Abstract

Title of Thesis: QUANTIFICATION OF FATTY ACIDS OVER THE GAMETOGENIC CYCLE OF STRIPED BASS (*MORONE SAXATILIS)* WITH VARYING DIETARY LEVELS OF DHA, EPA, AND AA.

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The purpose of this thesis was to investigate the effects of dietary treatments on the fatty acid composition of striped bass (*Morone saxatilis*) blood, liver and ovary over the course of the gametogenic cycle. Striped bass were fed experimental diets in which docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA) and arachidonic acid (AA) were increased in a stepwise fashion progressing from diets 1-4 respectively. A significant difference was seen in the tissues between fish from dietary treatment group 1 when compared to those fish from dietary treatment group 4 suggesting a direct impact on the tissues by the fatty acid composition of the diet.

The second purpose of this thesis was to explore the use of a novel method of fatty acid extraction from fish tissues. This method was compared (UMD method) was compared to the well-known Bligh and Dyer method commonly used for fatty

acid extraction. The UMD method proved to be superior quantitatively in terms of fatty acid extraction when compared to the Bligh and Dyer method. The UMD is also more cost affective and leaves less room for human error.

Quantification of fatty acids over the gametogenic cycle of striped bass (*Morone saxatilis)* with varying dietary levels of DHA, EPA and AA

by

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Dedication

Dedicated to my parents and husband

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I would like to give a special thanks to my advisor Dr. L. Curry Woods III. It's been a rough road and he has always found a way to encourage me and keep me on track. His countless hours of guidance and assistance are greatly appreciated and will never be forgotten.

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List of Abbreviations

Abbreviation	Word Equivalent
PUFA	polyunsaturated fatty acid
EFA	essential fatty acid
DHA	docosahexaenoic acid
EPA	eicosapentaenoic acid
AA	arachidonic acid
PC	phosphotidyl-choline
PI	phosphotidyl-inositol
PE	phosphotidyl-ethanolamine
DO	dissolved oxygen
FAME	fatty acid methyl ester

Introduction

History of Striped Bass

Striped bass, *Morone saxatilis*, is the largest and longest-lived species within the family Moronidae (Whitehurst and Stevens 1990). It has long been a popular sport fish and within the past two decades has become an important species for aquaculture. Originally the striped bass was found in the wild from the St. Lawrence River in Canada to northern Florida, and along the gulf coast of Florida to Louisiana (Raney et al., 1952). The popularity and demand for this fish led to the seining of 435 yearling fish in 1879 and 1881 from the Navesink and Shrewsbury Rivers in New Jersey where upon they were transported by train across country and released into San Francisco Bay (Whitehurst and Stevens, 1990). A fishery soon developed in central California. The striped bass can now be found from Southern California to the Columbia River in Oregon (Whitehurst and Stevens, 1990).

Excessive harvesting, destruction of habitats and pollution has greatly effected this species over the years. The first legislation to protect these fish dates back to 1639 when the Massachusetts Bay Colony passed legislation prohibiting the use of striped bass as fertilizer (Whitehurst and Stevens, 1990). Around 1973, the striped bass population began to plummet. Catches declined from 14.7 million pounds in 1973 to 3.7 million pounds in 1979 (Baker 1994). This led to the development and implementation of the Atlantic Striped Bass Conservation Act in 1984 (Fowler 1995). This act allowed for government monies to be set aside and used for striped bass research and management programs.

In 1985, states such as Maryland and Virginia implemented strict moratoriums severely limiting the capture striped bass (Baker 1994). In order to bring population numbers back to self-sustainable levels, hatchery programs were initiated (Fowler 1995). Due to the success of these programs the Maryland moratorium prohibiting the capture of these fish was lifted in 1990 (Baker 1994).

It became clear in the 1970's that the demand for the striped bass was greater than could be sustained by natural populations. Although a number of attempts were made to culture these animals in the 1970's, they met with no success due to the regulations prohibiting the use of wild fish for broodstock (Stickney 1996).

The industry's dependence on the use of wild fish for seedstock has become one of greatest obstacles in the success of striped bass aquaculture (Smith and Jenkins 1986; Smith 1989; Woods et al., 1990; Harrell, 1992; Woods et al., 1992). The number of fish a grower is allowed to capture and use for seedstock is often very limited (Woods et al., 1992; Woods and Sullivan 1993; Leffler 1999). This not only creates seasonal limitations but eliminates the ability of the grower to choose fish based on improved genetic performance for heritable traits of importance which is very important in developing quality seedstock (Woods and Sullivan 1993). In order for the striped bass aquaculture industry to become successful it is clear that domesticated broodstock must be developed (Smith 1989).

Success in domesticating striped bass will in part depend on determination of qualitative and quantitative dietary requirements (Tuncer and Harrell 1990; Harrell and Woods 1995). Nutrition is a critical factor in aquaculture due to its effect on fish growth and health as well as cost of production (Gatlin 1995). Growth within the

aquaculture industry has led to the increased use of formulated diets. The use of natural feed sources is both inefficient and costly. Natural food sources can be contaminated and nutritional value can often be inconsistent or unknown. Successful domestication of striped bass will not only depend on the formulation of good quality larval diets but on the creation of proper broodstock diets as well.

The Role of Nutrition in Aquaculture

Increase in use of formulated diets requires a full understanding as to the nutritional requirements of the cultured species. There must not only be full knowledge as to the nutritional make-up of that species natural diet but also the physiological importance of each of these nutrients. This is required so that formulated diets can nutritionally compensate for situations encountered daily in captive situations, which can often cause stress and disruption of the normal life cycles.

Broodstock nutrition not only effects the health of the parental fish, but nutrition has been suggested to directly effect the gamete quality as well (Watanabe et al., 1984a; Watanabe et al., 1984b; Mourente and Odriozola 1990; Cerda et al., 1994b; Harel et al., 1994; Bromage 1995; Bell et al., 1997; Almansa et al., 1999; Cavalli et al., 1999; Rainuzzo et al., 1997). It has been shown that while gross nutrient availability (i.e. food ration modifications), does influence various aspects of reproductive physiology, there is little evidence to show that such changes can effect egg and larval quality (Cerda et al., 1994a; Bromage 1995). Research has instead

concentrated on the individual components of the diet such as vitamins, amino acids and fatty acids.

Polyunsaturated Fatty Acids

Polyunsaturated fatty acids have been found to have a number of important physiologic and regulatory functions. In fact all vertebrate species that have been studied have a requirement for certain polyunsaturated fatty acids (PUFAs). If the diet of that species is deficient in PUFAs, the animal may stop growing and reproducing. It also can develop various pathologies and could even die (Sargent et al., 1995). Polyunsaturated fatty acids play a major role in membrane fluidity and permeability, transportation of lipids, activation of enzymes and act as precursors for prostaglandins all of which are essential to the health and well-being of the fish species involved (National Research Council 1993).

Three PUFAs in particular, have been found to be essential to several marine fish species such as red sea bream (*Pargus major*) (Watanabe 1993), yellowtail (*Seriola quinqueradiata*) (Watanabe 1993), turbot (*Scophthalmus maximus*) (Bell et al., 1985), gilthead sea bream (*Sparus aurata* L.) (Bessonart et al., 1999), sunshine bass (Nematipour and Gatlin 1993), Argentine prawn (*Artemesia longinaris*) (Petriella et al., 1984), Japanese flounder (*Paralichtys olivaceus*) (Tago et al., 1999), striped bass (*Morone saxatilis*) (Tuncer and Harrell 1992), palmetto bass (*Morone saxatilis* × *M. chrysops*) (Tuncer and Harrell 1992; Tuncer et al., 1993) and red drum (*Sciaenops ocellatus*) (Williams and Robinson 1988) and have been termed essential fatty acids (EFA). Two are of the omega-3 fatty acid family (docosahexaenoic acid (DHA), 22:6n-3 and eicosapentaenoic acid (EPA), 20:5n-3) and one is in the omega-6 fatty

acid family (arachidonic acid (AA), 20:4n-6). These fatty acids are termed essential, especially in the case of marine fish, due to the inability of these fish to self-produce quantities of these fatty acids sufficient enough to meet nutritional needs. It is felt that this inability to self-produce such fatty acids as DHA, EPA and AA arises from the fact that these fish, in the wild, consume large quantities of these fatty acids in their predominantly piscine natural diets (Sargent et al., 1995). Over time, through evolution, these fish have lost the ability to effectively convert shorter chain precursors such as alpha-linolenic acid (18:3(n-3)) and linoleic acid (18:2(n-6)) to their respective homologues DHA, EPA and AA (Owen et al., 1975; Tuncer et al., 1993; Sargent et al., 1995). The inability to produce long-chain PUFAs arises from a deficiency of Δ5-desaturase enzyme, which is required for the formation of 20:4(n-6), 20:5(n-3) and 22:6(n-3) from their C_{18} precursors (Sargent et al., 1995; Bell et al., 1997).

It is arguable as to whether or not marine fish possess the ability to convert EPA to DHA. Some researchers do believe that marine fish possess the ability to create DHA from EPA and AA via a pathway involving Δ6-desaturase, fatty acid elongases and possibly Δ-4 desaturase (Voss et al., 1991; Sargent et al., 1995; Brodtkorb et al., 1997). The exact details of this pathway are currently under investigation with many debates as to the existence of the Δ-4 desaturase (Sargent et al., 1995). It is felt, however, that this pathway is not efficient enough to produce the quantities needed to meet the nutritional needs of the marine fish for DHA. The inefficiency of the pathway may stem from the fact that both the conversion of C_{18} to C_{20} PUFA and the conversion of C_{20} to C_{22} PUFA involve the action of Δ-6 desaturase

therefore creating competition between the two pathways (Sargent et al., 1995). This especially makes sense when you consider the fact that a great deal of artificial and enriched marine fish diets are high in the C_{18} due to the potentially excessive use of vegetable source oils. Vegetable oils are less expensive and more readily available then a fish oils such as menhaden or squid oils and are often used as the lipid source in a diet. Others believe that EPA is not converted to DHA but is instead converted to 22:5n-3 and that DHA and EPA may serve different physiologic functions (Watanabe 1993). In either case it becomes clear that it is necessary to supplement diets with these fatty acids.

Polyunsaturated fatty acids have been shown to perform a number of functions within fish. One such function, as mentioned previously is the reduction of stress, which affect almost every aspect of fish physiologically. It has been observed, however, that by the addition of PUFA's to the diet of cultured fish that they may become more adaptable to stress therefore increasing survival (Tuncer and Harrell 1992; Tuncer et al, 1993; Tago et al, 1999). It has been argued that DHA is superior to EPA as a stress reliever in marine finfish (Watanabe 1993).

A variable often used in determining EFA conditioning of larval fish is a so-called "activity test". Fish are fed experimental diets, which vary the types and levels of specific fatty acids especially PUFAs. The fish are then submitted to an acute stress test in which fish are held out of the water for a short period of time and then placed back into a tank. Survival rate is determined 24 hours later. Research has shown that many species fed diets that are high in PUFA's such as DHA, EPA and/or AA tend to result in better survival rates (Petriella et al., 1984; Bell et al., 1985; Williams and

Robinson 1988; Tuncer and Harrell 1992; Nematipour and Gatlin 1993; Tuncer et al., 1993; Watanabe 1993; Bessonart et al., 1999; Tago et al, 1999). Experiments conducted by Watanabe (1993) were able to demonstrate that not only did the presence of PUFA's increase survivability but that DHA was actually superior to EPA when supplementing diets with one or the other fatty acid. In the case where diets were supplemented with EPA only, mortality rates were higher than in the DHA and DHA/EPA supplemented diets suggesting the importance of the ratio between DHA and EPA. Tago et al. (1999), working with Japanese flounder, also found DHA to be superior to EPA. The experiment set out to determine the effect of 1,2-di-20: 5-PC, 1,2-di-22: 6-PC (PC- phosphotidylcholine; a phospholipid form of EPA and DHA respectively) and 22:6 TG (TG- triglyceride form of DHA) on growth and stress tolerance of larval Japanese flounder. The results indicated that 1,2-di-22: 6-PC was more efficient than 1,2-di-20:5-PC and 22:6 TG in increasing levels of stress tolerated by Japanese flounder larvae when exposed to increased water temperature and reduced DO. It is felt that excessive amounts of EPA may promote an imbalance of the two fatty acids within the phospholipids of the biomembrane therefore affecting membrane fluidity (Watanabe 1993b).

A very important challenge in the aquaculture industry is larval nutrition. This is a time of rapid growth and tissue differentiation during which nutrition is critical in order to ensure proper development. A great deal of the research that has been done concerning PUFA's has centered on the importance of these fatty acids to the developing larvae. As stated previously, one of the major roles of the n-3 HUFA's are as components of membrane phospholipids. DHA has been found to be especially

abundant in developing neural tissue such as the eyes and brain and is considered essential (Tocher and Harvie, 1988; Bell and Dick, 1991). It becomes increasingly important to the developing larvae which rely on sight in order to obtain prey or food needed for growth.

A number of studies conducted on larval fish have been starvation studies. Lipid depletion during starvation has been shown to occur rapidly in larval fish after exhaustion of endogenous nutrients from the egg yolk sac (Rainuzzo et al., 1997). Some researchers believe these studies to be unsatisfactory in that the resulting growth limitations may reflect a deprivation of a nutrient other than fatty acids (Sargent et al., 1995). While this may be true, it is my opinion that these studies are helpful in that they allow the researcher to observe which types of fatty acids are conserved in a starvation situation, and may therefore be deemed necessary if not essential for the survival of the larvae. It has been shown that regardless of the species or tissue type, triglycerides (TG) are the predominant form of reserve lipid, which is always mobilized before the phospholipids (PL) during starvation (Sargent et al., 1989). It is believed that the larvae utilize the TG during starvation in order to meet energy requirements while conserving the PL because it is important in the structure and fluidity of cell membranes (Rainuzzo et al., 1997).

