Larviculture and Nutrition of Three of Florida's High Value Food and Stock Enhancement Finfish, Common Snook (*Centropomus undecimalis*), Florida Pompano (*Trachinotus carolinus*) and Red Drum (*Sciaenops ocellatus*)

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DECLARATION

This thesis has been composed in its entirety by the candidate, except where specifically acknowledged. The work described in this thesis has been conducted independently and has not been submitted for any other degree.

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DATE: 28th of March 2014

ABSTRACT

The main objective of this thesis was to gain new insights in three of Florida's high value food and stock enhancement finfish nutrition (Common snook, Florida pompano and red drum) to improve larviculture protocols.

The main bottleneck in snook production is the extremely low larval survival rate, which hinders subsequent research. This work first focused on the source of the larvae by looking at potential nutritional deficiencies in captive broodstock. The lipid composition of wild and captive common snook broodstock were compared to identify disparities and gain the information necessary for the formulation of a suitable diet for captive stocks. Results showed that captive snook lipid content was significantly higher than that of wild fish. However, cholesterol and arachidonic acid (ARA) levels were significantly lower compared to wild broodstock, with potential impact on steroid and prostaglandin production, reproductive behavior and gametogenesis. Eggs from captive broodstock incorporated high docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) levels and low ARA levels. Consequently, ARA/EPA ratio in captive eggs was more than half of that in wild eggs (2.3 ± 0.6 and 0.9 ± 0.1 respectively), with a probable perturbation in eicosanoid production and adverse effects on embryo and larval development. The large differences observed between wild and captive broodstock most likely contributed to the reproductive dysfunctions observed in captive snook broodstock (e.g. incomplete oocyte maturation, low milt production and poor egg quality). In addition, the presence of hydrocarbons was detected in the liver of most of the wild snook sampled. This requires further investigation to identify the source of the

contamination, monitor a potential impact on reproductive performances and protect the species habitat.

Another major bottleneck in marine fish rearing occurs during the transition from endogenous feeding to exogenous feeding, with mass mortality events linked to inadequate first feeding diets. To gain insight on the early fatty acid requirements and mobilization of pompano and snook larvae, the pattern of conservation and loss of fatty acids from the yolk sac during the endogenous feeding period and subsequent starvation was studied. In both species, fatty acids were utilized as an energy source after hatching. Mono-unsaturated fatty acids were catabolized, while saturated and poly-unsaturated fatty acids were conserved. High levels of arachidonic acid (ARA) in pompano and snook eggs (respectively 2.44 ± 0.1 and 5.43 ± 0.3 % of total fatty acids), as well as selective retention in the unfed larvae, suggested a high dietary requirement for this fatty acid during the early stages of larval development. The effect of an ARA supplementation was therefore investigated in snook larvae at the rotifer feeding stage. Larvae receiving the supplementation did incorporate higher levels of ARA, and DHA/EPA and ARA/EPA ratios were successfully modified to match those observed in wild eggs. No significant improvements in growth or survival were observed, however the success in fatty acid profile modification suggest a possible impact of the supplementation on a longer period of time and a possible effect on stress resistance.

Probiotics have been shown to enhance larval performances of several species and this strategy was therefore investigated to evaluate a potential impact on Florida pompano, red drum and common snook larvae. The effect of a commercial mix of *Bacillus* sp. was studied on larval survival, growth and digestive enzyme

activities. Larvae were fed either live feed enriched with Algamac 3050 (Control), Algamac 3050 and probiotics (PB), or the previous diet combined with a daily addition of probiotics to the tank water (PB+). Microbiological analyses were performed at the end of the pompano trial. Numbers of presumptive *Vibrio* sp. were low and not statistically different between treatments, therefore no additional microbiological analyses were performed on the system. At the end of the pompano and snook trial, standard lengths of larvae from the PB and PB+ treatments were significantly greater than for the control larvae. For both pompano and snook, trypsin specific activity was higher in PB and PB+ larvae compared to the control larvae. Similarly, alkaline phosphatase activity was higher for the pompano larvae fed the PB and PB+ treatments and for the snook larvae fed the PB+ treatment compared to the control larvae. No enhancement of growth or digestive enzymes activities was observed in red drum larvae. Yet, no negative effects were noticed and a longer trial period and the study of additional parameters could reveal different effects. In all three species, survival was not affected by the supplementation; however, stress exposure should be further investigated as the supplementation may strengthen the larvae, especially pompano and snook larvae where the *Bacillus* sp. supplementation appears to promote growth through an early maturation of the digestive system.

Another key challenge in marine fish larval rearing resides in weaning the larvae onto dry micro-diets. This step is commonly concurrent with larvae metamorphosis into juveniles, with extensive morphological and physiological changes that are likely to influence nutritional requirements. In the present project, three microdiets were tested on weaning of Florida pompano larvae: Otohime, Gemma and a reference diet LR803. The experimental system was stocked with 11-

day-old larvae, which were co-fed micro-diets and live food from 11 dph to 17 dph then micro-diets only until 28 dph. Survival from 11 dph to 28 dph was similar for all treatments, with an average of 33 %. At the end of the trial, the Gemma larvae were significantly longer and heavier than larvae fed the other diets. Fatty acid composition of the diets and larvae varied significantly between treatments. The Gemma larvae incorporated the lowest amount of eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and arachidonic acid (ARA). However, they presented the highest DHA/EPA and ARA/EPA ratios, supporting the concept that the proportions of polyunsaturated fatty acids are of greater importance than their absolute amount. Results from the enzyme analysis showed that fishmeal is a suitable main source of protein for Florida pompano larvae and demonstrated the full functionality of the pancreas at 16 days post hatch. These results provide the basis of a suitable weaning diet for pompano larvae and indicate the possibility of a weaning time prior to 16 days post hatch, which is of high interest in commercial production.

Overall, this research provides new data on common snook, pompano and red drum nutritional requirements with results that can be directly applied to help overcome major bottlenecks in the hatchery phase and improve rearing protocols

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ABBREVIATIONS

Below is a list of the most commonly used abbreviations in the text. Other abbreviated terms are explained in the text.

AN	Leucine aminopeptidase
AP	Alkaline phosphatase
ARA	Arachidonic acid (20:4n6)
CHOL	Cholesterol
DAG	Diacylglycerols
DHA	Docosahexaenoic acid (22:6n-3)
DPH	Day post hatch
EPA	Eicosapentaenoic acid (20:5n3)
FA	Fatty acid
FFA	Free fatty acids
GSI	Gonadosomatic index
HC	Hydrocarbons
HSI	Hepatosomatic index
LA	Linoleic acid
Leu-ala	Leucine–alanine peptidase
MUFA	Mono-unsaturated fatty acids
nd	Not detected.
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PI	Phosphatidylinositol
PS	Phosphatidylserine
PUFA	Poly-unsaturated fatty acid
SFA	Saturated fatty acids
TAG	Triacylglycerols
W+SE	Wax and sterol ester

Chapter 1 General Introduction

1.1 PRESENTATION OF THE SPECIES AND CURRENT STATUS IN AQUACULTURE

1.1.1 Florida pompano, Trachinotus carolinus

Figure 1.1. Florida pompano

The Florida pompano belongs to the Carangidae family also commonly referred as the Jack family. Pompano are found in small to large schools along sandy beaches and in estuarine waters along the coasts of the eastern Atlantic ocean and Gulf of Mexico, from Massachusetts down to Brazil, with the highest abundance along the coasts of Florida (Gilbert, 1986; Smith-Vaniz, 2002; Solomon and Tremain, 2009). They feed mainly on crustaceans, mollusks, and less frequently on small fish (Finucane, 1969; Wheeler et al., 2002). Adults can reach 64 cm and 3.7 kg with a most common size of 35 cm and 1.1 kg (Gilbert, 1986; Smith-Vaniz, 2002). There is no obvious morphological difference between males and females, though mature females tend to be larger (Main et al., 2007). Males mature during their first year while about 50 % of females mature during their first year and 100 % between their 2nd and 3rd year (Finucane, 1969). Their lifespan is relatively short with the oldest fish examined being 7 years old (Murphy et al., 2008). Spawning has never been observed but is thought to occur offshore, in spring and fall in Florida, and juveniles

are found in the surf-zone of sandy beaches after about one month (Finucane, 1969; Solomon and Tremain, 2009).

Florida pompano is a valuable commercial species and a popular game fish. In the United States, current recreational regulations limit the daily bag at 6 per harvester per day with a size limit of 28 cm (fork length) and a season open all year round. The commercial fishery is small and the demand is consistently higher than the supply (Weirich et al., 2006). Stocks of Florida pompano from the Gulf of Mexico coast are considered of moderate concern while stocks from the Atlantic coast are likely overfished (Murphy et al., 2008). In 2011 and 2012, with only 103 metric tons of commercial landing per year, the whole-fish dockside price reached US$ 8.9/kg (NOAA/NMFS, 2014). In addition to a high market value, Florida pompano has a fast growth rate and can withstand high densities, making it a great candidate for aquaculture production (Iversen and Berry, 1969; Moe et al., 1968; Weirich et al., 2006). In the 1960s and 1970s, research focused on developing production in ponds (Berry and Iversen, 1967; Moe et al., 1968), in-pond cages (Smith, 1973; Swingle, 1972; Tatum, 1973, 1972), and flow through tanks (Gomez and Scelzo, 1982; Iversen and Berry, 1969). However, survival was low and growth rate drastically slowed down past 200 g, hindering the production of market size fish (>450 g) (Weirich et al., 2009). Interest in the aquaculture production of the species diminished and research was put on hold for over a decade. Progress in recirculating system technology contributed to renewed interest in the species and Weirich et al. (2006) successfully overcame the growth rate lag by rearing juveniles in a maintained salinity of 23-28 ppt. Juveniles weighing 17 g reached 459 g in 4 to 5 months and over 700 g in 8 to 9 months with a survival exceeding 95 %. Despite the

recent progress, poor egg quality from captive spawns, combined with unreliable and costly larval rearing protocols, prevent the mass production of robust juveniles and very few aquaculture farms are currently producing Florida pompano.

1.1.2 Common snook, Centropomus undecimalis

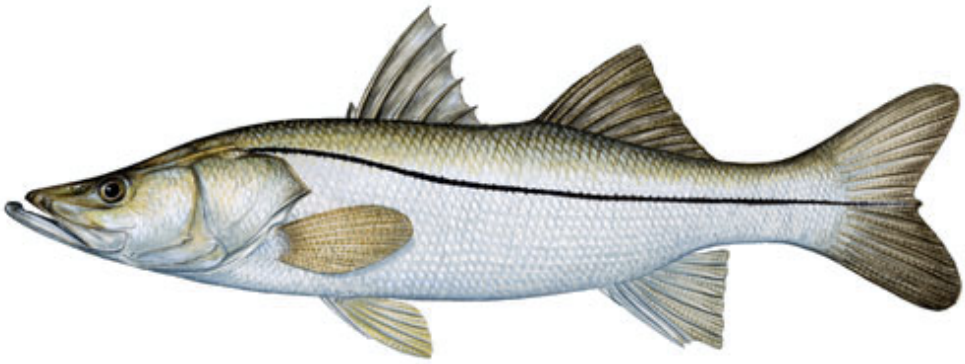

Figure 1.2 Common snook

The common snook belongs to the Centropomidae family, commonly referred to as the "snook" family. They are found in subtropical and tropical waters, around the Gulf of Mexico and along the western Atlantic coast from Cape Canaveral, Florida, down to Florianopolis, Brazil (Alvarez-Lajonchère and Tsuzuki, 2008; Rivas, 1986). They are a protandric hermaphrodite species with transitional fish observed up to 7 years of age (Muller and Taylor, 2006). They are ambush feeders and prey on fish, shrimp, and small crabs. Females can measure up to 120 cm and live 21 years, while males can measure up to 100 cm and live 15 years (Taylor et al., 2000). Puberty is reached in 50% of males at about 15 cm fork length (within their first year) while females can mature as early as 2.5 years (>43 cm) and all are mature by 7-8 years (about 63 cm) (Muller and Taylor, 2006). In Florida, adults spawn from April to September, along sandy beaches, inlets and tidal passes of estuaries (Taylor et al., 1998). The larval phase is planktonic then juveniles migrate to mangrove-lined

swamps, brackish creeks and freshwater rivers (Peters et al., 1998). Adults over-winter in upper estuaries and migrate to their spawning grounds in the spring with close to 100 % site fidelity (Adams et al., 2009, 2011), though it has been demonstrated that up to 40 % of adults do not migrate and skip one or more spawning seasons (Trotter et al., 2012).

The common snook is a popular commercial food fish in South America and Mexico (Alvarez-Lajonchère and Tsuzuki, 2008). In Florida, it is illegal to buy or sell common snook since 1957, however they remain one of the most popular game fish due to their spectacular jumping and fighting abilities (Muller and Taylor, 2012). Recreational regulations limit the bag to 1 per harvester per day with a size limit of no less than 71 cm and no more than 84 cm (total length). The season closes during the winter months (December to February) when snook become lethargic as the temperature drops and during the summer months (May to August) during the spawning season. Environmental changes including cold kills, habitat destruction and increased recreational fishing pressure contributed to a decline in common snook stocks in the Gulf of Mexico and in the Atlantic coast (McRae and McCawley, 2011; Muller and Taylor, 2006). In 2010, southwestern Florida experienced an unusual cold front for 13 days with the temperature of estuarine systems dropping down to 12°C for 10 days, causing extensive snook mortalities (Adams et al., 2012; McRae and McCawley, 2011). After the event, harvest season did not reopen until the end of August 2013 and snook are now a high-priority species which undergo regular stock assessments (Muller and Taylor, 2013, 2012). Therefore, research is carried out to develop the aquaculture production of common snook, with the objective to supplement local fisheries in Florida through stock enhancement, and increase

commercial production in South America (Alvarez-Lajonchère and Tsuzuki, 2008; Brennan et al., 2008). Despite recent progress in captive spawning (Ibarra-Castro et al., 2011; Neidig et al., 2000; Rhody et al., 2014, 2013; Yanes-Roca et al., 2009) and advances in larval rearing protocols (Barón-Aguilar et al., 2013; Ibarra-Castro et al., 2011; Rhody et al., 2010; Wittenrich et al., 2009), larval survival remains extremely low and there is currently no established large scale production of this species.

1.1.3 Red drum, Sciaenops ocellatus

Figure 1.3 Red drum

The red drum, also know as red fish, belong to the Sciaenidae family, often referred to as the drum family in relation to the drumming sound they can make by vibrating special muscles on their swim bladder. Red drums can tolerate a wide range of temperature and salinity, and are found along the Atlantic coast from Massachusetts down to Florida and around the Gulf of Mexico (Peters and McMichael, 1987). Red drum appear to move only short distances and further sub-populations occur among the Atlantic and Gulf groups (Gold and Richardson, 1991; Seyoum et al., 2000). They are mainly bottom feeders, preying on shrimps, small crabs and fish (Scharf and Schlight, 2000). Red drum have an extended life span and can reach a large size, the oldest red drum examined was 40 years old and the heaviest one ever caught was

43 kg and length of 150 cm (Murphy and Munyandorero, 2009). Males reach puberty and can mature between 1-3 years old and females between 3-6 years old and spawning occurs in late summer and fall, in passes and inlets (Murphy and Taylor, 1990). After about 3 weeks of planktonic phase, young juveniles settle in estuaries, primarily into seagrass meadows, where they remain until they reach sexual maturation and move to offshore waters (Rooker and Holt, 1997). Red drum is a very popular game and food fish. In 1986, depleted stocks led to an emergency rule prohibiting all harvest and sale of red drum. A couple of years later the fishery reopened under strict regulations for both commercial and recreational fisherman. Today, red drum stocks are recovering and Florida regulations for recreational anglers allow up to 2 fish per bag per day with a size between 46 - 69 cm and no closed season (Murphy and Munyandorero, 2009). Commercial harvest is still prohibited in all federal waters (outside three nautical miles from shore) and Florida state waters but small commercial fisheries (100 metric tons total) exist in North Carolina and Mississippi. In parallel to the protection of the existing stock, stock enhancement and research programs were initiated in Florida, Texas, Georgia and South Carolina (Carson et al., 2009). Red drum proved robust, fast growing and culture protocols were rapidly established (Chamberlain et al., 1990; Holt, 1993). Every year, several millions of hatchery-produced red drum fingerlings are released and contribute to the recovery of the stocks (Saillant et al., 2009; Willis et al., 1995). The development of culture protocol arising from stock enhancement programs contributed to the emergence of red drum commercial aquaculture production. Even though the vast majority of red drum populations and highest abundance is found along the Atlantic coast of North-America and around the Gulf of Mexico, the large

temperature and salinity tolerance, and the overall robustness of red drum allow for intensive production in a wide variety of tropical and sub-tropical waters. Currently, aquaculture production of red drum exists in the United States, the West Indies, Israel, Mayotte and Ecuador, though as of 2004, 94 % of the production was raised in China. In 2012, 68,000 metric tons of red drum were produced for a value of $USD 92 M (FAO, 2014). The main weakness of red drum aquaculture is the lack of a world wide established market. Indeed, since the natural range is limited to the West coast and Gulf coast of North America, the species is unknown for most consumers in the world and strong marketing campaigns are necessary to promote the species.

1.2 FACILITIES AND EXPERIMENTAL SYSTEMS

1.2.1 Mote Marine Laboratory

Mote Marine Laboratory (MML) is an independent non-profit laboratory founded in 1955 by Dr. Eugenie Clark with the Vanderbilt's family financial support. At the time the laboratory was in Placida, FL and called Cape Haze Marine Laboratory. Dr. Clark's work focused on the biology and behavior of sharks, including sharks ability to learn. Dr. Clark's work was world-renowned and the small laboratory expanded quickly, moving to a larger location on Siesta Key, Sarasota, FL in 1960. By 1965 the Vanderbilt family had lost interest in the project and Mr. William Mote, a rich entrepreneur from Tampa and an avid fisherman, became deeply involved in both research projects and provided financial support to the project. A couple of years later, the Cape Haze Marine Laboratory was renamed in honor of the Mote family, Mr. Mote playing an important role in the organization until his passing in 2000. In 1978 MML moved to his current location on City Island in Sarasota.

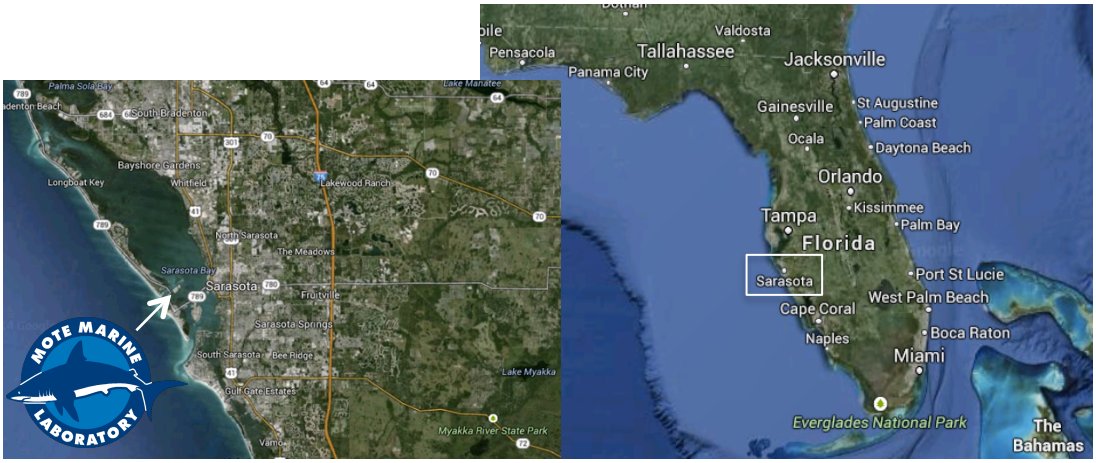

Figure 1.4 MML location

In the following 35 years, MML expanded, opening the public aquarium in 1980 and developing diverse research programs. Today, MML is composed of 22 research programs, a total staff of 192, including 81 research staff (31 Doctoral level), and over 1,600 volunteers contributing 215,000 hours/year.

In 2001, 200 acres of land were purchased for the establishment and development of the Mote Aquaculture Park (MAP). To benefit from lower land cost and protection from hurricane surges, MAP was established 17 miles inland, with state-of-the-art freshwater and saltwater filtrations allowing for zero-discharge recirculating systems. Three research programs are housed at MAP: the marine and freshwater aquaculture research program, the marine stock enhancement program and the sturgeon commercial demonstration program. Several levels of filtration are in place to maintain water quality with a final filtration system composed of a solids filter, a moving bed biofilter, a foam fractionator, a denitrification reactor, UV lamps, ozone and carbon filtration. Solids are dewatered and captured in a sand-drying bed or a geotube. In addition, research efforts are directed to use wastewater from freshwater

farming and solid waste from saltwater farming to grow wetland plants for habitat restoration projects.

Figure 1.5 MAP location

The marine aquaculture buildings include 7 broodstock rooms with temperature and light control, a live feed room for rotifer rearing, a hatchery composed of 3 commercial-size systems, and the two experimental systems used in the following studies.

1.2.2 Small experimental system

The small experimental setup included three identical independent systems. Each system was composed of four 100 L tanks with water recirculating from the tanks to a sump/biofilter and back to the tanks via a UV light. Water quality was maintained by the biofilters, regular syphoning and cleaning of the standpipes, and regular water changes. Heaters were placed in each sump to maintain water temperature. In addition, a cooling/heating air conditioning unit maintained a constant temperature in the room.

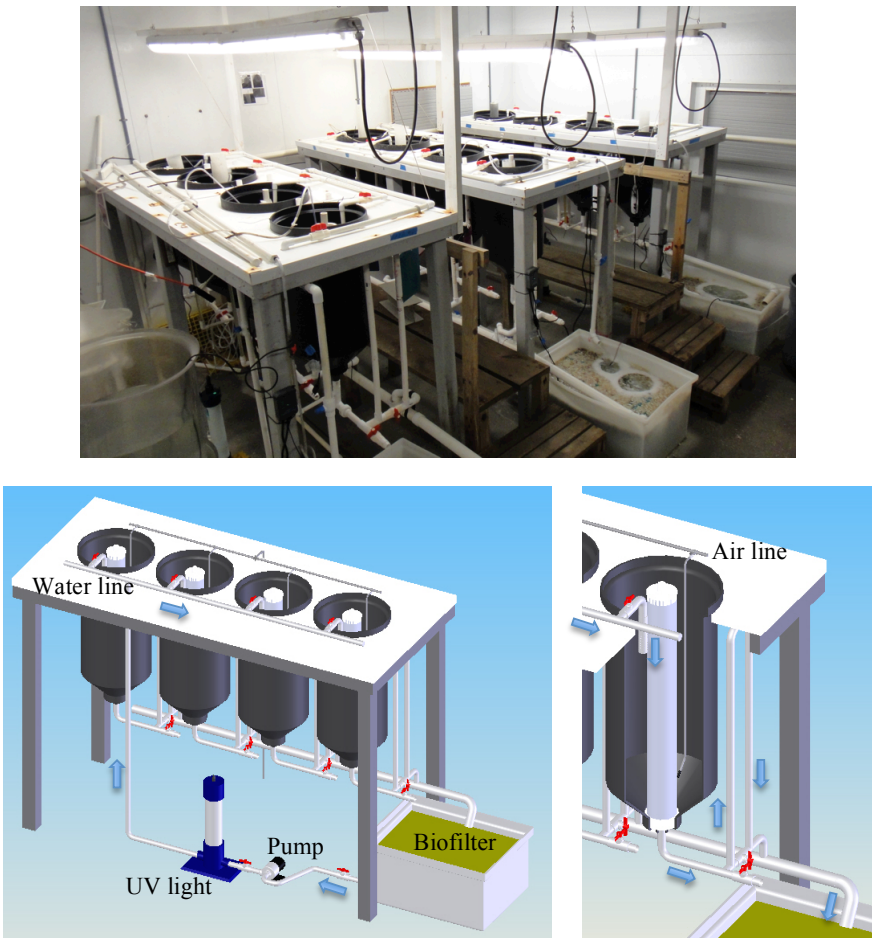

Figure 1.6 Small experimental system

1.2.3 Large experimental system

The large experimental setup was composed of up to twenty-four 130 L tanks, connected to a large filtration room. Filtration included a drum filter, a biofilter, a protein skimmer in a large sump, ozonation, a carbon filter and three UV lights. Two ORP (oxidation-reduction potential) meters insured the ozone regulation. Water temperature was maintained by three online heating units and a cooling/heating air conditioning unit. The large water volume (4 m^3), drum filter and ozonation maintained a high level of water quality.

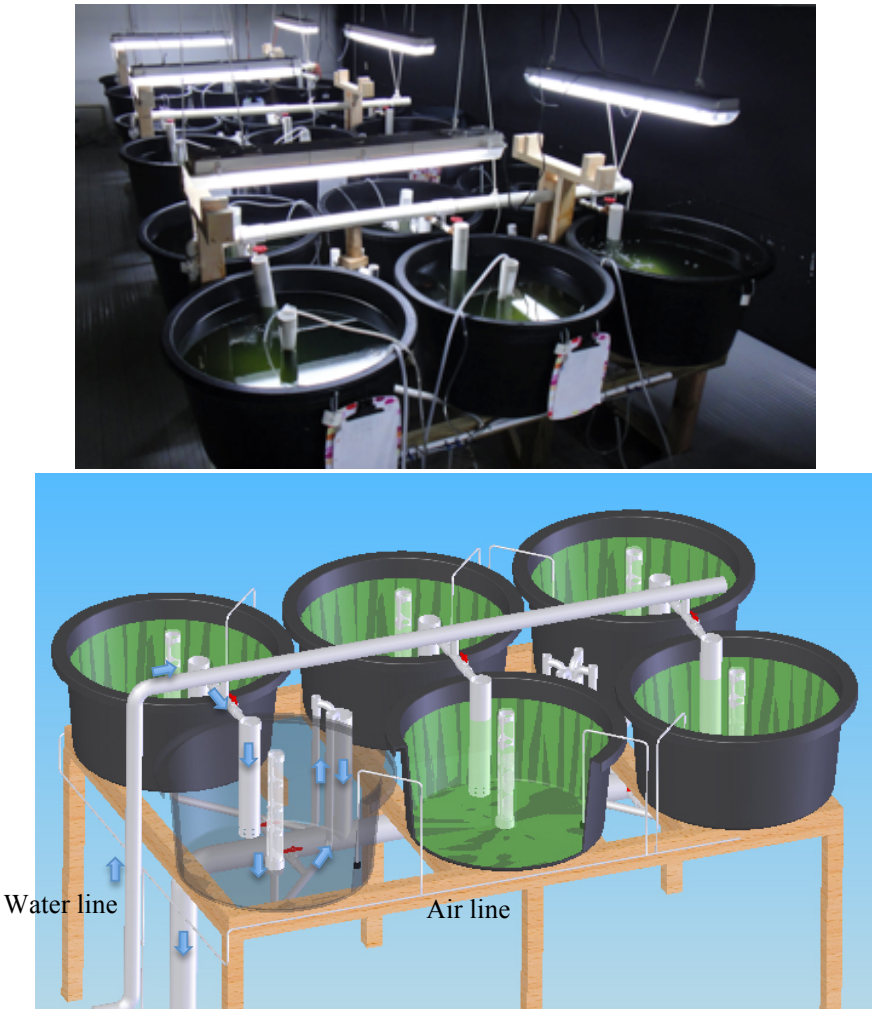

Figure 1.7 Large experimental system – Tanks

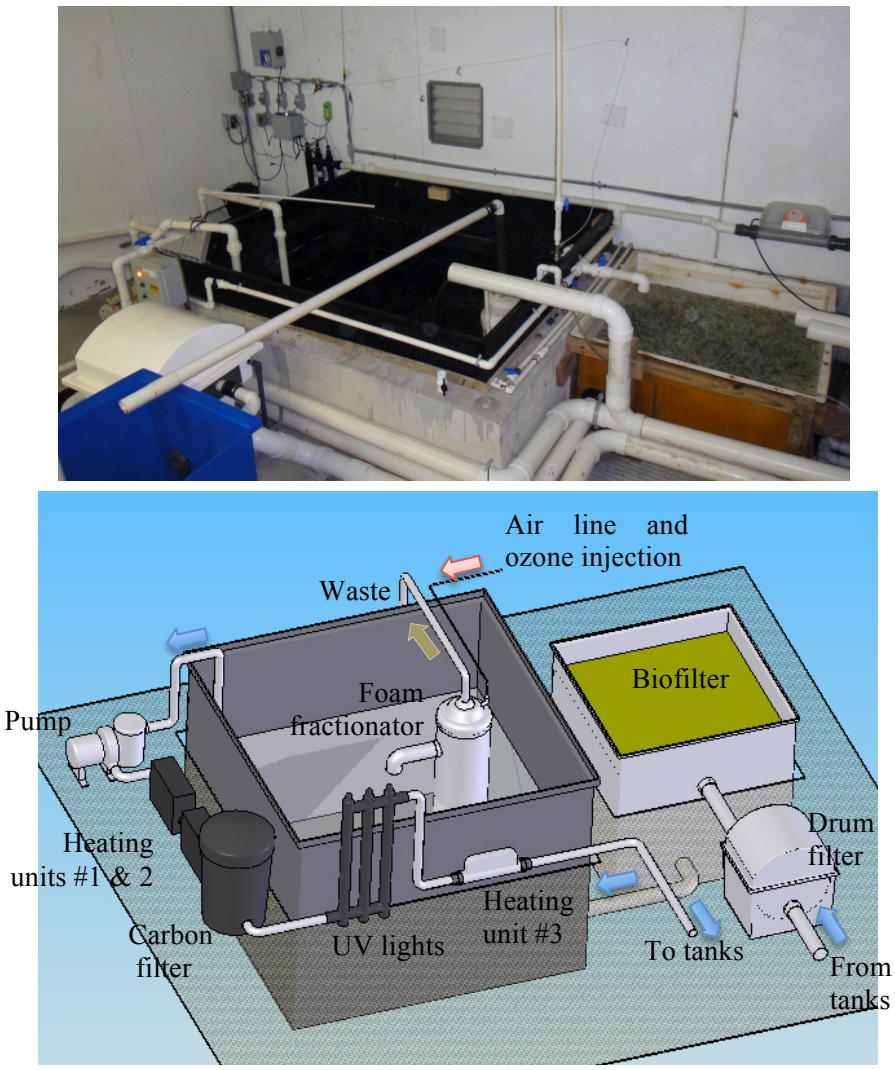

Figure 1.8 Large experimental system – Filtration room

1.3 LIPID NUTRITION IN MARINE FISH

1.3.1 Importance of dietary lipids

Lipids and fatty acids (FAs) have been extensively studied in fish for the past 20 years and remain the focus of a tremendous amount of research. It is indisputable that every class of nutrients (lipids, proteins, vitamins, minerals…) are of equal importance, regardless of proportions. However, dietary lipids have a greater influence due to the low enzyme-substrate interactions in lipid and FA metabolism

(Sargent et al., 1993). Enzyme–substrate interactions in other classes of nutrients depend predominantly on strong ionic and hydrogen bond interactions between substrate and product molecules and enzymatic proteins, resulting in highly specific enzyme-catalyzed reactions. For example, the amino acid composition of a protein will always be rigorously identical, independently of the composition of dietary proteins, reflecting the high specificity of coupling amino acids with transfer RNAs in protein synthesis. Contrastingly, enzyme–substrate interactions in lipid and FA metabolism depend on hydrophobic interactions based on van der Waals and dispersion forces, resulting in lower specificities in enzyme-catalyzed reactions involving lipids and FAs. For example, 16:1n-7, 18:1n-9, 18:2n-6 and 18:3n-3 are all substrates for the enzyme delta-6 fatty acid desaturase, while several C20 and C22 FAs are effective inhibitors of the enzyme. Therefore, their final concentrations and cellular functions are strongly influenced by the proportion of each FA in dietary lipids (Sargent et al., 2002, 1993).

1.3.2 Essential fatty acids

FAs are characterized by a carboxyl group at one end of their aliphatic chain, and a methyl group on the opposite end. FAs with a carbon chain incorporating fewer than six carbons are considered short chain FAs while the presence of 14 carbons or more characterize long chain FAs. Enzymes called desaturases allow for the introduction of one or several double bonds to form monounsaturated FAs or polyunsaturated FAs (PUFAs), the position(s) of the double bond(s) being determined by the type of desaturase. The concomitant presence of several double bonds and more than 14 carbons characterize the long chain - polyunsaturated Fatty Acids (LC-PUFAs)

category (Tocher, 2003). LC-PUFAs include the physiologically essential C20 and C22 LC-PUFAs, eicosapentaenoic acid (20:5n-3, EPA) and docosahexaenoic acid (22:6n-3, DHA), which provide important health benefits to both fish an human consumers while arachidonic acid (20:4n-6, ARA) is important in the stress response. Only algae and plants possess the delta-12 and delta-15 desaturases necessary to biosynthesize linoleic (18:2n-6) and linolenic (18:3n-3) acids, respectively, therefore these FAs are essential elements for all animals. In addition, marine fish have evolved in an environment rich in PUFAs and their abilities to elongate and desaturate have been repressed, thus requirements have to be fulfilled through the diet (Sargent et al., 2002).

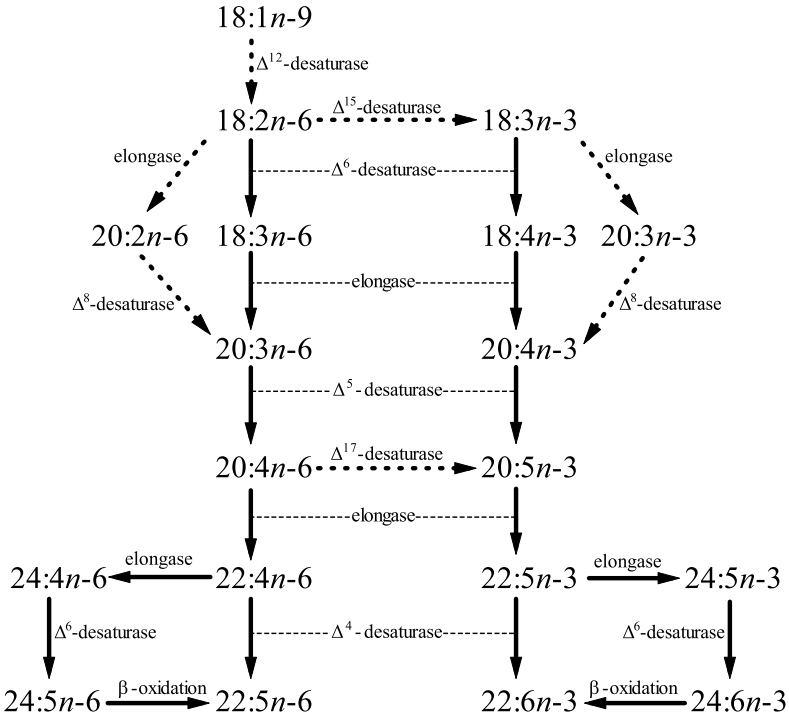

Figure 1.9 PUFA biosynthesis pathways in teleosts (solid lines) and pathways shown in other organisms but unconfirmed in teleosts (broken lines). From (Li et al., 2010)

Eicosanoids are a broad group of compounds (including prostaglandins and leukotrienes) derived from C20 FAs, mainly ARA and EPA. These FAs are released

from membrane phospholipids through the action of phospholipase A2. Even though EPA is the main C20 PUFA in fish tissues, ARA is the preferred substrate for eicosanoid production and the metabolites produced are of higher biological activity (Tocher et al., 1996) (Figure 1.10). EPA competitively inhibits the production of eicosanoids from ARA therefore eicosanoid actions are determined by the ARA/EPA ratio in the tissues (Sargent et al., 1999a).

