015	0.87 (18)

[1] Value in parentheses represents supplemental taurine level.
[2] R^2 value for linear regression of taurine content by immersion time, n (number of data points for each regression).
[3] All pellets were completely dissolved by 40min, analyses performed on 20 min values.

Leaching rate was strongly correlated to initial taurine content regardless of top coating ($F = 12.3$, $p = 0.013$) or not ($F = 214.8$, $p < 0.001$, Figure 2.1.2), or water type (D.I. $F = 102.08$, $p < 0.001$; Artificial Seawater, $F = 47.45$, $p = 0.020$, Figure 2.1.3). ANCOVA between D.I. water and artificial seawater leaching rates as a function of initial taurine

content showed no significant effect of water type (F = 0.56, p = 0.465). ANCOVA between the leaching rates resulting from top coated or not top coated feeds as a function of initial taurine content showed no significant effect of top coating or not (F = 0.11, p > 0.5). Overall, for all 16 leaching trials, leaching rate of taurine was linearly correlated to initial taurine content by the following equation (R^2=0.93):

Leaching rate = initial taurine content * (0.015732 ± 0.001) +

(-0.0054338 ± 0.0338);

Initial taurine concentrations for the non top coated EPP feeds gives estimates of the taurine lost during the mixing and manufacturing processes without top coating. Supplementation levels of 0.5 % of the diet resulted in initial taurine concentrations in the pellets of approximately 2.69 mg g^{-1}, indicating a loss of 2.31 mg g^{-1} of taurine during manufacturing. Supplementation levels of 5.0 % of the diet resulted in initial taurine concentrations in the pellets of approximately 47.09 mg g^{-1}, a loss of 2.91 mg g^{-1} of taurine during manufacturing. EPP3 from the top coated diet trials also gives an estimate of taurine lost during manufacturing with top coating since it is another fishmeal-free, all plant protein-based feed. Supplementation of taurine in this diet at 1.5% resulted in an initial taurine concentration of 10.57 mg g^{-1}, indicating a loss of 4.43 mg g^{-1} during the mixing and manufacturing of this diet.

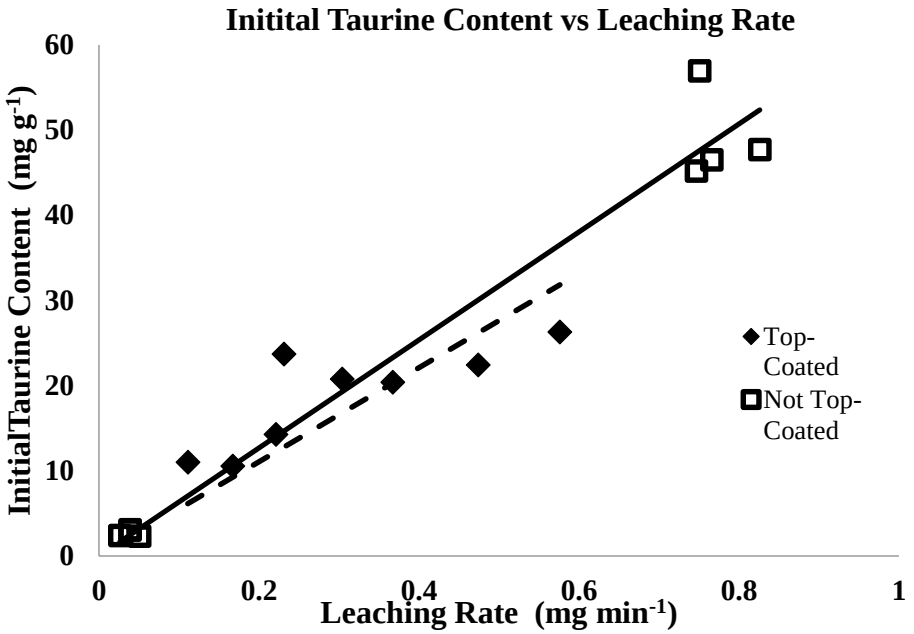

Figure 2.1.2. Initial taurine content (mg g^{-1}) as a function of leaching rate (mg min^{-1}) for all 16 leaching trials comparing the top coated feeds with not top coated feeds. Lines are linear regressions with the solid line corresponding to the not top-coated feeds (open squares) and the dashed line corresponding to the top-coated feeds (solid diamonds).

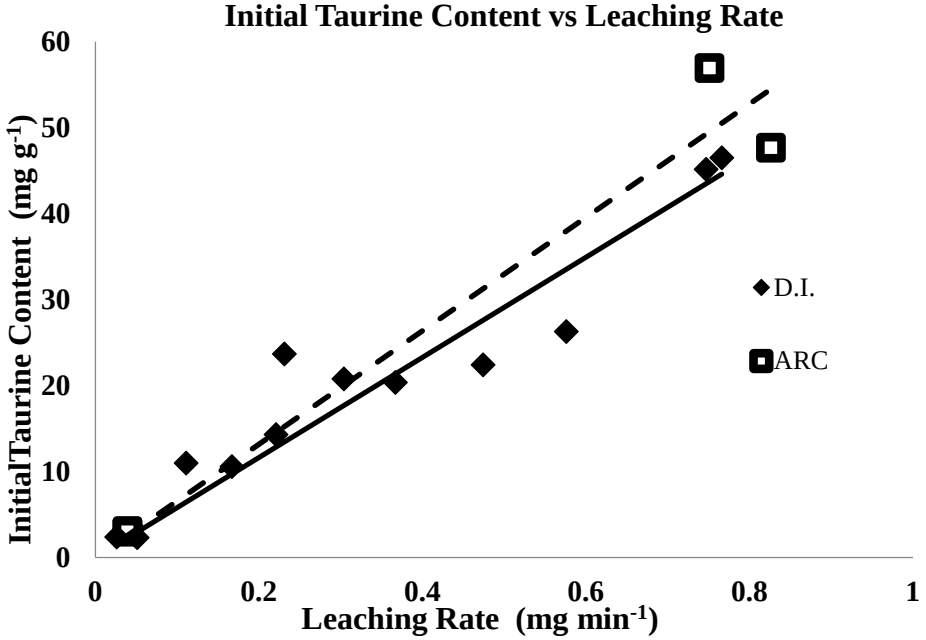

Figure 2.1.3. Initial taurine content (mg g^{-1}) as a function of leaching rate (mg min^{-1}) for all 16 leaching trials comparing the trials run in D.I. water compared to ARC water. Lines are linear regressions with the solid line corresponding to the trials run in D.I. water (solid diamonds) and the dashed line corresponding to trials run in ARC water (open squares).

Discussion

To our knowledge this is the first study to examine the leaching rates of taurine from pelleted fish feeds designed for juvenile grow-out phases. Among 12 different feeds, 16 feed trials, and 285 individually extracted pellet samples, this study found no difference in leaching rate between trials conducted in D.I. water (12) or artificial seawater (4), or between feeds that were top coated (8) during manufacturing and those that were not (8). There was a strong linear correlation between increasing initial taurine content and subsequent leaching rate across the 16 trials, which makes it possible to predict the amount of taurine remaining in feed pellets over time. This study also confirms the use of a simple extraction protocol and LC-MS method for detection and quantification of taurine from feed samples with 98 % similarity to AOAC method 994.12 performed by New Jersey Feed Labs, Inc and 98 % similarity to NIST standard material 1849a (Infant/Adult Nutritional Formula) performed with the LC-MS protocol in this study.

For all feeds, the average percent loss of taurine after 40 min immersion was 59.48 ± 16.49%, which is a much higher percent loss than Marchetti *et al.* (1999) found for crystalline vitamins leaching from pelleted or extruded feeds after 60 min, but is roughly similar to percent loss after 120 min. Yufera *et al.* (2002) found similar leaching rates after approximately 40 min for other free amino acids; alanine, glycine, and lysine from a gelatin microbound diet for larval fish. Kvåle *et al.* (2006) also found similar leaching rates of free serine from microparticulate diets.

Cobia are voracious feeders, often consuming full rations within seconds or minutes of feed being delivered to tanks, so significant loss of taurine or other soluble compounds due to leaching prior to consumption is usually not a concern with this species. However,

when we fed cobia a fishmeal-free, plant protein diet with no addition of taurine, the feed was poorly consumed (Watson *et al*., 2012). Tulli *et al.* (2007) found a significant increase in the length of time it took sea bass, *Dicentrarchus labrax,* to finish individual meals as the fishmeal content of feeds was reduced and the amount of vegetable protein in the feed was increased with diets not supplemented with taurine. Fish that are not as voracious feeders or consume their feed over longer periods of time may not be receiving the necessary amounts of taurine due to this leaching; however they may benefit from some initial leaching since taurine has been shown to act as a feed attractant (Martinez *et al.*, 2004; Qi *et al.*, 2012) and possible palatability enhancer due to its small, nitrogenous structure. We also noticed loss of taurine, from 5-47%, in the feeds tested between the mixing of ingredients through the completion of the pelleting process. These are all factors that should be considered when tailoring feeds for individual species or groups of species, to ensure leaching does not affect adequate nutrition, and in understanding potential effects that leaching may have on aquaculture systems and surrounding ecosystems.

Acknowledgments

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Taurine in prey items and fish eggs

Introduction

One of the most important aspects of designing species specific diet formulations for aquaculture is determining the minimum and optimal inclusion levels for every ingredient. The ability or inability to synthesize amino and fatty acids from precursor molecules can be used to determine when nutrients are considered essential or semi-essential. The protein and lipid content in the diet often varies between species, and the specific requirements for the different amino and fatty acids can vary substantial between species (Carter *et al*., 2003; Glencross *et al*., 2007; Sales, 2008; Zhou *et al*., 2007). All of these must meet minimum requirements for each species, but maximizing growth while reducing waste and redundancy of components that can be biosynthesized requires the specific knowledge of inclusion levels of many components, especially those that are or should be considered essential.

In an effort to better understand dietary requirements, researchers often analyze the prey items or eggs of a species. Understanding the natural diet of species targeted for aquaculture can give researchers a better idea of amino and fatty acid requirements. Analyzing wild spawned eggs of a species targeted for aquaculture reveals the maternally derived nutrition that eggs and non-feeding larvae begin with. This can be incredibly helpful in designing diets for larvae and juveniles as well as designing enrichment procedures for the live feeds fed to larvae in aquaculture. Another step that is often taken is to catch and analyze the tissues of wild individuals of the species of interest. This can be difficult however as dietary patterns can change throughout the year, or as with cobia, there

may not be a commercial fishery to acquire specimens from when they are needed. The analysis of a variety of prey items however, also gives a much better picture of what is being consumed, as opposed to just what is in the tissues. Any components that are routinely used and depleted, or chemically altered prior to deposition in tissues, will produce discrepancies if only the species of interest is analyzed.

Assessing potential prey items also has multiple applications for assessing wild populations and in fisheries management. Understanding what the nutritional sources in prey are and being able to assess prey populations can give researchers and fisheries managers better predictions of how certain year classes will perform and can give insight into how particular years may develop in terms of recruitment of juveniles or spawning production.

In collaboration with Dr. Jessica Miller's lab at Oregon State University and Adam Peer, an UMCES graduate student, we have endeavored to begin analyzing prey items from the Pacific Northwest and striped bass, *Morone saxatilis*, eggs from the Chesapeake Bay, respectively. Analysis of these potential prey items and eggs for taurine content will help determine the natural dietary intake of taurine of the animals consuming these prey items and eggs, and for the eggs it will also determine the amount of taurine that is maternally deposited for early growth and development.

Methods and Materials

Striped bass eggs were collected from the Roanoke and Patuxent River tributaries to the Chesapeake Bay as well as the Chesapeake Bay proper. The two spawning sites are different in temperature, salinity, and local prey sources for the adult fish. Eggs were

collected and stored in 70% EtOH in 20ml glass jars. Due to the eggs being stored in the same solvent used for extraction, preparation of samples was altered from the typical procedures described earlier in chapter 2.1. Six jars were selected from each treatment group and treated as individual replicates. Samples were left uncovered in a fume hood until all solvent had evaporated. Once dry, samples were homogenized and weighed prior to lyophilization. Once lyophilized samples were treated as described in chapter 2.1, with taurine quantified based on a dry weight of eggs.

Samples of potential prey items from the Pacific Northwest were collected by Dr. Jessica Miller's lab and shipped frozen to IMET. Upon arrival, three individuals of each species were individually weighed, homogenized and lyophilized prior to taurine extraction.

Results

Taurine content of the wild striped bass eggs are shown in Table 2.2.1. Taurine was significantly higher (t-test) in eggs from the Roanoke River tributary (51.96 ± 15.56 mg $100g^{-1}$) than from the main Chesapeake Bay (22.27 ± 2.43 mg $100g^{-1}$).

Table 2.2.1. Taurine content of wild striped bass eggs.

Sample Group	Taurine (mg $100g^{-1}$)	Number of samples (n)
KQF (Chesapeake)	22.27 ± 2.43	19
F (Roanoke)	51.96 ± 15.56	15

Taurine content of the prey items are shown in Table 2.2.2. Since taurine was measured from dry samples after lyophilization, yet these are consumed while alive and

containing water, the taurine content was also estimated with the assumption that when alive, these organisms would contain ~75% water.

Table 2.2.2. Taurine content (dry and wet weight estimate) of various prey items from the Pacific Northwest.

Prey Item	Dry Weight Taurine (mg $100g^{-1}$)	Wet Weight Estimate (75% water) Taurine (mg $100g^{-1}$)
Squid	5724.3 ± 306.6	1340.6 ± 79.4
Rockfish	1204.2 ± 69.6	301.0 ± 17.4
Osmeridae	120.7 ± 6.1	30.2 ± 1.5
C. magister	1428.3 ± 175.8	357.1 ± 44.0
Anchovy	721.1 ± 23.3	180.3 ± 5.8
Seabaste	702.4 ± 47.6	175.6 ± 11.9
Euphasid	950.0 ± 48.1	237.7 ± 12.0

Discussion

The taurine content of the various prey items examined ranged widely from 30.2 – 1340.6 mg $100g^{-1}$, as a wet weight estimate. Overall, the invertebrates tended to have higher taurine concentrations (237.7-1340.6) than vertebrates (30.2-301.0). Both of these ranges are similar to those obtained by Satake *et al*. (1988) for prey items collected in the Western Pacific Ocean near Japan. In that study invertebrates ranged from 166.9-657.4 mg $100g^{-1}$ and vertebrates ranged from 20.7-983.2 mg $100g^{-1}$.

All of the prey items measured in this study, with the exception of the Osmeridae, represent substantial taurine sources for juvenile fish relying on these prey items. The invertebrate and many of the vertebrate values also relate to substantially higher taurine values than those found in larger fish species, fish meal, and pelleted feeds. This could be due to invertebrates requiring more taurine themselves than vertebrates, or more likely, a higher synthesis capacity in the lower invertebrates resulting in higher concentrations in their tissues. Further work is continuing in examining taurine concentration in a wider

variety of prey items, and will hopefully extent to the larvae and juvenile fish species that utilizes these prey sources in an effort to determine dietary requirements.

Taurine content of striped bass eggs differed between the two spawning sites where they were collected. However, whether this is due to differences in local salinity (0.0 vs. 0.8 ppt), maternal deposition, or maternal nutrition prior to spawning is unclear. Analyses to determine if the differences in taurine in eggs between the two sites is also correlated with other potential differences in egg size, yolk quantity, etc. are underway as a portion of Adam Peer's research. Correlating the taurine content in eggs to subsequent growth and survival of the larvae would extend this research and reveal whether or not this maternally derived taurine significant enhances the probability of survival. As important as taurine has been shown to be, it is not often looked at as a component of eggs or larval nutrition as it may relate to growth and survival. Maternal nutrition is critical for proper growth and survival of larvae and juveniles, both in the wild and in aquaculture settings. As shown in chapter 2.1, taurine is often lacking in commercially prepared feeds, many of which are utilized for broodstock diets. Many aquaculture facilities also utilize more "natural" diets of fish and squid leading up to and during spawning season. However these sources may not be providing enough taurine during critical spawning periods if the broodstock have been receiving inadequate taurine levels the remainder of the year. As an example of this, Lanes *et al*. (2012) examined the biochemical composition and subsequent performance of batches of eggs from farmed and wild broodstock Atlantic cod, *Gadus morhua*. Wild broodtsock eggs contained significantly more taurine, among many components, and performed significantly better than their farm broodstock counterparts. The authors argue

that parameters such as these can be used, as discussed here, in determining minimum and optimal dietary requirements for larvae.

Chapter 3: Taurine Biosynthesis in Teleosts

Chapter 3 is a discussion of the synthetic capacity for taurine that a variety of teleost species possess to begin testing whether or not taurine is an essential amino acid. The first section of this chapter deals with several species raised in our facility and the development of a method (RT-qPCR) to measure transcript abundance of the genes in taurine synthesis. The second and third sections utilize RT-qPCR methods to measure the synthetic capacity for taurine in zebrafish, *Danio rerio*, and sablefish, *Anoplopoma fimbria*, respectively. The objective of this chapter in relation to the hypotheses of this dissertation is to determine which, if any, species may be capable of synthesizing sufficient taurine.

Taurine biosynthesis in species fed commercial aquaculture feeds

Initial attempts to assay the taurine biosynthetic capacity of marine species began with a variety of species reared in the Aquaculture Research Center (ARC) at IMET. This first approach was a "shotgun" approach attempting to design primers that would work on multiple species, under the assumption that many marine species would have closely related mRNA sequences for the genes of interest. Cysteine dioxygenase was not included initially due to its primary role in regulating cellular cysteine levels, discussed previously, as it would be expected to be expressed to some degree in all species. Alignments from available teleost species in the NCBI database were generated, and Primer3 (Rozen and Skaletsky, 2000) was used to design primers for RT-qPCR based on the consensus sequence established by the alignment.

All fish sampled for this trial were adult fish maintained in the recirculating systems in ARC at species specific temperatures and salinities. No dietary manipulation was undertaken prior to this sampling, with all fish maintained on various commercial feeds for marine fish, with varying levels of fishmeal and taurine. Brain and liver tissues were extracted from three individuals of each species: cobia, *Rachycentron canadum*, gilthead sea bream, *Sparus aurata*, European seabass, *Dicentrarchus labrax*, and striped bass, *Morone saxatilis*. Primers for CSD, ADO, TauT, and beta actin were generated (Table 3.1.1) and tested on each of the species to verify accuracy.

Table 3.1.1. Primers used for genes of interest in taurine metabolism designed from consensus teleost sequences.

Target Gene	Primer Sequence	Tm
Cysteamine dioxygenase (ADO) Forward	5'-AGTGGCCCTTGTGTTTTGAG-3'	55.8
Cysteamine dioxygenase (ADO) Reverse	5'-AGCTTCATCATCACCGCTCT-3'	56.3
Cysteinesulfinate decarboxylase (CSD) Forward	5'-GCACCACGGATGGCTATATT-3'	55.0
Cysteinesulfinate decarboxylase (CSD) Reverse	5'-AACAGCGTCTTGGGATTTTG-3'	53.7
Taurine transporter (TauT) Forward	5'-CAAGAACAAGGGGTGGACAT-3'	55.1
Taurine transporter (TauT) Reverse	5'-CCAGAAGCAGCAGCATGATA-3'	55.1
Beta Actin Forward	5'-GCTACAGCTTCACCACCACA-3'	57.5
Beta Actin Reverse	5'-CACCGATCCAGACGGAGTAT-3'	56.0

Approximately 50 mg samples of liver as well as whole brain samples were homogenized, total RNA was extracted using tri-reagent, and RNA was quantified on a Nanodrop nd1000 spectrophotometer (Thermo Scientific, Wilmington, DE). 1000 ng total RNA was used for reverse transcription using Superscript II Reverse Transcriptase (Invitrogen, Life Technologies, Grand Island, NY) and the resultant cDNA was diluted to 10 ng μl^{-1} for and quantitative RT-PCR (RT-qPCR) assays. RT-qPCR was performed in triplicate per sample and tissue type for each gene of interest (n=3 for all tissues) alongside the reference gene on an ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Life Technologies, Grand Island, NY). Cycling parameters for the assays were as follows: initial denaturation at 95 ºC for 2 min, followed by 40 cycles of denaturing (95 ºC for 10 s), annealing (56 ºC for 30 s), and extension (72 ºC for 30 s) with a melting curve to determine presence of spurious products. Expression relative to the expression of the beta actin reference gene was determined by the following equation:

$$\text{Relative expression of target gene} = \left(\frac{(\text{target gene PE})^{(35-\text{target gene avg. Ct})}}{(\text{beta actin PE})^{(35-\text{beta actin avg. Ct})}} \right) * 100$$

A Ct value of 35 was used as the cut-off to eliminate any weak products that may have been produced by contamination or fluorescence artifacts (Caraguel *et al*., 2011), and the primer efficiency (PE) was assumed to be 1.8 for these preliminary measurements. Results of the RT-qPCR measurements are shown in Figure 3.1.1. CSD was only detected in the brains of European seabass at minimal levels compared to beta actin. TauT was detected in both tissue types for all species as expected for diets containing taurine. ADO was also detected in both tissue types in all species, although at much lower levels than the transporter.

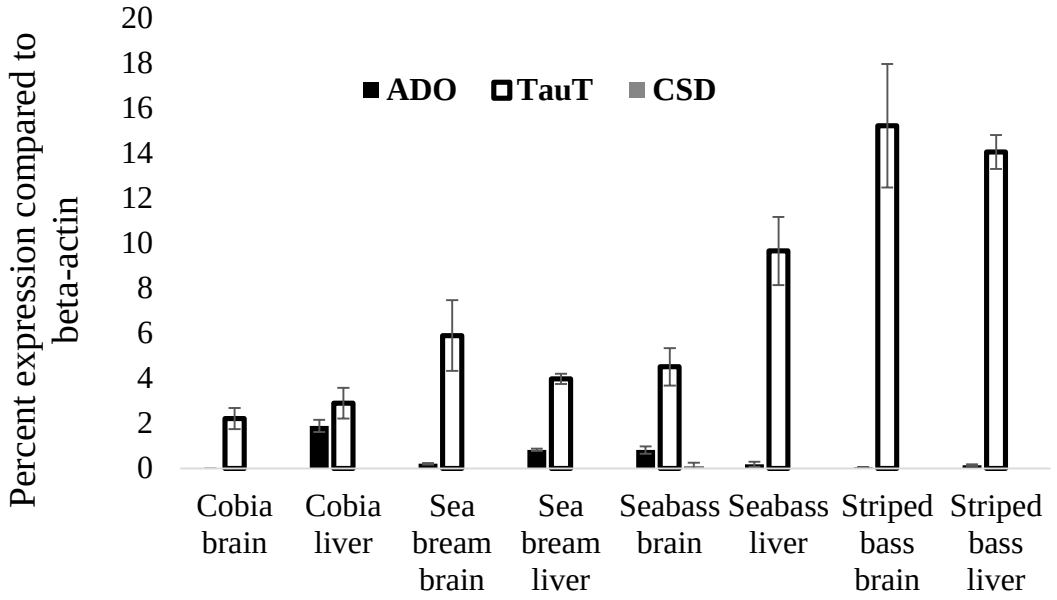

Figure 3.1.1. Relative transcript abundance of ADO, CSD, and TauT as a percentage of beta actin transcript level. Equivalent cDNA input (10 ng) for triplicate samples of each tissue with three fish sampled per tissue type.

This initial trial indicated that marine species express at least one of the genes, ADO, that can aid in taurine synthesis. These species also express appreciable levels of the taurine transporter, required for absorption, recycling, and transport of taurine into tissues

unable to synthesis taurine themselves. The anadromous striped bass has the highest transcript abundances for the transporter, possibly indicating a reliance on taurine as an osmolyte as this species moves between fresh and salt water. These preliminary measurements also indicate that CSD is minimally expressed, if it is detectable at all. Assuming this is directly correlated to protein abundance for these enzymes, this means these species are critically low in the rate-limiting step for taurine synthesis and may be unable to synthesize sufficient quantities. These results indicate the importance of taurine to these species in particular, and affirmed our ability to measure transcript expression levels of the genes responsible.

Zebrafish, Danio rerio, as a freshwater, omnivorous model species

Introduction

One of the main hypotheses of the work in this dissertation is that marine carnivores do not possess the ability to synthesize sufficient quantities of taurine to meet their physiological requirements. As a comparison to marine carnivores, we have used zebrafish, *Danio rerio,* as a freshwater omnivore example. The hypothesis for marine carnivores is that due to the high quantities of taurine in their prey, and the fact they rely on a fairly limited prey diversity, the need to synthesize taurine has been eliminated or diminished. The hypothesis for zebrafish on the other hand, is that due to the wide and varying nature of their diet, they have had to maintain the ability to synthesize sufficient quantities of taurine, since it may often be lacking in their diet. This hypothesis extends to all omnivores and herbivores, which are well represented among freshwater taxa. As mentioned previously, freshwater species have generally been the easiest to switch from fishmeal and meat based diets to plant-based feeds. This may potentially be due in part to the intact nature of synthesis pathways required by the varied and often deficient natural diets of these species, but it may also be due to a potentially higher ability to digest a wider range of ingredients due to the varied nature of their natural diets. This study was undertaken to examine the biosynthetic capacity for taurine in zebrafish and to assess the effects of feeding zebrafish diets containing no and high quantities of dietary taurine.

Methods

To examine the biosynthetic capacity of zebrafish for taurine, primers for RT-qPCR were developed from cDNA sequences of all four genes involved in taurine synthesis and

transport. These primers were designed and tested on a zebrafish liver cell line by Travonya Kenly, as a part of the LMRCSC summer intern program, working with Aaron Watson. The reference gene utilized for this study was a ribosomal protein, L13A, and primers for this gene were obtained from Tang *et al*. (2007). For sample acquisition for primers, adult zebrafish were sacrificed with an overdose of MS-222 and approximately 50 mg samples of liver tissue was homogenized, total RNA was extracted using tri-reagent, and RNA was quantified on a Nanodrop nd1000 spectrophotometer (Thermo Scientific, Wilmington, DE). 1000 ng total RNA was used for reverse transcription using Superscript II Reverse Transcriptase (Invitrogen, Life Technologies, Grand Island, NY) and the resultant cDNA was diluted to 10 ng μl^{-1} for PCR and RT-qPCR assays. Using the deposited and BLAST verified, sequences for cysteine dioxygenase (CDO), cysteinesulfinate decarboxylase (CSD), cysteamine dioxygenase (ADO), taurine transporter (TauT), and the reference gene L13A; primers for RT-qPCR (Table 3.2.1) were designed with the use of Primer3 (Rozen and Skaletsky, 2000). Verification that primers were indeed amplifying only a single product, and that the product was the desired target, end-point PCR was performed on limited samples in 10 µl reactions with 10 ng cDNA using Promega 2x Mastermix (Promega, Madison, WI) using the following parameters: initial denaturation (95 ºC for 2 min) followed by 35 cycles of denaturation (95 ºC for 30 s), annealing (56 ºC for 30 s), extension (72 ºC for 45 s), and a final polishing step (72 ºC for 5 min). Two µl of the end-point PCR products were used for gel electrophoresis to assess product size and number with the remaining 8 ul purified and sequenced to determine accuracy of selected primers.

Table 3.2.1. Primers used for RT-qPCR in this study. Designed and tested in a zebrafish liver cell line by Travonya Kenly.

Target Gene	Primer Sequence	Tm
Cysteamine dioxygenase (ADO) Forward	5'-TTACAGACTGCTGGGAAAAA-3'	51.8
Cysteamine dioxygenase (ADO) Reverse	5'-GGCTTGAAACAAGCAAATAA-3'	49.4
Cysteine dioxygenase (CDO) Forward	5'-GAACCTGATGGAGTCCTACC-3'	54.3
Cysteine dioxygenase (CDO) Reverse	5'-AACTTTCCGTTTCCTTCATC-3'	50.6
Cysteinesulfinate decarboxylase (CSD) Forward	5'-AGCTGAGATCTCTCCTGGAC-3'	55.5
Cysteinesulfinate decarboxylase (CSD) Reverse	5'-TGGTATTGAGGGTTTCAGTG-3'	52.1
Taurine transporter (TauT) Forward	5'-ATCACCTGTTGGGAGAAACT-3'	53.7
Taurine transporter (TauT) Reverse	5'-CAGGTAGTACAAGCCACAGG-3'	55.0
Ribosomal protein L13A (L13A) Forward[a]	5'-TCTGGACTGTAAGAGGTATGC-3'	57.6
Ribosomal protein L13A (L13A) Reverse[a]	5'-AGACGCACAATCTTGAGAGCAG-3'	57.3

[a] From (Tang *et al.*, 2007).

Upon selection of appropriate primer pairs for each gene of interest, primer efficiencies were determined with cDNA from zfl cells, a zebrafish fetal liver cell line (provided by Jerren Liu) and consisted of triplicate measurements made at five different dilutions of cDNA (40, 20, 10, 5, and 2.5 ng cDNA per reaction) based on the following equation:

$$\text{Primer efficiency (PE)} = 10^{-\frac{1}{\text{slope}}},$$

with slope determined from the plot of cDNA content (ng) vs. average Ct for each dilution. RT-qPCR was performed in triplicate per sample and tissue type for each gene of interest (n=5 for all tissues) alongside the reference gene on an ABI 7500 Fast Real-Time System (Applied Biosystems, Life Technologies, Grand Island, NY). Cycling parameters for the assays were as follows: initial denaturation at 95ºC for 2 min, followed by 40 cycles of denaturing (95 ºC for 10 s), annealing (56 ºC for 30 s), and extension (72 ºC for 30 s) with a melting curve to determine presence of spurious products. Expression relative to that of the L13A reference gene was determined by the following equation:

$$\text{Relative expression of target gene} = \left(\frac{(\text{target gene PE})^{(38\text{-target gene avg. Ct})}}{(\text{L13A PE})^{(38\text{-L13A avg. Ct})}} \right) * 100$$

A cut-off Ct of 38 was used to eliminate any weak products that could potentially result from contamination or fluorescence artifacts (Caraguel *et al*., 2011). Following the transcript measurements made by Travonya Kenly in zfl cells, which are maintained in medium containg taurine, we endeavored to determine if juvenile zebrafish can utilize this pathway to synthesize sufficient taurine.

For this phase of the study, roughly 20 juvenile zebrafish were placed into each of eight tanks in the zebrafish facility of ARC once they were fully weaned onto a dry feed. Four of these tanks were fed a plant-based flake diet with zero supplemented taurine with the other four tanks being fed the same diet but with a 5% supplementation of taurine. Formulations of these two diets can be seen in Table 3.2.2. These diets are identical in formulation to those used in later chapters, however they were produced as a flake version as opposed to the pelleted feeds produced for trials of the larger marine species. Zebrafish were fed to satiation twice daily for eight weeks prior to sampling. Three individual fish from each tank (n=12) were flash-frozen on dry ice, and whole livers and brains were extracted from each fish. Sample processing to acquire cDNA for RT-qPCR, and the RT-qPCR proceeded as previously described with primer efficiencies were determined on liver tissue as opposed to using efficiencies determined on the cell line due to the significant difference in sample types. Dietary and whole body taurine of zebrafish at the conclusion of the trial were also measured.

Table 3.2.2. Dietary formulations for the four plant-based diets with graded levels of taurine used in this study.

Ingredient (g kg^{-1})	PP1	PP4
Soy Protein Concentrate	269	269
Soy Protein Concentrate HP300	0.0	0.0
Corn Protein Concentrate	193.4	193.4
Wheat Flour	175.5	125.5
Soybean meal, solvent extracted	90	90
Wheat Gluten meal	22	22
Menhaden Fish Oil	120	120
Mono-Dical Phosphate	42.5	42.5
Vitamin Pre-mix[1]	20	20
Lecithin	20	20
L-Lysine	19.9	19.9
Choline CL	6	6
Potassium Chloride	5.6	5.6
DL-Methionine	5	5
Threonine	2.8	2.8
Sodium Chloride	2.8	2.8
Stay-C	2	2
Trace mineral pre-mix[2]	1	1
Magnesium Oxide	0.5	0.5
Mycozorb	2	2
Taurine	0	50

[1]Contributed per kg diet; vitamin A, 9650 IU; vitamin D, 6.6 IU; vitamin E, 132 IU; menadione sodium bisulfite, 4.7 mg; thiamine mononitrate, 9.1 mg; riboflavin, 9.6 mg; pyridoxine hydrochloride, 13.7 mg; pantothenate, DL-calcium, 101.1 mg; cyanocobalamine, 0.03 mg; nictonic acid, 21.8 mg; biotin, 0.33 mg; folic acid, 2.5 mg.
[2]Contributed in mg kg^{-1} of diet; zinc 37; manganese, 10; iodine, 5; copper, 1.

Results

Dietary taurine and whole body taurine of the zebrafish are shown in Table 3.2.3. There was a significant difference (t-test, $p<0.05$) between the whole body taurine contents of fish fed the PP1 diet compared to fish fed the PP4 diet, with higher dietary taurine resulting in higher whole body taurine.

Table 3.2.3. Dietary and whole body taurine concentrations from this study. Values with different superscripts within a column are significantly different ($p<0.05$).

Diet	Dietary Taurine (%)	Whole Body Taurine (%)
PP1	0.02 ± 0.001	1.37 ± 0.03
PP4	4.08 ± 0.21[a]	2.04 ± 0.28[a]

Initial transcript measurements made in the zebrafish liver cell line indicate the presence of transcripts for all four genes of interest (Figure 3.2.1). Not only are all four genes expressed at the transcript level, but they are at appreciable amounts in comparison to the reference gene transcript expression.

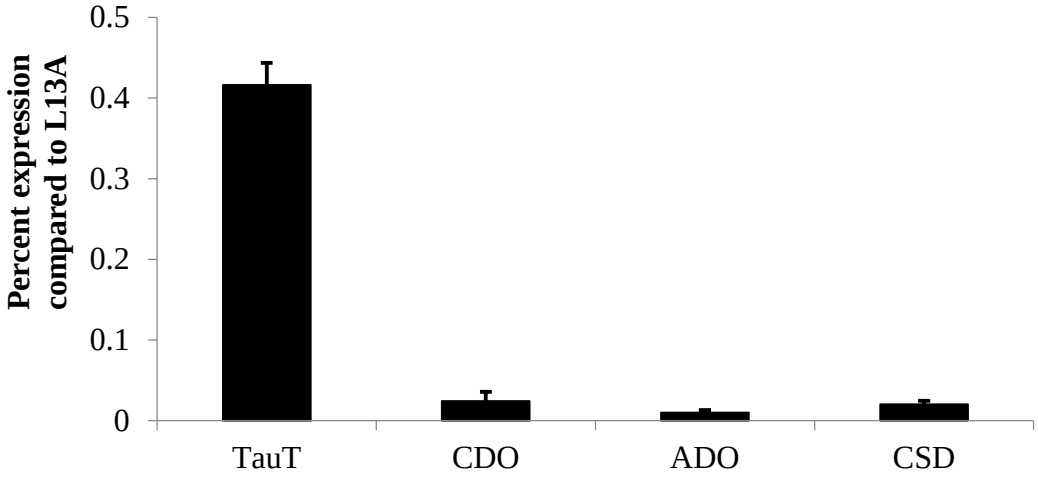

Figure 3.2.1. Transcript expression as a percentage of L13A transcript abundance in a zebrafish cell line. Values represent mean ± S.D. for three replicates with identical cDNA input (10ng) per gene. Measurements made by Travonya Kenly, LMRCSC summer intern 2012.

Survival and growth of the juvenile zebrafish fed diets PP1 and PP4 were equivalent, although due to multiple infections in the zebrafish facility unrelated to these tanks or dietary treatments, these specific data are unreliable. Expression data for the genes of interest from this trial in liver and brain of juvenile zebrafish is shown in Figure 3.2.2. There was no significant difference in the measured Ct values of the L13A reference gene between the two dietary treatments (Table 3.2.4, t-test, $p>0.05$). There were no significant differences (t-test, $p>0.05$) between the dietary treatments in brain or liver tissue for the transcript levels TauT. ADO was significantly up-regulated in both the livers and brains of fish fed the PP1 diet. CDO was up-regulated in the brains but not the livers of fish fed the PP1 diet. CSD was up-regulated in the livers of fish fed the PP1 diet compared to fish fed the PP4 diet.

Table 3.2.4. L13A reference gene Ct values (average ± S.D.) for liver and brain tissue from each treatment. No significant differences between treatments for each tissue type (t-test, $p>0.05$).

	PP1	PP4
Liver	23.70 ± 2.08	22.73 ± 2.39
Brain	25.20 ± 1.57	23.84 ± 1.70

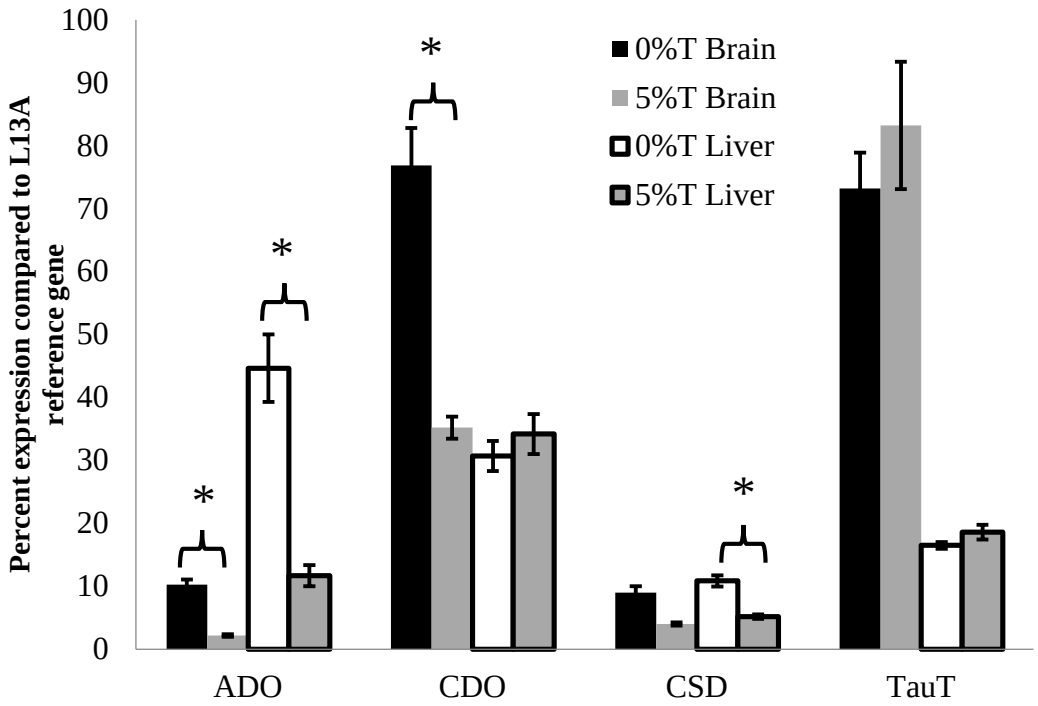

Figure 3.2.2. Relative transcript expression of cysteamine dioxygenase (ADO), cysteine dioxygenase (CDO), cysteinesulfinate decarboxylase/cysteine sulfinic acid decarboxylase (CSD/CSAD), and the taurine transporter (TauT) in the liver and brain of zebrafish, *Danio rerio* fed either a 0 % taurine or 5 % taurine supplemented flake diet for 6 weeks. Shown as percent expression (mean ± S.D.) compared to the reference gene L13A. Equivalent cDNA input (10ng) run in triplicate per sample with three individual fish sampled per tank with four tanks per dietary treatment (n=12 for each measurement).

Discussion

Zebrafish can clearly synthesize sufficient quantities of taurine to meet physiological requirements (Table 3.2.3). Despite being on a taurine free diet for 8 weeks, whole body taurine levels still average greater than 1 %. This is clear due to the presence and expression of all four genes required for synthesis in an isolated cell line, as well as the brains and livers of juveniles. Whole body taurine concentrations in fish fed the PP1 diet were substantially higher than that of the diet, although still significantly lower than the whole body concentrations of fish fed the PP4 diet. There is potential for some of this taurine to have been the result of the pool available prior to the switch to the no taurine

diet, however the lack of differences in appearance, growth, and survival between the treatments seems to indicate that the fish are not deficient in taurine. The flake feed used for these fish from the time of weaning until the start of these trials contains only 0.22 ± 0.07 % taurine, so the possibility of a large enough taurine pool to last for the entirety of the 8 week trial is unlikely.

Not only are zebrafish able to synthesize taurine but they are able to up-regulate expression of the genes involved in its synthesis and transport when dietary taurine levels are not adequate. Interestingly, the increase in transcripts, and assumedly the increase in synthesis, follows separate pathways in the brain compared to the liver in fish fed the PP1 diet. In the liver, CSD and ADO are both significantly increased, whereas CDO is not. In the brain however ADO is increased, CDO is increased at an even greater percentage, and CSD is not when compared to the PP4 fish. This is most likely due to other uses of cysteine and cysteamine, the precursors for the CDO-CSD and ADO pathways to taurine, respectively. Cysteamine has been shown to play multiple roles in signaling pathways in the brain of mammals (Figueiredo *et al*., 2009; Gibrat and Cicchetti, 2011; Rech *et al.*, 2008; Sun *et al*., 2010), so animals may actively avoid using this compound for other synthesis pathways, which could explain why cysteine appears to be the preferred substrate for taurine production in the brain.

Fish fed the PP1 diet exhibited similar growth and survival as those fed the PP4 diet and did not exhibit any signs or symptoms of taurine deficiency (Kim *et al*., 2005b; Maita *et al*., 1997; Takagi *et al.*, 2011). Whether all of the synthetic capacity, or its increase, is due to an increase in transcript abundance alone is not clear. Both CDO and CSD in mammals can be regulated post-transcriptionally, so increases at the mRNA level are not

often observed at significant levels. Although the same mechanisms have not been identified specifically in any teleosts, this may certainly also be the case, however the results here indicate that at least to some degree zebrafish rely on an increase in transcripts in order to increase synthesis.

Acknowledgements

I would like to thank Dr. Shaojun (Jim) Du and his lab for donating the zebrafish embryos and assisting in the rearing of the zebrafish for these trials, as well as Jerren Liu for providing the liver cell line samples. Travonya Kenly designed, verified, and conducted the cell line work for this study as a part of the LMRCSC summer internship program under the mentorship of Aaron Watson.

Sablefish, Anoplopoma fimbria, as a cold water carnivore model species

Introduction

Sablefish, *Anoplopoma fimbria,* is a slow growing marine carnivore native to the Pacific Northwest whose late maturity (~5 years) and relatively low fecundity (~200K-1000K eggs female^{-1} season^{-1}) puts them at risk for overfishing (King *et al*., 2001). Sablefish do however have long lifespans (55-100 years) so lifetime fecundity is quite high, although juvenile sablefish serve an important trophic function as food for several species of halibut (Trumble *et al*., 1993). Juvenile sablefish, prior to sexual maturity, display rapid growth rates and are found in shallow, warmer, prey-rich waters while adults are adapted to cold temperatures, low oxygen levels, and limited food availability and are often found at depths >200 m (Sullivan and Smith Jr., 1982). There is an economically important fishery off the west coast of Canada and the United States for sablefish, valued at ~$41 million with approximately ~8 million tons landed in 1998 in Canada (King *et al*., 2001) and 42.8 million pounds worth approximately $128.6 million in the United States. In Canada, individual vessel quotas have been put in place since 1990, and poor year-classes have been experienced from 1989-1997 indicating that the current and future outlook for the fishery is poor (King *et al*., 2001).

Due to the high demand for sablefish and marine protein sources in general, and the potential advantages to developing an aquaculture industry for sablefish, NOAA-Northwest Fisheries Science Center (NWFSC) researchers and others have undertaken a variety of studies aimed at improving all aspects of sablefish aquaculture (Hannah *et al*., 2013; Smith *et al*., 2013; Sumaila *et al*., 2007). From a dietary perspective, as with many species either anticipated or currently utilized for intensive large scale production, there

exists the desire to identify alternative protein sources for this species. Dr. Ron Johnson (NOAA-NWFSC) leads a NOAA funded project to determine to what extent fishmeal can be replaced for sablefish, utilizing taurine supplemented plant protein-based feeds. An initial trial was planned with a low fishmeal (~9 %) diet and approximately 1500 juvenile sablefish (~5 g) hatched at the NOAA Manchester Laboratory, Port Orchard, WA were transported to the indoor recirculation system (12.5 ºC) at the NOAA Montlake Laboratory, Seattle, WA in late August 2011. Fish growth in the first month after transport was slow and at ~10 g, fish were transitioned to a "zero" taurine conditioning feed (Table 3.3.1) for a twelve week pre-trial wash-out phase.

Table 3.3.1. Dietary formulation of "zero" taurine, plant-based feed utilized in juvenile sablefish growth study.

Ingredient	g kg^{-1}
Taurine	0
Soy protein concentrate	300
Corn protein concentrate	260
Wheat flour	155
Fishmeal, anchovy	90
Fish oil	127
Fish gelatin (cod)	20
Trace minerals	1
Vitamin premix	15
Vitamin C	1
Choline	5
Betaine	2.5
L-methionine	1.6
L-lysine	2
Dicalcium phosphate	20

At the conclusion of the pre-trial wash-out phase conducted at NOAA-NWFSC, attempting to wash-out the juveniles of any existing taurine pool prior to the initiation of a graded dietary taurine study, it was apparent the fish were not performing well. Low feed consumption, low growth, and a general unhealthy appearance were recorded in fish being fed the plant-based feed with no supplemental taurine when compared to con-specifics being reared on a standard, commercial trout diet. The full-scale trial on the plant-based diet with graded levels of taurine was subsequently postponed due to the effects observed during this pre-trial wash-out phase.

To explore whether a lack of taurine due to limited dietary input coupled with poor synthesis was the cause, as opposed to otherwise poor acceptance or performance of the feed itself, this study aimed to assist NOAA-NWFSC researchers by assessing the biosynthetic capacity for taurine in sablefish resulting from being fed the low fishmeal, un-supplemented diet in comparison to fish fed the commercial trout diet in an effort to help identify minimal and optimal dietary taurine supplementation levels.