Although triglycerides have been shown to be utilized before phospholipids there is evidence that within the specific classes of lipids certain fish species show a preferential utilization of specific fatty acids. A number of marine fish species such as turbot (Rainuzzo et al. 1994), red sea bream (Tandler et al., 1989), gilthead sea bream (*Sparus aurata*) (Koven et al., 1989; Koven et al., 1993; Rodriquez 1994) and cod

(van der Meeren et al., 1991) have been shown to preferentially conserve the polyunsaturated fatty acids DHA and AA. Koven et al, (1989) demonstrated that gilthead seabream larvae (*Sparus aurata*) tended show not only a pattern for (n-3) conservation but a preference for DHA conservation over EPA. This was also found to be the case with starved turbot larvae (*Scophthalmus maximus*). One such study observed a 50% reduction of EPA as well as the noted conservation of AA in addition to DHA. The authors were able to establish a pattern of fatty acid conservation in which EPA was the least conserved fatty acid followed by the monounsaturated fatty acids, then the saturated fatty acids. Docosahexaenoic and AA were the most tightly conserved (Rainuzzo et al., 1994). Starved turbot larvae were found to have mainly catabolized TG and steryl esters. There was also a noted increase in phosphotidylethanolamine or PE, which is a type of phospholipid. This is significant because it has been shown that there are relatively higher levels of DHA within PE than in the other fish phospholipids such as phosphotidylinositol (PI) and phosphotidylcholine (PC) (Bell et al., 1985).

In addition to starvation trials, a great deal has been learned about the beneficial effects and necessity of supplementing marine larvae diets with PUFA's through feeding trials that vary the levels of essential fatty acids. The feeding of marine fish larvae artificial diets or live foods such as *Artemia* or rotifers, deficient in essential fatty acids has resulted in reduced growth for a number of species such as striped bass (Tuncer and Harrell, 1992), sunshine bass (Fair et al., 1993; Nematipour and Gatlin 1993), red sea bream (Izquierdo et al., 1989; Watanabe 1993b), gilthead sea bream (Koven et al., 1990; Rodriquez et al., 1993; Rodriquez et al., 1994; Salhi et

al., 1994), turbot (Bell et al., 1985), red drum (Williams and Robinson 1988; Craig et al., 1994), yellowtail (Watanabe 1993a), striped jack (Watanabe 1993a), and coho salmon (Yu and Sinnhuber 1979). Researchers are able to determine the ability of a fish to convert their dietary C_{18} precursors to longer chained, more highly unsaturated fatty acids by the presence of specific fatty acids in the tissues of the fish. The presence of 20:3(n-9), 20:2(n-9) and 18:2(n-9), detectable through high resolution capillary gas-liquid chromatography, indicates the inability of the fish to convert C_{18} precursors to longer chained homologues such as DHA, EPA and AA. The presence of the aforementioned fatty acids has been labeled as an indicator of EFA deficiency in fish (Sargent et al., 1995)

In a number of the larval studies that compared lipid oil sources high in PUFA's to those high in saturated and monounsaturated fatty acids such as vegetable oils, results were similar. In almost all cases the type of oil used, whether vegetable or fish, had a direct effect in the lipid composition of the body tissues (Bell et al., 1985; Williams and Robinson 1988; Nematipour and Gatlin 1993; Fair et al., 1993; Koven et al., 1993; Harel et al., 1994; Bell et al., 1995; Xu et al., 1996; Brodtkorb et al., 1997; Olsen and Henderson 1997; Peres and Oliva-Teles 1999). Diets high in 18:3 (n-3),18:2 (n-6) or saturated fatty acids tended to change the composition of body tissues, such as muscle, to directly reflect what was fed. Tissue samples collected and evaluated for fatty acid composition from these fish reflected the fatty acid composition of the diet. The same is true for diets containing high levels of PUFAs such as DHA, EPA and AA. As stated before marine fish appear to have a very limited ability if any, to convert 18:3 (n-3) to essential fatty acids such as 22:6(n-3)

(DHA) or 20:5(n-3) (EPA) (Nematipour and Gatlin 1993). The same holds true for the conversion of 18:2(n-6) to 20:4(n-6) (AA). Studies comparing the fatty acid composition of eggs from domestic striped bass and wild striped bass found arachidonic acid levels to be significantly lower in the domestic striped bass eggs (Gallagher et al., 1998). Levels of 18:2(n-6) have been found to be greater in domestic striped bass, which may be attributed to high levels of this fatty acid in the diet and an inefficient ability to convert this fatty acid via elongation and desaturation to AA (Harrell and Woods 1995).

Early studies conducted with mammals were able to demonstrate that AA (20:4(n-6)) had an essential role in the maintenance and structural integrity of the mammalian cell membrane (Sargent et al., 1995). In cases where this fatty acid was deficient, cell membranes became mechanically fragile and osmotically unstable. This resulted in components such as erythrocytes being very susceptible to lysis and mitochondria isolated from EFA deficient animals increasingly susceptible to uncoupling; a process by which the outer membrane of the mitochondria is interrupted resulting in electron transport without ATP synthesis eventually exhausting energy resources (Sargent et al., 1995; Mathews and van Holde 1996). Fish generally have much greater levels of DHA and EPA in their phospholipids than AA as compared to terrestrial animals. Due to this fact it is believed that DHA and EPA may play the role of AA in the membrane phospholipids of fish, which would explain the greater requirement that has been observed for omega-3 fatty acids over omega-6 fatty acids (Sargent et al., 1995).

It is believed that DHA is an important part of the PE molecular molarity due to the fact that not only is DHA highly conserved within the PE, but the enzyme responsible for PE formation apparently prefers substrates containing DHA (Tinoco, 1982). Koven et al. (1993) was able to demonstrate a higher assimilation of DHA over EPA into the PE fraction of larval gilthead seabream. This preferential assimilation was shown to be highly correlated with growth in 5-36 day old larvae. This implies the possibility that DHA may have a higher biological value than EPA. In addition it may also be true that the PE fraction may be important in growth. Higher assimilation of DHA over EPA into fish lipids has been demonstrated in a number of larval fish species such as gilthead seabream (Koven et al., 1993; Koven et al., 1989), red seabream (Watanabe et al., 1989a), and juvenile striped jack (Watanabe et al., 1989b). It has also been found that like DHA, AA seems to be conserved for the biosynthesis of PI (Castell et al., 1994; Rainuzzo et al., 1994). It is quite evident that this type of biological strategy allows for the preservation of valuable biological membrane components.

The importance DHA plays in the body of the fish is evident in its markedly increased concentration in membranes contained in the brain, eyes and testis (Tocher et al., 1992; Koven et al., 1993; Sargent et al., 1995; Brodtkorb et al., 1997). In neural membranes such as those of the retina, the di-22:6(n-3) form of DHA, which is a phospholipid form of DHA, can be found to account for 70% of the phosphatidylethanolamine and 60% of the phosphotidylserine found in this tissue (Bell and Dick 1991). It is believed that this phospholipid form of DHA may play a structural role by forming a highly ordered and unique bilayer that serves two specific

functions in tissue membranes such as the outer rod segment of the retina or the brain synaptosomal membranes. First it would sustain and facilitate very fast conformational changes undergone by membrane signaling proteins such as rhodopsin. Secondly it could remain relatively constant when faced with changes in environmental temperature and pressure (Sargent et al., 1993). With the exception of those membranes which appear to specifically require DHA in order to produce the special di-22:6(n-3) phospholipid form, it is believed that EPA and AA and DHA all have the sufficient structure required to maintain cell membranes both mechanically and osmotically (Sargent 1995).

Sight for obvious reasons is very important to the developing larvae. Marine fish eggs are often very small in size resulting in a limited endogenous food source i.e. the yolk, for the larval fish. Striped bass eggs for example, only provide sufficient nutrition for about 4-5 days. After that period the larvae must rely on sight in order to consume exogenous food. The size of the eggs also results in the production of very small larvae, which have very rapidly developing tissues and organs such as the brains and eyes. The eyes in particular have been found to make up a substantial portion of the total body mass of the larvae (Sargent, 1995). The importance of di-22:6(n-3) in the neural and ocular tissues can again be noted in the correlation between the appearance of rods in the retina of developing herring larvae and the appearance of the di-22:6(n-3) within the developing fish phospholipids (Bell and Dick 1993). Bell et al. (1995) were further able to demonstrate that herring larvae fed diets deficient in DHA had decreased predation efficiency at low, yet natural, light intensities. These are the light intensities where rods become operational. Further investigation was able

to show decreased levels of di-22:6(n-3) in those larvae fed DHA deficient diets. A number of studies support the finding that dietary levels of DHA have a direct effect on the levels found in the brain and neural tissues of developing larvae (Mourente et al., 1991; Tocher et al., 1992).

Studies also have shown no effect on DHA levels in the eye or brain when varying the levels of this fatty acid in the diet. Studies conducted on Atlantic salmon juveniles found DHA to be the most abundant fatty acid in the brain even though this fatty acid was not the dominating fatty acid in the diet (Brodtkorb et al., 1997). This study actually found no difference in brain levels of DHA when comparing the effects of varying levels of DHA in the experimental diets pointing to a selective incorporation of DHA into the developing salmon brain. It is possible however that the levels of DHA fed in these diets were not varied enough to cause an effect. Brodtkorb et al., (1997) acknowledged the fact that in order to produce major changes in the fatty acid composition of the brain and eye, a DHA deficiency must be present. Tocher et al. (1992) concluded in their study on juvenile turbot, that strictly carnivorous marine fish are heavily dependent on a substantial dietary input of DHA for normal neural and visual development.

Additional data supporting the importance of DHA can be found when looking at the pineal gland of fish. It has been shown that the pineal gland in fish contains rhodopsin which is important in vision. This gland has also been found to be a production sight for melatonin which is important in fish coloration (Falcon et al., 1992). Henderson et al. (1993) and Sargent (1995) were able to demonstrate that the pineal gland also contains levels of di-22:6(n-3) intermediate to those levels found in

the brain and eyes of fish. This information demonstrates another possible area where deficient levels of DHA could not only effect sight, but skin coloration as well which is important in aquaculture for the presentation of the fish to the consumer. More research is needed in this area.

The role that EPA plays in marine fish nutrition may seem diminished when compared to the role of DHA. EPA does appear to play a significant role in the regulation of AA and it's uptake into tissues. The role of arachidonic acid in the diets of marine fish is currently receiving increased attention. Comparison studies with fish often find significantly lower arachidonic acid levels in domesticated fish when compared to their wild counterparts (Harrell and Woods 1995; Gallagher et al., 1998). The lower levels of AA may stem from the use of vegetable oils in formulated diets and the inability to elongate 18:2 (n-6) to AA in certain fish species. Bell et al. (1997) reported significantly lower AA/EPA ratios in sea bass (*Dicentrarchus labrax*) fed pelleted diets when compared with those fish fed a local trash fish, Bogue (*Boops boops*). AA has been found to play a significant role in prostaglandin formation and eicosanoid production, both of which are physiologically necessary (Castell et al., 1984; Sargent et al., 1989; Sargent 1995). Prostaglandin's are a type of eiconsanoid and are produced from C_{20} fatty acids (Mathews and van Holde 1996). These compounds are considered locally acting hormones and have been found to play roles in the signaling of such physiological processes as egg shedding in ripe fish (Sargent 1995), the inhibition of platelet aggregation (reduction of blood clotting) and stimulation of vasodilation (PGE 1) and muscle contraction (Mathews and van Holde 1996). In addition, certain forms of prostaglandins have been found to decrease

gastric secretions (Mathews and van Holde 1996). Bell et al. (1985) found that turbot (*Scophthalmus maximus*) fed a diet low in n-3 and/or n-6 PUFA developed gross changes in gill structure involving the disappearance of chloride cells, a 'sloughing off' of the epithelium along the primary and secondary filaments and an accumulation of cellular material in the inter-lamellar spaces which led to the eventual disintegration of the tissue and exposure of the connective tissue. The fish were also noted as showing a pink suffusion on their skin, which was most noticeable on their pale undersides and in extreme cases bled from the edge of the fins and the operculum. EPA (20:5n-3) has been found to play an indirect role in the regulation of eicosanoid production. EPA competes with the enzyme systems which converts AA to eicosanoids (Sargent 1995; Bell et al., 1994; Bromage 1995). It is for this reason that the AA/EPA ratios must be considered when formulating diets. Experimental evidence has shown a decreased uptake of AA in such critical phospholipids as phosphotidylinositol (PI). Phosphotidylinositol has been shown to play a major role in cell membrane signal transduction (Sargent 1995). Studies have shown that fish fed diets in which the EPA/AA ratio was high tended to have significantly lower levels of AA in phospholipids such as PI (Bell et al., 1997; Bessonart et al., 1999). Bell et al. (1997) determined in a study with sea bass eggs that diet formulations for sea bass broodstock should contain DHA/EPA ratios > 1 and EPA/AA ratios <3 as well as the exclusion of 18:2n-6 vegetable oils in order to increase larval quality and survivability. The ratio of n-3/n-6 fatty acids is very important when considering eiconsanoid production. Due to the fact that eicosanoids are produced in response to a range of external stressors, it is possible that a diet fed with a high ratio of n-6/n-3 PUFA can

actually increase susceptibility to external stressors by the exaggeration of stress responses (Sargent 1995). Salmon fed diets rich in n-6 PUFA with sufficient levels of DHA and EPA were noted as having severe cardiac disorders (Bell et al., 1990, 1992). These disorders became apparent only when fish were stressed by transporting and they were correlated with increased eicosanoid production due to increased levels of AA in the fish. Although more research is needed in this area it is of special concern in the formulation of broodstock diets. It is believed that any excess of n-6 PUFA in the broodstock fish will be readily transferred to the egg and result in an elevated production of eicosanoids during subsequent embryogenesis and larval development. The elevation of eicosanoid production in the larvae as stated previously, is likely is likely to increase susceptibility to external stressors (Sargent 1995).