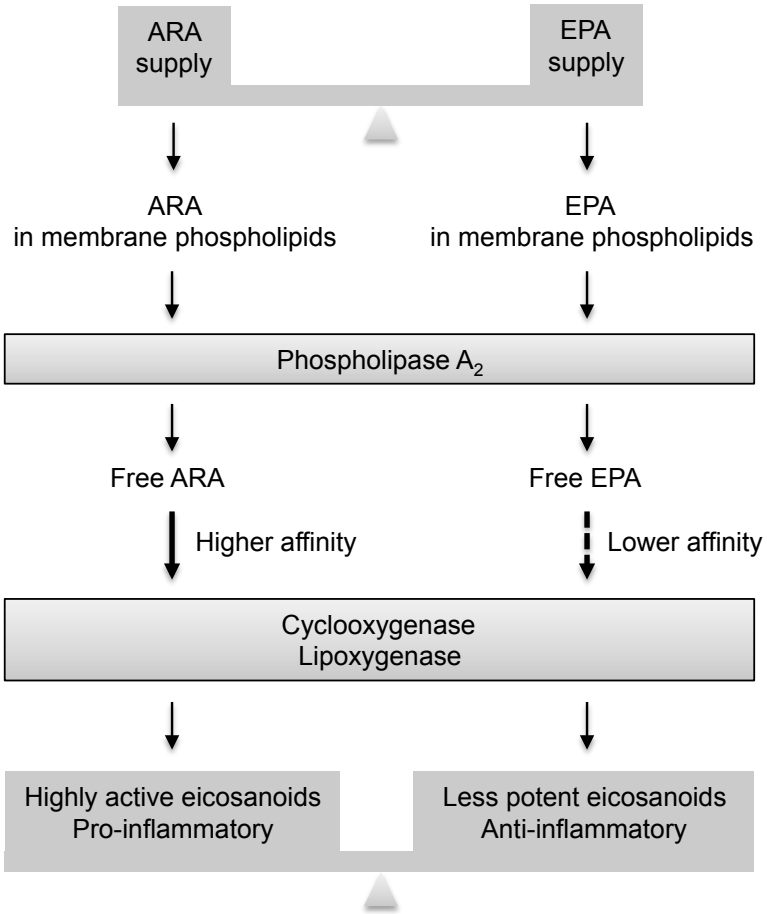

Figure 1.10 Eicosanoids biosynthesis pathway from ARA (arachidonic acid) or EPA (eicosapentaenoic acid).

Similarly, DHA and EPA compete in the formation of phospholipid structures, specifically for the enzymes that esterify fatty acids onto the glycerophospho-base structure, with a higher biological value for DHA than EPA (Glencross, 2009;

Sargent et al., 1999b). Therefore, determining the optimal DHA:EPA:ARA ratio is challenging as changing the percentage of one PUFA alters the ratio (Sargent et al., 1999a).

1.4 BROODSTOCK LIPID NUTRITION AND IMPACT ON REPRODUCTIVE SUCCESS

Improvements in broodstock lipid nutrition have been shown to increase egg and sperm quality as well as fertilization success (Almansa et al., 2001; Izquierdo et al., 2001; Migaud et al., 2013). First, dietary lipids are the main source of energy used to fuel reproductive processes and dietary restrictions can inhibit or delay gamete production. In European sea bass *Dicentrarchus labrax* broodstock, a food ration divided by half for six months delayed the spawning period and reduced the size of eggs and newly-hatched larvae, compared to broodstock fed the full ration (Cerdá et al., 1994). Food intake also impacted on total fecundity in herring *Clupea harengus* (Ma et al., 1998), plaice *Pleuronectes platessa* (Horwood et al., 1989), tilapia *Tilapia zilli* (Coward and Bromage, 1999), cod *Gadus morhua* (Kjesbu et al., 1998, 1991) and three-spined stickleback *Gasterosteus aculeatus* (Ali and Wootton, 1999; Fletcher and Wootton, 1995).

In addition, dietary lipids and FAs impact the regulation of reproductive functions. As mentioned above, ARA and EPA are precursors of prostaglandins, with ARA being the main precursor of 2-series prostaglandins providing an inflammatory response while EPA, the main precursors of the less biologically active and antagonistic 3-series prostaglandins provides a lower-inflammatory response (Tocher, 2003). Prostaglandins of the 2-series are involved in the stimulation of ovarian and testicular steroidogenesis and follicle maturation in fish, acting

hormonally to trigger female sexual behavior and milt production in males (Diotel et al., 2011; Kobayashi et al., 2002; Tokarz et al., 2013). Studies on juvenile turbot (Scophthalmus maximus) have shown a modification in the profile of prostaglandins in heat, brain, gills and kidney tissues following a change in dietary FAs (J. G. Bell et al., 1995). Moreover, ARA is involved in the regulation of cholesterol, which has been identified as the main precursor of sex steroid hormones in fish (Hu et al., 2010; Norambuena et al., 2013). ARA and its metabolites regulate the steroidogenic acute regulatory protein (StAR protein), which controls the transfer of cholesterol from the outer membrane of mitochondria to the inner membrane, a prerequisite to all steroid synthesis (Hu et al., 2010). In fish, ARA stimulates testosterone production by increasing cyclic adenosine monophosphate (cAMP) levels in a dose dependent manner, however, high doses of ARA may inhibit steroidogenesis through the limitation of cholesterol availability (Mercure and Kraak, 1995; Mercure and Van Der Kraak, 1996). Sex steroid hormones are the ultimate effectors of gonad development; therefore, inadequate FA proportions in the broodstock diet can disrupt the reproductive processes (Mylonas et al., 2010).

Beyond gamete maturation, FAs also impact on gametes quality. The FA composition of male gonads and milt reflects the FA composition of the broodstock diet and affects milt quality (Izquierdo et al., 2001). In particular, ARA content in male gametes has been correlated with fertilization success in halibut *Hippoglossus hippoglossus* (Mazorra et al., 2003) and sea bream *Sparus aurata* (Atalah et al., 2011b). However, ARA above the optimal level has been shown to reduce reproductive performances (Atalah et al., 2011b; Furuita et al., 2003; Norambuena et al., 2013). Lipids and FAs also determine oocyte quality. Indeed, during

vitellogenesis, lipids necessary for embryonic development and the yolk-sac period are incorporated and stored in large amounts (Lubzens et al., 2010). Part of the stored lipids is catabolized for energy but PUFAs are mainly used for structural development. DHA is the main fatty acid in neural and visual tissues and a deficiency has been shown to hinder larval development (M. V Bell et al., 1995; Benítez-Santana et al., 2007; Neuringer et al., 1988). In addition, as mentioned previously, ARA and EPA are involved in eicosanoid production with impacts on the modulation of neural transmission, hypothalamic functions and many immune and stress functions (Bell et al., 1996; Sargent et al., 1999b; Tocher et al., 1996). Therefore, FAs play a major role in the formation of good quality eggs exhibiting high fertilization and hatching rates and producing healthy larvae.

1.5 LARVAL NUTRITION AND IMPACT ON SURVIVAL AND DEVELOPMENT

1.5.1 Development of the digestive system

At hatching, the digestive system of marine fish larvae is undifferentiated, consisting of a straight tubular segment, lined by a single layer of columnar epithelial cells (future enterocytes), lying dorsally to the yolk sac. During the first weeks of life, larvae undergo major morphological and cellular changes with the development and differentiation of the different regions and organs including the bucco-pharynx, esophagus, intestine, pancreas, liver, and stomach depending on the species involved (Zambonino-Infante et al., 2008). The main difference between the development of freshwater and saltwater fish larvae is in the timing of the gastric glands emergence and the onset of acidic digestion. This process takes place during the transition to

exogenous feeding in freshwater larvae while it does not take place until metamorphosis in marine species (Govoni et al., 1986). Prior to metamorphosis, marine larvae present particular digestive characteristics. As the secretory function of the exocrine pancreas progressively develops, amylase expression decreases while other pancreatic enzymes expression (trypsin, lipase…) increase until the end of the larval period, reflecting different nutrient requirements at different stages of life (Krogdahl and Sundby, 1999). In the intestine, the early mode of digestion is intracellular via pinocytosis, with high expression of enzyme from the enterocyte cytosol (especially peptidase) (Govoni et al., 1986). As the intestine evolves and the enterocytes develop their brush border membrane, the cytosolic activities decrease to the profit of membranous enzyme activities of the brush border, such as alkaline phosphatase or aminopetidase N, and this reflects the onset of the adult mode of digestion (Zambonino-Infante and Cahu, 2001).

1.5.2 Feeding strategies to promote growth and survival

The onset of exogenous feeding is a crucial moment in the life of marine fish larvae. Larvae are particularly vulnerable at this stage as the yolk reserves reach exhaustion and no other stored energy is available. Any food deprivation or inadequacy at this point leads to massive mortality events and represent a major bottleneck in the research of new aquaculture species. In the wild, fish larvae feed mainly on copepods (Hunter, 1981), which provide an adequate nutritional profile with a variety of sizes to suit all larval stages (van der Meeren et al., 2008). However, copepod production is currently not economically viable and hatcheries have to rely on the traditional live preys: rotifers and Artemia (Stottrup, 2000). Relative success in the rearing of marine

fish larvae was achieved using these live preys as they are relatively easy to produce, they are easily detected and captured due to their movements in the water column, and their nutritional value can be manipulated to a certain extent through enrichment. Indeed, rotifers and Artemia have nutritional deficiencies for marine larvae, in particular in PUFAs whose essentiality was discussed above (Sargent et al., 1999b). Table 1.1 presents FA profiles from wild plankton and enriched cultured rotifers and Artemia, highlighting these differences. Rotifers are naturally rich in EPA and Navarro et al. (1999) demonstrated the ability of Artemia nauplii to catabolize and assimilate the PUFAs from the enrichment as well as retro-converting DHA into EPA. Therefore, delivering a specific and adequate FA profile to the larvae using these live feeds is difficult and makes larval nutritional studies challenging.

Table 1.1: Individual size (dry weight: DW), dry matter content (% of wet weight: WW), ash content, and FAs from copepods, copepod nauplii, zooplankton (copepods and decapod zoeae), rotifers, and Artemia (1-day or 3-day after hatching). Modified from (van der Meeren et al., 2008).

	Wild plankton				Intensive live feed			
	Copepods	Copepods	Copepod nauplii	Zooplankton	Rotifers	Artemia		
	2000	2001	2001	2001	IMR[a]	1-day[b]	1-day[c]	3-day[c]
Dry matter and ash								
Dry weight (µg/individual)	9.4[d] ± 2.5	8.1 ± 2.7	0.63[e] ± 0.7	9.9	0.61	2.1	2.1	2.5
Dry matter (% of WW)	14.9 ± 1.1	15.3 ± 1.5	15.2 ± 1.9	17.7	13.2	10.2	10.8	8.9
Ash content (% of DW)	10.3 ± 1.2	10.5 ± 1.3	9.9 ± 0.5	15.3	9.6	10.4	9.6	9.5
FAs (% of total lipid)								
Myristic acid	3.4[d] ± 1.7	1.7 ± 1.1	1.3 ± 0.8	3.8	6.7	1.7	1.5	2.4
Palmitic acid	14.5 ± 1.9	14.4 ± 1.4	13.7 ± 2.5	14.1	19.7	14.9	14.4	15.8
Palmitoleic acid	3.4 ± 1.8	4.4 ± 4.7	1.8 ± 1.4	7.6	9.2	4.8	1	3
Stearic acid	3.5 ± 1.0	3.7 ± 0.7	3.9 ± 1.0	4.1	3.9	5	5	5.4
Oleic acid	2.3 ± 1.1	2.6 ± 1.4	1.3[e] ± 0.7	7.3	7.8	23.3	22.8	17.8
Vaccenic (Asclepic) acid	2.7 ± 0.6	2.9 ± 0.7	2.0[e] ± 0.5	3.1	4.9	5.5	6.3	5.4
Linoleic acid	1.5[d] ± 0.5	2.3 ± 0.7	1.5[e] ± 0.5	2.2	15.3	6.6	5.8	4.2
α-Linolenic acid	1.9[d] ± 1.0	2.4 ± 1.1	1.5[e] ± 0.9	1.4	1.2	12.2	16.2	10.2
Stearidonic acid	2.3[d] ± 1.4	4.1 ± 2.9	4.5 ± 5.7	5.2	2	2.8	3.2	1.7
Arachidonic acid (ARA)	0.8 ± 0.5	0.9 ± 0.7	0.6 ± 0.3	1.6	1.9	2	2	3.2
Eicosapentaenoic acid (EPA)	17.4 ± 3.1	16.2 ± 3.4	16.3 ± 6.4	16.4	7.1	7.8	7.8	9.2
Docosahexaenoic acid (DHA)	34.4 ± 4.6	32.9 ± 6.8	40.5[e] ± 2.4	17.3	12.4	10.6	11.1	20
Other saturated FAs	3.1 ± 1.1	3.3 ± 1.2	3.9 ± 2.7	9.7	n.d.	n.d.	n.d.	n.d.
Other monounsaturated FAs	3.7[d] ± 1.0	2.8 ± 0.9	2.7 ± 1.2	2	4.2	1.2	1.5	1.7
Other polyunsaturated FAs	5.1 ± 1.2	5.4 ± 1.5	4.6 ± 1.0	4.2	3.8	1.6	1.4	n.d.
Total amounts of FA groups (%)								
Saturated FAs	24.6[d] ± 2.9	23.1 ± 2.2	22.7 ± 2.9	31.7	30.3	21.6	20.9	23.7
Monounsaturated FAs	12.1 ± 2.1	12.7 ± 6.1	7.8 ± 3.2	20.1	26.1	34.8	31.6	27.8
Polyunsaturated FAs	63.3 ± 3.7	64.2 ± 6.8	69.4 ± 5.8	48.3	43.7	43.6	47.5	48.5
Highly unsaturated (n-3) FAs	51.8[d] ± 4.5	49.1 ± 6.8	56.8[e] ± 6.8	33.6	19.4	18.4	19	29.2
(n-3)/(n-6)	11.3[d] ± 2.7	9.8 ± 2.5	12.5[e] ± 3.0	7	1.5	3.9	4.2	5.5
DHA/EPA	2.1 ± 0.5	2.2 ± 1.0	2.8 ± 1.3	1.1	1.7	1.4	1.4	2.2
EPA/ARA	24.7 ± 9.2	23.2 ± 10.1	27.7 ± 4.0	10.3	3.7	4	4	2.9

Data are given as mean±SD when number of samples N>1. Values below detection are denoted n.d.. [a]: Rotifers grown on Rotimac and Isochrysis galbana algae. [b] Artemia enriched with DC-DHA Selco. [c] Artemia fed DC-DHA Selco and Algamac 2000.

Formulated diets allow for a better control of the nutrient quality and concentration, delivering a constant nutritional profile that can be accurately adjusted in nutritional studies. However, the first attempts to feed compound diets to marine fish larvae were unsuccessful, and the observation of dead larvae with a gut full of food suggested that larvae did not possess the necessary enzymes to digest an inert diet at the onset of exogenous feeding (Dabrowski, 1984; Kolkovski et al., 1993; Lauff and Hofer, 1984). It was later demonstrated that larvae possess a wide range of enzyme from hatching although their digestive capacities are different from that of juveniles (Cahu and Zambonino-Infante, 2001). In particular, larvae growth was promoted with the incorporation of phospholipids and protein hydrolysates while these components are not essential for juveniles (Coutteau et al., 1997; Zambonino-Infante et al., 1997). Phospholipids biosynthesis is limited during fish larval stages and a dietary supplementation was shown to increase the efficiency of absorption of dietary FAs and lipids from the gut, through enhanced lipoprotein synthesis (Tocher et al., 2008). Protein hydrolysates consist of low molecular-weight peptides resulting from protein pre-digestion, which are more easily absorbed by enterocytes than high-molecular-weight macromolecules. They also act as feed attractants as they contain digested protein components such as free amino acids and peptides. Therefore they enhance the digestibility and appetence of microdiets, increasing feeding success, growth and survival (Gisbert et al., 2012; Zambonino-Infante et al., 1997). It is necessary to take these specificities into consideration when developing compound diets for young larvae, as an inadequate diet can delay or prevent the maturation process of the digestive system. European sea bass larvae were successfully reared

with a compound micro-diet from mouth opening, however, most marine species require a period of live feed (C. L. Cahu et al., 2003).

An approach combining both micro-diets and live food was shown to improve larval performances compared to feeding microdiets or live food alone. This feeding strategy benefits from the visual, chemical and nutritional stimulation of live prey, improving the ingestion and digestion of the inert particles, and habituating the larvae early to facilitate weaning (Canavate and Fernandez-Diaz, 1999; W Koven et al., 2001; Rosenlund et al., 1997).

Alongside the development of micro-diets, another approach to improve larval growth and survival explores the use of probiotics. Since the digestive tract of fish larvae is sterile at hatching, the initial colonization depends on the environment and live feed ingested (Grisez et al., 1997). This microflora is of major importance as osmoregulation requires constant ingestion of the surrounding water and this microflora constitutes the first barrier (Gatesoupe, 1999). In addition, in both human and fish, probiotics have been shown to promote nutrient absorption and influence expression of genes involved in growth metabolism (Hooper et al., 2001; Lazado et al., 2012; Suzer et al., 2008). Improvement in growth and stimulation of the digestive system was observed in several marine fish species including Japanese flounder *Paralichthys olivaceus* (Ye et al., 2011), orange-spotted grouper *Epinephelus coioides* (Sun et al., 2013), rohu *Labeo rohita* (Mohapatra et al., 2012) and sea bream (Avella et al., 2010). The interest in the use of probiotics in aquaculture is somewhat recent and the mechanisms of actions are not fully understood, nevertheless, they open new opportunities to assist in the rearing of new delicate species.

1.6 AIMS OF THIS THESIS

The objective of this thesis was to improve the larviculture of three of Florida's high value food and stock enhancement finfish, common snook, Florida pompano and red drum, through the study of their nutritional requirements.

Common snook larviculture is hindered by extremely low and inconsistent larval survival; therefore, the studies conducted on this species in this thesis focused on the early stages of larval rearing. This included gamete and egg quality with a study comparing the lipid and FA profile of muscle, liver and eggs, from wild and captive broodstock to highlight potential imbalances and allow for the correct adjustment of the captive fish diet (Chapter 2).

The next step was to gain knowledge on the early FAs requirements of common snook and Florida pompano to determine the most critical components necessary at first feeding and allow for the elaboration of adequate enrichment formulations (Chapter 3).

In addition to the study of lipid enrichment of live feed, the supplementation of live feed with a mix of probiotics was tested on all three species to study a potential increase in larvae growth and survival, and both tank bacteriology and enzyme secretion were studied to gain some insight on the mechanisms of action of probiotics (Chapter 4).

The transition from live feed to dry diet was studied with the comparison of three micro-diets on Florida pompano weaning success, with a focus on FA incorporation and enzyme secretion, to allow for the formulation or selection of an adequate diet and evaluate early weaning possibilities (Chapter 5).

Chapter 2 Comparative study of lipids and fatty acids in liver, muscle, and eggs of wild and captive common snook broodstock

RESEARCH ARTICLE

In preparation

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Contributions: The present manuscript was compiled and written in full by the author of this thesis. Sampling, lab and statistical analyses have been carried out by the candidate except for the fatty acid analyses that were performed by Cynthia Faulk from the Fisheries and Mariculture Laboratory at the University of Texas Marine Science Institute. Co-authors provided assistance with the proofreading of the manuscript.

2.1 ABSTRACT

In this study, the lipid composition of wild and captive common snook broodstock were investigated to identify potential nutritional deficiencies and formulate suitable diets for captive stocks. Results showed captive snook incorporated significantly more lipid than their wild counterparts. However, cholesterol and arachidonic acid (ARA) levels were significantly lower compared to wild fish, which may impact on steroid and prostaglandin production, reproductive behavior and gametogenesis. In eggs obtained from captive broodstock, high docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) levels, associated with low ARA contents were found. As a result, ARA/EPA ratio in captive eggs was less than half of that in wild eggs with the potential for negative consequences on embryo and larval development. In conclusion, large differences were noticed between wild and captive broodstock that most likely contribute to the reproductive dysfunctions observed in captive snook broodstock (e.g. incomplete oocyte maturation, low milt production and poor egg and larval quality). The wild snook survey also identified the presence of hydrocarbons in the liver, which should be further studied to identify a potential impact on the reproductive performances of a vulnerable population like common snook.

Keywords: Snook, broodstock, arachidonic acid, egg quality, fatty acid, flesh, liver

2.2 INTRODUCTION

Dietary lipids and in particular polyunsaturated fatty acids (PUFAs) play a critical role in the successful production of high quality gametes and eggs of marine fish (Izquierdo et al., 2001; Sargent et al., 2002). While a large proportion of dietary lipids is catabolized to fuel reproductive processes, they are also deposited into gametes, especially as yolk reserve in the oocytes (Tocher, 2003). Yolk fatty acid composition directly affects the optimal development of the embryo and yolk-sac larvae by providing docosahexaenoic acid (DHA), essential in neural and visual development, as well as eicosapentaenoic acid (EPA) and arachidonic acid (ARA) which serve as precursors of eicosanoids involved in the modulation of neural, hypothalamic, and immune functions (Bell, 2003; Kamler, 2007; Migaud et al., 2013; Tocher, 2010). ARA is a key PUFA for fish reproduction through the production of prostaglandins that stimulates ovarian and testicular steroidogenesis, final oocyte maturation, ovulation and milt production (Lister and Van Der Kraak, 2008; Norambuena et al., 2013; Sorbera et al., 2001; Wade, 1994). ARA-derived prostaglandins also act as pheromones and influence sexual behavior (Stacey and Sorensen, 2011).

Marine teleosts have lost their ability to synthesize PUFAs, thus, DHA, EPA and ARA are essential fatty acids that must be provided by the diet (Sargent et al., 1997). The low substrate specificity in fatty acid metabolism (several fatty acids are substrates for the same enzyme) explains the greater direct influence of dietary lipids on final concentrations and cellular functions compared to any other class of nutrients. As a result, the fatty acid profile from fish tissues and eggs reflects the fatty acid profile supplied through the diet (Alasalvar et al., 2002; Sargent et al.,

2002, 1993). The comparison of tissues and/or eggs from wild and captive fish allows to identify potential nutritional deficiencies, which is essential for the development of suitable broodstock diets (Migaud et al., 2013). This strategy has been successful in many species including striped trumpeter *Latris lineata* (Morehead et al., 2001), sea bass *Dicentrarchus labrax* (Alasalvar et al., 2002), white seabream *Diplodus sargus* (Cejas et al., 2004b, 2003), black seabream *Spondyliosoma cantharus* (Rodriguez et al., 2004), Japanese eel *Anguilla japonica* (Oku et al., 2009), black sea bass *Centropristis striata* (Seaborn et al., 2009), highfin amberjack *Seriola rivoliana* (Saito, 2012), greater amberjack *Seriola dumerili* (Rodriguez-Barreto et al., 2012; Saito, 2012) and Senegalese sole *Solea senegalensis* (Norambuena et al., 2012a).

The common snook *Centropomus undecimalis* is an estuarine species found in subtropical and tropical waters, around the Gulf of Mexico and along the western Atlantic coast from Cape Canaveral, Florida, down to Florianopolis, Brazil (Alvarez-Lajonchère and Tsuzuki, 2008). Snook support a valuable recreational fishery in the southeastern United States and are a popular food fish in South America and Mexico. It is a protandric hermaphrodite species with transitional fish observed up to 7 years of age (Muller and Taylor, 2006). On the east coast of Florida, the spawning season extends from April to September, with spawning events typically occurring along sandy beaches, inlets and tidal passes of estuaries (Taylor et al., 1998). Factors such as environmental changes including cold kills, habitat destruction and increased recreational fishing pressure contributed to a decline in common snook stocks in the Gulf of Mexico (McRae and McCawley, 2011; Muller and Taylor, 2006). Therefore, additional fishery management tools, such as stock enhancement, are being

investigated to supplement local fisheries in Florida (Brennan et al., 2008). Intensive aquaculture production is also of interest to increase market availability in South America (Alvarez-Lajonchère and Tsuzuki, 2008).

Despite recent breakthroughs in the spawning of captive common snook broodstock (Ibarra-Castro et al., 2011; Neidig et al., 2000; Rhody et al., 2014, 2013; Yanes-Roca et al., 2009) and advances in larval rearing protocols (Barón-Aguilar et al., 2013; Ibarra-Castro et al., 2011; Rhody et al., 2010; Wittenrich et al., 2009), to date, there is still no established large scale production of this species for food or restocking. Major reproductive bottlenecks of captive snook broodstock include the failure of females to ovulate without hormonal manipulation, reduced milt production in males and inconsistent supply of high quality eggs and larvae.

The aim of this study was to compare the lipid composition of muscle, liver and eggs from wild and common snook broodstock maintained in captivity for 3 years, to gain information on broodstock dietary requirements and improve captive spawn quality.

2.3 MATERIALS AND METHODS

2.3.1 Captive fish and egg collection

Captive broodstock were captured in Sarasota Bay, Florida, in Fall 2009, and held indoors in a 4.6 m diameter, 25 m^3, fiberglass tank equipped with a filtration unit. Fish were fed a 50 % shrimp, 50 % herring diet (Table 2.1) at 2.5 % body weight every other day, and maintained under simulated natural conditions. In May 2012, female broodstock reproductive development was assessed by ovarian biopsy and individuals with oocytes classified in the later stages of the oogenetic cycle (e.g.

Secondary Growth Stage, Full-grown Step, Grier et al., 2009; Rhody et al., 2013) were hormonally induced to spawn with gonadotropin-releasing hormone (GnRHa implants, 50 μg/Kg bodyweight, Institute of Marine and Environmental Technologies, University of Maryland, Baltimore, MD, USA). Fish then spawned spontaneously by 32 hours post implantation. Eggs were gathered into a collector via skimming of the tank's surface. After collection, eggs were transferred into a conical tank and after 4 hours of incubation (past the blastula stage) the non-viable sinking eggs were removed and discarded (fertilization rate 64.1 ± 4.2 %). Three viable buoyant egg aliquots were then sampled and rinsed with deionized water before storage at -70°C. Eggs hatched after 19 hours of incubation at 28°C (hatching rate 82.6 ± 2.8 %). In addition, 6 males with poor milt production and 6 females presenting non mature oocytes, were sacrificed with an overdose of tricaine methanesulfonate (MS 222), weighed, measured, the otoliths were extracted for age determination, and flesh and liver samples were stored at -70°C. Hepatosomatic index (HSI) and gonadosomatic index (GSI) were calculated as: (liver or gonad weight (g) / body weight (g)) x100 (Table 2.2).

Table 2.1 Fatty acid profile (% of total FA) and total fatty acid content (mg/g of dry weight) of the diet fed to the captive broodstock (n=3).

	Captive broodstock diet		
	Herring	**Shrimp**	**50/50**
14:0	4.7 ± 0.1	1.5 ± 0.1	3.9 ± 0.4
15:0	1.2 ± 0.1	1.0 ± 0.0	1.0 ± 0.1
16:0	20.5 ± 0.2	11.6 ± 0.4	18.8 ± 0.9
17:0	1.5 ± 0.0	1.6 ± 0.1	1.5 ± 0.0
18:0	6.0 ± 0.2	6.9 ± 0.3	6.9 ± 0.0
Σ SFA[1]	**34.0 ± 0.3**	**22.8 ± 0.9**	**32.3 ± 1.4**
16:1n-7	5.9 ± 0.1	5.2 ± 0.4	5.5 ± 0.1
18:1n-9	6.4 ± 0.1	7.0 ± 0.2	6.1 ± 0.4
18:1n-7	4.3 ± 0.2	6.0 ± 0.3	5.1 ± 0.4
20:1n-9	0.4 ± 0.1	1.0 ± 0.2	0.8 ± 0.1
Σ MUFA[2]	**17.0 ± 0.2**	**19.4 ± 0.4**	**17.6 ± 0.1**
16:3n-4	0.5 ± 0.0	1.1 ± 0.0	0.5 ± 0.0
18:2n-6	1.4 ± 0.0	1.5 ± 0.1	1.5 ± 0.0
20:4n-6	**3.1 ± 0.2**	**9.2 ± 0.4**	**4.3 ± 0.2**
22:5n-6	1.5 ± 0.1	1.0 ± 0.2	1.6 ± 0.1
20:5n-3	**8.6 ± 0.4**	**15.3 ± 0.3**	**9.5 ± 0.3**
22:5n-3	1.6 ± 0.1	2.1 ± 0.1	1.8 ± 0.1
22:6n-3	**22.2 ± 0.6**	**8.7 ± 0.7**	**19.9 ± 0.9**
Σ n-6[3]	7.5 ± 0.3	13.3 ± 0.4	8.6 ± 0.3
Σ n-3[4]	34.8 ± 1.0	27.1 ± 1.0	33.0 ± 1.1
Σ PUFA[5]	**43.7 ± 0.9**	**42.1 ± 1.0**	**42.9 ± 1.3**
DHA/EPA	**2.6 ± 0.1**	**0.6 ± 0.0**	**2.1 ± 0.0**
ARA/EPA	**0.4 ± 0.0**	**0.6 ± 0.0**	**0.5 ± 0.0**
n-3/n-6	4.7 ± 0.3	2.0 ± 0.1	3.8 ± 0.0
Total FA	**117.3 ± 6.9**	**26.0 ± 1.3**	**71.8 ± 11.2**

[1] Includes 12:0.
[2] Includes 15:1.
[3] Includes 18:3n-6, 20:2n-6, 20:3n-6.
[4] Includes 18:3n-3, 18:4n-3, 20:3n-3, 20:4n-3.
[5] Includes 16:2n-4, 18:3n-4.
SFA: saturated fatty acids, MUFA: mono-unsaturated fatty acids; PUFA: poly-unsaturated fatty acids; DHA: docosahexaenoic acid (22:6n-3); EPA: eicosapentaenoic acid (20:4n-3); ARA: arachidonic acid (20:4n-6).

2.3.2 Wild fish tissue and egg collection

Wild fish were collected from two close spawning sites (Emerson Point or Rattlesnake Key depending) in waters around Sarasota, once each in April, June, July and August 2012. Fish were captured with a seine net and held in floating pens until processed. Fish were measured, weighed and their sex and reproductive status assessed. At each time point, 6 sexually mature females (visual observation of mature oocytes after stripping or canulation biopsy) and 6 males (visual observation of milt expression after stripping or canulation) were sacrificed with an overdose of MS 222, placed on ice and quickly brought back to the laboratory where gonad, liver and total weight were recorded, the otoliths were extracted for age determination, and flesh and liver samples stored at -70°C. In June and August, no mature males were captured and therefore only female samples could be analyzed. In July, milt was collected from 6 males using syringes and stored on ice and eggs were stripped from 6 females. Eggs from 2 females were pooled and the 3 batches of eggs were fertilized in sterile seawater using a drop of milt from each male. After fertilization, eggs were rinsed and stored in sterile seawater in a bag under pure oxygen, secured in a cooler and quickly brought back to the laboratory and transferred to conical tanks to separate viable and non-viable eggs before sampling of 3 aliquots and storage as described previously. The average fertilization rate and hatching rate for the 3 batches were 78.3 ± 6.3 % and 83.1 ± 5.1 % respectively.

All fish were collected under a Florida Fish and Wildlife Conservation Commission Special Activity License (Contract No.10087, Permit # SAL 09-522-SR). Animals were sacrificed in accordance with United States legislation concerning the protection of animals used for experimentation. All methods were conducted in

accordance with Mote Marine Laboratory's Institutional Animal Care and Use Committee approved protocols (IACUC Approval No. 12-03-KM1).

2.3.3 Proximate, fatty acid and lipid classes analyses

Proximate compositions of flesh and liver samples were determined according to standard procedures (AOAC, 2000). Prior to analysis, samples were minced and blended to ensure homogeneity. Moisture content was determined by drying the samples at 105 °C for 24 h. Ash content was determined after 24 h in crucibles at 600 °C. Crude protein content (Nx6.25) was determined using the automated Kjeldahl method (Tecator Kjeltec Auto 1030 analyzer, Foss, Warrington, U.K). Crude lipid content was determined after extraction according to Folch et al. (1957).

Separation of lipid classes was performed by double development high-performance thin- layer chromatography using methyl acetate/ isopropanol/ chloroform/ methanol/ 0.25% aqueous KCl (25:25:25:10:9, by volume) as first development to separate polar lipids and isohexane/diethyl ether/acetic acid (85:15:1, by volume) as a secondary development to separate neutral lipids (Henderson and Tocher, 1992). Lipid classes were visualized by charring at 160°C for 20 min after spraying with 3% (w/v) aqueous cupric acetate in 8% (v/v) phosphoric acid and quantified by densitometry with a tungsten lamp at 370 nm using a CAMAG-3 TLC scanner (CAMAG, Muttenz, Switzerland) with winCATS Planar Chromatography Manager software. Identification of individual classes was confirmed by comparison and reference to retention factors of authentic standards run alongside samples.

Fatty acid composition was determined by gas-liquid chromatography after preparation of fatty acid methyl esters (FAMEs) according to Morrison and Smith

(1964) . FAMEs were separated and quantified on a gas chromatograph (Shimadzu GC-2014, Shimadzu Scientific Instruments, Columbia, MD, USA) equipped with a Phenomenex ZB-WAX plus capillary column (30 m long, 0.53 mm internal diameter, 1.0 μm thickness; Phenomenex, Torrance, CA, USA) with on-column injection and flame ionization detection, using helium as carrier gas (4 mL min^{-1}) and injector and detector temperatures of 250 and 260 °C respectively. Temperature was held at 160°C for 5 min then increased up to 220°C at 3°C per minute and maintained at this temperature for 30 minutes. FAME peaks were identified by comparison with known standards (Supelco, Inc., Bellefonte, Pennsylvania, USA).