Methods and Materials

Liver and muscle tissue samples (~100mg) along with whole brains were taken from five individuals from both the trout diet and plant protein-based diet. Samples were flash frozen in liquid nitrogen and stored at -80 ºC prior to shipping on dry ice to the Institute of Marine and Environmental Technology (IMET) in Baltimore, MD. Approximately 50 mg samples of liver and muscle, as well as whole brain samples were homogenized, total RNA was extracted using tri-reagent, and RNA was quantified on a Nanodrop nd1000 spectrophotometer (Thermo Scientific, Wilmington, DE). 1000 ng total

RNA was used for reverse transcription using Superscript II Reverse Transcriptase (Invitrogen, Life Technologies, Grand Island, NY) and the resultant cDNA was diluted to 10 ng μl^{-1} for PCR and RT-qPCR assays. As of this work, three of the four genes involved in the synthesis and transport of taurine had been identified and deposited into the Genbank database at NCBI along with a commonly used reference gene for RT-qPCR, L13A, a ribosomal protein encoding gene. Using these deposited and BLAST verified sequences for cysteine dioxygenase (CDO), cysteinesulfinate decarboxylase (CSD), taurine transporter (TauT), and the reference gene L13A for sablefish; primersfor RT-qPCR (Table 3.3.2) were designed with the use of Primer3 (Rozen and Skaletsky, 2000). Verification that primers were indeed amplifying only a single product, and that the product was the desired target, end-point PCR was performed on limited samples in 10 µl reactions with 10 ng cDNA using Promega 2x Mastermix (Promega, Madison, WI) using the following parameters: initial denaturation (95ºC for 2 min) followed by 35 cycles of denaturation (95 ºC for 30 s), annealing (56 ºC for 30 s), extension (72 ºC for 45 s), and a final polishing step (72 ºC for 5 min). Two µl of the end-point PCR products were used for gel electrophoresis to assess product size and number with the remaining 8 µl purified and sequenced to determine accuracy of selected primers.

Table 3.3.2. Primers used for RT-qPCR in this study.

Target Gene	Primer Sequence	Tm	PE
Cysteine dioxygenase (CDO) F	5´-AAGTCTTCGAGAGCGACAGC-3´	57.1	
Cysteine dioxygenase (CDO) R	5´-CAATGGCAGTCTGTGTGGTC-3´	56.3	1.74
Cysteinesulfinate decarboxylase (CSD) F	5´-CAGAGGGCCAACTCTTCTTG-3´	55.6	
Cysteinesulfinate decarboxylase (CSD) R	5´-TTGACGCTGTACTTCGCAAC-3´	55.8	1.74
Taurine transporter (TauT) F	5´-GGACACTCTGAAGCCGTCTC-3´	57.5	
Taurine transporter (TauT) R	5´-CCAGACGTTCCCTAAACCAA-3´	54.7	1.74
Ribosomal protein L13A (L13A) F	5´-ACAGCCACTCTGGAGGAGAA-3´	57.9	
Ribosomal protein L13A (L13A) R	5´-TTATTGGCCACAGACAACCA-3´	54.5	1.67

Upon selection of appropriate primer pairs for each gene of interest, primer efficiencies were determined with cDNA from a single liver tissue sample from a control, trout diet fed individual and consisted of triplicate measurements made at five different dilutions of cDNA (20, 10, 5, 2.5, and 1.25 ng cDNA per reaction) based on the following equation:

$$\text{Primer efficiency (PE)} = 10^{-\frac{1}{\text{slope}}},$$

with slope determined from the plot of cDNA content (ng) vs. average Ct for each dilution. RT-qPCR was performed in triplicate per sample and tissue type for each gene of interest (n=5 for all tissues) alongside the reference gene on an ABI 7500 Fast Real-Time RT-rPCR System (Applied Biosystems, Life Technologies, Grand Island, NY). Cycling parameters for the assays were as follows: initial denaturation at 95 ºC for 2 min, followed by 40 cycles of denaturing (95 ºC for 10 s), annealing (56 ºC for 30 s), and extension (72 ºC for 30 s) with a melting curve to determine presence of spurious products. Expression relative to the expression of the L13A reference gene was determined by the following equation:

$$\text{Relative expression of target gene} = \left(\frac{(\text{target gene PE})^{(38-\text{target gene avg. Ct})}}{(\text{L13A PE})^{(38-\text{L13A avg. Ct})}} \right) * 100$$

Paired t-tests (p=0.05) were used to assess the differences in relative expression of the target genes in tissues from fish fed the plant-based diet compared to fish fed the commercial trout diet. Taurine was also measured directly from the diets, livers, and muscle tissues of fish from both treatments using the LC-MS method described in Chapter 2.1. Water content was determined for liver and muscle tissues from five individual fish from each dietary treatment by lyophilization to constant weight.

Results

Results of the taurine measurements and water content are shown in Table 3.3.3. Livers from fish fed the commercial trout diet had significantly higher liver taurine concentrations (13.36 ± 1.46 μmol g^{-1}) and significantly lower liver water content (65.23 ± 1.20 %) than livers from fish fed the plant-based diet (6.36 ± 1.26 μmol g^{-1} liver taurine and 73.26 ± 1.37 % liver water content; t-test, $p<0.05$). There were no significant differences in muscle taurine or water content between the two dietary treatments.

Table 3.3.3. Diet, liver, and muscle taurine and water content. Mean ± S.D. of five sampled individuals for liver and muscle tissues. Values with different superscripts (liver or muscle) indicate significant difference within the tissue type (t-test, $p<0.05$).

Diet	Tissue	Taurine Content	Water Content (% dw)
Trout diet	Diet	0.46 (g $100g^{-1)}$	~7%
Plant-based diet	Diet	0.16 (g $100g^{-1)}$	~1.4%
Trout diet	Liver	13.36 ± 1.46 (μmol g^{-1})[a]	65.23 ± 1.20[a]
Plant-based diet	Liver	6.36 ± 1.26 (μmol g^{-1})	73.26 ± 1.37
Trout diet	Muscle	4.32 ± 1.58 (μmol g^{-1})	72.79 ± 4.33
Plant-based diet	Muscle	3.32 ± 0.80 (μmol g^{-1})	76.62 ± 1.02

Results of the RT-qPCR assays are displayed as mean percent expression (± s.e.) compared to the mean expression level of the L13A reference gene (Figure 3.3.1) for the five individuals measured in triplicate per tissue and treatment. There were no significant differences in L13A Ct values between treatment types within each tissue type (Table 3.3.4, t-test, $p>0.05$). There were no significant differences in relative expression of CDO, CSD, or TauT in the brains or muscles of fish fed either diet (t-test, $p>0.05$). There were significant differences in the relative expression of all three genes in the livers however, with fish fed the plant-based diet consistently displaying higher transcript expression than that of fish fed the trout diet (Figure 3.3.1; t-test, $p<0.05$). CDO had the highest transcript

expression difference between the two diets with fish fed the plant diet displaying transcript levels ~281% times higher than that of L13A while fish fed the trout diet had transcript levels ~ 41% of L13A expression. The taurine transporter (TauT) had transcript levels ~219% of L13A expression in fish fed the plant diet, while fish fed the trout diet had TauT transcript levels ~83% of L13A levels. CSD, the rate limiting step of the synthesis pathway from cysteine to hypotaurine and taurine had transcript levels that were 58% of that of L13A in fish fed the plant diet compared to transcript levels that were ~28% of L13A in fish fed the trout diet.

Table 3.3.4. L13A reference gene Ct values (average ± S.D.) for each dietary and tissue type. No significant difference within each tissue type between dietary treatments (t-test, $p>0.05$).

	Trout Diet	Zero Taurine Diet
Liver	25.13 ± 1.59	25.04 ± 2.25
Brain	26.06 ± 4.28	24.43 ± 4.79
Muscle	24.64 ± 2.25	25.15 ± 3.69

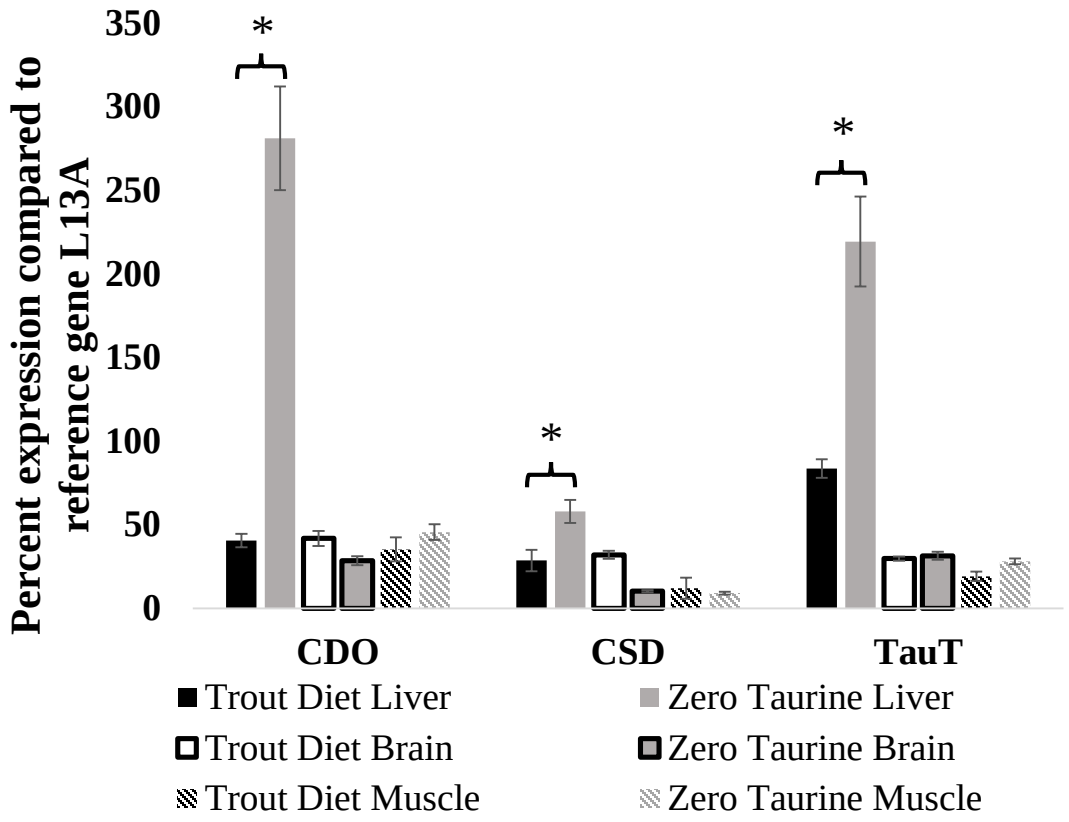

Figure 3.3.1. Relative transcript expression of genes involved in taurine synthesis and transport in juvenile sablefish, *Anoplopoma fimbria,* as a percent expression compared to reference gene L13A expression. Equivalent cDNA input (10 ng) for triplicate samples of each tissue and five fish sampled per treatment group and tissue (n=5 per data point).

Discussion

The RT-qPCR results of this study indicate that sablefish have an ability to respond to insufficient dietary taurine and increase the transcripts of genes involved in taurine synthesis. The major site of synthesis in juvenile sablefish is clearly the liver (Figure 3.3.1), and an increase in the transporter for taurine (TauT) was also observed in this tissue for fish fed the plant-based diet. Although transcript levels were higher in the livers of sablefish fed the plant-based diet in this study, their poor performance on the diet indicates that there may still not be sufficient taurine synthesis to meet physiological needs. Taurine has been

shown to play multiple important physiological roles in teleosts (Schuller-Levis and Park, 2003), although dietary requirements are often poorly understood even in species that have established aquaculture industries (Goto *et al*., 2001b).

The plant-based diet used in this study still maintained a 9% fishmeal inclusion, although this clearly did not provide the needed quantities of taurine. Subsequent trials with juvenile sablefish at NOAA-NWFSC have been conducted with a shortened wash-out period that resulted in much better acceptance and performance on the zero taurine added plant-based diet, although transcript levels from that study have yet to be analyzed. It was concluded by NOAA-NWFSC researchers that the initial trial's wash-out period was too long, and that the fish subsequently fed the low taurine plant-based diets had had their taurine pool reduced too significantly to recover from, regardless of the switch to higher taurine diets or the ability to potentially synthesize sufficient taurine.

Overall it is clear that unlike cobia, *Rachycentron canadum* discussed in subsequent chapters, sablefish have an ability to respond to low dietary taurine, although it may not be a sufficient enough response to actually result in an adequate increase in taurine availability. There are several potential explanations for this difference between these two marine carnivores. Cobia are a much faster growing species than sablefish even though they are at fairly similar positions in their respective food webs and with similar diets consisting of smaller fish, crabs, and squid. This could lead to cobia depleting their taurine pools much faster than sablefish, subsequently resulting in higher taurine requirements that have been evolutionarily satisfied by their diets, leading to a loss of synthesis capacity that was not fatal. Another, and more plausible explanation may be the change in life history strategy that sablefish undergo as they mature into adults and move to colder, deeper waters

that are much more scarce in food availability than the shallower, warmer waters that juveniles inhabit (King *et al*., 2001). This life history change could result in adults maintaining the ability to synthesize taurine due to low prey availability and being adapted to long periods of starvation.

In future studies of plant-based diets utilizing graded levels of taurine supplementation and low or no fishmeal, methods like the ones employed in this study will be utilized to help determine minimum and optimal dietary taurine requirements. Finding a balance in fast growth, low feed conversion ratio, and minimal need to expend energy in the expression of taurine synthesis genes will aid the development of optimal diets for sablefish aquaculture.

Chapter 4: Utilizing Taurine Supplementation with Plant Protein Sources to Replace Fishmeal

Chapter 4 combines methods developed in the previous two chapters to develop a fishmeal free, plant protein-based diet for cobia, *Rachycentron canadum*. The first two sections of this chapter examine the utilization of graded levels of taurine in a traditional, fishmeal-based formulation and the utilization of high inclusion levels of non-GM soybean strains in reduced fishmeal formulations. The third section discusses the digestibility of plant proteins and the development of the first fishmeal free, all plant protein-based diet. Finally, the fourth section of the chapter discusses two additional fishmeal free, plant-based formulations with graded levels of taurine inclusion. This chapter provides evidence that completely replacing fishmeal with plant proteins is not only a possibility, but can result in improved production in cobia as long as taurine is supplemented.

Effects of graded taurine on juvenile cobia, Rachycentron canadum, growth

Abstract

Taurine has been shown to have multiple important physiological roles in teleosts and mammals and is an amino acid not found in many of the alternative protein sources that are not derived from animals. Although taurine is found in fishmeal-based feeds, its high water solubility leads to lower taurine levels in reduction process based feeds than marine carnivores such as cobia, *Rachycentron canadum*, are adapted to in their natural diets. Graded taurine supplementation (0, 0.5, 1.5, and 5.0 %) to a traditional fishmeal-based formulation was examined in two trials, one initiated with 10g individuals and the second initiated with 120 g individuals. Growth during the first trial ranged from 1,313 % to 1,514 % with increasing dietary taurine along with a decrease in feed conversion ratio from 1.04 to 0.99. During the second trial, growth ranged from 220 % to 243 % with fish fed the diet with 1.5 % supplement of taurine having the best growth, although there were no significant differences in these performance characteristics during either trial between dietary treatments. Transcript levels for two of the genes involved in taurine synthesis, CDO and ADO, as well as the membrane bound transporter of taurine, TauT, did not vary in response to dietary taurine levels. Increasing dietary taurine resulted in increased production characteristics and significantly increased fillet, liver, and plasma taurine levels.

Introduction

Taurine is a free amino acid found in high concentrations in the natural prey of many marine species that are cultured (Satake *et al.*, 1988). However, the high water solubility of taurine can lead to losses during processing of fishmeal and other by-product meals used in feed formulation (Kousoulaki *et al*., 2009). Unlike lysine, threonine and methionine, which are supplemented in diets to balance amino acid requirements; taurine is often not supplemented in dietary formulations, and it is not yet approved as an additive for fish feeds in the United States. However, multiple important physiological roles for taurine in fish have been identified such as its strong antioxidant properties, conjugation to bile salts, photoreceptor protection, cardiac function, and as an intracellular osmolyte in many species, including humans (Schuller-Levis and Park, 2003). Taurine supplementation to diets for a variety of species have been conducted, with the general conclusion that increased levels of taurine result in increased growth, improved feed conversion, and normal behavior when compared to no or low taurine control diets (Kim *et al*., 2005a, 2005b; Park *et al.*, 2002; Takagi *et al.*, 2008).

Cobia, *Rachycentron canadum*, is a fast growing marine carnivore that is highly fecund, amenable to intensive tank, pond and cage culture, is naturally highly disease resistant, and found tropically and sub-tropically around the world except the Eastern Pacific (Holt *et al*. 2007). These traits along with a lack of major commercial fisheries and their high quality flesh make them an ideal candidate for further marine aquaculture development, especially in recirculating systems.

The objective of the current study was to examine the effects of graded levels of supplemented taurine (0.0, 0.5, 1.5, and 5.0 %) to a defined commercial-like fishmeal-

based diet to assess the effects of increasing taurine levels on cobia growth performance. Our hypothesis is that marine carnivores, such as cobia, have either lost or have a greatly diminished capacity for the biosynthesis of taurine due to high concentrations in their natural prey (Satake *et al.,* 1988). Therefore, increasing levels of taurine in the diet should result in improved growth and feed conversion ratios. Also, expression of genes potentially involved in the taurine biosynthesis were measured to assess the results of dietary taurine modulation on their expression. Cysteine dioxygenase (CDO), cysteamine dioxygenase (ADO), and the taurine transporter (TauT) are all involved in either the synthesis or transport of taurine in vertebrates. CDO has been shown in mammals to be regulated post-transcriptionally (Dominy *et al.,* 2006), but little work has been done assessing the transcript regulation, if any, in this pathway in teleosts under varying dietary taurine inputs. Increases in transporter transcripts may also be expected after prolonged periods of feeding sub-optimal taurine levels if this species maintains the ability to regulate transport at the transcript level. ADO has been shown to be important in reducing cysteamine in the mammalian brain, where it can inhibit multiple pathways (Figueiredo *et al.*, 2009; Jiang *et al*., 2004; Rech *et al.*, 2008; Sun *et al*., 2010), which if allowed to reach high levels could become detrimental or toxic. The terminal step in taurine biosynthesis catalyzed by cysteinesulfinate decarboxylase (CSAD), has proved to be difficult to amplify in cobia and will be the subject of a future manuscript.

Methods

Diet preparation

Formulations of the experimental diets are shown in Table 4.1.1 with amino acid profiles and proximate compositions of the diets shown in Tables 4.2.2 and 4.2.3, respectively. For all diets, ingredients were ground using an air-swept pulverizer (Model 18H, Jacobsen, Minneapolis, MN) to a particle size of <200 μm. All ingredients were mixed prior to extrusion. Pellets were prepared with a twin-screw cooking extruder (DNDL-44, Buhler AG, Uzwil, Switzerland) with an 18 second exposure to 127 °C in the extruder barrel. Pressure at the diet head was approximately 26 bar, and a die head temperature of 71 °C was used. The pellets were dried for approximately 15 min to a final exit air temperature of 102 ºC using a pulse bed drier (Buhler AG, Uzwil, Switzerland) followed by a 30 min cooling period to product temperature less than 25 °C. Final moisture levels were less than 10 % for each diet. Diets were stored in plastic lined paper bags at room temperature, and were fed within six months of manufacture. Portions of each diet were analyzed by New Jersey Feed Labs, Inc. (Trenton, NJ) for proximate and amino acid composition.

Experimental fish and systems

This study was carried out in accordance with the guidelines of the International Animal Care and Use Committee of the University of Maryland Medical School (IACUC protocol # 0610015). Approximately 500 juvenile (~2 g) cobia, *Rachycentron canadum*, were obtained from the Virginia Agricultural Experiment Station, Virginia Tech, Hampton, VA. Juveniles were housed at the Institute of Marine and Environmental Technology's

Aquaculture Research Center, Baltimore, MD. Fish were fed the FM2 diet until they reached an average weight of ~10 g at which point 18 fish were stocked into each of 12 identical tanks and randomly assigned one of the four experimental diets for three replicate tanks per dietary treatment.

Table 4.1.1. Dietary formulations for the diets with graded levels of taurine used in this study.

Ingredient (g kg^{-1})	FM1	FM2	FM3	FM4
Menhaden Fish Meal	345	345	345	345
Corn Protein concentrate	44.3	44.3	44.3	44.3
Poultry by-product meal	118	118	118	118
Wheat Flour	242.7	237.7	227.7	192.7
Soybean meal, solvent extracted	90	90	90	90
Blood meal, spray dehydrated	39	39	39	39
Menhaden Fish Oil	90	90	90	90
Vitamin Pre-mix[1]	20	20	20	20
Choline CL	6	6	6	6
Stay-C	2	2	2	2
Trace mineral pre-mix[2]	1	1	1	1
Mycozorb	2	2	2	2
Taurine	0	5	15	50

[1] Contributed per kg diet; vitamin A, 9650 IU; vitamin D, 6.6 IU; vitamin E, 132 IU; menadione sodium bisulfite, 4.7 mg; thiamine mononitrate, 9.1 mg; riboflavin, 9.6 mg; pyridoxine hydrochloride, 13.7 mg; pantothenate, DL-calcium, 101.1 mg; cyanocobalamine, 0.03 mg; nictonic acid, 21.8 mg; biotin, 0.33 mg; folic acid, 2.5 mg.
[2] Contributed in mg kg^{-1} of diet; zinc 37; manganese, 10; iodine, 5; copper, 1.

The first trial was conducted for 8 weeks, with tank weights recorded and feeding rates adjusted weekly to 5 % body weight (bw) day^{-1}. At the conclusion of the first trial, fish being fed diets FM1 and FM2 as well as those unused in the first trial were re-pooled, fed the FM2 diet for one week, and subsequently restocked at 5 fish per tank at

approximately 120 g average weight for a second trial. The second trial was also conducted for 8 weeks, with tank weights recorded weekly and feeding rates adjusted from 3.5 % bw day^{-1} to 2.5 % bw day^{-1}, with a bi-weekly 0.25 % bw day^{-1} reduction throughout the trial.

Table 4.1.2. Amino acid profiles of the diets used in this study.

Amino Acid[1]	FM1	FM2	FM3	FM4
Methionine	1.06	1.01	1.05	1.03
Cysteine	0.50	0.49	0.50	0.48
Lysine	2.98	3.21	3.27	3.10
Phenylalanine	2.13	2.16	2.16	2.10
Leucine	3.82	3.94	4.00	3.85
Isoleucine	1.31	1.45	1.52	1.39
Threonine	1.59	1.67	1.65	1.69
Valine	1.87	2.05	2.11	2.00
Histidine	1.28	1.43	1.66	1.31
Arginine	2.72	2.85	2.94	2.85
Glycine	2.80	2.87	2.94	2.94
Aspartic Acid	4.04	4.03	4.24	4.19
Serine	1.76	1.69	1.81	1.93
Glutamic Acid	7.26	7.30	7.27	7.10
Proline	3.00	3.31	3.28	3.23
Hydroxyproline	0.50	0.54	0.58	0.54
Alanine	2.70	2.71	2.78	2.76
Tyrosine	1.34	1.43	1.44	1.42
Taurine	0.24	0.44	1.41	4.55

[1] New Jersey Feed Labs analysis.

Six, 340-liter tanks connected to bubble-bead and biological filtration as well as protein skimmers constituted the recirculating systems used with four replicate systems occupied simultaneously during the trials with a photoperiod maintained at 14L:10D throughout. There were no significant differences in water quality parameters between the four systems used in either trial. During the first trial water quality parameters (mean ± SD)

were: temperature, 26.58 ± 1.06 °C; salinity, 25.55 ± 1.40 ppt; pH, 8.15 ± 0.19; total ammonia nitrogen, 0.07 ± 0.11 mg l^{-1}; nitrite, 0.15 ± 0.20 mg l^{-1}; nitrate, 24.95 ± 11.32 mg l^{-1}; and alkalinity, 121.31 ± 19.75 mEq l^{-1}. During the second trial mean ± SD water quality parameters were: temperature, 25.72 ± 1.15 °C; salinity, 24.97 ± 2.44 ppt; pH, 7.98 ± 0.17; total ammonia nitrogen, 0.23 ± 0.12 mg l^{-1}; nitrite, 0.46 ± 0.36 mg l^{-1}; nitrate, 29.83 ± 4.41 mg l^{-1}; and alkalinity, 116.34 ± 22.65 mEq l^{-1}.

Table 4.1.3. Proximate compositions and measured taurine values of the diets used in this study.

Proximate Composition[1]	FM1	FM2	FM3	FM4
Protein (% DM)	49.2	48.9	49.5	51.4
Lipid (% DM)	12.28	12.11	12.29	12.66
Fiber (% DM)	0.54	0.61	0.42	0.53
Carbohydrate, (% DM by difference)	27.36	28.01	27.24	24.9
Moisture (%)	2.15	2.76	3.38	2.43
Ash (% DM)	10.62	10.37	10.55	10.51
Taurine (%)	0.24	0.44	1.41	4.55

[1] New Jersey Feed Labs analysis.

Analytical procedures

At the conclusion of the first trial two individual fish from each tank were sacrificed for analysis. Bile was removed aseptically directly from the gall bladder using a tuberculin syringe with a 27 gauge x 1/2" needle. The bile volume was measured and diluted 1:1 (v/v) with methanol, centrifuged to remove biliary proteins, and kept frozen at -20°C until HPLC analysis. The sodium salts of glycocholic acid (GCA), glycochenodeoxycholic acid (GCDA), glycodeoxycholic acid (GDCA), taurocholic acid (TCA), taurochenodeoxycholate (TCDC), and tauro-deoxycholic acid (TDCA) were supplied by

SIGMA (St. Louis MO, USA). Water was purified with an EASY System and a NANOpure® Diamond Life Science (UV/UF) ultrapure water system from Barnstead/Thermoline (Dubuque, IA, USA). Methanol was HPLC grade from Burdick and Jackson (Honeywell, Morristown, NJ, USA). All other chemicals were of analytical grade. LC-MS was performed using an Agilent 1100 Series LC-MSD system, consisting of a binary pump system, autosampler and diode array detector (DAD) with a micro high-pressure flow cell (6 mm path length, 1.7 µl volume), fraction collector, and a quadrupole mass spectrometer (G1956A SL) equipped with an electrospray ionization (ESI) interface. The HPLC method was performed under isocratic conditions at room temperature. Analyses were performed on a reversed-phase C-18 column: LiChrosorb RP-18, 5 µm, 250 x 4.6 mm from HiChrom (Novato, CA, USA). Acetate buffer was prepared daily with 0.5 M sodium acetate, adjusted to pH 4.3 with o-phosphoric acid, and filtered through a 0.22 µm filter (Whatman R, England). The flow rate was 1.0 ml min^{-1} and the detection was performed at 205 nm. The injection loop was set to 20 µl. The eluate from the DAD was split (1/3 to 1/6) using a graduated micro-splitter valve (Upchurch Scientific). The major portion of the eluate was collected in multiple fractions while the remaining portion was subjected to MS analysis under the following spray chamber conditions: drying gas (N2) flow rate 10 l min^{-1}, pressure 60 psi, temperature 350 °C, fragmentor voltage 350 V, capillary voltage 4000 V. A 5 mM ammonium acetate solution in water (0.1 ml min^{-1}) was added post-column via a T-connector to provide higher pH conditions for enhanced negative mode ionization. Total ion chromatograms for ions in the mass range from 300 to 600 were collected. Total bile salts were assayed with 3 α-hydroxysteroid dehydrogenase (Coleman *et al*. 1979). Blood samples were taken from the caudal vein with heparinized

needles, plasma was separated by centrifugation (16,000 RCF for 20 min) and total plasma protein was quantified after 1:600 dilution utilizing a Micro BCA™ Protein Assay Kit (Product# 23235, Thermo Scientific, Rockford, IL).

At the conclusion of the second trial two fish from each tank, six per dietary treatment, were randomly selected for sampling. Fish were anesthetized with tricaine methanosulfonate (MS-222, 70 mg l^{-1}, Finquel, Redmond, WA), blood samples were taken from the caudal vein with heparinized needles, after which fish were euthanized with MS-222 (150 mg l^{-1}) and gall bladders removed with bile analyzed as in Trial 1. Liver and fillet samples were also taken. Blood plasma was separated by centrifugation (16,000 RCF for 20 min) and plasma osmolality measured in triplicate (10 µl) on a Vapro™ Model 5520 vapor pressure osmometer (Wescor, Logan, UT). Plasma samples from three fish per dietary treatment were sent to the Pathology and Laboratory Medicine Services department at the University of California at Los Angeles for constituent analysis. Remaining plasma, fillet and liver samples were frozen and stored at -80 °C and portions of each were lyophilized to constant weight for water and taurine content analyses. Triplicate samples each of liver (~10 mg), fillet (~50 mg), plasma (~10 µL), and diet (~50 mg) sample were used for taurine extractions based on Chaimbault *et al.* (2004), with samples being homogenized in cold 70 % EtOH, sonicated for 20 min, dried, and re-suspended in 1 ml H_2O prior to injection into the LC-MS. Taurine was quantified in all samples based on the methods described in Chapter 2.

Production characteristics were determined as follows:

$$\text{Weight gain (\%)} = 100 * \left(\frac{\text{final wieght (g)} - \text{initial weight(g)}}{\text{initial wieght (g)}}\right)$$

$$\text{Feed conversion ratio (FCR)} = \left(\frac{\text{food fed (g)}}{\text{weight gained (g)}}\right)$$

$$\text{Specific growth rate (SGR)} = 100 * \left(\frac{\ln \text{final weight (g)} - \ln \text{initial weight(g)}}{\text{days of trial}}\right)$$

$$\text{Protein efficiency ratio (PER)} = \left(\frac{\text{weight gained (g)}}{\text{protein fed (g)}}\right)$$

$$\text{Hepatosomatic index (HSI)} = 100 * \left(\frac{\text{liver weight (g)}}{\text{body weight (g)}}\right)$$

Primers for RT-qPCR (Table 4.1.4) were designed for each of the genes of interest; CDO, ADO, and TauT based on consensus sequences of alignments derived from teleost species in the NCBI Genbank database. Liver, brain, and muscle tissue from each fish sampled (6 per dietary treatment) were used for q-PCR assays. Primer3 was used for primer design (Rozen and Skaletsky, 2000). To verify that primers were indeed amplifying only a single product, and that the product was the desired target, end-point PCR was performed on limited samples in 10 µl reactions with 10 ng cDNA using Promega 2x Mastermix (Promega, Madison, WI) using the following parameters: initial denaturation (95ºC for 2 min) followed by 35 cycles of denaturation (95 ºC for 30 s), annealing (56 ºC for 30 s), extension (72 ºC for 45 s), and a final polishing step (72 ºC for 5 min). Two µl of the end-point PCR products were used for gel electrophoresis to assess product size and number of products with the remaining 8 ul purified and sequenced to determine accuracy of selected primers.

Upon selection of appropriate primer pairs for each gene of interest, primer efficiencies were determined with cDNA from a single liver tissue sample and consisted of triplicate measurements made at five different dilutions of cDNA (20, 10, 5, 2.5, and 1.25 ng cDNA per reaction) based on the following equation:

$$\text{Primer efficiency (PE)} = 10^{-\frac{1}{\text{slope}}},$$

with slope determined from the plot of cDNA content (ng) vs. average Ct for each dilution.

Table 4.1.4. Primers used for genes of interest in taurine metabolism.

Gene Name	Primer Sequence	Tm
Cysteamine dioxygenase (ADO) Forward	5'-AGACCTCGCTCATCCAGAAA-3'	55.7
Cysteamine dioxygenase (ADO) Reverse	5'-AGGGGAGGATGTGGAGACTT-3'	57.5
Cysteine dioxygenase (CDO) Forward	5'-AGGGTCAGCTGAAGGAGACA-3'	57.9
Cysteine dioxygenase (CDO) Reverse	5'-GCACCCTCTGTGTGGCTATT-3'	57.4
Taurine transporter (TauT) Forward	5'-GCTTCATGGCACAAGAACAA-3'	54.0
Taurine transporter (TauT) Reverse	5'-TCAACAAACTGGCTGTCGAG-3'	55.3
Beta Actin Forward	5'-TGCGTGACATCAAGGAGAAG-3'	54.9
Beta Actin Reverse	5'-AGGAAGGAAGGCTGGAAGAG-3'	56.4

RT-qPCR was performed in triplicate per sample and tissue type for each gene of interest (n = 6 for all tissues) alongside the reference gene on an ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Life Technologies, Grand Island, NY). Cycling parameters for the assays were as follows: initial denaturation at 95 ºC for 2 min, followed by 35 cycles of denaturing (95 ºC for 10 s), annealing (56 ºC for 30 s), and extension (72 ºC for 30 s) with a melting curve to determine presence of spurious products. A cut-off Ct value of 38 was used to eliminate weak products potentially resulting from contamination or fluorescence artifacts (Caraguel *et al.*, 2011) and samples run in triplicate with subsequent Ct values that had deviations >0.8 were re-analyzed. Expression of target genes

relative to expression of beta actin, reference gene, was determined by the following equation:

$$\text{Relative expression of target gene} = \left(\frac{(\text{target gene PE})^{(38\text{-target gene avg. Ct})}}{(\text{beta actin PE})^{(38\text{-beta actin avg. Ct})}} \right) * 100$$

Statistics

All statistical tests were run using Aabel v.3.0.6 (Gigawiz Ltd., OK, USA) with significance values of $p < 0.05$. ANOVA with Tukey's HSD post-hoc analyses were used to determine differences between dietary treatments. Q-PCR expression data were square root transformed prior to homogeneity of variance (Bartlett's test) and ANOVA analyses. A Mann-Whitney U test was used when expression values failed homogeneity of variance.

Results

Weight gain during the first trial ranged from 1,313 % to 1,514 % with a gradual, but not significant increase as dietary taurine increased (10g initial weight). Feed conversion ratio, FCR, was approximately 1 for fish fed each of the four diets. Specific growth rate (SGR) ranged from 4.72 to 4.97, again increasing with the increase in dietary taurine, although not significantly (Table 4.1.5). There were no significant differences in total plasma protein (ANOVA, $F=0.18$, $P>0.5$) or total bile salts (ANOVA, $F=2.46$, $P=0.092$) among the fish in dietary treatments during the first trial.

During the second trial there were no significant differences in weight gain, FCR, SGR, or total bile salt concentrations among the fish (120g initial weight) in the dietary treatments (Table 4.1.6, ANOVA, $P>0.05$). Consistent with other analyses for carnivorous

fish bile (Une *et al*., 1991), cobia bile salts were predominantly taurine conjugates of the primary bile salts cholic (133 ± 39 mM) and chenodeoxycholic acids (97.1 ± 52 mM). No secondary bile salts (*e.g.* deoxycholic acid) or glycine conjugates were observed. Small quantities (3.58 ± 1.8 mM) of N-cholyl-D-cysteinolic acid were detected as had been observed by Une *et al.* (1991). No differences in bile salt composition or concentration were observed in fish fed the different diets. There were several trends within each parameter with FM2 and FM3 slightly outperforming FM1 and FM4. Weight gain, mean ± SD, was highest for cobia fed FM2 (242.35 ± 6.91 %) and FM3 (243.03 ± 28.24 %) compared to fish fed FM1 (220.48 ± 20.49 %) and FM 4 (227.93 ± 15.78 %). FCR and SGR had similar trends with cobia fed FM2 and FM3 having slightly lower FCR's (1.42, 1.43) and slightly higher SGR's (2.20, 2.19) than cobia fed FM1 (FCR 1.52, SGR 2.07) and FM4 (FCR 1.51, SGR 2.12).

Table 4.1.5. Performance characteristics of cobia in the first trial (9.12 g initial weight). Within a row, means with different letters are significantly different (P<0.05).

Diet (Taurine %)	FM1	FM2	FM3	FM4
Survival (%)	100 ± 0.00	100 ± 0.00	98.03 ± 1.97	98.03 ± 1.97
Weight Gain (%)	1313.31 ± 95.24	1467.23 ± 64.11	1514.73 ± 105.16	1514.98 ± 55.33
FCR	1.04 ± 0.02	0.97 ± 0.02	0.97 ± 0.01	0.99 ± 0.03
SGR	4.72 ± 0.12	4.91 ± 0.07	4.96 ± 0.12	4.97 ± 0.06
PER	2.03 ± 0.01 z	2.04 ± 0.01 z	2.02 ± 0.01 z	1.95 ± 0.01 y
TPP[1] (g dL^{-1})	3.41 ± 0.23	3.52 ± 0.16	3.38 ± 0.17	3.33 ± 0.08
Bile Salts (mM)	43.48 ± 0.62	42.44 ± 0.11	42.44 ± 0.16	42.29 ± 0.10

[1] Total plasma protein.

There were several significant differences observed in the second trial with regards to fillet and liver characteristics. Feeding FM4 resulted in significantly higher fillet taurine content (65.58 ± 19.69 µmol g^{-1}), higher liver water content (50.37 ± 0.77 %), and higher liver taurine content (20.81 ± 2.89 µmol g^{-1}) than FM1 (Table 4.1.7, ANOVA, P<0.05)

with tissue taurine increasing with dietary taurine (Figure 4.1.1). There were no significant differences between the diets in fillet water content, fillet yield, fillet lipid, or hepatosomatic index (Table 4.1.7, ANOVA, $P>0.05$).

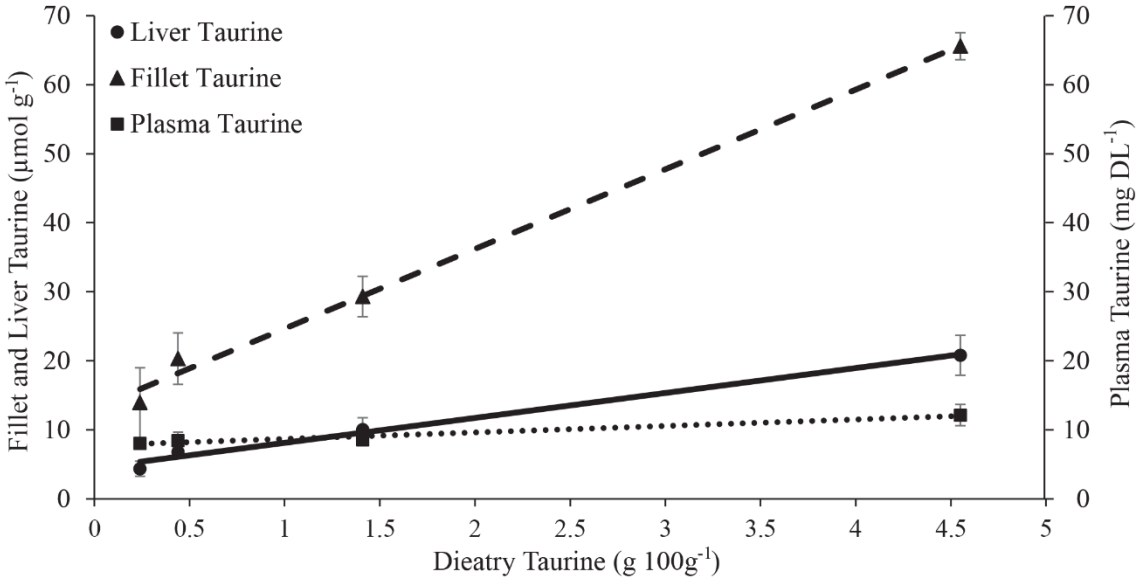

Figure 4.1.1. Liver (µmol g^{-1}), fillet (µmol g^{-1}), and plasma taurine (mg DL^{-1}) increase with increasing dietary taurine (g $100g^{-1}$).

Table 4.1.6. Performance characteristics of cobia in the second trial (127 g initial weight). Within a row, means with different letters are significantly different ($P<0.05$).

Diet (Taurine %)	FM1	FM2	FM3	FM4
Survival (%)	100	100	100	100
Weight Gain (%)	220.48 ± 20.49	242.35 ± 6.91	243.03 ± 28.24	227.93 ± 15.78
FCR	1.52 ± 0.10	1.42 ± 0.02	1.43 ± 0.11	1.51 ± 0.05
SGR	2.07 ± 0.12	2.20 ± 0.04	2.19 ± 0.14	2.12 ± 0.09
PER	1.35 ± 0.15	1.44 ± 0.04	1.26 ± 0.43	1.29 ± 0.07
HSI	3.14 ± 0.14	2.99 ± 0.18	2.78 ± 0.45	2.72 ± 0.19
Total Bile Salts (mM)	28.48 ± 4.79	32.69 ± 6.04	30.02 ± 4.34	29.59 ± 2.32

Table 4.1.7. Fillet and liver characteristics from the second trial. Values represent the mean ± standard error for six fish per dietary treatment. Within a row, means with different letters are significantly different (P<0.05).

Diet (Taurine %)	FM1	FM2	FM3	FM4
Fillet Water Content (%)	73.73 ± 0.23	72.79 ± 0.45	72.74 ± 0.23	73.09 ± 0.36
Fillet Taurine (μmol g^{-1})	13.94 ± 5.07 z	20.31 ± 3.73 z	29.32 ± 3.02 zy	65.58 ± 19.69 y
Fillet Yield (%)[1]	25.75 ± 0.83	26.63 ± 1.22	27.22 ± 1.63	27.07 ± 1.19
Fillet Lipid (% dw)	19.07 ± 0.33	19.32 ± 1.07	19.21 ± 1.46	17.73 ± 2.14
Liver Water Content (%)	42.32 ± 0.58 z	45.08 ± 0.87 z	43.96 ± 1.20 z	50.37 ± 0.77 y
Liver Taurine (μmol g^{-1})	4.36 ± 1.11 z	6.79 ± 0.87 z	10.02 ± 1.74 z	20.81 ± 2.89 y
Hepatosomatic Index	3.13 ± 0.06	2.98 ± 0.07	2.79 ± 0.18	2.71 ± 0.08

[1]Fillet yield = fillet weight (g)*100/body weight(g).

Analysis of plasma (Table 4.1.8) from fish in the second trial revealed a significant difference in plasma taurine among the fish (ANOVA, F=0.26, P=0.018), with fish fed FM4 (969.13 ± 120.96 nmol ml^{-1}) having significantly higher plasma taurine than fish fed FM1 (641.59 ± 56.86 nmol ml^{-1}). Significant differences were also observed in many other constituents including plasma cholesterol (ANOVA, F=7.15, P=0.012), glucose (ANOVA, F=5.87, P=0.02), magnesium (ANOVA, F=4.32, P=0.043), sodium (ANOVA, F=7.75, P=0.009), and potassium (ANOVA, F=5.23, P=0.027). Trends within these parameters appear to either be related directly or inversely to dietary taurine level, or to the overall production characteristic with fish fed the FM3 and FM2 diets having improved production characteristics than fish fed FM4.

At the conclusion of the first trial, there were no significant differences in final fish weight or bile salt concentration between the dietary treatments (ANOVA, F=2.46, P=0.092), with an overall mean weight ± SD of 140.70 ± 6.72 g and bile salt concentration ± SD of 42.66 ± 0.55 mM. The concentration of bile slats was lower for the larger fish at the conclusion of the second trial to 30.20 ± 1.79 mM when the overall average of fish was

421.78 ± 16.25 g, with no significant differences in weight or bile salt concentration among the dietary treatments (ANOVA, F=1.09, P=0.373).

Results of the quantitative PCR (q-PCR) analyses of liver, brain, and muscle tissues of fish from the second trial are shown in Figure 4.1.2A-C, respectively. High variance among measured expression values was due to high inter-animal variability (biological variability), not high variability in q-PCR measurements between triplicates (technical variability). There were no significant differences in expression among dietary treatments for CDO and TauT in any of the three tissues (ANOVA, P>0.05), and there was no significant difference in expression of ADO in the liver or muscle tissues among dietary treatments (ANOVA, P>0.05). Only ADO expression in the brain failed homogeneity of variance analysis between the dietary treatments and was analyzed with the non-parametric Mann-Whitney U test, which found no significance among the treatments (P=0.063).

Table 4.1.8. Plasma analysis from fish from the second trial. Values represent the mean ± SD for three fish per dietary treatment. Within a row, means with different letters are significantly different ($P<0.05$).

Diet (Taurine %)	FM1	FM2	FM3	FM4
Water Content (%)	94.49 ± 0.13	94.42 ± 0.11	94.48 ± 0.06	93.96 ± 0.05
Osmolality (Osm L^{-1})	314.50 ± 23.03	336.33 ± 4.54	337.33 ± 2.78	341.17 ± 9.08
Taurine (nmol ml^{-1})	641.59 ± 56.86 z	684.42 ± 97.57 z	675.91 ± 19.80 z	969.13 ± 120.96 y
Albumin (g dL^{-1})[1]	0.90 ± 0.00	0.87 ± 0.03	0.87 ± 0.03	0.96 ± 0.03
Total Bilirubin (mg dL^{-1})[1]	0.30 ± 0.00	0.30 ± 0.06	0.37 ± 0.03	0.27 ± 0.03
Calcium (mg dL^{-1})[1]	11.56 ± 0.22	11.00 ± 0.23	10.97 ± 0.09	11.30 ± 0.21
Cholesterol (mg dL^{-1})[1]	88.33 ± 2.91 z	92.00 ± 2.64 z	94.00 ± 0.58 zy	104.00 ± 3.06 y
Creatine Kinase (U L^{-1})[1]	510.33 ± 92.89	177.00 ± 67.08	304.00 ± 97.39	279.33 ± 28.75
Creatinine (mg dL^{-1})[1]	0.23 ± 0.03	0.20 ± 0.00	0.20 ± 0.00	0.20 ± 0.00
Glucose (mg dL^{-1})[1]	49.33 ± 1.45 z	46.67 ± 1.33 zy	43.00 ± 0.58 y	45.67 ± 0.67 zy
Phosphorous (mg dL^{-1})[1]	10.13 ± 0.22	9.13 ± 0.23	9.70 ± 0.31	9.50 ± 0.45
Magnesium (mg dL^{-1})[1]	2.10 ± 0.06 zy	1.90 ± 0.10 z	2.30 ± 0.06 y	2.27 ± 0.12 zy
Triglycerides (mg dL^{-1})[1]	143.00 ± 11.59	121.67 ± 15.39	147.67 ± 17.02	134.67 ± 11.39
Sodium (mmol L^{-1})[1]	176.43 ± 0.74 z	174.00 ± 0.17 zy	172.17 ± 0.95 y	176.90 ± 1.01 y
Potassium (mmol L^{-1})[1]	10.11 ± 0.18 z	9.23 ± 0.19 zy	10.09 ± 0.67 z	8.28 ± 0.23 y
Chloride (mmol L^{-1})[1]	169.83 ± 1.29	168.03 ± 0.62	165.10 ± 1.49	169.03 ± 3.07

[1] UCLA DLAM analysis.