Broodstock Nutrition

It is clear from the evidence presented that polyunsaturated fatty acids mainly DHA, EPA and AA are essential to the well being and growth of the larval fish. In recent years the direction of research concerning fish larval nutrition has begun to concentrate on the nutrition of the broodstock female. This appears to be the next logical step in closing the life cycle for domestic stocks of many aquaculture species. A fish culturist may have the best larval diet on the market, proven to provide all the nutrients needed for growth and development, however if the eggs from the culturists broodfish are low in nutritional quality the larvae may never hatch or survive long enough post-hatch to make it to first feeding, which may in some fish, such as striped bass, be as long as 4-5 days. During this period the larvae must rely on the endogenous nutrition within the yolk sac given to them by their mother during egg

maturation. Variable egg quality has been regarded as one of the limiting factors for successful mass production of fish fry (Kjorsvik et al., 1990). It has also been stated that poor egg quality will probably have a great impact on larval survival specifically during the transition from endogenous to exogenous feeding (Rainuzzo et al., 1997). Broodstock nutrition has been shown to clearly influence egg quality production (Watanabe 1985; Watanabe et al., 1984 a; Watanabe et al, 1984 b; Rainuzzo et al., 1997; Mourente and Odriozola 1990; Bell et al., 1997).

In terms of fatty acids, two things must be considered during the development of a broodstock diet. The first consideration must be for the general health and well being of the brood female. Due to the role they play as precursors to eicosanoids and fish prostaglandins, which are physiologically necessary in many of the vital organs as previously mentioned, it is clear that fatty acids such as DHA, EPA and AA, play an essential role in maintaining the health of the adult fish (Castell et al., 1984; Sargent et al., 1989; Sargent 1995). Many of the same neuro-endocrine functions previously described for larval fish, such as signal transduction and stress adaptation, are applicable to the adult fish as well.

The second and most important consideration in formulating a broodstock diet is that lipid levels must not only be adequate to satisfy the general nutrition needs of the adult fish but must also be adequate to meet the additional needs of a mature female undergoing oocyte maturation. It has been stated that among the nutritional constituents of the broodstock diet, lipids are the chemical components that affect the composition of the eggs most dramatically (Watanabe et al., 1984c; Watanabe 1985). Lipids are the source of metabolic energy for gonadogenesis in the female fish and are

essential for the formation of cell and tissue membranes (Sargent 1995; Ronnestad et al., 1994).

During gonadogenisis, fatty acids are mobilized from the neutral lipid reserves of fish adipose tissue and transferred via the serum to the liver where they are assembled into vitellogenin, an egg-specific lipoprotein (Sargent 1995). During the mobilization of these lipids, saturated and monounsaturated fatty acids are catabolized in order to provide the metabolic energy required for egg lipoprotein biosynthesis. The polyunsaturated fatty acids, especially DHA, are incorporated into the phospholipid-rich vitellogenin, which is transferred via the serum to the developing eggs (Sargent 1995).

In general, the levels of omega-3 PUFA's within marine fish egg phospholipids and triglycerols have been found to be 50% and 30% respectively (Sargent 1995). Sargent (1995) points out that of the omega-3 PUFA's the largest percentage is made up of DHA and EPA found in a 2:1 ratio. Sargent (1995) also points out that although some of the omega-3 PUFA's are used to provide metabolic energy, most of the (n-3)PUFA, mainly DHA, is used in the formation of neural cell membranes which constitute a large fraction of the embryonic and larval body mass. Within the marine fish egg most of the AA is located within phosphotidylinositol and is believed to function mainly in eicosanoid production (Sargent 1995).

There have been a number of studies, which have looked at the affects of the broodstock diet on the quality of eggs produced. Two separate experiments conducted with red sea bream (*Pargus major*) concluded that the fatty acid composition of the eggs was greatly affected by the dietary fatty acids supplied to the broodstock

(Watanabe et al., 1984a; Watanabe et al., 1984b). The proportion of n-3 PUFA was high in the eggs of the broodstock given a diet high in n-3 polyunsaturated fatty acids. Those eggs produced from fish fed a diet deficient in n-3 PUFA's and enriched with corn oil were high in 18:2n-6 and were of lower quality. Buoyancy of the eggs of red sea bream was markedly reduced by replacing cuttlefish liver oil with corn oil in the diets of the broodstock female. In studying the compartmental changes in the content of total lipid, lipid classes and their associated fatty acids in the developing yolk-sac larvae of Atlantic halibut, *Hippoglossus hippoglossus* (L.), Ronnestad et al. (1995) found that out of the fatty acids released from lipid hydrolysis and used as energy substrates by the growing halibut larvae, DHA was quantitatively one of the most important fatty acid fuels in energy metabolism. They also noted a preferential retention of DHA within PE, which they felt, pointed to DHA's high biological value. Freshwater species, as previously mentioned, generally do not require high levels of PUFA's in their diet due to the lack of these lipids in the natural diet. The EFA requirements of most freshwater species are generally met by 18:3(n-3) due to the fact that they have the ability to elongate shorter chain fatty acids to the polyunsaturated fatty acids needed in membrane formation (Sargent 1995). Cavalli et al. (1999), however, found that by feeding freshwater prawn broodstock, (*Macrobrachium rosenbergi*), a diet rich in 18:2(n-6) as well as n-3 PUFA's they were able to improve fecundity, egg hatchability and overall quality of the larvae.

Mourente and Odriozola (1990) fed two different diets to broodstock gilthead sea bream (*Sparus aurata* L.) in order to determine the effect of the diets on the fatty acid compositions of the eggs. They determined that the fatty acid composition of the total

lipids and the main lipid classes, (i.e. phospholipids and triglycerides), in the two groups of eggs showed significant differences which reflected the influence of the dietary fatty acid levels. The contrast between the two types of eggs was greatest between the n-3 and n-6 fatty acids. Fish fed the diet high in n-3 PUFA's produced the eggs with the highest levels of n-3 PUFA's, mainly DHA. The same was true for those fish fed the diet high in n-6 fatty acids with the predominant fatty acids being 18:2n-6 and 20:4n-6 (AA). The lowest amounts of n-3 PUFA were found in the triglycerides (TG's) of eggs produced from the n-3 PUFA deficient diet. It is believed that the TG's are reserve lipids, storing not only saturated fatty acids for energy reserves but polyunsaturates as well (Tocher et al., 1985). Mourente and Odriozola (1990) felt that the low levels of n-3 PUFA's within the TG may be a result of compensation or directed placement of the n-3 PUFA's within the phospholipid classes which are mainly utilized for biomembrane formation within the developing organism (Sargent et al., 1995). The feeding of diets to broodstock low in n-3 PUFA may not only result in eggs with a low n-3 PUFA content in their phospholipids, but in their triglycerides as well. It was theorized by the authors that this may result in a poor reserve of structural polyunsaturated fatty acids critical to larval growth, during the starvation period between endogenous and exogenous feeding.

Almansa et al. (1999) looked at the influence of broodstock dietary lipids on the egg fatty acid composition and the egg quality of gilthead seabream eggs. Gilthead seabream display non-synchronous ovarian development and spawn daily for a period of 3-4 months. Unlike some marine teleosts, gilthead seabream broodstock feed throughout the spawning season. Eggs collected throughout the spawning season

showed no significant difference in fatty acid composition as well as egg fertilization and hatching rates in those fish fed a diet enriched with n-3 PUFA's. A second group of fish were fed a diet enriched with corn oil which resulted in deficient levels of n-3 PUFA but rich in oleic (18:1n-9) and linolenic (18:3n-3) acids. Although eggs produced early in the season showed no effect from the diet, those eggs produced in the middle and late season clearly reflected the influence of the dietary fatty acids. Egg fatty acid composition clearly reflected that of the diet with a significant loss of EPA and DHA from the neutral lipid fraction of the eggs. EPA and DHA both appeared to be conserved within the polar fraction of the egg which the authors felt reflected a biochemical strategy to preserve these lipids for their roles both structurally and functionally in cellular membranes. Fertility rates were negatively effected by the n-3 PUFA deficient diet as well.

The competitive interaction between AA and EPA must also be taken into account when formulating broodstock diets. Previous analyses of eggs and/or newly hatched larvae from seven species of marine teleost eggs have found considerably higher levels of AA than in the normal body lipids of these fish suggesting a high biological importance of this fatty acid especially in eicosanoid production (Tocher and Sargent 1984; Falk-Petersen et al., 1989). Eggs collected from both cultured and wild striped bass have shown greater levels of AA in the eggs from the wild fish when compared to eggs from the cultured fish (Harrell and Woods 1995; Gallagher et al., 1998). Bell et al. (1997) looked at the difference in fatty acid composition of eggs from sea bass broodstock (*Dicentrarchus labrax*) fed a formulated pelleted feed containing both corn oil and fish oil or a local fish (*Boop boops*). The pelleted feed

contained an AA/EPA ratio of 0.1 while the trash fish contained a ratio of AA/EPA of 0.7. Levels of DHA were similar in each diet. The fatty acid compositions of the phospholipids PC, PE and PI from the eggs of the fish fed the fish diet contained significantly more AA and DHA compared to the fish fed the formulated diet. In PI, a phospholipid which characteristically is high in AA, the AA/EPA ratios were 1.5 and 8.6 in the eggs from broodstock fish fed the formulated pelleted diet and fish respectively. The authors of this study suggest that broodstock diets which increase the ratio of DHA/EPA and AA/EPA should be beneficial in improving quality and survivability of sea bass larvae. This consideration should also be acknowledged in the formulation of all marine broodstock diets.

Objectives

The primary objective of this study is to quantify the levels of specific unsaturated fatty acids in important tissues of the adult, domestic female striped bass throughout the process of oogenesis when fed experimental diets with varying levels of DHA, EPA and AA. This study sets out to observe any trends in the tissue levels of fatty acids which may be related to dietary n-3/n-6 ratio. The secondary objective of this thesis will be to evaluate the use of a novel method of fatty acid analyses for fish tissue which is more cost efficient and less time consuming than currently used methods.

Chapter 2

Quantification of fatty acids over the gametogenic cycle fed varying dietary levels of DHA, EPA and AA

Abstract

A study was conducted in order to quantify levels of specific fatty acids in liver, eggs and blood of the domestic female striped bass (*Morone saxatilis*) throughout the process of oogenesis when fed experimental diets with varying levels of DHA, EPA and AA. Statistical differences between dietary treatment groups were detected in the tissues suggesting the direct dietary influence of 18:2n-6, DHA and EPA. A significant positive correlation was found between liver levels of EPA and dietary levels of n-3/n-6 fatty acids, which may indicate the influence of greater levels of EPA within the dietary treatments and elimination of corn oil from the diets. A positive correlation was also found between DHA and EPA levels in eggs and dietary n-3/n-6 fatty acids. In both cases tissue levels of each fatty acid increased and n-3/n-6 ratios increased. A trend was observed in the decreasing blood serum levels of DHA and EPA during the course of the experiment. This may serve as an indication of vitellogenisis.

Introduction

Striped bass, *Morone saxatilis*, is the largest and longest-lived species within the family Moronidae (Whitehurst and Stevens, 1990). It has long been a popular sport fish and within the past two decades has become an important species for aquaculture. A major challenge within the aquaculture industry today is the formulation of good quality diets. Nutrition is a critical factor in aquaculture due to its effect on fish growth and health as well as cost of production (Gatlin 1995). The use of natural feed sources is both inefficient and costly. Natural food sources can be contaminated and nutritional value can often be inconsistent or unknown. Success in domesticating striped bass will in part depend on determination of qualitative and quantitative dietary requirements (Tuncer and Harrell 1990; Harrell and Woods 1995). It has been shown that while gross nutrient availability (i.e. food ration modifications), does influence various aspects of reproductive physiology, there is little evidence to show that such changes can effect egg and larval quality (Bromage 1995; Cerda et al., 1994a). Research has instead concentrated on the individual components the diet such as vitamins, amino acids and fatty acids.

Polyunsaturated fatty acids (PUFAs) have been found to have a number of important physiologic and regulatory functions. Polyunsaturated fatty acids play a major role in membrane fluidity and permeability, transportation of lipids, activation of enzymes and act as precursors for prostaglandins all of which are essential to health and well-being of the fish species involved (National Research Council 1993). Three PUFAs in particular have been found to be essential to several fish species such as red sea bream (*Pargus major*) (Watanabe 1993), yellowtail (*Seriola quinqueradiata*)

(Watanabe 1993), turbot (*Scophthalmus maximus*) (Bell et al., 1985), gilthead sea bream (*Sparus aurata* L.) (Bessonart et al., 1999), sunshine bass (Nematipour and Gatlin 1993), Argentine prawn (*Artemesia longinaris*) (Petriella et al., 1984), Japanese flounder (*Paralichtys olivaceus*) (Tago et al., 1999), striped bass (*Morone saxatilis*) (Tuncer and Harrell 1992), palmetto bass (*Morone saxatilis* × *M. chrysops*) (Tuncer and Harrell 1992; Tuncer et al., 1993) and red drum (*Sciaenops ocellatus*) (Williams and Robinson 1988) and have been termed essential fatty acids (EFA). Two are of the omega-3 fatty acid family (docosahexaenoic acid (DHA), 22:6n-3 and eicosapentaenoic acid (EPA), 20:5n-3) and one is in the omega-6 fatty acid family (arachidonic acid (AA), 20:4n-6). These fatty acids are termed essential, especially in the case of marine fish, due to the inability of these fish to self-produce quantities of these fatty acids sufficient enough to meet nutritional needs. Striped bass, like other marine teleosts, have been shown to lack the ability to chain elongate shorter chain fatty acids into essential polyunsaturated fatty acids (Sargent et al., 1997). The inability to produce long-chain PUFA's arises from a deficiency of Δ5-desaturase enzyme which is required for the formation of AA, EPA and DHA from their C_{18} precursors (Bell et al., 1997; Sargent et al., 1995).