2.3.4 Statistics

Statistical analysis was performed using MINITAB® version 16.0. Data were analyzed by one-way ANOVA followed by a Tukey's post hoc test with 95% confidence. Non-homogenous data were arcsine square root transformed before analysis. No statistical analysis was performed on hydrocarbon data as this lipid class was not observed in all samples and more data would be required. All results are presented as means ± SEM. Only fatty acids contributing to at least 1% in one group are represented.

2.4 RESULTS

2.4.1 Broodstock morphometrics and proximate composition

Morphometric and lipid class composition data are presented in Table 2.2 for females and Table 2.3 for males. There was no statistical difference in age among the groups used in this study. Weight and fork length were significantly lower in captive females compared to wild females while no differences were observed between male groups. HSI from July males was the lower (0.3±0.0) than that of April males (0.8±0.1) and not statistically different from captive males (0.6±0.1). HSI from August females was the greatest (1.2±0.1) of all female groups yet not statistically different from April and June females. GSI was the lowest for captive females and males (0.7±0.2). Highest GSI values were observed for July and August females (5.2±1.5 and 5.0±0.8 respectively) though not statistically different from June females.

In flesh samples, lipid content was significantly higher for captive females (2.2±0.5 %) compared to all the other female groups (average of 0.7±0.1 %), as well as significantly higher in captive males (1.0±0.1 %) compared to July males (0.5±0.0 %) and not different from April males (0.7±0.1 %). Liver lipid content was statistically higher for captive males compared to wild males, however captive females liver lipid content was not statistically different from that of wild females. Liver protein content was significantly higher in wild June females and wild July males and females. In contrast, in these same three groups, flesh protein content was lower, though not statistically different from April males and females.

Table 2.2 Proximate composition (% of wet weight) of flesh and liver, weight (kg), fork length (cm), hepatosomatic index (HSI), gonadosomatic index (GSI) and age (years) from wild and captive female common snook broodstock (n=6). Superscript letters indicate significant differences within a row.

		Female				
		Wild April	**Wild June**	**Wild July**	**Wild August**	**Captive**
Lipid	Flesh	1.0 ± 0.1^{b}	0.7 ± 0.1^{ab}	0.5 ± 0.0^{a}	0.7 ± 0.1^{ab}	2.2 ± 0.5^{c}
	Liver	6.0 ± 1.0^{a}	9.4 ± 3.2^{b}	6.3 ± 1.8^{a}	15.8 ± 4.6^{c}	9.8 ± 2.0^{b}
Protein	Flesh	20.1 ± 0.3^{ab}	19.4 ± 0.2^{a}	19.2 ± 0.5^{a}	21.1 ± 0.5^{b}	22.0 ± 0.2^{b}
	Liver	14.8 ± 0.8^{ab}	17.3 ± 1.3^{b}	18.0 ± 1.6^{b}	13.0 ± 0.9^{a}	13.3 ± 0.9^{a}
Moisture	Flesh	78.2 ± 0.4^{b}	79.3 ± 0.2^{b}	79.5 ± 0.3^{b}	76.2 ± 0.3^{a}	75.0 ± 0.4^{a}
	Liver	73.6 ± 0.9^{b}	69.8 ± 3.1^{ab}	75.0 ± 1.8^{b}	65.9 ± 4.1^{a}	66.6 ± 1.5^{a}
Ash	Flesh	1.2 ± 0.1	1.2 ± 0.0	1.2 ± 0.1	1.2 ± 0.0	1.4 ± 0.1
	Liver	1.4 ± 0.1	1.5 ± 0.1	1.4 ± 0.1	1.3 ± 0.1	1.2 ± 0.1
Weight		3.04 ± 0.55^{b}	4.32 ± 0.84^{b}	3.32 ± 0.77^{b}	3.79 ± 0.48^{b}	2.05 ± 0.17^{a}
Fork Length		668 ± 39^{b}	722 ± 64^{b}	604 ± 64^{b}	726 ± 27^{b}	593 ± 18^{a}
HSI		0.9 ± 0.1^{bc}	0.9 ± 0.1^{bc}	0.7 ± 0.1^{ab}	1.2 ± 0.1^{c}	0.7 ± 0.1^{ab}
GSI		1.6 ± 0.6^{ab}	4.1 ± 0.7^{bc}	5.2 ± 1.5^{c}	5.0 ± 0.8^{c}	0.7 ± 0.2^{a}
Age (mean)		6.3 ± 0.9	5.7 ± 0.8	6.0 ± 1.0	6.0 ± 0.7	6.7 ± 0.5
Age (range)		4 - 10	3 - 8	3 - 9	5 - 9	5 - 8

Table 2.3 Proximate composition (% of wet weight) of flesh and liver, weight (kg), fork length (cm), hepatosomatic index (HSI), gonadosomatic index (GSI) and age (years) from wild and captive male common snook broodstock (n=6). Superscript letters indicate significant differences within a row.

		Male		
		Wild April	**Wild July**	**Captive**
Lipid	Flesh	0.7 ± 0.1^{ab}	0.5 ± 0.0^{a}	1.0 ± 0.1^{b}
	Liver	3.4 ± 0.3^{a}	4.5 ± 0.9^{ab}	6.6 ± 1.0^{b}
Protein	Flesh	20.1 ± 0.4^{ab}	18.9 ± 0.2^{a}	21.9 ± 0.2^{b}
	Liver	13.2 ± 0.4^{a}	18.4 ± 2.1^{b}	12.9 ± 0.7^{a}
Moisture	Flesh	78.6 ± 0.3^{b}	79.8 ± 0.2^{b}	76.4 ± 0.4^{a}
	Liver	74.2 ± 0.6^{b}	74.4 ± 0.7^{b}	69.6 ± 1.1^{a}
Ash	Flesh	1.3 ± 0.0	1.2 ± 0.1	1.3 ± 0.0
	Liver	1.3 ± 0.0	1.3 ± 0.1	1.1 ± 0.1
	Weight	2.22 ± 0.24	3.22 ± 0.91	2.19 ± 0.19
	Fork Length	617 ± 20	556 ± 44	583 ± 25
	HSI	0.8 ± 0.1^{b}	0.3 ± 0.0^{a}	0.6 ± 0.1^{ab}
	GSI	0.9 ± 0.4^{ab}	1.2 ± 0.3^{b}	0.7 ± 0.2^{a}
	Age (mean)	6.3 ± 0.4	7.5 ± 1.2	8.8 ± 1.3
	Age (range)	5 - 8	4 - 12	6 - 14

2.4.2 Lipid classes

Lipid class composition of flesh is presented in Tables 2.4 for females and 2.5 for males. In flesh, captive females presented significantly lower total polar lipid content, and higher total neutral lipid content compared to wild females. Likewise, captive males presented lower total polar lipid content and higher total neutral lipid content compared to wild males. In addition, levels of phosphatidylcholine (PC), phosphatidylethanolamine (PE) and cholesterol (CHOL) were significantly lower, and levels of triacylglycerols (TAG) significantly higher in captive females flesh samples compared to wild females and captive males compared to wild males. Among wild males, significantly lower total polar lipid content and higher total neutral lipid content were observed in April males compared to July males. Among wild females, highest total polar lipid content and lowest total neutral lipid content were observed in July females though not significantly different than levels in June females.

Lipid class composition of liver is presented in Tables 2.6 for females and 2.7 for males. In liver, captive males presented significantly lower total polar lipid content, and higher total neutral lipid content compared to wild males. However, levels were more variable among wild females with highest total polar lipid content observed in July females though not significantly different from that of April females. July females also presented the lowest total neutral lipid content though not significantly different from that of April females. In addition, presence of hydrocarbons was detected in wild liver samples while no observation was made in captive liver samples and flesh samples from both wild and captive fish.

Table 2.4 Lipid class composition (%) of flesh from wild and captive female common snook broodstock (n=6). Superscript letters indicate significant differences within a row.

	Female				
	Wild April	**Wild June**	**Wild July**	**Wild August**	**Captive**
PC	25.3 ± 1.6^{c}	27.3 ± 1.8^{cd}	29.7 ± 0.5^{d}	19.9 ± 1.2^{b}	15.4 ± 1.1^{a}
PS	3.2 ± 0.3^{ab}	3.6 ± 0.2^{b}	5.0 ± 0.2^{c}	4.5 ± 0.3^{c}	2.3 ± 0.6^{a}
PI	4.8 ± 0.4^{ab}	5.9 ± 0.4^{b}	6.1 ± 0.2^{b}	5.8 ± 0.3^{b}	3.5 ± 0.5^{a}
PE	11.7 ± 0.7^{bc}	12.7 ± 0.8^{c}	13.4 ± 0.5^{c}	9.5 ± 0.7^{b}	7.3 ± 0.3^{a}
Total polar*	**51.1 ± 3.4^{b}**	**55.8 ± 2.7^{bc}**	**64.3 ± 1.3^{c}**	**51.9 ± 2.5^{b}**	**33.9 ± 3.2^{a}**
DAG	2.2 ± 0.3^{b}	1.6 ± 0.4^{ab}	0.4 ± 0.2^{a}	2.0 ± 0.1^{b}	2.7 ± 0.2^{b}
CHOL	12.1 ± 0.7^{b}	14.4 ± 1.2^{bc}	17.7 ± 0.7^{c}	12.3 ± 0.4^{b}	8.6 ± 0.5^{a}
FFA	4.9 ± 0.4^{a}	4.5 ± 0.4^{a}	5.3 ± 0.5^{a}	14.9 ± 0.7^{b}	4.8 ± 0.5^{a}
TAG	24.1 ± 4.1^{bc}	19.8 ± 4.3^{b}	8.5 ± 1.3^{a}	15.7 ± 3.0^{b}	43.9 ± 3.7^{d}
W+SE	5.6 ± 0.6^{b}	3.9 ± 0.7^{a}	3.9 ± 0.6^{a}	3.2 ± 0.2^{a}	6.3 ± 1.9^{b}
HC	nd	nd	nd	nd	nd
Total neutral	**48.9 ± 3.4^{c}**	**44.2 ± 2.7^{bc}**	**35.8 ± 1.3^{a}**	**48.1 ± 2.5^{c}**	**66.1 ± 3.2^{d}**

PC: phosphatidylcholine; PS: phosphatidylserine; PI: phosphatidylinositol; PE: phosphatidylethanolamine; DAG: diacylglycerols; CHOL: cholesterol; FFA: free fatty acids; TAG: triacylglycerols; W+SE: wax and sterol ester; HC: hydrocarbons; nd: not detected.
*: includes lysophosphatidylcholine, spingomyelin, phosphatidylglycerol and pigmented material.

Table 2.5 Lipid class composition (%) of flesh from wild and captive male common snook broodstock (n=6). Superscript letters indicate significant differences within a row.

	Male		
	Wild April	**Wild July**	**Captive**
PC	29.3 ± 1.6^{b}	31.5 ± 0.2^{b}	20.7 ± 1.3^{a}
PS	3.8 ± 0.2^{a}	5.3 ± 0.2^{b}	3.1 ± 0.3^{a}
PI	6.4 ± 0.5^{b}	8.2 ± 0.3^{c}	5.1 ± 0.3^{a}
PE	14.1 ± 1.0^{b}	14.3 ± 0.3^{b}	9.3 ± 0.6^{a}
Total polar*	**59.7 ± 3.1^{b}**	**69.1 ± 0.2^{c}**	**45.3 ± 2.4^{a}**
DAG	2.0 ± 0.2^{b}	0.8 ± 0.3^{a}	2.4 ± 0.3^{b}
CHOL	13.8 ± 0.6^{b}	16.6 ± 0.5^{c}	10.1 ± 0.8^{a}
FFA	5.0 ± 0.6	5.6 ± 0.1	4.7 ± 0.7
TAG	14.6 ± 3.4^{b}	4.9 ± 0.6^{a}	28.8 ± 2.1^{c}
W+SE	5.0 ± 0.6^{b}	3.0 ± 0.2^{a}	8.8 ± 1.8^{c}
HC	nd	nd	nd
Total neutral	**40.4 ± 3.1^{b}**	**30.9 ± 0.2^{a}**	**54.7 ± 2.4^{c}**

Abbreviations as in table 2.4.
*: includes lysophosphatidylcholine, spingomyelin, phosphatidylglycerol and pigmented material.

Table 2.6 Lipid class composition (%) of liver from wild and captive female common snook broodstock (n=6). Superscript letters indicate significant differences within a row.

	Female				
	Wild April	**Wild June**	**Wild July**	**Wild August**	**Captive**
PC	16.0 ± 1.4^{bc}	13.3 ± 2.2^{b}	21.7 ± 2.6^{c}	5.8 ± 0.9^{a}	9.1 ± 1.5^{ab}
PS	1.9 ± 0.3^{ab}	1.3 ± 0.5^{a}	3.0 ± 0.7^{b}	1.1 ± 0.2^{a}	1.2 ± 0.5^{a}
PI	2.8 ± 0.5^{b}	2.5 ± 0.6^{b}	5.2 ± 0.9^{c}	0.7 ± 0.1^{a}	1.7 ± 0.6^{ab}
PE	9.4 ± 0.9^{b}	8.8 ± 1.4^{b}	11.9 ± 1.4^{b}	3.6 ± 0.9^{a}	5.7 ± 1.0^{a}
Total polar*	**43.0 ± 3.2^{bc}**	**37.6 ± 6.6^{b}**	**51.0 ± 6.2^{c}**	**22.2 ± 2.6^{a}**	**28.8 ± 5.0^{a}**
DAG	2.7 ± 1.0^{ab}	3.7 ± 1.0^{bc}	1.7 ± 0.8^{a}	4.9 ± 0.6^{c}	5.3 ± 0.8^{c}
CHOL	12.4 ± 1.7^{b}	8.7 ± 1.6^{ab}	13.4 ± 1.9^{b}	5.5 ± 0.5^{a}	8.1 ± 1.1^{ab}
FFA	20.6 ± 1.8^{b}	20.6 ± 0.7^{b}	15.7 ± 1.4^{ab}	28.4 ± 0.8^{c}	20.1 ± 1.5^{b}
TAG	9.8 ± 2.8^{a}	18.9 ± 6.9^{b}	10.2 ± 5.1^{a}	29.3 ± 4.1^{c}	27.1 ± 5.9^{c}
W+SE	11.0 ± 2.1^{b}	7.4 ± 0.4^{a}	6.8 ± 1.1^{a}	7.6 ± 1.8^{ab}	10.7 ± 1.7^{b}
HC	0.5 ± 0.9	3.1 ± 0.5	1.3 ± 0.3	2.1 ± 0.7	nd
Total neutral	**57.0 ± 5.5^{ab}**	**62.4 ± 6.6^{b}**	**49.0 ± 6.2^{a}**	**77.8 ± 2.6^{c}**	**71.2 ± 5.0^{bc}**

Abbreviations as in table 2.4.
*: includes lysophosphatidylcholine, spingomyelin, phosphatidylglycerol and pigmented material.

Table 2.7 Lipid class composition (%) of liver from wild and captive male common snook broodstock (n=6). Superscript letters indicate significant differences within a row.

	Male		
	Wild April	**Wild July**	**Captive**
PC	17.6 ± 0.7^{b}	14.3 ± 1.5^{ab}	10.0 ± 1.7^{a}
PS	2.8 ± 0.3^{ab}	3.9 ± 0.6^{b}	1.6 ± 0.5^{a}
PI	3.2 ± 0.3^{b}	3.6 ± 0.6^{b}	1.6 ± 0.5^{a}
PE	10.3 ± 0.7^{b}	10.0 ± 1.2^{b}	6.3 ± 1.2^{a}
Total polar*	**48.7 ± 1.9^{b}**	**45.6 ± 4.8^{b}**	**31.7 ± 5.0^{a}**
DAG	2.1 ± 0.2^{ab}	0.9 ± 0.9^{a}	4.6 ± 1.0^{c}
CHOL	13.3 ± 0.7^{b}	13.8 ± 1.7^{b}	8.9 ± 1.3^{a}
FFA	18.1 ± 0.8^{a}	18.4 ± 1.5^{a}	20.6 ± 1.2^{b}
TAG	7.0 ± 4.1^{ab}	4.9 ± 4.9^{a}	23.0 ± 4.4^{c}
W+SE	10.3 ± 1.7	10.9 ± 1.8	11.2 ± 1.5
HC	0.3 ± 0.1	3.2 ± 0.4	nd
Total neutral	**51.2 ± 4.1^{a}**	**52.1 ± 5.4^{a}**	**68.3 ± 5.0^{b}**

Abbreviations as in table 2.4.
*: includes lysophosphatidylcholine, spingomyelin, phosphatidylglycerol and pigmented material.

2.4.3 Fatty acid profiles

Fatty acid profiles and total fatty acid content of flesh samples are presented in Tables 2.8 for females and 2.9 for males. In flesh samples, total saturated fatty acids (SFA) and mono-unsaturated fatty acid (MUFA) were significantly higher in captive males compared to wild males and in captive females compared to April, June and July females. No significant difference in total SFA was observed among wild females and among wild males. Total MUFA were not statistically different between wild males, while it was significantly higher in August females compared to the other wild female groups. Levels of ARA in captive females and captive males were significantly lower than that of wild females and wild males respectively. No significant difference was observed in ARA levels among wild males while in wild females ARA levels were significantly higher in July females compared to other wild females groups. There was no significant difference in EPA levels between captive and wild fish. In addition, no difference was observed among wild males and among wild females. DHA levels were not different in males of both origins while, among females a significantly lower level of DHA was observed in August females compared to July and captive females, with April and June presenting intermediate levels. DHA/EPA ratio was not statistically different among males from both origins and not significantly different among females with the exception of August females presenting a significantly lower DHA/EPA ratio than that of April and July. Captive females and wild June females presented an intermediate ratio. ARA/EPA ratio was significantly lower in captive males (1.6±0.2) compared to wild males (average of 3.1±0.4). It was significantly lower also for captive females (1.6±0.1) compared to June and July females (average of 2.6±0.4) and not

statistically different from April and August females. Total PUFA levels were significantly lower in captive males (40.6±2.2 %) compared to April and July males (46.0±2.9 and 46.3±2.8 % respectively). In females, lower total PUFA was observed in August (40.4±3.2 %) and captive females (39.7±1.4 %) compared to July females (47.4±0.7 %) and content was intermediate in April and June females.

Fatty acid profiles and total fatty acid content of liver samples are presented in Tables 2.10 for females and 2.11 for males. In liver, no difference in total SFA was observed among males, while captive females showed levels similar to that of all other groups except August females that showed significantly higher total SFA. Total MUFA were significantly greater in captive males compared to wild males, while captive females presented a total MUFA level not statistically different from April, June and August females, but significantly higher that that of July females. As in flesh, liver ARA levels in captive males (4.3±1.0 %) were significantly lower than that of wild males (average of 8.6±0.9 %). ARA levels in captive females (3.3±0.8 %) were not different from that of August females but significantly lower than that of other female groups (average of 6.1±0.9 %). EPA levels in captive males were not statistically different from that of wild males, while EPA levels in captive females were significantly lower than that in June, July and August females, with April females showing an intermediate level. As in flesh, ARA/EPA ratio of captive males (2.1±0.5 %) was significantly lower than that of April and July males (3.8±0.7 and 6.4±1.2 % respectively). ARA/EPA ratio in captive females was significantly lower than that of July females but not different from that of other wild female groups. DHA/EPA ratio was not statistically different among males from all groups while it was significantly higher in captive females compared to wild

females. Total PUFA was not significantly different among males while only August females presented a significantly lower total PUFA compared to other female groups.

Table 2.8 Fatty acid profile (% of total FA) and fatty acid content (mg/g of dry weight) of flesh from wild and captive female common snook broodstock (n=6). Superscript letters indicate significant differences within a row.

	Female				
	Wild April	**Wild June**	**Wild July**	**Wild August**	**Captive**
14:0	1.8 ± 0.3^{ab}	1.6 ± 0.2^{a}	1.4 ± 0.1^{a}	2.0 ± 0.2^{ab}	2.6 ± 0.2^{b}
15:0	1.3 ± 0.3^{b}	1.0 ± 0.1^{ab}	0.6 ± 0.1^{a}	1.1 ± 0.1^{ab}	0.6 ± 0.0^{a}
16:0	21.6 ± 0.9^{ab}	20.9 ± 0.4^{a}	20.5 ± 0.5^{a}	21.9 ± 0.9^{ab}	23.8 ± 0.2^{b}
18:0	5.2 ± 0.2^{a}	5.7 ± 0.2^{b}	5.6 ± 0.3^{b}	5.3 ± 0.3^{a}	5.4 ± 0.2^{ab}
Σ SFA[1]	**30.9 ± 1.3^{a}**	**30.0 ± 0.7^{a}**	**28.8 ± 0.5^{a}**	**31.2 ± 1.2^{ab}**	**33.1 ± 0.2^{b}**
16:1n-7	5.8 ± 0.7^{b}	4.8 ± 0.5^{ab}	4.2 ± 0.4^{a}	6.2 ± 0.8^{b}	5.5 ± 0.5^{b}
18:1n-9	10.9 ± 1.0^{a}	10.8 ± 0.9^{a}	10.1 ± 0.3^{a}	12.0 ± 0.8^{ab}	13.5 ± 0.6^{b}
18:1n-7	3.3 ± 0.3^{b}	2.8 ± 0.3^{ab}	2.5 ± 0.1^{a}	3.4 ± 0.4^{b}	2.8 ± 0.2^{ab}
Σ MUFA[2]	**20.7 ± 1.8^{ab}**	**18.9 ± 1.6^{a}**	**17.2 ± 0.5^{a}**	**22.3 ± 2.1^{b}**	**22.7 ± 1.2^{b}**
16:3n-4	1.6 ± 0.3^{b}	1.4 ± 0.1^{ab}	1.6 ± 0.1^{b}	1.6 ± 0.1^{b}	1.1 ± 0.1^{a}
18:2n-6	1.4 ± 0.1^{b}	1.1 ± 0.1^{a}	1.5 ± 0.2^{b}	1.2 ± 0.1^{a}	1.2 ± 0.0^{a}
20:4n-6	**7.4 ± 0.7^{b}**	**8.9 ± 1.0^{bc}**	**11.4 ± 0.7^{c}**	**7.7 ± 1.1^{b}**	**5.6 ± 0.6^{a}**
22:5n-6	2.6 ± 0.4^{a}	3.0 ± 0.3^{ab}	3.9 ± 0.3^{b}	2.5 ± 0.4^{a}	2.5 ± 0.2^{a}
20:5n-3	**3.7 ± 0.3^{a}**	**4.4 ± 0.1^{b}**	**3.8 ± 0.3^{ab}**	**4.9 ± 0.4^{b}**	**3.6 ± 0.1^{b}**
22:5n-3	3.7 ± 0.4^{b}	3.7 ± 0.3^{b}	3.0 ± 0.2^{a}	3.5 ± 0.4^{b}	2.8 ± 0.2^{a}
22:6n-3	**18.5 ± 2.7^{ab}**	**18.7 ± 1.6^{ab}**	**19.5 ± 1.4^{b}**	**15.5 ± 2.0^{a}**	**20.0 ± 0.8^{b}**
Σ n-6[3]	12.3 ± 1.0^{b}	14.1 ± 1.2^{c}	17.8 ± 1.0^{d}	12.5 ± 1.3^{b}	10.2 ± 0.8^{a}
Σ n-3[4]	27.3 ± 2.4^{ab}	27.7 ± 1.4^{ab}	27.2 ± 1.2^{ab}	25.1 ± 2.0^{a}	27.4 ± 0.7^{ab}
Σ PUFA[5]	**42.2 ± 3.1^{ab}**	**44.1 ± 2.2^{ab}**	**47.4 ± 0.7^{b}**	**40.4 ± 3.1^{a}**	**39.7 ± 1.4^{a}**
DHA/EPA	**5.2 ± 0.9^{bc}**	**4.3 ± 0.3^{ab}**	**5.4 ± 0.8^{bc}**	**3.2 ± 0.3^{a}**	**5.6 ± 0.2^{bc}**
ARA/EPA	**2.0 ± 0.3^{ab}**	**2.1 ± 0.3^{b}**	**3.1 ± 0.4^{c}**	**1.6 ± 0.2^{a}**	**1.6 ± 0.1^{a}**
n-3/n-6	2.2 ± 0.1^{ab}	2.0 ± 0.2^{ab}	1.6 ± 0.2^{a}	2.1 ± 0.2^{ab}	2.7 ± 0.1^{b}
Total FA	37.4 ± 4.1^{bc}	27.2 ± 2.1^{ab}	23.3 ± 1.5^{a}	40.1 ± 9.2^{bc}	56.7 ± 13.7^{cd}

Abbreviations as in table 2.1. [1] Includes 12:0. [2] Includes 15:1, 20:1n9. [3] Includes 18:3n6, 20:2n6, 20:3n6. [4] Includes 18:3n3, 18:4n3, 20:3n3, 20:4n3. [5] Includes 16:2n4, 18:3n4.

Table 2.9 Fatty acid profile (% of total FA) and fatty acid content (mg/g of dry weight) of flesh from wild and captive male common snook broodstock (n=6). Superscript letters indicate significant differences within a row.

	Male		
	Wild April	**Wild July**	**Captive**
14:0	1.4 ± 0.2[a]	1.3 ± 0.3[a]	2.8 ± 0.2[b]
15:0	0.7 ± 0.1	0.7 ± 0.1	0.6 ± 0.0
16:0	21.9 ± 0.4[ab]	20.9 ± 0.8[a]	23.1 ± 0.8[b]
18:0	5.9 ± 0.4[b]	6.2 ± 0.3[b]	5.5 ± 0.2[a]
Σ SFA[1]	**30.7 ± 0.8[a]**	**29.8 ± 1.2[a]**	**32.7 ± 0.9[b]**
16:1n-7	3.9 ± 0.5[a]	3.3 ± 0.6[a]	5.4 ± 0.5[b]
18:1n-9	11.0 ± 1.3[ab]	10.6 ± 1.2[a]	12.9 ± 0.8[b]
18:1n-7	2.5 ± 0.3[ab]	2.3 ± 0.2[a]	2.7 ± 0.2[b]
Σ MUFA[2]	**18.0 ± 1.9[a]**	**16.8 ± 2.1[a]**	**22.1 ± 1.6[b]**
16:3n-4	1.2 ± 0.1[ab]	1.4 ± 0.2[b]	1.0 ± 0.1[a]
18:2n-6	1.3 ± 0.1	1.2 ± 0.1	1.3 ± 0.1
20:4n-6	**8.8 ± 1.0[bc]**	**10.8 ± 1.0[c]**	**5.6 ± 0.6[a]**
22:5n-6	3.5 ± 0.4[b]	4.2 ± 0.4[b]	2.6 ± 0.3[a]
20:5n-3	**3.5 ± 0.2[ab]**	**3.1 ± 0.2[a]**	**3.5 ± 0.1[ab]**
22:5n-3	3.6 ± 0.2[b]	2.9 ± 0.2[a]	3.2 ± 0.2[ab]
22:6n-3	**21.2 ± 2.0**	**20.3 ± 1.7**	**20.5 ± 1.3**
Σ n-6[3]	14.6 ± 1.3[b]	17.0 ± 1.4[b]	10.4 ± 0.9[a]
Σ n-3[4]	29.5 ± 1.8	27.1 ± 1.7	28.2 ± 1.4
Σ PUFA[5]	**46.0 ± 2.9[b]**	**46.3 ± 2.8[b]**	**40.6 ± 2.2[a]**
DHA/EPA	**6.1 ± 0.8**	**6.9 ± 1.0**	**5.9 ± 0.3**
ARA/EPA	**2.6 ± 0.4[bc]**	**3.6 ± 0.4[c]**	**1.6 ± 0.2[a]**
n-3/n-6	2.1 ± 0.1[ab]	1.6 ± 0.1[a]	2.8 ± 0.2[b]
Total FA	31.0 ± 3.6[b]	22.3 ± 3.8[a]	70.7 ± 35.7[c]

Abbreviations as in table 2.1. [1] Includes 12:0. [2] Includes 15:1, 20:1n9. [3] Includes 18:3n6, 20:2n6, 20:3n6. [4] Includes 18:3n3, 18:4n3, 20:3n3, 20:4n3. [5] Includes 16:2n4, 18:3n4.

Table 2.10 Fatty acid profile (% of total FA) and fatty acid content (mg/g of dry weight) of liver from wild and captive female common snook broodstock (n=6). Superscript letters indicate significant differences within a row.

	Female				
	Wild April	**Wild June**	**Wild July**	**Wild August**	**Captive**
14:0	1.7 ± 0.3^{a}	1.8 ± 0.3^{a}	1.4 ± 0.3^{a}	2.4 ± 0.3^{ab}	3.8 ± 0.6^{b}
15:0	1.3 ± 0.5^{ab}	2.1 ± 0.6^{b}	0.8 ± 0.2^{a}	1.7 ± 0.1^{b}	0.9 ± 0.1^{a}
16:0	23.7 ± 1.5^{a}	24.6 ± 1.7^{ab}	22.5 ± 2.0^{a}	29.6 ± 0.4^{b}	23.1 ± 1.1^{a}
17:0	1.0 ± 0.2^{a}	1.8 ± 0.2^{b}	1.4 ± 0.2^{ab}	1.7 ± 0.1^{b}	1.0 ± 0.0^{a}
18:0	5.8 ± 0.5^{a}	8.3 ± 1.6^{b}	10.6 ± 1.2^{bc}	6.2 ± 0.6^{ab}	5.5 ± 0.2^{a}
Σ SFA[1]	**33.6 ± 1.7^{ab}**	**38.5 ± 1.4^{bc}**	**36.9 ± 1.3^{b}**	**41.5 ± 0.6^{c}**	**34.4 ± 1.4^{ab}**
16:1n-7	7.5 ± 1.2^{bc}	6.5 ± 1.3^{b}	4.4 ± 1.1^{a}	9.9 ± 0.6^{c}	7.3 ± 0.7^{b}
18:1n-9	12.7 ± 1.4^{a}	12.5 ± 1.5^{a}	13.1 ± 0.9^{a}	16.0 ± 0.5^{b}	13.5 ± 1.4^{a}
18:1n-7	5.0 ± 1.1^{bc}	3.9 ± 0.5^{b}	3.7 ± 0.6^{ab}	5.5 ± 0.3^{c}	4.2 ± 0.2^{b}
Σ MUFA[2]	**26.0 ± 1.9^{b}**	**23.5 ± 2.6^{ab}**	**21.7 ± 2.6^{a}**	**32.1 ± 0.9^{bc}**	**26.2 ± 2.1^{b}**
16:3n-4	1.4 ± 0.4^{b}	1.9 ± 0.4^{b}	0.9 ± 0.2^{a}	1.7 ± 0.1^{b}	0.9 ± 0.1^{a}
18:2n-6	1.2 ± 0.2^{a}	1.0 ± 0.1^{a}	1.3 ± 0.2^{ab}	1.1 ± 0.1^{a}	1.5 ± 0.1^{b}
20:4n-6	**5.4 ± 0.8^{b}**	**5.6 ± 0.8^{b}**	**7.4 ± 1.1^{bc}**	**2.9 ± 0.2^{a}**	**3.3 ± 0.8^{a}**
22:5n-6	1.4 ± 0.2^{bc}	1.1 ± 0.2^{b}	2.1 ± 0.5^{c}	0.5 ± 0.0^{a}	1.4 ± 0.1^{bc}
20:5n-3	**2.0 ± 0.2^{ab}**	**2.7 ± 0.3^{bc}**	**2.3 ± 0.3^{b}**	**2.6 ± 0.4^{bc}**	**1.8 ± 0.3^{a}**
22:5n-3	3.0 ± 0.4^{bc}	1.8 ± 0.1^{ab}	1.8 ± 0.2^{ab}	1.3 ± 0.1^{a}	3.0 ± 0.2^{bc}
22:6n-3	**15.3 ± 2.5^{b}**	**13.4 ± 3.3^{b}**	**15.1 ± 2.7^{b}**	**5.9 ± 0.9^{a}**	**16.6 ± 2.0^{bc}**
Σ n-6[3]	9.0 ± 0.8^{b}	8.7 ± 0.8^{b}	11.9 ± 1.6^{bc}	5.5 ± 0.3^{a}	7.2 ± 0.9^{ab}
Σ n-3[4]	21.7 ± 2.7^{b}	19.1 ± 3.0^{b}	20.1 ± 2.6^{b}	10.8 ± 0.7^{a}	23.4 ± 2.0^{bc}
Σ PUFA[5]	**33.1 ± 3.2^{b}**	**30.7 ± 3.3^{b}**	**33.7 ± 3.7^{b}**	**19.3 ± 0.9^{a}**	**32.9 ± 2.7^{b}**
DHA/EPA	**7.3 ± 0.8^{b}**	**5.8 ± 1.8^{ab}**	**8.3 ± 2.6^{bc}**	**2.7 ± 0.9^{a}**	**11.0 ± 2.1^{cd}**
ARA/EPA	**2.6 ± 0.2^{ab}**	**2.3 ± 0.5^{ab}**	**4.1 ± 1.4^{bc}**	**1.3 ± 0.3^{a}**	**2.1 ± 0.5^{a}**
n-3/n-6	2.4 ± 0.1^{b}	2.2 ± 0.2^{ab}	1.7 ± 0.1^{a}	2.0 ± 0.1^{ab}	3.3 ± 0.2^{c}
Total FA	165.5 ± 36.0^{a}	240.6 ± 63.7^{ab}	169.9 ± 39.8^{a}	352.7 ± 68.7^{b}	277.7 ± 67.4^{ab}

Abbreviations as in table 2.1. [1] Includes 12:0. [2] Includes 15:1, 20:1n9. [3] Includes 18:3n6, 20:2n6, 20:3n6. [4] Includes 18:3n3, 18:4n3, 20:3n3, 20:4n3. [5] Includes 16:2n4, 18:3n4.

Table 2.11 Fatty acid profile (% of total FA) and fatty acid content (mg/g of dry weight) of liver from wild and captive male common snook broodstock (n=6). Superscript letters indicate significant differences within a row.