Discussion

Overall, growth and feed conversion of the cobia were excellent when fed any of the four diets during both trials. Although not statistically significant, there were improvement trends in all production characteristics with taurine supplemented diets when compared to FM1, a diet containing 0.24 % taurine from the fishmeal component. However, significant increases were observed in fillet taurine content as dietary taurine increased. This represents a potential added human health benefit for taurine supplemented feeds. A consumer would be ingesting higher levels of a semi-essential nutrient similar to the benefits of n3 and n6 polyunsaturated fatty acids that marine fish are known to provide (Herold and Kinsella, 1986).

Work with plasma constituents in marine fish has focused on levels of sodium, potassium, osmolarity (Nordlie, 2009), glucose, and stress responses. Normal versus abnormal ranges for many species have not been established for many other components such as cholesterol, creatinine, albumin, *etc*. It is our hope that plasma values measured in this study will help in establishing normal ranges for some of the constituents measured, as fish in this study performed very well on all four diets and overall were healthy individuals.

Besides the significant increase in plasma taurine as dietary taurine increased, cholesterol also increased significantly as dietary taurine increased, which may be related to the role of taurine as a bile salt conjugate. This is different from what is seen in mammals however, where increasing plasma taurine is correlated with decreasing cholesterol levels (Militante and Lombardini, 2004). Plasma glucose, sodium, and potassium were significantly lower in fish with higher growth rates, which may be due to increasing taurine

transport utilizing sodium and chloride as chloride levels were also lower in high performers, although not significantly. Magnesium was significantly higher in FM3 than FM2, and was the only component with significant differences that did not relate to dietary taurine or production characteristics, so although significant, all values may fall within normal ranges. The lack of significant differences in total bile salts, of which taurine is a main component in teleosts, and no significant decrease in growth and production characteristics indicates that at a minimum, the 0.0 % addition of taurine, which contained ~0.24 % taurine from other dietary components, met requirements during the length of this study. The slight increases in production with increasing dietary taurine however, indicate that some supplementation may be beneficial for optimal growth and performance.

Cobia juveniles do not appear to regulate CDO, ADO, or TauT transcript levels in response to dietary input of taurine above that provided by fishmeal (~0.24 %) as no significant differences in the transcript levels for these three genes were observed in liver, brain, or muscle. There were no significant differences in the levels of beta actin expression in any of the three tissues examined between dietary treatments (Table 4.1.9, ANOVA, $p>0.05$).

Table 4.1.9. Reference gene beta actin Ct values (average ± S.D.). No significant differences between dietary treatments within each tissue type (ANOVA, $p>0.05$).

	FM1	FM2	FM3	FM4
Liver	24.66 ± 1.32	25.26 ± 3.82	23.35 ± 4.71	23.76 ± 5.27
Muscle	11.19 ± 1.01	12.03 ± 0.93	12.48 ± 0.80	13.56 ± 2.32
Brain	16.36 ± 1.61	16.58 ± 6.33	17.18 ± 0.36	18.89 ± 3.52

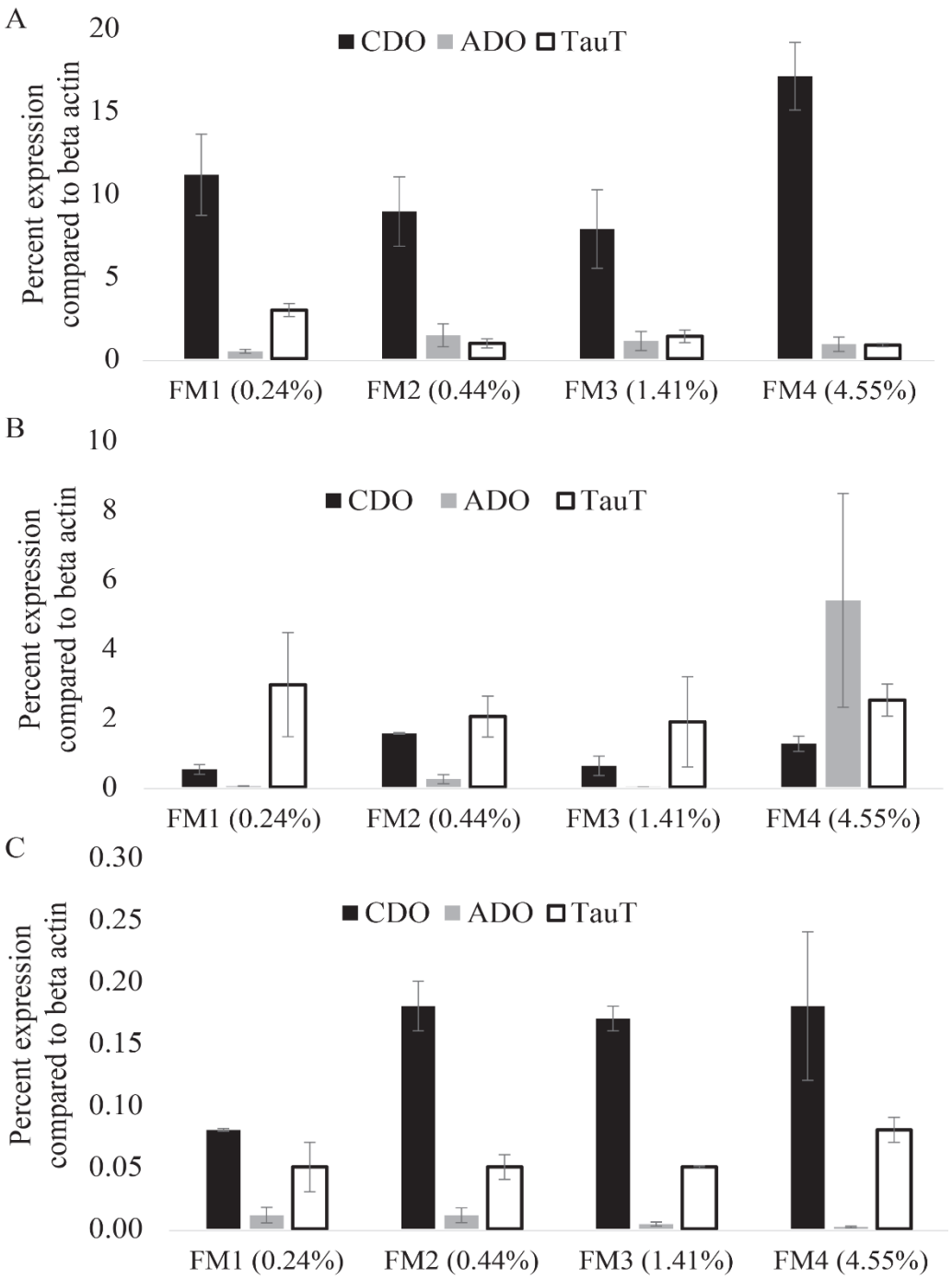

Figure 4.1.2. Relative transcript expression of genes involved in taurine synthesis and transport in juvenile cobia, *Rachycentron canadum*, as a percent expression compared to reference gene beta actin for each diet (% taurine) in liver (A), brain (B), and muscle (C). Equivalent cDNA input (10 ng) for triplicate samples of each tissue and six fish sampled per treatment group and tissue (n = 6 per data point). Numbers in parenthesis indicate measured taurine in each diet.

Although CDO has been shown to be regulated post transcriptionally in mammals (Dominy *et al.,* 2006), TauT has been shown to be regulated at the transcript level as well as the protein level based on taurine availability in mammals and zebrafish embryos (Bitoun and Tappaz, 2000; Han and Chesney, 2003; Kozlowski *et al.*, 2008). Expression levels in comparison to beta actin were also found to be very low, with the highest expression of any of the genes of interest in any tissues being ~15 % in the liver for CDO, where the majority of synthesis would be expected. CDO is also responsible for reducing high cysteine levels and has two resultant pathways, only one of which leads to taurine, so this result is not necessarily indicative of potential taurine synthesis. It is possible that synthesis is taking place in other tissues, or that the transporter may be up-regulated in other tissues to facilitate the recycling of taurine at the lower levels of dietary input, and these possibilities deserve more attention in order to continue to optimize diets for performance. Cysteine sulfinic acid decarboxylase is another enzyme in the taurine synthesis pathway that is responsible for the conversion of the product of CDO, cysteine sulfinic acid, to hypotaurine, which devolves to taurine. Multiple attempts to design primers for this enzyme were unsuccessful for cobia tissues, and enzymatic assays in other marine species have shown no or minimal activity of this enzyme (Goto *et al.*, 2001). Results from this study and attempts to detect mRNA for this enzyme appear to indicate that cobia do not express it in appreciable amounts in the tissues examined here, which would significantly impact the ability of this species to synthesize taurine. Overall, the slight increases in production characteristics with 0.5 and 1.5% additions of taurine seem to indicate that adequate dietary input is available, as minimal increases in dietary input result in subsequent increases in production; increases that would not be anticipated if cobia

maintained the ability to synthesize even small quantities of taurine. The drop in production at the 5 % addition level may indicate that some other limiting amino acid is being spared with that level of taurine inclusion. Although, the continued increase in plasma, liver, and fillet taurine levels indicate that this dietary input level does not negatively impact physiology, as multiple routes exist to remove excess taurine through urinary or bile salt excretion. Taurine was the only conjugate of bile salts observed, consistent with other studies of teleost bile (Une *et al.*, 1991) and confirming another critical role for this amino acid in cobia. The ability of cobia to thrive on low lipid diets may be due to this role of taurine. High protein (49-51 % CP) and low lipid (12 % CL) diets in both eight week trials of this study (9.12 g initial weight in trial 1, 127 g initial weight in trial 2) resulted in excellent feed conversion (≤ 1.04 in trial 1, ≤1.52 in trial 2), growth rates (≥ 4.72 in trial 1, ≥ 2.07 in trial 2), and high survival (≥ 98 % in trial 1, 100 % in trial 2) expected of fully developed juvenile cobia on appropriate dietary formulations. Closed formulation commercial feeds have not performed as well in recirculating aquaculture systems with larger (26.7 g initial weight) juvenile cobia (Wills *et al.*, 2013) generally resulting in lower survival (56-80 %) and growth rates (≤ 3.6 SGR) when compared to the diets in this study. Although utilizing a larger initial weight (29.2 g), Weirich *et al.* (2010) reported similar growth rates (4.23 % d^{-1}) and survival (97.9%) on their lowest lipid commercial diet (9.9% CL) when compared to those observed in this study. The previous study also utilized two higher lipid diets (13.8% and 16.4% CL) that did not perform as well as the 9.9% CL diet. Cobia clearly have different dietary requirements for optimal growth and feed efficiencies than other marine species, which explains the poor performance of cobia when fed commercial feeds developed for freshwater or anadromous species.

In conclusion, increased dietary taurine input slightly increased production characteristics as has been observed previously in cobia and other species (Gaylord *et al*., 2007; Lunger *et al.*, 2007; Pinto *et al.*, 2010) but resulted in a significant increase in tissue taurine levels, a potential added human health benefit of such dietary supplementation for aquaculture species.

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Analysis of Schillinger Genetics, Inc. non-genetically modified (non-GM) cultivars 3010 and 3032 as fishmeal replacements for cobia, Rachycentron canadum

Abstract

Two non-genetically modified cultivars of soybeans developed by Schillinger Genetics, Inc. were examined as potential fishmeal replacement candidates for juvenile cobia, *Rachycentron canandum*. Cultivars 3010 and 3032 were used to replace either 50, 60, or 70 % or 40, 50, or 60 %, respectively, of protein supplied by fishmeal compared to a commercial-like reference diet during a twelve week trial. 50 % replacement of fishmeal with 3010 performed significantly better than the 50 or 60 % replacements with 3032 in terms of weight gain and feed conversion, with none of the experimental diets performing significantly different than the reference. There was however a trend within each meal type of decreasing performance as inclusion level increased, although not significant. Despite the reduction of the anti-nutritional factors known to be present in soy meals through the marker assisted selection techniques utilized by Schillinger Genetics, this trend potentially indicates a negative effect of utilizing too high a quantity of these individual soy ingredients. Organoleptic analysis of fillets from all seven diets revealed no significant differences in major flavor or aromatic groups. Taken together, these results indicate that both the 3010 and 3032 cultivars are acceptable fishmeal replacements alone at relatively high inclusion levels without significant impact on performance characteristics or fillet quality.

Introduction

As the world's population and protein demands continue to increase, more pressure is put on the aquaculture industry to supply safe, sustainable seafood. However, with catch fisheries at their maximum potential yield, or with declining yields, fishmeal and fish oil production has remained static over much of the last decade (Tacon and Metian, 2009). Without increasing production of fishmeal and fish oil, the use of these ingredients as the staple protein and lipid sources in diets for aquaculture indicates that the aquaculture industry will be unable to expand to meet current and anticipated global needs.

The solution to this issue is to reduce the use of fishmeal and fish oil in feeds by replacing them with suitable alternatives. There are many potential protein replacements that can be derived from marine and terrestrial sources such as by-product meals from animal production, by-product meals from other seafood processing, algal meals, and algal biomass as a by-product from the growing biofuels industries (FAO, 2012). The most abundant, accessible, and sustainable source of proteins however are derived from terrestrial plants. Several issues arise with replacing fishmeal with any alternative protein source. Amino acid profile in comparison to fishmeal, crude protein and lipid levels, and potential negative effects of anti-nutritional factors are a few of the challenges that must be addressed when utilizing high percentages of certain plant proteins.

Protease inhibitors such as those of trypsin, chemotrypsin and elastase, hormonal inhibitors like glucosinolates, unavailable phosphorous complexed as phytic acid, high fiber levels, and complex carbohydrates can all have negative effects on palatability, digestibility, and growth in fish. This is especially true of the high-value marine carnivores targeted by aquaculture that may not possess the required digestive capabilities to handle

these otherwise foreign protein sources. Several of the effects of anti-nutritional factors can be eliminated or mediated during processing, or with extra processing steps while producing feed grade meals from various plant sources. However, in deactivating or eliminating anti-nutrients additional processing steps such as steaming, roasting, and acid or alcohol percolation also damage nutrients that otherwise could contribute to the final formulation.

Another route to producing feed grade meals from plant sources that are suitable for fishmeal replacement has been through genetic modification (GM). Most GM in soy have focused on increasing agronomic yield and indeed have succeeded in bringing the bushels/acre from 28 in 1984 to ~44 in 2010. The dominating GM variety is Roundup Ready® soy, which is modified to be tolerant to the herbicide glyphosate (Padgette *et al.*, 1995). Many of the modifications introduced into different crop strains have aimed at achieving the same objective, making the plants resistant to certain herbicides, insecticides and fungicides. This significantly reduces observed mortality in crop fields, but may have many unintended negative effects on surrounding plant and animal life. However, a few genetic modifications have not been designed for resistance but instead have been aimed at increasing the nutritional value on the crop itself through increased amino acid production, reduction in anti-nutritional factors, or increased production of fatty acids. However, the potential occurrence of unintended effects of GM is one issue that remains to be adequately addressed from a safety perspective in plants and animals intended as feed ingredients or directly as food (Kuiper and Kleter, 2003; Kuiper *et al.*, 2002). Today, transgene insertion is an imprecise and poorly understood event, and introduction of superfluous DNA, as well as deletions and rearrangements of host DNA at the insertion

site, are common occurrences (Latham *et al*., 2006; Somers and Makarevitch, 2004). An insertion might disrupt transcription of endogenous genes, resulting in unintended changes in levels of macro/micronutrients, anti-nutritional factors or production of toxic compounds (Cellini *et al.*, 2004).

Although GM technology can currently help produce suitable, sustainable protein sources, political and economic considerations prevent its global use as many GM organisms are tightly regulated in many countries and are prohibited in some depending on the source of the modification and its objectives. The research, development, and approval process for GM products can also be lengthy and very costly, reducing the economic suitability of GM seeds for many crops. Also, due to patent and proprietary reasons, many GM seeds are designed to produce sterile adult plants, requiring farmers to purchase new seeds every year. For these reasons, GM crops may not be viable options in many developing parts of the world where utilizing GM plants in feed production as opposed to direct human consumption is potentially cost prohibitive, or in many developed countries with strict regulations.

Selective breeding techniques of only selecting individuals for propagation that exhibit high levels of targeted traits has been the traditional method of increasing production in the majority of agricultural fields. Increasing yield, disease and pathogen resistance, enhancing flavors, and many other traits have been modified in current strains of cultured plants and animals. This process is a much slower one than genetically modifying an organism with specific target genes, however, when coupling the traditional selection method with genetic marker assisted selection the length of time needed to develop strains with target traits can be greatly reduced. This marker assisted selection is

the process Schillinger Genetics, Inc. has used to develop multiple cultivars of soybean strains with potential as fishmeal replacements in diets for aquaculture. The biggest impact these selections have had in terms of developing strains suitable as feed ingredients has been the reduction of the anti-nutritional factors raffinose, stachyose, and trypsin inhibitors.

Even with reduced anti-nutritional factors, species specific determination of digestibility and acceptability of these ingredients at increasing levels is needed to maximize protein and amino acid utilization and determine the extent to which an individual ingredient can be utilized to replace fishmeal. To this end, this study was undertaken to examine the effects of utilizing non-GM soybeans at high fishmeal protein replacement levels (40-70%) in juvenile cobia, *Rachycentron canadum*. Cobia are a naturally carnivorous, hardy species with great aquaculture potential, and have shown the ability to thrive on many types of fishmeal replacement sources (Chou *et al*., 2004; Craig *et al.*, 2006; Lunger *et al.*, 2006; Salze *et al*., 2010; Watson *et al*., 2012; Zhou *et al*., 2005).

Methods and Materials

Diet Preparation

Formulations of the seven experimental diets are shown in Table 4.2.1. Due to differences in total protein and lipid content, diets were formulated to replace 50, 60, or 70 % of protein supplied by fishmeal with SG's 3010 (referred to as 3010 50%, 3010 60%, and 3010 70%) or 40, 50, or 60 % of fishmeal protein with SG's 3032 (referred to as 3032 40%, 3032 50%, and 3032 60%). 3010 is a cooked, solvent-extracted meal containing 0.07 % raffinose, 0.50 % stachyose, and ~1,700 TIU (trypsin inhibitor units) while the 3032 ingredient is a full fat, cold pressed cake product containing <0.05 % raffinose, 1.31 %

stachyose, and ~3,300 TIU. Amino acid profiles of the 3010, 3032, and typical menhaden fishmeal are shown in Table 4.2.2. Diets were formulated to be isonitrogenous, isocaloric, and contain similar amino acid profiles. For all diets, ingredients were ground using an air-swept pulverizer (Model 18H, Jacobsen, Minneapolis, MN) to a particle size of <200µm. All dry ingredients were mixed prior to extrusion. Pellets were prepared using a twin-screw cooking extruder (DNDL-44, Buhler AG, Uzwil, Switzerland) with an 18 second exposure to 127°C in the extruder barrel. Pressure at the diet head was approximately 26 bar, and a die head temperature of 71 °C was used. The pellets were dried for approximately 15 min to a final exit air temperature of 102 ºC using a pulse bed drier (Buhler AG, Uzwil, Switzerland) followed by a 30 min cooling period to product temperature less than 25 °C before top coating the oil component. Final moisture levels were less than 10 % for each diet. Diets were stored in plastic lined paper bags at room temperature, and were fed within six months of manufacture. Portions of each diet were analyzed by New Jersey Feed Labs, Inc. (Trenton, NJ) for proximate composition (Table 4.2.1).

Table 4.2.1. Diet formulations and proximate compositions of the seven experimental feeds.

Ingredient (g $100g^{-1}$)	Reference	3010 50	3010 60	3010 70	3032 40	3032 50	3032 60
Menhaden Meal	45.5	22.9	18.0	13.4	27.4	23.5	18.4
Poultry meal	7.5	3.8	3.0	2.3	4.5	3.8	3
Wheat Flour	16	15.0	14.0	9.9	15.0	15	14
Soy Protein Concentrate	7.5	3.8	3.0	2.3	4.5	3.8	3
SG-3010	--	35.1	42.5	50.7	--	--	--
SG-3032	--	--	--	--	31.2	39	48
Corn	17	9.4	8.6	8.4	8.0	6.1	4.5
Menhaden Oil	3.9	6.4	7.3	9.2	6.2	5.6	5.8
Vitamin pre-mix	1	1.0	1.0	1.0	1.0	1	1
Trace mineral pre-mix	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Taurine	1.5	1.5	1.5	1.5	1.5	1.5	1.5
Lysine HCL	--	0.1	0.1	0.3	0.1	0.1	0.1
DL-Methionine	--	0.8	0.9	1.1	0.5	0.5	0.6
Proximate Composition							
Water (g $100g^{-1}$)	4.41	4.87	4.77	4.53	4.74	4.78	5.19
Protein (g $100g^{-1}$ dm)[1]	47.7	45.6	44.5	45.5	46.1	45.4	44.9
Fat (g $100g^{-1}$ dm)[1]	8.02	9.92	10.12	9.99	9.54	9.85	9.90
Fiber (g $100g^{-1}$ dm)[1]	1.64	1.19	1.32	1.30	1.42	1.27	1.48
Ash (g $100g^{-1}$ dm)[1]	9.51	9.41	8.20	7.49	7.29	8.33	7.72
Carbohydrate (g $100g^{-1}$ dm)[2]	28.72	29.01	31.09	31.19	30.91	30.37	30.81
Energy (MJ Kg^{-1})	18.65	18.97	19.22	19.29	19.19	19.19	19.29

[1] New Jersey Feeds Labs analysis.
[2] Calculated by difference (100-Water-Protein-Ash-Fat-Fiber).

Table 4.2.2. Amino acid profiles of main protein ingredients utilized.

Amino Acid	SG 3010	SG 3032	Fish Meal[1]
Arginine*	4.26	3.64	4.05
Glycine	2.29	2.03	3.87
Histidine*	1.45	1.28	2.03
Isoleucine*	2.52	2.14	3.20
Leucine*	4.19	3.71	5.51
Lysine*	3.60	3.21	5.73
Methionine*	0.73	0.69	1.95
Cysteine	0.76	0.74	0.53
Phenylalanine*	2.82	2.42	3.27
Tyrosine	1.82	1.58	2.58
Serine	2.96	2.60	1.95
Threonine*	2.07	1.79	2.97
Tryptophan*	0.70	0.75	0.54
Valine*	2.59	2.25	3.68
Aspartic Acid	6.31	5.60	6.22
Glutamic Acid	10.34	9.12	9.04
Proline	2.83	2.46	-
Alanine	2.33	2.09	-

* Essential amino acid.

[1] Menhaden, Omega Protein.

Experimental Fish and Systems

This study was carried out in accordance with the guidelines of the International Animal Care and Use Committee of the University of Maryland Medical School (IACUC protocol # 0610015). Approximately 500 juvenile (~2 g) cobia were obtained from the University of Miami, Miami, FL. Juveniles were housed at the Institute of Marine and Environmental Technology's Aquaculture Research Center, Baltimore, MD. Fish were “cold-banked”; maintained at 20 °C and fed a maintenance ration 5 days a week at 1.5 % bw daily for 6 weeks prior to acclimation, with cold-banking parameters based on Schwarz *et al.* (2007). Acclimation to study conditions at 27 °C and 5 % bw feeding daily occurred

over the course of two weeks, increasing 1 °C per day and 0.5 % bw per day for one week, followed by maintenance at 27 °C for one week prior to grading and stocking. Fish were maintained on the reference diet until they reached an average weight of ~18 g at which point 12 fish were stocked into each of 21 identical tanks and randomly assigned one of the seven experimental diets in three replicate tanks per dietary treatment.

Six, 340-liter tanks connected to bubble-bead and biological filtration constituted the recirculating systems used, with four replicate systems occupied simultaneously during the trial on a photoperiod of 14L:10D. During the trial water quality parameters were: temperature, 26.88 ± 1.06 °C; salinity, 26.78 ± 1.81 ppt; pH, 7.77 ± 0.22; total ammonia nitrogen, 0.23 ± 0.24 mg l^{-1}; nitrite, 0.39 ± 0.21 mg l^{-1}; and alkalinity, 139.75 ± 48.10 mEq l^{-1}, with no significant differences in water quality parameters between systems utilized ($p > 0.05$).

The trial was conducted for 12 weeks, with tank weights recorded and feeding rates adjusted weekly to 5 % bw day^{-1} for the first 6 weeks, reduced to 3.5 % from 6 weeks through 10 weeks, and 3.0 % for the final 2 weeks of the trial as feed conversion ratio gradually increased. Fish were fed by hand four times daily to maintain apparent satiation and avoid overfeeding.

Sampling

Three fish from each tank were removed, bled and euthanized 8 weeks into the trial. The blood samples were immediately centrifuged (10,000 x g for 15 min) to separate plasma, which was stored at 4°C prior to shipping to NOAA-NWFSC, Seattle, WA. Two plasma samples from each tank (6 samples per dietary treatment) were analyzed on an

IDEXX VetTest Chemistry Analyzer (model 8008, software version 8.33A, IDEXX Labs, Westbrook, Maine, U.S.A.) at NOAA-NWFSC within 48 hours of shipping.

At the conclusion of the 12 week trial, six fish from each tank were weighed (g), measured (cm), bled, euthanized, and dissected for target tissues. Fish were fed to apparent satiation approximately 3 hr prior to sampling to ensure the presence of gut contents. Blood was centrifuged (10,000 x g for 15 min) to separate plasma and both plasma and blood pellet samples were shipped to Texas A&M University (TAMU). Kidneys from 3 fish from each tank were removed and placed in L15 media prior to shipping to TAMU. Intestines were extracted and delineated from the pyloric caeca to the anus. Three sets of intestines from each tank were immediately placed into Davidson's fixative and transferred to 70 % EtOH after 15 h prior to shipping to TAMU. The other three sets of intestines had their contents gently squeezed into micro-centrifuge tubes and were immediately frozen in liquid nitrogen prior to shipping to TAMU for denaturing gradient gel electrophoresis (DGGE) analysis. Three fillets for organoleptic analysis were taken from each tank along with three whole bodies for proximate composition and stored at -20 °C prior to shipping to the University of Arkansas and TAMU, respectively. Blind organoleptic analysis was performed by a panel of ten spectrum method trained panelists, with randomized sample presentation and fillets cooked to a consistent white, flaky state.

Production characteristics were determined as follows:

$$\text{Weight gain (\%)} = 100 * \left(\frac{\text{final wieght (g)} - \text{initial weight(g)}}{\text{initial wieght (g)}}\right)$$

$$\text{Feed conversion ratio (FCR)} = \left(\frac{\text{food fed (g)}}{\text{weight gained (g)}}\right)$$

$$\text{Feed efficiency (FE)} = \left(\frac{\text{weight gained(g)}}{\text{food fed (g)}}\right)$$

$$\text{Specific growth rate (SGR)} = 100 * \left(\frac{\ln \text{final weight (g)} - \ln \text{initial weight(g)}}{\text{days of trial}}\right)$$

$$\text{Protein efficiency ratio (PER)} = \left(\frac{\text{weight gained (g)}}{\text{protein fed (g)}}\right)$$

$$\text{Condition factor (CF)} = \left(\frac{\text{weight (g)} * 100}{\text{length}^3}\right)$$

Statistics

All statistical tests were run using Aabel v.3.0.6 (Gigawiz Ltd., OK, USA) with significance values of $p < 0.05$. ANOVA with Tukey's HSD post-hoc analyses were used to determine differences between dietary treatments.

Results

Production Characteristics

No negative effects of the "cold-banking" were observed as growth rates from all seven diets fell within previously observed ranges for these size ranges and rearing conditions at IMET (Watson *et al*., 2012). At the conclusion of the 12 week trial, weight gain as a percent increase from initial weight was 1,491 % for the reference diet, which

was not significantly different from any of the six experimental feeds with varying levels of SG soy protein ingredients. Inclusion of the 3010 ingredient at levels of 50, 60, and 70 % resulted in weight gains of 1,723 %, 1,517 %, and 1,459 %, respectively. Inclusion of the 3032 ingredient at levels of 40, 50 and 60% resulted in weight gains of 1,446 %, 1,296 %, and 1,279 %, respectively. Only the 3010 50% resulted in significantly higher weight gain than 3032 50% and 3032 60%, no other significant differences in weight gain between diets was observed (Table 4.2.3). The general trend within both sets of diets (3010 and 3032) was a decrease in weight gain as inclusion level increased.

Feed efficiency (FE) and feed conversion ratio (FCR) are the inverse of one another, although different groups within aquaculture prefer one over the other, so both are reported here (Table 4.2.3). FE values ranged from a high of 0.83 from the reference diet to 0.65 from the 3032 60% diet. FCR ranged from a low of 1.20 in the reference diet to 1.53 in the 3032 60%. There were varying significant differences between the diets in both FE and FCR with the general trend of increasing performance within each diet type (3010 and 3032) with the 3010 diets outperforming the 3032 diets.

Specific growth rate (SGR) for the 12 week trial ranged from 3.12 in the 3032 60% diet to 3.45 in the 3010 50% diet. Only the 3010 50% diet was significantly higher than the 3032 50% and 3032 60% diets, with no other significant differences between diets or when compared to the reference diet (Table 4.2.3). Again, the general trend within each diet type (3010 and 3032) was an increase in SGR as inclusion level decreased, although there were no significant differences in SGR within each diet type.

Table 4.2.3. Production Characteristics (mean ± S.D.). Values with different superscript letters are significantly different from one another ($p<0.05$).

Diet	Weight Gain (%)	SGR	FE	PER	FCR	CF
Reference	$1491.25 \pm 107.41^{a,b}$	$3.29 \pm 0.08^{a,b}$	0.83 ± 0.02^{a}	$1.74 \pm 0.04^{a,b}$	1.20 ± 0.03^{a}	0.637 ± 0.05^{a}
3010 50%	1723.13 ± 130.03^{a}	3.45 ± 0.08^{a}	$0.82 \pm 0.03^{a,b}$	1.79 ± 0.07^{a}	1.23 ± 0.05^{a}	0.715 ± 0.04^{b}
3010 60%	$1517.50 \pm 112.71^{a,b}$	$3.31 \pm 0.08^{a,b}$	$0.75 \pm 0.03^{b,c}$	$1.69 \pm 0.08^{a,b}$	$1.33 \pm 0.06^{a,b}$	$0.688 \pm 0.06^{a,b}$
3010 70%	$1459.63 \pm 147.63^{a,b}$	$3.27 \pm 0.11^{a,b}$	$0.76 \pm 0.02^{a,c}$	$1.68 \pm 0.05^{a,b}$	$1.31 \pm 0.04^{a,b}$	$0.702 \pm 0.06^{a,b}$
3032 40%	$1446.73 \pm 23.77^{a,b}$	$3.26 \pm 0.02^{a,b}$	$0.73 \pm 0.02^{c,d}$	$1.58 \pm 0.05^{b,c}$	$1.37 \pm 0.04^{b,c}$	$0.688 \pm 0.05^{a,b}$
3032 50%	1296.63 ± 85.32^{b}	3.14 ± 0.07^{b}	0.68 ± 0.03^{d}	1.49 ± 0.07^{c}	$1.48 \pm 0.07^{c,d}$	$0.711 \pm 0.06^{a,b}$
3032 60%	1279.18 ± 84.62^{b}	3.12 ± 0.07^{b}	0.65 ± 0.02^{d}	1.46 ± 0.05^{c}	1.53 ± 0.05^{d}	$0.704 \pm 0.05^{a,b}$
Pooled SE	60.93	0.46	0.02	0.03	0.03	0.02
P>F	0.003	0.003	<0.001	<0.001	<0.001	0.046

Table 4.2.4. Whole body proximate compositions from experimental fish (average ± S.D. for 6 fish per diet). Values with different superscript letters are significantly different from one another ($p<0.05$).

Diet	Moisture	Protein	Lipid	Ash
Reference	71.63 ± 0.42^{a}	59.48 ± 1.26	8.20 ± 0.34^{a}	2.39 ± 0.19
3010 50%	$70.46 \pm 0.72^{a,b}$	57.09 ± 1.47	$8.97 \pm 0.30^{a,b}$	2.48 ± 0.20
3010 60%	69.06 ± 0.46^{b}	55.49 ± 0.58	10.07 ± 0.22^{b}	2.91 ± 0.07
3010 70%	$70.96 \pm 0.20^{a,b}$	57.20 ± 0.97	$9.28 \pm 0.24^{a,b}$	2.86 ± 0.10
3032 40%	$70.42 \pm 0.23^{a,b}$	56.29 ± 0.66	8.83 ± 0.37^{a}	2.97 ± 0.09
3032 50%	$70.11 \pm 0.42^{a,b}$	56.21 ± 0.64	$9.27 \pm 0.15^{a,b}$	2.92 ± 0.18
3032 60%	$70.95 \pm 0.36^{a,b}$	57.35 ± 0.59	8.68 ± 0.16^{a}	2.78 ± 0.05

Protein efficiency ratio (PER) for the 12 week trial ranged from 1.46 in the 3032 60% diet to 1.79 in the 3010 50% diet. The reference diet produced a PER of 1.74, which was significantly higher than the 3032 50% and 3032 60% diets (Table 4.2.3). There were no significant differences within each diet type (3010 and 3032) when comparing inclusion rates of each protein, although in both diet types PER increased as inclusion level decreased.

Condition factor (CF) for the 12 week trial ranged from 0.637 for the reference diet to 0.715 for the 3010 50% diet. This was the only significant difference observed (Table 4.2.3) with no other diets being different from each other, the reference, or within each diet type (3010 or 3032). CF did not follow the pattern of the other production characteristics in that there was no general trend within each diet type with respect to inclusion level.

Whole body proximate compositions are presented in Table 4.2.4. There were no significant differences between the seven diets in whole body protein or ash content (ANOVA $p>0.05$). The 3010 60% diet was significantly lower in whole body moisture percent (69.06 ± 0.46) than the reference diet (71.63 ± 0.42), though no other significant differences were observed in moisture content. The 3010 60% diet was also significantly higher in whole body lipid content (10.07 ± 0.22) when compared to the reference diet (8.20 ± 0.34) and the 3032 diets at the 40 % (8.83 ± 0.37) and 50 % (8.68 ± 0.16) inclusion levels.

Plasma Analysis

The results of the plasma analysis performed after 8 weeks of the trial are in Table 4.2.5. Of the 13 analytes measured, the only analyte that differed significantly between any of the diets was glucose, which ranged from 3.24 mM in the 3032 50% diet to 6.25 mM in the reference diet. The reference diet was significantly higher than all other diets except the 3010 60% and 3032 60% diets. No other significant differences were found between diets. Urea (mM) and phosphorous (mM) were the only two analytes where slight trends were observed within each diet type (3010 and 3032) with urea increasing and phosphorous decreasing within each diet type as inclusion of the target soy ingredient increased.

Organoleptic Analysis

Results of the organoleptic analysis can be seen in Figure 4.2.1. Panel A presents the basic taste parameters reported by the testers with panel B showing the aromatics. No significant differences were detected between any of the seven diets in regards to basic tastes or aromatics (ANOVA, $p>0.05$).

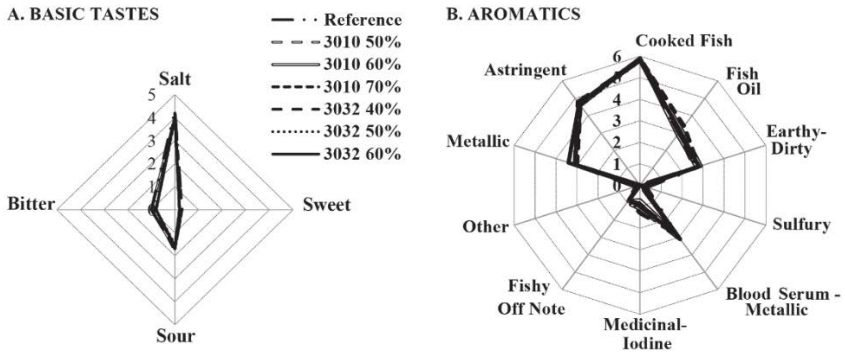

Figure 4.2.1. Results of blind organoleptic analysis of seven diets used in a trial with juvenile cobia. No significant differences were determined between any of the diets in terms of basic tastes or aromatics (ANOVA, $p>0.05$).

Table 4.2.5. Plasma analysis performed at NOAA-NWFSC. Values with different superscript letters are significantly different from one another ($p<0.05$).

	Reference	3010 50%	3010 60%	3010 70%	3032 40%	3032 50%	3032 60%
Albumin (g L^{-1})	5.67 ± 0.23	6.17 ± 0.19	6.50 ± 0.17	5.67 ± 0.17	5.83 ± 0.19	5.17 ± 0.19	6.00 ± 0.24
Alkaline Phosphatase (U L^{-1})	18.67 ± 0.90	21.67 ± 1.20	20.33 ± 0.51	25.50 ± 2.28	22.17 ± 0.81	22.00 ± 1.09	18.80 ± 0.70
Amylase (U L^{-1})	103.33 ± 3.31	105.17 ± 1.91	112.00 ± 3.18	102.83 ± 1.88	110.33 ± 1.50	92.50 ± 2.63	107.80 ± 5.05
Urea (mM)	1.67 ± 0.10	1.28 ± 0.05	1.50 ± 0.06	1.67 ± 0.09	1.33 ± 0.03	1.48 ± 0.11	1.74 ± 0.05
Ca (mM)	2.92 ± 0.02	3.04 ± 0.03	3.05 ± 0.01	3.08 ± 0.03	2.99 ± 0.02	2.92 ± 0.03	3.02 ± 0.01
Cholesterol (mM)	1.23 ± 0.05	1.34 ± 0.02	1.43 ± 0.01	1.31 ± 0.04	1.41 ± 0.02	1.41 ± 0.04	1.39 ± 0.04
Globulin (mM)	27.50 ± 0.27	28.50 ± 0.23	29.67 ± 0.17	28.17 ± 0.27	28.80 ± 0.18	28.17 ± 0.25	28.20 ± 0.30
Glucose (mM)	6.25 ± 0.33^{a}	3.48 ± 0.15^{b}	4.78 ± 0.19^{a,b}	3.97 ± 0.17^{b}	3.30 ± 0.19^{b}	3.24 ± 0.13^{b}	4.18 ± 0.23^{a,b}
Mg (mM)	1.06 ± 0.02	1.01 ± 0.01	1.08 ± 0.03	1.10 ± 0.03	1.02 ± 0.02	1.04 ± 0.03	1.03 ± 0.02
Phosphorous (mM)	4.38 ± 0.09	4.58 ± 0.07	4.48 ± 0.09	4.30 ± 0.06	4.71 ± 0.06	4.39 ± 0.10	4.24 ± 0.09
Total Bilirubin (umol L^{-1})	2.17 ± 0.07	2.00 ± 0.00	2.00 ± 0.00	2.00 ± 0.00	2.33 ± 0.14	2.00 ± 0.00	2.00 ± 0.00
Total Protein (g L^{-1})	33.00 ± 0.49	34.67 ± 0.43	36.33 ± 0.34	33.83 ± 0.37	34.60 ± 0.38	33.17 ± 0.44	34.20 ± 0.41
Triglycerides (mM)	0.68 ± 0.03	0.60 ± 0.02	0.56 ± 0.01	0.64 ± 0.02	0.70 ± 0.01	0.71 ± 0.03	0.68 ± 0.04

Discussion

This feeding trial was undertaken to investigate the effects of different inclusion levels of two non-GM soy protein products from Schillinger Genetics, Inc. as fishmeal replacement sources. The 3010 and 3032 ingredients were included in dietary formulations to replace 50, 60, 70% or 40, 50, 60% of fishmeal protein, respectively. A reference diet was formulated containing 45.5% fishmeal, and fishmeal inclusion varied in the experimental diets from 13.4% to 27.4%. Poultry meal, wheat flour, soy protein concentrate, corn, and fish oil were all varied between diets to maintain isonitrogenous, isolipidic, and isocaloric qualities. There was a difference in total lipid content with the 3010 60% diet having slightly higher lipid than the other six diets, a difference that translated to a significantly higher whole body lipid content in fish fed this diet when compared to the reference diet and the 3032 40% and 50% diets (Table 4.2.4).

Few difficulties were encountered during the feeding trial, but it is important to note that with the rapid growth and high feeding rates for all diets, densities within the four systems approached 10kg m^3, which is a significant accomplishment for these systems considering their lack of drum filtration, protein skimming, ozone or UV systems. This speaks to the high tolerance cobia have over a wide range of conditions. The cobia were very tolerant to the subsequent high turbidity that occasionally occurred in the tanks, and flow rate was partially hindered by the build-up of irremovable solids and the “sticky” nature of the waste and solids that result from diets high in plant ingredients, especially soy ingredients. Although water quality parameters (ammonia, nitrite, pH, and alkalinity) in all four systems were maintained at levels significantly lower than hazardous, low dissolved oxygen levels were occasionally encountered due to the biomass load on the systems, the

slowing of flow, and the buildup of an organic biofilm on the air stones. All of these system issues were easily remedied by regular maintenance but it is important to note the issue of solids building up in the plumbing of recirculating systems when using diets with such high soy inclusion levels without the use of powerful mechanical filtration such as drum filters.

Overall, the diets utilizing the 3010 outperformed the diets utilizing the 3032 ingredient in terms of weight gain (%), specific growth rate (SGR), feed efficiency (FE) and protein efficiency ratio (PER) with no overlap. Although not significant within each diet type (3010 or 3032) there was a trend for each production characteristic to improve as inclusion level of the soy protein of interest decreased. Other than the 3010 50% diet significantly outperforming the 3032 50% and 3032 60% diets in all characteristics measured, there were few significant differences between diet types or comparing individual dietary performance to the reference diet.

The 3010 50% and 3010 60% diets were the only two to outperform the reference diet in weight gain and SGR, although FE, FCR, and PER were not improved compared to the reference.

Condition factor was the only index measured that did not show a trend within or between diet types, with the only significant difference being between the reference diet (0.637) and the 3010 50% diet (0.715) which were the minimum and maximum CF's during the trial. It is noteworthy that the reference diet with 45.5% fishmeal resulted in the lowest CF and all diets with high soy inclusion levels had CF's above 0.688. These relatively low CF values compared to other species are not unusual for juvenile cobia, as this species is long and lean until reaching larger sizes (800+g).

The plasma analysis during this trial was conducted at NOAA-NWFSC in Seattle, WA. Although there is not a wealth of data on the plasma parameters measured in this study for a wide range of teleost species, the data collected in this trial agree with previously analyzed cobia plasma samples from our lab (UCLA analysis; unpublished data) and will help in the validation of using this particular analyzer for future studies. Only glucose showed a significant difference in any of the experimental diets when compared to the reference diet, potentially due to the increased digestibility of fishmeal when compared to plant proteins utilized in the experimental feeds, although this did not appear to affect growth. The significant inclusion of single soy ingredients in the experimental diets also did not have a significant impact on the majority of plasma components analyzed, potentially indicating no negative kidney function effects of the high inclusions, as many of the components measured are directly correlated to kidney function in higher vertebrates and mammals that the analysis panels are designed for.

In summary, none of the experimental diets performed significantly differently than the reference diet in terms of weight gain or SGR, however the higher inclusion levels (50 and 60%) of 3032 resulted in significantly poorer performance in FE, FCR, and PER. Organoleptic analysis also failed to discern differences between the fillets from fish fed any of the experimental diets when compared to each other or the reference diet, indicating no negative effects on flavor or aromatics to the consumers of fish fed these high non-GM soy ingredient diets. Although not significantly, the 3010 diets outperformed the reference diet in most characteristics at the 50 and 60% inclusion levels before seeing a drop-off in performance at the highest inclusion level (70%). These results indicate that the 3010 cultivar may be a more promising ingredient than 3032 for high levels of fishmeal and total

protein replacement for juvenile cobia. Both of these ingredients are ideal candidates for use in plant protein blends utilizing multiple sources of protein ingredients where no single ingredient is included at levels higher than 30% of the total diet to minimize potential negative impacts of specific anti-nutritional factors.

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Developing a plant-based diet for cobia, Rachycentron canadum

Watson, A.M., Kissil, G. Wm., Barrows, F.T., Place, A.R. 2012. Developing a plant-based diet for cobia *Rachycentron canadum.* International Aquafeed. 15:1, 34-38.

Aquaculture reached a landmark in 2009, supplying greater than half of the total fish and shellfish for human consumption (Naylor *et al*., 2009). With global fisheries in decline and human population increasing, the gap between protein supply and protein demand is widening. Aquaculture must continue to expand to meet these growing needs, and it must do so in a safe, sustainable manner that decreases the world's reliance on harvesting fish for fishmeal while still producing a high quality product. There are several difficult hurdles the aquaculture industry now faces if this needed growth is to occur. These include, but are not limited to; the continued heavy reliance upon capture and reduction fisheries to supply fishmeal and fish oil as the major base components for aquatic feeds, build-up of contaminants from these wild caught ingredients in the final products, and public perception that aquaculture in its current state is not sustainable and is a detriment to local ecosystems (Naylor *et al*., 2009). Tacon and Metian (2008) reported that 36.2 % of total worldwide catch in 2006 was destined for non-human consumption, meaning the reduction to fishmeal and fish oil for aquaculture diet formulation, the pet food industry, or as bait. The aquaculture industry currently consumes roughly 68.2 % of global fishmeal production and 88.5 % of global fish oil production (Tacon and Metian, 2008). These trends are not sustainable given the state of the world's fisheries and alternatives to fishmeal and fish oil must be found to ensure the sustainability and expansion of the industry as well as the conservation of wild populations and ecosystems.