Much of the research in this area has centered on larval nutrition. It has been suggested that optimal formulations for first-feeding larvae should simulate that which is found in the yolk (Hemming and Buddington 1988). In recent years, the direction of research concerning fish larval nutrition has begun to concentrate on the nutrition of the broodstock female. Broodstock nutrition has been shown to clearly influence egg quality production (Watanabe 1985; Watanabe et al., 1984a; Watanabe et al., 1984b;

Rainuzzo et al., 1997; Mourente and Odriozola 1990; Bell et al., 1997). Variable egg quality has been reqarded as one of the limiting factors for the successful mass production of fish fry (Kjorsvik et al., 1990). It has also been stated that poor egg quality will probably have a great impact on larval survival specifically during the transition from endogenous to exogenous feeding (Rainuzzo et al., 1997). Fish such as striped bass, must rely on endogenous nutrition for as long as 4-5 days post-hatch.

It has been stated that among the nutritional constituents of the broodstock diet, lipids are the chemical components that affect the composition of the eggs most dramatically (Watanabe et al., 1984c; Watanabe 1985). Lipids are the source of metabolic energy for gonadogenisis in the female fish and as stated before, are essential for the formation of cell and tissue membranes (Sargent 1995; Ronnestad et al., 1994). During gonadogenisis, fatty acids are mobilized from the neutral lipid reserves of fish adipose tissue and transferred via the serum to the liver where they are assembled into vitellogenin, an egg specific lipoprotein (Sargent 1995). Saturated and monounsaturated fatty acids are catabolized in order to provide the metabolic energy required for egg lipoprotein biosynthesis. Polyunsaturated fatty acids, especially DHA, are incorporated into the phospholipid-rich vitellogenin which is transferred via the serum to the developing eggs (Sargent 1995).

In general, the levels of omega-3 PUFAs within marine fish egg phospholipids and triglycerols have been found to be 50% and 30% respectively (Sargent 1995). Sargent (1995) points out that of the omega-3 PUFAs the largest percentage is made up of DHA and EPA found in a 2:1 ratio. Sargent (1995) also points out that although some of the omega-3 PUFAs are used to provide metabolic energy, most of the (n-

3)PUFA, mainly DHA, is used in the formation of neural cell membranes which constitute a large fraction of the embryonic and larval body mass. Within the marine fish egg most of the AA is located within phosphotidylinositol and is believed to function mainly in eicosanoid production (Sargent 1995).

Comparisons of the eggs produced by the wild striped bass female and the domestic broodstock female have shown differences in the levels of DHA, EPA and AA with cultured eggs being significantly lower in the amounts of these fatty acids (Harrell and Woods 1995; Gallagher et al, 1997). Lower levels of these fatty acids may be attributed to the feeding of pelleted diets heavily supplemented with vegetable oils as observed in studies of several species (Bell et al., 1985; Williams and Robinson 1988; Nematipour and Gatlin 1993; Fair et al., 1993; Koven et al., 1993; Harel et al., 1994; Bell et al., 1995; Xu et al., 1996; Brodtkorb et al., 1997; Olsen and Henderson 1997; Peres and Oliva-Teles 1999). Vegetable oils tend to be high in 18:2(n-6) (linoleic acid). Striped bass, as previously stated, have been shown to lack the ability to chain elongate this shorter chained fatty acid to the essential polyunsaturated fatty acids they require. It is believed, therefore, that by feeding broodstock females diets supplemented with lipid sources high in DHA, EPA and AA, a direct effect may be seen in the lipid content of the egg produced by these females. The objective of this study is to quantify the levels of specific unsaturated fatty acids in important tissues of the adult, domestic female striped bass throughout the process of oogenesis when fed experimental diets with varying levels of DHA, EPA and AA.

Materials and Methods

The experiment used 2m diameter, 2500L tanks (n = 15), each containing a total 5-6 striped bass at the Department of Animal and Avian Science Animal Research Wing (College Park, Maryland). Fish were of the 1995 year class and originally produced by the Crane Aquaculture Facility (Baltimore, Maryland). One known mature female was placed into each tank. Additionally 4-5 unsexed striped bass were placed into tanks for beneficial behavioral reasons and/or to provide access to tissue samples. Fish were sexed at sampling. Before the start of feeding of the experimental diets all fish were weighed (to the nearest gram) and measured (total length to the nearest 0.1cm). Initial fish weights ranged between 1476g and 6144g. Each fish was identified with a dorsal subcutaneous passive integrated transponder (PIT) tag so that surviving fish could be measured for growth rate over the 41 week period. The tanks were supplied with dechlorinated city water set up as a flow system with a rate of 2 turnovers per day. To reduce stress a 9:1 solution of CaCl: NaCl was directly injected into the incoming water in order to maintain calcium concentration at 50 ppm. Photoperiod was maintained at 13L : 11D at the onset of the experiment July 6, 1998 until October 29, 1998 at which time it was adjusted to 12L : 12D. The photoperiod was gradually adjusted over a period of one week to 10L : 14D (November 18, 1998) in order to emulate changing environmental photoperiod. Oocyte growth was discovered March 1, 1999 and photoperiod was re-adjusted to 12L : 12D. Photoperiod was increased to 14L : 12D (March 25, 1999) where it was maintained throughout the rest of the experiment April 24, 1999. Dissolved oxygen,

ammonia, chlorine, and pH as well as temperature were monitored weekly and kept at acceptable levels for striped bass intensive culture systems (Nicholson et al., 1990).

Table 2.1 Dietary level of DHA, EPA and AA in formulated diets fed to striped bass broodstock[a].

Fatty Acid	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5
DHA[a]	1%	1%	1%	2%	1.16%
EPA[a]	0.5%	1%	1%	1%	1.71%
AA[a]	0	0	0.11%	0.11%	0
n-3/n-6[b]	1.34	2.24	2.82	8.41	9.42

[a]Values are expressed as % of diet as fed and are estimations based upon known FAME evaluations of lipid sources used in diet formulations.
[b]Estimations of dietary n-3/n-6 ratios are based upon known FAME evaluations of lipid sources used in diet formulations.

Four treatment diets with varying levels of DHA, EPA and AA were examined and compared with each other and a control diet (NRC salmonid diet) (Tables 2.1 and 2.2). Diet were fed to fish during the entire course of the experiment, which ran from July 6, 1998-April 24, 1999 (41 weeks). All diets were analyzed for total lipid and the amount of each fatty acid present. For two months prior to the feeding of experimental diets all fish were fed a common commercial diet. In December, prior to the onset of vitellogenisis, which occurs when both water temperature and day length decrease rapidly (Woods and Sullivan 1993), all striped bass were individually anesthetized with buffered MS-222 (70mg/L w/ 500mg/L $NaHCO_3$), weighed (to the nearest gram) and measured (total length to the nearest 0.1cm). One striped bass was randomly chosen from each tank (1 fish per tank, 3 replicates per treatment and control, n = 15) and exposed to a lethal overdose of MS-222 (200mg/L w/ 500mg/L

$NaHCO_3$). Five ml of blood was drawn from the caudal vein of each euthanized female using heparin-coated syringes fitted with 21-gauge needles. Whole blood from each individual was immediately centrifuged at 10,000 rpm for five minutes before being purged with nitrogen gas and stored at -80°C until lipid analysis. In addition, two transverse sections of liver tissue, approximately 1cm wide, across all three lobes of each liver were collected from the same striped bass, placed in an airtight vial purged with nitrogen and stored for lipid analysis. A second blood sample was scheduled to take place prior to the onset of oocyte growth (ca March 1) which occurs when both water temperature and day length both begin to increase rapidly (Woods and Sullivan 1993). Fifteen striped bass (1 fish from each tank, 3 replicates per treatment and control, n = 15) were individually anesthetized and blood plasma collected and processed as described above. The ovaries of random striped bass were catheterized periodically in order to determine sex and to collect and measure the diameters of ova for validation that oocyte growth had been initiated. Ova were collected for lipid analyses when vitellogenisis had ended and final oocyte maturation was imminent (defined as: ova with a mean diameter $\geq$ 800*u*m). Three females per treatment were randomly chosen with the caveat that the fish must have grown a minimum of 5% during the course of the experiment. Females were euthanized (as described above) and 5ml of blood was drawn from the caudal vein of each euthanized fish as described above for lipid analysis. Two ovarian samples, each containing 10ml of ova from each ovarian lobe, were collected from all 15 striped bass, placed in a 50ml airtight vial purged with nitrogen, and stored at -80°C until analyzed for total lipids and fatty acid methyl esters.

Tissue, blood and diet samples (approximately 1g) were evaluated for fatty acid composition and quantitated using an internal standard. Lipids were extracted using the UMD Method (see Chapter 3). Samples were evaluated as fatty acid methyl esters (FAME) using a Hewlett Packard 5890 gas chromatograph equipped with a 30 M Sp 2380 capillary column. Appropriate response factors were applied.

Statistics

Data were analyzed by mixed model analysis of variance techniques. The model included the fixed effect of the five fatty acid levels. Residuals were examined for the assumptions of the analysis of variance. This examination indicated problems with the homogeneity of variances assumption. Variances were partitioned for the fatty acid levels into multiple residual variances to better represent the heterogeneity of variances. Goodness of fit statistics available in the Mixed Procedure (SAS, version 6.12) were used to select the model that best fit the residual variances. Mean fatty acid levels were compared using the Least Significant Difference test at the 5% level of significance. Dependent variable means for fatty acid levels with their standard error and significant differences are presented in tables 2.3-2.5. Correlation data were run using a non-parametric Spearman's Rank Correlation. Significance was set at the 5% level.

Table 2.2 Experimental diet formulations[a] used in quantifying levels of DHA, EPA and AA throughout the gametogenic cycle of striped bass female broodstock.

Ingredient	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5 (control)
Wheat middlings	18.0	18.0	18.0	18.0	30.0
Soybean meal dehul.	15.0	15.0	15.0	15.0	13.0
Corn glutten meal 60%	10.0	10.0	10.0	10.0	17.0
Isolated soy protein	9.0	10.0	10.0	10.0	0.0
Blood flour ap300	8.0	8.0	7.0	8.0	7.0
Rice Bran	8.0	8.25	9.25	4.65	0.0
DL methionine	1.6	1.6	1.6	1.6	0.0
Mineral mix[b]	1.2	1.2	1.2	1.2	1.2
Ligin Sulfonate	1.0	1.0	1.0	1.0	2.0
Vitamin mix[c]	1.0	1.0	1.0	1.0	1.0
Lysine HCl	0.3	0.3	0.3	0.3	0.0
Choline CL 60%	0.25	0.35	0.25	0.25	0.2
Corn Oil	5.0	3.0	2.0	0.0	0.0
Menhaden fish oil[d]	1.15	3.0	0.0	0.0	11.5
Squid oil[e]	1.5	3.3	0.0	0.0	0.0
Cod liver oil[f]	0.0	0.0	7.4	8.0	0.0
Algal DHA[g]	3.0	0.0	0.0	5.0	0.0
Herring fish meal	15.5	15.5	15.5	15.5	30.0
Defluorinated phosphate	0.5	0.5	0.5	0.5	0.5
Stay C	0.0	0.0	0.0	0.0	0.1

[a]Ingredient concentrations expressed as % fed.

[b]Contains(as mg kg^{-1} diet): KCl, 0.008; NaCl, 7.76; $MgSO_4$, 3.15; Fe citrate, 0.24; $MnSO_4$, 0.08; $ZnCO_3$, 0.12; $CuSO_4$, 0.004; KI, 0.0008; Na_2SeO_3, 0.00024.

[c]Contains (as mg kg^{-1} diet unless otherwise noted): choline chloride, 3465; inositol, 396; niacin, 153; α-tocopheryl acetate, 45; calcium pantothenate, 50.4, riboflavin, 20.7; menadione sodium bisulfate, 9.9; thiamin, 12.6; pyridoxine-HCl, 12.6; cyanocobalamin (3000 μg g^{-1}), 5.8; folic acid, 5.4; retinyl acetate, 3.9; biotin, 4.5; cholecalciferol, 5 μg kg^{-1}; anti-oxidant, (Monsanto Corp.) 125.

[d]Zapata Corp., Reedville, VA, USA.

[e]Ziegler Brothers, USA.

[f]U.S.B., Cleveland, OH, USA.

[g]Martech Corp., USA.

Results

Liver, egg and blood samples were evaluated for fatty acid methyl esters (FAME). FAME levels of saturated, monounsaturated and polyunsaturated fatty acids were compared for all tissues order to observe any effect caused by dietary treatments. Due to the inability to separate the peaks for 20:4(n-6), 20:3(n-3), 22:1(n-11) and 22:1(n-9) using the 30M Sp2380 capillary column levels of AA were not able to be reported. Lipid extraction from dietary samples was incomplete (refer to Chapter 3 results and discussion). Analysis of fish oils used in dietary treatment corresponded with the assumed values used in formulation. Estimations of dietary n-3/n-6 ratios were based upon known FAME evaluations of lipid sources used in diet formulations.