	Male		
	Wild April	**Wild July**	**Captive**
14:0	1.3 ± 0.2[a]	1.2 ± 0.2[a]	3.3 ± 0.4[b]
15:0	0.7 ± 0.1	1.0 ± 0.2	0.8 ± 0.0
16:0	22.0 ± 1.1[b]	22.8 ± 1.1[b]	20.8 ± 0.7[a]
17:0	0.9 ± 0.1[a]	1.2 ± 0.1[ab]	1.0 ± 0.0[a]
18:0	6.1 ± 0.4[a]	7.7 ± 0.6[ab]	6.0 ± 0.1[a]
Σ SFA[1]	**31.0 ± 1.5[a]**	**33.9 ± 1.3[ab]**	**31.8 ± 1.0[a]**
16:1n-7	4.3 ± 0.6[a]	3.4 ± 0.6[a]	6.1 ± 0.6[b]
18:1n-9	9.7 ± 1.4[a]	8.2 ± 1.2[a]	13.4 ± 1.9[b]
18:1n-7	3.2 ± 0.3[a]	2.9 ± 0.3[a]	3.9 ± 0.4[b]
Σ MUFA[2]	**17.9 ± 2.3[a]**	**15.0 ± 2.0[a]**	**24.7 ± 3.0[b]**
16:3n-4	0.8 ± 0.1	0.9 ± 0.2	0.8 ± 0.1
18:2n-6	1.0 ± 0.1[a]	1.1 ± 0.1[a]	1.4 ± 0.1[b]
20:4n-6	**8.0 ± 0.9[b]**	**9.1 ± 0.9[b]**	**4.3 ± 1.0[a]**
22:5n-6	2.4 ± 0.3[b]	2.6 ± 0.2[b]	1.6 ± 0.2[a]
20:5n-3	**2.3 ± 0.2[b]**	**1.7 ± 0.3[a]**	**2.1 ± 0.2[ab]**
22:5n-3	3.5 ± 0.3[b]	2.2 ± 0.3[a]	3.1 ± 0.2[b]
22:6n-3	**23.4 ± 2.6[b]**	**23.2 ± 2.3[b]**	**20.2 ± 3.1[ab]**
Σ n-6[3]	12.3 ± 1.1[b]	13.7 ± 1.0[b]	8.2 ± 1.0[a]
Σ n-3[4]	30.2 ± 2.0	28.0 ± 1.9	27.2 ± 2.8
Σ PUFA[5]	**44.1 ± 2.8[b]**	**43.3 ± 2.5[b]**	**37.4 ± 3.7[ab]**
DHA/EPA	**11.2 ± 1.9[a]**	**16.0 ± 2.5[b]**	**10.2 ± 1.8[a]**
ARA/EPA	**3.8 ± 0.7[b]**	**6.4 ± 1.2[c]**	**2.1 ± 0.5[a]**
n-3/n-6	2.5 ± 0.1[b]	2.1 ± 0.1[ab]	3.4 ± 0.1[c]
Total FA	94.5 ± 16.2[a]	106.2 ± 23.4[a]	187.7 ± 34.6[b]

Abbreviations as in table 2.1. [1] Includes 12:0. [2] Includes 15:1, 20:1n9. [3] Includes 18:3n6, 20:2n6, 20:3n6. [4] Includes 18:3n3, 18:4n3, 20:3n3, 20:4n3. [5] Includes 16:2n4, 18:3n4.

Fatty acid profile and total fatty acid content of flesh, liver and egg samples from July females and captive females are compared in Table 2.12. No significant differences were observed in flesh and liver total SFA between wild and captive females, however captive eggs contained significantly lower SFA levels compared to wild eggs (25.4±0.3 and 30.6±0.6 % respectively). Total MUFA were significantly lower in captive eggs compared to wild eggs (23.3±0.3 and 29.5±0.2 % respectively) although not different between wild and captive liver tissue and significantly higher in captive flesh tissue compared to wild flesh tissue (22.7±1.2 % and 17.2±0.5 % respectively). ARA contents were significantly higher in wild fish tissues and eggs compared to captive fish tissues and eggs with 11.4±0.7 and 5.6±0.6 % respectively in flesh, 7.4±1.1 and 3.3±0.8 % respectively in liver, and 5.4±0.3 and 3.8±0.2 % respectively in eggs. No significant differences were observed between wild and captive fish flesh and liver EPA contents; however, EPA contents were significantly lower in wild eggs than in captive eggs (2.4±0.4 and 4.2±0.2 % respectively). A similar pattern was observed in DHA incorporation with no significant difference in flesh and liver DHA content between wild and captive fish, however, DHA contents were significantly lower in wild eggs compared to captive eggs (14.5±0.2 and 27.3±0.4 % respectively). Consequently, ARA/EPA ratio in wild eggs was significantly higher than that in captive eggs (2.3±0.6 and 0.9±0.1 respectively) while there was no significant difference between DHA/EPA ratios. Total PUFA was not significantly different in wild and captive liver tissue, however, total PUFA in wild flesh tissue was significantly higher than that in captive flesh tissue (46.4±0.7 and 39.7±1.4 % respectively) and total PUFA in wild eggs was significantly lower than that in captive eggs (33.6±0.5 and 47.0±0.3 respectively).

Table 2.12 Fatty acid profile (% of total FA) and fatty acid content (mg/g of dry weight) of flesh, liver and eggs from wild and captive common snook female broodstock (n=6 for flesh and liver, n=3 for eggs). Superscript letters indicate significant differences within a row.

	Wild July			Captive		
	Flesh	Liver	Eggs	Flesh	Liver	Eggs
14:0	1.4 ± 0.1^{a}	1.4 ± 0.3^{a}	1.9 ± 0.1^{ab}	2.6 ± 0.2^{b}	3.8 ± 0.6^{c}	2.4 ± 0.1^{b}
16:0	20.5 ± 0.5^{ab}	22.5 ± 2.0^{b}	21.7 ± 0.7^{b}	23.8 ± 0.2^{c}	23.1 ± 1.1^{bc}	17.6 ± 0.3^{a}
17:0	0.7 ± 0.1^{a}	1.4 ± 0.2^{c}	1.0 ± 0.0^{b}	0.7 ± 0.0^{a}	1.0 ± 0.0^{b}	0.8 ± 0.0^{a}
18:0	5.6 ± 0.3^{b}	10.6 ± 1.2^{c}	5.2 ± 0.1^{b}	5.4 ± 0.2^{b}	5.5 ± 0.2^{b}	4.0 ± 0.1^{a}
Σ SFA[1]	**28.8 ± 0.5^{ab}**	**36.9 ± 1.3^{c}**	**30.6 ± 0.6^{b}**	**33.1 ± 0.2^{bc}**	**34.4 ± 1.4^{bc}**	**25.4 ± 0.3^{a}**
16:1n7	4.2 ± 0.4^{a}	4.4 ± 1.1^{a}	7.6 ± 0.3^{c}	5.5 ± 0.5^{ab}	7.3 ± 0.7^{bc}	6.2 ± 0.1^{b}
18:1n9	10.1 ± 0.3^{a}	13.1 ± 0.9^{b}	16.9 ± 0.3^{c}	13.5 ± 0.6^{b}	13.5 ± 1.4^{b}	12.9 ± 0.2^{b}
18:1n7	2.5 ± 0.1^{a}	3.7 ± 0.6^{ab}	4.6 ± 0.2^{bc}	2.8 ± 0.2^{a}	4.2 ± 0.2^{b}	3.8 ± 0.1^{b}
Σ MUFA[2]	**17.2 ± 0.5^{a}**	**21.7 ± 2.6^{b}**	**29.5 ± 0.2^{c}**	**22.7 ± 1.2^{b}**	**26.2 ± 2.1^{bc}**	**23.3 ± 0.3^{b}**
16:2n4	0.6 ± 0.0^{a}	0.5 ± 0.0^{a}	0.5 ± 0.0^{a}	0.8 ± 0.0^{ab}	1.1 ± 0.1^{b}	0.9 ± 0.0^{b}
16:3n4	1.6 ± 0.1^{b}	0.9 ± 0.2^{a}	1.4 ± 0.0^{b}	1.1 ± 0.1^{ab}	0.9 ± 0.1^{a}	0.9 ± 0.0^{a}
18:2n6	1.5 ± 0.2^{ab}	1.3 ± 0.2^{a}	2.2 ± 0.5^{b}	1.2 ± 0.0^{a}	1.5 ± 0.1^{ab}	1.9 ± 0.0^{b}
20:4n6	**11.4 ± 0.7^{c}**	**7.4 ± 1.1^{bc}**	**5.4 ± 0.3^{b}**	**5.6 ± 0.6^{b}**	**3.3 ± 0.8^{a}**	**3.8 ± 0.2^{a}**
22:5n6	3.9 ± 0.3^{c}	2.1 ± 0.5^{ab}	2.1 ± 0.2^{ab}	2.5 ± 0.2^{b}	1.4 ± 0.1^{a}	1.9 ± 0.0^{a}
20:5n3	**3.8 ± 0.3^{c}**	**2.3 ± 0.3^{ab}**	**2.4 ± 0.4^{ab}**	**3.6 ± 0.1^{c}**	**1.8 ± 0.3^{a}**	**4.2 ± 0.2^{cd}**
22:5n3	3.0 ± 0.2^{b}	1.8 ± 0.2^{a}	2.7 ± 0.1^{b}	2.8 ± 0.2^{b}	3.0 ± 0.2^{b}	3.2 ± 0.1^{b}
22:6n3	**19.5 ± 1.4^{b}**	**15.1 ± 2.7^{a}**	**14.5 ± 0.2^{a}**	**20.0 ± 0.8^{b}**	**16.6 ± 2.0^{ab}**	**27.3 ± 0.4^{c}**
Σ ω6[3]	17.8 ± 1.0^{c}	11.9 ± 1.6^{b}	10.6 ± 1.0b	10.2 ± 0.8^{b}	7.2 ± 0.9^{a}	8.7 ± 0.1^{ab}
Σ ω3[4]	27.2 ± 1.2^{b}	20.1 ± 2.6^{a}	20.8 ± 0.6^{a}	27.4 ± 0.7^{b}	23.4 ± 2.0^{ab}	36.3 ± 0.3^{c}
Σ PUFA[5]	**47.4 ± 0.7^{c}**	**33.7 ± 3.7^{a}**	**33.6 ± 0.5^{a}**	**39.7 ± 1.4^{b}**	**32.9 ± 2.7^{a}**	**47.0 ± 0.3^{c}**
DHA/EPA	**5.4 ± 0.8^{a}**	**8.3 ± 2.6^{b}**	**6.5 ± .7^{b}**	**5.6 ± 0.2^{a}**	**11.0 ± 2.1^{bc}**	**6.6 ± 0.3^{b}**
ARA/EPA	**3.1 ± 0.4^{c}**	**4.1 ± 1.4^{cd}**	**2.3 ± 0.6^{bc}**	**1.6 ± 0.1^{b}**	**2.1 ± 0.5^{bc}**	**0.9 ± 0.1^{a}**
ω3/ω6	1.6 ± 0.2^{a}	1.7 ± 0.1^{a}	2.1 ± 0.3^{ab}	2.7 ± 0.1^{b}	3.3 ± 0.2^{bc}	4.2 ± 0.1^{c}
Total FA	23.3 ± 1.5^{a}	169.9 ± 39.8^{cd}	193.4 ± 17.2^{d}	56.7 ± 13.7^{b}	277.7 ± 67.4^{de}	169.0 ± 13.9^{c}

Abbreviations as in table 1. [1] Includes 12:0, 15:0. [2] Includes 15:1, 20:1n9. [3] Includes 18:3n6, 20:2n6, 20:3n6. [4] Includes 18:3n3, 18:4n3, 20:3n3, 20:4n3. [5] Includes 18:3n4.

2.5 DISCUSSION

Results from this study highlighted numerous differences in lipids between wild and captive snook broodstock with potential consequences on reproductive success and egg quality.

Captive fish presented a significantly higher flesh lipid content compared to their wild counterparts. This is the likely consequence of feeding a high lipid diet combined with reduced physical activity as already reported in several other marine fish species including white seabream (Cejas et al., 2004b, 2003), black seabream (Rodriguez et al., 2004), greater amberjack (Rodriguez-Barreto et al., 2012; Saito, 2012) and Senegalese sole (Norambuena et al., 2012a). In fish, excess energy is mainly stored as neutral lipid and more particularly as TAG (Sargent et al., 2002), explaining the high TAG content (>40%) in the flesh of captive females. The estrogen activation of the hormone-sensitive lipase (HSL) during gonadogenesis leads to the mobilization of this lipid reserve to fuel reproductive processes, including sexual maturation and egg production (Tocher, 2003). In this study, despite the significantly higher lipid and TAG content of captive females, captive eggs contained a significantly lower total FA content compared to wild eggs (13 % reduction). Rodriguez-Barreto et al. (2012) made a similar observation between wild and cultured greater amberjack with cultured fish presenting higher total lipid content in flesh and liver but lower content in gonads. A disturbance in the activation of HSL might be involved and should be investigated in future studies. Among wild fish, no clear trend of TAG utilization during the reproduction period was detected, even though lowest TAG levels in the flesh were observed in July, which is considered the peak of the snook spawning season. Wild snook keep feeding

throughout the spawning season and though Almansa et al., (2001) demonstrated the use of lipid reserve during ovarian maturation of captive seabream fed during the spawning period, the wild snook diet seems to cover the nutritional needs of brooders. This would explain the lack of depletion of TAG reserves, the low flesh lipid content and low levels of perivisceral fat. Additional data would be necessary to investigate the mobilization of reserves during the spawning season. Another difference in lipid classes among wild and captive fish was noticed with regards to CHOL levels. CHOL is a simple lipid that does not contain any fatty acid and teleost fish have the ability to synthesize it (Leaver et al., 2008; Tocher, 2003). ARA is involved in the regulation of CHOL levels and in Senegalese sole ARA and CHOL levels in blood were correlated with dietary ARA levels (Norambuena et al., 2013). CHOL has been identified as the main precursor of sex steroid hormones in fish which play major roles in final oocyte maturation, meiosis resumption and sexual behavior (Diotel et al., 2011; Tokarz et al., 2013). Therefore, the lower levels of CHOL observed in captive fish may be a consequence of the lower ARA levels and could contribute to the reproductive dysfunction reported in captive snook (e.g. incomplete ovarian maturation, low sperm production and low quality eggs). In wild fish, the presence of hydrocarbons in the liver is of concern. Hydrocarbon contaminants have been found to have detrimental effect on vitellogenesis with repercussion on circulating hormones and plasma vitellogenin, estrogenic and antiestrogenic effects as well as delay in oocyte maturation (Nicolas, 1999). In vulnerable populations such as common snook, any reduction in reproductive success can seriously impact on wild stock recruitment and further investigation is therefore critical. Previous research has demonstrated the existence of hydrocarbon

detoxification mechanisms in fish (Lee et al., 1972) and the lack of hydrocarbons in captive fish samples suggest a successful detoxification after three years in captivity unless the contamination of wild fish occurred after the acquisition of the captive broodstock (e.g. BP Deepwater Horizon oil spill in April 2010) (Weisberg et al., 2014).

Dietary fatty acids and their cyclooxygenase and lipoxygenase metabolites are known to impact on oocyte maturation and spermatogenesis as well (Cerda et al., 1997; Sorbera et al., 2001). Lower ARA contents in captive broodstock, as observed in the present study, have also been reported in captive broodstock of white sea bream (Cejas et al., 2004b), black sea bream (Rodriguez et al., 2004), Senegalese sole (Norambuena et al., 2012a) and greater amberjack (Rodriguez-Barreto et al., 2012; Saito, 2012). As mentioned previously, ARA is a precursor of prostaglandins that is thought to stimulate the later stages of gametogenesis (e.g. ovulation) as well as influencing mating behavior (e.g. pheromones). ARA and EPA compete for the same enzymes involved in the production of prostaglandins (Sargent et al., 1999a). ARA forms 2-series prostaglandins while EPA forms the less biologically active and antagonistic 3-series prostaglandins (Bell et al., 1994; Tocher et al., 1996). Therefore, in addition to absolute content, the relative proportion of each fatty acid should be taken into consideration (Izquierdo et al., 2001, 2000; Sargent et al., 1999a). Indeed, in turbot, *Scophthalmus maximus*, changes in the dietary ARA/EPA ratio modified the proportion of prostaglandins produced (Bell et al., 1994; J. G. Bell et al., 1995). In addition, significantly higher levels of 2-series prostaglandins and lower levels of 3-series prostaglandins were measured in wild Senegalese sole compared to captive broodstock that had lower ARA content, lower ARA/EPA ratio

and presented reproductive dysfunctions (Norambuena et al., 2012b). Therefore, the lower ARA content and ARA/EPA ratios in captive snook broodstock may impact on prostaglandin synthesis with potential negative consequences on captive snook reproduction. In addition, in Senegalese sole, increased ARA levels and ARA/EPA ratios were correlated with increased plasma steroid levels in males (11-ketotestosterone and testosterone) but no effect was observed in females (estradiol) (Norambuena et al., 2013). Moreover, in sea bass, it was demonstrated that a diet high in n-3 fatty acids promoted female reproductive performances while a diet with a higher level of ARA and lower n-3 content improved fertilization rate (Asturiano et al., 2001). Therefore, dietary ARA levels and ARA/EPA ratios seem to be of particular importance in male gonad maturation and quality, and the lower values observed in the captive males in this study may contribute to the poor milt production (quantity and quality) reported in captivity. Among wild fish, no clear seasonal variation in flesh and liver fatty acid profiles was observed during the spawning season even though ARA content was significantly higher in July during the peak of the natural spawning season. Fuiman and Faulk (2013) studied the transfer of dietary ARA to the eggs in red drum *Sciaenops ocellatus* and demonstrated a rapid diet-egg connection, supporting the hypothesis that batch-spawners migrate to their spawning ground to take advantage of a diet promoting gonad maturation and quality. Therefore, it seems as though snook spawning ground diets are able to sustain gamete production throughout the spawning season with potentially a higher supply of ARA during the peak of the spawning season.

In addition to their impact on gonad maturation, spawning behavior and sperm quality, dietary fatty acids also influence egg quality and larval survival. Indeed,

many studies demonstrated the importance of egg and yolk-sac lipid reserves for both energy and structural development of embryos and larvae from warm and temperate waters, including red drum (Vetter et al., 1983), red sea bream *Pagrus major* (Koven et al., 1989), gilthead sea bream *Sparus aurata* (Koven et al., 1989; Rønnestad et al., 1994), common dentex *Dentex dentex* (Mourente et al., 1999), white seabream (Cejas et al., 2004a) and Atlantic Bluefin tuna *Thunnus thynnus* (Morais et al., 2011). After hatching, MUFAs are preferentially used for energy while SFAs and PUFAs are incorporated into structural phospholipids (Kamler, 2007; Sargent et al., 2002). DHA is the main fatty acid in neural and visual membranes and a deficiency has been shown to strongly impair larval development (M. V Bell et al., 1995; Benítez-Santana et al., 2007; Neuringer et al., 1988). DHA and EPA compete in the formation of phospholipid structures with a higher biological value for DHA than EPA (Rodriguez et al., 1998; Sargent et al., 1999b). Therefore, as for ARA and EPA, the DHA:EPA ratio needs to be considered in addition to absolute content. In this experiment, DHA and EPA levels were significantly higher in captive eggs, however DHA/EPA ratios were similar in the eggs. It is interesting to note that flesh and liver DHA and EPA levels were not different between wild and captive females. The selective transfer and accumulation of DHA and to a lesser extent EPA into fish eggs has been demonstrated and the DHA and EPA rich captive diet probably leads to this large deposition in captive eggs (Johnson, 2009; Sargent et al., 2002; Wiegand, 1996). The higher level of EPA incorporated into the eggs, combined with the lower ARA content, lead to an ARA/EPA ratio less than half that of wild eggs, leading to possible modification in

eicosanoid production and subsequent modulation of neural transmission, hypothalamic and immune functions as well as stress resistance (Bell, 2003).

Overall, the present results support an ARA supplementation to improve male and female reproduction in captivity including gametogenesis and reproductive behavior, as well as egg and larval quality. Additional studies are required to determine the optimal level of supplementation and to achieve an adequate ARA/EPA ratio taking into account a probable rapid diet-egg transfer. In addition, lowering the dietary EPA content would most likely benefit egg quality as well. The presence of hydrocarbon in the liver of wild fish should be further investigated to identify the source and potential impact on fish reproduction. The study of spawning grounds diet would also be of interest, allowing for the monitoring of the resource as a shift in prey availability due to changing environmental conditions could impact snook reproductive success.

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Chapter 3 Fatty acid utilization during the early larval stages of Florida pompano (*Trachinotus carolinus*) and Common snook (*Centropomus undecimalis*)

RESEARCH ARTICLE

Submitted to Aquaculture Research

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Contributions: The present manuscript was compiled and written in full by the author of this thesis. Egg collection, larval rearing, rotifer rearing, sampling, lab and statistical analyses have been carried out by the candidate except for the fatty acid analyses that were performed by Cynthia Faulk from the Fisheries and Mariculture Laboratory at the University of Texas Marine Science Institute. Co-authors provided assistance with the proofreading of the manuscript.

3.1 ABSTRACT

The pattern of conservation and loss of fatty acids from the yolk sac during the endogenous feeding period and subsequent starvation was studied in pompano and snook larvae. Fundamental information on the early fatty acid requirements and mobilization of pompano and snook larvae was collected. In both species, fatty acids were utilized as an energy source after hatching. Mono-unsaturated fatty acids were catabolized, while saturated and poly-unsaturated fatty acids were conserved. High levels of arachidonic acid (ARA) in pompano and snook eggs, as well as selective retention in the unfed larvae suggest a high dietary requirement for this fatty acid during the early stages of larval development. The effect of an ARA supplementation was therefore investigated in snook larvae at the rotifer feeding stage. The fatty acid profile of the larvae was successfully influenced to match that of wild eggs; however, no significant improvement in growth or survival was observed. Future research should be carried out over a longer period of time and include factors related to stress resistance.

Keywords: fatty acid, starvation, fish, larvae, arachidonic acid, enrichment

3.2 INTRODUCTION

In the wild, fish larvae feed mainly on copepods (Hunter, 1981), which provide adequate nutrition and the large variety of prey sizes essential for successful development (van der Meeren et al., 2008). Nevertheless, more research is needed before mass copepod production can be economically viable and hatcheries have to rely on the traditional live preys: rotifers and Artemia (Stottrup, 2000). Despite the convenience of their production, rotifers and Artemia have a main drawback which resides in their poor nutritional profile (Conceição et al., 2010). Both prey types are deficient in essential polyunsaturated fatty acids (PUFAs), which have long been identified as one of the most critical nutritional factors to impact marine fish larval development and survival (Sargent et al., 1997; Watanabe et al., 1983). Indeed, due to their inability to elongate and desaturate 18:3n-3 and 18:2n-6 to the PUFAs eicosapentaenoic acid (20:5n-3, EPA), docosahexaenoic acid (22:6n-3, DHA) and arachidonic acid (20:4n-6, ARA), marine fish depend solely on their diet to fulfill their requirements (Sargent et al., 1999b; Tocher, 2003).

PUFA deficiencies strongly impact growth and normal development of fish larvae (Mourente et al., 1993; Watanabe, 1993). DHA is highly concentrated in the neural and visual cell membrane and a deficiency has been shown to impact visual development (Bell et al., 1995; Benítez-Santana et al., 2007; Neuringer et al., 1988), skeletal development (C. Cahu et al., 2003; Roo et al., 2009) and stress and immune responses (Ganga et al., 2006; Montero et al., 2003). EPA and ARA are both precursors of eicosanoids, and compete for the cyclo-oxygenases and lipoxygenases that generate the local hormones prostaglandins, thromboxanes and leukotrienes that are involved in the modulation of neural transmission, hypothalamic functions and

many immune functions (Bell et al., 1996; Sargent et al., 1999b; Tocher et al., 1996). Even though EPA is the main C20 PUFA in fish tissues, ARA seems to be the preferred substrate for eicosanoid production and the metabolites produced are of higher biological activity (Tocher et al., 1996). EPA competitively inhibits the production of eicosanoids from ARA therefore eicosanoid actions are determined by the ARA/EPA ratio in the tissues (Sargent et al., 1999a). Similarly, DHA and EPA compete for the formation of phospholipid structures with a higher biological value for DHA than EPA (Glencross, 2009; Rodriguez et al., 1998; Sargent et al., 1999b). Determining the optimal DHA:EPA:ARA ratio has proven difficult since changing the percentage of one PUFA alters the ratio (Sargent et al., 1999a). Considerable research has been carried out on cold and temperate water species where a DHA/EPA ratio equal or greater than 2 has been considered adequate (Sargent et al., 1999a). An ARA/EPA ratio close to 0.25 was found necessary for good growth and survival of gilthead sea bream *Sparus aurata* and European sea bass larvae *Dicentrarchus labrax* (Atalah *et al.*, 2011a, b). It has been shown that an excess of ARA can cause malpigmentation in flatfish and an ARA/EPA ratio of 0.25 for turbot *Scophthalmus maximus*, Atlantic halibut *Hippoglossus hippoglossus* and Japanese flounder *Paralichthys olivaceus* and 0.5 for Senegalese sole *Solea senegalensis* gave the best percentage of normal pigmentation (J. G. Bell et al., 1995; Castell et al., 1994) gilthead sea bream (W. Koven et al., 2001) and striped bass *Morone saxatilis* (Harel et al., 2001). Bessonart et al., (1999) have shown that in gilthead sea bream, the effect of an ARA supplementation is enhanced if associated with a high DHA/EPA ratio, which highlights the necessity to consider FA proportions in addition to absolute amount (Izquierdo et al., 2000; Sargent et al., 1999b).

On the other hand, little is known about the PUFA requirement of marine finfish from subtropical/tropical waters, as most of them are new emerging species in aquaculture. As a general trend, these species tend to have intermediate to high DHA and ARA levels and low EPA levels, leading to high DHA/EPA and ARA/EPA ratios (Faulk and Holt, 2003; Fogerty et al., 1986; Gibson, 1983; Ogata et al., 2004; Yanes-Roca et al., 2009). This higher ARA content suggests that larvae from tropical species may require more ARA than cold/temperate species to ensure normal development (Faulk and Holt, 2005; Ogata et al., 2004).

Common snook and Florida pompano are species commonly found in the tropical and subtropical western Atlantic Ocean including the Gulf of Mexico and previous work has shown that eggs of both species have a high ARA/EPA ratio (Main et al., 2010; Yanes-Roca et al., 2009). Since fish eggs contain all the essential nutrients required for the successful development of the embryo and the yolk-sac larvae, it is believed that their composition reflects the optimal first feeding diet (Heming and Buddington, 1988). The study of the pattern of conservation and loss of fatty acids (FAs) from the yolk sac during the endogenous feeding period has proven a useful approach to investigate the requirements of fish larvae with the most critical FAs spared to the detriment of others catabolized as an energy source (Hamre et al., 2013; Izquierdo, 1996).

The aim of the present study was to examine the changes occurring in the FA profiles of common snook and pompano larvae deprived of food after hatching to gain a better understanding of their FA requirements. In addition, an experiment was carried out to investigate the effectiveness of an ARA supplementation to produce

larvae with PUFA ratios close to that of natural eggs and to study the impact on early larval development of common snook larvae.

3.3 MATERIALS AND METHODS

3.3.1 Starvation trial

Pompano broodstock were captured off of Florida's west coast and held inland in a zero-discharge recirculating system at the Mote Marine Laboratory Center for Marine and Freshwater Aquaculture Research, in Sarasota, Florida. Spontaneous spawning was obtained after photo-thermal conditioning in a 25 m^3 tank, and hormonal induction of mature females with sGnRHa (50μg/kg). Snook broodstock were collected in estuarine waters around Sarasota, Florida with seines in mid-July during the ambient spawning window. Capture and strip spawning procedures were similar to those described in Yanes-Roca et al., 2009.

Collected eggs from the captive pompano (fertilization rate= 54.3%) and wild snook (fertilization rate= 68.2%) broodstocks were placed into small conical tanks with aeration. Once the aeration was stopped, the buoyant fertilized eggs accumulated at the surface and the poor, unfertilized eggs were drained from the hatchers. The aeration was then turned back on and three 5ml samples were taken and counted to estimate the egg concentration. Approximately 750 eggs were volumetrically stocked in sieves made of a 100 mm diameter PVC pipe sealed at one end with a 330 μm mesh. The sieves were set on a grid in a 340 liter water table (salinity 35 g/L ±1, dissolved oxygen 5 mg/L ±1, pH 8.5 ± 0.5, temperature 27.5°C ±1) equipped with a 25 W UV light and a 50 L/min pump. In addition, three egg aliquots were sampled

from the conical tank, drained on a 100 µm sieve and rinsed with deionized water before storage at -70°C.

Larvae hatched after 24 hours for pompano (hatch rate=73.2 %) and 18 hours for snook (hatch rate=96 %). Three sieves were removed each day and the larvae anesthetized with MS222 and carefully separated from egg casings and any dead eggs or larvae. The larvae from each sieve were pooled and rinsed with deionized water then stored at -70 °C. The trials were stopped when no living larvae were observed in the sieves, at 5 days post hatch (DPH) for pompano, and 6 DPH for snook.

3.3.2 Live food trial

Two trials were run to study the effect of an ARA supplementation on snook larvae. Eggs for the first trial were obtained from captive broodstock captured off the west coast of Florida and held in a $45m^3$ tank at the Mote Marine Laboratory Center for Marine and Freshwater Aquaculture Research. Spawning was induced by photo-thermal conditioning and sGnRHa implantation (female only, 50 µg/kg) Fish spawned volitionally (fertilization rate= 65.8 %). Eggs for the second trial were collected in the wild in mid-August, using the technique described above (fertilization rate=49.2 %). In both trials, the eggs were stocked at 150 $eggs.L^{-1}$ in 130 L tanks in a recirculating system equipped with drum filtration, biological filtration, ozonation, carbon filtration, UV lights and an inline heater unit (temperature 27.5 ± 1 °C, salinity 35 ± 1 $g.\ L^{-1}$, dissolved oxygen of 6 ± 1 $mg.L^{-1}$ and pH of 8 ± 0.5).

From three days after hatching (hatching rate: trial 1 = 69.8 %, trial 2 = 47.6 %), larvae were fed twice a day with enriched rotifers (5/ml) after shading with RotiGrow *plus* (Reed Mariculture Inc, CA, USA) at 500,000 cells per liter.

During the first trial, three enrichments were tested in quadruplicate: Algamac 3050 (A1) (Aquafauna Bio-Marine Inc, USA), and two experimental formulations (Fa and Fb) (Reed Mariculture Inc, CA, USA) while during the second trial only two enrichments were tested due to limited egg availability: Algamac 3050 (A2) and one experimental formulation (Fc) (Reed Mariculture Inc, CA, USA). Rotifers (*Brachionus plicatilis*) were batch cultured in six 200 L tanks at 27 °C (salinity 35 g/l) and fed concentrated *Nannochloropsis* (Nanno 3600, Reed Mariculture Inc, CA, USA). One tank was harvested daily and the rotifers were rinsed and transferred to 18L buckets (one per enrichment) and enriched for 7 hours, then rinsed and fed to the larvae or stored at 8 °C overnight for the following morning feeding. Samples of enriched rotifers were preserved for FA analysis by sieving on a 55 µm mesh and rinsing with deionized water before storage at -70°C.

At 1, 7, 12 and 15 DPH for the first trial and 1, 5, 9 and 14 DPH for the second trial, 10 larvae per tank were photographed and standard length and eye diameter were measured using ImageJ (U. S. National Institutes of Health, Bethesda, Maryland, USA). At the end of each trial, all larvae were counted to determine survival and 50 larvae per tank were preserved for FA analysis. In addition to standard length and eye diameter, swim bladder inflation was also assessed at that point.

3.3.3 Fatty acid analysis

Lipids were extracted according to Folch et al. (1957) and the FA composition was determined by gas-liquid chromatography after preparation of fatty acid methyl esters (FAMEs) according to Morrison and Smith (1964). FAMEs were analyzed on a gas chromatograph (Shimadzu GC-2014, Shimadzu Scientific Instruments, Columbia, MD, USA) equipped with a flame ionization detector using a Phenomenex ZB-WAX plus capillary column (30 m long, 0.53 mm internal diameter, 1.0 μm thickness; Phenomenex, Torrance, CA, USA). The flow rate of the carrier gas helium was 4 mL min^{-1}. Temperature was held at 160 °C for 5 min then increased up to 220 °C at 3 °C per minute and maintained at this temperature for 30 minutes. Injector and detector temperatures were 250 and 260 °C respectively. FAMEs peaks were identified by comparison with known standards (Supelco, Inc., Bellefonte, Pennsylvania, USA). During lipid extraction, tricosanoic acid (23:0) was added as an internal standard for subsequent quantification of FAs.

3.3.4 Statistical analyses

Statistical analysis was performed with MINITAB® version 16.0 (Minitab Ltd., Coventry, UK). Growth and eye diameter data were compared using a General Linear Model (GLM) analyzing all time and treatment interactions. Significant differences between treatment means were tested by Tukey's test ($p<0.05$). Non-homogeneous data (survival, swim bladder inflation and FA composition) were arcsine square root transformed before a one-way ANOVA followed by a Tukey post hoc test ($p<0.05$). All data are presented as mean ± standard error of the mean.

3.4 RESULTS

3.4.1 Fatty acid utilization

FA data are presented in Table 3.1 for pompano and Table 3.2 for snook. In addition the change in concentration of DHA, EPA, ARA, total saturated, total mono-saturated and total poly-unsaturated from the egg to the end of the starvation period are represented in Figure 3.1 for pompano and Figure 3.2 for snook.

Table 3.1 Fatty acid profile of fertilized eggs and unfed pompano larvae up to 4 days post hatch (DPH). Only fatty acids contributing to at least 1% at one point are reported, all fatty acids are included in totals and ratios. Means are ± SEM, n = 3. Letters indicate significant differences within a same row (Tukey test, $p<0.05$).