Replacement of fishmeal and fish oil in aquaculture diets has been a goal for several decades but has met with limited success often due simply to the cost and inconsistency in the quality and quantity of the product produced. Replacing fishmeal and fish oil for freshwater species without loss in production is easier to accomplish than it is with marine species. This may be due in part to the fact that many freshwater fish are extensively cultured and enjoy a much deeper knowledge and experience base than their marine counterparts, but it may also be a result of most freshwater species in culture being herbivores, omnivores, or scavengers in their natural systems. Most marine species that are sought for intensive culture on the other hand, are carnivorous, which precludes different dietary habits and requirements.

Our research has centered on replacing fishmeal with a blend of plant protein sources to completely eliminate the need for fishmeal in diets for cobia, *Rachycentron canadum*, and other high-value marine carnivores. Cobia are a highly carnivorous species (Franks *et al*., 1996; Arendt *et al*., 2001) found tropically and sub-tropically around the world except for the eastern Pacific, are highly fecund and can be spawned both naturally and through artificial induction in captivity, display rapid growth rates and high natural disease resistance, and are adaptable to a variety of culture and tank conditions (Holt *et al*., 2007). This species is a prime target in the need to increase aquaculture production and serves as an excellent model species due to its rapid growth and limited competition from a wild fishery.

Several physiological issues are presented however, with the use of plant proteins as opposed to other alternative protein sources such as animal meals. Digestibility of plant proteins, possible anti-nutritional factors present, palatability, and lack of essential amino

acids all must be solved to successfully replace fishmeal with plant proteins. Digestibility can be examined on a species specific basis, one protein source at a time as we have done with juvenile cobia utilizing an inert marker such as chromium oxide (Table 4.3.1). This process involves feeding experimental diets containing a fishmeal base along with each individual protein source, gently stripping feces and analyzing them for protein, lipid, and energy content in relation to the concentration of the inert marker, and comparing results to those obtained from diets only containing the fishmeal base (Lupatsch *et al*., 1997). Through this process, digestible protein, lipid, and overall energy can be determined for the test ingredient. It is important to note however, that the ability to digest plant proteins may be different at different developmental stages depending upon the species' complement of digestive enzymes and intestinal flora.

In our examination of six plant proteins (wheat gluten, barley meal, soy protein concentrate, corn gluten, soybean meal, and wheat flour) with juvenile cobia (400-700g), only one plant source (barley meal) was deemed to have too low a digestibility to be considered a viable replacement candidate, with the rest of the plant proteins having digestibility's similar to fishmeal sources (Table 4.3.2), indicating that for the most part, digestibility itself is not a primary obstacle. The lack of known essential amino acids from plant protein sources can easily be remedied by their addition during the formulation and manufacture of the diet, a common practice in the industry already for lysine, methionine, and threonine, along with other components known to be lacking in fishmeal replacement sources, or as additives simply to enhance growth, health, and palatability. The biggest issues have arisen when attempting complete fishmeal replacement as opposed to simply reducing the amount of fishmeal utilized in favor of plant proteins. Many researchers and

growers have encountered lower growth and survival rates when reducing the percentage of fishmeal inclusion in diets for marine fish below 10-20%, depending on the species. There appears to be at least one essential component found in fishmeal and other animal meals that is lacking in plant sources that is responsible for the inability to formulate plant-based diets with complete fishmeal replacement.

Table 4.3.1. Composition of diets used for determination of individual ingredient digestibility.

	Diet							
	FM1	FM2	WG	BM	CG	SPC	SM	WF
Component (g kg^{-1})								
Fish Meal 1	978		678	678	678	678		
Fish Meal 2		978					678	678
Wheat Gluten			300					
Barley Meal				300				
Corn Gluten					300			
Soy Protein Concentrate						300		
Soybean Meal							300	
Wheat Flour								300
Algal Meal								
Vitamin Pre-Mix[a]	14	14	14	14	14	14	14	14
Chromium Oxide	8	8	8	8	8	8	8	8
Proximate Analysis (g kg^{-1} DM)								
Crude Protein	593	656	647	456	642	599	611	515
Crude Lipid	165	95	191	103	75	77	73	73
Ash	200	160	130	148	130	135	157	152
Gross Energy (MJ kg^{-1})	20.27	19.38	19.17	20.05	20.92	19.1	13.61	13.95

[a]Contributed per kg diet; vitamin A, 13510 IU; vitamin D, 9.2 IU; vitamin E, 184.4 IU; menadione sodium bisulfite, 6.6 mg; thiamine mononitrate, 12.7 mg; riboflavin, 13.4 mg; pyridoxine hydrochloride, 19.2 mg; pantothenate, DL-calcium, 141.5 mg; cyanocobalamine, 0.04 mg; nictonic acid, 30.5 mg; biotin, 0.46 mg; folic acid, 3.5 mg.

Table 4.3.2. Apparent digestibility coefficients (ADC) of individual ingredients.

Apparent Digestibility (%)	Ingredient							
	FM1	FM2	WG	BM	CG	SPC	SM	WF
Crude Protein	91	84	83	53	92	85	76	89
Crude Lipid	97	91	52	16	37	25	29	32
Gross Energy	90	84	62	27	86	43	38	37
DCP[a] (g kg^{-1})	540	567	685	96	736	558	387	152
DL[b] (g kg^{-1})	155	85	24	5	19	5	6	6
DE[c] (MJ kg^{-1})	18	15	13	5	19	9	7	6

[a]Digestible crude protein
[b]Digestible lipid.
[c]Digestible energy.

Taurine, an amino acid that is not incorporated into any proteins but plays critical roles in lipid metabolism, oxidative stress responses, muscle activity, and photoreceptor protection (Schuller-Levis and Park, 2003) is found in high concentrations in many tissue types in carnivorous fish and their prey (Satake *et al*., 1988), as well as fishmeal (Kim *et al*., 2005). Taurine is not found in high concentrations however, in many fishmeal replacement sources, most notably plant protein sources such as wheat flour, soy protein concentrate, and corn gluten. Due to its water solubility, taurine is also often found in low concentrations even in fishmeal-based diets and other fishmeal replacement sources, as large quantities of taurine are often lost in the processing of these ingredients. The re-addition of the stickwater by-product, which is high in taurine and other free amino acids, back to the manufacturing of diets has been shown to increase growth in Atlantic salmon (Kousoulaki *et al*., 2009). Several researchers have noted increased feeding and growth rates in marine fish fed diets supplemented with taurine, especially when attempting to replace fishmeal either partially or completely (Martinez *et al*., 2004; Lunger *et al*., 2007; Gaylord *et al*., 2007; Matsunari *et al*., 2008).

Based on the digestibility of the individual ingredients examined, two experimental plant protein-based diets (EPP1 and EPP2) were formulated (Table 4.3.3) with equivalent protein (~45%) and energy (~20Mj Kg^{-1}) digestibility to commercially available feeds. Grow-out trials were conducted at the Institute of Marine and Environmental Technology (IMET) in eight foot diameter, four cubic meter, recirculating systems sharing mechanical and bio-filtration as well as life support systems. Both trials were conducted at 27°C and 25 ppt, with 120 fish per tank in the first trial and 60 fish per tank in the second. The results of the first trial with EPP1 resulted in poor feed conversion, poor percent weight gain, and poor specific growth rate (4.66, 199%, 1.09; respectively, Table 4.3.3). Top coating EPP1 pellets with attractants did not improve acceptance. Fish being fed the commercial feed had normal performance indices (FCR 1.32, % weight gain 900, and SGR 3.65) that indicated that this batch was healthy and grew at similar rates as other batches of cobia

Table 4.3.3. Diet formulations and performance indices for plant-based diets.

	Diet	
Ingredient (g kg^{-1})	**EPP1[a]**	**EPP2[b]**
Soy Protein Concentrate	364.3	269.3
Corn Gluten	201.0	211.0
Wheat Flour	-	226.5
Barley Meal	104.5	-
Soybean Meal, Solvent Extracted	-	121.0
Wheat Gluten	82.3	-
Menhaden Oil	146.0	84.0
Di-calcium Phosphate	40.7	23.7
Vitamin Pre-mix[c]	10.0	10.0
Lysine-HCL	21.5	15.5
Choline CL	6.0	6.0
Trace Mineral Pre-mix[d]	1.0	1.0
Magnesium Oxide	0.5	0.5
Stay-C	3.0	3.0
DL-Methionine	3.4	5.8
Threonine	2.1	2.1
Potassium Chloride	5.6	5.6
Taurine	-	15.0
Proximate Composition[e]	Calculated	Measured
Lipid, % dm	15.1	7.87 ± 1.07
Ash, % dm	4.5	4.98 ± 0.03 (5.15)
Protein, % dm	47.4	49.50 (47.3)
Carbohydrate, % dm by difference	32.67	35.14
Fiber, % dm	(0.33)	(2.51)
Moisture, %	5.3	7.14 (9.96)
Energy Content, MJ Kg^{-1}	20.7	19.30 ± 0.77
Performance Indices	EPP1[i]	EPP2[j]
FCR[f]	4.66	1.35
Weight Gain (%)	199	379
Hepatosomatic index[g]	nt	2.34 ± 0.001
Specific Growth Rate[h]	1.09	2.36
Survival	95%	98%

[a] Experimental Plant Protein 1

[b] Experimental Plant Protein 2

[c] Contributed per kg diet; vitamin A, 9650 IU; vitamin D, 6.6 IU; vitamin E, 132 IU; menadione sodium bisulfite, 4.7 mg; thiamine mononitrate, 9.1 mg; riboflavin, 9.6 mg; pyridoxine hydrochloride, 13.7 mg; pantothenate, DL-calcium, 101.1 mg; cyanocobalamine, 0.03 mg; nictonic acid, 21.8 mg; biotin, 0.33 mg; folic acid, 2.5 mg.

[d] Contributed in mg kg^{-1} of diet; zinc 37; manganese, 10; iodine, 5; copper, 1.

[e] Values in parentheses were determined by New Jersey Feed Labs, Inc.

[f] Feed conversion ratio (g fed/g gained).

[g] Liver weight/body weight*100 ± standard deviation

[h] SGR=specific growth rate= $((\ln BW_2 - \ln BW_1)*(\text{days of trial}^{-1}))*100$.

[i] Initial Weight 30 g, final weight 62 g, 27 °C, 25 ppt, 8 week trial.

[j] Initial Weight 120 g, final weight 572 g, 27 °C, 25 ppt, 8 week trial.

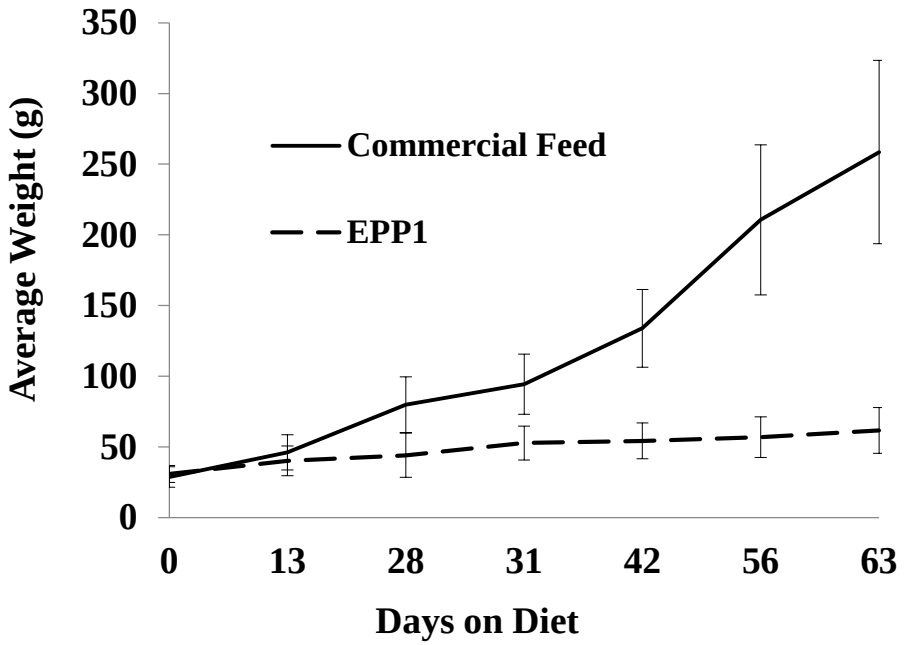

Figure 4.3.1 Growth of juvenile cobia (30 g initial weight) during 9 week trial. 120 fish per tank, 27 °C, 25 ppt salinity. Average weight ± S.D.

raised in our facility, and were larger upon completion of the trial (ANCOVA, $p < 0.001$, with diet as covariate, Figure 4.3.1) than fish fed EPP1.

In the second trial with EPP2, a plant-based trout diet (Gaylord *et al*., 2007) was modified for use with marine species. The changes in formulation between EPP1 and EPP2 include reducing the lipid content from 15 % to 8 %, replacing barley meal with wheat flour because of the low digestibility of barley meal, and replacing wheat gluten with solvent extracted soybean meal. Taurine was absent in the formulation of EPP1, and due to taurine's known physiological roles and that it has been shown to increase growth in a variety fish species (Kim *et al*., 2005; Gaylord *et al*., 2007; Takagi *et al*. 2008), including cobia (Lunger *et al*., 2007), it was included in the formulation of EPP2 at 1.5 %. Fish fed EPP2 performed better than fish fed EPP1, with better feed conversion, higher percent

weight gain, and higher specific growth rates (1.35, 379 %, 2.36 respectively for the EPP2; Table 4.3.3), even given the larger starting size of individuals in the second trial. Fish fed the commercial diet during the second trial had significantly lower growth (FCR 1.85, % weight gain 255, and SGR 1.93) and were smaller upon completion of the trial compared to those from EPP2 (ANCOVA, p=0.018, with diet as covariate, Figure 4.3.2).

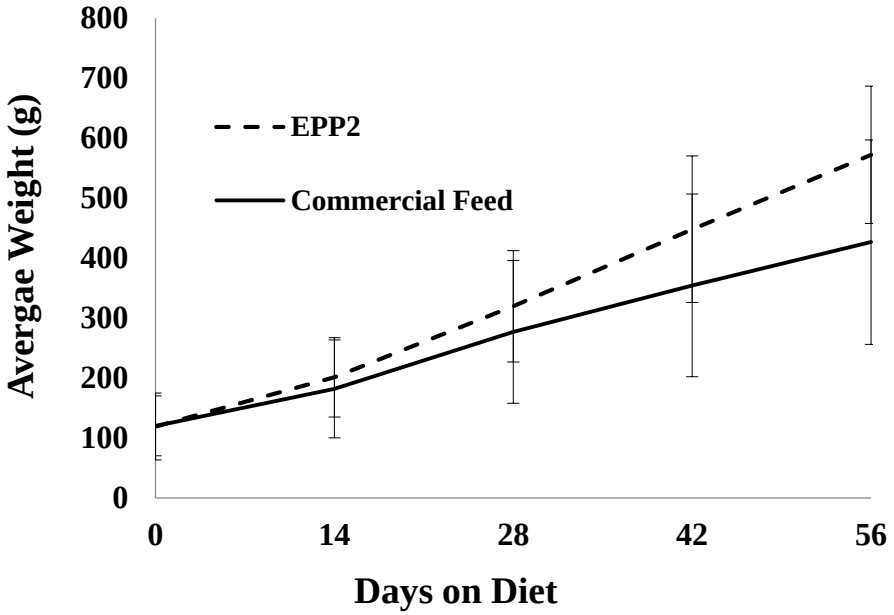

Figure 4.3.2. Growth of juvenile cobia (120 g initial weight) during 8 week trial. 60 fish per tank, 27 °C, 25 ppt salinity. Average weight ± S.D.

During the first trial with diet EPP1 fish grew very poorly as evidenced by the slow growth rate and high feed conversion. This poor performance clearly suggests an issue outside of protein digestibility since several highly digestible protein sources are included in the blend. Although poor palatability is another possibility, the addition of feeding stimulants to EPP1 did not alter feeding behavior.

Growth on EPP2 resulted in much higher feeding rates and greatly increased performance characteristics such as fillet yield and lower feed conversion ratios (Table

4.3.3). Fish in the other tank of the paired system being fed the in-house, commercially available feed had slightly and significantly lower FCR, SGR, and percent weight gain from 120 g to 355 g during the trial. Growth and FCR observed on EPP2 are equivalent to results found by other researchers with various sizes of juvenile cobia, using diets based on fishmeal as well as several fishmeal replacement trials (Lunger *et al*., 2007; Salze *et al*., 2010).

Although there were several differences in the plant protein blends used for the two experimental diets in the current study (barley meal and wheat gluten in EPP1 replaced by wheat flour and soybean meal in EPP2) other differences in the two formulations include the addition of taurine and reduced lipid content of EPP2. Due to the roles that taurine has been shown to play, such as a possible feed attractant (Brotons Martinez *et al*., 2004) and its involvement in bile salt conjugation (Kim *et al*., 2007), it is our opinion that the most important difference in the formulations of the diets in this study is the addition of taurine to EPP2. Taurine is not incorporated into any known proteins and therefore is only considered semi-essential in most species but is considered essential for at least one strict carnivore, felines.

The findings from the digestibility portion of our study demonstrate that several plant protein sources are highly digestible and suitable fishmeal replacements for cobia, which are strict carnivores. The results of the grow out trials present evidence that taurine needs to be added to diets for carnivorous marine fishes, especially when attempting to completely replace fishmeal with alternate sources that may be naturally devoid of taurine. In addition, the growth rates observed with EPP2, an 8 % lipid diet, were equivalent to growth seen on the commercial diet, a 15 % lipid diet, indicating that cobia may be able to

utilize lower lipid diets, helping to reduce the overall cost of feed required to reach market size. Interestingly, regardless of lipid content of the diet, fillets from fish fed either EPP2 or the commercial diet maintained equivalent lipid levels within their fillets (~12-13 % dry weight).

Upon completion of these pilot-scale trials, several more questions involving the use of plant proteins and taurine have arisen that are currently being examined in our lab with juvenile cobia as well as other high-value species such as gilthead seabream and striped bass. The next hurdles are to determine what the effects may be on the final fillet in terms of taste and texture when eliminating fishmeal in favor of plant proteins. Can the fish oil component of the diet also be replaced without detrimental effects to production characteristics or final fillet quality? Will raising farmed fish on plant-based diets reduce contaminants such as mercury and PCBs that are known to accumulate in fish raised on traditional, fishmeal-based diets as well as found in wild-caught fish brought to market? Is taurine an essential amino acid for marine carnivores?

Although our research is now focused primarily on taurine and its biosynthesis pathway in an effort to establish taurine as an essential amino acid for marine carnivores, encouraging results to all of these questions have been obtained in our work so far. This work, and that of many others in the field is indicating that complete fishmeal replacement is possible with marine carnivores in intensive aquaculture systems. Reducing the industry's reliance upon the reduction fisheries to supply fishmeal and fish oil for diets will not only allow the needed expansion of aquaculture to supply the world's growing protein demands, but will also immensely benefit the recovery and sustainability of the oceans

forage and food fishes and the ecosystems that have decimated by decades of over fishing and poor fishing practices.

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Effects of graded taurine supplementation in plant-based diets on juvenile cobia, Rachycentron canadum

Abstract

Three separate trials were undertaken with two different plant protein diets, one with four graded levels of taurine (0, 0.5, 1.5, and 5%) and the other with a single 1.5% taurine level. The first two trials were conducted with 8g and 120g initial weight juvenile cobia, *Rachycentron canadum*. The third trial utilized a different plant protein blend lacking wheat gluten, an ingredient found to impose negative effects in the graded taurine formulation used in the first two trials. At the conclusion of the second trial, RT-qPCR measurements were made to assess the transcript abundance of three genes, cysteine dioxygenase (CDO), cysteamine dioxygenase (ADO), taurine transporter (TauT), involved in taurine synthesis and transport in the brain, liver, and muscle tissue of individuals from all four dietary treatments. Vision tests on live individuals from the lowest and highest taurine supplementation treatments revealed potential differences in vision capabilities. Taurine appears to be an essential amino acid for juvenile cobia as fish fed low and zero taurine supplemented diets exhibited significantly lower growth and survival than fish fed high taurine feeds. There is also no evidence at the transcript level that juvenile cobia possess the ability to synthesize taurine as there is no up or down regulation of relevant gene transcripts regardless of dietary taurine level. Overall, it is evident that taurine supplementation is required for juvenile cobia for maximum growth and survival on plant protein diets, and that not all plant protein blends perform provide adequate nutrition for cobia.

Introduction

Replacing fishmeal with plant proteins is a high priority for the aquaculture industry and the development of optimized diets is underway for species in intensive culture (Rust *et al*., 2011). Plant protein concentrates provide high protein and low fat content when seeking to spare fishmeal as a protein source (Hardy, 2000). However, commercial feed formulations often vary based on the availability and cost of ingredients, with different batches containing significantly different proportions or levels of quality of ingredients, or different ingredients all together. For this reason, the assessment of multiple ingredients and ingredient combinations is necessary. In light of this we formulated a diet based on available and cost effective plant ingredients, all of which have previously been shown to be highly digestible by cobia (*Rachycentron canadum*) (Watson *et al*., 2012) and to be effective fishmeal replacements in rainbow trout (*Oncorhyncus mykiss*) (Barrows *et al*., 2010; Palti *et al*., 2006). However, supplementation of essential amino acids lacking in plant proteins is required such as lysine, methionine, threonine and many vitamins and minerals are also routinely supplemented to plant-based diets for this reason (Barrows *et al*., 2008; Gaylord *et al*., 2007; Zhou *et al.*, 2007). Taurine is a component in fishmeal that is totally lacking in plant protein sources and has been shown to play multiple important physiological roles (Schuller-Levis and Park, 2003). Supplementation of taurine has also previously been shown to increase growth in fishmeal-free diets for cobia (Lunger *et al.*, 2007b; Watson *et al.*, 2012).

We initially examined a plant protein-based diet with four levels of taurine supplementation (0, 0.5, 1.5, and 5.0 %) to examine growth, feed conversion, and physiological effects of increasing dietary taurine. We also measured transcript abundance

for two of the genes involved in taurine synthesis and the taurine transporter to help in determining whether this species is capable of synthesizing sufficient quantities of taurine and if these pathways are regulated at the transcript level based on dietary input. Effects on vision in the highest and lowest levels of taurine supplementation were also examined. However, after observing sub-optimal results from the plant protein formulation in comparison to previously successful formulations, we formulated a similar plant protein diet lacking wheat gluten, the major difference in the poor performing diets, which resulted in significantly increased performance.

Materials and Methods

Diet preparation

Formulations of the five experimental diets used between the three trials are shown in Table 4.4.1. For all diets, ingredients were ground using an air-swept pulverizer (Model 18H, Jacobsen, Minneapolis, MN) to a particle size of <200 μm. All ingredients for diets PP1, PP2, PP3, and PP4 were mixed prior to extrusion; while EPP3 was top-coated with the oil ingredient after extrusion. Pellets were prepared with a twin-screw cooking extruder (DNDL-44, Buhler AG, Uzwil, Switzerland) with an 18 second exposure to 127 °C in the extruder barrel. Pressure at the diet head was approximately 26 bar, and a die head temperature of 71 °C was used. The pellets were dried for approximately 15 min to a final exit air temperature of 102 ºC using a pulse bed drier (Buhler AG, Uzwil, Switzerland) followed by a 30 min cooling period to product temperature less than 25°C. Final moisture levels were less than 10% for each diet. Diets were stored in plastic lined paper bags at room temperature, and were fed within six months of manufacture. Portions of each diet

were analyzed by New Jersey Feed Labs, Inc. (Trenton, NJ) for proximate and amino acid composition.

Table 4.4.1. Dietary formulations for the four plant-based diets with graded levels of taurine used in this study.

Ingredient (g kg^{-1})	PP1	PP2	PP3	PP4	EPP3
Soy Protein Concentrate	269	269	269	269	269
Soy Protein Concentrate HP300	0.0	0.0	0.0	0.0	233.3
Corn Protein Concentrate	193.4	193.4	193.4	193.4	153.4
Wheat Flour	175.5	170.5	160.5	125.5	150.4
Soybean meal, solvent extracted	90	90	90	90	0.0
Wheat Gluten meal	22	22	22	22	0.0
Menhaden Fish Oil	120	120	120	120	59.5
Mono-Dical Phosphate	42.5	42.5	42.5	42.5	39.5
Vitamin Pre-mix[1]	20	20	20	20	20
Lecithin	20	20	20	20	30
L-Lysine	19.9	19.9	19.9	19.9	7.5
Choline CL	6	6	6	6	6
Potassium Chloride	5.6	5.6	5.6	5.6	5.6
DL-Methionine	5	5	5	5	4.5
Threonine	2.8	2.8	2.8	2.8	0.0
Sodium Chloride	2.8	2.8	2.8	2.8	2.8
Stay-C	2	2	2	2	2
Trace mineral pre-mix[2]	1	1	1	1	1
Magnesium Oxide	0.5	0.5	0.5	0.5	0.5
Mycozorb	2	2	2	2	0.0
Taurine	0	5	15	50	15

[1]Contributed per kg diet; vitamin A, 9650 IU; vitamin D, 6.6 IU; vitamin E, 132 IU; menadione sodium bisulfite, 4.7 mg; thiamine mononitrate, 9.1 mg; riboflavin, 9.6 mg; pyridoxine hydrochloride, 13.7 mg; pantothenate, DL-calcium, 101.1 mg; cyanocobalamine, 0.03 mg; nictonic acid, 21.8 mg; biotin, 0.33 mg; folic acid, 2.5 mg.

[2]Contributed in mg kg^{-1} of diet; zinc 37; manganese, 10; iodine, 5; copper, 1.

Experimental fish, systems and trials

This study was carried out in accordance with the guidelines of the International Animal Care and Use Committee of the University of Maryland Medical School (IACUC protocol # 0610015). Approximately 500 juvenile (~2 g) cobia were obtained from the Virginia Agricultural Experiment Station, Virginia Tech, Hampton, VA for the first and second trials Approximately 500 juveniles (~2 g) were obtained from the University of Miami, Miami, FL for the third trial. Juveniles were housed at the Institute of Marine and Environmental Technology's Aquaculture Research Center, Baltimore, MD. Fish for the first and second trials were maintained on the FM2 diet until they reached an average weight of ~10 g at which point 18 fish were stocked into each of 12 identical tanks and randomly assigned one of the four experimental diets using three replicate tanks per dietary treatment.

Six, 340 liter tanks connected to bubble-bead and biological filtration as well as protein skimmers constituted the recirculating systems used with four replicate systems occupied simultaneously during trials with photoperiod maintained at 14L:10D throughout the trials. The first trial was conducted for 8 weeks, with tank weights recorded and feeding rates adjusted weekly to 5 % bw day^{-1}. At the conclusion of the first trial, fish being fed diets FM1 and FM2 as well as those unused in the first trial were re-pooled, maintained on FM2 for one week, and subsequently restocked at 5 fish per tank and approximately 120 g average weight for the second trial. The second trial was conducted for 8 weeks, with tank weights recorded weekly and feeding rates adjusted from 3.5 % bw day^{-1} to 2.5 % bw day^{-1}, with a bi-weekly 0.25 % bw day^{-1} reduction throughout the trial. The third trial was initiated with 18 g average weight individuals, with 12 fish stocked per tank. The third trial

was conducted for 12 weeks, with tank weights recorded and feeding rates adjusted weekly to 5 % bw day^{-1} for the first 6 weeks, reduced to 3.5 % from 6 weeks through 10 weeks, and 3.0 % for the final 2 weeks of the trial as feed conversion ratio gradually increased. Fish for all trials were fed by hand four times daily to maintain apparent satiation and avoid overfeeding.

Production characteristics were determined as follows:

$$\text{Weight gain (\%)} = 100 * \left(\frac{\text{final wieght (g)} - \text{initial weight(g)}}{\text{initial wieght (g)}}\right)$$

$$\text{Feed conversion ratio (FCR)} = \left(\frac{\text{food fed (g)}}{\text{weight gained (g)}}\right)$$

$$\text{Specific growth rate (SGR)} = 100 * \left(\frac{\ln \text{final weight (g)} - \ln \text{initial weight(g)}}{\text{days of trial}}\right)$$

$$\text{Hepatosomatic index (HSI)} = 100 * \left(\frac{\text{liver weight (g)}}{\text{body weight (g)}}\right)$$

Analytical procedures

At the conclusion of the first trial two individuals from each tank were sacrificed for intestinal analysis. Portions of the anterior intestine were preserved in 4% paraformaldehyde and dehydrated from 70 % to 90 % EtOH in 10 % increments over eight hours. Dehydrated samples were sent to AML Laboratories (Baltimore, MD) for sectioning, mounting and H&E staining. Slides were analyzed for pathologies and abnormalities with the aid of the acknowledged pathologist, Dr. Renate Reimschuessel (FDA/CVM/Vet-LRN, Laurel, MD). Gall bladders were removed and bile was extracted and stored at -20 °C prior to bile salt analysis. Total bile salts were assayed with 3 α-

hydroxysteroid dehydrogenase (Coleman *et al*., 1979). Blood samples were taken from the caudal vein with heparinized needles, plasma was separated by centrifugation (16,000 RCF for 20 min), and total plasma protein was quantified after a 1:600 dilution utilizing a Micro BCA™ Protein Assay Kit (Product# 23235, Thermo Scientific, Rockford, IL).

At the conclusion of the second trial two fish from each tank, six per dietary treatment, were randomly selected for sampling. Fish were anesthetized with Tricaine methanosulfonate (MS-222, 70 mg l^{-1}, Finquel, Redmond, WA), blood samples were taken from the caudal vein with heparinized needles, after which fish were euthanized with MS-222 (150 mg l^{-1}) and gall bladders removed with bile analyzed as in trial 1. Liver and fillet samples were also taken. Blood plasma was separated by centrifugation (16,000 RCF for 20 min) and plasma osmolality measured in triplicate (10 µl) on a Vapro™ Model 5520 vapor pressure osmometer (Wescor, Logan, UT). Plasma samples from three fish per dietary treatment were sent to the Pathology and Laboratory Medicine Services department at the University of California at Los Angeles for constituent analysis. Remaining plasma, fillet and liver samples were frozen and stored at -80 °C and portions of each were lyophilized to constant weight for water and taurine content analysis. Triplicate samples of each liver (~10 mg), fillet (~50 mg), plasma (~10 µL), and diet (~50 mg) sample were used for taurine extractions based on Chaimbault *et al*. (2004), with samples being homogenized in cold 70 % EtOH, sonicated for 20min, dried, and re-suspended in 1 ml H_2O prior to injection into the LC-MS. Taurine was quantified in all samples based on methods described in Chapter 2.

Methods for the vision assays are from Horodysky *et al*. (2010, 2008). Briefly, whole-animal corneal electroretinography (ERG) was conducted to assess the absolute

sensitivities, temporal properties, and spectral sensitivities of cobia visual systems. Teflon-coated, chlorided 0.5 mm silver wire (Ag–AgCl2) electrodes were used to measure and record ERG potentials: the active electrode was placed on the corneal surface and a reference electrode was placed subdermally in the dorsal musculature. ERG recordings and stimulus presentations were controlled using software written in LabVIEW (National Instruments, Austin, TX, USA). All subjects were dark-adapted for a minimum of 30min prior to stimulus exposure.

Absolute sensitivity of cobia visual systems was assessed by intensity–response (V $logI^{-1}$) experiments. Six orders of magnitude of stimulus intensity were presented to subjects. V $logI^{-1}$ experiments progressed from subthreshold to saturation intensity levels in 0.2 log unit steps. At each intensity step, ERG b-waves were recorded from a train of five 200 ms flashes, each separated by 200 ms rest periods. This process was repeated three times. ERG responses of the final averaged flashes (Vresponse) were recorded at each intensity step and subsequently normalized to the maximum voltage response (Vmax). Mean V $logI^{-1}$ curves were created by averaging the V $logI^{-1}$ curves of all individuals from each treatment. Dynamic ranges, defined as the log irradiance range between the limits of 5–95 % Vmax, were also calculated (Frank, 2003).

The temporal resolution of cobia visual systems was assessed via flicker fusion frequency (FFF) experiments using methods developed elsewhere (Fritsches *et al.*, 2005). FFF experiments monitored the ability of a visual system to track light flickering in logarithmically increasing frequencies. Sinusoidally modulated white light stimuli ranging in frequency from 1 Hz (0 log units) to 100 Hz (2.0 log units) were presented to subjects in 0.2log unit frequency steps. The voltage offset and the amplitude of the sinusoidal light

stimulus signal were always equal (contrast=1). At each frequency step, light stimuli were presented for 5 s, followed by 5 s of darkness (i.e. rest). This stimulus train was repeated three times at each frequency, and b-wave responses were averaged for each subject. For each subject, seven total FFF experiments were conducted: one at 25% (I25) of maximum stimulus intensity (Imax) from the V $\log I^{-1}$ curve, and one in each of log10 step intervals over six orders of magnitude of light intensity. A subject's FFF threshold at a given intensity increment was determined by analyzing the power spectrum of the averaged responses from 1–100 Hz and comparing the power of the subject's response frequency (signal) to the power of a neighboring range of frequencies (noise). FFF was therefore defined as the frequency at which the power of the response signal fell below the power of the noise, as determined by graphical analysis of normalized power amplitudes as a function of frequency. We considered the FFF at Imax as the probable maximum flicker fusion frequency attainable by the visual system, and FFF at I25 to be a proxy for ambient environmental light intensity.

Spectral sensitivity experiments were conducted to assess the ability of cobia visual systems to respond to colored light stimuli. The output of a Cermax Xenon fiberoptic light source (ILC Technology, Sunnydale, CA, USA) was controlled by a CM110 monochromator, collimated, and passed through each of two AB301 filter wheels containing quartz neutral density filters (CVI Laser Spectral Products, Albuquerque, NM, USA). The first wheel allowed light attenuation from 0 to 1 log units of light intensity in 0.2 log unit steps, the second from 0 to 4log units in 1log unit steps. In concert, the two wheels allowed the attenuation of light from 0 to 5 log units in 0.2 log unit steps. Stimuli were delivered by a LabVIEW program that controlled a Uniblitz LS6 electronic shutter

(Vincent Associates, Rochester, NY, USA) using the analog and digital output of the DAQ card and the computer's serial RS232 interface. A cylindrical lens focused the attenuated light beam onto the entrance slit of the monochromator to produce colored light. The 1cm diameter quartz light guide was placed within 10mm of a subject's eye. Approximately isoquantal spectral stimuli were presented to subjects via the selective use of neutral density filters. Light stimuli covering the spectral range from UV (300 nm) to the near infrared (800 nm) were presented sequentially in 10 nm steps during spectral response experiments. Subjects were presented with five single 40ms stimulus flashes at each experimental wavelength, each followed by 6 s rest. The amplitudes of ERG b-wave responses were recorded and averaged to form raw spectral response curves for each individual. A spectral V $\log I^{-1}$ recording was then conducted for each subject at the wavelength (λmax) that generated its maximum ERG response (Vmax). This allowed the subsequent calculation of the subject's spectral sensitivity curve. V $\log I^{-1}$ experiments exposed the subject to five individual monochromatic 200 ms flashes at each intensity. Intensities increased in 0.2 log unit increments over five orders of magnitude. The amplitudes of these flashes were recorded and averaged to create each subject's spectral V $\log I^{-1}$ curve. To transform spectral response voltages to spectral sensitivities for each subject, the former were converted to equivalent intensities through the V $\log I^{-1}$ curve using the following equation:

$$S = 100 * 10^{-[\mathrm{Imax}-\mathrm{IN}]}$$

where S is the sensitivity, Imax is the intensity at maximum response voltage and IN is the intensity at response voltage. Spectral sensitivity curves for each individual were expressed on a percentage scale, with 100 % indicating maximum sensitivity. To obtain the final spectral sensitivity curve for each species, we averaged the sensitivity curves of all subjects

and normalized to the maximum resulting value such that maximum sensitivity equaled 100 %.

V $\log I^{-1}$ and FFF data were analyzed separately using two-way repeated measures ANOVAs with Tukey's post-hoc comparisons to assess whether ERG responses varied between treatments. All statistical analyses were conducted using SAS v 9.1 (SAS Institute, Cary, NC, USA).

Quantitative PCR primers (Table 4.4.2) were designed for each of the genes of interest: CDO, ADO, and TauT based on consensus sequences from alignments derived from teleost species in the NCBI database. Primr3 was used for primer design (Rozen and Skaletsky, 2000). To verify that primers were indeed amplifying only a single product, and that the product was the desired target, end-point PCR was performed on limited samples in 10 µl reactions with 10 ng cDNA using Promega 2x Mastermix (Promega, Madison, WI) using the following parameters: initial denaturation (95 ºC for 2 min) followed by 35 cycles of denaturation (95 ºC for 30 s), annealing (56 ºC for 30 s), extension (72 ºC for 45 s), and a final polishing step (72 ºC for 5 min). Two µl of the end-point PCR products were used for gel electrophoresis to assess product size and number with the remaining 8 ul purified and sequenced to determine accuracy of selected primers.

Upon selection of appropriate primer pairs for each gene of interest, primer efficiencies were determined using cDNA from a single liver tissue sample and consisted of triplicate measurements made at five different dilutions of cDNA (20, 10, 5, 2.5, and 1.25 ng cDNA per reaction) based on the following equation:

$$\text{Primer efficiency (PE)} = 10^{-\frac{1}{\text{slope}}},$$

where slope was determined from the plot of cDNA content (ng) vs. average Ct for each dilution. RT-qPCR was performed in triplicate per sample and tissue type for each gene of interest (n=5 for all tissues) alongside the reference gene on an ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Life Technologies, Grand Island, NY). Cycling parameters for the assays were as follows: initial denaturation at 95ºC for 2 min, followed by 35 cycles of denaturing (95 ºC for 10 s), annealing (56 ºC for 30 s), and extension (72 ºC for 30 s) with a melting curve to determine the presence of spurious products. Expression relative to the expression of the beta actin reference gene was determined by the following equation:

$$\text{Relative expression of target gene} = \left(\frac{(\text{target gene PE})^{(38-\text{target gene avg. Ct})}}{(\text{beta actin PE})^{(38-\text{beta actin avg. Ct})}}\right) * 100$$

Table 4.4.2. Primers used for genes of interest in taurine metabolism.

Gene Name	Primer Sequence	Tm
Cysteamine dioxygenase (ADO) Forward	5'-AGACCTCGCTCATCCAGAAA-3'	55.7
Cysteamine dioxygenase (ADO) Reverse	5'-AGGGGAGGATGTGGAGACTT-3'	57.5
Cysteine dioxygenase (CDO) Forward	5'-AGGGTCAGCTGAAGGAGACA-3'	57.9
Cysteine dioxygenase (CDO) Reverse	5'-GCACCCTCTGTGTGGCTATT-3'	57.4
Taurine transporter (TauT) Forward	5'-GCTTCATGGCACAAGAACAA-3'	54.0
Taurine transporter (TauT) Reverse	5'-TCAACAAACTGGCTGTCGAG-3'	55.3
Beta Actin Forward	5'-TGCGTGACATCAAGGAGAAG-3'	54.9
Beta Actin Reverse	5'-AGGAAGGAAGGCTGGAAGAG-3'	56.4

Statistics

All statistical tests were run using Aabel v.3.0.6 (Gigawiz Ltd., OK, USA) with significance values of $p < 0.05$. ANOVA with Tukey's HSD post-hoc analyses were

used to determine differences between dietary treatments. RT-qPCR expression data were square root transformed prior to homogeneity of variance (Bartlett's test) and ANOVA analyses.

Results

Proximate compositions of the diets are presented in Table 4.4.3. Performance characteristics for the first grow out (initial weight 10 g) as well as the total biliary protein concentration are shown in Table 4.4.4. Due to low survival in several replicates of PP1 and PP2 resulting in 0 individuals in some tanks, these diets were not included in statistical analyses other than survival for the first grow out. Survival curves from the first grow out are shown in Figure 4.4.1, with all four diet displaying type III survival curves. Growth curves are shown in Figure 4.4.2. There were no significant differences among surviving individuals in percent weight gain, feed conversion ratio (FCR), specific growth rate (SGR), total plasma protein concentration, or total bile salt concentration (ANOVA, $p>0.05$). Cannibalism was not observed to be a contributing factor to the low survival and dead individuals were promptly removed from the tanks so as to not be a nutritional/taurine source for remaining fish.

Table 4.4.3. Proximate compositions and measured taurine values of the diets used in the three trials.

Proximate Composition[1]	PP1	PP2	PP3	PP4	EPP3
Protein (% DM)	47.6	48.5	48.6	50.3	46.4
Lipid (% DM)	12.16	11.73	12.06	11.91	7.92
Fiber (% DM)	1.25	1.09	1.28	1.21	2.39
Carbohydrate (% DM by difference)	30.82	30.84	30.04	28.81	35.16
Moisture (%)	2.14	1.73	2.15	2.13	6.56
Ash (% DM)	8.17	7.84	8.02	7.77	8.13
Taurine (%)	0.02	0.39	1.35	4.08	1.05

[1] New Jersey Feed Labs analysis.

Table 4.4.4. Performance characteristics from the first trial (8.86 g initial weight). Within a row, values that share common superscripts are not significantly different from one another (P >0.05).

Diet (Taurine %)	PP1 (0.02)	PP2 (0.39)	PP3 (1.35)	PP4 (4.08)
Weight Gain (%)	23.35 ± 22.82^{a}	80.57 ± 60.24^{a}	130.87 ± 25.20^{b}	133.82 ± 10.83^{b}
FCR	6.38 ± 1.49^{a}	2.97 ± 1.17^{b}	1.98 ± 0.19^{b}	2.12 ± 0.23^{b}
SGR	0.57 ± 0.12^{a}	1.31 ± 0.26^{b}	1.47 ± 0.19^{b}	1.51 ± 0.09^{b}
Total Bile Salts (mM)	38.76 ± 7.40	28.37 ± 1.94	36.30 ± 5.69	28.75 ± 3.22

[1]Not included in statistical analyses due to lack of replicates due to survival.

Table 4.4.5. Performance characteristics from the second trial (128.37 g initial weight). Within a row, values that share common superscripts are not significantly different from one another (P >0.05).

Diet (Taurine %)	PP1 (0.02)	PP2 (0.39)	PP3 (1.35)	PP4 (4.08)
Survival (%)	1.96 ± 1.96^{a}	$9.80 \pm 5.18^{a,b}$	$9.83 \pm 1.97^{a,b}$	19.6 ± 7.06^{b}
Weight Gain (%)	315.55[1]	1194.73 ± 21.22[1]	1155.92 ± 410.70	1200.68 ± 214.22
FCR	1.93[1]	0.96 ± 0.02 [1]	1.17 ± 0.44	0.95 ± 0.11
SGR	2.54[1]	4.57 ± 0.03 [1]	4.26 ± 0.72	4.53 ± 0.31
Total Plasma Protein ($g\ dL^{-1}$)	3.49[1]	3.40 ± 0.17[1]	3.35 ± 0.17	3.48 ± 0.23
Total Bile Salts (mM)	40.84[1]	42.17 ± 0.46[1]	41.37 ± 1.99	41.50 ± 1.36

Performance characteristics from the second trial (120 g initial weight) are shown in Table 4.4.5 and growth curves are shown in Figure 4.4.3. Survival during the second trial was 100% in all dietary treatments. Percent weight gain was significantly lower in diets PP1 and PP2 than the other four fishmeal-based diets ($p<0.05$), but not significantly different than diets PP3 and PP4 ($p>0.05$). Diet PP1 resulted in significantly lower FCR and SGR than the other diets ($p<0.05$). Diets PP2, PP3, and PP4 did not result in significantly different SGRs than one another ($p>0.05$). Total bile salt concentration was not significantly different between any of the 4 dietary treatments ($p>0.05$). Table 4.4.6 shows the performance characteristics of the third trial. Although only one diet was run in the third trial, weight gain, FCR, and SGR were much improved from the first two trials, and were an improvement over all previous plant protein diets tested in our laboratory.

Table 4.4.6. Performance characteristics from the third trial (17.95 g initial weight).

Diet (Taurine %)	EPP3
Weight Gain (%)	1673.52 ± 192.75
FCR	1.22 ± 0.04
SGR	3.42 ± 0.13

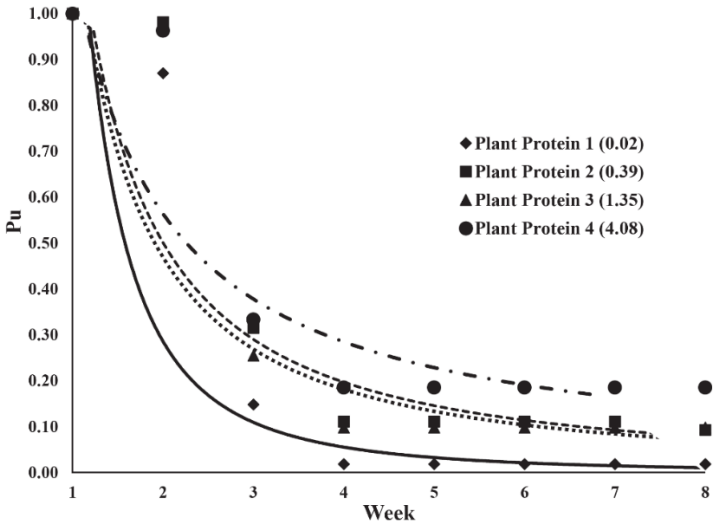

Figure 4.4.1. Survival analysis of juvenile cobia trial #1. Ten-g initial weight, 8 week trial, 25 ppt, 27 °C. Number in parentheses indicates measured dietary taurine level.