Blood serum samples collected in December (figure 2.3) from striped bass fed dietary treatment 4 showed significantly greater levels of DHA when compared with to those fish fed diet 1. Levels of linoleic acid (18:2n-6) decreased significantly from diets 1-4 respectively. Diets 1 and 2 blood serum samples had significantly greater levels of total polyunsaturated fatty acids when compared to diets 3 and 4.

Liver tissue levels of fatty acids were significant between some dietary treatments (Table 2.4). Liver levels of saturated fatty acids were significantly greater in those fish fed diet 4 when compared to diets 1 and 3. Mean levels of polyunsaturated fatty acids was greatest in those fish fed diet 1 and were significantly greater than diets 4 and 5. DHA and EPA liver levels were significantly greater for fish in dietary treatments 2-4 when compared to diet 1. A significant positive correlation (R=0.76) was found between liver levels of EPA and dietary n-3/n-6 ratios.

EPA levels increased with increasing dietary n-3/n-6 ratios. No significant correlation was found between liver DHA levels and dietary n-3/n-6 ratio.

Blood serum samples collected in March and April (Tables 2.5 and 2.6) both showed significant decreases in linoleic acid (18:2n-6) levels in fish fed diets 1-4 respectively. April serum levels from fish from dietary treatments 2, 3 and 4 had significantly greater levels of DHA and EPA when compared with diet 1.

Evaluation of FAME levels collected from eggs of experimental fish (Table 2.7) showed eggs collected from dietary treatment 4 to be significantly greater in saturated fatty acids when compared to those eggs from dietary treatments 1 and 3. Mean EPA and palmitoleic (16:1) fatty acid levels were significantly greater in those eggs from dietary treatment 4 when compared to diet 1. Linoleic fatty acid levels decreased significantly from diets 1-4 respecitively. Correlation statistics did show a significant positive correlation (R=0.56) for DHA levels in eggs when compared with dietary n-3/n-6 ratios. Fish eggs from dietary treatment 4 contained significantly greater levels of EPA when compared with those eggs from dietary treatment group 1. A significantly positive correlation (R=0.77) was found between egg EPA levels and dietary n-3/n-6 ratios.

Table 2.3 FAME levels of blood serum 24 weeks (December) when fed diets containing varying levels of DHA, EPA and AA measured in g/100g FAME. Data are presented as means±SEM.

Fatty Acid	Diet 1	Diet 2	Diet 3	Diet 4	Control
14:0	0.03 ± 0.01^{bc}	0.04 ± 0.001^{b}	0.03 ± 0.004^{c}	0.03 ± 0.01^{bc}	0.09 ± 0.01^{a}
16:0	0.38 ± 0.04^{ab}	0.37 ± 0.01^{a}	0.25 ± 0.03^{c}	0.27 ± 0.04^{bc}	0.42 ± 0.05^{a}
18:0	0.11 ± 0.02^{a}	0.11 ± 0.02^{a}	0.06 ± 0.01^{b}	0.01 ± 0.02^{ab}	0.11 ± 0.01^{a}
Tot. Sat.[e]	0.54 ± 0.05^{c}	0.54 ± 0.06^{b}	0.35 ± 0.05^{c}	0.40 ± 0.05^{c}	0.64 ± 0.06^{a}
16:1	0.07 ± 0.02^{b}	0.07 ± 0.002^{b}	0.06 ± 0.01^{b}	0.08 ± 0.02^{b}	0.15 ± 0.01^{a}
18:1	0.59 ± 0.09^{a}	0.54 ± 0.02^{a}	0.27 ± 0.08^{b}	0.36 ± 0.09^{ab}	0.45 ± 0.03^{ab}
20:1	0.09 ± 0.02^{a}	0.09 ± 0.01^{a}	0.04 ± 0.02^{a}	0.08 ± 0.02^{a}	0.09 ± 0.01^{a}
Tot. Mono.[f]	0.77 ± 0.14^{a}	0.73 ± 0.04^{a}	0.40 ± 0.09^{b}	0.56 ± 0.15^{ab}	0.76 ± 0.04^{a}
18:2 n-6	0.49 ± 0.03^{a}	0.28 ± 0.01^{b}	0.16 ± 0.05^{c}	0.08 ± 0.03^{d}	0.10 ± 0.01^{cd}
18:3 n-3	0.02 ± 0.001^{a}	0.01 ± 0.001^{a}	0.01 ± 0.003^{a}	0.01 ± 0.003^{a}	0.01 ± 0.001^{a}
20:5 n-3	0.12 ± 0.01^{c}	0.20 ± 0.02^{b}	0.11 ± 0.02^{c}	0.12 ± 0.02^{c}	0.30 ± 0.02^{a}
22:5 n-3	0.02 ± 0.001^{c}	0.03 ± 0.004^{bc}	0.03 ± 0.01^{b}	0.04 ± 0.01^{ab}	0.06 ± 0.004^{a}
22:6 n-3	0.24 ± 0.01^{c}	0.34 ± 0.04^{ab}	0.28 ± 0.02^{abc}	0.25 ± 0.02^{bc}	0.34 ± 0.03^{a}
Tot. Poly.[g]	1.04 ± 0.07^{a}	0.96 ± 0.08^{a}	0.66 ± 0.09^{b}	0.64 ± 0.07^{b}	0.98 ± 0.08^{a}
Tot. FAME	2.35 ± 0.27^{ab}	2.25 ± 0.15^{ab}	1.41 ± 0.24^{c}	1.58 ± 0.27^{bc}	2.41 ± 0.15^{a}

Dietary treatment levels expressed as % of diet as fed of DHA:EPA:AA respectively: Diet 1 (1.0:0.5:0.0), Diet 2 (1.0:1.0:0.0), Diet 3 (1.0:1.0:0.11), Diet 4 (2.0:1.0:0.11), Control (1.16:1.71:0.0).

[a,b,c,d]Means (n=3) in the same row not sharing similar superscripts are statistically different ($P<0.05$).

[e]Total saturated fatty acids.

[f]Total monounsaturated fatty acids.

[g]Total polyunsaturated fatty acids.

Table 2.4 FAME levels of liver tissue after 24 weeks (December) when fed diets containing varying levels of DHA, EPA and AA measured in g/100g FAME. Data are presented as means±SEM.

Fatty Acid	Diet 1	Diet 2	Diet 3	Diet 4	Control
14:0	0.28±0.02^{c}	0.36±0.15^{abc}	0.30±0.06^{bc}	0.49±0.06^{a}	0.42±0.06^{ab}
16:0	2.46±0.30^{b}	3.32±1.10^{ab}	1.65±0.40^{b}	3.93±0.09^{a}	2.36±0.30^{b}
18:0	0.62±0.10^{a}	0.93±0.29^{a}	0.38±0.10^{a}	0.85±0.10^{a}	0.56±0.10^{a}
Tot. Sat.^{e}	3.41±0.22^{b}	4.67±1.55^{ab}	2.38±0.56^{b}	5.35±0.22^{a}	3.39±0.56^{b}
16:1	0.71±0.13^{c}	1.04±0.43^{abc}	0.78±0.13^{bc}	1.77±0.04^{a}	1.15±0.13^{b}
18:1	5.52±0.57^{a}	8.32±2.35^{a}	3.42±1.92^{a}	8.22±1.91^{a}	4.79±0.57^{a}
20:1	0.75±0.08^{a}	1.26±0.42^{a}	0.52±0.34^{a}	1.59±0.34^{a}	0.69±0.08^{a}
Tot. Mono.^{f}	7.14±0.65^{a}	10.9±4.57^{a}	4.97±2.02^{a}	11.8±1.97^{a}	6.83±0.85^{a}
18:2 n-6	2.31±0.12^{a}	1.62±0.72^{abc}	1.49±0.58^{abc}	0.95±0.12^{b}	0.47±0.05^{c}
18:3 n-3	0.08±0.01^{a}	0.10±0.03^{a}	0.08±0.02^{a}	0.11±0.01^{a}	0.01±0.02^{a}
20:5 n-3	0.24±0.04^{c}	0.50±0.05^{b}	0.38±0.04^{b}	0.39±0.04^{b}	0.68±0.04^{a}
22:5 n-3	0.01±0.004^{a}	0.02±0.006^{a}	0.01±0.003^{a}	0.02±0.01^{a}	0.04±0.01^{a}
22:6 n-3	0.44±0.03^{c}	0.73±0.06^{a}	0.78±0.05^{a}	0.58±0.05^{b}	0.67±0.05^{ab}
Tot. Poly.^{g}	3.67±0.22^{a}	3.67±1.06^{ab}	3.35±0.86^{ab}	2.85±0.24^{b}	2.60±0.19^{b}
Tot. FAME	14.3±0.86^{a}	19.4±7.27^{a}	10.8±3.47^{a}	19.7±1.95^{a}	13.0±1.38^{a}

Dietary treatment levels expressed as % of diet as fed of DHA:EPA:AA respectively: Diet 1 (1.0:0.5:0.0), Diet 2 (1.0:1.0:0.0), Diet 3 (1.0:1.0:0.11), Diet 4 (2.0:1.0:0.11), Control (1.16:1.71:0.0).

^{a,b,c,d}Means (n=3) in the same row not sharing similar superscripts are statistically different ($P<0.05$).

^{e}Total saturated fatty acids.

^{f}Total monounsaturated fatty acids.

^{g}Total polyunsaturated fatty acids.

Table 2.5 FAME levels of blood serum 33 weeks (March) when fed diets containing varying levels of DHA, EPA and AA measured in g/100g FAME. Data are presented as means±SEM.

Fatty Acid	Diet 1	Diet 2	Diet 3	Diet 4	Control
14:0	0.02±0.003[b]	0.04±0.006[ab]	0.04±0.006[ab]	0.04±0.003[a]	0.07±0.04[ab]
16:0	0.26±0.04[a]	0.31±0.04[a]	0.29±0.04[a]	0.30±0.04[a]	0.31±0.10[a]
18:0	0.11±0.01[a]	0.11±0.01[a]	0.09±0.01[a]	0.09±0.02[a]	0.08±0.01[a]
Tot. Sat.[e]	0.40±0.05[a]	0.47±0.07[a]	0.43±0.05[a]	0.44±0.07[a]	0.47±0.15[a]
16:1	0.05±0.01[a]	0.07±0.01[a]	0.08±0.01[a]	0.07±0.03[a]	0.13±0.07[a]
18:1	0.39±0.05[a]	0.40±0.05[a]	0.41±0.05[a]	0.36±0.05[a]	0.35±0.15[a]
20:1	0.04±0.01[a]	0.05±0.01[a]	0.06±0.03[a]	0.07±0.01[a]	0.06±0.03[a]
Tot. Mono.[f]	0.52±0.06[a]	0.57±0.08[a]	0.61±0.09[a]	0.60±0.08[a]	0.61±0.27[a]
18:2 n-6	0.35±0.05[a]	0.26±0.05[ab]	0.19±0.05[bc]	0.12±0.01[c]	0.10±0.05[c]
18:3 n-3	0.01±0.002[a]	0.01±0.002[a]	0.01±0.004[a]	0.01±0.005[a]	0.01±0.004[a]
20:5 n-3	0.10±0.02[a]	0.16±0.03[a]	0.08±0.03[a]	0.11±0.01[a]	0.20±0.09[a]
22:5 n-3	0.02±0.003[a]	0.02±0.003[a]	0.02±0.01[a]	0.04±0.01[a]	0.04±0.02[a]
22:6 n-3	0.19±0.03[a]	0.30±0.04[a]	0.15±0.05[a]	0.25±0.08[a]	0.27±0.11[a]
Tot. Poly.[g]	0.74±0.10[a]	0.82±0.15[a]	0.51±0.12[b]	0.63±0.10[a]	0.72±0.34[a]
Tot. FAME	1.67±0.20[a]	1.87±0.31[a]	1.56±0.23[a]	1.67±0.15[a]	1.81±0.77[a]

Dietary treatment levels expressed as % of diet as fed of DHA:EPA:AA respectively: Diet 1 (1.0:0.5:0.0), Diet 2 (1.0:1.0:0.0), Diet 3 (1.0:1.0:0.11), Diet 4 (2.0:1.0:0.11), Control (1.16:1.71:0.0).

[a,b,c,d]Means (n=3) in the same row not sharing similar superscripts are statistically different ($P<0.05$).

[e]Total saturated fatty acids.

[f]Total monounsaturated fatty acids.

[g]Total polyunsaturated fatty acids.

Table 2.6 FAME levels of blood serum 41 weeks (April) when fed diets containing varying levels of DHA, EPA and AA measured in g/100g FAME. Data are presented as means±SEM.