% total FA	Fertilized egg	0 DPH	1 DPH	2 DPH	3 DPH	4 DPH
14:0	1.81 ± 0.0^{c}	2.02 ± 0.1^{c}	1.60 ± 0.1^{bc}	1.14 ± 0.1^{ab}	1.09 ± 0.0^{a}	0.78 ± 0.1^{a}
16:0	21.48 ± 0.1^{b}	17.05 ± 0.8^{a}	18.15 ± 0.1^{a}	17.72 ± 0.7^{a}	18.32 ± 0.1^{a}	17.46 ± 0.6^{a}
18:0	2.89 ± 0.0^{a}	2.47 ± 0.1^{a}	4.20 ± 0.4^{b}	5.84 ± 0.1^{c}	7.50 ± 0.2^{d}	8.25 ± 0.4^{d}
Total saturated	27.09 ± 0.1^{bc}	22.53 ± 0.8^{a}	24.94 ± 0.3^{ab}	25.68 ± 0.8^{bc}	28.09 ± 0.4^{c}	27.65 ± 0.7^{bc}
16:1n-7	4.43 ± 0.0^{d}	4.96 ± 0.1^{d}	4.81 ± 0.0^{d}	3.79 ± 0.2^{c}	3.01 ± 0.1^{b}	2.11 ± 0.1^{a}
18:1n-9	17.86 ± 0.2^{d}	14.98 ± 0.5^{c}	14.38 ± 0.5^{c}	13.69 ± 0.6^{bc}	12.02 ± 0.1^{ab}	10.25 ± 0.4^{a}
18:1n-7	2.66 ± 0.0^{c}	2.35 ± 0.0^{bc}	2.36 ± 0.1^{bc}	2.29 ± 0.1^{b}	2.09 ± 0.1^{ab}	1.82 ± 0.1^{a}
Total mono-unsaturated	25.56 ± 0.1^{d}	22.75 ± 0.7^{c}	22.00 ± 0.5^{c}	20.32 ± 0.9^{c}	17.76 ± 0.2^{b}	15.09 ± 0.4^{a}
18:2n-6	1.22 ± 0.0^{b}	1.31 ± 0.0^{b}	1.27 ± 0.1^{b}	1.04 ± 0.0^{a}	1.31 ± 0.4^{b}	0.88 ± 0.1^{a}
20:4n-6	2.44 ± 0.1^{a}	3.18 ± 0.1^{b}	3.72 ± 0.1^{c}	3.90 ± 0.1^{c}	4.70 ± 0.1^{d}	5.49 ± 0.2^{e}
20:5n-3	3.22 ± 0.0^{a}	4.74 ± 0.2^{c}	4.56 ± 0.2^{c}	3.90 ± 0.2^{b}	3.30 ± 0.1^{a}	2.97 ± 0.0^{a}
22:5n-6	0.87 ± 0.0^{a}	1.15 ± 0.1^{ab}	1.15 ± 0.1^{ab}	1.36 ± 0.1^{b}	1.35 ± 0.1^{b}	1.26 ± 0.0^{b}
22:5n-3	3.39 ± 0.0^{c}	3.35 ± 0.2^{c}	2.70 ± 0.0^{b}	2.55 ± 0.1^{b}	1.97 ± 0.0^{a}	1.75 ± 0.0^{a}
22:6n-3	28.76 ± 0.2^{a}	33.72 ± 0.5^{b}	31.96 ± 0.2^{ab}	33.10 ± 0.3^{b}	33.42 ± 0.6^{b}	35.22 ± 0.9^{b}
Total poly-unsaturated	43.38 ± 0.3^{a}	51.08 ± 0.8^{b}	48.78 ± 0.5^{b}	49.05 ± 0.5^{b}	48.78 ± 0.7^{b}	50.35 ± 0.8^{b}
Total n-3	36.82 ± 0.2^{a}	43.24 ± 0.9^{b}	40.35 ± 0.4^{ab}	40.51 ± 0.5^{ab}	39.30 ± 0.8^{ab}	40.43 ± 0.8^{ab}
Total n-6	5.02 ± 0.1^{a}	6.17 ± 0.0^{b}	6.73 ± 0.2^{c}	7.00 ± 0.1^{c}	7.95 ± 0.1^{d}	8.49 ± 0.1^{d}
n-3/ n-6 ratio	7.35 ± 0.1^{c}	7.01 ± 0.3^{c}	6.00 ± 0.1^{b}	5.78 ± 0.2^{b}	4.95 ± 0.2^{a}	4.76 ± 0.1^{a}
ARA/EPA	0.76 ± 0.0^{a}	0.67 ± 0.0^{a}	0.82 ± 0.0^{a}	1.00 ± 0.0^{b}	1.42 ± 0.1^{c}	1.85 ± 0.1^{d}
DHA/EPA	8.92 ± 0.1^{b}	7.11 ± 0.1^{a}	7.03 ± 0.3^{a}	8.50 ± 0.2^{b}	10.12 ± 0.2^{c}	11.87 ± 0.4^{d}
Total FA (mg FA/g dry weight)	190.7 ± 3.0^{c}	181.5 ± 1.1^{bc}	179.4 ± 2.3^{b}	173.5 ± 1.2^{b}	147.5 ± 2.7^{a}	146.3 ± 0.7^{a}

Table 3.2 Fatty acid profile of fertilized eggs and unfed snook larvae up to 5 days post hatch (DPH). Only fatty acids contributing to at least 1% at one point are reported, all fatty acids are included in totals and ratios. Means are ± SEM, n = 3. Letters indicate significant differences within a same row (Tukey test, $p<0.05$).

% total FA	Fertilized egg	0 DPH	1 DPH	2 DPH	3 DPH	4 DPH	5 DPH
14:0	1.92±0.1^{bc}	2.42±0.0^{c}	1.89±0.1^{bc}	1.86±0.2^{bc}	0.89±0.2^{a}	1.32±0.1^{a}	1.55±0.1^{b}
16:0	21.70±0.7	19.69±0.2	19.43±0.1	19.86±0.3	20.16±0.5	20.32±0.2	20.57±0.4
17:0	1.00±0.0	0.88±0.0	0.90±0.0	0.90±0.0	0.89±0.0	0.88±0.1	0.88±0.1
18:0	5.23±0.1^{a}	4.47±0.1^{a}	5.25±0.3^{a}	6.84±0.5^{a}	9.16±0.7^{b}	9.14±0.1^{b}	8.52±0.1^{b}
Total saturated	30.60±0.6^{b}	28.35±0.4^{a}	28.36±0.3^{a}	30.31±0.3^{b}	31.79±0.4^{bc}	32.47±0.8^{bc}	32.44±0.4^{c}
16:1n-7	7.59±0.3^{b}	9.00±0.1^{b}	7.37±0.5^{b}	5.62±0.4^{b}	3.00±0.4^{a}	3.83±0.2^{a}	4.10±0.4^{a}
18:1n-9	16.89±0.3^{b}	15.12±0.2^{b}	13.88±0.2^{b}	11.78±0.3^{b}	9.23±0.3^{a}	9.42±1.0^{a}	9.73±1.4^{a}
18:1n-7	4.58±0.2^{b}	4.26±0.1^{b}	3.93±0.1^{b}	3.01±0.1^{b}	2.41±0.1^{a}	2.54±0.2^{a}	2.61±0.3^{a}
Total mono-unsaturated	29.53±0.2^{b}	28.85±0.2^{b}	25.68±0.1^{b}	20.88±0.6^{b}	14.96±0.8^{a}	16.28±0.8^{a}	17.05±0.8^{a}
16:3n-4	1.36±0.0^{b}	1.42±0.0^{b}	1.41±0.0^{b}	1.12±0.0^{a}	1.06±0.1^{a}	1.01±0.6^{a}	0.95±0.1^{a}
18:2n-6	2.16±0.5^{b}	2.70±0.2^{c}	2.72±0.0^{c}	2.02±0.0^{b}	1.36±0.1^{a}	2.07±0.2^{b}	2.11±0.8^{b}
20:4n-6	5.43±0.3^{a}	6.45±0.2^{ab}	7.73±0.1^{b}	8.37±0.1^{b}	9.47±0.1^{c}	9.44±0.1^{c}	9.35±0.2^{c}
20:5n-3	2.38±0.4^{b}	3.01±0.1^{a}	2.29±0.2^{b}	2.33±0.1^{b}	2.41±0.1^{b}	2.38±0.0^{b}	2.34±0.0^{b}
22:5n-6	2.08±0.2^{ab}	1.75±0.0^{a}	2.23±0.1^{ab}	2.82±0.2^{ab}	3.03±0.3^{bc}	3.19±0.1^{bc}	3.34±0.2^{c}
22:5n-3	2.69±0.1^{c}	2.63±0.0^{c}	2.36±0.0^{b}	2.01±0.1^{a}	1.95±0.1^{a}	2.12±0.3^{ab}	2.24±0.7^{b}
22:6n-3	14.53±0.2^{a}	16.21±0.2^{b}	17.54±0.2^{b}	20.01±0.3^{b}	26.05±0.6^{c}	26.01±0.3^{c}	25.82±0.5^{c}
Total poly-unsaturated	33.57±0.5^{a}	37.41±0.4^{b}	39.30±0.4^{b}	41.36±0.2^{b}	47.65±0.4^{c}	49.36±0.9^{c}	48.71±0.7^{c}
Total n-3	20.83±0.6^{a}	23.25±0.3^{a}	23.33±0.4^{a}	25.28±0.4^{a}	31.06±0.6^{b}	31.35±0.8^{b}	31.39±0.9^{b}
Total n-6	10.55±0.5^{a}	11.82±0.3^{ab}	13.67±0.2^{ab}	14.10±0.3^{ab}	14.68±0.4^{b}	15.50±0.4^{b}	15.61±0.9^{b}
n-3/ n-6 ratio	2.10±0.3^{b}	1.97±0.1^{ab}	1.71±0.1^{a}	1.79±0.1^{a}	2.12±0.1^{b}	2.02±0.0^{ab}	2.02±0.1^{ab}
ARA/EPA ratio	2.83±0.6^{b}	2.15±0.1^{a}	3.41±0.3^{c}	3.59±0.2^{c}	3.94±0.2^{d}	3.97±0.1^{d}	4.00±0.1^{d}
DHA/EPA ratio	7.14±0.7^{ab}	5.41±0.2^{a}	7.74±0.6^{ab}	8.59±0.2^{ab}	10.82±0.1^{b}	10.93±0.0^{b}	11.05±0.1^{b}
Total FA (mg FA/g dry weight)	193.3±3.5^{e}	187.3±4.1^{de}	163.4±2.2^{d}	147.7±1.9^{cd}	134.1±1.2^{bc}	119.6±2.3^{ab}	110.1±2.2^{a}

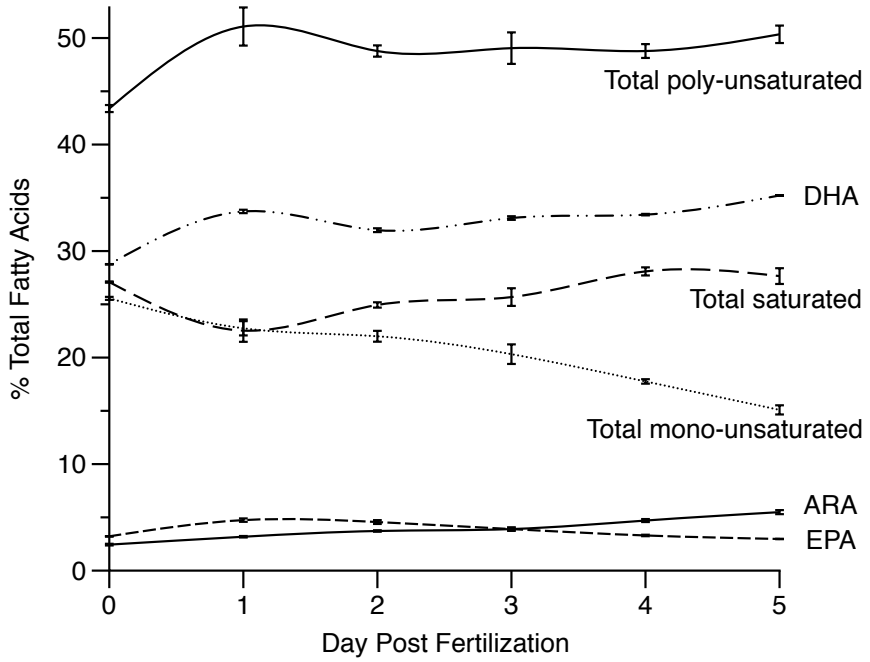

Figure 3.1 Evolution of selected fatty acids in eggs (0 days post fertilization) and unfed pompano larvae (1 to 5 day post fertilization). Means are ± SEM , n=3.

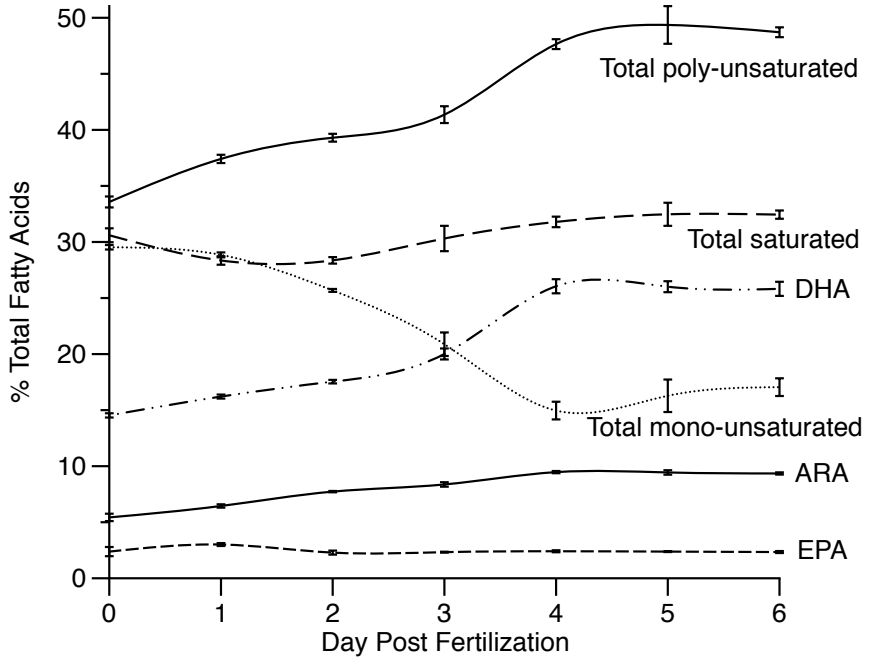

Figure 3.2 Evolution of selected fatty acids in eggs (0 days post fertilization) and unfed snook larvae (1 to 5 day post fertilization). Means are ± SEM , n=3.

In both pompano and snook, the four predominant FAs in the eggs and newly hatched larvae were DHA, 16:0, 18:1n9 and 16:1n7, contributing to over 50 % of total FA. For pompano DHA was the main FA throughout the experiment (4 DPH), while for snook 16:0 was the main FA in the egg and larvae up to 2 DPH when its proportion decreases, while DHA increases. At the end of the trial, DHA, 16:0 and 18:1n9 were the predominant FAs, followed by 18:0 then ARA for pompano and ARA followed by 18:0 for snook.

In pompano, from hatching to the end of the trial period, proportions of DHA and ARA increased from 33.7±0.5 up to 35.2±0.9 % and from 3.2±0.1 up to 5.5±0. 2% respectively, while EPA decreased from 4.7±0.2 down to 2.9±0.0 %. In snook, these FAs followed a similar trend with the proportion of DHA and ARA increasing from 16.2±0.2 up to 25.8±0.5% and from 6.5±0.2 up to 9.4±0.2% respectively, while EPA decreased from 3.0±0.2 down to 2.3±0.0%. Accordingly, DHA/EPA and ARA/EPA ratios increased from 7.1±0.1 up to 11.9±0.4 and from 0.7±0.0 up to 1.9±0.1 respectively for pompano, and from 5.4±0.2 up to 11.1±01 and from 2.2±0.1 up to 4.0±0.1 respectively for snook.

Overall, the proportion of saturated FAs increased in both species between hatching and the end of the experiment with an increase in pompano larvae from 22.5±0.8 up to 27.7±0.7% and an increase in snook larvae from 28.4±0.4 up to 32.4±0.4%. In addition, the total proportion of n-6 increased for pompano from 6.2±0.0 up to 8.5±0.1%, and for snook from 11.8±0.3 up to 15.6±0.9%. In contrast, the proportion of mono-unsaturated FAs decreased in pompano larvae from 22.8±0.7 down to 15.1±0.4%, and in snook larvae from 28.9±0.2 down to 17.1±0.8%. The proportion of poly-unsaturated FAs and total proportion of n-3 increased from 37.4±0.4 up to

48.7±0.7 % and from 23.3±0.3 up to 31.4±0.9 % respectively in snook larvae ,while contrastingly they did not vary significantly in pompano larvae with an average of 49.7±0.2 % and 40.8±0.7 % respectively. Consequently, the n-3/n-6 ratio decreased from 7.0±0.3 down to 4.8±0.1 in pompano, while it did not vary significantly in snook larvae with an average of 1.9±0.8.

The total proportion of lipid decreased by 23 % in pompano larvae with 190.7±3 mg in the eggs and 146.3±0.7 in the larvae at the end of the trial, and by 43 % in snook larvae with 193.3±3.5 mg in the eggs and 110.1±2.2 mg at the end of the trial.

3.4.2 Live feed trials

At the end of the trials, survival rates from hatching for the A1 and A2 larvae and the Fc larvae were similar with an average of 5.9±0.6 %, significantly higher than that of Fa larvae with 2.4±0.5 % and Fb larvae with 2.1±0.4 % (Table 3.3). No significant difference was observed in the proportion of functional swim bladder in A1 and A2 larvae with an average of 78.9±1.7 %, however it was significantly higher compared to the other treatments that averaged 53.4±2.2 % (Table 3.3).

At the end of the first trial, standard length was significantly higher for the Fa and Fb larvae compared to the A1 larvae (4.26±0.12, 4.29±0.11 and 3.98±0.09 mm, respectively) while there was no significant difference in eye diameter with an average of 0.38±0.08 mm (Fig. 3.3). At the end of the second trial, there were no significant differences in standard length and eye diameter between treatments (Fig. 3.3).

Table 3.2 Percent survival and percent of functional swim bladder at the end of live feed trials with snook larvae fed rotifers enriched with Algamac 3050 (A1), Formulation a (Fa) or Formulation b (Fb) during the first trial, and Algamac 3050 (A2) or Formulation c (Fc) during the second trial. Means are ± SEM, n = 4 in trial 1, n=3 in trial 3, 10 larvae per tank. Superscript letters indicate significant differences within a same column (Tukey test, $p<0.05$).

		Survival (%)	Functional swim bladder (%)
Trial 1	A1	6.9^{b}±0.9	80.2^{b}±2.1
	Fa	2.4^{a}±0.5	50.9^{a}±2.8
	Fb	2.1^{a}±0.4	52.1^{a}±3.4
Trial 2	A2	5.2^{b}±1.2	77.1^{b}±3.1
	Fc	5.4^{b}±0.9	58.4^{a}±5.2

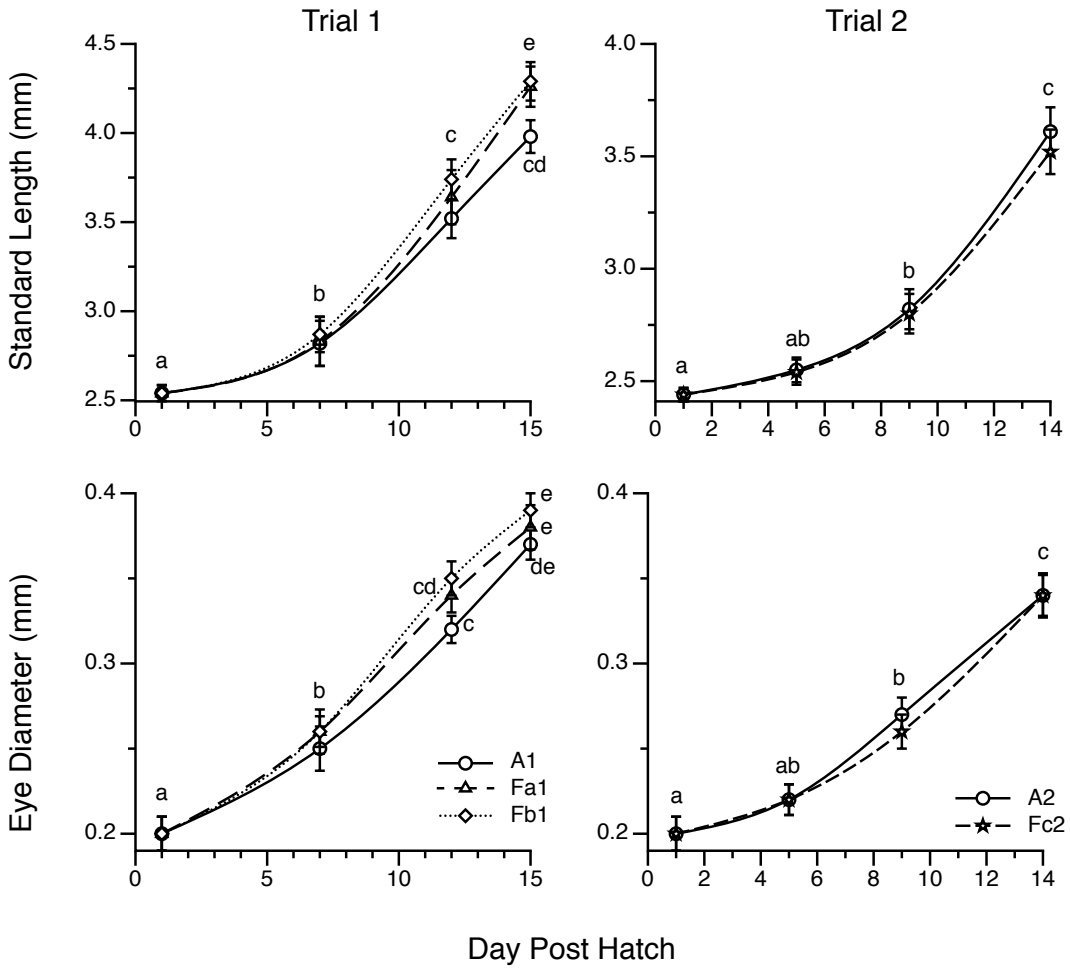

Figure 3.3 Standard length (A) and eye diameter (B) of snook larvae fed rotifers enriched with Algamac 3050 (A1), Formulation a (Fa1) or Formulation b (Fb1) during the first trial, and Algamac 3050 (A2) or Formulation c (Fc2) during the second trial. Means are ± standard error of the mean, n= 4 in trial 1, n=3 in trial 2, 10 larvae per tank and sampling point. Letters indicate significant differences between treatments and time points (Tukey test, $p<0.05$).

FA composition of eggs, enrichment formulations, enriched rotifers and larvae at the end of the trial are presented in Table 3.4 for the first trial and Table 3.5 for the second trial. In addition, absolute PUFAs content and ratios of the enrichment formulations, enriched rotifers and larvae at the end of the trial are represented in Figure 3.4 for trial 1 and Figure 3.5 for trial 2.

In the first trial, there was no significant difference in total PUFA between the different enrichments with an average of 59.1±1 % of total FA. However, total PUFA differed in the rotifers with the greatest amount in A1 rotifers (66.2±1.0 % of total FA) followed by Fa rotifers (57.6±1.0% of total FA) and Fb rotifers (51.5±0.2 % of total FA). At the end of the trial, total PUFA in the larvae was significantly higher for A1 and Fa larvae with an average of 47.4±0.5 % of total FA compared to Fb larvae (43.1±0.7 % of total FA). In trial 2, no significant differences were observed in PUFA contents between treatments with an average of 63.6±1.0 % of total FA in the enrichment, 54.0±0.5 % of total FA in the rotifers and 43.5±0.4 % of total FA in the larvae.

DHA content in trial 1 was greatest in the A1 enrichment and rotifers (40.8±0.8 and 38.2±0.8 % of total FA), followed by the Fa enrichment and rotifers (29.8±0.7 and 25.0±0.6 % of total FA) then the Fb enrichment and rotifers (28.0±0.4 and 20.6±0.3 % of total FA). However, at the end of the trial there was no significant difference in larvae DHA content with an average of 24.3±0.4 % of total FA. In trial 2, DHA content was significantly higher for the A2 enrichment, rotifers and larvae (40.8±0.6, 27.3±0.4 and 22.7±0.1 % of total FA respectively), than in their equivalent from the Fc treatment (22.9±0.2, 15.3±0.4 and 19.4±0.3 % of total FA respectively).

ARA content in trial 1was the greatest in the Fb enrichment (17.5±0.1 % of total FA) then in the Fa enrichment (14.7±0.1 % of total FA) followed by the A1 enrichment (1.6±0.3 % of total FA). Likewise, highest ARA content was observed in the Fb rotifers (11.1±0.6 % of total FA) then in the Fa rotifers (9.3±0.3 % of total FA) followed by the A1 rotifers (2.8±0.1 % of total FA). At the end of the trial, the ARA content was significantly lower in the A1 larvae (4.4±0.9 % of total FA) than in the other treatments (average of 7.1±0.4 % of total FA). In trial 2, ARA content was significantly higher in the Fc enrichment, rotifers and larvae (20.8±0.2, 14.0±0.7 and 8.7±0.3 % of total FA respectively) than in their equivalent from the A2 treatment (1.6±0.3, 3.3±0.2 and 5.2±0.2 % of total FA respectively).

At the end of the first trial, the highest DHA/EPA ratio was observed in the A1 larvae (8.9±0.1) and the lowest ratio was observed in the Fa larvae (7.0±0.3) while the Fb ratio was not significantly different from the other treatments (7.9±0.6). In the second trial, the DHA/EPA ratio in the A2 larvae (6.5±0.1) was significantly higher than that of the Fc larvae (5.1±0.3). The highest ARA/EPA ratio in trial 1 was observed in the Fb larvae (2.4±0.1), the lowest ratio in the A1 larvae (1.6±0.2) while the ratio in the Fa larvae was not significantly different from the other treatments (2.0±0.1). In the second trial, the Fb larvae presented an ARA/EPA ratio significantly higher (2.3±0.2) than that of the A2 larvae (1.5±0.2).

Table 3.3 Fatty acid profile of fertilized eggs, Algamac 3050 (A1), Formulation a (Fa), and Formulation b (Fb) and their enriched rotifers during the trial (6 sampling times) as well as their resulting snook larvae at the end of the trial. Only fatty acids contributing to at least 1% in one of the treatments are reported, all fatty acids are included in totals and ratios. Means are ± SEM, n = 3 for the eggs, enrichment and larvae (50 larvae per tank), n=6 for enriched rotifers. Superscripted letters indicate significant differences within a same row (Tukey test, $p<0.05$). nd means not detected.

% total FA	Fertilized Eggs	A1			Fa			Fb		
		Enrichment	Enriched Rotifers	Larvae	Enrichment	Enriched Rotifers	Larvae	Enrichment	Enriched Rotifers	Larvae
12:0	0.0±0.0^{a}	0.3±0.1^{b}	0.1±0.0^{ab}	0.2±0.1^{b}	0.9±0.0^{c}	0.2±0.0^{b}	0.2±0.0^{b}	1.1±0.0^{c}	0.3±0.0^{b}	0.3±0.1^{b}
14:0	3.1±0.1^{b}	9.8±0.6^{e}	4.4±0.2^{c}	1.3±0.1^{a}	6.0±0.2^{d}	3.2±0.1^{b}	0.9±0.2^{a}	6.0±0.1^{d}	3.4±0.2^{bc}	0.9±0.1^{a}
16:0	17.9±0.8^{c}	25.9±0.7^{d}	15.2±0.8^{bc}	16.5±1.2^{c}	11.7±0.3^{ab}	12.0±0.3^{ab}	14.6±0.3^{bc}	11.8±0.2^{ab}	11.0±0.5^{a}	16.2±0.6^{c}
17:0	0.7±0.0^{cd}	nd	0.5±0.0^{c}	1.2±0.2^{ef}	0.1±0.0^{b}	0.9±0.0^{de}	1.2±0.1^{ef}	0.1±0.0^{b}	0.7±0.1^{cd}	1.3±0.1^{f}
18:0	4.8±0.1^{c}	0.5±0.0^{a}	1.5±0.1^{b}	8.4±0.4^{d}	1.7±0.6^{b}	2.7±0.1^{b}	7.9±0.5^{d}	2.2±0.6^{b}	2.6±0.2^{b}	9.3±0.7^{d}
Total saturated	27.1±0.8^{c}	37.0±0.6^{d}	22.0±0.8^{ab}	27.9±0.7^{c}	20.4±0.2^{a}	19.2±0.4^{a}	25.0±0.7^{bc}	21.3±0.9^{ab}	18.2±0.8^{a}	28.4±1.5^{c}
16:1n-7	8.0±0.2^{f}	nd	3.2±0.2^{c}	3.0±0.2^{c}	2.2±0.0^{b}	5.9±0.2^{e}	3.2±0.4^{c}	1.7±0.0^{b}	4.8±0.4^{d}	2.9±0.5^{c}
18:1n-9	11.1±0.4^{f}	0.1±0.0^{a}	0.6±0.1^{b}	4.9±0.3^{c}	14.5±0.2^{g}	7.7±0.3^{de}	6.9±0.3^{d}	13.9±0.3^{g}	8.4±0.3^{e}	7.1±0.3^{de}
18:1n-7	6.6±0.1^{d}	nd	1.8±0.1^{b}	3.2±0.2^{c}	nd	3.8±0.2^{c}	3.7±0.1^{c}	nd	3.1±0.3^{c}	2.9±0.3^{bc}
20:1n-9	0.5±0.0^{bc}	nd	0.5±0.1^{bc}	0.7±0.1^{c}	0.3±0.0^{bc}	1.4±0.2^{d}	1.4±0.1^{d}	0.2±0.0^{b}	1.4±0.1^{d}	1.3±0.1^{d}
Total mono-unsaturated	26.4±0.5^{f}	0.1±0.0^{a}	6.2±0.4^{b}	11.9±0.7^{e}	17.0±0.3^{de}	18.9±0.4^{e}	15.3±0.4^{d}	15.9±0.3^{d}	17.8±0.4^{de}	14.6±0.6^{d}
18:2n-6	1.8±0.0^{c}	0.1±0.0^{a}	0.9±0.1^{b}	1.5±0.1^{c}	4.6±0.1^{e}	4.4±0.1^{e}	2.4±0.1^{d}	4.9±0.1^{e}	4.6±0.1^{e}	2.6±0.2^{d}
18:3n-6	0.2±0.1^{a}	0.2±0.0^{a}	0.3±0.0^{a}	0.5±0.0^{b}	0.8±0.0^{c}	0.7±0.0^{c}	0.5±0.1^{b}	1.1±0.1^{d}	0.8±0.0^{c}	0.6±0.1^{b}
18:4n-3	0.5±0.1^{a}	0.3±0.1^{a}	0.4±0.1^{a}	1.1±0.8^{ab}	2.4±0.1^{c}	1.7±0.2^{bc}	0.4±0.1^{a}	0.7±0.0^{ab}	0.8±0.1^{ab}	0.4±0.1^{a}
20:3n-6	0.6±0.1^{bc}	0.4±0.0^{a}	0.8±0.1^{c}	0.8±0.1^{c}	0.5±0.2^{b}	1.6±0.1^{e}	1.0±0.1^{d}	1.1±0.2^{d}	1.7±0.2^{e}	0.8±0.1^{c}
20:4n-6	5.1±0.0^{c}	1.6±0.3^{a}	2.8±0.1^{b}	4.4±0.9^{c}	14.7±0.1^{g}	9.3±0.3^{e}	7.1±0.2^{d}	17.5±0.1^{h}	11.1±0.6^{f}	7.0±0.6^{d}
20:4n-3	0.4±0.0^{bc}	0.8±0.0^{cd}	1.0±0.1^{d}	0.4±0.1^{bc}	nd	0.7±0.1^{cd}	0.3±0.1^{bc}	nd	0.5±0.0^{c}	0.2±0.1^{b}
20:5n-3	4.5±0.1^{c}	1.0±0.5^{a}	5.8±0.5^{d}	2.8±0.3^{b}	1.0±0.0^{a}	7.7±0.4^{e}	3.6±0.3^{bc}	1.2±0.0^{a}	6.4±0.2^{d}	3.0±0.3^{b}
22:5n-6	1.5±0.1^{c}	14.6±0.5^{e}	12.8±0.3^{e}	8.8±0.2^{d}	0.2±0.0^{ab}	0.6±0.1^{b}	1.1±0.4^{bc}	0.1±0.0^{a}	0.5±0.1^{b}	1.3±0.9^{bc}
22:5n-3	3.3±0.1^{cd}	0.3±0.0^{a}	2.7±0.3^{c}	1.9±0.1^{b}	2.5±0.1^{bc}	4.4±0.2^{d}	3.2±0.3^{cd}	3.0±0.0^{cd}	3.5±0.4^{cd}	2.3±0.4^{bc}
22:6n-3	22.3±0.8^{ab}	40.8±0.8^{e}	38.2±0.8^{e}	24.5±0.4^{b}	29.8±0.7^{d}	25.0±1.0^{b}	25.1±0.6^{b}	28.0±0.4^{c}	20.6±0.3^{a}	23.4±0.3^{ab}
Total poly-unsaturated	42.6±0.9^{a}	60.4±1.4^{d}	66.2±1.0^{e}	48.2±0.7^{b}	58.2±1.0^{d}	57.6±1.0^{d}	46.7±0.4^{b}	58.7±0.5^{d}	51.5±0.2^{c}	43.1±0.7^{a}
Total n-3	31.8±0.6^{b}	43.4±0.6^{cd}	48.4±0.9^{d}	31.2±3.2^{b}	37.1±0.8^{c}	35.8±1.0^{bc}	31.2±1.2^{b}	33.8±0.5^{b}	36.8±0.6^{c}	26.4±1.5^{a}
Total n-6	9.4±0.1^{a}	16.8±0.4^{c}	17.7±0.3^{cd}	16.2±1.1^{c}	21.0±0.2^{d}	17.0±0.4^{c}	12.6±0.3^{b}	24.9±0.0^{e}	19.0±0.5^{cd}	12.5±1.6^{b}
n-3/ n-6	3.4±0.2^{f}	2.6±0.1^{e}	2.7±0.0^{e}	1.9±0.2^{c}	1.8±0.0^{b}	2.1±0.1^{d}	2.5±0.2^{de}	1.4±0.0^{a}	1.9±0.1^{c}	2.1±0.0^{d}
ARA/EPA	1.1±0.0^{b}	1.5±0.2^{c}	0.5±0.0^{a}	1.6±0.2^{cd}	14.9±0.1^{f}	1.2±0.1^{b}	2.0±0.1^{de}	14.7±0.6^{f}	1.8±0.2^{d}	2.4±0.1^{e}
DHA/EPA	5.0±0.2^{ab}	39.4±1.5^{f}	6.6±0.8^{b}	8.9±0.1^{c}	30.2±0.5^{e}	3.3±0.2^{a}	7.0±0.3^{b}	23.5±0.4^{d}	3.2±0.2^{a}	7.9±0.6^{bc}
Total FA (mg FA/g dry weight)	151.4±5.0^{d}	398.3±5.0^{g}	153.9±6.3^{d}	88.5±6.3^{b}	254.8±1.2^{e}	82.7±3.9^{b}	64.2±3.5^{a}	328.7±4.1^{f}	106.2±8.4^{c}	94.5±2.2^{b}

Table 3.4 Fatty acid profile of fertilized eggs, Algamac 3050 (A2) and Formulation c (Fc), and their enriched rotifers during the trial (6 sampling times) as well as their resulting snook larvae at the end of the trial. Only fatty acids contributing to at least 1% in one of the treatments are reported, all fatty acids are included in totals and ratios. Means are ± SEM, n = 3 for the eggs, enrichment and larvae (50 larvae per tank), n=6 for enriched rotifers. Superscripted letters indicate significant differences within a same row (Tukey test, $p<0.05$). nd means not detected.