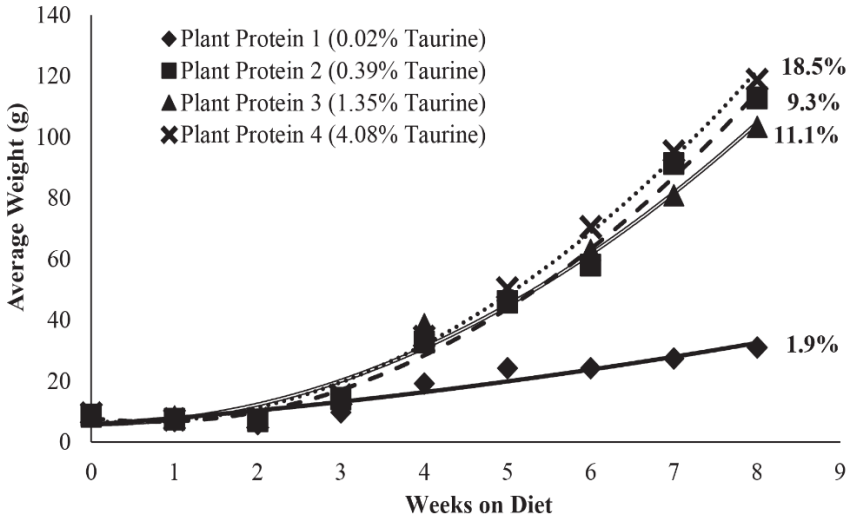

Figure 4.4.2. Growth of juvenile cobia (10 g initial weight) during first 8 week trial. Systems maintained at 25 ppt and 27 °C.

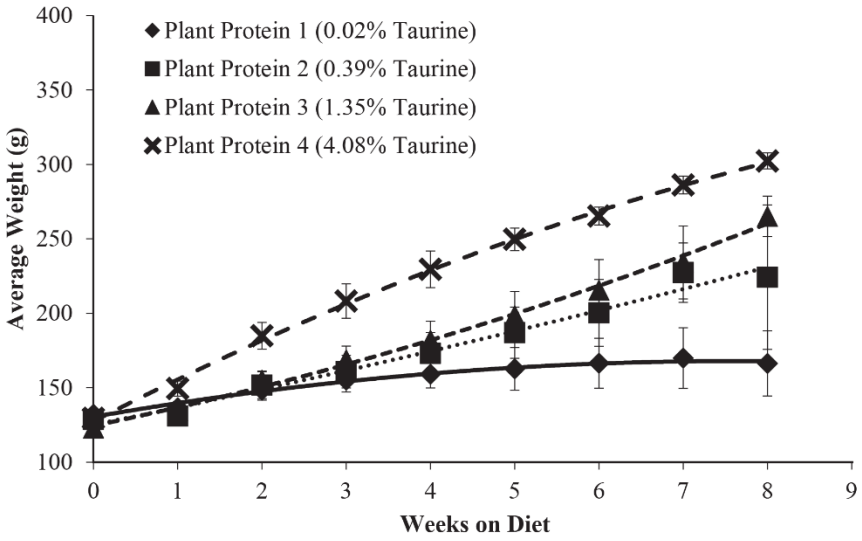

Figure 4.4.3. Growth of juvenile cobia (120 g initial weight) during second 8 week trial. Systems maintained at 25 ppt and 27 °C.

RT-qPCR results are shown in Figure 4.4.4 (A-C). There were no significant differences in beta actin expression between dietary treatments within each of the three tissue types examined (Table 4.4.7, ANOVA, $p>0.05$). There were no significant differences in transcript expression levels in liver (A), brain (B), or muscle (C) for CDO, ADO, or TauT (ANOVA, $p>0.05$). Expression of all three genes in comparison to beta actin transcript levels was highest in the liver and lowest in the muscle, with the general trend of CDO>TauT>ADO within each tissue. The only exception to this was ADO expression levels in the brain. This data set did not pass the homogeneity of variance test, so was analyzed with the non-parametric Mann Whitney U test, where no significant difference in expression between dietary treatments was observed ($p>0.05$).

Table 4.4.7. Reference gene beta actin Ct values (average ± S.D.). No significant differences between dietary treatments within each tissue type (ANOVA, $p>0.05$).

	PP1	PP2	PP3	PP4
Liver	22.31 ± 2.64	24.98 ± 2.83	24.88 ± 3.35	22.13 ± 2.65
Muscle	13.05 ± 2.07	14.20 ± 1.67	11.61 ± 0.92	12.99 ± 1.45
Brain	13.75 ± 2.39	16.20 ± 1.58	14.58 ± 0.90	15.04 ± 3.43

Fillet and liver characteristics from the second and third trials are shown in Table 4.4.8. Fillet water content was highest in fish from PP1 with a gradual reduction in fillet water content as dietary taurine level increased. Hepatosomatic index showed a similar trend of reduction as dietary taurine level increased. Fillet yield, liver water, fillet taurine, and liver taurine contents all showed increasing trends with increasing dietary taurine level. Results of the plasma analysis are shown in Table 4.4.9. Plasma water content significantly decreased as dietary taurine level increased ($p<0.05$). Plasma taurine levels were significantly lower in diets PP1 and PP2 than the other diets ($p<0.05$), plasma cholesterol, phosphorous, and albumin all showed similar trends of significantly increasing with dietary taurine level ($p<0.05$)

Vision analysis on living animals from the PP1 and PP4 dietary treatments is shown in Figure 4.4.5. There were no significant differences in the response of cobia to bright light (A) with animals fed the high taurine diet (PP4) responding with a higher percentage of photoreceptors than fish fed the zero taurine supplemented diet (PP1) after bright light stimulus. There was no significant difference in flicker fusion frequency between the two dietary treatments (B). Response to colored light was significantly lower in fish fed PP1 in terms of percent response of photoreceptors in the range of ~500-570 nm (C). Figure 4.4.6 is a microscope image of a preserved retina from the PP4 treatment. The important layers

and components of the retina are labeled and Table 4.4.10 shows the measurements of individual layers and important ratios of layers to one another for six individuals from each dietary treatment. Although there were no significant differences in the thicknesses of any of the individual layers between the treatments, there were significant differences in several of the ratios. The outer nuclear layer: cone layer (ON:C), cone layer: outer photoreceptor layer (C:OS), and total retinal thickness: cone layer (T:C) with the high taurine diet resulting in higher ratios for the ON:C and T:C comparisons and the zero taurine diet resulting in a higher C:OS ratio.

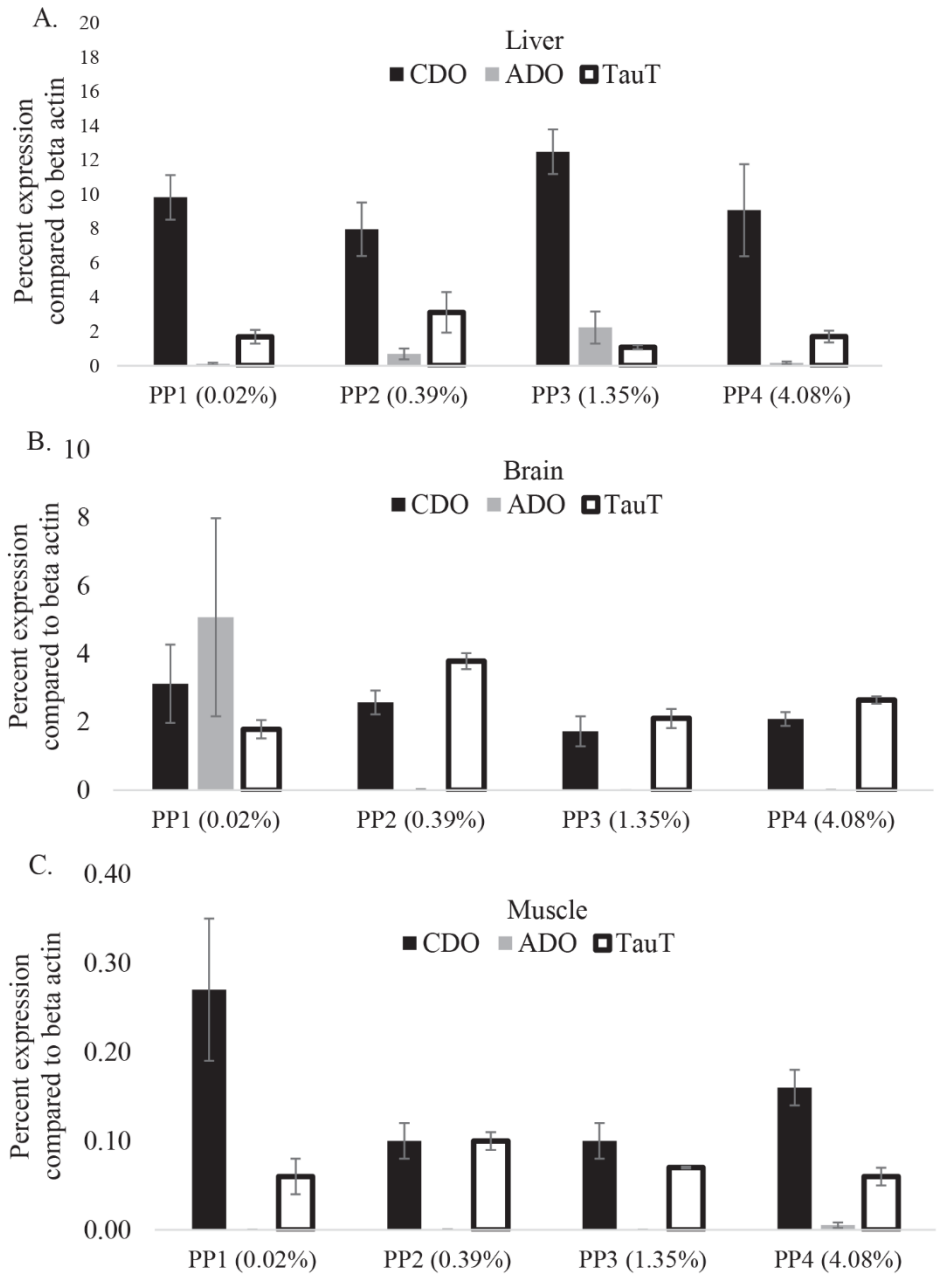

Figure 4.4.4. Relative transcript expression of genes involved in taurine synthesis and transport in juvenile cobia, *Rachycentron canadum*, as a percent expression compared to reference gene beta actin for each diet (% taurine) in liver (A), brain (B), and muscle (C). Equivalent cDNA input (10 ng) for triplicate samples of each tissue and six fish sampled per treatment group and tissue (n = 6 per data point). Numbers in parenthesis indicate measured taurine in each diet.

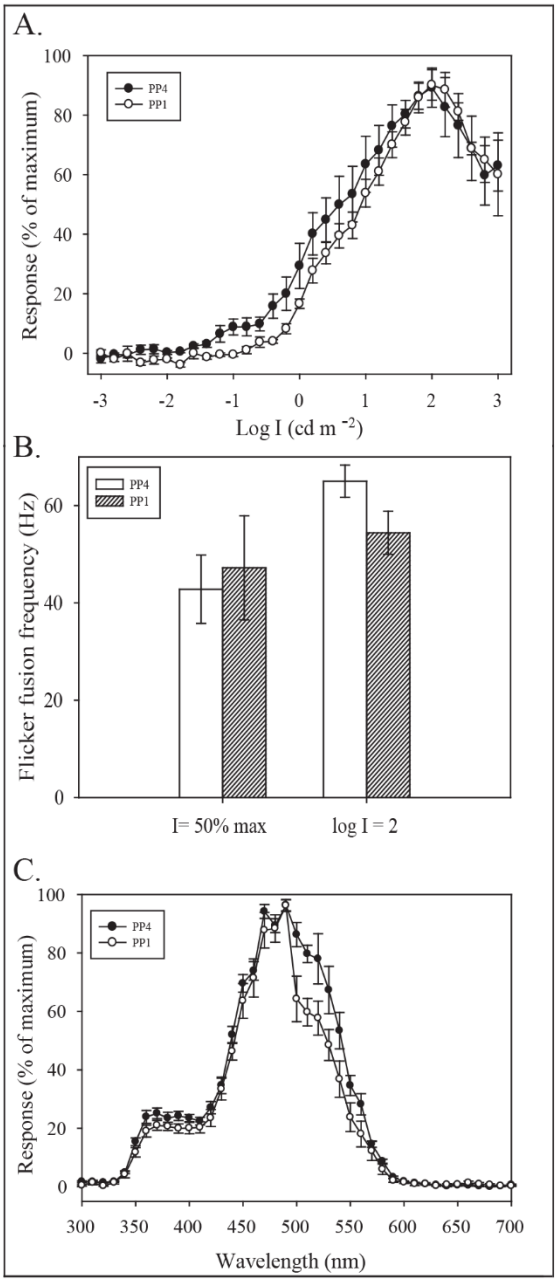

Figure 4.4.5. Response to bright light stimulus (A), flicker fusion frequency (B), and response to color light stimulus (C) of juvenile cobia fed either PP1 or PP4 diets for 8 weeks. Measurements made on living animals.

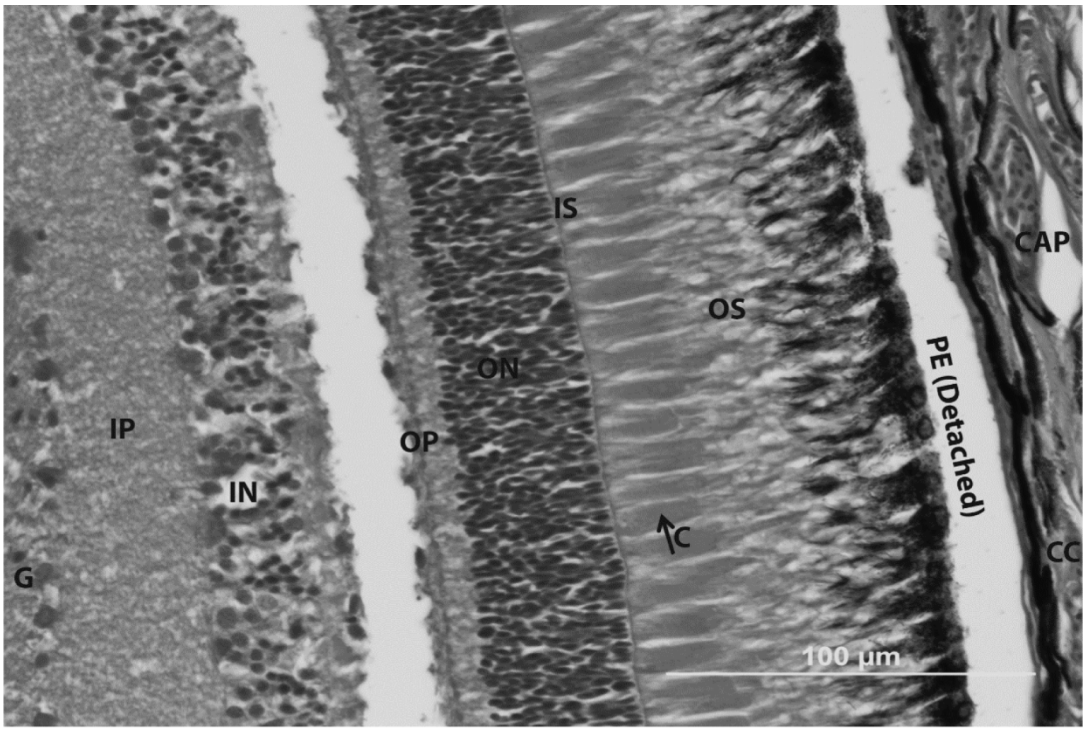

Figure 4.4.6. Retinal layers in juvenile cobia. G – Ganglion cell layer; IP – inner plexiform layer; IN – inner nuclear layer; OP – outer plexiform layer; ON – outer nuclear layer; IS – inner segments of photoreceptor layer; OS – outer segments of photoreceptor layer; C – Cone photoreceptors; PE – pigment epithelium; CC – choriocapillaries; CAP – choroid capillaries.

Table 4.4.8. Fillet and liver characteristics from the second and third trials. Values represent the mean ± standard error for six fish per dietary treatment. Within a row, values that share common superscripts are not significantly different from one another (P >0.05).

Diet (Taurine %)	PP1 (0.02)	PP2 (0.39)	PP3 (1.35)	PP4 (4.08)	EPP3
Fillet Water Content (%)	79.21 ± 2.46	75.34 ± 0.29	74.19 ±0.17	75.16 ± 0.67	76.30 ± 0.19
Fillet Taurine (μmol g^{-1})	2.14 ± 0.46^{a}	5.65 ± 0.90^{a,b}	19.55 ± 11.32^{b}	67.29 ± 18.15^{c}	9.25 ± 0.62^{b}
Fillet Yield (%)[1]	19.86 ± 1.84	23.54 ± 0.87	25.07 ± 2.18	23.79 ± 0.43	nd
Fillet Lipid (% dw)	14.57 ± 3.33	16.04 ± 0.86	15.83 ± 1.18	12.93 ± 3.48	nd
Liver Water Content (%)	55.97 ± 7.81	48.54 ± 3.79	53.46 ± 1.80	61.40 ± 1.57	51.89 ± 4.67
Liver Taurine (μmol g^{-1})	2.03 ± 0.99^{a}	10.62 ± 1.13^{b}	20.36 ± 2.78^{c}	42.21 ± 5.02^{d}	11.06 ± 4.17^{b}
Hepatosomatic Index	2.29 ± 0.41	2.23 ± 0.16	2.23 ± 0.21	2.05 ± 0.19	2.20 ± 0.36

[1]Fillet Yield = fillet weight (g)*100/body weight(g).

Table 4.4.9. Plasma analysis from fish from the second and third trials. Values represent the mean ± standard error for three fish per dietary treatment. Within a row, values that share common superscripts are not significantly different from one another (P >0.05).

Diet (Taurine %)	PP1 (0.02)[1]	PP2 (0.39)[1]	PP3 (1.35)[1]	PP4 (4.08)[1]	EPP3[2]
Water Content (%)	96.75 ± 0.19^{a}	94.75 ± 0.21^{b}	95.19 ± 0.29^{a,b}	94.73 ± 0.13^{b}	94.94 ± 1.21^{a,b}
Osmolality (Osm L^{-1})	329.17 ± 5.57	343.00 ± 5.57	325.17 ± 18.94	336.33 ± 4.60	332.84 ± 9.65
Taurine (nmol ml^{-1})	421.25 ± 41.03^{a}	565.79 ± 17.91^{a}	658.47 ± 50.92^{b}	723.86 ± 89.62^{b}	601. 29 ± 47.97^{a,b}
Albumin (g dL^{-1})	0.53 ± 0.12^{a}	0.70 ± 0.06^{a,b}	0.80 ± 0.00^{a,b}	0.87 ± 0.03^{b}	0.52 ± 0.07^{a}
Total Bilirubin (mg dL^{-1})	0.40 ± 0.10^{a}	0.30 ± 0.00^{a}	0.23 ± 0.03^{a}	0.23 ± 0.03^{a}	0.12 ± 0.00^{b}
Calcium (mg dL^{-1})	9.70 ± 0.88	10.90 ± 0.61	11.10 ± 0.10	11.37 ± 0.27	11.69 ± 0.30
Cholesterol (mg dL^{-1})	49.33 ± 12.17^{a}	68.33 ± 1.20^{a,b}	80.33 ± 3.18^{b}	79.33 ± 4.26^{a,b}	46.59 ± 11.43^{a}
Creatine Kinase (U L^{-1})	96.67 ± 46.77	386.67 ± 276.01	301.33 ± 81.63	552.00 ± 113.15	nd
Creatinine (mg dL^{-1})	0.17 ± 0.03	0.20 ± 0.00	0.17 ± 0.03	0.17 ± 0.03	nd
Glucose (mg dL^{-1})	48.00 ± 4.36^{a}	51.33 ± 3.48^{a}	48.33 ± 3.92^{a}	41.67 ± 3.93^{a}	109.13 ± 19.13^{b}
Phosphorous (mg dL^{-1})	7.13 ± 1.34^{a}	8.37 ± 0.69^{a}	8.87 ± 0.38^{a}	9.33 ± 0.23^{a}	13.82 ± 1.78^{b}
Magnesium (mg dL^{-1})	2.37 ± 0.22	1.97 ± 0.07	2.13 ± 0.03	2.23 ± 0.08	2.77 ± 0.18
Triglycerides (mg dL^{-1})	66.67 ± 27.43	112.00 ± 19.67	91.00 ± 36.12	109.67 ± 20.96	75.73 ± 17.35
Sodium (mmol L^{-1})	169.10 ± 3.56	178.67 ± 2.89	177.33 ± 2.32	174.83 ± 3.89	nd
Potassium (mmol L^{-1})	8.78 ± 1.09	8.07 ± 0.36	8.92 ± 0.53	9.35 ± 0.23	nd
Chloride (mmol L^{-1})	169.97 ± 1.93	173.97 ± 2.08	168.83 ± 0.88	169.43 ± 3.85	nd

[1] UCLA DLAM analysis.

[2] NOAA-NWFSC analysis by A.W. (excluding water content, osmolality, and taurine).

Table 4.4.10. Retinal thickness measurements for juvenile cobia from vision analyses. Values are mean ± S.E. for six individuals per treatment. Values with different superscripts are significantly different from one another (t-test, $P<0.05$).

Retinal Layer	PP1 (0.02)	PP4 (4.08)
Total Retina Thickness, T (µm)	140.72 ± 1.32	145.75 ± 2.6
Outer Nuclear Layer, ON (µm)	50.92 ± 0.49	52.61 ± 1.04
Cone Layer, C (µm)	36.31 ± 0.73	32.36 ± 0.58
Outer Photoreceptor Layer, OS (µm)	54.24 ± 0.63	60.61 ± 1.34
Photoreceptor Layer, P (µm)	91.42 ± 1.37	93.49 ± 1.69
Ratios		
ON:C	1.42 ± 0.03^{a}	1.63 ± 0.01^{b}
ON:P	0.56 ± 0.01	0.56 ± 0.01
ON:OS	0.94 ± 0.01	0.87 ± 0.02
C:OS	0.67 ± 0.01^{a}	0.54 ± 0.01^{b}
T:ON	2.77 ± 0.02	2.78 ± 0.03
T:C	3.91 ± 0.06^{a}	4.51 ± 0.04^{b}
T:OS	2.59 ± 0.02	2.42 ± 0.03
T:P	1.54 ± 0.01	1.56 ± 0.01

Discussion

Negative effects observed with the first trial; low growth, poor survival, and high feed conversion ratios may have been due to the size of the fish utilized, the specific cohort used, or the dietary formulation itself. Fish from the same cohort were used for a separate study involving fishmeal-based formulations and did not grow any different than other cohorts of cobia grown at the IMET facility on commercial feeds, so a poor cohort is not suspected in this study. The formulation utilized in this study, outside of the differing taurine levels, is different than previously successful formulations. The possibility exists that an ingredient utilized in the formulations of the first two trials of this study was having a negative impact on palatability or digestibility on either these size fish or cobia in general. Regardless of the poor performance overall, diets PP2, PP3, and PP4 performed

significantly better than PP1 in the first study. The increasing dietary taurine partially remediated the negative overall impacts of this particular formulation in terms of growth and survival. Palatability issues with plant protein diets have been well described for many species (Glencross *et al*., 2007; Gómez-Requeni *et al*., 2004), and taurine has the potential to serve as a feed attractant due to its small nitrogenous structure. Increased feed palatability may be the sole explanation for the increase in growth and survival during the first trial, although the cause of the poor palatability and performance in these small fish is still elusive. For this reason and the concern over the formulation itself, the second trial was initiated with an initial size that has readily accepted and performed well on plant protein formulations. Although growth and feed conversion in the second trial were lower than previously observed with plant protein formulations, survival was 100 % in all treatments, a significant improvement over the first trial. As with the first trial, there was a significant increase in performance with increasing dietary taurine, clearly indicating that taurine is at least partially remediating the negative impacts of this formulation, which were extended to these larger fish. Feed consumption was not an issue during the second trial, indicating palatability of this diet was no longer an issue with this size fish. The mechanism of taurine remediating the negative impacts of this diet are not clear, however several potentials exist. Taurine has been shown to be a powerful antioxidant, and if some form of intestinal enteritis is occurring due to the wheat gluten inclusion, free radical production during inflammation would be expected. Intestinal enteritis has been observed in several species with specific plant protein inclusion such as soy ingredients in salmon and carp (Romarheim *et al*., 2011; Urán *et al*., 2008). Taurine's most important role may be that of an antioxidant, especially in mitochondria where it has been shown to prevent the diversion

of electrons into superoxide generation by improving the function of the electron transport chain (Jong *et al*., 2012). Reducing energy spent in recovery from free radical damage allows for more energy for growth.

Wheat gluten has been shown to have negative impacts on humans, a result of celiac disease, and other mammalian models (Briani *et al*., 2008; Kuiper and Kleter, 2003; Penttila *et al*., 1991; Rivabene *et al.*, 1999). We have previously shown wheat gluten to have relatively high digestibility in cobia with an apparent digestibility coefficient of 0.83, roughly equivalent to other plant ingredients which are major constituents in fishmeal replacement: soy protein concentrate (0.85), wheat flour (0.89) and soybean meal (0.76) (Watson *et al*., 2012). However, due to the relatively high stress to the animals involved in a digestibility study (regular handling, anesthetizing, and stripping of feces) growth and other physiological parameters on these individual plant protein diets was not tracked. Wheat gluten has been used successfully as a fishmeal replacement in several species without negative impact when compared to control fishmeal formulations (Glencross *et al*., 2011; Helland and Grisdale-Helland, 2006), however species specific effects of wheat gluten with many species, including cobia have not been examined.

Based on the RT-qPCR results measuring the transcript levels of ADO, CDO, and TauT it is apparent that cobia do not possess the capacity to regulate this synthesis pathway at the transcript level. This is evident since the expression levels are not up- or down-regulated with low or high dietary taurine supply, respectively. Coupling this with the growth and survival data from the first two trials of this study makes it apparent that juvenile cobia do not synthesize sufficient quantities of taurine, if any at all, to meet their physiological requirements. Small juveniles fed low or no taurine supplemented feeds have

very poor survival and growth (Figures 1 and 2). Even larger juveniles fed no and low taurine supplemented feeds do not grow very well (Figure 3). The low growth but higher survival observed in the second trial may have been a function of the animal's ability to recycle the taurine pool acquired prior to the transition onto the plant protein diets. Juveniles of this size should have a sufficient taurine pool to last during the short duration of the trials here, however it is apparent that any taurine present in the fish fed the PP1 diet during the second trial was minimal at best. Even without accurate measurements of cysteine sulfinic acid decarboxylase (CSD) and with only transcript abundance measured without protein quantification or activity measure, these results further indicate that taurine should be considered an essential amino acid for juvenile cobia, as they clearly do not survive or grow appropriately when not supplied with taurine in their diets.

Along with the significant increase in growth observed in the second trial between the PP1 and PP4 treatments, there were significant difference in vision capacity between these treatments. Overall, vision parameters recorded from cobia under the PP4 dietary treatment are very similar to those recorded by Horodysky *et al.* (2010) for wild caught cobia. Although there was no significant difference detected in overall light sensitivity, interestingly, after only an eight week trial significant differences were observed in color vision with wavelength ranges resulting in lessened response in fish fed the PP1 diet. However, these differences may not be biologically significant, as they represent the difference between ~80 % (PP4) and ~60 % (PP1) of maximum. The difference in color vision as compared to bright light may be due to the cones of the retina being more sensitive to light, and the lack of sufficient taurine leaves the PP1 fed fish unable to deal with the damage. Over longer periods of time these differences would only be magnified, with cones

and eventually rods becoming more and more damaged and irreparable. The role of taurine as a photoreceptor protectant, most likely through its role as an antioxidant, is apparent in these studies with living animal vision tests. This is evidence that although cobia have the ability to recycle taurine through the taurine transporter and biliary recycling pathways, constant dietary supply is required to maintain proper functions throughout multiple tissue types. Both the growth and effects on vision incurred by the low taurine diet would be anticipated to be further exacerbated the longer dietary taurine requirements are not met.

Acknowledgements

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Chapter 5: Fish Oil Replacement with Taurine Supplemented Fishmeal-free Diets

Chapter 5 is the logical next-step after successfully developing fishmeal-free formulations. This chapter examines the effects on production and fillet fatty acid profiles of fishmeal and fish oil-free diets. The results of studies utilizing a thraustochytrid meal plus soybean oil or a canola oil plus exogenous EFAs with juvenile cobia, *Rachycentron canadum*, and juvenile gilthead sea bream, *Sparus aurata*, are discussed in the first and second sections, respectively. This chapter provides further evidence of the importance of taurine in feeds and its role as a bile salt conjugate. This function may be the reason these species are able to thrive on diets utilizing alternative lipid sources and diets lower in total lipid than what many commercial produces are using for these species.

Taurine supplementation of plant derived protein and n-3 fatty acids are critical for the optimal growth and development of cobia, Rachycentron canadum

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Abstract

We examined growth performance and lipid content in juvenile cobia, *Rachycentron canadum,* fed a taurine supplemented (1.5 %), plant protein-based diet with two fish oil replacements. The first fish oil replacement was a thraustochytrid meal (TM+SOY) plus soybean oil (~9 % CL) and the second was a canola oil supplemented with the essential fatty acids (EFAs) docosahexaenoic acid (DHA) and arachidonic acid (ARA) (~8 % CL). The diet using the thraustochytrid meal plus soybean oil performed equivalently to the fish oil diet; both resulting in significantly higher growth rates, lower feed conversion ratios, and higher survival than the supplemented canola oil diet, even though all three diets were similar in overall energy and met known protein and lipid requirements for cobia. The poor performance on the canola oil diet was attributed to insufficient addition of EFAs in the supplemented canola oil source. Increasing levels of EFAs in the supplemented canola oil above 0.5 g EFA kg^{-1} would likely improve results with cobia. When fish fed either of the fish oil replacement diets were switched to the fish oil control diet, fatty acid profiles of the fillets were observed to transition toward that of the fish oil diet and could be predicted based on a standard dilution model. Based on these findings, a formulated diet for cobia can be produced without fish products providing 100 % survivorship, specific growth rates greater than 2.45 and feed conversion ratios less than 1.5, as long as taurine is added and EFA levels are above 0.5 g EFA kg^{-1}.

Introduction

Aquaculture produced 63.6 million tonnes in 2011 and was responsible for supplying approximately 47% of the total fish and shellfish for human consumption in 2010 (FAO, 2012). With increased population growth on the horizon, the aquaculture industry now faces several hurdles in order to continue expanding. One of the most pressing issues has been the industry's heavy reliance upon capture and reduction fisheries to supply fish meal and fish oil as the major base components for aquatic feeds. Aquaculture currently consumes 68.2% of global fish meal production and 88.5% of global fish oil production (Naylor *et al*., 2009). Other major concerns include xenobiotic contaminants in the final products and public perception that aquaculture in its current state is not sustainable and is a detriment to local ecosystems (Tacon and Metian, 2009).

Replacement of fish meal and fish oil in aquaculture diets has been a goal for several decades but has met with limited success often due simply to the cost and inconsistency in quality and quantity of available fish meal alternatives. Replacing fish meal and fish oil without loss of production for freshwater species has been more successful than with marine species. This may be due to increased experience and knowledge involved with some of the extensively cultured freshwater species in comparison to marine species, but it may also be a result of some of the intensively cultured freshwater species being omnivorous in their natural systems. Most marine species that are sought for intensive culture on the other hand, are carnivorous, which dictates significantly different dietary habits and therefore, dietary requirements. Taurine, an amino acid not used for protein synthesis, but playing critical roles in lipid metabolism, oxidative stress responses, muscle activity, and photoreceptor protection (Schuller-Levis and Park, 2003)

is found in high concentrations in carnivorous fish and their prey (Satake *et al*., 1988), as well as fish meal (Kim *et al*., 2005b). However, taurine is not found in many fish meal replacement sources, most notably plant protein sources such as wheat flour, soy protein concentrate, and corn gluten (Gaylord and Barrows, 2009). Several researchers have noted increased feeding and growth rates in marine fish fed diets supplemented with taurine, especially when attempting to replace fish meal either partially or completely (Martinez *et al*., 2004; Gaylord *et al*., 2007; Kim *et al*., 2005b; Lunger *et al*., 2007a; Matsunari *et al*., 2008; Watson *et al*., 2012).

Recently, fish oil replacements have been summarized (Turchini *et al*., 2011) and several commercially viable alternatives have emerged. The necessity for fish oil derives from the essential fatty acids (EFA) it supplies (Turchini *et al*., 2011). Docosahexaenoic acid (DHA; 22:6n-3) has consistently been shown to provide the greatest EFA value to most species for promoting growth. However, in addition to DHA, eicosapentaenoic (EPA; 20:5n-3) and arachidonic (ARA; 20:4n-6) acids serve critical roles in membranes and as precursors molecules in multiple signaling pathways (Glencross, 2009). Although the potential for synthesis or inter-conversion of these EFAs from DHA or other long chain fatty acids varies by species, dietary intake is generally regarded as the most important source for all three of these EFAs. Requirement levels for these acids individually and combined is poorly understood in many species, although the importance of these EFAs is significantly greater than that exhibited by linolenic (LA; 18:3n-3) and linoleic (LNA; 18:2n-6) acids (Glencross, 2009), which are the predominant polyunsaturated fatty acids (PUFA) in seed oils.

Very few studies have attempted to replace both fish meal and fish oil in the same diet. Torstensen *et al*. (2008) used plant meal and vegetable oil blends in an attempt to increase the sustainability of Atlantic salmon, *Salmo salar*, production by reducing the amount of wild fish product needed to produce an equivalent amount of farmed product. They found that at 80% fish meal and 70% fish oil replacement, growth was depressed.

Cobia, *Rachycentron canadum*, is a promising candidate for aquaculture, especially in recirculating systems, because it is a fast growing, highly fecund species found tropically and sub-tropically throughout the world with the exception of the Eastern Pacific (Holt *et al.*, 2007). Boosting the appeal of cobia for intensive aquaculture is the fact that they are typically not a schooling fish and there is little wild commercial competition via a managed fishery. Cobia are cultured extensively in several Asian countries as well as some Caribbean nations, and a great deal of research has been performed on their ability to digest and grow on fish meal replacement protein sources (Lunger *et al*., 2007a, 2007b, 2006; Webb *et al*., 2010; Zhou *et al*., 2005; Zhou *et al*., 2004). Chou *et al.* (2001) determined the optimal dietary protein and lipid levels for juvenile cobia to be around 45% protein and 8% lipid. Salze *et al*. (2010) were able to completely eliminate fish meal and fish oil from cobia diets using soy protein concentrate, a marine worm meal, a yeast protein extract, and mannan oligosaccharides without significant decreases in production characteristics (i.e. feed efficiency, specific growth rate, and survival). Moreover, Trushenski *et al*. (2011) examined the effectiveness of replacing fish oil solely with soybean oil for juvenile cobia and found that a substantial portion of fish oil (~66%) can be replaced without reduced growth. Negative effects on feed intake and final weight were only observed with complete fish oil replacement. In terms of overall dietary lipid, high levels, i.e. >15%, have been

shown to detrimentally affect the health and growth of juvenile cobia (Fraser and Davies, 2009), with a minimum level, 5.76%, identified by Chou *et al.* (2001).

In general, replacement of high percentages of fish meal in diets for cobia with sustainable plant proteins has been unsuccessful without taurine supplementation (Watson *et al*., 2012). Similar trends in lipid replacement have been observed when terrestrial alternatives to fish oil have been utilized without supplementation of exogenous EFAs, most notably DHA (Trushenski *et al*., 2011; Trushenski *et al*., 2012). Non-fish oil sources of these EFAs, such as those utilized for this study, are in critical demand for the success of reducing the reliance of aquaculture on wild feed sources. Genetically engineered crop usage is highly controversial, however few other sources of exogenous EFAs currently exist, and those that do through the use of bacterial or algal production vectors are currently too expensive to be considered viable long-term solutions for the aquaculture industry. In order to develop truly sustainable diets for aquaculture, and break the reliance on dwindling reduction fisheries, both fish meal and fish oil replacement must be accomplished.

The main objective of the current study was to examine the effects of alternative lipid sources in combination with a successful taurine supplemented, plant protein-based diet, on the growth, feed conversion ratio, survival, and proximate compositions of juvenile cobia reared in recirculating aquaculture systems. We chose two alternative lipid sources: a canola based oil supplemented with ARA and DHA to mimic an engineered oil seed crop that will be commercially available in the near future (Domergue *et al*., 2005; Qi *et al.*, 2004; Venegas-Calerón *et al*., 2010) and a single cell microbial meal with high DHA levels (Lewis *et al*., 1999; Lippmeier *et al*., 2009) plus soybean oil. Additional objectives were to examine the fatty acid profiles in the fillets of fish reared on these alternative lipid diets,

determine whether the amino acid profiles of fillets from fish reared on the alternative lipids varied, and whether finishing diets with fish oil can be utilized to recover a fatty acid profile more typical of wild caught fish.

Materials and Methods

Diet Formulation

The formulations for the three fish meal free diets using a blend of plant protein sources are presented in Table 5.1.1. The plant protein blend supplementation for all three diets is based on a proven formulation for rainbow trout (Barrows *et al.*, 2010) with slight modifications to meet known minimum protein (44.5 %) and lipid (5.76 %) levels for cobia (Chou *et al.*, 2001). Lysine, methionine, threonine, magnesium, and potassium chloride were all supplemented to mimic concentrations commonly found in fillet tissues (Barrows *et al.*, 2010). All ingredients were ground using an air-swept pulverizer (Model 18H, Jacobsen, Minneapolis, MN) to a particle size of <200 μm. All ingredients were mixed before extrusion except for the menhaden oil. Pellets were prepared with a twin-screw cooking extruder (DNDL-44, Buhler AG, Uzwil, Switzerland) with an 18 s exposure to 127 °C in the extruder barrel. Pressure at the die head was approximately 26 bar, and a die head temperature of 71 °C was used. The pellets were dried for approximately 15 min to a final exit air temperature of 102 ºC using a pulse bed drier (Buhler AG, Uzwil, Switzerland) followed by a 30 min cooling period to a product temperature less than 25 °C. Final moisture levels were less than 10 % for each diet. Oils were top-coated after cooling using a vacuum pressure of 25 bar (A7J Mixing, Ontario, CA). Diets were stored in plastic lined paper bags at room temperature, and were fed within 4 months of manufacture.

Table 5.1.1. Diet formulations and fatty acid compositions of the diets in the trial.

Ingredient (g kg^{-1})	Diet		
	TM+SOY	**CAN+EFA**	**ARS Control**
Soy Protein Concentrate	269.3	269.3	269.3
Corn Gluten	211	211	211
Wheat Flour	226.5	226.5	226.5
Soybean Meal Solvent Extracted	121	121	121
Menhaden Oil	0	0	84
Soybean Oil	5	0	0
Algamac 3050	79	0	0
Canola Oil + DHA + ARA	0	84	0
Dicalcium Phosphate	23.7	23.7	23.7
Vitamin Pre-mix[1]	10	10	10
Lysine-HCL	15.5	15.5	15.5
Choline CL	6	6	6
Trace Mineral Pre-mix[2]	1	1	1
Magnesium Oxide	0.5	0.5	0.5
Stay-C	3	3	3
DL-Methionine	5.8	5.8	5.8
Threonine	2.1	2.1	2.1
Potassium Chloride	5.6	5.6	5.6
Taurine	15	15	15
Fatty Acid (g $100g^{-1}$)[1]			
12:0	0.15	0.34	0.10
14:0	4.33	1.21	4.88
16:0	17.17	7.73	22.89
17:0	0.16	0.15	0.47
18:0	2.77	3.49	5.31
20:0	0.28	0.82	0.30
22:0	0.24	0.38	0.14
SFA[3]	25.10	14.12	34.08
16:1n-7	0.23	0.46	5.44
18:1n-7	0.87	2.58	2.46
18:1n-9+6	13.86	48.28	19.53
20:1n-15+cis-8	0.06	0.03	0.22
20:1n-9	0.14	0.76	1.21
24:1n-9	0.0	0.13	0.32
MUFA[4]	15.15	52.24	29.18
16:3n-4	0.05	0.06	0.57
16:4n-1	0.04	0.06	0.79
18:2n-6	33.27	22.86	12.74
18:3n-3	3.94	6.25	1.19
20:2n-6	0.04	0.13	0.18
20:4n-6	0.70	0.32	0.84
20:5n-3	0.42	0.27	8.90
22:5n-6	6.09	0.66	0.31
22:5n-3	0.16	0.16	1.51
22:6n-3	14.93	2.85	8.57
PUFA[5]	59.75	33.64	36.75
n-3[6]	19.45	9.53	20.17
n-6[7]	40.10	23.97	14.07
n-3:n-6	0.49	0.40	1.43

[1] Contributed per kg diet; vitamin A, 13510 IU; vitamin D, 9.2 IU; vitamin E, 184.4 IU; menadione sodium bisulfite, 6.6 mg; thiamine Mononitrate, 12.7 mg; riboflavin, 13.4 mg; pyridoxine hydrochloride, 19.2 mg; pantothenate, DL-calcium, 141.5 mg; cyanocobalamine, 0.04 mg; nictonic acid, 30.5 mg; biotin, 0.46 mg; folic acid, 3.5 mg.

[2] Contributed in mg kg^{-1} of diet; zinc 37; manganese, 10; Iodine 5; copper, 1.

[3] Saturated fatty acids = sum of all fatty acids without double bonds.

[4] Monounsaturated fatty acids = sum of all fatty acids with a single double bond.

[5] Polyunsaturated fatty acids = sum of all fatty acids with two or more double bonds.

[6] Sum of all n-3 fatty acids.

[7] Sum of all n-6 fatty acids.

The Plant Protein Cobia Diet formulated by the USDA's Agricultural Research Service ("ARS") served as the control diet for this study as it relies on fish oil as the lipid source, and its palatability and effectiveness as a feed has previously been examined in our lab showing excellent performance with juvenile cobia (120-500 g) (Watson *et al*., 2012). The experimental diets for this study examined two possible fish oil replacement sources in the fish meal free, plant protein-based diet. A thraustochytrid based meal (Algamac 3050; 35.58 % CL, 16.25 CP, 9.36 % ash, 0.89 % fiber) with additional soybean oil (Aquafauna Biomarine, Hawthorne, CA) ("TM+SOY") constituted the lipid source for one diet, and a commercially produced canola oil plus DHA, which was further supplemented with DHA (DHAsco, Martek BioSciences, Columbia, MD) and ARA (ARAsco, Martek BioSciences, Columbia, MD) in an attempt to meet known fish requirements ("CAN+EFA") (Glencross, 2009), constituted the lipid source for the second experimental diet. This formulation with limited addition of ARA and DHA to the CAN+EFA diet was chosen to mimic currently available levels of these EFA's in genetically modified canola oil products. Neither experimental diet was supplemented with additional EPA due to the presence of the precursor lipid, alpha-linolenic acid (ALA). EPA has also been shown to be expendable for juvenile cobia, possibly only needed in trace amounts when using soybean oil and DHA (Trushenski *et al*., 2012). Proximate compositions of the three diets are presented in Table 5.1.2. To examine whether oxidation of the lipids occurred during the feed manufacturing process or storage and experiment duration, samples were sent to New Jersey Feed Labs (Trenton, NJ) for peroxide analysis.

Table 5.1.2. Proximate compositions (mean ± S.D.) of the three tested diets.

	TM+SOY	CAN+EFA	ARS Control
Lipid, g $100g^{-1}$ dm^{1}	9.07 ± 0.54	7.32 ± 0.46	7.82 ± 1.05
Ash, g $100g^{-1}$ dm^{1}	5.66 ± 0.04	5.10 ± 0.26	5.04 ± 0.14
Protein, g $100g^{-1}$ dm^{1}	49.00	51.38	44.88
Carbohydrate, g $100g^{-1}$ dm^{2}	35.55	35.22	39.75
Fiber, g $100g^{-1}$ $dm^{1,3}$	(0.72)	(0.98)	(2.51)
Moisture, g $100g^{-1}$ dm	4.02	7.70	5.11
Energy Content, MJ Kg^{-1}	20.69 ± 0.15	20.29 ± 0.13	19.30 ± 0.77

[1] After lyophilization.
[2] After lyophilization (100-% lipid, ash, protein, fiber).
[3] Values from New Jersey Feed Labs Analysis.

Growth trial

This study was carried out in accordance with the guidelines of the International Animal Care and Use Committee of the University of Maryland Medical School (IACUC protocol # 0610015). Cobia eggs were obtained from the University of Miami and reared at the University of Maryland Center for Environmental Science's (UMCES) Institute for Marine and Environmental Technology (IMET) in Baltimore, Md. USA. Fish were reared on a combination of live feed (rotifers and Artemia), Otohime (Reed Mariculture, Campbell, CA), and Zeigler Marine Grower (Zeigler Bros, Gardners, PA) until reaching approximately 130 g. Temperature and salinity were maintained at 27 °C and 25 ppt throughout larval and juvenile rearing. Twenty-five fish each were placed into six identical two cubic meter tanks, connected in pairs as recirculating systems that share filtration and life support systems which include protein skimming, ozonation, mechanical filtration in the form of bubble-bead filters, and biological filtration. With two replicate tanks per diet, tanks were assigned a diet so that no system contained both replicates of any single diet. Water quality samples were randomly taken from each system 2-3 times per week during the study and analyzed by the National Aquarium in Baltimore (NAIB) water quality lab located at IMET. Water quality was not significantly different between systems utilized

(ANOVA, $p>0.05$) during the study and overall parameters (Standard Methods #) were: dissolved oxygen, 5.69 ± 1.62 mg l^{-1}; temperature, 26.85 ± 1.77 °C; pH (4500-H^+), 7.61 ± 0.27; total ammonia nitrogen (4500-NH_3), 0.06 ± 0.06 mg l^{-1}; nitrite (4500-NO_2^-), 0.12 ± 0.08 mg l^{-1}; nitrate (4500-NO_3^-), 49.28 ± 8.87 mg $l^{-1,}$ alkalinity (2320), 95.77 ± 23.11 mEq l^{-1}; and salinity (2510) 24.91 ± 1.65 ppt.