Fatty Acid	Diet 1	Diet 2	Diet 3	Diet 4	Control
14:0	0.02±0.002[a]	0.03±0.002[a]	0.02±0.004[a]	0.02±0.002[a]	0.06±0.02[a]
16:0	0.25±0.01[a]	0.24±0.01[a]	0.22±0.03[a]	0.20±0.02[a]	0.32±0.11[a]
18:0	0.12±0.01[a]	0.10±0.01[ab]	0.08±0.02[b]	0.08±0.01[b]	0.10±0.02[ab]
Tot. Sat.[e]	0.41±0.04[a]	0.38±0.03[a]	0.33±0.05[a]	0.67±0.36[a]	0.50±0.15[a]
16:1	0.05±0.03[a]	0.07±0.03[a]	0.05±0.02[a]	0.04±0.02[a]	0.11±0.03[a]
18:1	0.34±0.05[a]	0.28±0.01[a]	0.29±0.05[a]	0.21±0.05[a]	0.31±0.11[a]
20:1	0.06±0.01[a]	0.04±0.01[a]	0.04±0.003[a]	0.03±0.004[a]	0.05±0.03[a]
Tot. Mono.[f]	0.47±0.03[a]	0.40±0.03[a]	0.35±0.09[a]	0.66±0.33[a]	0.53±0.20[a]
18:2 n-6	0.33±0.03[a]	0.20±0.01[b]	0.14±0.03[bc]	0.07±0.03[c]	0.09±0.04[c]
18:3 n-3	0.01±0.0002[a]	0.01±0.001[a]	0.01±0.002[a]	0.004±0.001[a]	0.01±0.003[a]
20:5 n-3	0.07±0.01[c]	0.12±0.01[a]	0.11±0.01[ab]	0.10±0.01[b]	0.17±0.05[abc]
22:5 n-3	0.01±0.002[c]	0.02±0.002[c]	0.03±0.002[a]	0.02±0.003[b]	0.03±0.01[abc]
22:6 n-3	0.13±0.02[c]	0.23±0.02[ab]	0.26±0.02[a]	0.19±0.02[b]	0.25±0.08[abc]
Tot. Poly.[g]	0.60±0.06[a]	0.62±0.03[a]	0.59±0.04[a]	0.53±0.11[a]	0.64±0.22[a]
Tot. FAME	1.48±0.10[a]	1041±0.08[a]	1.27±0.18[a]	1.87±0.81[a]	1.68±0.58[a]

Dietary treatment levels expressed as % of diet as fed of DHA:EPA:AA respectively: Diet 1 (1.0:0.5:0.0), Diet 2 (1.0:1.0:0.0), Diet 3 (1.0:1.0:0.11), Diet 4 (2.0:1.0:0.11), Control (1.16:1.71:0.0).

[a,b,c,d]Means (n=3) in the same row not sharing similar superscripts are statistically different ($P<0.05$).

[e]Total saturated fatty acids.

[f]Total monounsaturated fatty acids.

[g]Total polyunsaturated fatty acids.

Discussion

Broodstock nutrition has been suggested to directly effect the quality of gamete produced (Watanabe et al., 1984a; Watanabe et al., 1984b; Mourente and Odriozola 1990; Cerda et al., 1994b; Harel et al., 1994; Bromage 1995; Bell et al., 1997; Almansa et al., 1999; Cavalli et al., 1999; Rainuzzo et al., 1997). It has been stated that among the nutritional constituents of the broodstock diet, lipids are the chemical components that affect the composition of the eggs most dramatically (Watanabe et al., 1984c; Watanabe 1985). In comparative studies on the essential fatty acid requirements of marine organisms n-3 PUFA's, in particular EPA and DHA were superior to shorter-chain fatty acids (i.e. linoleic (18:2n-6) and linolenic (18:3n-3) in terms of feeding efficiency ratios, survival and growth enhancement (Watanabe 1982). PUFAs play a major role in membrane fluidity and permeability, transportation of lipids, activation of enzymes and act as precursors for prostaglandins all of which are essential to the health and well-being of the fish species involved (National Research Council 1993). Striped bass is an anadromous species like salmon. This means it reproduces in freshwater however spends the majority of its' life in a marine environment. Similar to marine species, striped bass lack the ability to chain-elongate and desaturate shorter chain fatty acids to the necessary longer chain fattyacids (Owen et al., 1975; Tuncer et al., 1993; Sargent et al., 1995). Essential fatty acids must therefore be supplied within the diets.

Data collected during the course of this study supports the fact that an impact on fish tissue fatty acid levels was made by the dietary treatments. Levels of linoleic acid (18:2n-6) were found to significantly decrease in all fish tissues evaluated as

Table 2.8 Mean growth performance of striped bass broodstock fed diets containing varying levels of DHA, EPA and AA for 41weeks[a].

Tank #	Diet #	Weight gain (g)	FCR[b]	SGR[c]
81 (n=4)	1	2851	1.75	0.09±0.03
87 (n=3)	1	1366	2.85	0.03±0.08
92 (n=5)	1	3655	1.62	0.08±0.04
80 (n=5)	2	3134	1.45	0.08±0.06
86 (n=4)	2	1202	3.72	0.02±0.07
91 (n=4)	2	1871	2.41	0.07±0.04
83 (n=3)	3	1307	2.61	0.05±0.04
84 (n=4)	3	1128	2.47	0.03±0.08
94 (n=4)	3	618	5.89	0.03±0.01
82 (n=3)	4	1706	1.81	0.09±0.01
88 (n=4)	4	1551	2.94	0.07±0.01
90 (n=3)	4	1120	2.83	0.03±0.06
85 (n=3)	Control	1240	2.31	0.07±0.02
89 (n=2)	Control	496	7.15	0.005[d]
93 (n=3)	Control	1851	2.34	0.07±0.02
Average by Diet	1		2.07±0.68	0.07±0.03
	2		2.53±1.14	0.05±0.03
	3		3.66±1.94	0.04±0.01
	4		2.53±0.62	0.06±0.03
	Control		3.93±2.79	0.05±0.04

Results are presented as mean±SEM.
[a]Dietary treatment levels expressed as % of diet as fed of DHA:EPA:AA respectively: Diet 1 (1.0:0.5:0.0), Diet 2 (1.0:1.0:0.0), Diet 3 (1.0:1.0:0.11), Diet 4 (2.0:1.0:0.11), Control (1.16:1.71:0.0).
[b]Food conversion ratio = weight of food consumed (g as fed)/ wet weight gain × 100.
[c]Specific growth rate = [ln(final weight) – ln(initial weight)/(# of days)] × 100.
[d]Standard error could not be reported due to number of surviving fish in tank (n=2).

levels of corn oil used in the formulation of the diets decreased. Corn oil contains high levels of 18:2n-6 when compared to fish oils. The decrease in dietary levels of 18:2n-6 used in formulations if the dietary treatments and the stepwise increase in DHA(22:6n-3) and EPA(20:5n-3) dietary percent levels (Table 2.1) greatly contributed to the increase in dietary n-3/n-6 ratio seen within the experimental diets.

Harrell and Woods (1995) concluded that commercial diets high in corn oil played a major role in the low n-3/n-6 ratios in fish eggs produced by domestic striped bass when compared to eggs produced by wild striped bass. Gallagher et al. (1998) did not find significantly higher levels of 18:2n-6 when comparing eggs from wild and cultured striped bass but attributed this to the use of fish and canola oils in experimental diets fed to the cultured striped bass. These oils tend to be low in 18:2n-6 and contain greater levels of 18:3n-3.

Comparison of liver and blood serum samples taken in December from fish fed experimental diet 1 showed significantly greater levels of total polyunsaturated fatty acids when compared to diet 4. Qualitative examination of the polyunsaturated fatty acids between the diets 1 and 4 however show that the majority (47%) of the PUFA in diet 1 is 18:2n-6. Liver samples from dietary treatment group 4 had significantly greater levels of DHA and EPA when compared to dietary treatment group 1. The ratio of n-3/n-6 fatty acids within lipids sources used in the formulation of diets has been found to be directly reflected in fish tissues (Bell et al., 1985; Williams and Robinson 1988; Nematipour and Gatlin 1993; Fair et al., 1993; Koven et al., 1993; Harel et al., 1994; Bell et al., 1995; Harrell and Woods 1995; Xu et al., 1996; Brodtkorb et al., 1997; Olsen and Henderson 1997; Peres and Oliva-Teles 1999). The greater levels of DHA and EPA, both of which are n-3 fatty acids, within the liver tissues of the striped bass, indicates the direct influence of dietary fatty acid composition on striped bass tissues. A significant positive correlation was found between dietary n-3/n-6 ratio and EPA liver levels which indicates that as the dietary levels of n-3 fatty acids increased and 18:2n-6 levels decreased EPA also increased.

A great deal of debate has risen over the years as to what constitutes the optimal dietary ratio of n-3/n-6 PUFA in the formulated diets of marine fish. Arachidonic acid (20:4n-6), an omega-6 PUFA, has been found to play a significant role in prostaglandin formation and eicosanoid production (Castell et al., 1984; Sargent et al., 1989; Sargent 1995). EPA competes with AA for enzyme systems which convert AA to eicosanoids (Sargent 1995; Bell et al., 1994; Bromage 1995). Studies have shown that fish fed diets in which the EPA/AA ratio was high tended to have significantly lower levels of AA in their phospholipids (Bell et al., 1997; Bessonart et al., 1999). It might seem logical to simply add more AA to the diets therefore decreasing the n-3/n-6 ratio. Bell et al. (1991) strongly cautions against this and recommends that diets be formulated to emulate the n-3/n-6 ratios found in a marine species natural diet which range between 5:1-10:1 for n-3:n-6 respectively. Diets high in n-6 fatty acids, mostly due to the excess use of oils high in linoleic acid, have been shown to result in significant mortalities when fish are stressed due cardiac myopathy (Bell et al., 1990; Bell et al., 1991). It has been suggested that highly elevated levels of AA may actually exaggerate normal stress responses due to the overproduction of eicosanoids which could be significant in larvae produced by females fed diets with elevated levels of n-6 PUFA (Sargent 1995). Diets used in this experiment were formulated to range from 1.34:1.00 (Diet 1)- 8.41:1.00 (Diet 4) for n-3:n-6 respectively. Eggs collected from striped bass at the end of the experiment did show a significant positive correlation for both DHA and EPA when compared to dietary n-3/n-6 ratios. This again indicates a direct influence of the dietary n-3/n-6 ratio on the fish tissues. It would have been interesting to compare larvae hatched

from diets 1 and diets 4 for survival in order to see any influence the range of dietary n-3:n-6 ratios may have had.

Eggs collected from fish in dietary treatment 4 had significantly greater levels of EPA than in dietary treatment group 1. There was also a significant increase in the monounsaturated fatty acid 16:1 between diets 1,2 and 4 with diet 4 having the greatest levels. This may be attributed to the use of cod liver oil in diet 4. Diets 1 and 2 were supplemented with menhaden fish oil. Both cod liver oil and menhadden fish oil contain 16:1, however cod liver oil quantitatively contains more 16:1 than menhaden oil.

Most oocyte growth during oogeneis in striped bass is attributed to the uptake of vitellogenin (Tao et al., 1993). Vitellogenin is a specialized high-density serum lipoprotein (VHDL) consisting of approximately 80% protein and 20% lipid (Sargent 1995). Quantitatively most of the lipids carried by vitellogenin are phospholipids (about 2/3); the other 1/3 has been shown to be mainly triglycerides (Sargent 1995; Sullivan et al., 1997). Fatty acids are mobilized from the neutral lipid reserves of fish adipose tissue during gonadogenisis and transferred via serum to the liver where they are assembled in the vitellogenin (Sargent 1995). Vitellogenesis in striped bass occurs when both water temperature and day length decrease rapidly i.e. late fall, early winter (Woods and Sullivan 1993). This experiment did produce evidence which suggests that vitellogenisis was taking place. Figures 2.1 and 2.2 both show trends in DHA and EPA concentrations in blood plasma collected during the course of the experiment. With the exception of diet 3, mean levels of DHA and EPA, though not always significant from one another, did tend to decrease in mean concentration from

December to April. Increased concentrations of DHA and EPA in December could indicate the mobilization of these fatty acids from the adipose tissue for transfer to the liver and uptake by vitellogenin. Concentrations would decrease as vitellogenin was taken up by the eggs. Perhaps blood serum collections spaced further apart i.e. beginning of December, February and April may have produced more significant results. Trends observed in diet 3 may be the result fish sampled during the March sampling date. Figure 2.6 represents the mean growth performance of striped bass during the course of this experiment. The overall average FCR for diet 3 was high (3.66). This was due to the high FCR in tank # 94. Blood was collected from every fish however fatty acid analysis was performed randomly on only 3 samples from each diet. It is possible that the blood analyzed for fatty acids in diet 3 came from a fish that were not eating which would have resulted in lower mean levels of fatty acids. April sampling required the fish to have grown at least 5% during the course of the experiment. This would have eliminated fish that did not eat the diets.

In conclusion the data which resulted from this experiment did suggest a direct influence on striped bass tissues from the dietary treatments. The influence of varying levels of DHA and EPA as well as the decreased use of corn oil in the diets was directly reflected in the tissues sampled. Further experimentation would have to be done in order to see the influence these dietary n-3/n-6 ratios would have on the resulting larvae. Data however does support that the dietary intake of the broodstock female is reflected in the eggs. This is very significant to the aquaculture industry if good quality striped bass gametes are to be produced.

Figure 2.1 Mean DHA levels of blood serum from striped bass fed diets with varying levels of DHA, EPA and AA. Means within the same dietary treatment with different letter notation are significantly different (P<0.05).