% total FA	Fertilized Eggs	A2			Fc		
		Enrichment	Enriched Rotifers	Larvae	Enrichment	Enriched Rotifers	Larvae
14:0	2.2 ±0.1^{b}	9.8 ±0.6^{d}	5.1 ±0.1^{c}	1.2 ±0.0^{a}	5.8 ±0.3^{c}	3.0 ±0.1^{b}	1.2 ±0.1^{a}
16:0	22.9 ±0.5^{b}	25.9 ±0.7^{c}	18.0 ±0.3^{a}	23.8 ±0.1^{bc}	16.0 ±0.4^{a}	15.8 ±0.3^{a}	22.3 ±0.3^{b}
18:0	5.5 ±0.1^{d}	0.5 ±0.1^{a}	1.7 ±0.0^{b}	8.9 ±0.1^{e}	3.0 ±0.1^{c}	2.6 ±0.1^{c}	8.0 ±0.2^{e}
Total saturated	32.4 ±0.5^{c}	37.0 ±1.1^{d}	25.6 ±0.4^{b}	34.9 ±0.6^{c}	25.5 ±0.5^{b}	21.9 ±0.3^{a}	32.5 ±0.3^{c}
16:1n7	8.0 ±0.4^{c}	nd	7.7 ±0.2^{c}	4.0 ±0.1^{b}	0.6 ±0.1^{a}	8.2 ±0.2^{c}	5.1 ±0.2^{bc}
18:1n9	14.4 ±0.8^{e}	0.1 ±0.0^{a}	2.7 ±0.1^{b}	5.6 ±0.2^{c}	2.0 ±1.1^{b}	6.3 ±0.2^{c}	7.1 ±0.3^{d}
18:1n7	5.3 ±0.1^{d}	nd	2.3 ±0.1^{b}	2.9 ±0.2^{b}	3.9 ±1.1^{c}	2.3 ±0.1^{b}	2.8 ±0.1^{b}
20:1n9	0.2 ±0.0^{b}	nd	1.2 ±0.0^{c}	1.0 ±0.0^{c}	0.2 ±0.0^{b}	1.4 ±0.0^{c}	1.2 ±0.1^{c}
Total mono-unsaturated	28.2 ±0.6^{e}	0.1 ±0.0^{a}	13.9 ±0.3^{c}	13.6 ±0.1^{c}	6.7 ±0.3^{b}	18.2 ±0.3^{d}	16.3 ±0.3^{d}
16:3n4	1.5 ±0.1^{d}	0.2 ±0.0^{b}	0.2 ±0.0^{b}	0.3 ±0.0^{c}	nd	0.3 ±0.0^{c}	0.3 ±0.0^{c}
18:2n6	1.9 ±0.1^{bc}	0.1 ±0.0^{a}	1.6 ±0.1^{b}	1.7 ±0.1^{b}	4.7 ±0.1^{d}	4.8 ±0.1^{d}	2.6 ±0.2^{c}
18:3n6	0.6 ±0.0^{c}	0.2 ±0.0^{a}	0.4 ±0.1^{bc}	0.3 ±0.0^{ab}	1.1 ±0.0^{d}	1.0 ±0.0^{d}	0.5 ±0.0^{c}
18:3n3	1.1 ±0.1^{c}	0.1 ±0.1^{ab}	0.2 ±0.0^{ab}	0.1 ±0.0^{a}	0.2 ±0.0^{ab}	0.3 ±0.0^{b}	0.1 ±0.0^{a}
20:3n6	0.5 ±0.0^{b}	0.4 ±0.0^{a}	0.7 ±0.1^{bc}	0.6 ±0.0^{b}	1.8 ±0.1^{d}	1.6 ±0.0^{d}	0.9 ±0.1^{c}
20:4n6	4.7 ±0.2^{bc}	1.6 ±0.3^{a}	3.3 ±0.2^{b}	5.2 ±0.2^{c}	20.8 ±0.2^{f}	14.0 ±0.7^{e}	8.7 ±0.3^{d}
20:5n3	2.4 ±0.1^{b}	1.0 ±0.5^{a}	7.3 ±0.3^{d}	3.5 ±0.1^{c}	1.4 ±0.1^{a}	7.1 ±0.4^{d}	3.8 ±0.2^{c}
22:5n6	1.3 ±0.1^{a}	14.6 ±0.5^{e}	8.9 ±0.2^{d}	5.5 ±0.2^{c}	5.8 ±0.2^{c}	3.4 ±0.1^{b}	3.1 ±0.1^{b}
22:5n3	2.9 ±0.1^{b}	0.3 ±0.0^{a}	2.5 ±0.1^{b}	2.5 ±0.1^{b}	4.6 ±0.3^{d}	3.7 ±0.1^{c}	3.1 ±0.0^{bc}
22:6n3	9.5 ±0.4^{a}	40.8 ±0.6^{f}	27.3 ±0.4^{e}	22.7 ±0.1^{d}	22.9 ±0.2^{d}	15.3 ±0.4^{b}	19.4 ±0.3^{c}
Total poly-unsaturated	29.3 ±0.6^{a}	62.4 ±1.4^{d}	54.4 ±0.4^{c}	43.4 ±0.1^{b}	64.77 ±0.5^{d}	53.5 ±0.6^{c}	43.7 ±0.6^{b}
Total ω3	17.5 ±0.4^{a}	43.4 ±1.4^{d}	38.8 ±0.3^{d}	29.3 ±0.1^{c}	30.26 ±0.9^{c}	27.6 ±0.2^{bc}	26.9 ±0.1^{b}
Total ω6	9.3 ±0.2^{a}	16.8 ±0.4^{c}	15.0 ±0.1^{b}	13.4 ±0.1^{b}	34.44 ±0.3^{e}	25.1 ±0.7^{d}	16.1 ±0.6^{c}
ω3/ ω6	1.9 ±0.2^{cd}	2.6 ±0.1^{e}	2.6 ±0.2^{e}	2.2 ±0.4^{d}	0.88 ±0.0^{a}	1.1 ±0.1^{b}	1.7 ±0.1^{c}
ARA/EPA	2.0 ±0.1^{c}	1.5 ±0.2^{b}	0.5 ±0.1^{a}	1.5 ±0.2^{b}	14.53 ±0.3^{d}	2.0 ±0.1^{c}	2.3 ±0.2^{c}
DHA/EPA	4.0 ±0.3^{b}	39.4 ±0.5^{f}	3.7 ±0.2^{b}	6.5 ±0.1^{d}	15.95 ±0.4^{e}	2.2 ±0.1^{a}	5.1 ±0.3^{c}
Total FA (mg FA/g dry weight)	173.4 ±2.9^{c}	398.3 ±5.0^{d}	174.8 ±4.1^{c}	110.0 ±5.5^{a}	392.0 ±2.0^{d}	141.5 ±3.1^{b}	120.3 ±2.9^{ab}

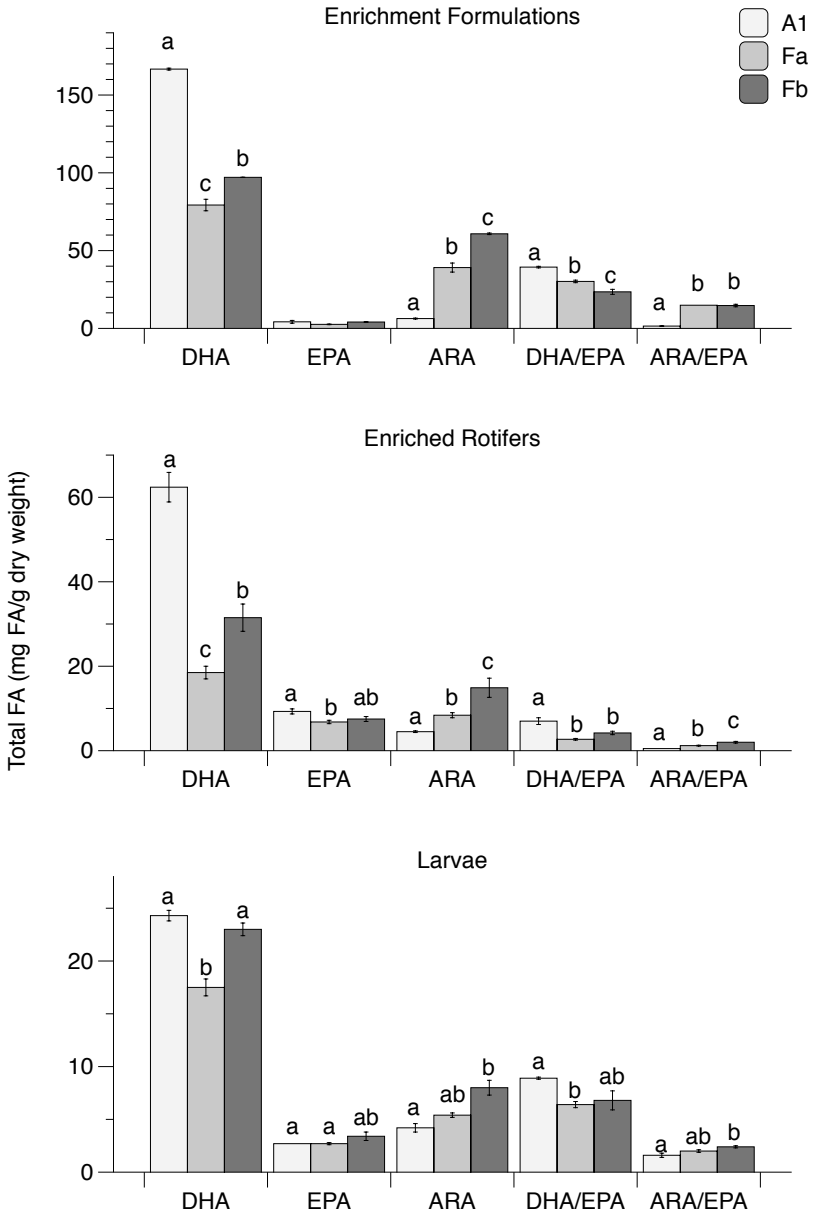

Figure 3.4 DHA, EPA, ARA, DHA/EPA and ARA/EPA ratios in the enrichment formulations, enriched rotifers and larvae at the end of the first trial. Means are ± SEM, n = 3 for the enrichment formulations and larvae (50 larvae per tank), n=6 for enriched rotifers. Letters indicate significant differences between treatments.

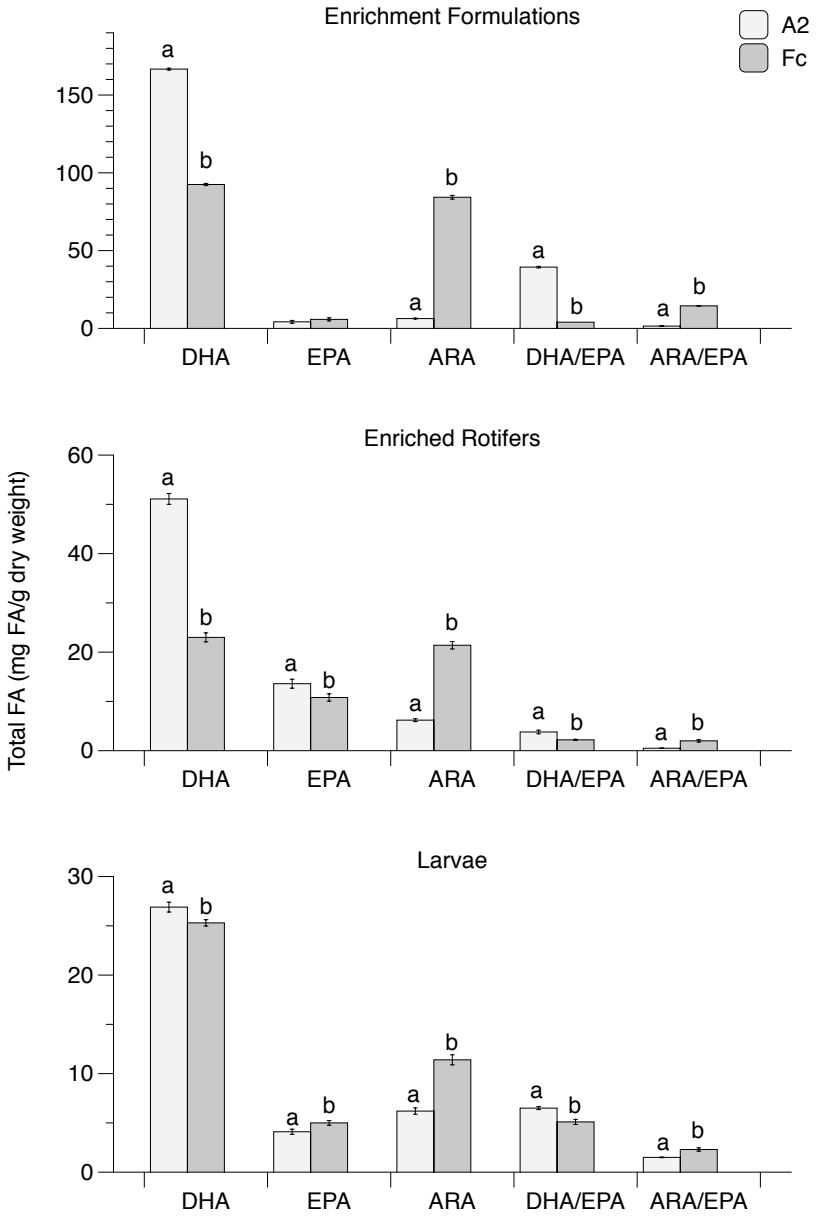

Figure 3.5 DHA, EPA, ARA, DHA/EPA and ARA/EPA ratios in the enrichment formulations, enriched rotifers and larvae at the end of the second trial. Means are ± SEM, n = 3 for the enrichment formulations and larvae (50 larvae per tank), n=6 for enriched rotifers. Letters indicate significant differences between treatments

3.5 DISCUSSION

The study of FA utilization in pompano and snook unfed larvae revealed clear patterns of lipid utilization and mobilization. Eggs from both species contained an oil globule and are classified as being high in lipids (>19% of dry weight)(Sargent et al., 1989). After hatching the decline in total FA reflected the utilization of lipids as an energy source. This is in accordance with previous studies in species such as red drum *Sciaenops ocellatus* (Vetter et al., 1983), red sea bream *Pagrus major* (Tandler et al., 1989), gilthead sea bream (Koven et al., 1989; Rønnestad et al., 1994), turbot (Rainuzzo et al., 1994), Senegal sole (Mourente and Vázquez, 1996), common dentex *Dentex dentex* (Mourente et al., 1999), white sea bream *Diplodus sargus* (Cejas et al., 2004a) and Atlantic bluefin tuna *Thunnus thynnus* (Morais et al., 2011) indicating that fast developing eggs from temperate and warm waters, with high lipid content and an oil globule, incorporate a large fraction of neutral lipids in the oil droplet which is utilized for energy soon after hatching. Contrastingly, species from cold water and with slower egg development such as Atlantic salmon *Salmo salar* (Cowey et al., 1985), Atlantic herring *Clupea harengus* (Tocher et al., 1985a) Atlantic cod *Gadus morhua* and plaice *Pleuronectes platessa* (Rainuzzo et al., 1992) or Atlantic halibut *Hippoglossus hippoglossus* (Rainuzzo et al., 1992; Ronnestad et al., 1995) mainly catabolize free amino acid after hatching (Rønnestad, 1999).

The neutral lipid fraction is generally rich in mono-unsaturated FAs that are preferentially used for energy (Kamler, 2007). It seems to be the case in this study as, even though no lipid class analyses were performed, a strong decrease in mono-unsaturated FAs and no increase in their elongation products were observed in both species after hatching. The selective retention of certain fatty acids has been

described as a biochemical strategy allowing the preservation of the most essential components of biological membranes during starvation periods (Izquierdo, 1996). It has been recently demonstrated in turbot that the expression profile of some genes involved in lipid metabolism (Hepatic lipase, FA synthetase and Diacylglycerol O-acyltransferase homolog 1) responded solely to starvation (Cunha et al., 2013). In the present study saturated fatty acids (SFAs) and PUFAs were preferentially retained. SFAs and PUFAs play important roles in respectively the sn-1 and sn-2 position of structural phospholipids with phosphatidylcholine containing high levels of 16:0, phosphatidylserine incorporating high levels of 18:0 associated with C22 PUFAs, and phosphatidylinositol including high levels of 18:0 associated with C20 PUFAs and more particularly ARA (Tocher, 1995). In both pompano and snook, the main PUFAs selectively retained were DHA and ARA, while low levels of EPA were observed. DHA is selectively retained in unfed larvae of several species, including Atlantic herring (Tocher et al., 1985b), gilthead sea bream (Koven et al., 1989), red sea bream (Tandler et al., 1989), cod (Van der Meeren et al., 1993), turbot (Rainuzzo et al., 1994), Senegal sole (Mourente and Vázquez, 1996) common dentex (Mourente et al., 1999), and Atlantic bluefin tuna (Morais et al., 2011). This reflects the importance of DHA in larvae structural development and in particular on neural and visual functions (M. V Bell et al., 1995; Sargent et al., 1999a). ARA is selectively retained along with DHA in fewer species, including turbot (Rainuzzo et al., 1994), Senegal sole (Mourente and Vázquez, 1996), and bluefin tuna (Morais et al., 2011). In addition, in Atlantic halibut, cod, plaice (Rainuzzo et al., 1992) and white sea bream (Cejas et al., 2004a), ARA solely was conserved, while utilization of DHA was observed, indicating a special role of ARA in the early larval development of

these species. Concurrently to the retention of ARA, a decrease in EPA level is observed throughout the starvation period in pompano while a significant decrease is only observed between 0 and 1 dph in snook. In the absence of information on bioconversion rates in these species, the decrease could be due to either catabolism or bioconversion to DHA.

Among the enrichment formulations tested in the second part of the study, the ARA supplementation did not increase survival or eye diameter. An increase in growth was observed in the first enrichment trial, yet, considering the lower survival and swim bladder inflation rates in the supplemented treatments, the difference can probably be attributed to the lower density in these treatments rather than the supplementation. In the second trial, no significant difference was observed in growth or survival between treatments even though the rate of functional swim bladder was significantly lower for the supplemented larvae. The oil-based formulation of the supplemented treatments seemed to negatively impact the swim bladder inflation success of the larvae compared to the flake formulation of Algamac 3050 despite efforts to reduce the oil film at the surface of the tanks. Future studies could benefit from supplementing the enrichment with ARA powder rather than oil.

Large differences were observed in the fatty acid profile of the larvae at the end of the trials. The main challenge in achieving a high ARA/EPA ratio is the relatively high natural EPA content of rotifers. Indeed, even when the proportion of EPA in the enrichment formulation was as low as 1 %, the proportion reached over 5 % in the enriched rotifers. At the end of the first trial, Fa and Fb larvae successfully incorporated ARA and EPA at a 2:1 ratio; however, the low 22:5n6 (Docosapentaenoic acid, DPA) content in these treatments compared to that of the

Algamac treatment was a concern. In the second trial, a higher level of DPA was provided in the Fc formulation, which might have played a role in the increase in survival from the Fa and Fb larvae. The Fc formulation appears to be adequate in providing the necessary amount and proportions of fatty acid to snook larvae in reference to the wild eggs; however, even though growth and survival were not different than that of Algamac 3050, the rate of functional swim bladder development was significantly lower. Additional studies should therefore investigate the use of ARA powder rather than oil as mentioned previously, or improve the stability of the oil emulsion. Bessonart et al. (1999) did not observe a significant improvement in growth after supplementing ARA to gilthead sea bream larvae for two weeks, but a difference was observed after three weeks. In addition, Koven et al. (2001) did not observe growth or survival improvement either after supplementing larvae from the same species for two weeks; nevertheless, a significant increase in stress resistance was observed in the supplemented larvae exposed to handling stress potentially suggesting more robust larvae. Therefore, future studies should investigate the effect of an ARA supplementation over a longer period of time and include the study of additional parameters such as cortisol production.

In conclusion, these studies bring fundamental information on the early fatty acid requirements of pompano and snook larvae. Findings also provided the first insight of snook larvae fatty acid incorporation during the live food period and the influence of an ARA supplementation on final fatty acid incorporation and ratios in snook larvae.

3.6 ACKNOWLEDGMENTS

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Chapter 4 Effects of a mix of Bacillus sp. as a potential probiotic for Florida pompano, common snook and red drum larvae performances and digestive enzyme activities.

RESEARCH ARTICLE
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Contributions: The present manuscript was compiled and written in full by the author of this thesis. Rotifer rearing, larval rearing, sampling, lab and statistical analyses have been carried out by the candidate. Co-authors provided assistance with the proofreading of the manuscript.

4.1 ABSTRACT

This study examined the effect of a commercial mix of *Bacillus* sp. on survival, growth and digestive enzyme activities of Florida pompano, red drum and common snook. Larvae were fed either live feed enriched with Algamac 3050 (Control), Algamac 3050 and probiotics (PB), or the previous diet combined with a daily addition of probiotics to the tank water (PB+). Survival was not affected by the treatments for any of the species. At the end of the pompano and snook trial, standard lengths of larvae from the PB and PB+ treatments were significantly greater than for the control larvae. Microbiological analyses were performed at the end of the pompano trial and numbers of presumptive Vibrio were not a concern in the system. For both pompano and snook, trypsin specific activity was higher in PB and PB+ larvae compared to the control larvae. Similarly, alkaline phosphatase activity was higher for the pompano larvae fed the PB and PB+ treatments and for the snook larvae fed the PB+ treatment compared to the control larvae. This experiment suggests that a mix of *Bacillus* sp. can promote growth through an early maturation of the digestive system during the early larval stages of pompano and snook.

Keywords: probiotic, *Centropomus undecimalis*, *Trachinotus carolinus*, *Sciaenops ocellatus*, fish larvae, digestive enzymes.

4.2 INTRODUCTION

Marine fish larvae undergo major physiological and morphological changes during the first weeks of their lives (Péres et al., 1997). Inadequate nutrition and larval rearing conditions during this crucial transitional period adversely affect the development and the future success of the larvae (Yúfera and Darias, 2007). Determining optimal rearing protocols has proven difficult in marine fish due to the small size and fragility of the larvae therefore, the mass production of robust juveniles remains a challenge for most species (Hamre et al., 2013). The external environment has a major impact on marine fish gastrointestinal microflora as osmoregulation requires constant ingestion of the surrounding water (Gatesoupe, 1999). Since the digestive tract of fish larvae is sterile at hatching, the initial colonization depends on the environment and live feed ingested (Grisez et al., 1997). The initial microflora established at the larval stage seems to persist after metamorphosis, it is therefore thought that providing probiotics as soon as possible after hatching can have beneficial effects (Ringø and Vadstein, 1998). In addition, the larvae immune system is immature and relies on nonspecific defense mechanisms, thus a healthy microflora constitutes a crucial primary barrier to which probiotics can, possibly, effectively contribute (Hansen and Olafsen, 1999).

Probiotics are 'live microorganisms which when administered in adequate amounts confer a health benefit to the host' (Aureli et al., 2011). Probiotics have various modes of actions including the competitive exclusion of pathogenic bacteria (Balcázar et al., 2004; Chythanya et al., 2002; Gomez-Gil et al., 2000; Moriarty, 1997; Vine et al., 2004), the improvement of water quality (Moriarty, 1997), the enhancement of the immune system (Balcázar et al., 2004; Gatesoupe, 1999;

Picchietti et al., 2009) and the stimulation of the digestive system (Lazado et al., 2012; Suzer et al., 2008).

In humans, Hooper *et al*. (2001) demonstrated that probiotics can modulate the expression of genes involved in nutrient absorption, mucosal barrier fortification and postnatal intestinal maturation. Bacteria communicate via the use of quorum-sensing molecules, which regulate gene expression mainly when the population has reached a high cell density (Williams et al., 2007). *B. subtilis* was found to produce a quorum-sensing pentapeptide, the competence and sporulation-stimulating factor (CSF), which activates two cellular survival pathways (protein kinase B and p38 mitogen-activated protein kinase) and induces the expression of the heat shock protein (e.g. Hsp27) in intestinal epithelial cells (Fujiya et al., 2007). Hsps protect cells against various stresses and this mechanism is highly conserved throughout evolution and across species (Parsell and Lindquist, 1993). When over expressed, Hsps increase intestinal epithelial cells viability and protect from oxidative injury, contributing to intestinal homeostasis (Tao et al., 2006).

Among the probiotics, *Bacillus* is of particular interest as it is a spore forming bacteria (Cutting, 2011). Spore production is triggered by nutrient depletion in the bacterial environment, allowing for long-term survival in conditions inadequate to vegetative bacteria. Even though spores are dehydrated and have an inactive metabolism, they are able to monitor the environment (Nicholson et al., 2000). Under appropriate conditions, germination occurs by allowing water to penetrate the spore and vegetative growth resumes (Moir, 2006). The spore surface layer confers outstanding resistance to extreme physical and chemical stress (Henriques and Moran, 2007). Spores are heat stable and can survive the low pH of the gastric

barrier (Spinosa et al., 2000). Therefore, they can be stored at room temperature in a desiccated form for a long period of time and all of the administrated spores will reach the intestinal tract (Cutting, 2011). In addition, production cost is low, making *Bacillus* particularly valuable for use in aquaculture production (Wang et al., 2008).

In fish, the administration of *Bacillus* was found to positively influence expression of genes involved in growth metabolism and animal welfare in sea bream (Avella et al., 2010). In the same species, *Bacillus* was shown to increase the expression of occludin, a trans-membrane component of tight junctions in the intestine, suggesting an improvement of cell junction integrity between enterocytes (Cerezuela et al., 2013). In addition, studies have demonstrated, but not explained, the ability for *Bacillus* to increase resistance to vibrio in black tiger shrimp *Penaeus monodon* (Vaseeharan and Ramasamy, 2003), common snook, *Centropomus undecimalis* (Kennedy et al., 1998) and sea bass *Dicentrarchus labrax* (Touraki et al., 2012); increase growth and stimulate the digestive system in Indian white shrimp *Fenneropenaeus indicus* (Ziaei-Nejad et al., 2006), Pacific white shrimp *Penaeus vannamei* (Wang, 2007), Japanese flounder *Paralichthys olivaceus* (Ye et al., 2011), orange-spotted grouper *Epinephelus coioides* (Sun et al., 2013), rohu *Labeo rohita* (Mohapatra et al., 2012) and common carp *Cyprinus carpio* (Wang and Zirong, 2006); promote growth and improve tolerance to rearing conditions in sea bream *Sparus aurata* (Avella et al., 2010).

The present study aimed to test the effects of a commercial mix of *Bacillus* on the growth and digestive enzyme activities in early larval stages of some of Florida's high-value marine food fish (Florida pompano *Trachinotus carolinus* and red drum

Sciaenops ocellatus) and stock enhancement species (common snook *Centropomus undecimalis*).

4.3 MATERIALS AND METHODS

4.3.1 Experimental animals

Snook and pompano eggs were obtained from broodstock captured on the southwest Florida coast and held at the Mote Marine Laboratory Aquaculture Research Park in Sarasota, Florida. Broodstock were conditioned through photothermal regimes in tanks (25 m^3 for pompano, 45 m^3 for snook) equipped with recirculating filtration systems. Spawning was induced by implanting mature pompano females with 50 $\mu g.kg^{-1}$ of sGnRHa (Ovaplant®) and mature snook females with 50 $\mu g.kg^{-1}$ of sGnRHa from the Institute of Marine and Environmental Technology of the University of Maryland. Red drum eggs were received from captive broodstock held at the Florida Fish and Wildlife Conservation Commission Stock Enhancement Research Facility.

Eggs for each species were incubated in a 100 L hatching tank with aeration and an upwelling water circulation from a $3m^3$ system with UV and bio-filtration. Fertilization and hatching rates were respectively 54.5 % and 73.2 % for pompano and 83.5 % and 85.2 % for snook. The fertilization rate of the red drum eggs was unknown while the hatching rate reached 90.2 %.

4.3.2 Experimental set up and treatments

The experimental set up included three identical independent systems. Each system was composed of four 100L tanks with water recirculating from the tanks to a

biofilter and back to the tanks via a UV light. Each independent system was assigned a treatment to avoid probiotic cross contamination.

After hatching, larvae were volumetrically counted and transferred to the experimental tanks at 100 larvae per liter for pompano and red drum, and 200 larvae per liter for snook according to standard procedures at the research park. For all species, photoperiod was maintained at 12h dark: 12h light, salinity at 35 ± 1 $g.L^{-1}$, temperature at 27 ± 1 °C, pH at 8 ± 0.5, dissolved oxygen at 6 ± 2 $mg.L^{-1}$. From 2 days post hatch (DPH), rotifers were fed twice a day at 5 rotifers per mL. In addition, a microdiet (Gemma, Skretting, France) was delivered twice a day in between rotifer feeding.

Trials were stopped at the end of the rotifer-feeding period for pompano (9 DPH) and snook (12 DPH). The red drum trial was extended up to 21 DPH due to the lack of significant difference in growth at the end of the rotifer-feeding period (10 DPH). In this case, rotifer density was decreased to 3 per ml at 7 DPH and the fish weaned onto the microdiet from 10 DPH.

Three treatments were tested in quadruplicate for all trials. The first treatment (control) was rotifers enriched with Algamac 3050 (Aquafauna Bio-Marine Inc, USA). The second treatment (PB) was rotifers enriched with Algamac 3050 and a commercial mix of *Bacillus* spp. (0.5 g per liter of enrichment according to manufacturer's recommendations, Sanolife MIC-F, INVE Technologies, Belgium). The third treatment (PB+) was the second treatment, with additional probiotics (5 $g.m^{-3}$, according to manufacturer's recommendations) added daily directly to the tank water.

4.3.3 Sampling

Larvae growth was monitored through standard length measurement of 10 larvae from each tank (40 per treatment) at 1, 5 and 9 DPH for pompano; 1, 5, 9, and 12 DPH for snook; 1, 7, 14 and 21 DPH for red drum. Pompano larvae body depth was also recorded in consideration to the particular short, deep and compressed pompano body shape. At the end of the trials, 50 larvae from each tank (200 per treatment) were preserved at -70°C for enzyme analysis. In addition, at the end of the pompano trial, 25 larvae from each tank (100 per treatment) were preserved at 4°C for bacterial analyses performed the following day.

4.3.4 Enzyme and bacterial analyses

Enzyme analyses were performed at the Functional Physiology of Marine Organisms unit at Ifremer in Brest, France. Larvae from each tank were pooled and homogenized prior to all analyses. Trypsin, amylase, alkaline phosphatase (AP) and leucine–alanine peptidase (Leu-ala) activities were assayed according to Holm et al. (1988), Métais and Bieth (1968), Bessey et al. (1946) and Nicholson and Kim 1975, respectively. Enzyme activity results are expressed as specific activities, i.e. $U.mg^{-1}$ protein. Protein was determined by the Bradford procedure (Bradford, 1976). Due to technical difficulties, red drum larvae sampled at the end of the rotifer feeding period (7 dph) could not be processed and enzyme analyses were performed at 21 dph only, therefore, results from the red drum trial are presented separately.

Bacterial analyses were performed at the Mote Marine Laboratory, Center for Marine Microbiology. Larvae from each tank were pooled and rinsed three times with sterile

seawater then ground using a PowerSoil® DNA isolation kit (MO-BIO Laboratories, Inc., USA). Serial dilutions of the homogenates were then plated on marine agar (promoting the growth of all marine heterotrophs) and TCBS (medium selective of *Vibrio* sp.) media. The petri dishes were incubated at 22 °C and the number of colony-forming units were counted 48 hours after plating.

4.3.5 Probiotic strains identification

One gram of the commercial mix was diluted in 99 ml of Phosphate-Buffered Saline (PBS) and mixed thoroughly. An inoculating loopful of the suspension was then plated following the quadrant method on Trypticase Soy Agar (TSA) media. The plate was incubated inverted at 37 °C overnight. Colonies showing distinct morphologies were sub-cultured on TSA media following the same method. Isolated strains were sent for 16S rDNA sequencing identification to Accugenix, Inc. (Newark, DE, USA).

4.3.6 Statistical analysis

Statistical analyses were performed using MINITAB® version 16.0. Normality and homogeneity of variance were confirmed using Kolmogorov–Smirnov test. Growth and body depth data were analyzed using a General Linear Model (GLM) with all time and treatment interactions being analyzed and significant differences tested by a Tukey post-hoc test with 95 % confidence. Bacterial counts and enzyme activities were compared by a one-way ANOVA followed by a Tukey post hoc test with 95 %

confidence. Survival data was arcsine square root transformed before a one-way ANOVA followed by a Tukey post-hoc test with 95 % confidence.

4.4 RESULTS

4.4.1 Bacillus strains identification

Three *Bacillus* strains were isolated from the commercial mix. The 16S rDNA sequence-based analysis identified the following species: *Bacillus licheniformis*, *Bacillus amyloliquefaciens plantarum/methylotrophicus* and *Bacillus pumilus/safensis*. In the two later cases, the strain matched two closely related species that cannot be differentiated by 16S rDNA (Fig. 4.1).