Fish were anesthetized with tricaine methanosulfonate (MS-222, 70 mg l^{-1}, Finquel, Redmond, WA.) and weighed bi-weekly to record growth and reevaluate feed amounts according to total weight. Feed amounts were gradually reduced from 5% to 3% bw during the 8 week trial. At the conclusion of the eight weeks, six fish from each tank were randomly selected and euthanized with an overdose of MS-222 (150 mg l^{-1}). Three fish from each trial were used for fillet and whole body analyses.

Since lipid composition and source play important roles in final flavor and acceptance by consumers, we examined the changes that take place in a four week "finisher" period following the initial eight week trial. For four additional weeks after the initial eight week trial, tanks that had been fed the experimental diets (TM+SOY or CAN+EFA diets) during the initial eight weeks were switched to the fish oil ARS control diet, and were fed 3% bw daily with bi-weekly weighing. At the conclusion of the four week "finisher" period, three fish from each tank were randomly selected and sampled for fillet lipid analysis to assess the change in fatty acid profile following the diet switch.

Whole body and fillet samples were prepared by lyophilization to constant weight followed by homogenization by blending in a Waring blender (Waring Products, CT, USA). Ash percentage was determined by combustion at 600 °C for a minimum of 8 h (AOAC 942.05). Energy content was determined through bomb calorimetry (6772

calorimeteric thermometer and 1341 bomb calorimeter, Parr Instrument Company, Moline, IL) with calibration using benzoic acid every 20 samples. Crude protein was determined by CHN analysis (EPA method 440.0, Chesapeake Biological Laboratory, University of Maryland Center for Environmental Science, %N X 6.25). Amino acids (including taurine, AOAC 994.12) were determined by New Jersey Feed Labs, Inc. (Trenton, NJ) and examined for differences in amino acid profiles based on lipid source to determine if lipid source affected amino acid synthesis or deposition in fillet tissues.

Contaminant analysis

Samples of fillets were frozen, lyophilized, and sent for total mercury (Hg) and polychlorinated biphenyl (PCB) concentration quantification. The methods for total-Hg analysis in fish is derived from EPA Method 1631. Fillet samples were digested prior to analysis via hot reflux in a 7:4 HNO_3:H_2SO_4 acid mix. Total Hg in samples is reduced to elemental Hg with $SnCl_2$, stripped into the gas phase via a gas-liquid separator, trapped onto gold-coated bead columns and heated into the an-atomic fluorescence detector (Tekran 2600). Detection limits for most matrices are <0.05 ng g^{-1}. Routine QA/QC includes 10% blanks and duplicates/replicates, and analysis of CRMs (CRC DORM III) in every run.

For PCB analysis, approximately 20 g of freeze dried fillet tissue was pulverized in a clean mortar and pestle and transferred to a 100 ml glass beaker. A surrogate spike of PCBs 14 and 65 was added to all the samples before extraction to check for PCB recovery. The samples were then extracted by sonication using a mixture of hexane:acetone 1:1 (v/v) following EPA method SW846 3550B. The extracts were concentrated to 5 ml and treated

with concentrated sulfuric acid (method 3665A) for removal of lipids followed by activated copper treatment for removal of sulfur (method 3660B). The extracts were further cleaned to remove interfering organics by passing through a glass column layered with 20 g of acidified silica gel (30 % concentrated sulfuric acid by weight), 24 g of 3 % deactivated Florisil©, and 1 inch of anhydrous sodium sulfate (top to bottom). 150 ml of hexane was used for complete elution of PCBs from the column. The eluate was concentrated to 1 ml under a gentle stream of nitrogen for analysis. PCB congener analysis was done using a gas chromatograph with a micro electron-capture detector (6890N, Agilent Technologies, Santa Clara, CA, USA). A 60 m X 0.25 mm X 0.25 µm fused silica capillary column (RTX-5MS, Restek US, Bellefonte, PA) was used with helium as the carrier gas at constant flow of 1 ml min^{-1}. The oven temperature program began at 100 °C and was increased at the rate of 2 °C min^{-1} to 280 °C, followed by an increase of 10 °C min^{-1} to 300 °C and was held at this temperature for 6 min. Quantification of the target PCB compounds was performed using a multi-level calibration. Identification of PCB congeners was carried out by comparison of retention times in the chromatogram with that of PCB standards purchased as hexane solutions from Ultra Scientific (North Kingstown, RI). PCB 30 and 204 were used as the two internal standards because they are not present in commercial Aroclor mixtures. Using this method 89 PCB congeners, including some coeluting peaks were identified and quantified. Coeluting peaks were quantified as the sum of the congeners.

Lipid extraction and analysis of fatty acid methyl esters (FAMEs)

Total lipids were extracted as described in Harvey *et al.* (1987), a modified version of Bligh and Dyer (1959). Samples were subjected to three extractions with 4 ml of 2:1

methylene chloride (MeCl):methanol (MeOH), 1:1 methylene chloride (MeCl):methanol (MeOH) and 1:2 methylene chloride (MeCl):methanol (MeOH) in a sonicating water bath, the supernatant being removed each time to a clean round-bottom glass test tube. Water was added (0.25 vol) to the lipid extracts. After vortex mixing and phase separation, the organic layer was recovered and dried under N_2 gas. The residue was brought to 1 ml in 1:1 MeCl:MeOH, flushed with N_2 gas, and stored at –20 °C for less than 1 month.

10 % (vol.) of the lipid extract was used to generate fatty acid methyl esters (FAMEs) according to procedures described in Ederington *et al*. (1995). Five μg of C19:C21 internal standard (2.5 μg of each FA) and the lipid extract were added to glass tubes and dried under N_2 gas. Saponification of lipids was accomplished by adding 2.5 ml MeOH, 1.5 ml KOH-saturated MeOH, and 0.5 ml deionized water to each tube, which was then capped with a teflon-lined screw cap under a stream of N_2 gas and incubated at 70 °C for 1 h. After cooling, 0.5 ml of deionized water was added and neutral lipids (such as sterols) were removed by extracting 3x with hexane:ether (9:1). Fatty acids were then recovered by dropping the pH of the mixture remaining in the tubes to <2 by drop-wise addition of concentrated HCl, and repeating the extraction (3x) with hexane:ether (9:1). Neutral lipids were stored frozen or discarded and fatty acids were dried under N_2 gas. Methylation of fatty acids was accomplished using 10% (w/w) BF3 in methanol (Sigma-Aldrich, Bellefonte, PA USA). To each dried sample, 1 ml of BF3/methanol were added, the tubes were flushed with N_2 gas, capped with teflon-lined screw caps, and incubated at 70 °C for 30 min. After cooling, 1 ml of methanol was added to each tube and the samples were mixed by vortexing. The FAMEs were extracted with hexane:diethyl ether (9:1) three times. The upper organic phase containing FAMEs was recovered to new glass tubes, dried

under N_2 gas, resuspended in 500 µl hexane, transferred to crimp-top GC sample vials containing small-volume sample inserts, capped under N_2 gas, and stored at –80 °C until analysis.

Identification of FAMEs by gas chromatography retention with authentic quantitative standards (stds 3B, GLC-68D, GLC-17AA') from NU-CHECK, Inc. (Elysian, MN, USA) and qualitative standards (PUFA No. 1 – Marine Source) from Matreya (Pleasant Gap, PA, USA). Peaks in some samples were also confirmed by GC-MS. The Hewlett-Packard 6890 GC we used was equipped with a 30 m x 0.25 mm I.D. capillary column with 0.25 mm film thickness (DB Wax, J & W Scientific, Folson CA), and a flame ionization detector at 300 °C. The GC was run in 'constant flow rate' mode at 1.5 ml min^{-1} with H_2 as the carrier gas. The column-temperature profile was as follows: 50 °C for 0.5 min, hold at 195 °C for 15 min after ramping at 40 °C min^{-1}, and hold at 220 °C for 7 min after ramping at 2 °C min^{-1}. Total runtime was 38.13 min. The mass of FAMEs was determined by comparison with the internal C19:C21 standards run with each sample. The relative distribution (% FAMEs) was calculated based on the peak area of a given peak divided by the total peak area of identified FAMEs in a sample.

Dilution model

Control comparisons were made between fatty acid compositions of fillets from fish raised on a commercial diet from 30 g to 130 g average weight before beginning the trial in order to determine if fatty acid profiles were likely to change simply due to the growth of the fish in general.

The dilution model of fatty acid turnover in fish is expressed as:

$$\mathrm{Pt} = \mathrm{Pr} + (\mathrm{Pr} - \mathrm{Pi})/(\frac{\mathrm{Qt}}{\mathrm{Qi}}),$$

where Pt is the percentage of a specific fatty acid in an experimental fillet at time T following the dietary change, Pi and Pr are the percentages of the same fatty acid before the diet change (initially) and in a reference fillet (fish fed the ARS diet throughout), and Qi and Qt are the total lipid content initially and at time T, respectively (Robin *et al*., 2003; Trushenski and Boesenberg, 2009). We compared the fillet fatty acid compositions from fish fed the three plant protein-based test diets, Pr, with the predicted values obtained from the model using the fatty acid profiles of the commercial diet fed to the fish prior to the trial as the initial, Pi. We then compared the fatty acid compositions of the fillets from fish that were involved in the four week switch to the ARS control diet, Pr, to the fatty acid profiles predicted by the dilution model using the TM+SOY and CAN+EFA fillet profiles as the initial, Pi.

Statistics

All statistical tests were run using Aabel v2.4.2 (Gigawiz Ltd., OK, USA) with significance values of $p < 0.05$. The experimental design was treated as a balanced incomplete block design with systems treated as blocks with not all diets represented within each block. ANOVA analysis was used to test differences in means between groups and two-way ANOVA was used to test differences in the growth curves from the trials. Means were averaged across each treatment if no significant difference was found within each treatment. Statistics were not performed on the carbohydrate and fiber content pool of the

tissues since all other components were measured directly, therefore dramatically increasing and pooling the error of this one group's measurement.

Results

Diet formulation

The protein content of the two fish oil replacement diets are slightly higher (Table 5.1.2) than the plant protein fish oil reference diet (ARS). Crude protein was lower in the ARS diet than either of the experimental diets. There were no differences between the diets in overall energy, lipid, or moisture content (Table 5.1.2). The canola oil prior to and post addition of DHA and ARA had peroxide values of 5.2 and 2.0 mep kg^{-1}, respectively. The plant protein diet containing thraustochytrid meal and soybean oil had a peroxide value of 18.0 mep kg^{-1}, the CAN+EFA diet had a peroxide value of 2.2 mep kg^{-1}, and the ARS diet with fish oil as the lipid source had a peroxide value of 10.0 mep kg^{-1}. All these measures are below the accepted rancid values of 20 Mep kg^{-1} (Turchini *et al*., 2011).

Growth trial

At the end of the eight week trial, growth rates and average weights were significantly higher in fish fed the TM+SOY and ARS diets than for fish fed the CAN+EFA diet (ANOVA, $p<0.001$, Figure 5.1.1),with no significant difference between the growth rates of fish fed the TM+SOY and ARS diets (ANOVA, $p=0.115$). Survival (58 %) was significantly lower for fish fed the CAN+EFA diet compared to 100 % and 98 % on the TM+SOY and ARS diets, respectively (ANOVA, $p<0.05$, Table 5.1.3). At the end of the four week period following the initial trial when fish were switched to the ARS diet,

specific growth rates approached a common value of around 1.35 regardless of prior dietary history.

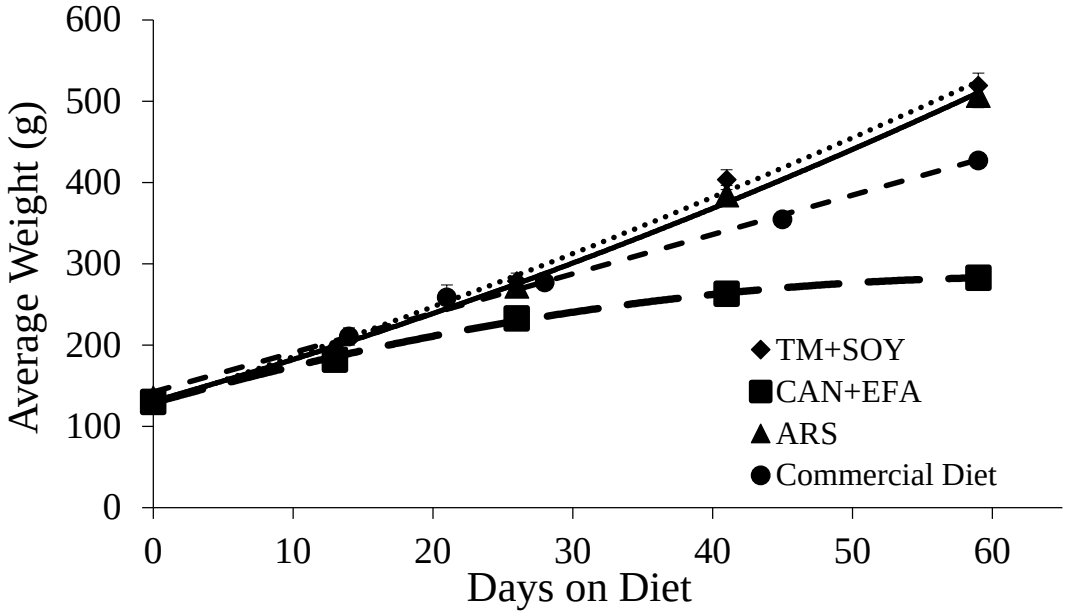


Figure 5.1.1. Growth of juvenile cobia (130 g initial weight) during 8 week trial. 25 fish per 2 m^3 tank, 2 tanks per dietary treatment, maintained at 27 °C and 25 ppt salinity. Symbols represent means ± standard deviations.

Table 5.1.3. Production characteristics from the eight week grow out trial. Data from a previous grow out with juvenile cobia on a fish meal and fish oil based, commercially produced diet are included to show overall effects of fish meal and fish oil replacement.

	TM+SOY	CAN+EFA	ARS Control	Commercial Diet (2009 grow out data)[6]
Weight Gain (%)[1]	288[a]	117[b]	275[a]	218
Fillet Yield (%)[2]	25.93 ± 3.34	20.88 ± 4.34	25.71 ± 1.90	20.12 ± 0.01
FCR[3]	1.42[a]	2.98[b]	1.46[a]	1.85
Plasma Osmolality	358.25 ± 30.59[a]	311.75 ± 36.82[b]	327.58 ± 13.83[a,b]	na
PCB Content (ng g^{-1})	8.40 ± 1.54	9.30 ± 3.41	13.2 ± 4.2	45.5 ± 3.8
Mercury Content (ng g^{-1})	30.20 ± 3.53	48.17 ± 16.24	20.14 ± 2.96	71.37 ± 3.56
Hepatosomatic Index[4]	1.83 ± 0.19[a]	2.93 ± 0.79[b]	2.66 ± 0.55[a,b]	3.15 ± 0.002
Specific Growth Rate[5]	2.48[a]	1.42[b]	2.36[a]	1.93
Survival	100 %[b]	58 %[a]	98 %[b]	100 %

[1] Weight gain=(final tank weight - initial tank weight)/ initial tank weight*100.
[2] Fillet yield=(fillet weight/body weight)*100.
[3] FCR=Feed conversion ratio= grams fed/grams gained.
[4] Hepatomsomatic index=liver weight/ body weight.
[5] SGR=specific growth rate= $((\ln BW_2 - \ln BW_1)*(\text{days of trial}^{-1}))*100$.
[6] Initital weight 120g, eight week trial (Watson *et al.* 2011).
Values in the same row with different superscripts are significantly different ($p<0.05$), no superscript indicates no significant difference within a category.

The proximate compositions for the whole body and fillet tissues from fish fed each of the three diets are presented in Tables 5.1.4 and 5.1.5. Using final body weight as a covariate, there was no significant difference in whole body lipid, ash, or moisture content but whole body protein was significantly lower in the ARC fed fish than the TM+SOY fed fish (Table 5.1.4, ANCOVA, $p<0.05$). There were no significant differences in fillet lipid or protein between the diets however there were significant differences in ash with CAN+EFA being higher than TM+SOY and in fillet moisture content with CAN+EFA being higher than the other two diets (Table 5.1.5, ANOVA). There were several significant differences in the overall production characteristics of percent weight gain and feed conversion ratio (FCR) as well as the previously mentioned differences in survival and specific growth rate, all of which favored the TM+SOY and ARS diets over the CAN+EFA diet (Table 5.1.3).

Table 5.1.4. Proximate compositions (mean ± S.D) of whole body tissues from animals fed the three experimental diets.

	TM+SOY	CAN+EFA	ARS Control
Lipid, g 100 g^{-1} dm[1]	11.39 ± 3.06	7.73 ± 2.45	11.15 ± 1.91
Ash, g 100 g^{-1} dm[1]	9.86 ± 0.68	12.64 ± 1.95	10.37 ± 1.49
Protein, g 100 g^{-1} dm[1]	71.84 ± 6.53[a]	70.34 ± 7.20[a,b]	59.90 ± 2.24[b]
Carbohydrate/Fiber, g 100 g^{-1} dm[2]	6.91	9.29	18.58
Moisture, g 100 g^{-1} dm	69.13 ± 0.03	74.75 ± 0.02	71.08 ± 0.03
Energy Content, MJ Kg^{-1}	23.61 ± 0.62[b]	21.72 ± 1.03[a]	23.36 ± 0.90[b]
Body Weight[3]	516.67 ± 77.55	332.00 ±72.15	543.50 ± 105.28

[1] After lyophilization.
[2] After lyophilization (100-% lipid, ash, protein).
[3] For fish used in analyses, g.
Values in the same row with different superscripts are significantly different ($p<0.05$), no superscript indicates no significant difference within a category. Final body weight used as covariate for proximate analyses in ANCOVA.

Contaminant analysis

Fillet PCB levels ranged from 8.4-13.2 ng g^{-1} dry weight, with no significant difference between the diets, however the ARS fish oil diet resulted in the highest PCB levels (13.2 ± 4.2 ng g^{-1}). Mercury levels ranged from 20.14-48.17 ng g^{-1} with no significant differences between treatments. Mercury levels were highest in the CAN+EFA fed fish (48.17 ± 16.24 ng g^{-1}).

Fatty acid analysis

Fatty acid compositions did not differ significantly between fish raised on the commercial fish meal and fish oil based diet from 30 g to 130 g average weight prior to the start of this study, indicating that growth alone did not alter fatty acid composition of fillet tissue within these size ranges (linear regression, R^2=0.992). Table 5.1.6 presents the fatty acid profiles for the fillet and whole body tissues after the initial eight week grow-out on the three diets as well as the fillet profiles obtained after the additional four weeks when switched to the ARS diet containing fish oil. Figure 5.1.2 presents a radial diagram comparing the fatty acid profiles of the fillet and whole body tissues for fish from each as a ratio of the same fatty acid concentrations within the diet. Deviations from the line of equality represent enhancement or depletion of each fatty acid when compared to the diet fed, with values greater than 1 indicating an enrichment in the fillet compared to the diet, and values less than 1 indicating a depletion in the fillet compared to the diet. Erucic acid (22:1n9) is a toxic fatty acid present in canola oil, however it was not detected in the diets, whole body, or fillet tissues of any of the animals fed the CAN+EFA diet.

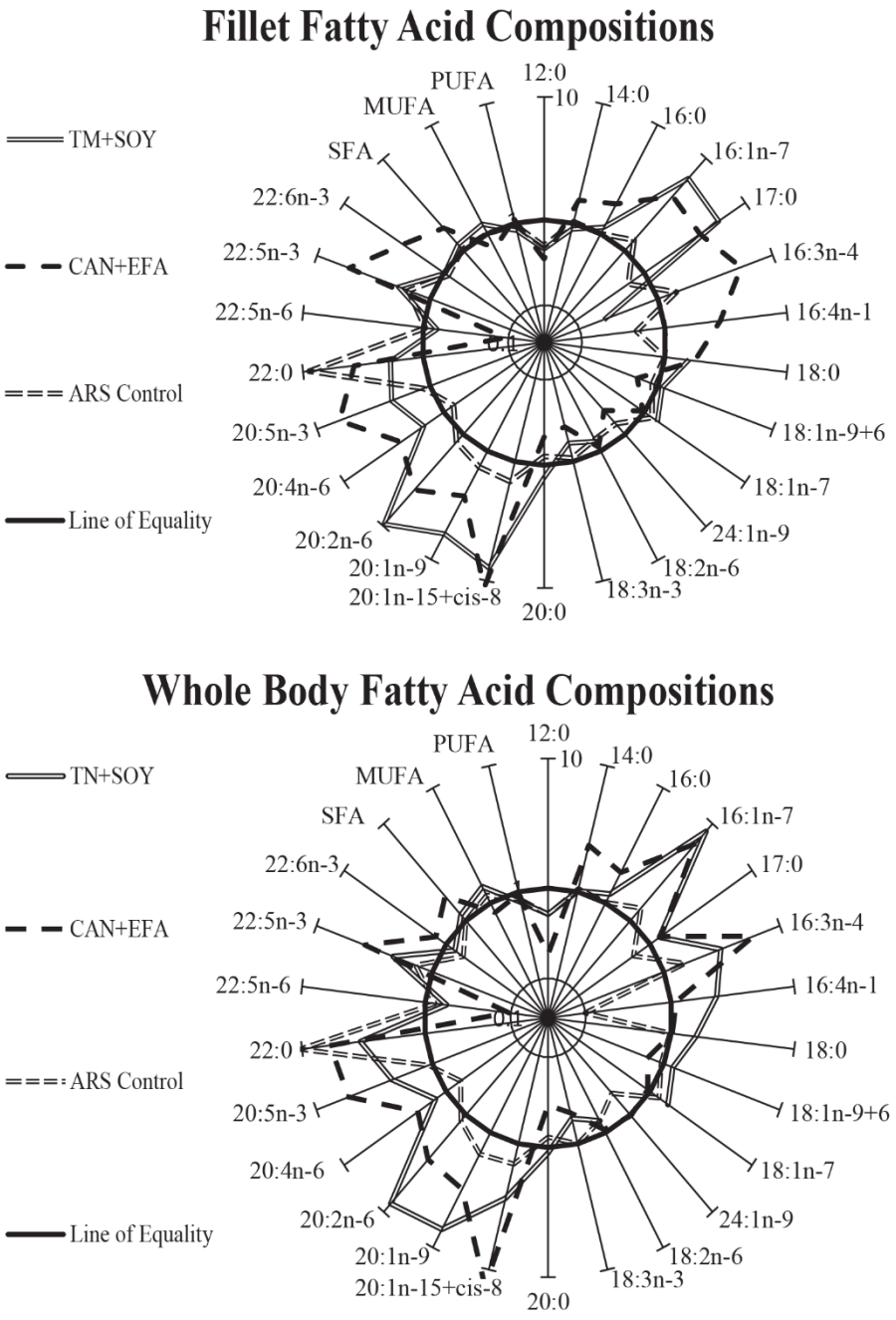


Figure 5.1.2. Fatty acid compositions of fillet and whole body tissues from juvenile cobia fed experimental fish oil replacement diets expressed as a fraction of dietary total lipid profile. Values were calculated from relative fatty acid methyl ester (FAME) composition (Fillet or whole body fatty acid concentration/Diet fatty acid concentration). Based on this calculation, a value of 1 represents equality between fillet and dietary fatty acid composition.

Dilution model

Figure 5.1.3 presents the fatty acid profiles of fillets from fish fed the control ARS diet throughout the study as compared to fillets from fish fed either experimental diet for the 8 week grow out phase as well as after switching to the ARS diet with fish oil for the 4 week recovery period. The dilution model accurately predicted the change in fatty acid composition as percentages of individual fatty acids (% dw) in fillets of fish fed either experimental diet (CAN+EFA diet switched to the ARS diet, linear regression, R^2=0.9513; TM+SOY diet switch to the ARS diet, linear regression, R^2=0.8564). The dilution model overestimated the concentrations of 16:0, 20:5n-3, and 16:1n-7 and underestimated the concentrations of 18:1n-9+6, 18:2n-6, and 22:6n-3 in the CAN+EFA predictions. The model also overestimated 20:5n-3 and 16:1n-7 and underestimated 18:2n-6 and 22:6n-3 in the TM+SOY predictions.

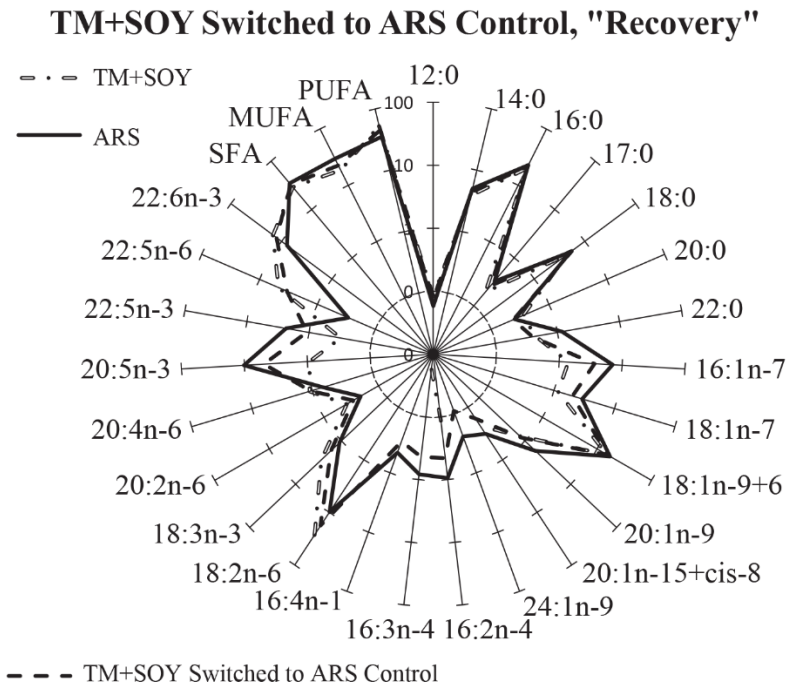
Table 5.1.5. Proximate compositions (mean ± S.D.) of fillet tissue from animals fed the three experimental diets.

	TM+SOY	CAN+EFA	ARS Control
Lipid, g 100 g^{-1} dm[1]	5.81 ± 0.77	5.53 ± 3.15	7.90 ± 1.75
Ash, g 100 g^{-1} dm[1]	5.40 ± 0.32[a]	6.45 ± 0.94[b]	5.46 ± 0.34[a,b]
Protein, g 100 g^{-1} dm[1]	78.75 ± 5.80	83.85 ± 6.01	80.21 ± 3.37
Taurine, g 100 g^{-1} dm[2]	(0.81)	(0.79)	(0.76)
Carbohydrate/Fiber, g 100 g^{-1} dm[3]	10.04	4.17	6.43
Moisture, g 100 g^{-1} dm	74.14 ± 0.02[a]	78.40 ±0.02[b]	74.10 ± 0.01[a]
Energy Content, MJ Kg^{-1}	22.630 ± 0.42[a]	21.720 ± 1.04[b]	22.83 ± 0.63[a]
Body Weight[4]	547.00 ± 132.98	321.00 ± 98.32	530.17 ± 79.87

[1] After lyophilization.
[2] New Jersey Feed Labs analysis.
[3] After lyophilization (100-% lipid, ash, protein).
[4] For fish used in analyses, g.
Values in the same row with different superscripts are significantly different ($p<0.05$), no superscript indicates no significant difference within a category.

Table 5.1.6. Fatty acid compositions (g 100 g^{-1}) of fillet and whole body tissues from the eight week grow out as well as recovery fillets from the additional four week period where animals that were on the two fish oil replacement diets were fed the control, fish oil based diet.

Fatty Acid (g $100g^{-1}$)	Fillets			Whole Body			Recovery Fillets	
	TM+SOY	**CAN+EFA**	**ARS Control**	**TM+SOY**	**CAN+EFA**	**ARS Control**	**TM+SOY**	**CAN+EFA**
12:0	0.08 ± 0.01	0.16 ± 0.29	0.06 ± 0.002	0.09 ± 0.01	0.11 ± 0.01	0.06 ± 0.01	0.08 ± 0.01	0.03 ± 0.04
14:0	3.86 ± 0.12	1.88 ± 0.43	5.05 ± 0.24	4.41 ± 0.29	2.82 ± 0.67	5.16 ± 0.25	4.40 ± 0.34	3.71 ± 0.61
16:0	19.78 ± 0.49	14.61 ± 1.07	22.85 ± 0.78	20.64 ± 0.74	14.19 ± 0.87	23.01 ± 0.87	21.84 ± 0.29	20.04 ± 0.66
17:0	0.20 ± 0.02	0.22 ± 0.11	0.32 ± 0.01	0.21 ± 0.01	0.19 ± 0.03	0.33 ± 0.01	0.27 ± 0.02	0.32 ± 0.04
18:0	4.36 ± 0.65	6.13 ± 3.27	5.25 ± 0.31	4.42 ± 0.31	3.55 ± 0.46	5.23 ± 0.36	5.55 ± 1.13	5.99 ± 0.88
20:0	0.35 ± 0.18	0.49 ± 0.19	0.26 ± 0.04	0.31 ± 0.08	0.38 ± 0.15	0.25 ± 0.05	0.24 ± 0.05	0.27 ± 0.16
22:0	0.45 ± 0.47	1.40 ± 0.37	1.18 ± 0.29	0.80 ± 0.30	2.23 ± 1.01	1.40 ± 0.37	0.40 ± 0.05	0.69 ± 0.12
SFA	29.08 ± 0.82	24.90 ± 4.34	34.96 ± 0.65	30.88 ± 1.01	23.46 ± 2.82	35.44 ± 0.97	32.78 ± 0.72	31.05 ± 0.66
16:1n-7	1.40 ± 0.30	1.78 ± 0.54	7.07 ± 0.28	1.91 ± 0.29	2.82 ± 0.52	7.37 ± 0.31	3.65 ± 0.38	4.93 ± 0.81
18:1n-7	1.18 ± 0.06	2.48 ± 0.16	2.94 ± 0.07	1.31 ± 0.06	2.46 ± 0.06	3.00 ± 0.04	2.02 ± 0.12	2.94 ± 0.05
18:1n-9+6	15.12 ± 0.49	31.98 ± 7.15	17.31 ± 0.66	16.51 ± 1.18	35.39 ± 4.21	18.60 ± 0.50	15.71 ± 0.95	20.58 ± 1.38
20:1n-15+cis-8	0.23 ± 0.25	0.34 ± 0.20	0.32 ± 0.07	0.16 ± 0.13	0.40 ± 0.14	0.32 ± 0.07	0.17 ± 0.03	0.35 ± 0.06
20:1n-9	0.78 ± 0.35	1.97 ± 0.39	1.69 ± 0.26	0.94 ± 0.35	2.38 ± 1.12	1.87 ± 0.30	0.84 ± 0.15	1.39 ± 0.24
24:1n-9	0	0.07 ± 0.11	0.24 ± 0.06	0	0.13 ± 0.14	0.19 ± 0.06	0.09 ± 0.07	0.09 ± 0.14
MUFA	18.70 ± 0.86	38.55 ± 7.29	29.33 ± 0.65	20.88 ± 1.26	43.57 ± 2.91	31.34 ± 0.28	22.39 ± 1.13	30.18 ± 1.95
16:3n-4	0.02 ± 0.06	0.32 ± 0.17	0.82 ± 0.04	0.16 ± 0.84	0.35 ± 0.15	0.86 ± 0.04	0.43 ± 0.04	0.59 ± 0.18
16:4n-1	0	0.18 ± 0.21	0.46 ± 0.47	0.08 ± 0.06	0.07 ± 0.06	0.16 ± 0.03	0.30 ± 0.11	0.51 ± 0.35
18:2n-6	26.84 ± 0.68	21.02 ± 1.50	9.87 ± 0.77	26.33 ± 0.79	21.44 ± 1.13	10.03 ± 1.03	17.94 ± 1.40	12.95 ± 1.26
18:3n-3	2.76 ± 0.09	3.20 ± 1.14	1.10 ± 0.08	2.48 ± 0.85	3.55 ± 1.31	1.19 ± 0.10	1.71 ± 0.45	1.33 ± 0.19
20:2n-6	0.38 ± 0.18	0.53 ± 0.19	0.21 ± 0.07	0.33 ± 0.17	0.38 ± 0.09	0.20 ± 0.08	0.29 ± 0.08	0.30 ± 0.16
20:4n-6	1.11 ± 0.18	0.87 ± 0.50	0.67 ± 0.08	0.89 ± 0.17	0.61 ± 0.25	0.60 ± 0.07	1.07 ± 0.33	1.00 ± 0.20
20:5n-3	0.92 ± 0.24	1.71 ± 0.75	10.15 ± 0.79	0.93 ± 0.16	1.43 ± 0.34	9.60 ± 0.70	4.91 ± 0.31	8.21 ± 0.98
22:5n-6	4.69 ± 0.36	0.14 ± 0.32	0.29 ± 0.11	4.05 ± 0.23	0.14 ± 0.18	0.25 ± 0.04	3.35 ± 0.64	0.85 ± 0.88
22:5n-3	0.31 ± 0.33	0.83 ± 0.28	2.33 ± 0.11	0.36 ± 0.11	0.62 ± 0.15	2.09 ± 0.16	1.08 ± 0.10	1.95 ± 0.12
22:6n-3	14.71 ± 0.83	7.27 ± 3.74	7.80 ± 0.65	12.21 ± 0.60	3.59 ± 0.68	5.83 ± 0.53	12.63 ± 1.13	9.43 ± 1.93
PUFA	52.22 ± 1.38	36.55 ± 3.99	35.70 ± 1.21	48.21 ± 1.67	32.96 ± 1.20	33.33 ± 1.17	44.83 ± 0.77	38.77 ± 2.34
n-3	18.70 ± 0.73	13.00 ± 3.56	21.86 ± 0.77	15.90 ± 1.06	9.04 ± 1.31	18.06 ± 1.14	20.90 ± 0.65	21.79 ± 0.75
n-6	33.38 ± 1.03	22.56 ± 1.12	11.37 ± 0.95	31.60 ± 1.03	22.57 ± 0.91	11.10 ± 1.11	22.65 ± 0.78	15.10 ± 1.82
n-3:n-6	0.56 ± 0.02	0.58 ± 0.16	1.94 ± 0.20	0.50 ± 0.03	0.40 ± 0.06	1.65 ± 0.23	0.92 ± 0.05	1.46 ± 0.15



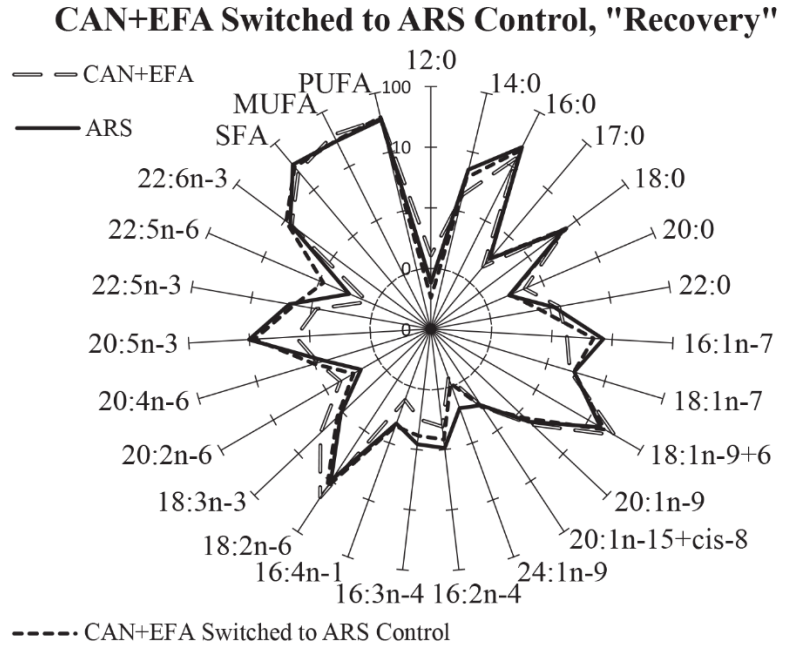


Figure 5.1.3. Fatty acid compositions of fillet tissues from juvenile cobia after eight weeks on experimental lipid replacement diets, eight weeks on fish oil based control diet, and eight weeks on experimental fish oil replacement diet with four "recovery" weeks on fish oil based control diet.

Amino acid analysis

Overall fillet amino acid profiles of fish fed the CAN+EFA diet were unaffected by the low growth and poor survival observed on this diet, and none of the three lipid sources used in the study appear to have had an effect on fillet amino acid composition (ANOVA, $P>0.05$), indicating limited interactions between amino acid and fatty acid uptake and deposition within the fish. At a dietary taurine level of 1.5 % inclusion, taurine accumulates in fillets to ~0.80 % (Table 5.1.5).

Discussion

Aquaculture of cobia has risen dramatically over the last 10 to 15 years primarily due to its favorable production characteristics, most notably being the ability to reach market size (4-6 kg) within a year (Nhu *et al*., 2010). However, only recently has work been published on the specific nutritional requirements of this species due in part to the relative success of commercial compounded feeds (Chou *et al*., 2001). Fraser and Davies (2009) recently summarized these studies for cobia and determined optimum dietary protein and lipid levels in juvenile cobia to be 45 and 5-15 % dry weight, respectively. Zhou *et al*. (2006) determined the methionine requirement to be 2.64 % of dietary protein dry weight and lysine requirement to be 5.3 % of dietary protein (Zhou *et al.*, 2007). Moreover, cobia exhibits a low capacity for protein sparing through dietary lipid, preferring mainly protein as the primary dietary energy source to lipid. Excess lipid levels (>12 %) reduce growth (Craig *et al.*, 2006). Using the fatty acid profile of cobia eggs, Fraser and Davies (2009) estimate that juvenile cobia require high amounts of long-chain polyunsaturated fatty acids (LC-PUFA) DHA, EPA and ARA as these account for

approximately 80 % of the PUFAs in cobia eggs and yolk sac larvae. Considering these requirements and the success with a plant protein-based formulation for trout (Gaylord *et al*., 2007) we formulated a fish meal free diet for cobia (ARS). An important addition to this formulation was taurine, which is proving to be essential for efficient use of plant protein diets (Gaylord *et al.*, 2006; Kim *et al.*, 2005a; Lunger *et al*., 2007b; Watson *et al*., 2012). We extended this formulation by replacing the fish oil with plant-based oils containing sufficient LC-PUFAs through either addition of DHA and ARA rich oils (Martek) or addition of thraustochytrid meal (TM+SOY) which is rich in DHA and the n-6 fatty acid, docosapentaenoic acid (DPA, 22:5n-6). No addition of EPA was made to either of the fish oil replacements since EPA has been shown to be expendable, potentially only needed in trace amounts (Trushenski *et al*., 2012).

As clear from the growth and production characteristics, the plant protein diet with the microbial single cell meal and soybean oil source is equivalent in performance to the ARS Cobia diet with fish oil. The protein and ash content differences between the diets had no effect on growth rates, FCRs, or fillet characteristics. The unique fatty acid profiles for the diets are reflected in the fatty acid profiles of the whole body and fillets of each dietary treatment with patterns of enrichment, a comparison of individual fatty acid concentrations found in the diet to the concentrations found in the fish tissues, in whole body tissues and fillet tissues are very similar for each of the three diets (Figure 5.1.2). Both experimental lipid sources result in similar patterns of enrichment, with the exception of a greater enrichment of DHA in the fillets of fish fed the CAN+EFA diet, which would be expected in comparison to either of the other diets, due to the low concentration of DHA in the CAN+EFA diet. ARA and EPA are both enriched in the fillets compared to the diets

for both of the experimental diets, which would be expected considering there was no supplemental addition of EPA to either experimental diet, and low levels of ARA in both. With few exceptions, the enrichment pattern observed for fish fed the control ARS diet is very close to the 1:1 line of equality for both whole body tissues and fillet tissues, reinforcing how important matching the fatty acid profile of fish oil is for any replacement source.

Removal of fish meal and fish oil from dietary formulations resulted in drastically reduced PCB and mercury levels compared to similarly sized fish also reared for eight weeks on a commercial diet. Replacing fish meal alone and still utilizing fish oil resulted in PCB levels 71 % lower and mercury levels 72 % lower than utilizing both fish derived ingredients (Table 5.1.3). Further removal of fish oil from the formulation did not result in any greater reduction in PCBs, and the alternate lipid sources resulted in levels higher than the ARS fish oil diet used in this study, but levels still much lower than those in fish fed the commercial diet. This represents a significant consumer added advantage of utilizing both fish meal and fish oil replacements, in addition to the potential for more sustainable diets.

Figure 5.1.3 represents the recovery of the fillet fatty acid profile towards the fatty acid profile observed when fish were fed the ARS diet throughout the study. The four week switch to the ARS diet was not enough to fully recover the ARS fatty acid profile, however changes have taken place and many of the fatty acids have started to transition towards the ARS concentrations. Interestingly the concentration of DHA stays high relative to the ARS level, even though the fish have spent four recovery weeks on the lower DHA content ARS diet. A simple dilution model accurately predicted the fatty acid profile for the switch from

either experimental diet to the ARS fish oil control diet. The model had trouble predicting concentrations of 18:2n-6, 16:1n-7, 20:5n-3, and 22:6n-3 in both dietary switches. This is most likely due to these four fatty acids undergoing the most modification and conversion as opposed to simple dilution by different concentrations in the ARS diet. Understanding the dilution and rate of change in fatty acid profiles of fillet tissues can help reduce production costs by utilizing cheap lipid sources, followed by a short period on a replete finishing diet to obtain more appealing fatty acid profiles in the final product. This process allows for the maintenance of a healthy source of essential fatty acids for the consumer without substantially altering taste.

Both the TM+SOY and ARS diets performed better than the CAN+EFA based diet. The low survival and poor growth along with the production of a fillet with higher ash content and lower energy content lead us to the conclusion that sufficient EFAs were not added to canola oil to meet the requirements for cobia. The minimum EFA requirements for cobia are currently not known, however the g EFA kg^{-1} diet for marine fish has been suggested to be in the range of 0.5-1 (Glencross, 2009). As mentioned previously, the addition of DHA and ARA to the canola oil source was selected to mimic current genetically modified crop levels. This resulted in an EFA level of 0.25 g kg^{-1} diet for the CAN+EFA diet. In contrast the TM+SOY diet has an EFA level of 1.21 g kg^{-1} diet and the ARS diet has an EFA level of 1.70 g kg^{-1} diet. Plotting specific growth rate vs. g EFA kg^{-1} (Figure 5.1.4) reveals our CAN+EFA diet may contain insufficient EFA to meet requirements for maximum growth. Concentrations of 0.5 g EFA kg^{-1} is considered minimum for other marine fish and 0.8 to 1.2 g EFA kg^{-1} has been suggested by Chou *et al.* (2001) for cobia. Although the CAN+EFA diet appears to be too low in overall EFA

content (Figure 5.1.4 insert), this short term trial with 130 g fish on a diet not completely devoid of EFAs should not alone have resulted in such poor survival (58 %), as the existing pool of fatty acids should be sufficient for survival. Potential explanations for the low survival include dilution of EFAs below minimum requirements, the presence of high dietary oleic acid which has been shown to have negative effects on leukocytes and respiratory organs in mammals (Brinkmann *et al.*, 2013; de Albuquerque *et al.*, 2012), an inhibition of even minimal conversion of precursors to EPA and DHA as evidenced in the rat model with ALA (Gibson *et al.*, 2013), or an overall loss of EFAs throughout the whole body tissues of the cobia. Whole body g EFA kg^{-1} was 1.56 ± 0.24 at the initiation of the study, with the final concentrations being 1.36 ± 0.36 and 1.53 ± 0.34 for the TM+SOY and ARS diets, respectively. In contrast to this, the g EFA kg^{-1} in whole body tissue of the CAN+EFA fish dropped significantly to 0.35 ± 0.07 at the conclusion of the eight week trial (Figure 5.1.4 insert). Prior work with juvenile cobia (50-160 g) found that up to 66 % of fish oil can be replaced with soybean oil in diets with 10-12 % crude lipid, without significant reduction in production characteristics (Trushenski *et al.*, 2011) and that addition of DHA, EPA, and combinations of the two resulted in higher concentrations of these in tissues (Trushenski *et al.*, 2012). Using the EFA levels reported in these studies with the reported specific growth rates, we see a range of EFA concentrations utilized (Figure 5.1.4), with a general trend of increasing SGR as dietary EFAs are increased. However, supplementing diets with exogenous EFAs can be expensive, and little added advantage of overall EFA levels above ~1.2 is observed (Figure 5.1.4). Reducing the EFA levels to 1.2 g EFA kg^{-1} could be a considerable savings in cost in future formulations.