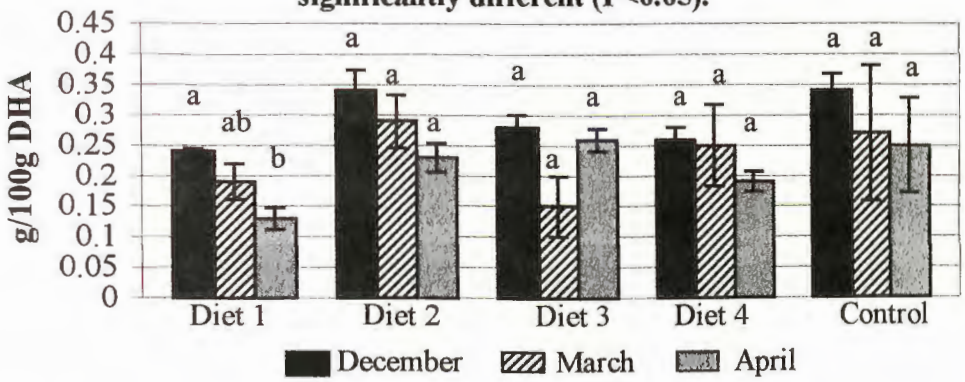
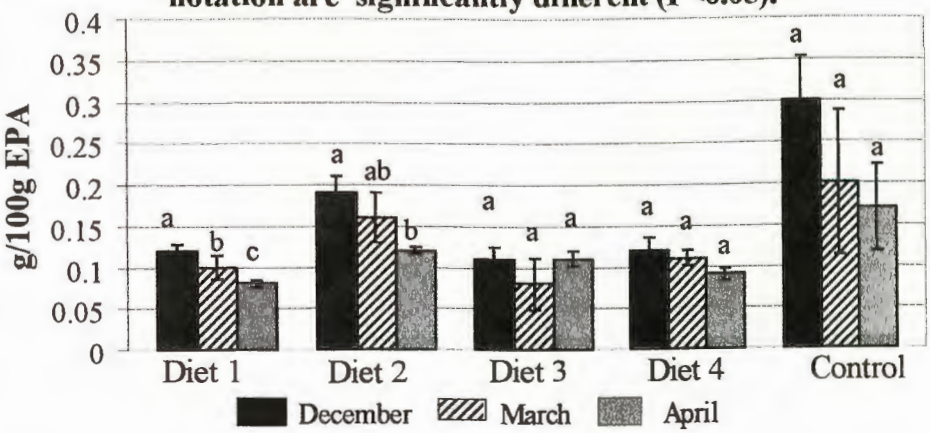


Figure 2.2 Mean EPA levels of striped bass blood serum when fed diets with varying levels of DHA, EPA and AA. Means within the same dietary treatment group with different letter notation are significantly different (P<0.05).



Chapter 3

Quantitative and qualitative comparison of two methods of fatty acid extraction: UMD method vs. Bligh and Dyer method.

Abstract

Fatty acid methyl esters (FAME) were extracted from samples of striped bass (*Morone saxatilis*) liver, egg and a formulated pelleted diet used in striped bass aquaculture in order to compare two methods of fatty acid extraction. The two methods used were the Bligh and Dyer method and the UMD method. The UMD method is a modified version of the Direct method. FAME was evaluated and compared as both a norm % and g/100g of total FAME. Total saturated, monounsaturated and polyunsaturated fatty acid classes were examined. In addition to total polyunsaturated fatty acids individual levels of DHA, EPA and AA were quantified as well for each method. There was no significant difference between methods when evaluating liver and egg samples as a norm %, which indicates no selective extraction of specific fatty acid classes. The UMD method did significantly than the Bligh and Dyer method when comparisons were done quantitatively using g/100g FAME for all of the above fatty acids and fatty acid classes with the exception of AA. Quantitatively the amounts of AA may have been too small to detect a difference between the methods. Both the Bligh and Dyer and the UMD method were found to have sub-optimal levels of fatty acid extraction on dietary samples when compared to formulated dietary levels of polyunsaturated fatty acids. This may have been due to oxidation and polymerization of the fatty acids during the extrusion and pelleting proccess. Further investigation is needed in order to determine which method is more appropriate for diet analysis.

Introduction

Nutrition is a critical factor in aquaculture due to its effect on fish growth and health as well as cost of production (Gatlin 1995). Growth within the aquaculture industry has led to the increased use of formulated diets. The increase in the use of formulated diets requires a full understanding as to the nutritional requirements of the cultured species. There must be a complete knowledge of the nutritional make-up of that species natural diet as well as the physiological importance of each of these nutrients. Diets can then be formulated to nutritionally compensate for the situations encountered daily in a captive situation which can often cause stress and disruption of the normal life cycles. Research in the area of aquaculture nutrition has concentrated on the individual components of the diet such as vitamins, amino acids and fatty acids.

Fatty acids, specifically polyunsaturated fatty acids (PUFAs), have been found to have a number of important physiologic and regulatory functions. In fact all vertebrate species that have been studied have a requirement for certain PUFAs. In the absence of these lipids within the diet, the animal may stop growing and reproducing and can even develop serious pathologies which may lead to death (Sargent et al., 1995). PUFAs play a major role in membrane fluidity and permeability, transportation of lipids, activation of enzymes and act as precursors for prostaglandins, all of which are essential to the health and well-being of the fish species being cultured (National Research Council 1993). Determination of fatty acid requirements relies on the determination of fatty acid compositions of lipids within fish tissues and diets.

Classic techniques for the determination of fatty acid composition of lipids requires solvent extraction, purification, hydrolysis and derivatization procedures (Lepage and Roy 1986). One of the first and very commonly used methods of lipid isolation and identification is called the Folch. Folch et al. (1957) developed this method for the preparation and purification of brain lipids. This method involved two very lengthy and cumbersome operations. The first step required that the lipids be extracted by homogenization of the tissue with a 2:1 chloroform-methanol (v/v) solution. The homogenate was then filtered. In the second step, the filtrate, which contained tissue lipids as well as non-lipid substances, was washed by being placed in contact with at least 5-fold its volume of water. During this washing step an estimated loss of about 1% of the lipids could be expected (Folch et al., 1957). Modifications were eventually made to the washing step of this procedure. The new method, still considered the Folch method, required the filtrate to be mixed with 0.2 its volume of water. The result was a bi-phasic system in which the upper phase contained all of the non-lipid substances and the lower phase contained all of the tissue lipids. The modified method was stated as being easier and applicable to any size sample desired. It was also said to be more exact in that losses experienced during the washing process were substantially decreased.

Due to the rapid deterioration of lipids in frozen fish samples Bligh and Dyer (1959) developed a new method for lipid extraction known as the Bligh and Dyer method. The researchers felt that the Folch method was too time consuming for routine investigation especially in the case of fish lipids. Due to their high concentration of PUFA's they are increasingly susceptable to oxidative

decomposition (Bligh and Dyer 1959). It was also felt that the Folch method required large and inconvenient volumes of solvents. This method too required the use of chloroform, methanol and water. It was felt that once tissue was homogenized with an appropriate volume of a mixture of chloroform and methanol it would mix with the water present already in the tissue to form a mono-phasic solution. The resulting homogenate could then be diluted with chloroform and/or water to produce a bi-phasic system in which the chloroform layer would contain the lipids needed for analysis (Bligh and Dyer 1959). Through experimentation with different volumes of solvent, tissue and water the optimum conditions were discovered for lipid extraction. The method was originally developed to be applicable to tissues that contained 80 ± 1% water such as fish muscle (Bligh and Dyer 1959). The procedure was adaptable to other sample types as long as the volumes of chloroform: methanol: water were kept in proportions of 1:2:0.8 and 2:2:1.8 before and after partitioning respectively. This meant that those samples which were not 80% water could either be adjusted weight-wise in order to contain 80g of water or the volumes of chloroform and methanol could be adjusted to give the above described proportions for lower weight samples (Bligh and Dyer 1959). Though faster than the Folch method both methods still required solvent extraction, drying and in some cases hydrolysis and purification of the sample in order to obtain the lipid needed for transesterification so that quantitative analysis could be done by gas-liquid chromatography (GLC) on the fatty acid methyl esters (FAME).

Lepage and Roy (1986) developed a procedure which called for the direct transesterification of samples in methanol-benzene 4:1 with acetyl chloride. This

method was found to be applicable to all lipid types both complex (cholesterol esters, phospholipids, and sphingomyelin) and simple (triglycerides). The experiment used samples of human blood plasma, rat liver homogenate, human bile and fecal homogenate from a pre-mature human infant. In comparison with the Folch method the Lepage and Roy (1986) technique increased recovery percentage of total fatty acids 20.1% for blood plasma, 3.9% for feces, 7.4% for bile and 9.7% for rat liver. As a result of the direct transesterification of samples, steps such as solvent extraction, purification and hydrolysis(Lepage and Roy 1986). The result is a technique that is not only faster but more precise due to greater FAME recoveries (Lepage and Roy 1986). Due to the elimination of costly solvents this method is more cost efficient as well.

The UMD (Univeristy of Maryland) method (Kalscheur et al., 1997) described in this chapter is a modified version of the direct method described by Lepage and Roy (1986). This method uses 1.5N MeOH: HCl (1.5 Normal- (1.5g/mol. hydrochloric acid gas)/ (liter methanol) for transesterification. The volume of 1.5N MeOH: HCl is based on the water content of the sample used. Final volume of sample and 1.5N MeOH:HCL should contain less than 10% water. This experiment will compare the use of the UMD method to the commonly used Bligh and Dyer method in the quantitative fatty acid analysis of fish diet, liver and ovary samples. Data from this comparison will be part of a set of data comparing the UMD method to classical ones for food analysis, dairy samples, animal feeds and tissue samples that is currently unpublished.

Materials and Methods

Samples of striped bass, (*Morone saxatilis*), eggs, liver and a NRC salmonid diet were homogenized and divided into 12 samples (approximately 1g) for each type of tissue and the feed. Four additional aliquots from each sample type were weighed and dried in order to determine % water.

Bligh and Dyer

Six replicate samples for each tissue were processed using the methods described by Bligh and Dyer (1959). Once the lipid layer was extracted it was filtered through anhydrous sodium sulfate in order to remove any remaining water. All samples were brought up to volume (10ml) in a graduated cylinder with a solution of 1:1 MeCl: MeOH. Volumetrically, 2ml from each graduated cylinder was placed into a pre-weighed pan and set out to dry in order to determine lipid weight. A second volume of each sample was additionally removed from the graduated cylinder and prepared for transesterification. Each sample received 1.0ml of an internal standard (15:0 ME w/ $MeCl_2$ total concentration- 10.859mg/ml) and 1 drop of butyl- hydroxy toluene (BHT concentration- 1.0g BHT/ 1000ml MeOH). Samples were transesterified using 1.5N MeOH: HCl. 1.5 N MeOH: HCl was added based on the premise that the final volume of sample and 1.5 N MeOH: HCl must contain less than 10% water. All samples were tightly sealed and placed in an oven overnight at 80°C for transesterification.

Direct Method

Six replicate samples for each tissue type or diet were precisely weighed into clean glass test tubes pre-rinsed with $MeCl_2$. To each sample 1.0ml of internal

standard (15:0 ME w/ $MeCl_2$ total concentration- 10.859mg/ml) was added along with 1 drop of BHT (concentration- 1.0g BHT/ 1000ml MeOH) in order to prevent oxidation of tissue or diet fatty acids. 1.5 N MeOH: HCl was added based on the premise that the final volume of sample and 1.5 N MeOH: HCl must contain less than 10% water. All samples were tightly sealed and placed in an oven overnight at 80°C for transesterification.

FAME Extraction

All samples from both procedures were taken out of the oven and cooled to room temperature. To each sample 2ml of hexane, 1ml KCl and 1ml of distilled H_2O were added. All samples were vortexed and centrifuged. Top layer (lipid in hexane) was removed. All steps were repeated three times with the exception of the addition of KCl and H_2O. The extract was dried under a gentle stream of N_2 gas in a 30°C water bath. Dried samples were diluted with 0.25ml hexane. Samples were purified for GLC (gas liquid chromatography) using a mini-column (glass Pasteur pipette filled with glass wool and silicic acid) in order to remove as much color as possible from samples. Mini-columns were rinsed with 6ml of hexane then 3ml of 95:5 hexane: Ethyl ether solution in order to insure the recovery of all PUFA from the sample. All samples were dried as described above under N_2 gas and prepared for GLC analysis. Dried samples were diluted with iso-octane and placed into vials then sealed.

Samples were evaluated as FAME using a Hewlett Packard 5890 gas chromatograph equipped with 30M Sp2380 capillary column. Appropriate response factors were applied. Due to the inability to separate 20:4(n-6), 20:3(n-3), 22:1(n-11) and 22:1(n-9) samples were re-run using a Hewlett Packard 5890 A cross link DMS

12M non-polar gas chromatograph. Confirmation of identification was done by running samples on silver nitrate TLC (thin layer chromatography). Plates were composed of 5% silver nitrate on salicic acid, developed in chloroform then sprayed with 2,4-di-chlorofluroscein. Bands corresponding to saturated, monounsaturated and polyunsaturated fatty acid methyl esters were recovered, eluted with $MeCl_2$, dried and prepared for GLC analysis. Samples were evaluated as FAME using a 30M Sp2380 capillary column.

Statistical Analysis

Total saturated, monounsaturated and polyunsaturated fatty acid methyl esters were quantified for each method and compared. Total FAME, DHA (docosahexanoic acid, 22:6(n-3), EPA (eicosapentanoic acid, 20:5(n-3) and AA (arachidonic acid, 20:4(n-6) were quantified as well and compared between methods. Total n-6/n-3 ratio was quantified and compared. A comparison will also be done between the total lipid extracted by the Bligh and Dyer method for each type of sample used and quantitative FAME recovery for the same method. Data were analyzed by mixed model analysis of variance techniques (SAS, version 6.12). The model included the fixed effects of each method of extraction. The random portion of the model contained variation among pairs of fatty acids. Dependent variable means for fatty acid and levels with their standard deviation are presented in tables.

Results

Samples of striped bass egg, liver and a NRC salmonid diet were evaluated for total saturated fatty acids, total monounsaturated fatty acids and total polyunsaturated fatty acids using two different methods for fatty acid transesterification and recovery. Fatty acid recoveries were also compared for DHA, EPA and AA. Results were evaluated as both a norm % (# GC counts per selected FAME in sample ÷ # GC counts of total FAME in sample) and g/100g {(wt. of FAME from GLC counts ÷ wt. of sample) × 100}. In addition recovery comparisons between the Bligh and Dyer method (1959) and the UMD method were done for total FAME and the ratio of n-6/n-3 polyunsaturates. Results were statistically evaluated as g/100g. Results are summarized in table 3.1.