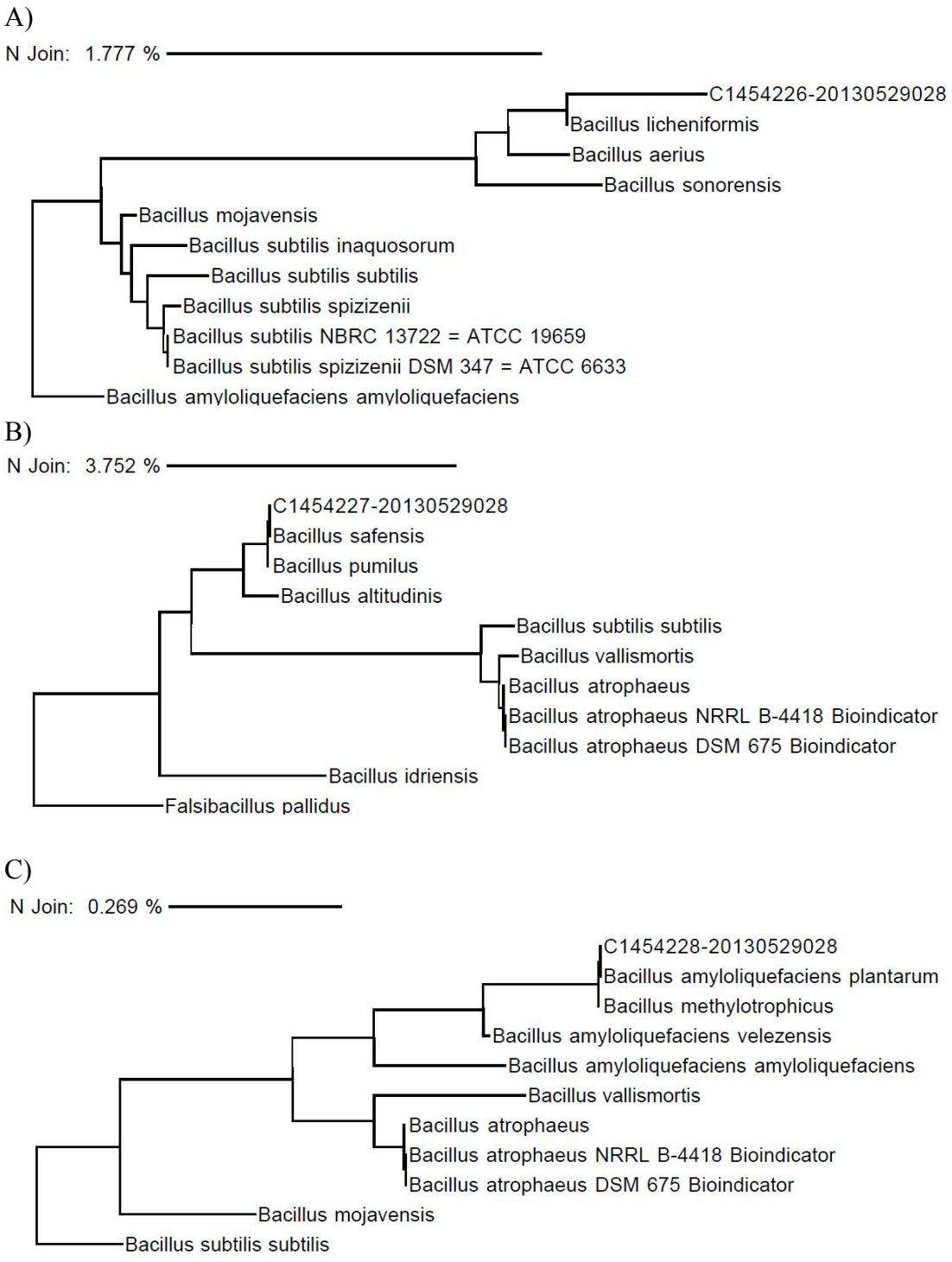

Figure 4.1 Phylogenetic trees constructed using the neighbor-joining method for each of the isolated strains. The N join scale bar provides a horizontal distance scale.

4.4.2 Survival

No significant difference in survival from hatching to the end of the trial was observed between treatments regardless of the species. However, survival was significantly higher in pompano (7.6 ± 1.9 %) and red drum (9.9 ± 0.8 %) compared to snook (2.4 ± 0.7 %) (Fig. 4.2). During the snook trial, poor survival led to the termination of one PB tank at 7 DPH, as well as one Control and one PB+ tank at DPH 9.

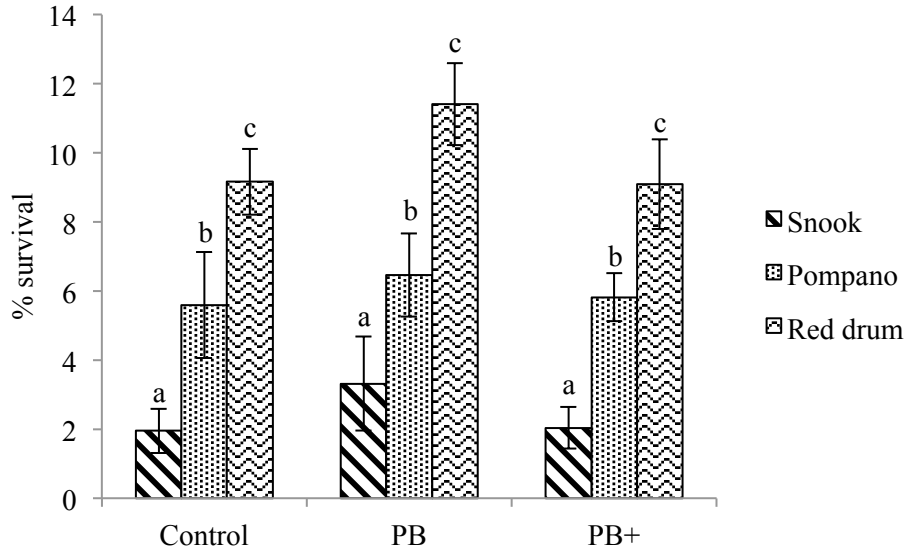

Figure 4.2 Survival from hatching for snook, pompano and red drum at the end of the trial. Mean ± standard error (n=3 for snook, n=4 for pompano and red rum). Letters indicate significant differences.

4.4.3 Growth

At the end of the pompano trial, PB and PB+ larvae had significantly greater standard length (Fig. 4.3A) and body depth (Fig. 4.3B) than the control larvae with 4.34 ± 0.10, 4.22 ± 0.07 and 3.89 ± 0.09 mm, respectively for standard length and 0.88 ± 0.01, 0.83 ± 0.03 and 0.66 ± 0.01, respectively for body depth. The same was true for snook larvae standard length with 3.69 ± 0.02, 3.60 ± 0.03 and 3.29 ± 0.03 mm, respectively for PB, PB+ and control larvae (Fig. 4.3C). However, no significant difference was observed for body depth with an average of 0.71 ± 0.20 mm at the end of the experiment (Fig. 4.3D). At the end of the red drum trial, no difference was observed between treatments with an average of 5.44 ± 0.07 mm (Table 4.1).

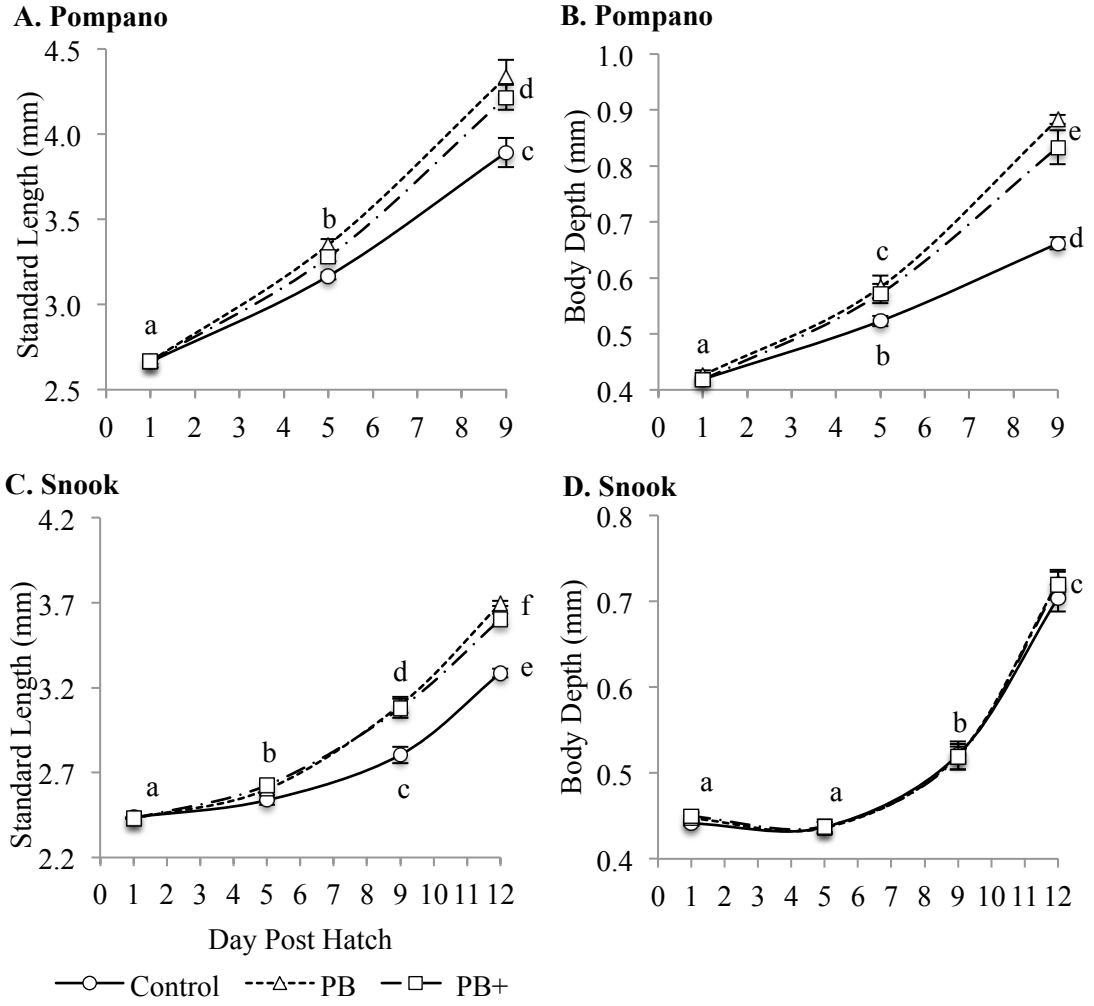

Figure 4. 3 Body depth and standard length of pompano and snook larvae during the trial. Mean ± standard error (n=4 for pompano, n=4 for snook at 1 and 5 DPH then n=3, 10 larvae per tank and time point). Letters indicate significant differences between times and treatments.

Table 4.1 Effects of a probiotic supplementation (PB and PB+) on standard length (mm) and specific activities ($U.mg^{-1}$ of protein) of trypsin, amylase, alkaline phosphatase (AP) and leucine-alanine peptidase (leu-ala) of red drum larvae. Mean ± standard error (n=4 tanks, 50 larvae per tank). No statistical differences were observed.

	Control	PB	PB+
Growth			
1 DPH	2.82 ± 0.01	2.81 ± 0.02	2.79 ± 0.01
7 DPH	3.51 ± 0.02	3.55 ± 0.01	3.58 ± 0.01
14 DPH	4.67 ± 0.01	4.48 ± 0.03	4.58 ± 0.02
21 DPH	5.54 ± 0.03	5.45 ± 0.08	5.32 ± 0.08
Specific activity at 21 DPH			
Trypsin	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
Amylase	0.56 ± 0.04	0.42 ± 0.07	0.51 ± 0.03
AP	0.09 ± 0.01	0.08 ± 0.01	0.10 ± 0.00
Leu-ala	145.8 ± 4.2	118.9 ± 3.2	143.1 ± 5.8

4.4.4 Bacterial analyses

Results from the microbiological analyses on the pompano larvae showed significantly higher counts of colony-forming units (CFU) per larvae on the marine agar media for the larvae fed the probiotics supplementation ($38.10^3 \pm 8.10^3$ CFU for PB and $18.10^3 \pm 22.10^3$ CFU for PB+) compared to the control larvae ($10^3 \pm 0.6.10^3$ CFU). Numbers of presumptive *Vibrio* on the TCBS media were low and not significantly different between treatments with an average of 0.06 ± 10^3 CFU per larvae (Table 4.2)

Table 4.2 Number of colony-forming units ($x10^3 \pm$ standard error of the mean) per pompano larvae (n=4 tank, 10 larvae per tank) fed without probiotic supplementation (Control), with probiotic supplementation in the live food (PB) or with probiotic supplementation in the live food and tank water (PB+). Superscript letters indicate significant differences within the same column (Tukey test, $p<0.05$).

	Marine Agar	TCBS
Control	1.0 ± 0.6^a	0.01 ± 0.00^a
PB	38 ± 8^b	0.15 ± 0.01^a
PB+	18 ± 22^b	0.02 ± 0.01^a

4.4.5 Enzyme activities

For both pompano and snook, trypsin specific activities at the end of the trial were significantly higher in larvae fed the PB and the PB+ treatments compared to the control larvae. Trypsin activities of snook larvae from the PB and PB+ treatments were respectively 37.3 % and 29.6 % higher than that of control larvae, while trypsin

activities of pompano larvae from the same treatments were respectively 45.1 % and 46.8 % higher than that of control larvae (Fig. 4.4A).

Difference in amylase activity was only observed for snook larvae, with activities of larvae from the PB+ treatment 65.2 % higher than that of control larvae (Fig. 4.4B).

AP activities of pompano larvae from the PB and PB+ treatments and snook larvae from the PB+ treatment were higher than that of control larvae. AP activity of snook larvae from the PB+ treatments was 27.1 % higher than that of control larvae while AP activities of pompano larvae from the PB and PB+ treatments were respectively 27.9 % and 28.0 % higher than that of control larvae. For all treatments, AP activities of pompano larvae were significantly higher than that of snook larvae (Fig. 4.4C).

For both snook and pompano, no significant differences were observed in Leu-ala activities between treatments or between species (Fig. 4.4D).

No significant differences were observed in the activities of the enzymes tested for red drum at the end of the trial (Table 4.1).

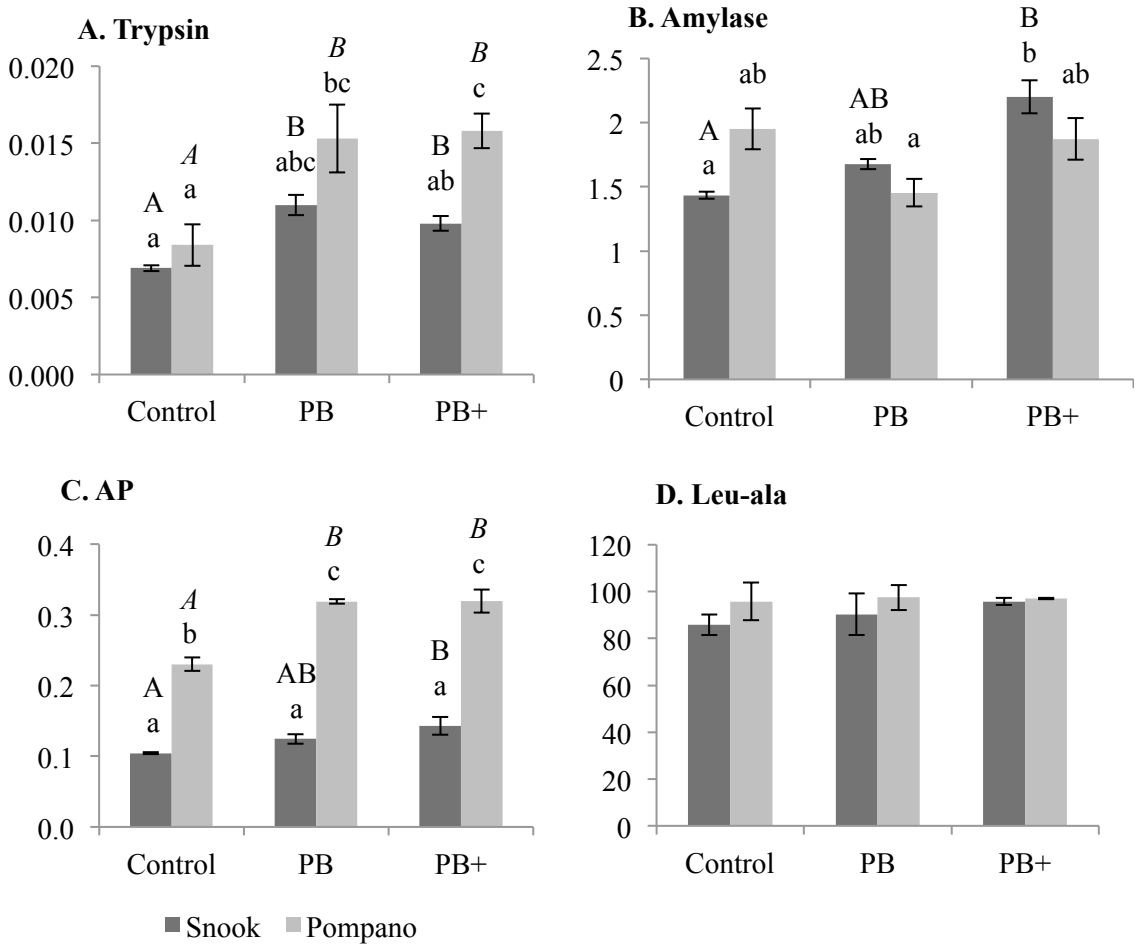

Figure 4.4 Specific activities (U.mg^{-1} of protein) of trypsin, amylase, alkaline phosphatase (AP) and leucine-alanine peptidase (leu-ala) of snook and pompano larvae at the end of the trial. Mean ± standard error (n=3 and 4 respectively for snook and pompano, 50 larvae per tank). Letters indicate significant differences between treatments for snook (uppercase letters), pompano (italic uppercase letters) and between treatment and species (lower case letter).

4.5 DISCUSSION

This series of experiments suggested a beneficial effect of *Bacillus* probiotic on growth and digestive enzyme activity of Florida pompano and Common snook larvae when supplemented during the early larval stages. Similar results have been observed with Indian white shrimp (Ziaei-Nejad et al., 2006), common carp (Wang and Zirong, 2006), Pacific white shrimp (Wang, 2007), Japanese flounder (Ye et al., 2011), rohu (Mohapatra et al., 2012) and orange-spotted grouper (Sun et al., 2013).

The three strains identified in the commercial mix are species closely related to *Bacillus subtilis* however, they differ metabolically and secrete different enzymes (Priest et al., 1987). *B. pumilus* isolated from the gut of rohu fingerlings was found to produce amylase and cellulase (Ghosh et al., 2002) and it demonstrated strong inhibition against several strains of *Vibrio* sp. when isolated from the gut of black tiger shrimp (Hill et al., 2009). *B. licheniformis* has been reported to have antiviral properties through the induction of cytokines (Arena et al., 2006) and to produce an antimicrobial peptide with a broad inhibitory spectrum (Cladera-Olivera et al., 2004). In addition it was shown to produce phytase when isolated from several freshwater cultured fish (Dan and Ray, 2013). *B. amyloliquefaciens* is closely related to *B. subtilis* and used to be given a subspecies status (*B. subtilis* subsp. *amyloliquefaciens)* before additional studies demonstrated the numerous physiological and biochemical specificities of *B. amyloliquefaciens* (Priest et al., 1987). One of the main differences between the two species is the ability of *B. amyloliquefaciens* to produce more extracellular enzyme than *B. subtilis*, including between 50 and 150 times more α-amylase (Priest et al., 1987; Welker and Campbell, 1967). In fish, *B. amyloliquefaciens* was found to have an inhibitory effect

on pathogenic *Aeromonas hydrophila* associated with the eel *Anguilla anguilla* (Cao et al., 2011), and improved growth, feed conversion ratio and immunological parameters in Nile tilapia *Oreochromis niloticus* (Ridha and Azad, 2012). The addition of either *B. subtilis*, *B. licheniformis* or *B. pumilus* to the diet of olive flounder *Paralichthys olivaceus*, led to different effects with *B. subtilis* increasing growth, *B. subtilis* and *B. pumilus* increasing survival rate, and *B. pumilus* and *B. licheniformis* increasing superoxide dismutase activity and disease resistance (Cha et al., 2013). These results highlight the interest of supplementing several strains of probiotics simultaneously. Nonetheless, it is likely that the inclusion rate of each strain will impact on the final effect of the product and therefore manufacturers should communicate not only on the qualitative characteristics of their product, but also on the quantitative characteristics.

Several modes of action are proposed to explain the positive effect of probiotics, including antagonism towards pathogens, competition for adhesion sites and competitions for nutrients (Ray et al., 2012). However, the microbiological analyses on the pompano larvae at the end of the trial did not show high vibrio counts in any of the treatments. Therefore, it was assumed that in the experimental system used for these trials, pathogenic bacteria were not a major issue and no microbiological analyses were performed for the other species. Counts of heterotrophic bacteria were significantly higher in the PB and PB+ treatments compared to the control, confirming the presence of the probiotics in the gut of the larvae.

Probiotics can also act on the digestive system of their host. Poorly developed at hatching, the digestive system of marine fish larvae matures progressively, evolving from an intracellular mode of digestion via pinocytosis, to an adult mode of digestion

involving membrane transport with the development of the brush border membrane (Govoni et al., 1986). Alkaline phosphatase, an enzyme mainly located in the brush border membrane of enterocytes, is therefore a good indicator of intestinal development (Cahu and Zambonino-Infante, 1995a). Simultaneously to the intestine maturation, the functional maturation of the pancreas occurs, with an activity increase of proteolytic enzymes, including trypsin, and a decrease in the carbolytic enzyme amylase (Cahu and Zambonino-Infante, 1994). In this study, results from the enzyme analyses showed an increase in trypsin specific activity for the pompano and snook larvae fed the probiotic supplementation. In addition, the specific activity of alkaline phosphatase was significantly higher for pompano larvae fed the PB and PB+ treatments and for snook larvae fed the PB+ treatment, suggesting an early maturation of the digestive system. The higher amylase specific activity in snook larvae fed the PB+ treatment might be due to a higher capacity of snook to utilize carbohydrates, which could be stimulated by the important extracellular amylase production by *B. amyloliquefaciens.* Very little is known about the natural diet of the early larval stage of snook and pompano and more research is needed to understand such variations.

It was hypothesized that the increase in enzyme activities observed with the use of probiotics could be due to the exoenzymes produced by the bacteria (Bairagi et al., 2002; Balcázar et al., 2006). Nonetheless, Ziaei-Nejad et al. (2006) demonstrated that the proportion of enzyme synthesized by the probiotics could only contribute to a very small amount of the total enzyme activity of the gut and suggested that instead, the probiotics stimulate the production of endogenous enzymes.

In the present study, the increased level of maturation of the enterocytes of the snook and pompano larvae fed the probiotic supplementation suggested an increased absorptive capacity of the brush-border membrane leading to more efficient feed utilization and better growth.

Pompano and snook larvae are not as robust as red drum larvae and seem to benefit more from the probiotic supplementation. However, no detrimental effect was observed for the red drum larvae and the probiotics might have influenced factors other than growth and digestive enzyme activity, such as disease and stress resistance, intestinal epithelium structure or general welfare as discussed above. In addition, a longer trial period might have revealed some differences in growth as Ridha and Azad (2012) did not observe any growth differences after 99 days when juvenile Nile tilapia were fed a diet enriched with *Bacillus* but observed differences 61 day after the end of the treatment compared to the control treatment. Moreover, a higher level of probiotic supplementation might be necessary. Indeed, Merrifield et al. (2010) showed that high intestinal levels of *B. subtilis* and *B. licheniformis* (>80 %) are required to improve rainbow trout growth performance and feed utilization.

No difference in survival was observed between treatments for all species although an increase in survival would be expected alongside the advanced digestive system maturation and improved growth, especially for snook where survival is very low. Many factors participate in the survival of young fish larvae and snook being a relatively new species in aquaculture, many rearing aspects still need to be explored and improved. Even though the probiotic supplementation did not increase survival it is likely that larvae with improved growth and digestive capabilities could be more

robust and a difference in survival might be observed after critical life events such as metamorphosis and weaning.

In conclusion, these experiments demonstrated positive effects from the mix of *Bacillus* on the development of pompano and snook larvae through an early maturation of the digestive tract. To obtain optimal effects, a supplementation through both the live feed and the tank water seems recommended. Further research is needed to better understand the mode of action of probiotics and the mechanisms involved during the ontogeny of the digestive system.

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Chapter 5 Impacts of three different microdiets on Florida Pompano, *Trachinotus carolinus*, weaning success, growth, fatty acids incorporation and enzyme activity

RESEARCH ARTICLE
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Contributions: The present manuscript was compiled and written in full by the author of this thesis. Larval rearing, sampling, lab and statistical analyses have been carried out by the candidate except for the fatty acid analyses that were performed by Cynthia Faulk from the Fisheries and Mariculture Laboratory at the University of Texas Marine Science Institute. Co-authors provided assistance with the proofreading of the manuscript.

5.1 ABSTRACT

In this study, three microdiets were tested on weaning of Florida pompano larvae: Otohime, Gemma and a reference diet LR803. The experimental system was stocked with 11-day-old larvae, which were co-fed micro-diets and live food from 11 dph to 17 dph then micro-diets only until 28 dph. Survival from 11 dph to 28 dph was similar for all treatments, with an average of 33%. At the end of the trial, the Gemma larvae were significantly longer and heavier than larvae fed the other diets. Significant differences were observed in fatty acid composition of the diets and larvae between treatments. The Gemma larvae incorporated the lowest amount of eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and arachidonic acid (ARA). However, they had the highest DHA/EPA and ARA/EPA ratios, which is in agreement with the concept that the proportions of polyunsaturated fatty acids could be of greater importance than their absolute amount. Results from the enzyme analysis suggest that fishmeal is suitable as the main protein source for Florida pompano larvae compared to krill meal. This study gives new insights on Florida pompano early nutritional requirements and demonstrated the full functionality of the pancreas at 16 days post hatch, opening possibilities of an earlier weaning time.

Keywords: Co-feeding, Enzyme, Fatty acids, Fish larvae, Florida pompano, Microdiets, *Trachinotus carolinus*, Weaning

5.2 INTRODUCTION

The Florida pompano, *Trachinotus carolinus*, is found along the coasts of the eastern Atlantic ocean and Gulf of Mexico, from Massachusetts down to Brazil, with the highest abundance along the coast of Florida (Gilbert, 1986; Smith-Vaniz, 2002). Belonging to the Carangidae family, it is a popular food and game fish (Iversen and Berry, 1969; Weirich et al., 2006). The commercial fishery in the United States has remained small and the demand is continuously increasing and higher than the supply (Weirich et al., 2006). In 2011, with only 102.4 metric tons of commercial landing, the whole-fish dockside price reached US$ 8.92/kg (NOAA/NMFS, 2013). In addition to a high retail price and consumer demand, the Florida pompano has a fast growth rate and can withstand high densities, making it a prime candidate for aquaculture production (Iversen and Berry, 1969; Moe et al., 1968; Weirich et al., 2006). However, difficulties in producing large quantities of juveniles have hindered the development of Florida pompano farming and the development of reliable hatchery protocols is essential (Cavalin and Weirich, 2009; Riley et al., 2009).

One of the main challenges of larval rearing is to provide adequate nutrition to support the fast growth and development of larvae. Traditionally, most marine finfish larvae are first fed rotifers then *Artemia* until the end of metamorphosis when they are slowly weaned on to a commercial dry diet (Rosenlund et al., 1997). However, live food production is costly, time consuming and *Artemia* nutritional value varies tremendously depending on strain, origin and even batches from the same location (Conceição et al., 2010; Lavens and Sorgeloos, 2000). Rotifers and *Artemia* are both deficient in essential fatty acids, which are a fundamental source of energy and structural components for larval development (Sargent et al., 1997). Indeed, marine

fish larvae are unable to elongate and desaturate 18:3n-3 and 18:2n-6 to polyunsaturated fatty acids (PUFAs) and these PUFAs must therefore be supplied by the diet (Bell, 2003; Sargent et al., 1999b, 1989). In addition to a poor nutritional profile live food may also transfer harmful bacteria to the larvae and the microbial control of live food cultures is a difficult procedure (Olafsen, 2001).

To achieve an economical and reliable production of juveniles in marine finfish aquaculture it is critical to develop micro-particulate diets that provide the adequate nutrition to the larvae. Research from the past decade showed that unlike previously suggested (Dabrowski, 1984; Kolkovski et al., 1993; Lauff and Hofer, 1984), fish larvae do possess the necessary enzymes to digest an inert diet at the onset of exogenous feeding (Cahu and Zambonino-Infante, 2001). However, fish larvae have very different nutritional requirements compared to juveniles. For example, it was found that dietary lipids (Coutteau et al., 1997) and protein hydrolysates (Zambonino-Infante et al., 1997) are two crucial components for the growth and development of larvae but are not essential for juveniles. Therefore, larval diets must be specifically designed to meet these particular needs. In addition, the design of the diet must take into consideration the reduced attraction of larvae for inert particles (Cox and Pankhurst, 2000; Fernandez-Diaz et al., 1994), the risk of leaching of nutrients due to the high surface to volume ratio (Kvåle et al., 2006; Langdon, 2003) and the effects on water quality (Bonaldo et al., 2011; Fletcher et al., 2007). While Cahu et al. (2003) successfully reared seabass *Dicentrarchus labrax* on an experimental diet with total elimination of live food, a live food period is still required for most marine fish larvae.

Co-feeding microdiets and live food has been shown to improve larval performance compared to feeding microdiets or live food alone in many species such as red sea bream *Pagrus major* and Japanese flounder *Paralichthys olivaceus* (Kanazawa et al., 1989), yellow-finned black porgy *Acanthopagrus latus* (Leu et al., 1991), red drum *Sciaenops ocellatus* (Holt, 1993), Atlantic cod *Gadus morhua* (Callan et al., 2003), barramundi *Lates calcarifer* (Curnow et al., 2006) or Senegalese sole *Solea senegalensis* (Engrola et al., 2009). Two main factors contribute to the success of co-feeding protocols. First, live food visual and chemical stimulation facilitates the ingestion of the microdiet, conditioning the larvae to prey on inert particles and allowing for an earlier weaning to dry feed (Canavate and Fernandez-Diaz, 1999; Rosenlund et al., 1997). Second, live prey carry numerous nutritional factors stimulating pancreatic enzyme secretions and endocrine responses which contributes to the maturation of the digestive system (Kolkovski et al., 1997; W Koven et al., 2001). The maturation of the digestive system is characterized by the progressive decline of the early mode of protein digestion and absorption (through pinocytosis and intracellular digestion) and the simultaneous increase of extracellular digestion and membrane transport as the enterocytes brush border membrane develops (Govoni et al., 1986). The study of the pancreatic, cytosolic and brush border membrane enzymes give essential information on this maturation process (Zambonino-Infante and Cahu, 2001).

The aim of the present study was to gain knowledge on Florida pompano larvae nutritional requirements and digestive abilities by studying the impact of co-feeding three different microdiets on larval performances, lipid incorporation and digestive enzyme activity.

5.3 MATERIALS AND METHODS

5.3.1 Experimental set up

Eggs were obtained from a captive spawn at the Mote Marine Laboratory Aquaculture Research Park in Sarasota, Florida. The broodstock population was collected in 2008 off the southwest coast of Florida and conditioned using temperature and photoperiod control in a 25m^3 tank equipped with a recirculating filtration system. After 6 weeks under simulated natural conditions, fish were sampled to check their reproductive status and females with oocytes in late secondary growth (410 ± 21 μm, n=14 females, 50 oocytes per females) were implanted with Ovaplant® at 50μg/kg (Western Chemical Inc.). Eggs were incubated in a 100L conical hatching tank with an upwelling water flow recirculating with a 3.3m^3 tank equipped with a filtration system. After hatching, the water flow was reduced and the screen of the overflow pipe removed to allow for a gentle release of the larvae in the tank and the collection of dead embryos and egg casings at the bottom of the hatching tank.

Approximately 200,000 larvae were reared in the 3.3 m^3 tank (temperature 26 ± 1 °C, salinity 35 ± 1 g L^{-1}, dissolved oxygen of 6 ± 1 mg L^{-1} and pH of 8 ± 0.5) up to 11 days post hatch (dph). Rotifers enriched with Algamac 3050 (Aquafauna Bio-Marine Inc., Hawthorne, CA, USA) were fed to the larvae at 5 ml^{-1} from 2 to 11 dph and *Artemia* were introduced from 9 dph at 3 ml^{-1}. Up to 11 dph, the tank water was shaded with RotiGrow *plus* (Reed Mariculture Inc, CA, USA) at 500,000 cells L^{-1}. At dph 11, the water level in the production tank was lowered and the larvae were collected and transferred to twelve 130 L experimental tanks (870 larvae $tank^{-1}$,

counted manually, equivalent to 6-7 larvae L^{-1}) corresponding to three microdiets tested in quadruplicate: 1) Otohime (Commercial diet, Marubeni Nisshin Feed Co. Japan), 2) Gemma (Commercial diet, Skretting, France) and 3) Larval Reference Diet 803 – LR803 (Experimental diet, Agricultural Research Service, USA). The diet ingredients are listed in Table 5.1.

Table 5.1 Ingredients in the microdiets tested, as communicated by the manufacturer.

Microdiet	Ingredients
Otohime	Krill meal, fish meal, squid meal, potato starch, wheat flour, fish oil, brewers yeast, calcium phosphate, guar gum, soy lecithin, betaine, licorice plant, apple extract, wheat germ.
Gemma	Fish meal, algae, fish oils, lecithin, betaine, wheat gluten, vitamins, minerals.
LR803	Squid meal, krill meal, anchovy oil, lecithin, wheat gluten meal, vitamins, di-calcium phosphate, taurine, vitamin C, astaxanthin.

Larvae were co-fed one of three microdiets, rotifers at 3 ml^{-1} and *Artemia* at 2 ml^{-1} from 11 to 13 dph, followed by microdiet and *Artemia* at 1 ml^{-1} until 16 dph. From 17 to 28 dph larvae were only fed microdiets through automatic feeders. Microdiets were overfed to ensure larvae satiation and tanks were siphoned on a daily basis to maintain tank hygiene. The experiment was ended at dph 28 when fish had completed metamorphosis, had been fully weaned for over 10 days and required transfer to a larger system.

Larvae were sampled at 11, 16, 22, 26 and 28 dph to record standard length and body depth (10 larvae $tank^{-1}$, 40 $treatment^{-1}$). Body depth was measured from the insertion of the first dorsal spine to the to the most ventral point on the base of the body, as

pictured in Cavalin and Weirich, 2009. Wet weight was recorded on 22, 26 and 28 dph only due to the very low weight of the larvae prior to 22 dph and the lack of analytical precision (10 larvae tank^{-1}). At 16, 22 and 28 dph, 10 larvae from each tank were preserved for enzyme analysis. At the end of the trial (28 dph), all fish were counted to determine survival and 10 larvae from each tank were preserved for proximate and fatty acid analysis.

5.3.2 Enzyme analysis

Enzyme analyses were performed at the Functional Physiology of Marine Organisms Unit at Ifremer, Brest, France. Larvae were dissected under a microscope on a glass plate at 0 °C to separate the pancreatic segment from the intestinal segment as described in Cahu and Zambonino-Infante (1994). Trypsin and amylase activities were assayed according to Holm et al. (1988) and Métais and Bieth (1968) respectively. Enzymes of the brush border membrane, alkaline phosphatase (AP) and leucine aminopeptidase (AN), were assayed according to Bessey et al. (1946) and Maroux et al. (1973) respectively. Leucine–alanine peptidase (leu-ala) assays were performed using the method of Nicholson and Kim (1975). Enzyme activities are expressed as specific activities (i.e. U/mg protein). Protein was determined by the Bradford procedure (Bradford, 1976).