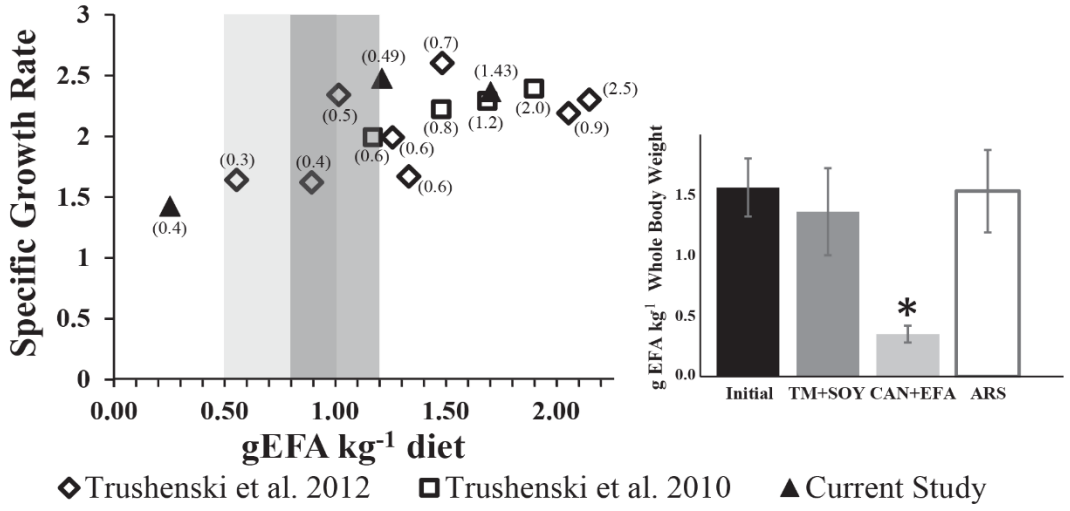


Figure 5.1.4. Grams of essential fatty acids per kg of diet (gEFA= (% dietary lipid * 0.85)*(% DHA+EPA+ARA/100) and the specific growth rates obtained by various lipid source diets for juvenile cobia. Fatty acids assumed to be ~85 % of total dietary lipid. Light grey shaded area represents 0.5-1.0 g EFA kg^{-1} minimum suggested for marine fish by Glencross (2009). Dark grey shaded area represents 0.8-1.2 g EFA kg^{-1} suggested for juvenile cobia by Chou *et al*. (2001). Data from Trushenski *et al*. (2011) with juvenile cobia (62g initial weight, 27.1°C, 6 week trial, 10.6-12.2% lipid diets) Trushenski *et al*. (2012) with juvenile cobia (52g initial weight, 26.4°C, 9.4-11.0% lipid diets). Numbers in parentheses' are the n-3:n-6 ratio of each diet. Insert bar graph is g EFA kg^{-1} whole body weight for initial fish (black) compared to the final g EFA kg^{-1} whole body weight of fish from each of the three experimental diets at the conclusion of the eight week trial. Star indicates significant difference (ANOVA, $p<0.05$).

While it could be argued that since we only added DHA and ARA and not EPA to the canola oil, the poor performance of this diet is due to EPA deficiency. Our excellent growth performance with the TM+SOY diet which contains no EPA addition indicates that perhaps EPA is not required in as high levels as previously though for sustained high growth in cobia, a result also observed by Trushenski *et al*. (2012). It could also be that DPA has partially taken over the role of EPA or that there is some inter-conversion or retro-conversion from other fatty acids. Further studies with higher levels of DHA addition to canola oil should help determine which explanation is correct. Because of the higher DPA

level in the fillet of fish fed TM+SOY diet, we examined whether switching to the ARS with fish oil would recover a more natural fatty acid composition, which was observed. The fatty acid profiles from fish raised on the CAN+EFA diet began to transition to that observed in the ARS fillets with survival maintained at 100 % during the recovery period and growth showing slight improvement. This indicates that whatever the cause of the poor performance during the eight week grow-out, potential remediation occurred after the switch to the fish oil based ARS diet.

The growth, FCR, and amino and fatty acid profiles of the fillets of fish fed the TM+SOY diet lead us to believe that this combination of lipid sources is a suitable fish oil replacement candidate. Carter *et al*. (2003) observed no significant decrease in production characteristics of Atlantic salmon fed a fish oil free diet containing 10 % of a thraustochytrid meal as part of the fish oil replacement source, but did observe lower survival after transfer to seawater and *Vibrio* challenges compared to fish fed fish oil. Further work with cobia and other high value marine species is needed to determine if any alternate protein and lipid sources or combinations result in similar immune deficiencies, especially considering the high rearing density in intensive recirculating aquaculture systems.

The ability of cobia to thrive on low lipid and alternative lipid sources may be due to the role of taurine as a bile salt conjugate, but may also be due in part to some EFA biosynthesis capacity. Monroig *et al*. (2011) have described a fatty acid elongase in cobia embryos that is similar to mammalian elongase of very long-chain fatty acids (Elovl4). *Elovl4, elovl5, and* Δ*6fad* were all observed between 18-36 h post fertilization in cobia embryos, indicating an ability to biosynthesize LC-PUFAs, including DHA (Monroig et

al., 2011). However, given the poor performance of the CAN+EFA diet, the biosynthetic capacity for LC-PUFAs must be limited in juvenile cobia.

New technology is advancing quickly to increase the content of EFAs in alternative sources, but more work must be done to determine these levels for the highly sought after marine carnivores that intensive aquaculture relies upon. With aquaculture on the verge of consuming virtually all of the fish meal and fish oil produced worldwide (Naylor *et al.*, 2009), and being relied upon to expand and support increasing demand for protein (Tacon and Metian, 2008), it is clear that current practices are unsustainable and do not allow for the needed expansion. However, complete fish product replacement is possible, even in fast growing marine carnivores like cobia, if care is taken to meet nutritional requirements. Based on these short term trial results, some currently available commercial alternative lipid sources appear sufficient for fish oil replacement. Through judicious selection of highly digestible plant protein sources, addition of essential amino acids including taurine (Gaylord *et al*., 2007) and adequate essential fatty acids (Glencross, 2009), a completely fish product free diet can be formulated to allow the expansion of aquaculture for future global protein needs.

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Taurine supplemented plant protein-based diets with alternative lipid sources for juvenile gilthead sea bream, Sparus aurata

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Abstract

Two lipid sources were examined as fish oil replacements in fishmeal-free, plant protein-based diets for juvenile gilthead sea bream, *Sparus aurata*. A thraustochytrid meal plus soybean oil (TM+SOY) and a canola oil (CAN+EFA) supplemented with docosahexaenoic (DHA) and arachidonic acids (ARA). A twelve week trial was undertaken to examine the performance of the diets and to assess whole body and fillet fatty acid profiles resulting from the use of alternative lipid sources was comparable to fish oil. A finishing period was also utilized in an attempt to recover a fish oil fatty acid profile in the fillets of fish fed the alternative lipid source diets. The TM+SOY diet significantly outperformed the fish oil control diet in terms of weight gain, feed conversion ratio, condition factor, and protein efficiency ratio while maintaining similar fillet yield while the CAN+EFA diet performed similarly to the control diet. Although fatty acid profiles in the whole bodies and fillets at the conclusion of the trial were representative of that of the diet fed, transition toward the fish oil profile was observed at the conclusion of the finishing period. Overall, both alternative lipid sources performed equivalently or better than the fish oil control, a significant finding for this species. Low dietary lipid (~7-9 %) was just as effective as high dietary lipid input, observed in other studies, and did not result in lowered

fillet lipid levels or growth performance. As a note taurine had to be supplemented to the plant protein diets.

Introduction

Fisheries, and to a growing extent aquaculture, are responsible for a major source of n-3 and n-6 polyunsaturated fatty acids (PUFA) for the human population (FAO, 2012; Gjedrem *et al*., 2012; Glencross, 2009). Species used for direct human consumption are often high in these essential and human health beneficial fatty acids, as are the species captured for reduction to fishmeal and fish oil, components utilized for the bulk of protein and lipid sources in many aquaculture diets. However, both capture and reduction fisheries have reached production plateaus, with continued decline predicted for most fisheries worldwide. Aquaculture already consumes the bulk of the world's production of both fishmeal (60.8 %) and fish oil (73.8 %) (FAO, 2012). In order for aquaculture to satisfy growing global population and protein requirements, alternative protein and lipid sources must be identified and evaluated to significantly reduce the quantities of fishmeal and fish oil used in diets for aquaculture. In fact, with the increase in aquaculture over the past few decades, the availability of fishmeal has steadily declined and costs have steadily increased (Tacon and Metian, 2008), with predictions that demand will soon be greater than supply.

Gilthead sea bream, *Sparus aurata*, is one of the most heavily cultured species in Europe with ~140,000 metric tons produced annually (FAO, 2012) and the ability to replace high quantities of fishmeal with plant proteins in the diets of juvenile gilthead sea bream has been well established (Benedito-Palos *et al*., 2007; Lupatsch *et al.*, 1997; Rigos *et al*., 2011; Robaina *et al.*, 1995; Sánchez-Lozano *et al.*, 2009; Sitjà-Bobadilla *et al*.,

2005), although complete fishmeal replacement often results in poorer growth and feed conversion when compared to traditional high fishmeal or commercial feeds for many species (Kader *et al*., 2012; Luo *et al*., 2006; Xie *et al*., 1998; Zhou *et al.*, 2005). Limited synthetic capacity of the essential arachidonic (ARA) and docosahexaenoic (DHA) acids, and their absence or low abundance in terrestrial oil sources has presented a formidable challenge in the development of complete fishmeal and fish oil free diets for many marine species (FAO, 2012; Rust *et al*., 2011). Eicosapentaenoic acid (EPA) has been considered an essential fatty acid for marine species, however recent evidence suggests that this may not be the case for all species (Trushenski *et al.*, 2012). Studies attempting to completely or substantially replace fish oil with terrestrial oils in diets for gilthead sea bream have generally observed lower growth, poorer feed conversion, increased stress responses, and poorer fillet quality when compared to full or only partially substituted fish oil diets. Much of this decline may be attributed to lack of essential fatty acids in the substituted oils.

The objectives of this study were to examine the effects on growth, feed conversion, survival, and proximate and fatty acid compositions of juvenile gilthead sea bream fed diets varying in lipid source. The base of the diets in the study consisted of a fishmeal-free, plant protein-based diet which has been used effectively for rearing juvenile cobia, *Rachycentron canadum*, in recirculating aquaculture systems (Watson *et al*., 2012). With fish oil serving as the sole lipid source in the control diet, two alternative lipid sources were selected for the experimental diets; a microbial single celled thraustochytrid derived meal high in DHA (Carter *et al*., 2003; Lewis *et al.*, 1999; Lippmeier *et al.*, 2009) plus soybean oil and a canola oil supplemented with ARA and DHA at levels to mimic potential genetically engineered canola oil levels (Qi et al., 2004; Venegas-calerón et al., 2010). In addition to

the twelve week trial on the complete fish product free experimental diets, a twelve week finishing period was implemented at the conclusion of the trial to determine the potential for fillet fatty acid profiles to return to a profile more similar to fish oil (i.e. farmed fish reared on traditional feeds or wild fish).

Materials and Methods

Diet formulation and growth trial

The formulations for the three fishmeal-free diets using a blend of plant protein sources are presented in Table 5.2.1. The plant protein blend supplementation for all three diets is based on a proven formulation for rainbow trout (Barrows *et al*., 2010) with slight modifications for cobia (Watson *et al*., 2012). Lysine, methionine, threonine, magnesium, and potassium chloride were all supplemented to mimic concentrations commonly found in fillet tissues (Barrows *et al.*, 2010). All ingredients were ground using an air-swept pulverizer (Model 18H, Jacobsen, Minneapolis, MN) to a particle size of <200 μm. All ingredients were mixed before extrusion except for the menhaden oil. Pellets were prepared with a twin-screw cooking extruder (DNDL-44, Buhler AG, Uzwil, Switzerland) with an 18 s exposure to 127 °C in the extruder barrel. Pressure at the die head was approximately 26 bar, and a die head temperature of 71 °C was used. The pellets were dried for approximately 15 min to a final exit air temperature of 102 ºC using a pulse bed drier (Buhler AG, Uzwil, Switzerland) followed by a 30 min cooling period to product temperature less than 25 °C. Final moisture levels were less than 10 % for each diet. Oils were top-coated after cooling using a vacuum pressure of 25 bar (A7J Mixing, Ontario,

CA). Diets were stored in plastic lined paper bags at room temperature, and were fed within 6 months of manufacture.

The control diet for this study utilizes the same fishmeal-free, plant protein base formulated by the USDA's Agricultural Research Service (“ARS Diet") with menhaden fish oil serving as the sole lipid source, and its palatability and effectiveness as a feed has previously been examined with positive results for juvenile cobia (120-500 g) (Watson *et al*., 2012). The experimental diets for this study examined two possible fish oil replacement sources in the fishmeal-free, plant protein-based diet. A thraustochytrid meal with additional soybean oil (Aquafauna Biomarine, Hawthorne, CA) ("TM+SOY Diet") constituted the lipid source for one diet, and a commercially produced canola oil plus DHA, which was further supplemented with DHA (DHAsco, Martek BioSciences, Columbia, MD) and ARA (ARAsco, Martek BioSciences, Columbia, MD) constituted the lipid source ("CAN+EFA Diet") for the second experimental diet. Proximate compositions of the three diets are presented in Table 5.2.2. To ensure limited oxidation of the lipids did not occur during the feed manufacturing process or storage and experiment duration, samples were sent to New Jersey Feed Labs (Trenton, NJ) for peroxide analysis.

Table 5.2.1. Diet formulations and fatty acid compositions of the diets.

	Diet		
Ingredient (g kg^{-1})	**TM+SOY**	**CAN+EFA**	**ARS Control**
Soy Protein Concentrate	269.3	269.3	269.3
Corn Gluten	199	211	211
Wheat Flour	198.5	226.5	226.5
Soybean Meal, extracted	121	121	121
Menhaden Oil	0	0	84
Soybean Oil	50	0	0
Algamac 3050	74	0	0
Canola Oil + DHA + ARA	0	84	0
Dicalcium Phosphate	23.7	23.7	23.7
Vitamin Pre-mix[a]	10	10	10
Lysine-HCL	15.5	15.5	15.5
Choline CL	6	6	6
Trace Mineral Pre-mix[b]	1	1	1
Magnesium Oxide	0.5	0.5	0.5
Stay-C	3	3	3
DL-Methionine	5.8	5.8	5.8
Threonine	2.1	2.1	2.1
Potassium Chloride	5.6	5.6	5.6
Taurine	15	15	15
Fatty Acid (g $100g^{-1}$)			
12:0	0.15	0.34	0.10
14:0	4.33	1.21	4.88
6:0	17.17	7.73	22.89
17:0	0.16	0.15	0.47
18:0	2.77	3.49	5.31
20:0	0.28	0.82	0.30
22:0	0.24	0.38	0.14
SFA	25.10	14.12	34.08
16:1n-7	0.23	0.46	5.44
18:1n-7	0.87	2.58	2.46
18:1n-9+6	13.86	48.28	19.53
20:1n-15+cis-8	0.06	0.03	0.22
20:1n-9	0.14	0.76	1.21
24:1n-9	0.0	0.13	0.32
MUFA	15.15	52.24	29.18
16:3n-4	0.05	0.06	0.57
16:4n-1	0.04	0.06	0.79
18:2n-6	33.27	22.86	12.74
18:3n-3	3.94	6.25	1.19
20:2n-6	0.04	0.13	0.18
20:4n-6	0.70	0.32	0.84
20:5n-3	0.42	0.27	8.90
22:5n-6	6.09	0.66	0.31
22:5n-3	0.16	0.16	1.51
22:6n-3	14.93	2.85	8.57
PUFA	59.75	33.64	36.75

[a]Contributed per kg diet; vitamin A, 13510 IU; vitamin D, 9.2 IU; vitamin E, 184.4 IU; menadione sodium bisulfite, 6.6 mg; thiamine mononitrate, 12.7 mg; riboflavin, 13.4 mg; pyridoxine hydrochloride, 19.2 mg; pantothenate, DI calcium, 141.5 mg; cyanocobalamine, 0.04 m nictonic acid, 30.5 mg; biotin, 0.46 mg; folic acid 3.5 mg.

[b] Contributed in mg kg^{-1} diet; zinc 37; manganese, 10; iodine, 5; copper, 1.

Table 5.2.2. Proximate compositions of the three tested diets.

	TM+SOY	CAN+EFA	ARS Control
	Mean ± S.D. (NJFL)[3]	Mean ± S.D. (NJFL)[3]	Mean ± S.D. (NJFL)[3]
Lipid, g $100g^{-1}$ dm^1	9.07 ± 0.54	7.32 ± 0.46	7.82 ± 1.05
Ash, g $100g^{-1}$ dm^1	5.66 ± 0.04[a]	5.10 ± 0.26[b]	5.04 ± 0.14[b]
Protein, g $100g^{-1}$ dm^1	49.00[a]	51.38[a]	44.88[b]
Carbohydrate, g $100g^{-1}$ dm^2	35.55	35.22	39.75
Fiber, g $100g^{-1}$ dm^1	(0.72)	(0.98)	(2.51)
Moisture, g $100g^{-1}$ dm	4.02	7.70	5.11
Energy Content, MJ Kg^{-1}	20.69 ± 0.15	20.29 ± 0.13	19.30 ± 0.77

[1] After lyophilization.
[2] After lyophilization (100-% lipid, ash, protein, fiber).
[3] Values from New Jersey Feed Labs Analysis.
Values in the same row with different superscripts are significantly different ($p<0.05$); no superscript indicates no significant difference within a category.

This study was carried out in accordance with the guidelines of the International Animal Care and Use Committee of the University of Maryland Medical School (IACUC protocol # 0610015). Sea bream eggs were spawned from in-house broodstock and reared at the University of Maryland Center for Environmental Science's (UMCES) Institute for Marine and Environmental Technology (IMET) in Baltimore, MD. USA. Fish were reared on a combination of live feed (rotifers and *Artemia*), Otohime (Reed Mariculture, Campbell, CA), and Zeigler Marine Grower (Zeigler Bros, Gardners, PA) until reaching approximately 11 g. Temperature and salinity were maintained at 25 °C and 15 ppt throughout larval and juvenile rearing. One hundred fish each were placed into six identical two cubic meter tanks, connected in pairs that share filtration and life support systems including protein skimming, ozonation, mechanical filtration in the form of bubble-bead filters, and biological filtration; and for this reason tanks were randomly assigned diets to avoid having both tanks of one system feed the same diet. Water quality parameters did not differ significantly between systems utilized during the feeding trial and were: salinity,

15.93 ± 2.42 ppt; pH, 7.70 ± 0.51; total ammonia nitrogen, 0.15 ± 0.14 mg l^{-1}; nitrite, 0.14 ± 0.12 mg l^{-1}; nitrate, 236.74 ± 167.72 mg l^{-1}; and alkalinity, 108.93 ± 33.45 mEq l^{-1}.

Fish were anesthetized with Tricaine methanosulfonate (MS-222, 70 mg l^{-1}, Finquel, Redmond, WA.) and weighed every six weeks to record growth with feed amounts gradually reduced from 4.4 % to 2.2 % bw during the 12 week trial. At the conclusion of the twelve weeks, six fish from each tank were euthanized with an overdose of MS-222 (150 mg l^{-1}). Three fish from each trial were used for fillet and whole body analyses. Since lipid composition and source plays such an important role in final flavor and acceptance by the consumer, we examined the changes that take place in a twelve week finishing period. For these additional weeks, tanks that had been fed the experimental diets during the trial were switched to the ARS control diet with fish oil. Afterwards, three fish from each tank were sampled for fillet lipid analysis to assess the change in fatty acid profile following the diet switch.

Whole body and fillet samples were prepared by lyophilization to constant weight followed by homogenization by blending in a Waring blender (Waring Products, CT, USA). Ash percentage was determined by ashing three replicate samples at 600 °C for a minimum of 8 h. Energy content was determined in triplicate for each sample through bomb calorimetry (Parr instrument company, Moline, IL.) with calibration using benzoic acid ever 20 samples. Crude protein was determined by CHN analysis (Chesapeake Biological Laboratory, Center for Environmental Science, University of Maryland, %N X 6.25).

Lipid extraction and analysis of fatty acid methyl esters (FAMEs)

Total lipids were extracted as described in Harvey *et al.* (1987), a modified version of Bligh and Dyer (1959). Samples were subjected to three extractions with 4 ml of 2:1 methylene chloride (MeCl):methanol (MeOH), 1:1 methylene chloride (MeCl):methanol (MeOH) and 1:2 methylene chloride (MeCl):methanol (MeOH) in a sonicating water bath, the supernatant being removed each time to a clean round-bottom glass test tube. Water was added (0.25 vol) to the lipid extracts. After vortex mixing and phase separation, the organic layer was recovered and dried under N_2 gas. The residue was brought to 1 ml in 1:1 MeCl:MeOH, flushed with N_2 gas, and stored at –20 °C for less than 1 month.

10% (vol.) of the lipid extract was used to generate fatty acid methyl esters (FAMEs) according to procedures described in Ederington *et al.* (1995). Five μg of C19:C21 internal standard (2.5 μg of each FA) and the lipid extract were added to glass tubes and dried under N_2 gas. Saponification of lipids was accomplished by adding 2.5 ml MeOH, 1.5 ml KOH-saturated MeOH, and 0.5 ml dH_2O to each tube, which was then capped with a teflon-lined screw cap under a stream of N_2 gas and incubated at 70 °C for 1 h. After cooling, 0.5 ml of dH_2O was added and neutral lipids (such as sterols) were removed by extracting 3x with hexane:ether (9:1). Fatty acids were then recovered by dropping the pH of the mixture remaining in the tubes to <2 by drop-wise addition of concentrated HCl, and repeating the extraction (3x) with hexane:ether (9:1). Neutral lipids were stored frozen or discarded and fatty acids were dried under N_2 gas. Methylation of fatty acids was accomplished using 10 % (w/w) BF_3 in methanol (Sigma-Aldrich, Bellefonte, PA USA). To each dried sample, 1 ml of BF_3:methanol were added, the tubes were flushed with N_2 gas, capped with teflon-lined screw caps, and incubated at 70 °C for

30 m. After cooling, 1ml of methanol was added to each tube and the samples were mixed by vortexing. The FAMEs were extracted with hexane:diethyl ether (9:1) three times. The upper organic phase containing FAMEs was recovered to new glass tubes, dried under N_2 gas, resuspended in 500 µl hexane, transferred to crimp-top GC sample vials containing small-volume sample inserts, capped under N_2 gas, and stored at –80 °C until analysis.

Identification of FAMEs was accomplished by comparing gas chromatography retention data with authentic quantitative standards (stds 3B, GLC-68D, GLC-17AA') from NU-CHECK, Inc. (Elysian, MN, USA) and qualitative standards (PUFA No. 1 – Marine Source) from Matreya (Pleasant Gap, PA, USA). Peaks in some samples were also confirmed by GC-MS. The Hewlett-Packard 6890 GC we used was equipped with a 30 m x 0.25 mm I.D. capillary column with 0.25 mm film thickness (DB Wax, J & W Scientific, Folson CA), and a flame ionization detector at 300 °C. The GC was run in 'constant flow rate' mode at 1.5 ml min^{-1} with H_2 as the carrier gas. The column-temperature profile was as follows: 50 °C for 0.5 min, hold at 195 °C for 15 min after ramping at 40 °C min^{-1}, and hold at 220 °C for 7 min after ramping at 2 °C min^{-1}. Total runtime was 38.13 min. The mass of FAMEs was determined by comparison with the internal C19:C21 standards run with each sample. The relative distribution (% FAMEs) was calculated based on the peak area of a given peak divided by the total peak area of identified FAMEs in a sample.

Dilution model

The dilution model of fatty acid turnover in fish is expressed as:

$$\mathrm{Pt} = \mathrm{Pr} + (\mathrm{Pr} - \mathrm{Pi})/(\frac{\mathrm{Qt}}{\mathrm{Qi}}),$$

where Pt is the percentage of a specific fatty acid in an experimental fillet at time T following the dietary change, Pi and Pr are the percentages of the same fatty acid before the diet change (initially) and in a reference fillet (fish fed the ARS diet throughout), and Qi and Qt are the total lipid content initially and at time T, respectively (Robin *et al.*, 2003; Trushenski and Boesenberg, 2009). We compared the fatty acid compositions of fillets from fish that were involved in the twelve week switch to the ARS control diet, Pr, to the fatty acid profiles predicted by the dilution model using the TM+SOY and CAN+EFA fillet profiles as the initial, Pi.

Statistics

All statistical tests were run using Aabel v2.4.2 (Gigawiz Ltd., OK, USA) with significance values of $p < 0.05$. Paired t-tests and ANOVA were used to test differences in means between groups and two-way ANOVA was used to test differences in the growth curves from the trials. Means were averaged across each treatment if no significant difference was found within each treatment. Statistics were not performed on the carbohydrate and fiber content pool of the tissues since all other components were measured directly, therefore dramatically increasing and pooling the error of this one groups measurement.

Results

Diet formulation

The protein content of the two fish oil replacement diets are slightly higher (Table 5.2.2) than the plant protein fish oil reference diet (ARS Plant Protein Cobia). Crude protein

was significantly lower in the ARS diet than either of the experimental diets (ANOVA, $p<0.05$). There were no significant differences between the diets in overall energy, lipid, or moisture content (Table 5.2.2). The canola oil prior to and post addition of DHA and ARA had peroxide values of 5.2 and 2.0 mep kg^{-1}, respectively. The plant protein diet containing thraustochytrid meal and soybean oil had a peroxide value of 18.0 mep kg^{-1}, the CAN+EFA diet had a peroxide value of 2.2 mep kg^{-1}, and the ARS diet with fish oil as the lipid source had a peroxide value of 10.0 mep kg^{-1}. All these measures are below the accepted rancid values of 20 Mep kg^{-1} (Turchini *et al*., 2011).

Growth trial

At the conclusion of the initial twelve week trial, weight gain, percent growth from initial weight, was significantly higher (ANOVA, $p<0.05$) on the TM+SOY diet (785.69 ± 3.84) than the ARS control diet (696.33 ± 0.05) and the CAN+EFA diet (680.34 ± 72.01), however weight gain on the CAN+EFA diet (680.34 ± 72.01) was not significantly different than the ARS control (Table 5.2.3, ANOVA, $p>0.05$). Feed conversion ratio (FCR) and hepatosomatic index (HSI) were both significantly lower on the TM+SOY diet than the CAN+EFA or the ARS control diets (ANOVA $p<0.05$). Protein efficiency ratio (PER) was significantly higher (ANOVA, $p<0.05$) on the TM+SOY (1.61 ± 0.04) and ARS control (1.64 ± 0.01) diets than the CAN+EFA diet (1.42 ± 0.04). There were no significant differences in fillet yield, condition factor, or survival between the three diets (Table 5.2.3, ANOVA $p>0.05$), with overall averages between the diets of 27.48 ± 1.79, 1.64 ± 0.14, and 95.8 % respectively.

Proximate compositions of the whole body and fillet tissues of three randomly selected individuals from each tank, six per diet, are seen in Tables 5.2.4 and 5.2.5, respectively. There were no significant differences between the diets in the whole body compositions in terms of lipid, ash, protein, moisture, energy content, or carbohydrates (Table 5.2.4, ANOVA $p>0.05$). There was a significant difference in fillet protein, g 100 g^{-1}, content between the diets with the ARS control diet (78.02 ± 2.51) resulting in significantly higher fillet protein than the TM+SOY (71.54 ± 5.43) or CAN+EFA (73.65 ± 2.64). There were no significant differences in fillet lipid, ash, moisture, or energy content between the diets (Table 5.2.5, ANOVA, $p>0.05$) between the diets.

Table 5.2.3. Production characteristics from the twelve week grow out trial.

	TM+SOY	CAN+EFA	ARS Control
Weight Gain (%)[1]	785.69 ± 3.84[a]	680.34 ± 72.01[b]	696.33 ± 0.05[b]
Fillet Yield (%)[2]	27.28 ± 1.30	27.50 ± 2.97	27.66 ± 1.09
FCR[3]	1.27 ± 0.01[a]	1.37 ± 0.04[b]	1.36 ± 0.01[b]
PER[4]	1.61 ± 0.04[a]	1.42 ± 0.04[b]	1.64 ± 0.01[a]
Hepatosomatic Index[5]	1.41 ± 0.18[a]	2.14 ± 0.39[b]	2.11 ± 0.50[b]
Specific Growth Rate[6]	2.48 ± 0.00	2.33 ± 0.11	2.36 ± 0.00
Condition Factor[7]	1.56 ± 0.15	1.67 ± 0.13	1.68 ± 0.13
Survival	97.5 %	94.5 %	95.5 %

[1] Weight gain = (final tank weight - initial tank weight)/ initial tank weight*100.
[2] Fillet yield = (fillet weight/body weight)*100.
[3] FCR=Feed conversion ratio = g fed/g gained.
[4] PER=Protein efficiency ratio = g gained /g protein fed.
[5] Hepatomsomatic index = (liver weight/ body weight)*100.
[6] Specific growth rate = $((\ln BW_F - \ln BW_I)/(\text{days of trial}))*100$.
[7] Condition Factor = $(\text{Weight}*100)/(\text{Length}^3)$.
Values in the same row with different superscripts are significantly different ($p<0.05$), no superscript indicates no significant difference within a category.

Table 5.2.4. Proximate compositions of whole body tissues from animals fed the three experimental diets.

	TM+SOY	CAN+EFA	ARS Control
	Mean ± S.D.	Mean ± S.D.	Mean ± S.D.
Lipid, g 100 g^{-1} dm^1	26.94 ± 5.55	27.40 ± 4.48	29.90 ± 6.65
Ash, g 100 g^{-1} dm^1	10.14 ± 1.66	10.51 ± 1.29	10.58 ± 1.22
Protein, g 100 g^{-1} dm^1	52.64 ± 5.88	51.42 ± 5.77	52.33 ± 4.52
Carbohydrate/Fiber, g 100 g^{-1} dm^2	10.28	10.67	7.19
Moisture, g 100 g^{-1} dm	62.88 ± 2.48	64.71 ± 0.81	65.59 ± 1.05
Energy Content, MJ Kg^{-1}	23.02 ± 1.07	24.11 ± 0.73	24.08 ± 0.84
Body Weight[3]	111.33 ± 8.76	94.75 ± 11.06	92.78 ± 13.79

[1] After lyophilization.
[2] After lyophilization (100-% lipid, ash, protein).
[3] For fish used in analyses, g.
Values in the same row with different superscripts are significantly different ($p<0.05$), no superscript indicates no significant difference within a category.

Table 5.2.5. Proximate compositions of fillet tissue from animals fed the three experimental diets.

	TM+SOY	CAN+EFA	ARS Control
	Mean ± S.D.	Mean ± S.D.	Mean ± S.D.
Lipid, g 100 g^{-1} dm^1	15.28 ± 4.54	16.33 ± 4.50	14.46 ± 3.93
Ash, g 100 g^{-1} dm^1	4.23 ± 0.87	4.07 ± 0.77	4.15 ± 1.15
Protein, g 100 g^{-1} dm^1	71.54 ± 5.43[a]	73.65 ± 2.64[a]	78.02 ± 2.51[b]
Carbohydrate/Fiber, g 100 g^{-1} dm^2	8.95	5.95	3.37
Moisture, g 100 g^{-1} dm	69.69 ± 2.92	71.61 ± 0.62	71.06 ± 2.31
Energy Content, MJ Kg^{-1}	25.39 ± 0.80	24.64 ± 0.59	24.70 ± 0.84
Body Weight[3]	118.72 ± 13.10	104.33 ± 12.52	113.47 ± 11.26

[1] After lyophilization.
[2] After lyophilization (100-% lipid, ash, protein).
[3] For fish used in analyses, g.
Values in the same row with different superscripts are significantly different ($p<0.05$), no superscript indicates no significant difference within a category.

Fatty acid analysis

Table 5.2.6 presents the fatty acid profiles for the fillet and whole body tissues after the twelve week trial and the fillet fatty acid profiles obtained after the additional twelve weeks when tanks fed either the TM+SOY or CAN+EFA diets were switched to the ARS diet containing fish oil. Figure 5.2.1 presents a radial diagram comparing the fatty acid profiles of the fillet and whole body tissues for fish from each diet at the conclusion of the twelve week trial as a ratio of fatty acid concentrations within the diet. Deviations from the line of equality represent enhancement or depletion of each fatty acid when compared to the diet fed, with values greater than 1 indicating an enrichment in the fillet or whole body compared to the diet, and values less than 1 indicating a depletion compared to the diet. Euricic acid (22:1n9) is a toxic fatty acid that can be present in canola oil, however it was not detected in the diets, whole body, or fillet tissues of any of the animals fed the CAN+EFA diet.

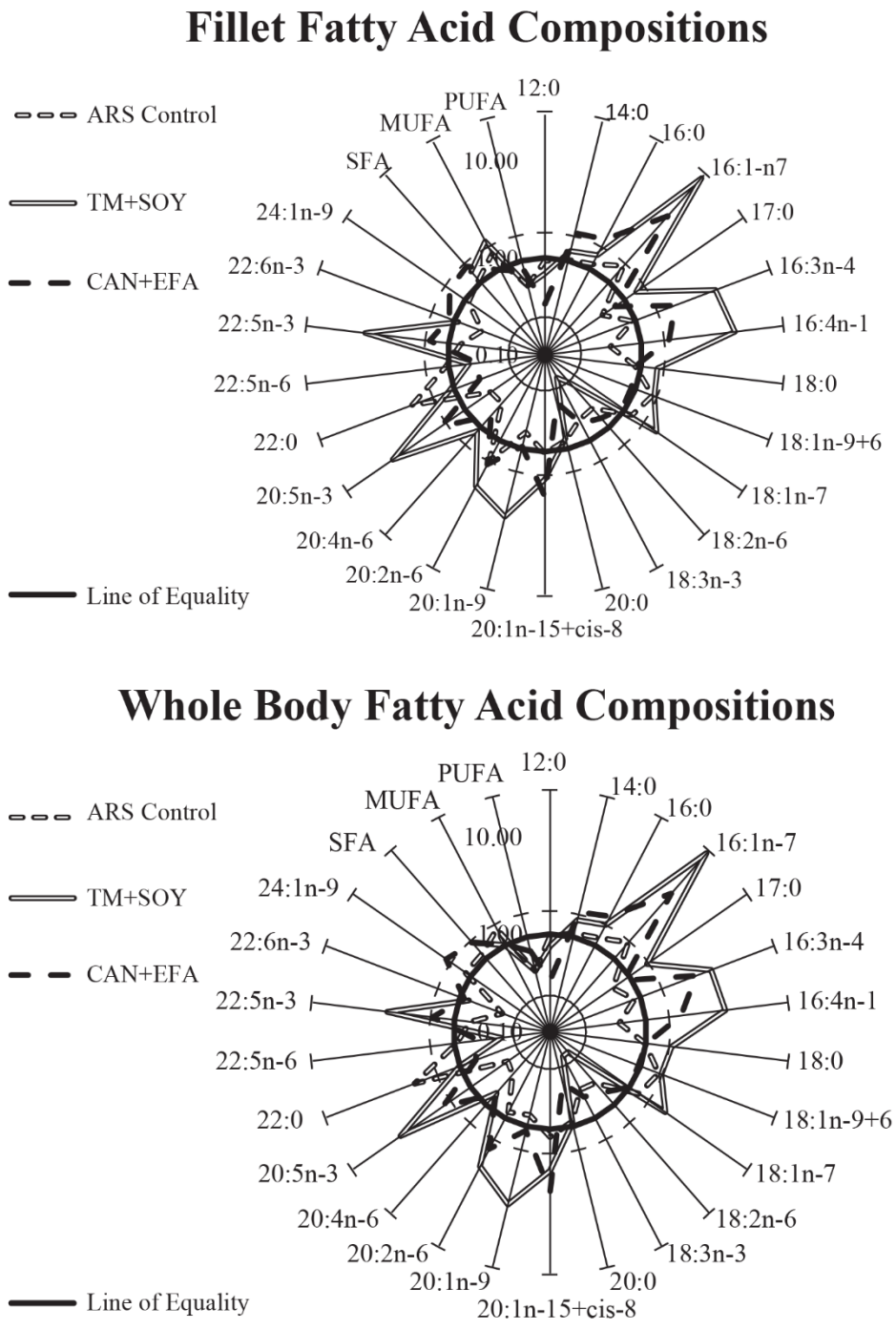

Figure 5.2.1. Fatty acid compositions of fillet and whole body tissues from sea bream experimental fish oil replacement diets expressed as a fraction of dietary total lipid profile. Values were calculated from relative fatty acid methyl ester (FAME) composition (fillet or whole body fatty acid concentration/diet fatty acid concentration). Based on this calculation, a value of 1 represents equality between fillet and dietary fatty acid composition.

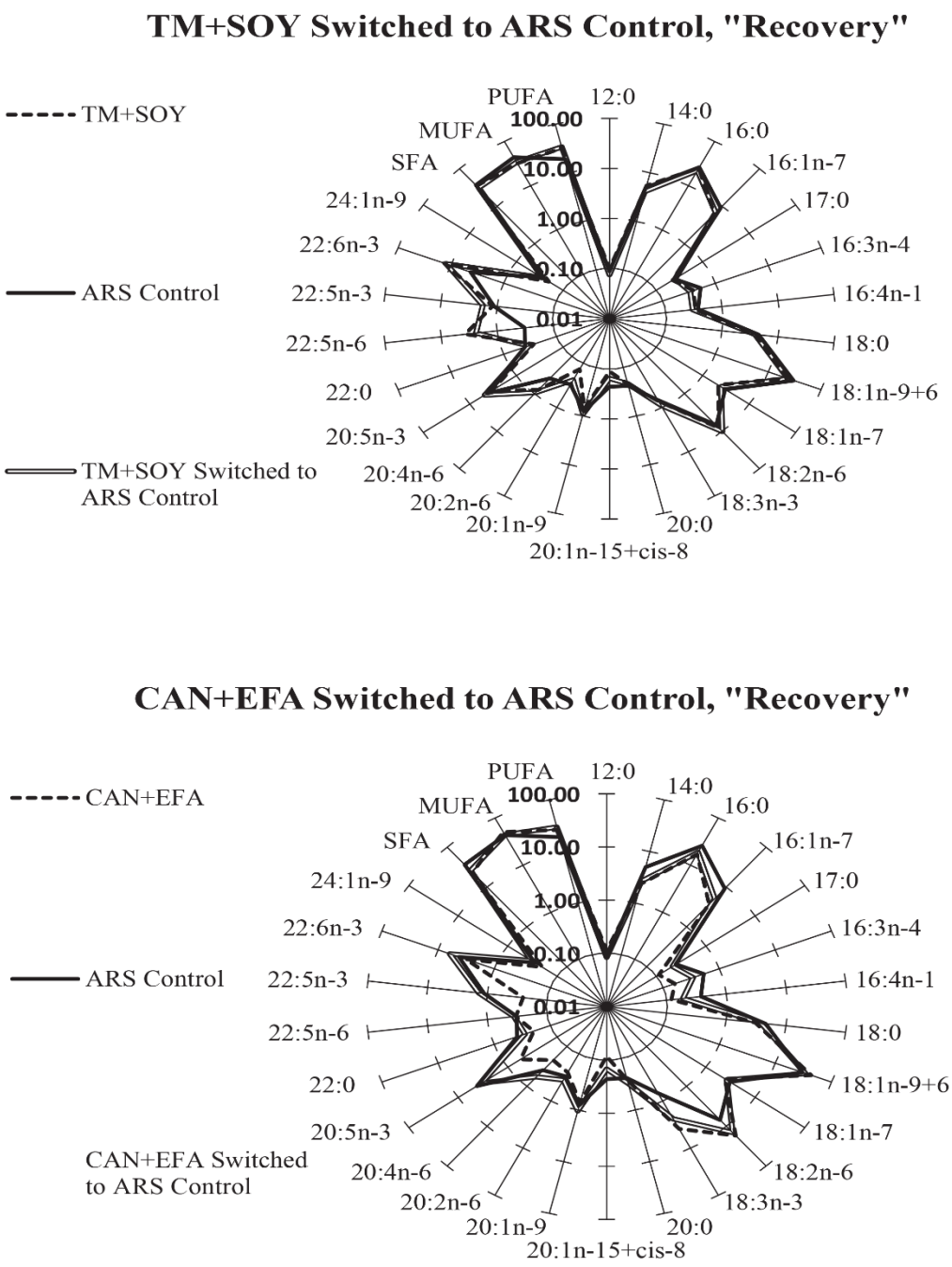

Figure 5.2.2. Fatty acid compositions of fillet tissues from sea bream after twelve weeks on experimental lipid replacement diets, twelve weeks on fish oil based control diet, and twelve weeks on experimental fish oil replacement diet with twelve "recovery" weeks on fish oil based control diet.

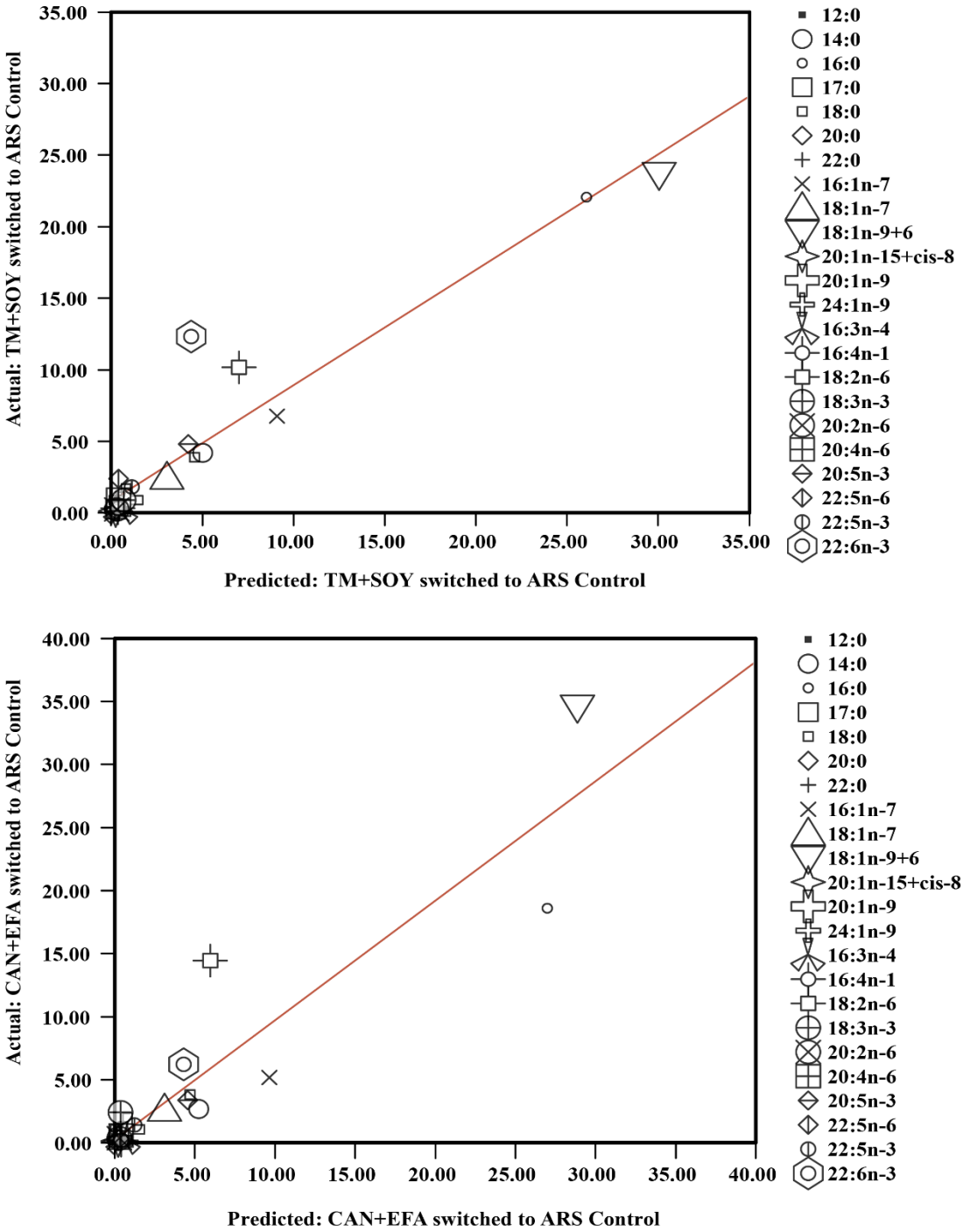

Figure 5.2.3. Relationship between the predicted and actual fillet fatty acid compositions (% dw) following switch from either the Plant Protein: CAN+EFA (A) or TM+SOY (B) diets to the fish oil based ARS control diet (12 week duration). The dilution model is described in materials and methods with predicted results based on a standard model (Robin *et al*., 2003).

Table 5.2.6. Fatty acid compositions (g 100 g^{-1}) of fillet and whole body tissues from the twelve week grow out as well as recovery fillets from the additional twelve week period where animals that were on the two fish oil replacement diets were fed the control, fish oil based diet.