Significant differences were found between methods for egg, liver and diet samples for total saturated, monounsaturated and polyunsaturated fatty acids when compared as g/100g of FAME recovery. In all samples FAME recovery was greater using the UMD method. FAME recoveries for total saturated fatty acids were increased an average of 20.0%, 31.1% and 22.8% for egg, liver and diet respectively. Total monounsaturated fatty acid recovery was increased by 18.4%, 30.5% and 20.9% for egg, liver and diet respectively. Total polyunsaturated FAME recoveries were increased by 20.7%, 32.5% and 22.8% respectively for egg, liver and diet samples.

Recoveries of DHA and EPA as g/100 FAME were significantly greater using the UMD method. Increases of 21.4%, 31.1% and 30.3% were seen for FAME recovery of DHA for fish, liver and diet respectively. FAME recovery of EPA increased an average of 19.6% for egg, 33.8% for liver and 28.1% for diet samples.

Recovery of total overall FAME for all samples increased by 20.2%, 31.3% and 24.5% with use of the UMD methods for eggs, liver and diet respectively.

No significant differences were seen between the two methods when samples were statistically evaluated using the values for norm % for any of the FAME types tested with the exception of total polyunsaturates and AA. Norm % of polyunsaturates increased in FAME recovery by 21% with use of the UMD method in evaluation of diet samples. Liver tissue AA levels increased using the UMD method by 84% when statistically evaluated as norm % and 88.7% when evaluated as g/100g. No significant differences were found between the n-6/n-3 ratios between the methods for egg, liver and diet samples. Comparison of total lipid extracted by the Bligh and Dyer method for each type of sample and quantitative FAME recovery for the same method were significantly different.

Table 3.1. Comparative fatty acid content of egg specimens using the Bligh and Dyer method and the UMD method.

Fatty Acid	UMD method	Bligh and Dyer method	Pooled SEM
	g/100g of FAME		
Total Saturated Fatty acids	0.72 [a]	0.58 [b]	0.02
Total Monounsaturated fatty acids	1.66 [a]	1.35 [b]	0.04
Total Polyunsaturated fatty acids	1.69 [a]	1.34 [b]	0.03
DHA	0.54 [a]	0.42 [b]	0.13
EPA	0.32[a]	0.26 [b]	0.01
AA	0.008 [a]	0.005 [a]	0.001
Total FAME	4.11[a]	3.28 [b]	0.07
	norm %		
Total Saturated Fatty acids	17.7 [a]	17.7 [a]	0.28
Total Monounsaturated fatty acids	40.2 [a]	41.1 [a]	0.44
Total Polyunsaturated fatty acids	41.0 [a]	40.8 [a]	0.69
DHA	13.1 [a]	13.0 [a]	0.40
EPA	7.76 [a]	7.83 [a]	0.26
AA	0.21 [a]	0.15 [a]	0.03

[ab]Means (n = 6) in the same row not sharing similar superscripts are statistically different ($P < 0.05$).

Table 3.2. Comparative fatty acid content of liver tissues using the Bligh and Dyer method and the UMD method.

Fatty Acid	UMD method	Bligh and Dyer method	Pooled SEM
	g/100g of FAME		
Total Saturated Fatty acids	3.88 [a]	2.67 [b]	0.11
Total Monounsaturated fatty acids	8.63 [a]	6.00 [b]	0.17
Total Polyunsaturated fatty acids	3.57 [a]	2.42 [b]	0.12
DHA	0.68 [a]	0.46 [b]	0.02
EPA	0.49 [a]	0.33 [b]	0.02
AA	0.175 [a]	0.0198 [a]	0.02
Total FAME	16.1 [a]	11.1 [b]	0.40
	norm %		
Total Saturated Fatty acids	53.5 [a]	54.1 [a]	0.28
Total Monounsaturated fatty acids	24.0 [a]	24.1 [a]	0.11
Total Polyunsaturated fatty acids	22.1 [a]	21.7 [a]	0.24
DHA	4.23 [a]	4.10 [a]	0.07
EPA	3.04 [a]	2.92 [a]	0.05
AA	1.09 [a]	0.18 [a]	0.14

[ab]Means (n = 6) in the same row not sharing similar superscripts are statistically different ($P < 0.05$).

Discussion

In general total FAME recoveries were greater using the UMD method. Significant increases were seen in FAME recoveries in tissue and diet samples for all fatty acid groups (i.e. saturated, monounsaturated and polyunsaturated) as well as for individual fatty acids. The differences between the methods may be attributed to two things. The first is a more complete extraction by the UMD method. Lepage and Roy (1986) concluded that the more complete recovery of fatty acids from complex lipids, such as membrane bound and protein bound fatty acids, could be attributed to the fact that they were more completely freed from the biological specimens. The second reason given for their increased recoveries was the direct nature of the liberation of fatty acids from the biological specimens (Lepage and Roy 1986). During the course of the Bligh and Dyer method many transfers of samples into different glassware are called for. With each transfer the possible loss of sample is possible. Although rinsing of glassware with solvent may aid in recovery of lost lipids it does not guarantee their full recovery. This also leaves more room for human error. Spillage of sample and incomplete rinsing of used glassware can make errors.

The exception to the increased recoveries with the UMD method was recovery of AA. Quantitatively, in comparison to the other individual fatty acids studied, the levels present in tissue and diet samples were low. It is possible that due to the small amounts present in the tissue and diet samples studied that recoveries would be low for both methods. Liver tissue however was found to have significantly greater FAME recoveries of AA using the UMD method. Recovery increases were not only evident as g/100g but as a norm % as well. There are a number of reasons why this

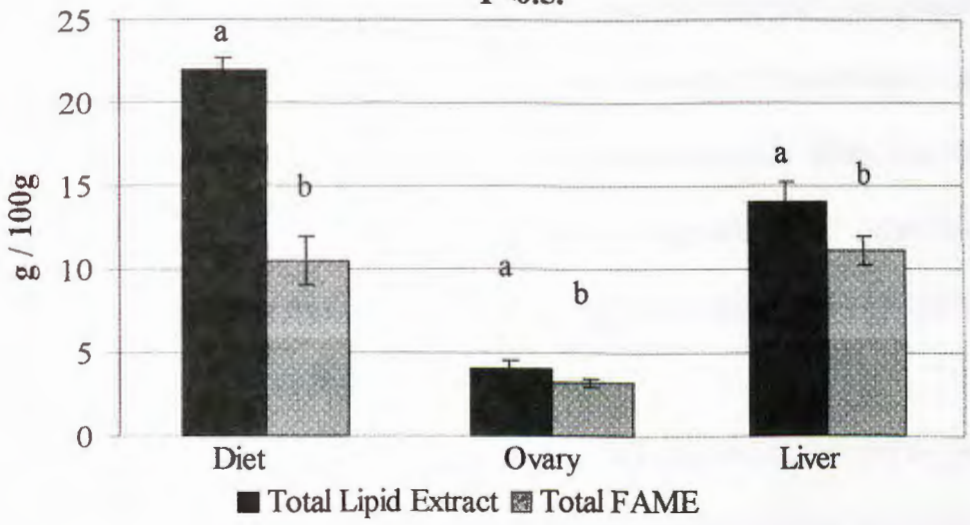
may be so. As stated previously it is possible that quantitative levels of AA were too small to detect any significant difference. In comparing quantitative amount of AA between the liver, egg and diet samples it is evident that liver tissue contained the most AA. Arachidonic acid is generally present in very small amounts in fish and is preferentially located in fish phospholipids, specifically phosphatidylinositol (Sargent et al., 1995). Comparisons of phosphatidylinositol levels within cod (*Gadus morhua*) show the highest levels of this phospholipid to be concentrated in the liver (Bell and Dick 1991). This creates the possibility that significance between the methods was only possible with greater concentrations of AA. One possible method of testing this theory would be to compare the methods using known levels of AA at different concentrations. In consideration of the increased amounts of AA within the liver homogenate the possible concentration of this fatty acid within fish phospholipids is important. Bolten et al. (1978) noted serious problems in extraction of phospholipids using the Folch method. It was proposed that due to the water-soluble nature of phospholipids that they may be lost in aqueous washes. The Bligh and Dyer method also calls for the use of water in order to create a bi-phasic system. Between this bi-phasic system is an interface between the organic layer, which contains the lipids, and the water layer. This interface is mainly made up of remaining tissue residue. It is quite plausible that phospholipids could get lost in the aqueous layer or be missed by incomplete recovery of the organic layer. It would be interesting to study the fatty acid content of the interface, which at times can be quite large, in order to see if phospholipids, due to their amphipathic nature, are present. Bligh and Dyer (1959) noted a recovery of 6% of the total lipid after re-blending and processing the

remaining tissue to the interface. This could prove significant especially in the case of AA, which is present in very small quantities.

The non-significant differences found between fatty acid groups when compared between the methods as a norm % is of great importance. This demonstrates the lack of selective recovery for specific groups of fatty acids. The fact that the norm % totals are not significantly different between the methods shows us that even though more FAME is recovered in terms of weight with the UMD method, the method is not selecting one group of fatty acid over another in recovery. If it were norm % totals would be expected to be different between methods.

A significant increase was also seen in the norm % of polyunsaturated fatty acids recovered from the dietary samples when using the UMD method. Animal diets in general are tough to extract using the Bligh and Dyer method. This is due to the increased presence of plant material such as wheat and corn. Due to their dry nature, a great deal of water must be added to the samples in order to achieve the water amounts called for by the method. Plant materials tend to absorb a good deal of solvents and water used by the Bligh and Dyer method as can be seen by their expansion in liquid. This may prevent solvents from being at the concentrations needed for complete digestion and extraction of fatty acids, especially in the case of polyunsaturates which tend to be more complex than highly unsaturated fatty acids due to their longer side chains. The UMD method gently heats the diet during digestion and extraction, which may help to break down plant material more effectively therefore reducing the amount of solvents absorbed. This would explain the increases in FAME extraction seen when using the UMD method. Comparisons for both methods with known lipid

Figure 3.1 Comparison of total lipid extract with total FAME for the Bligh and Dyer method. Data are presented as means+/- SEM. Values with different levels within groups are different from each other at P<0.5.



formulated lipid levels showed both methods to be sub-optimal in terms of fatty acid extraction. Bligh and Dyer (1959) in their method discuss the re-digestion of left over matter at the interface between the two phases. Perhaps re-digestion of the material left in the interface during this experiment would have provided better recoveries of fatty acids. Careful consideration would have to be given to fatty acid oxidation especially in the case of diets that contain fish oils. Fish oils are highly susceptible to oxidation due to the high PUFA content (Bligh and Dyer 1959). It is also possible that oxidation and polymerization if dietary fatty acids took place due to the heat of the extrusion process may have significantly reduced the amount of PUFA recovered from diet samples. Quantitative studies concerning the recovery of standards and FAME evaluation of the Bligh and Dyer interface will provide an answer to the question of incomplete recovery of fatty acids for both methods. FAME recoveries from the

interface by direct transesterification will provide a more complete recovery of fatty acids to which the UMD recoveries can be compared.

Figure 3.1 illustrates the difference between the average total lipid extracted for each type of sample and quantitatively how much lipid extract was actually detectable fatty acid methyl ester. This demonstrates one of the problems with the Bligh and Dyer method when forming a quantitative evaluation of the total fatty acid present within a sample. It is clear that although a large amount of "lipid soluble" material is extracted, a significant portion of the extract is extraneous material and is confounding and useless in FAME evaluations.

In conclusion the UMD method is the more appropriate method to use when the intent of the researcher is quantification of total FAME without separation of lipid classes. The Bligh and Dyer method often extracts a great deal of extraneous material and is more costly than the UMD method. The Bligh and Dyer method is also more time consuming requiring considerably more procedural steps than the UMD method leaving more room for human error and loss of sample due to frequent test tube transfers of sample.

Conclusions

The data presented in Chapter 2 did suggest a direct influence on striped bass tissues from the dietary treatments. The influence of varying levels of DHA and EPA as well as the decreased use of corn oil in the diets was directly reflected in the tissues sampled. Further experimentation would have to be done in order to see the influence these dietary n-3/n-6 ratios would have on the resulting larvae. Data however does support that the dietary intake of the broodstock female is reflected in the eggs. This is very significant to the aquaculture industry if good quality striped bass gametes are to be produced. Trends noted in the mean concentration levels of DHA and EPA in striped bass blood serum did suggest the process of vitellogenisis. During this process levels of fatty acids such as DHA and EPA are expected to decrease as vitellogenin is taken up by the egg. These trends, though not significant were seen and may have been significant if blood samples were collected further apart from one another. Isolation of vitellogenin and fatty acid analysis would provide more concrete evidence since it would eliminate the fatty acids that normally circulate in the blood.

Comparisons between the Bligh and Dyer and UMD method demonstrated the value in using direct transesterification processes. The UMD method is not only more cost effective but also recoveries were better quantitatively when compared to the Bligh and Dyer method. This is probably due to the elimination of multiple sample test tube transfers required by the Bligh and Dyer method. This creates more room for human error and allows for the possibility of sample loss. Both methods appear to be sub-optimal when extracting fatty acids from diet samples. Diets are typically extremely hard to extract due to the high content of plant materials. Incomplete

extraction is most likely due to incomplete digestion of the sample. The Bligh and Dyer method does allow the opportunity to re-digest undigested matter, which may make it better for diet extractions. It is also possible that heat created during the diet extrusion process in order to form pellets may be responsible for the oxidation of formulated polyunsaturated fatty acids. Further experimentation concerning the recovery of standards and FAME evaluation of the interface left behind during the Bligh and Dyer procedure will provide a clearer answer to this question by providing a more complete fatty acid analysis with which the UMD method can be compared.

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