Fatty Acid	Fillets			Whole Body			Recovery Fillets	
(g 100 g^{-1})	**TM+SOY**	**CAN+EFA**	**ARS Control**	**TM+SOY**	**CAN+EFA**	**ARS Control**	**TM+SOY**	**CAN+EFA**
12:0	0.10 ± 0.01	0.11 ± 0.01	0.09 ± 0.01	0.11 ± 0.03	0.11 ± 0.01	0.09 ± 0.01	0.08 ± 0.01	0.08 ± 0.01
14:0	5.48 ± 0.61	2.37 ± 0.20	4.99 ± 0.23	6.50 ± 1.14	2.20 ± 0.19	5.36 ± 0.59	4.18 ± 0.15	2.68 ± 0.11
16:0	24.93 ± 2.01	17.12 ± 0.55	26.08 ± 1.16	29.60 ± 4.21	16.74 ± 1.78	27.82 ± 3.13	22.05 ± 0.73	18.61 ± 0.61
17:0	0.23 ± 0.02	0.11 ± 0.01	0.25 ± 0.01	0.27 ± 0.03	0.13 ± 0.02	0.29 ± 0.03	0.26 ± 0.01	0.21 ± 0.02
18:0	4.08 ± 0.31	3.10 ± 0.13	4.57 ± 0.29	5.15 ± 0.70	3.54 ± 0.43	5.17 ± 0.51	3.87 ± 0.25	3.82 ± 0.11
20:0	0.22 ± 0.04	0.27 ± 0.02	0.24 ± 0.05	0.26 ± 0.04	0.29 ± 0.05	0.27 ± 0.04	0.22 ± 0.03	0.31 ± 0.05
22:0	0.27 ± 0.08	0.20 ± 0.12	0.40 ± 0.07	0.24 ± 0.11	0.25 ± 0.26	0.43 ± 0.05	0.33 ± 0.06	0.28 ± 0.06
SFA	35.32 ± 2.88	23.18 ± 0.60	36.61 ± 1.47	41.99 ± 6.10	23.10 ± 2.43	39.42 ± 4.24	30.98 ± 0.60	26.00 ± 0.79
16:1n-7	6.25 ± 0.55	3.71 ± 0.40	9.13 ± 0.41	6.37 ± 0.69	3.55 ± 0.48	9.08 ± 0.57	6.77 ± 0.61	5.16 ± 0.16
18:1n-7	2.17 ± 0.16	2.43 ± 0.05	3.07 ± 0.11	2.46 ± 0.19	2.58 ± 0.13	3.23 ± 0.16	2.48 ± 0.07	2.64 ± 0.07
18:1n-9+6	22.39 ± 1.48	43.53 ± 0.48	30.20 ± 0.69	23.65 ± 2.04	45.97 ± 2.71	31.13 ± 1.87	23.66 ± 0.85	34.57 ± 1.05
20:1n-15+cis-8	0.11 ± 0.02	0.09 ± 0.03	0.23 ± 0.06	0.16 ± 0.03	0.13 ± 0.07	0.26 ± 0.04	0.15 ± 0.03	0.15 ± 0.03
20:1n-9	0.71 ± 0.08	0.72 ± 0.12	0.81 ± 0.12	0.91 ± 0.08	0.84 ± 0.10	0.91 ± 0.13	0.91 ± 0.06	1.04 ± 0.08
24:1n-9	0.20 ± 0.04	0.20 ± 0.04	0.29 ± 0.07	0.33 ± 0.08	0.26 ± 0.06	0.37 ± 0.08	0.28 ± 0.06	0.33 ± 0.08
MUFA	31.84 ± 2.09	50.67 ± 0.42	43.72 ± 0.76	33.87 ± 3.02	53.34 ± 3.25	44.98 ± 2.46	34.24 ± 1.39	43.89 ± 1.12
16:3n-4	0.39 ± 0.04	0.16 ± 0.04	0.53 ± 0.04	0.31 ± 0.08	0.25 ± 0.10	0.49 ± 0.13	0.34 ± 0.12	0.35 ± 0.03
16:4n-1	0.34 ± 0.04	0.12 ± 0.04	0.38 ± 0.04	0.24 ± 0.05	0.14 ± 0.03	0.42 ± 0.03	0.31 ± 0.01	0.19 ± 0.02
18:2n-6	8.20 ± 0.60	17.12 ± 0.43	6.97 ± 1.99	6.35 ± 3.40	15.59 ± 3.13	6.40 ± 1.98	10.16 ± 0.35	14.45 ± 0.14
18:3n-3	0.73 ± 0.10	3.93 ± 0.13	0.69 ± 0.04	0.76 ± 0.12	3.56 ± 1.01	0.55 ± 0.21	0.89 ± 0.04	2.40 ± 0.08
20:2n-6	0.14 ± 0.03	0.25 ± 0.06	0.30 ± 0.39	0.15 ± 0.04	0.30 ± 0.07	0.15 ± 0.04	0.24 ± 0.03	0.36 ± 0.05
20:4n-6	0.79 ± 0.12	0.22 ± 0.03	0.39 ± 0.06	0.48 ± 0.38	0.23 ± 0.09	0.32 ± 0.13	0.93 ± 0.05	0.58 ± 0.08
20:5n-3	3.42 ± 1.09	0.55 ± 0.16	4.22 ± 0.17	3.21 ± 1.02	0.61 ± 0.35	3.04 ± 1.65	4.82 ± 0.38	3.39 ± 0.29
22:5n-6	3.73 ± 0.59	0.33 ± 0.12	0.33 ± 0.09	1.97 ± 1.66	0.37 ± 0.09	0.29 ± 0.08	2.40 ± 0.22	0.36 ± 0.06
22:5n-3	1.21 ± 0.17	0.25 ± 0.04	1.14 ± 0.14	0.82 ± 0.65	0.27 ± 0.10	1.04 ± 0.37	1.80 ± 0.11	1.39 ± 0.14
22:6n-3	13.46 ± 2.37	3.21 ± 0.41	4.23 ± 0.25	11.92 ± 2.81	2.53 ± 0.98	2.90 ± 1.58	12.33 ± 1.04	6.21 ± 0.57
PUFA	32.84 ± 4.38	26.16 ± 0.74	19.67 ± 2.03	24.14 ± 9.01	23.56 ± 5.55	15.60 ± 6.62	34.78 ± 1.84	30.11 ± 1.13

Dilution model

Figure 5.2.2 presents radial diagrams of the fillet fatty acid (% dw) profiles from this study. The top panel represents the "recovery" of the fatty acid profile of fish fed the TM+SOY diet when switched to the ARS control diet for the twelve week finishing period, and the bottom panel represents the "recovery" of the profile of fish fed the CAN+EFA diet when switched to the ARS control for the finishing period. Table 5.2.6 contains the fatty acid percentages found in the fillets of fish switched from the TM+SOY and CAN+EFA diets to the ARS control diet. Figure 5.2.3 presents the linear regression of the dilution model as applied to either the TM+SOY switch (top panel) or the CAN+EFA switch (bottom panel) to the ARS control diet. Linear regression analyses for the comparisons of predicted fatty acid percentages based on the model to actual fatty acid percentages for the individual fatty acids measured resulted in R^2 values of 0.91 and 0.86 for the TM+SOY and CAN+EFA switches to the ARS diet, respectively.

Discussion

Both experimental complete fish oil replacement diets performed as well or better than the fish oil, ARS, control diet. All three diets are also completely fishmeal-free, with this study representing one effective fishmeal-free and two effective complete fish product replacement diets for juvenile sea bream resulting in excellent growth rates, feed conversion ratios, and survival from 11-100 g over the initial twelve week trial. Performance of all three diets are similar to the results obtained by other researchers utilizing partial fish oil replacement in low fishmeal diets (Benedito-Palos *et al*., 2007; Fountoulaki *et al*., 2009; Glencross *et al*., 2003; Montero *et al*., 2008). However in this

study, complete fish oil replacement in a fishmeal-free diet was just as effective as full fish oil. It is important to note that the feeds used in this study were relatively low in overall lipid level (7.32-9.07 %), a significant reduction from the 14-16 % range utilized for many diets for this species (Dias *et al*., 2009; Fountoulaki *et al*., 2009; Izquierdo *et al.*, 2005, 2003; Liu *et al.*, 2002). The low dietary lipid level did not result in lower lipid levels in the fillet when compared to other studies, with gilthead sea bream in this study maintaining 14-16 % lipid in the fillet tissue, potentially indicating a strong ability for this species to modulate its tissue lipid content compared to dietary input.

In addition to the performance of the fish on the diets, the fatty acid profiles obtained for fillet and whole body tissues display unique characteristics similar to those of the diets, with the ARS fish oil diet being the most similar to the 1:1 line of equality for both fillets and whole body tissues (Figure 5.2.1). This indicates the importance of attempting to match a fish oil profile when utilizing alternative oil sources, even if the supplementation with exogenous EFAs is necessary. However, even given the differences observed due to dietary input, results from the finishing period (Figure 5.2.2) give another indication that gilthead sea bream may have a strong ability to regulate the fatty acids being deposited in their tissues. For most fatty acids measured, there is little difference between the concentration in fillets of fish fed the ARS control diet compared to either the TM+SOY or CAN+EFA diets, and where large differences were detected, after the twelve week finishing period, concentrations had transitioned towards the ARS fish oil type profile. One major exception to this trend was the effect of DHA in the TM+SOY fed fish. The TM+SOY diet is very high in DHA (~14.93 % of fatty acids), and after the twelve week finishing period, DHA remained high in the fillets (12.33 % vs 13.46 %), while the ARS

diet only contains ~8.57 % DHA. This is potentially an added human health related benefit to the TM+SOY diet, with or without the use of a finishing period. The same trend is seen in docosapentaenoic acid (DPA), an intermediary between EPA and DHA that has been shown to have its own roles in benefiting human health (Herold and Kinsella, 1986; Hino *et al.*, 2004; Sun *et al.*, 2008). Even with these exceptions, the simple dilution model accurately predicted the overall transitions observed when switching fish fed either the TM+SOY or CAN+EFA diets to the ARS control diet (Figure 5.2.3). The minimum g EFA kg^{-1} (EPA+ARA+DHA in this instance) provided in the diet for marine fish has been suggested to be in the range of 0.5-1, with requirement levels for various species falling in the 5-10 g kg^{-1} range depending on overall lipid level (Glencross, 2009). As mentioned previously, the addition of DHA and ARA to the canola oil source was selected to mimic potentially available genetically modified crop levels. This resulted in an EFA level of 0.26 g kg^{-1} diet for the CAN+EFA diet. In contrast the TM+SOY diet has an EFA level of 1.37 g kg^{-1} and the ARS diet has an EFA level of 1.93 g kg^{-1}. Even with the low level of overall EFAs in the CAN+EFA diet, growth was equivalent to the ARS control diet, indicating the EFA requirement for this species may be lower than other marine species, potentially due to some synthetic capacity. The fishmeal-free, plant protein-based diets in this study rely upon, and success with these and similar plant protein blends have largely been attributed to, the supplementation of taurine in the absence of fishmeal. Taurine is a non-protein amino acid that has multiple important physiological roles (Schuller-Levis and Park, 2003), however it is not found in any terrestrial plant sources, and must therefore be supplemented to feeds in the same manner as lysine and other essential components. Taurine has been shown to be the only amino acid used by marine teleosts as a bile conjugate, with

conjugated bile salts being critical for efficient lipid digestion (Kim *et al*., 2008). This may be a partial explanation as to why the low lipid diets in this study were still effective, especially considering the use of alternative lipid sources in these fishmeal-free diets.

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Chapter 6: Potential consumer concerns of plant protein utilization in aquaculture

Fillet assessment of fish fed fishmeal-free, plant protein diets: organoleptic and contaminant analysis of cobia, Rachycentron canadum, gilthead sea bream, Sparus aurata, and striped bass, Morone saxatilis

Abstract

This study was undertaken to examine the organoleptic characteristics and organic contaminant loads of fish raised on plant protein-based diets compared to commercial fishmeal-based diets. Cobia, *Rachycentron canadum*, gilthead sea bream, *Sparus aurata*, and striped bass, *Morone saxatilis*, were raised for equivalent periods of time on either a commercial fishmeal-based diet or a plant protein formulation developed in our lab and produced by a commercial manufacturer. A panel of organoleptic analysts were unable to discern the difference within any of the three species between fillets from fish raised on the plant protein diet compared to those raised on the commercial diets. Polychlorinated biphenyl (PCB) and total mercury concentrations were also significantly lower in fillets of fish raised on the plant protein diet for all species except striped bass, where the reduction was present but not significant. This represents a highly significant advantage in developing more sustainable diets for aquaculture, as replacing fishmeal with plant proteins will result in lowered public concern over contaminants while producing fillets that are identical in taste and texture to what consumers expect from fish.

Introduction

As the aquaculture industry continues to expand globally in total volume and diversity of species, several critical issues have been encountered. One of the most important is how to expand and become more sustainable when one of the main ingredients historically for fish feeds, fishmeal, is declining in production and increasing in price (FAO, 2012). A partial solution to this problem has been to replace fishmeal with alternative protein sources such as animal by-product meals or plant proteins. Species specific formulations are currently being developed worldwide for virtually every species in intensive culture (Gatlin *et al.*, 2007; Rust *et al.*, 2011) but two very important aspects of fishmeal replacement have not yet received the attention they deserve for their potential impacts on the industry. Replacing fishmeal with plant proteins creates more sustainable feeds for the expansion of the industry, but their effect on organoleptic qualities has been under scrutinized. Producing aquacultured products more sustainably is futile if it results in unacceptable products to the consumer due to organoleptic differences from wild caught products. However, producing products that are lower in organic contaminants through the reduction of fishmeal use could potentially add value to aquacultured products when compared to their wild caught counterparts.

Wild and farm raised fish have come under scrutiny for potentially containing high levels of organic contaminants (Du *et al*., 2012; Hayward *et al*., 2007; Kim *et al*., 2012; Stewart *et al.*, 2011). This issue has been extensively explored in farmed Atlantic salmon, *Salmo salar*, a high trophic level carnivore raised in varying locations worldwide (Hites *et al*., 2004). Biomagnification of these contaminants, such as polychlorinated biphenyls (PCBs) and mercury, occurs in these high level predators as a result of their carnivorous

feeding habits. In aquaculture scenarios these species are reared in high densities on diets that rely heavily on fishmeal and fish oil, products of the reduction of wild caught stocks of species such as anchovy and menhaden which are not usually consumed directly by humans. The concentrations of contaminants is further increased during the reduction process, leading to the high levels observed in farmed fish raised on this type of feed. Therefore, it is much more important to consider the source and contaminant load of the feed ingredients used as opposed to the source or location of the fish farm itself. Reducing fishmeal and fish oil use in diets for aquaculture has resulted in lower contaminant loads in trials with Atlantic salmon (Bell *et al*., 2005; Berntssen *et al*., 2005).

Mercury, most notably methylmercury, toxicity can lead to neurodevelopmental deficits, development of cardiovascular disease, and neurological deficits (Burger and Gochfeld, 2013). Although PCB production was halted decades ago, approximately 1.3 million tons of the carcinogenic material was produced, with potential effects lasting for centuries (Borchers *et al.*, 2010). The biggest route of contamination for both of these types of compounds to animals and especially humans is through ingestion.

The purpose of this study was to examine organoleptic qualities and contaminant concentrations in fish raised on fishmeal-free, plant protein-based diets compared to fish raised on traditional commercial diets. Gilthead sea bream, *Sparus aurata*, and striped bass, *Morone saxatilis*, are both extensively cultured in Europe and North America, respectively. Cobia, *Rachycentron canadum*, is heavily cultured in several Asian countries and production is increasing in Central America and the Caribbean.

Methods and Materials

Organoleptic analysis

To examine the organoleptic differences of fillets from fish raised on plant-based diets compared to commercial, fishmeal-based diets, we raised three different species on either a plant-based diet (ARS) previously developed in our lab (Watson *et al*., 2012) or commercially available fishmeal-based diets. The plant-based diet used in this study is identical in formulation to that used by Watson *et al.* (2012) however, IMET had 5 tons produced commercially by Silvercup Fish Feeds (now Skretting USA, Murray, UT). Cobia were reared on the diets from 130 to approximately 500 g prior to harvesting with Zeigler Marine Grower used as the commercial comparison. Gilthead sea bream were reared from 11 to 400 g prior to harvesting with Skretting Europa 18 used as the commercial comparison. Striped bass were reared from 530 to 1200 g prior to harvesting with Skretting's Europa 18 used as the commercial comparison. After harvesting, fillets were removed, packed on ice, randomly labeled with numbers by type and sent to the University of Maryland, Eastern Shore's Food Science and Technology Center for double-blind organoleptic analysis. Fillet samples were trimmed to similar sized pieces for each species and cooked in a 160 ºF convection oven to a consistent white, flaky state.

Organoleptic analysis was performed by panels of 17 volunteer, un-paid judges with 34 judgments made per species comparing fillets from fish raised on the plant-based diet to those raised on the commercial diet, intra-species comparisons only. A triangle test was performed to determine if judges could determine which of 3 cooked fish samples differed from the other 2 presented. If differences were determined, panelists were asked to define and rate the intensity of the difference. Statistics were run to determine if the

number of correct responses for each comparison differed significantly from expected random guessing (33 % correct). Peak shear force, the amount of pressure needed to tear the fillet, was measured on fresh and cooked samples of the sea bream and striped bass on a Kramer shear press. There was not enough fillet material from the cobia trial to assess shear force as well as the organoleptic properties.

Contaminant analysis

Samples of fillets from each species and dietary treatment were frozen, lyophilized, and sent for total mercury (Hg) and PCB quantification. Total mercury and total PCB concentration were compared by t-test within each species between fish fed the plant-based diet and fish fed the commercial diet. The methods for total-Hg analysis in fish is derived from EPA Method 1631. Fillet samples were digested prior to analysis via hot reflux in a 7:4 HNO_3:H_2SO_4 acid mix. Total Hg in samples is reduced to elemental Hg with $SnCl_2$, stripped into the gas phase via a gas-liquid separator, trapped onto gold-coated bead columns and heated into the an-atomic florescence detector (Tekran 2600). Detection limits for most matrices are <0.05 ng g^{-1}. Routine QA/QC includes 10 % blanks and duplicates/replicates, and analysis of CRMs (CRC DORM III) in every run.

For PCB analysis, approximately 20 g of freeze dried fish tissue was pulverized in a clean mortar and pestle and transferred to a 100 ml glass beaker. A surrogate spike of PCBs 14 and 65 was added to all the samples before extraction to check for PCB recovery. The samples were then extracted by sonication using a mixture of hexane:acetone 1:1 (v/v) following EPA method SW846 3550B. The extracts were concentrated to 5 ml and treated with concentrated sulfuric acid (method 3665A) for removal of lipids followed by activated copper treatment for removal of sulfur (method 3660B). The extracts were further cleaned

to remove interfering organics by passing through a glass column layered with 20 g of acidified silica gel (30 % concentrated sulfuric acid by weight), 24 g of 3 % deactivated Florisil©, and 1 inch of anhydrous sodium sulfate (top to bottom). 150 ml of hexane was used for complete elution of PCBs from the column. The eluate was concentrated to 1 ml under a gentle stream of nitrogen for analysis. PCB congener analysis was done using a gas chromatograph with a micro electron-capture detector (6890N, Agilent Technologies, Santa Clara, CA, USA). A 60 m X 0.25 mm X 0.25 µm fused silica capillary column (RTX-5MS, Restek US, Bellefonte, PA) was used with helium as the carrier gas at constant flow of 1 ml min^{-1}. The oven temperature program began at 100 °C and was increased at the rate of 2 °C min^{-1} to 280 °C, followed by an increase of 10 °C min^{-1} to 300 °C and was held at this temperature for 6 min. Quantification of the target PCB compounds was performed using a multi-level calibration. Identification of PCB congeners was carried out by comparison of retention times in the chromatogram with that of PCB standards purchased as hexane solutions from Ultra Scientific (North Kingstown, RI). PCB 30 and 204 were used as the two internal standards because they are not present in commercial Aroclor mixtures. Using this method 89 PCB congeners, including some co-eluting peaks were identified and quantified. Co-eluting peaks were quantified as the sum of the congeners.

Results

There were no significant differences detected by the organoleptic analysis panels for any of the three species with no more than 33 % of the panelists correctly choosing which of three fillet samples presented to them was different from the other two in the triangle test. This means that the average consumer cannot detect the differences in odor,

color, or flavor in fillets from fish raised on this plant-based diet when compared side by side with fillets from fish raised on traditional fishmeal-based diets. Peak shear force was roughly equivalent in both species for fresh fillets, however after cooking peak force was roughly twice as high in fillets from fish raised on the commercial feeds (Table 6.1.1) compared to fillets from plant protein fed fish. Shear force also tended to increase with cooking, an expectation of the reduction in water brought about by cooking.

Table 6.1.1. Peak shear force (g) of fresh and cooked fillets of sea bream and striped bass raised on commercial and plant-based diets. Analysis by Thomas Rippen, UMES.

Species	Diet	Status	Peak Shear Force (g)
Sea Bream	Commercial	Fresh	11,641
Sea Bream	Plant Protein	Fresh	10,820
Sea Bream	Commercial	Cooked	19,298
Sea Bream	Plant Protein	Cooked	7,295
Striped Bass	Commercial	Fresh	17,299
Striped Bass	Plant Protein	Fresh	12,922
Striped Bass	Commercial	Cooked	45,532
Striped Bass	Plant Protein	Cooked	22,689

Mercury and PCB concentrations were significantly higher (t-test, $p<0.05$) in fillets from fish raised on the commercial diets for all three species (Table 6.1.2) with the only exception being PCB concentrations in striped bass. Although the PCB concentration in striped bass was higher (12.05 ± 6.88) in fish fed the commercial diet than fish fed the plant-based diet (7.04 ± 5.12), the difference was not significant.

Table 6.1.2. Measured PCB and mercury concentrations (ng g^{-1} dry weight) in fillets of cobia, sea bream, and striped based raised at IMET. Numbers in parentheses are the initial and final average weights of fish used for fillet analyses. Values with different superscripts between each dietary type for each species and contaminant are significantly different from one another ($p<0.05$).

	PCB (ng g^{-1} dw)	Mercury (ng g^{-1} dw)
FDA limit in edible fish	2000 ng day^{-1}	1000 ng day^{-1}
Cobia (130-500 g)		
ARS Diet	3.60 ± 1.15^{a}	20.14 ± 2.96^{a}
Commercial Diet	45.5 ± 3.8^{b}	71.37 ± 3.56^{b}
Sea Bream (11-400 g)		
ARS Diet	6.31 ± 0.41^{a}	7.82 ± 1.94^{a}
Commercial Diet	12.19 ± 1.05^{b}	115.76 ± 40.07^{b}
Striped Bass (530-1200 g)		
ARS Diet	7.04 ± 5.12	36.92 ± 18.17^{a}
Commercial Diet	12.05 ± 6.88	94.58 ± 5.05^{b}

PCB congener analysis of both the diets (A) and fillets (B) are shown in Figure 6.1.1. As expected, the higher PCB loads in the diets are carried through to reveal higher PCB concentrations of the fillets of fish raised on the commercial feeds. Several trends are apparent when comparing the congener profiles of the diets and fillets. The commercial diets have higher concentrations of the larger congeners (tetra, penta, hexa, and hepta) whereas the plant-based diet has higher concentrations of the smaller congeners (di, tri, tetra). This produces two different signature patterns for the diet types that is carried through to the fillet profiles (Figure 6.1.1 B). The length of time the fish were raised on the plant-based diets also has the effect of increasing the PCB concentrations, as cobia, who were raised for the shortest amount of time have some of the lowest concentrations while sea bream and striped bass tend to have higher concentrations of all congeners and total PCB concentrations.

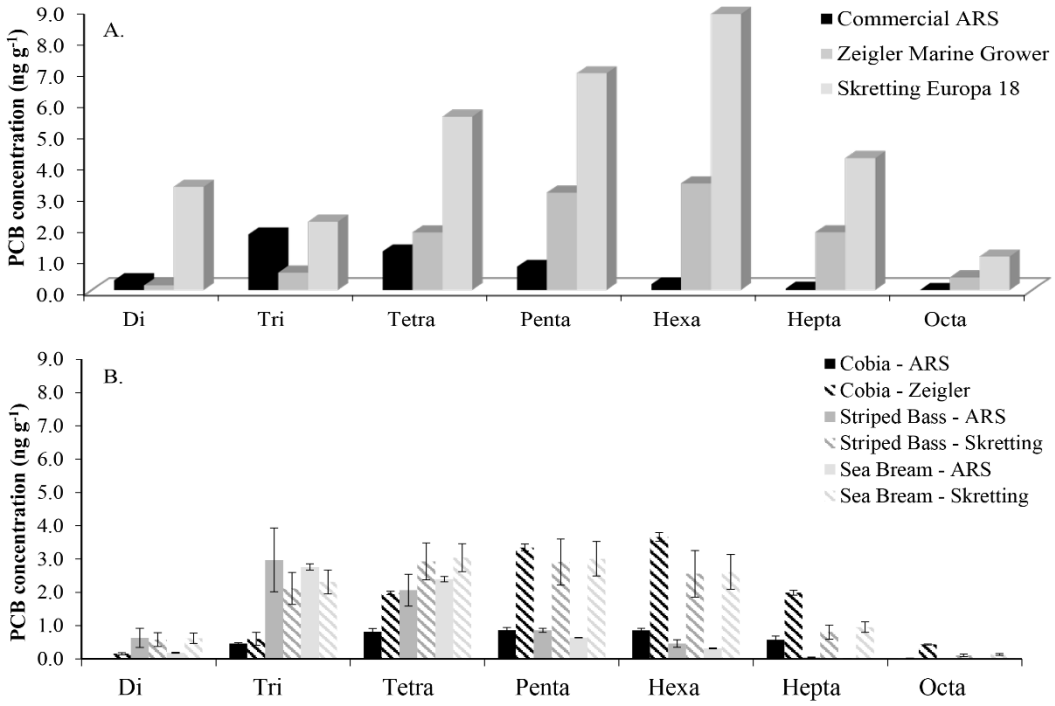

Figure 6.1.1. PCB congener concentrations (di-octa) for diets (A) and fillets (B) of fish used for contaminant and organoleptic analysis. PCB quantification performed by Dr. Upal Ghosh, UMBC.

Discussion

Elimination of fishmeal from diets for aquaculture results in significantly reduced contaminant loads in the fillets of fish (Table 6.1.2). Although values for fish raised on either the commercial feeds or the ARS plant-based diet were all much lower than FDA recommended limits, full-scale grow out cycles are longer than those used here, so values would go up slightly in a full-scale production setting. FDA advises intake of up to 0.4 micrograms mercury per kilogram body weight per day. This means a 150 lb person can consume up to 27 µg mercury daily. Our mercury measurements have been made on dry weight tissues, so the wet fillet weight that would be consumed would result in concentrations roughly 75 % lower than those reported in Table 6.1.2. PCB concentrations

would also be roughly 75 % lower, although dry weight contaminant levels are already significantly lower than the 2000 ppb FDA advisory.

The unique signature produced by the different type of protein sources is also an advantage for raising fish on plant-based feeds as the smaller congeners found in the plant-based fed fish fillets are both less toxic and easier to break down than the larger congeners found in higher concentrations in the fillets of fish fed the commercial fishmeal-based feeds. In contrast to the perceived line of thought that these contaminants are found and biomagnificate mostly through the lipid portion, removal of only the fishmeal protein source and still relying on fish oil, as all these diets do, resulted in significant reduction of PCBs and mercury. As previously discussed in Chapter 5, replacing fish oil with alternative oils further reduces, but does not totally eliminate contaminants. This indicates that these contaminants are not only found in the lipid sources, but are incredibly pervasive in our environment.

The differences observed in shear force in both sea bream and striped bass are interesting, as it appears the plant protein inclusion has effects on both water content and how that water behaves when cooked. However, these differences did not result in differences in taste or texture to the panel. The inability of potential consumers to detect differences in taste, odor, or smell of fillets from fish raised on the plant-based ARS diet compared to the commercial diets is a huge advantage for aquaculture. Just as replacing fishmeal with plant proteins would be ineffectual for aquaculture if fish do not consume and grow adequately, replacing fishmeal with plant proteins would be useless if it resulted in an inferior fillet product at market. Taken together, the lowered contaminant concentrations and similar organoleptic qualities of fish raised on the plant-based diet

represent a huge advantage in increasing the sustainability of aquaculture, feeds in particular. PCB concentrations from both dietary treatments of this study are significantly lower than concentrations observed in wild caught striped bass from both the Chesapeake Bay and Atlantic Ocean (Dr. Upal Ghosh, UMBC unpublished data). The lower contaminant concentrations also represents an added advantage to the consumer, and could be used to partially justify the currently higher prices incurred by this particular plant protein formulation.

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Chapter 7: Conclusions and Future Directions

Overarching conclusions

Through this research, a major roadblock to replacing fishmeal has been overcome with the use of taurine as a supplement in dietary formulations using plant protein sources, especially for marine carnivores. Plant protein blends can be used effectively to fully replace fishmeal as long as taurine is supplemented adequately, in addition to other constituents known to be insufficient in plant sources. However, not all plant proteins will work effectively in all species, underlining the importance of species-specific work to maximize production while minimizing negative environmental impacts and waste. In these studies, barley meal and wheat gluten were identified as ingredients that are not appropriate for cobia, either due to low digestibility or negative impacts most likely due to anti-nutritional factors. Both of these ingredients have been used effectively in other species, as previously discussed, and although gluten issues have been identified in other vertebrate species, the lack of digestibility of barley meal is curious, and may be related to the types of storage proteins utilized in barley compared to the other plant proteins. Although these studies have shown significantly reduced PCB and mercury concentrations in the fillets of fish raised on fishmeal-free, plant protein-based feeds, there exist similar concerns with plant meals. Mycotoxins are incredibly toxic to animals and may become a serious concern when considering large-scale production of plant based feeds (Balbus *et al*., 2013; Berthiller *et al*., 2013). The future of aquafeeds may see regular analysis of feeds for mycotoxin concentrations or possibly reduced shelf-life of feeds in comparison to traditional meat meal based feeds currently being produced.

If taurine is not available or becomes too expensive as a single ingredient, other sustainable ingredients high in taurine are available as potential replacements. Clam byproduct meal, which currently largely goes to waste (Henderson and Strombom, 1990), can be utilized to achieve similar results in fishmeal replacement (Begum *et al*., 1994). Squid and krill meals have often been supplemented in small quantities as "feed attractants" (Barrows *et al*., 2007; Gaylord *et al.*, 2007; Kim *et al.*, 2007; Welch *et al.*, 2010); however, these ingredients are more volatile than fishmeal in terms of quantities available and sustainability. Squid and krill meal in particular are very high in taurine, which may explain their effectiveness at small inclusion rates. However, they should be replaced with other invertebrate meals high in taurine to increase sustainability. Some meals such as earthworm meal can be utilized to achieve similar growth as fishmeal (Stafford and Tacon, 1985). Development of sustainable invertebrate meals from sources such as polychaete worms grown in the sludge sumps of marine, recirculating systems could become a dietary source of taurine, as well as an important secondary product for these expensive systems. These high taurine meals considered for inclusion in diets at low levels to meet taurine requirements may also help spare supplemental methionine requirements, as fish that are capable of synthesizing taurine would not have to rely on methionine or cysteine input to meet their taurine needs.

Effective fish oil replacements have also been identified for cobia and sea bream, although research needs to continue to identify and determine requirement levels of the essential fatty acids for each species, as there are clear differences in the applicability of the lipid sources used here. The CAN+EFA diet produced drastically different growth and survival results between cobia and sea bream, indicating significant differences between

these two species in either requirement level or synthesis capacity for specific fatty acids. Similar to what has been observed with some protein sources, some lipid sources may cause negative effects in different species. The potential presence of anti-nutritional factors in terrestrial oil sources needs to be examined closer as full fish product replacement becomes a necessity. The emerging biofuels industry may become a significant competitor with feed manufacturers for access to algal oils, which are the best suited for direct fish oil replacement. However, great potential for collaborative research exists in the development of sustainable algal oil sources.

The diets developed from these research projects often out-performed commercial diets. Although fishmeal and fish oil prices have not yet risen to the point of forcing manufacturers to find alternatives, many have begun seeking ways to reduce fishmeal, and several fish farms have already switched to having their own fishmeal-free, proprietary formulations manufactured. The diets developed here are anywhere from 10-20 % more expensive than current, commercially produced fishmeal-based diets depending on fishmeal and plant meal prices. Although the increased growth rates and reduced feed conversion ratios achieved by our diets make up for some of the cost differences by providing the ability to reach market size quicker with a lower volume of feed used, the differences are not currently economical on a large-scale. There also has yet to be enough research performed with these formulations on a wide enough range of species to justify their production and marketing on a wide scale. Feed manufacturers are unwilling, and farmers of species others than those tested here are too apprehensive, for these diets to be implemented on a large scale without further work.

An interesting phenomena occurs when compiling the fillet water content (% dw) versus dietary taurine values. Plotting all data points from the cobia growth studies described in the previous chapters based on the primary protein source of the diet (plant, fishmeal, or mixed) reveals opposing relationships with water content and dietary taurine (Figure 7.1.1). Plant-based diets result in decreasing water content in the fillet as dietary taurine increase, while fishmeal or mixed based diets have no effect on water content as a function of dietary taurine. This is clearly a response beyond simple taurine inclusion, and may be related to plant proteins affecting the overall physiology, water balance, or anti-inflammatory/antioxidant effects of taurine. The relationship within the plant-based diets is being driven by the zero taurine supplementation diets resulting in the highest fillet water content, so it may be as simple as an inflammatory response that only requires minimal taurine to counteract, since removing these data from the comparison results in similar trends as the fishmeal and mixed base diets. Although rarely significant between fishmeal diets and plant-based diets, this was a recurring trend with few exceptions. This trend was also observed in sablefish during our collaboration with NOAA-NWFSC. Fish fed a zero taurine, plant protein-based diet resulting in 76.62 ± 1.02 % water in their fillets compared to 72.79 ± 4.33 % water for fish fed a commercial fishmeal-based trout diet.

It is difficult to predict if this would be further exacerbated in longer trials and whether this is physiologically significant. This did not have an effect on any of the organoleptic tests with cobia, but it may be an explanation of the difference in shear forces recorded for sea bream and striped bass, where no organoleptic differences were detected. Higher water content of fresh fillets of fish raised on plant protein diets could be an added

advantage for farmers, as similar amounts of feed will produce an increased weight of product if all other growth characteristics are similar.

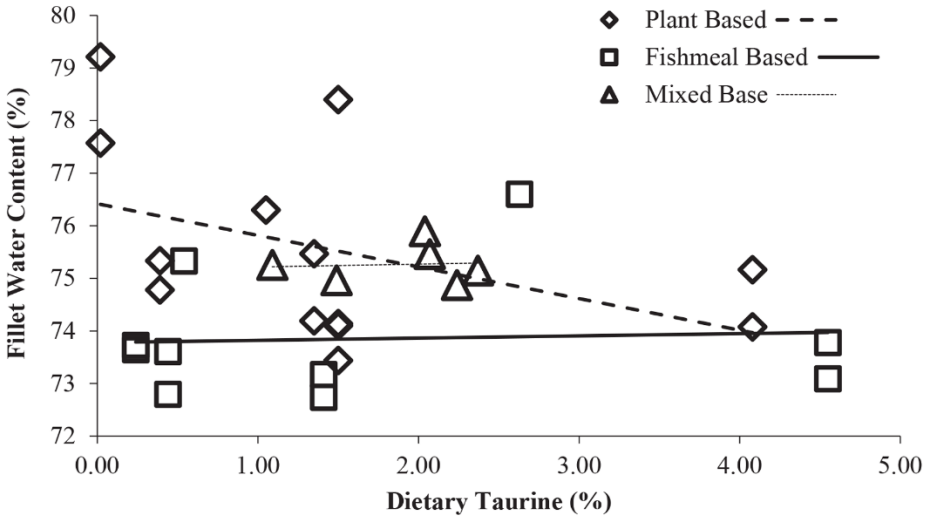

Figure 7.1.1. Fillet water content (% dw) vs. dietary taurine (%) for cobia trials. Water content decreases with increasing dietary taurine for plant-based diets (diamonds), however there is no effect on water content with increasing dietary taurine for fishmeal-based (squares) or mixed base (triangles) diets.

Taurine synthesis and CSD

Dozens of primer sets designed from database deposited sequences for individual teleost species, as well as degenerate, non-degenerate, and iCODEHOP primer sets from various consensus sequences of teleosts and non-teleosts were designed during the course of this work. All work was attempted with cDNA sequences to identify the presence of the transcript. None of these primer sets produced products that were similar to CSD using BLAST. Possible explanations for this include no expression, too low expression for the CSD transcript to be the primary product, an insertion or deletion in the genomic DNA in cobia that has rendered it unable to be transcribed, enough divergence in the sequence that the primers designed are ineffectual, or the possibility that although other synthesis genes are expressed in the tissues examined in this work, CSD may not be. Not discussed in the

previous chapters was that in attempts to find CSD expression; kidney, intestine, and pyloric caeca were all examined with similar methods. Expression of ADO, CDO, and TauT was observed in all of these other tissues, but CSD transcripts were never detected.

Although the lack of expression of CSD fits the hypothesis that cobia are unable to synthesize sufficient taurine, the inability to locate expression through these methods alone is insufficient to definitively conclude that issues with CSD are the reason cobia cannot synthesize taurine. After the attempts to isolate CSD expression and the variety of primer sets, primer design programs used, and PCR parameters tested, it is my opinion that CSD is not expressed in cobia. I believe that a mutation, insertion, or deletion in the CSD gene has occurred that has resulted in CSD either not being transcribed, or this change has been so significant that the product is no longer CSD and does not perform the same function. Due to the high dietary input of taurine in the natural diet of this carnivore, a change such as this would not have been lethal. Similar to the work done with cDNA sequences, research is currently underway with genomic DNA in an attempt to resolve this. However, it is clear from coupling the RT-qPCR results of these studies with the growth studies of cobia with zero taurine diets that cobia do not synthesize sufficient taurine, regardless of whether CSD is functional or not. Although there are significant increases in the transcripts of CDO, CSD, and TauT in sablefish fed a zero taurine supplemented plant protein-based diet, these fish were also apparently unable to synthesize sufficient taurine, as their performance was still significantly lower than that of conspecifics on a commercial trout diet (Dr. Ron Johnson, personal communication).

Figure 7.1.2 diagrams the major conclusions reached from the molecular work completed on the taurine synthesis genes. Overall, cobia do not show an ability to respond at the transcript level to low dietary taurine, unlike zebrafish.

There are differences in the ability to synthesize taurine among marine carnivores from the two (cobia and sablefish) examined here, but it is apparent that neither are capable of synthesizing sufficient taurine. Taurine must therefore be considered essential and be provided through dietary supplementation. The only freshwater omnivore, zebrafish, examined here however is capable of synthesizing sufficient taurine. These results agree with the history of many freshwater omnivorous, herbivorous, or scavenging based feeders being able to transition off of fishmeal-based feeds without taurine supplementation. An interesting comparison would be to analyze the taurine synthesis potential of anadromous and catadromous species to determine if habitat or diet is the more critical driving factor of taurine requirement and synthesis capacity.

These results and potential explanation also agree with decades of work done with dozens of species regarding fishmeal replacement. Many researchers have observed drop-offs in production characteristics when reducing fishmeal inclusion below 10-15% without taurine supplementation. Taurine supplementation often partially or completely recovers these loses in production, indicating just how critical taurine is and how many species may have lost the ability to synthesize sufficient quantities.

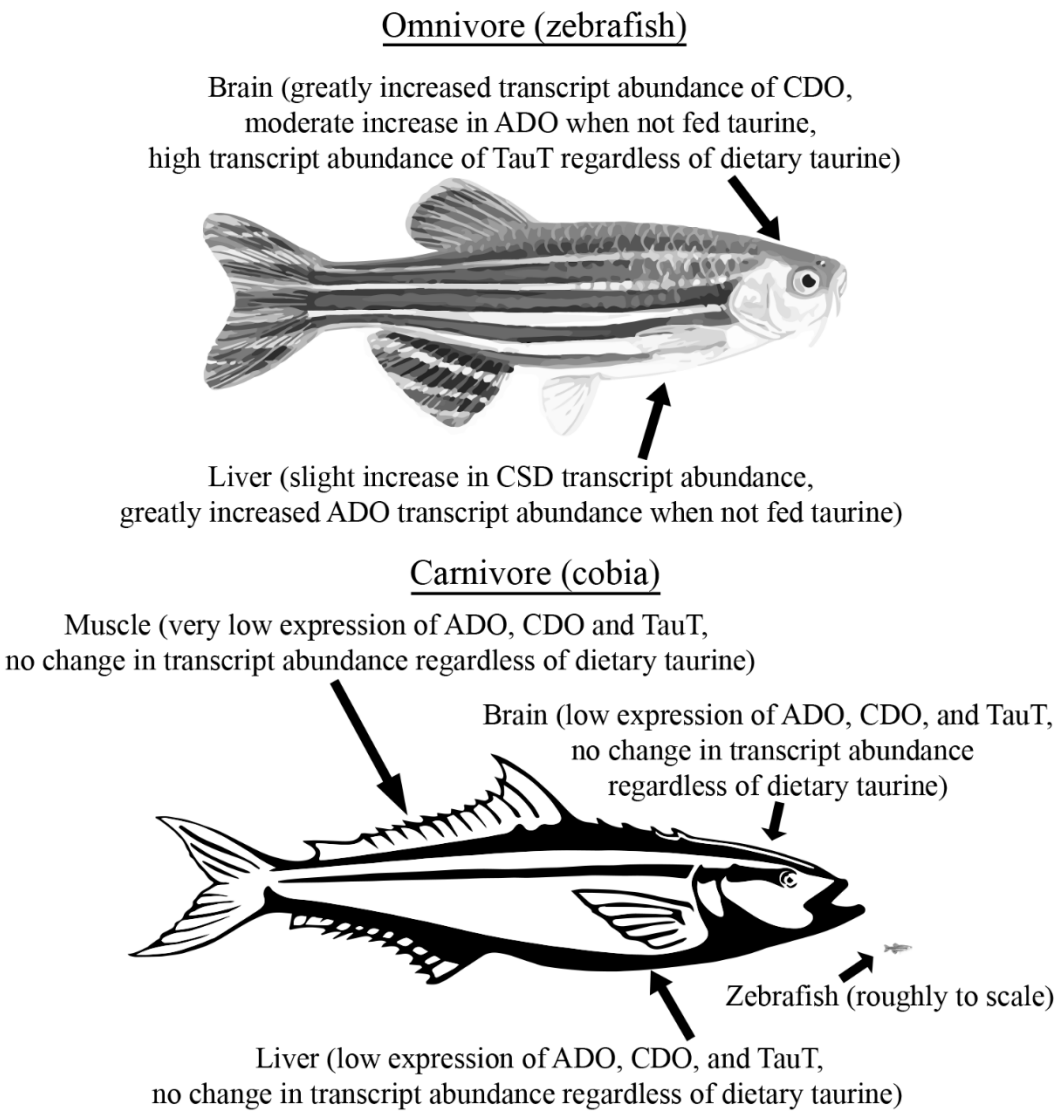

Figure 7.1.2. Major sites of potential taurine synthesis in a freshwater omnivore (top) and a marine carnivore (bottom). Zebrafish respond, although through different mechanisms in different tissues, to low taurine at the transcript level, whereas cobia do not. Zebrafish image modified from http://animaldiversity.ummz.umich.edu/, cobia image modified by Alexandra Casmer.

An interesting hypothesis to potentially explain the importance of taurine would be that when fish are fed reduced taurine feeds, the taurine present in the body is utilized for the most critical functions. Examining the whole body taurine pool over time after a switch to reduced taurine diets could answer this question. Tracking the different tissue taurine

levels (e.g. plasma, liver, muscle, bile) over time would help identify which roles of taurine are the most important physiologically, and this could be an explanation as to why lower taurine concentrations were observed in these studies with low taurine feeds, yet there was no change in bile salt concentration.

Future considerations

The next logical step in this research, which funding for was applied for but never obtained, is to look at protein expression through both western and enzymatic assays. Several published assays describe methods to determine the enzyme activity of ADO, CDO, and CSD (Goto *et al*., 2003, 2001a, 2001c; Ueki *et al*., 2012; Worden and Stipanuk, 1985) through various methods including production of CO_2 as a byproduct, measuring taurine production directly, or potentially through the incorporation of radiolabeled S^{35} methionine for the entire synthesis pathway. Teleost specific antibodies for these proteins do not currently exist, but having them generated or testing anti-mammalian antibodies would allow for the identification and quantification of these proteins in various potential synthesis sites.

Utilizing these assays as the next steps in this line of research would help answer the question of whether or not cobia express CSD, and would determine the specific enzyme activities in the taurine synthesis pathway. These tools could be applied to similar dietary studies, as done here with graded levels of taurine to truly determine if cobia, and other species, are capable of responding to insufficient dietary taurine and in defining dietary requirements.

Developmental stage also plays a major role in the ability or inability of various species to synthesize certain compounds. Embryos and early developing larvae have been shown to have limited capacity for essential fatty acid synthesis, and maternally deposited mRNAs for various enzymes are common up until the point the larvae can generate its own (Chang *et al*., 2013; Monroig *et al.*, 2009). Developing the knowledge of when target species are capable or incapable of synthesizing essential components and how they gain or lose these abilities would be incredibly beneficial in developing and maximizing feed formulations. Taurine supplementation to the live feeds, rotifers and *Artemia*, has been shown to increase growth in larval cobia and Senegalese sole (Pinto *et al*., 2010; Salze *et al*., 2012). However, this method can be challenging due to taurine's high water solubility so the development of improved enrichment methods could greatly enhance larval growth and survival for many species. Consistent supply of adequate fingerlings is a bottleneck for much of the industry with many species, and taurine may help alleviate this issue.

Parallels can be drawn to broodstock in terms of providing diets that are adequate for maximizing reproductive potential based on synthetic capacity of the species and providing needed components for deposition in eggs. Broodstock are often fed "natural" diets of whole squid and fish during spawning seasons, as these have long been believed to be better nutritional sources than pelleted feeds. The specific reasons behind these feeding habits are often elusive, however taurine may play a major role in explaining why whole fish and squid are better nutritional sources than pelleted feeds.

Taurine has been shown to play roles in spermatogenesis in eels (Higuchi *et al*., 2012a, 2012b) and fatty acids are critical during vitellogenesis, which are just two examples of components that broodstock would need increased quantities of prior to and

during spawning season as opposed to basal levels provided through traditional feeds. If high taurine concentrations in eggs and embryos can be linked to subsequent increases in growth or survival, then broodstock diets may need to have higher taurine supplementation than grow-out diets.

It has been my goal in the final stages of this work to develop not only complete, sustainable diets for aquaculture, but also a set of simple molecular tools (RT-qPCR primers) that can be used in short term trials to aid in determining taurine requirements. Through the applied approaches taken in this work, both of these objectives have been accomplished.

It is also my hope that additional sets of similar tools can be designed in the future for other synthesis pathways, such as those for essential, semi-essential, and potentially essential constituents. This could greatly enhance the optimization of diets for specific species as well as significantly reduce the costs associated with traditional routes of determining minimal and optimal requirements. This work represents advances in multiple areas of sustainable aquaculture, but much work remains to develop the aquaculture industry, especially in the United States, into one that can supply the world with safe, sustainable protein.

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