5.3.3 Proximate and lipid analyses

Proximate analyses were performed at the University of Stirling Institute of Aquaculture Nutrition Group, in Stirling, Scotland. Proximate composition of

microdiets and larvae at the end of the trial were determined according to standard procedures (AOAC, 2000). Prior to analysis, the larvae from each tank were pooled and minced. Moisture content was determined by drying the samples at 105 °C for 24 h. Ash content was determined after 24 h in crucibles at 600 °C. Crude protein content (Nx6.25) was determined using the automated Kjeldahl method (Tecator Kjeltec Auto 1030 analyzer, Foss, Warrington, U.K). Crude lipid content was determined after extraction according to Folch et al. (1957).

Lipid analyses were performed by the Fisheries and Mariculture Laboratory at the University of Texas Marine Science Institute (UTMSI) in Port Aransas, Texas, USA. Lipids were extracted according to Folch et al. (1957) and the fatty acid composition was determined by gas-liquid chromatography after preparation of fatty acid methyl esters (FAMEs) according to Morrison and Smith (1964). FAMEs were separated and quantified on a gas chromatograph (Shimadzu GC-2014, Shimadzu Scientific Instruments, Columbia, MD, USA) equipped with a Phenomenex ZB-WAX plus capillary column (30 m long, 0.53 mm internal diameter, 1.0 μm thickness; Phenomenex, Torrance, CA, USA) with on-column injection and flame ionization detection, using helium as carrier gas (4 mL min^{-1}) and injector and detector temperatures of 250 and 260 °C respectively. Temperature was held at 160°C for 5 min then increased up to 220°C at 3°C per minute and maintained at this temperature for 30 minutes. FAMEs peaks were identified by comparison with known standards (Supelco, Inc., Bellefonte, Pennsylvania, USA).

5.3.4 Statistical analysis

Statistical analysis was performed with MINITAB® version 16.0 (Minitab Ltd., Coventry, UK). Normality and homogeneity of variance were confirmed using Kolmogorov‑Smirnov test. Growth, body depth, wet weight and enzyme activities were compared using a General Linear Model (GLM) with all time and treatment interactions being analyzed and significant differences grouped by a Tukey post hoc test with 95 % confidence. Survival, proximate analysis and fatty acid data were arcsine square root transformed before a one-way ANOVA followed by a Tukey post hoc test with 95 % confidence. Linear regression was performed to evaluate the incorporation of selected fatty acids from the diet into larval tissues. All data are presented as mean ± standard error of the mean (SEM) and level of statistical significance was set at $P<0.05$.

5.4 RESULTS

5.4.1 Survival

Survival during the experiment (from 11 to 28 dph) was similar for all treatments with 32.1 ± 1.2 %, 32.6 ± 0.8 % and 33.3 ± 0.9 % for the fish fed the Gemma, LR803 and Otohime diets, respectively.

5.4.2 Growth

From 11 to 22 dph, larvae standard length was similar for all treatments (Fig. 5.1A). By 26 dph, the Gemma larvae were significantly longer than the larvae fed the other

diets. This trend continued at 28 dph with Gemma > LR803 > Otohime larvae. In addition, from 26 to 28 dph, the Otohime larvae exhibited a slower growth compared to the other treatments with an average length at 28 dph not statistically different from that of 26 dph (Fig. 5.1A).

The first significant difference in body depth was observed at 16 dph with the Gemma larvae being larger than the LR803 and Otohime larvae (Fig. 5.1B). The same trend was observed at 28 dph.

From 26 dph onwards, the Gemma larvae were significantly heavier than the LR803 and Otohime larvae (Fig. 5.1C). No difference was observed between the other two diets.

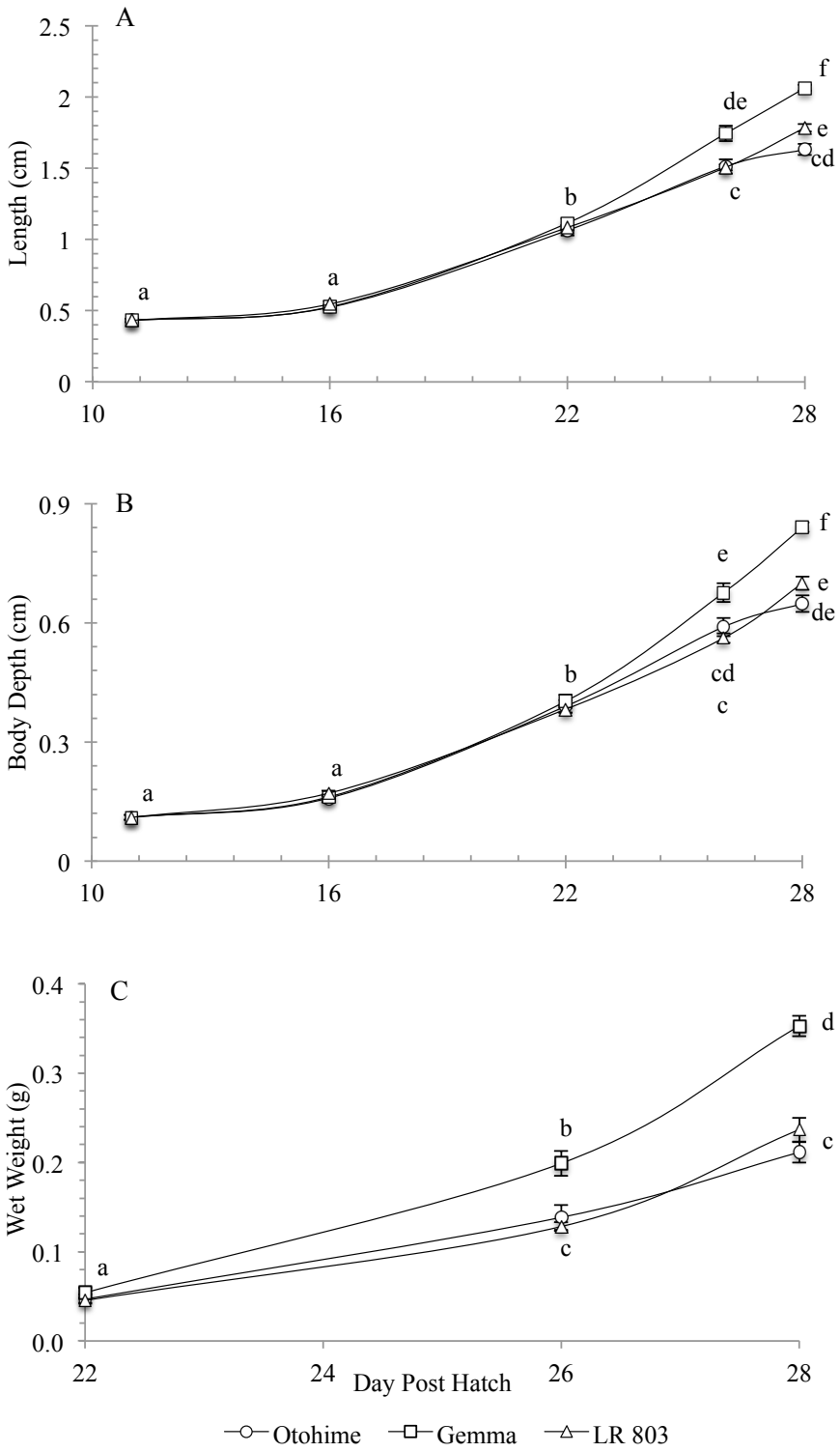

Figure 5.1 Standard length (A) Body depth (B) and wet weight (C) of Florida pompano larvae fed three different diets. Values are mean ± SEM (n = 4 tanks, 10 larvae per tank and time point). Letters indicate significant differences between treatments and time points (Tukey test, $p<0.05$).

5.4.3 Proximate analysis

Lipid content was significantly different between microdiets (Table 5.2). Highest lipid content was found in the LR803 diet (26.8 ± 0.1 %), compared to the Otohime diet (21.2 ± 1.6 %) and the Gemma diet (18.9 ± 0.5 %). At the end of the trial, no difference was observed in the lipid content of the larvae.

Protein content was significantly different between diets, with the Gemma diet containing 57.6 ± 0.1 % of protein, a significantly higher content than that of the Otohime diet (52.3 ± 0.1 %) and the LR803 diet (50.9 ± 0.1 %). The LR803 diet had a significantly lower protein content compared to the two other diets. At the end of the trial, the Gemma and Otohime larvae had similar protein contents (16.3 ± 0.3 % and 16.1 ± 0.3 % respectively), significantly higher than that of the LR803 larvae (14.5 ± 0.8 %).

Moisture and ash content were significantly lower for the LR803 diet (respectively 5.6 ± 0.1 and 3.7 ± 0.1%) compared to the other diets. Moisture content was similar for the Gemma and Otohime diets (6.4 ± 0.1 %) while ash content was significantly higher for the Otohime diet compared to the Gemma diet (respectively 12.8 ± 0.1 % and 10.8 ± 0.1 %). However, at the end of the trial no difference was observed in the moisture and ash content of the larvae fed the different treatments with an average moisture content of 61.2 ± 0.3 % and an average ash content of 10.6 ± 0.4 % (Table 5.2).

Table 5.2 Proximate analysis (% of wet weight) of the different microdiets and larvae (mean ± SEM, n = 4 tanks, 10 pooled larvae per tank) at the end of the trial. Letters indicate significant differences within the same column (Tukey test; $p<0.05$).

Sample	% Lipid	% Protein	% Moisture	% Ash
Gemma diet	18.9^{b} ± 0.5	57.6^{d} ± 0.1	6.4^{b} ± 0.1	10.8^{b} ± 0.1
Otohime diet	21.2^{b} ± 1.6	52.3^{c} ± 0.1	6.4^{b} ± 0.1	12.8^{c} ± 0.1
LR803 diet	26.8^{c} ± 0.1	50.9^{c} ± 0.1	5.6^{a} ± 0.1	3.7^{a} ± 0.1
Larvae fed Gemma diet	9.9^{a} ± 0.8	16.3^{b} ± 0.3	61.4^{c} ± 0.3	10.2^{b} ± 0.2
Larvae fed Otohime diet	10.5^{a} ± 0.9	16.1^{b} ± 0.3	60.6^{c} ± 0.3	10.8^{b} ± 0.6
Larvae fed LR803 diet	10.7^{a} ± 0.6	14.5^{a} ± 0.8	61.6^{c} ± 0.2	10.8^{b} ± 0.3

5.4.4 Fatty acid analysis

Fatty acid profile of the larvae at the end of the trial was strongly influenced by the fatty acid profile of the microdiets (Table 5.3). The main differences were observed in linoleic acid (LA), ARA, EPA and DHA content.

LA content contrasted greatly between the diets, with LR803 and Gemma containing 19.84 ± 0.04 and 30.40 ± 0.05 % of LA respectively, as opposed to only 4.54 ± 0.02 % for Otohime diet. LA content in the larvae was correlated (R^2=0.96) with the diet content. The LR803 and Gemma larvae incorporated respectively 20.06 ± 0.06 % and 24.40 ± 0.32 % of LA, against 4.51 ± 0.13 % for the Otohime larvae.

There was also a strong correlation (R^2=0.98) between the ARA content of the microdiets and the ARA content of the larvae at the end of the trial. Highest ARA content was observed in the LR803 diet and larvae with respectively 0.55 ± 0.00 % and 0.59 ± 0.01 %. The Otohime diet had a similar ARA content (0.54 ± 0.01 %), however the Otohime larvae incorporated only 0.52 ± 0.01 % of ARA, significantly

less than the LR803 larvae. A significantly lower ARA content was observed in the Gemma diet and larvae, with respectively 0.34 ± 0.00 % and 0.30 ± 0.00 %.

The Otohime and LR803 diet and larvae contained the highest proportion of EPA with respectively 10.79 ± 0.05 % and 9.01 ± 0.08 % for the diet and 7.28 ± 0.08 % and 6.10 ± 0.13 % for the larvae. The Gemma diet and larvae contained the lowest proportion of EPA with respectively 4.12 ± 0.03 % and 2.21 ± 0.04 %.

The Otohime diet contained the highest DHA content (9.57 ± 0.01 %), significantly greater than the Gemma diet DHA content (7.32 ± 0.05 %) and the LR803 DHA content (5.36 ± 0.01 %). The Otohime larvae incorporated the highest DHA content with 11.22 ± 0.29 %. However, even though the Gemma diet contained significantly higher DHA content than the LR803 diet, the larvae fed these diets incorporated similar DHA contents (7.37 ± 0.11 and 7.12 ± 0.13 %) for the Gemma and LR803 larvae respectively.

Table 5.3 Fatty acid profiles of microdiets and larvae sampled at the end of the trial (means are ± SEM, n = 4 tanks, 10 pooled larvae per tank). Letters indicate significant differences within a same row (Tukey test, $p<0.05$).

Fatty acids as % of total fatty acids	Gemma diet	Otohime diet	LR803 diet	Larvae fed the Gemma diet	Larvae fed the Otohime diet	Larvae fed the LR803 diet
Saturated fatty acids						
12:0	$0.06^{c} \pm 0.00$	$0.14^{e} \pm 0.00$	$0.11^{d} \pm 0.00$	$0.03^{a} \pm 0.00$	$0.06^{c} \pm 0.00$	$0.05^{d} \pm 0.00$
14:0	$2.97^{ab} \pm 0.02$	$7.40^{b} \pm 0.05$	$6.83^{ab} \pm 0.04$	$2.36^{a} \pm 0.02$	$4.64^{ab} \pm 0.07$	$3.72^{ab} \pm 1.23$
15:0	$0.28^{a} \pm 0.00$	$0.46^{d} \pm 0.00$	$0.43^{cd} \pm 0.00$	$0.35^{b} \pm 0.01$	$0.33^{b} \pm 0.01$	$0.42^{c} \pm 0.00$
16:0	$17.93^{a} \pm 0.06$	$18.34^{a} \pm 0.05$	$19.94^{b} \pm 0.07$	$21.25^{c} \pm 0.17$	$20.77^{bc} \pm 0.28$	$18.87^{a} \pm 0.28$
17:0	$0.23^{a} \pm 0.00$	$0.23^{a} \pm 0.00$	$0.33^{c} \pm 0.00$	$0.28^{b} \pm 0.01$	$0.25^{ab} \pm 0.01$	$0.36^{d} \pm 0.01$
18:0	$3.16^{a} \pm 0.00$	$3.09^{a} \pm 0.01$	$3.54^{a} \pm 0.01$	$5.55^{bc} \pm 0.20$	$5.90^{c} \pm 0.31$	$4.80^{b} \pm 0.11$
Total	$24.62^{a} \pm 0.08$	$29.67^{bc} \pm 0.1$	$31.17^{bc} \pm 0.1$	$29.81^{bc} \pm 0.4$	$31.95^{c} \pm 0.6$	$28.22^{b} \pm 1.3$
Mono-unsaturated fatty acids						
15:1	$0.04^{a} \pm 0.00$	$0.09^{c} \pm 0.00$	$0.07^{b} \pm 0.00$	$0.04^{a} \pm 0.00$	$0.07^{b} \pm 0.00$	$0.07^{b} \pm 0.01$
16:1n7	$2.19^{a} \pm 0.01$	$5.26^{d} \pm 0.02$	$6.28^{e} \pm 0.02$	$2.40^{b} \pm 0.02$	$4.84^{c} \pm 0.06$	$6.38^{e} \pm 0.06$
18:1n9	$11.72^{b} \pm 0.02$	$12.25^{b} \pm 0.03$	$9.92^{a} \pm 0.02$	$13.92^{c} \pm 0.27$	$15.59^{d} \pm 0.51$	$11.24^{b} \pm 0.07$
18:1n7	$1.47^{a} \pm 0.01$	$4.21^{c} \pm 0.02$	$3.11^{b} \pm 0.02$	$1.42^{a} \pm 0.06$	$4.01^{c} \pm 0.17$	$3.46^{b} \pm 0.03$
20:1n9	$3.23^{b} \pm 0.00$	$5.37^{c} \pm 0.05$	$0.98^{a} \pm 0.01$	$3.53^{b} \pm 0.04$	$3.75^{b} \pm 0.26$	$0.68^{a} \pm 0.18$
Total	$18.66^{a} \pm 0.02$	$27.18^{d} \pm 0.06$	$20.36^{b} \pm 0.06$	$21.30^{c} \pm 0.2$	$28.26^{e} \pm 0.1$	$21.84^{c} \pm 0.15$
Poly-unsaturated fatty acids						
16:2n4	$0.45^{b} \pm 0.00$	$0.75^{d} \pm 0.01$	$1.42^{f} \pm 0.00$	$0.38^{a} \pm 0.01$	$0.64^{c} \pm 0.01$	$1.03^{e} \pm 0.01$
16:3n4	$0.29^{a} \pm 0.00$	$0.52^{c} \pm 0.00$	$0.99^{d} \pm 0.00$	$0.27^{b} \pm 0.01$	$0.46^{c} \pm 0.02$	$0.74^{e} \pm 0.01$
18:2n6	$30.40^{d} \pm 0.05$	$4.54^{a} \pm 0.02$	$19.84^{b} \pm 0.04$	$24.40^{c} \pm 0.32$	$4.51^{a} \pm 0.13$	$20.06^{b} \pm 0.06$
18:3n6	$0.03^{a} \pm 0.01$	$0.14^{b} \pm 0.00$	$0.21^{b} \pm 0.00$	$0.06^{a} \pm 0.00$	$0.17^{b} \pm 0.00$	$0.15^{b} \pm 0.02$
18:3n4	$0.07^{a} \pm 0.00$	$0.16^{b} \pm 0.00$	$0.27^{c} \pm 0.00$	$0.12^{a} \pm 0.01$	$0.17^{b} \pm 0.01$	$0.34^{c} \pm 0.02$
18:3n3	$3.59^{e} \pm 0.01$	$1.18^{b} \pm 0.01$	$3.08^{d} \pm 0.01$	$2.81^{c} \pm 0.05$	$1.07^{a} \pm 0.01$	$2.77^{c} \pm 0.02$
18:4n3	$1.63^{b} \pm 0.01$	$2.53^{d} \pm 0.01$	$1.48^{b} \pm 0.01$	$0.92^{a} \pm 0.03$	$1.73^{bc} \pm 0.02$	$0.83^{a} \pm 0.21$
20:2n6	$0.15^{a} \pm 0.00$	$0.15^{a} \pm 0.00$	$0.09^{a} \pm 0.00$	$0.78^{b} \pm 0.04$	$0.22^{a} \pm 0.01$	$0.30^{a} \pm 0.10$
20:3n6	$0.05^{a} \pm 0.00$	$0.09^{b} \pm 0.00$	$0.20^{d} \pm 0.02$	$0.12^{bc} \pm 0.01$	$0.14^{c} \pm 0.01$	$0.21^{d} \pm 0.01$
20:4n6	$0.34^{a} \pm 0.00$	$0.54^{bc} \pm 0.01$	$0.55^{bc} \pm 0.00$	$0.30^{a} \pm 0.01$	$0.52^{b} \pm 0.01$	$0.59^{c} \pm 0.01$
20:3n3	$0.10^{a} \pm 0.00$	$0.16^{b} \pm 0.00$	$0.16^{b} \pm 0.00$	$0.26^{d} \pm 0.01$	$0.18^{b} \pm 0.01$	$0.21^{c} \pm 0.01$
20:4n3	$0.30^{a} \pm 0.00$	$0.56^{b} \pm 0.01$	$0.56^{b} \pm 0.03$	$0.56^{b} \pm 0.02$	$1.03^{d} \pm 0.03$	$0.87^{c} \pm 0.03$
20:5n3	$4.12^{b} \pm 0.03$	$10.79^{f} \pm 0.05$	$9.01^{e} \pm 0.08$	$2.21^{a} \pm 0.04$	$7.28^{d} \pm 0.08$	$6.10^{c} \pm 0.13$
22:5n6	$0.17^{c} \pm 0.00$	$0.10^{a} \pm 0.01$	$0.16^{bc} \pm 0.00$	$0.18^{c} \pm 0.01$	$0.14^{b} \pm 0.00$	$0.20^{d} \pm 0.00$
22:5n3	$0.59^{a} \pm 0.01$	$0.95^{b} \pm 0.00$	$1.18^{c} \pm 0.00$	$1.01^{b} \pm 0.04$	$2.58^{d} \pm 0.06$	$2.55^{d} \pm 0.05$
22:6n3	$7.32^{b} \pm 0.05$	$9.57^{c} \pm 0.01$	$5.36^{a} \pm 0.01$	$7.37^{b} \pm 0.11$	$11.22^{d} \pm 0.29$	$7.12^{b} \pm 0.13$
Total	$49.60^{d} \pm 0.09$	$32.69^{a} \pm 0.04$	$44.54^{c} \pm 0.11$	$41.75^{b} \pm 0.51$	$32.06^{a} \pm 0.34$	$44.06^{c} \pm 0.37$
Total ω3	$17.66^{b} \pm 0.09$	$25.73^{d} \pm 0.06$	$20.82^{c} \pm 0.09$	$15.14^{a} \pm 0.21$	$25.09^{d} \pm 0.35$	$20.44^{c} \pm 0.23$
Total ω6	$31.13^{d} \pm 0.04$	$5.55^{a} \pm 0.01$	$21.04^{b} \pm 0.05$	$25.84^{c} \pm 0.36$	$5.70^{a} \pm 0.12$	$21.51^{b} \pm 0.15$
ω3/ω6 ratio	$0.57^{a} \pm 0.00$	$4.63^{c} \pm 0.02$	$0.99^{b} \pm 0.00$	$0.59^{a} \pm 0.01$	$4.40^{c} \pm 0.13$	$0.95^{b} \pm 0.01$
ARA/EPA ratio	$0.08^{d} \pm 0.00$	$0.05^{a} \pm 0.00$	$0.06^{b} \pm 0.00$	$0.14^{f} \pm 0.00$	$0.07^{c} \pm 0.00$	$0.1^{e} \pm 0.00$
DHA/EPA ratio	$1.78^{e} \pm 0.01$	$0.89^{b} \pm 0.00$	$0.59^{a} \pm 0.01$	$3.33^{f} \pm 0.08$	$1.54^{d} \pm 0.05$	$1.17^{c} \pm 0.03$

5.4.5 Enzyme analyses

Trypsin activity in the pancreatic segment did not vary for the Otohime larvae (Fig. 5.2A). However, for the Gemma and LR803 larvae, the activity increased two-fold from 16 to 22 dph, and then decreased from 22 to 28 dph to levels similar to 16 dph. In the intestinal segment, trypsin activity remained steady in the LR803 larvae throughout the trial period, while a two-fold increase was observed for the Gemma larvae (Fig. 5.2B). A strong increase (4 fold) was observed in the Otohime larvae during the first half of the trial followed by a decrease in activity from 22 to 28 dph.

Amylase activity in the pancreatic segment exhibited a similar pattern for the larvae from all treatments with a sharp decrease between 16 and 22 dph and stabilization from 22 and 28 dph (Fig. 5.2C). In the intestinal segment, amylase specific activity did not vary significantly in the Gemma and LR803 larvae (Fig. 5.2D). However, a two-fold increase was observed between 16 and 22 dph in the Otohime larvae, that decrease between 22 and 28 dph, and reach levels similar to 16 dph.

In the brush border membrane, AP specific activity decreased by half between 22 and 28 dph in the Otohime larvae (Fig. 5.3A). In contrast, AP activity in the Gemma larvae appeared to increase between 22 and 28 dph though it was not statistically significant (40%, $p=0.087$). Activity levels did not vary significantly for the LR803 larvae.

Activity levels of AN increased more than two-fold between 22 and 28 dph in the Gemma larvae (Fig. 5.3B). A 50 % increase was also observed for the Otohime larvae, while no significant difference was found in the LR803 larvae. During the trial period, an increase in leu-ala activity was observed in larvae from all treatments although only significantly for the Otohime and Gemma larvae (Fig. 5.3C). Between

16 and 22 dph, a two-fold increase was observed in the Gemma larvae and a three-fold increase in the Otohime larvae. Between 22 and 28 dph, no significant variations were observed in the Gemma larvae while a 20 % decrease was observed in the Otohime larvae.

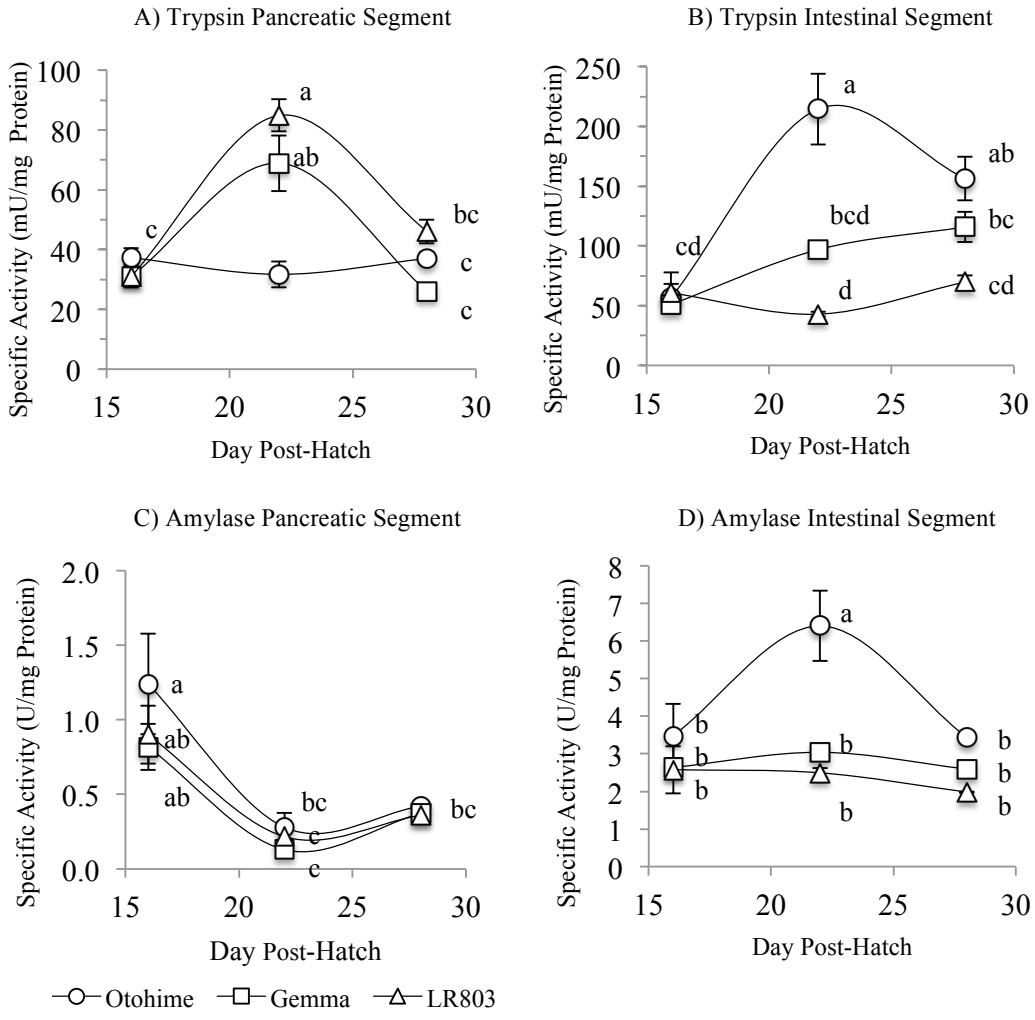

Figure 5.2 Specific activity (mU/mg protein) of trypsin and amylase in the pancreatic segment and intestinal segment of Florida pompano larvae fed different microdiets. Values are means ± SEM (n = 4 tanks, 10 pooled larvae per tank and time point). Letters indicate significant differences between treatments and time points (Tukey test, $p<0.05$).

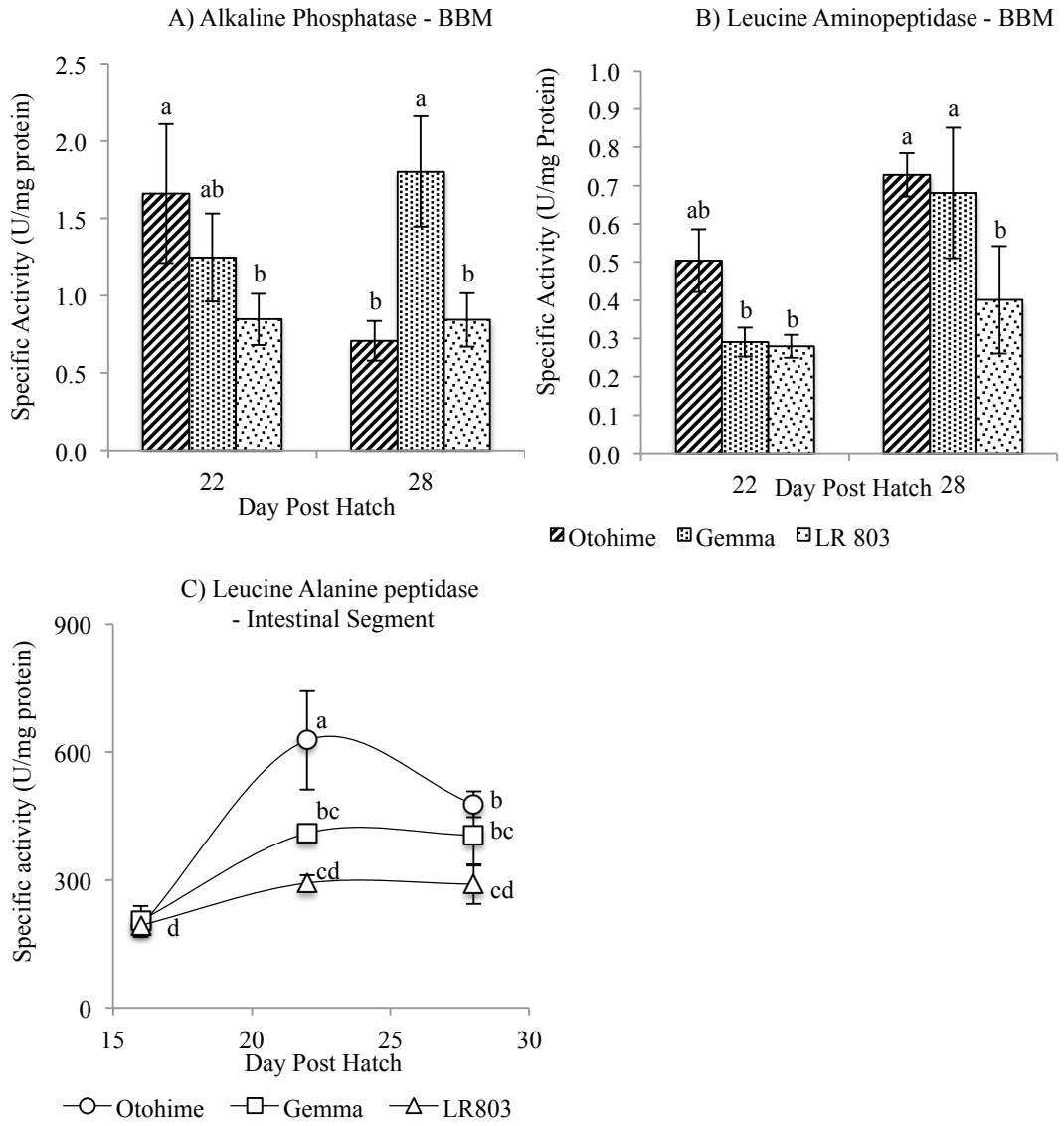

Figure 5.3 Specific activity (mU/mg protein) of alkaline phosphatase and leucine aminopeptidase in the brush border membrane (A and B), and of leucine alanine peptidase in the intestinal segment (C) of Florida pompano larvae fed different microdiets. Values are mean ± SEM (n = 4 tanks, 10 pooled larvae per tank and time point). Letters indicate significant differences between treatments and time points (Tukey test, $p<0.05$).

5.5 DISCUSSION

This study showed that microdiet composition had a major impact on Florida pompano larvae, influencing growth, fatty acid incorporation and activity of digestive enzymes. Standard length, body depth and wet weight were all enhanced by the Gemma diet compared to the Otohime and LR803 diet. Survival was similar for all treatments hence all diets were able to support the growth of larvae after the end of the live food period.

No differences were observed in the lipid, moisture and ash contents of the larvae despite significant differences in the proximate composition of the microdiets. This showed that the minimal lipid requirement was met and demonstrates the ability of Florida pompano larvae to tolerate some variations in the lipid dietary intake. However, significant differences were observed in the fatty acid profiles of the diets and larvae.

As previously mentioned, marine fish are unable to synthesize LC-PUFAs *de novo*. The dietary importance of PUFAs has been widely studied and reviewed (Sargent et al. 1989; Watanabe and Kiron 1994; Izquierdo 1996; Sargent et al. 1999b; Sargent et al. 2002). However, major uncertainties remain in PUFA dietary requirements as they vary between species and are determined not only by the absolute amount of PUFAs, but also by their relative ratios (Hamre et al., 2013; Izquierdo et al., 2000; Sargent et al., 1997).

DHA plays a critical role in the structure of cell membrane, especially for the correct development of the visual and immune systems (Bell *et al*., 1995; Rodríguez *et al*., 1997; Sargent, Bell, *et al*., 1999; Koven, 2003; Benítez-Santana *et al*., 2007). DHA and EPA compete for the same enzyme to esterify fatty acid into phospholipid

structures (Sargent, Mcevoy, *et al*., 1999). Several studies have demonstrated that DHA has a more dominant role in growth and membrane structure than EPA, suggesting that the relative proportions of DHA and EPA may have a greater impact than the absolute amount, with the necessity of a greater proportion of DHA than EPA (Glencross and Rutherford, 2011; Glencross, 2009; Reitan et al., 1994; Rodriguez et al., 1998; Watanabe, 1993). Our results agree with that hypothesis, where the Otohime diet and larvae displayed the highest DHA and EPA contents but also displayed the lowest growth. In contrast, the Gemma larvae, with the lowest DHA and EPA contents but with a DHA:EPA ratio more than double of that of the Otohime or LR803 treatments, exhibited the fastest growth. At the end of the experiment, DHA:EPA ratios in the larvae were significantly different from the ratios in the diet. The amount of EPA in the larvae was significantly lower than that of the diet for all treatments. In contrast, the amount of DHA was significantly higher in the Otohime and LR803 larvae than in the diets while it did not vary in the Gemma treatment. This resulted in significantly higher DHA:EPA ratios in the larvae than in the diet for all treatments. This suggests the ability of the larvae to modulate, to some degree, the incorporation of EPA and some activity of the Δ6 desaturase, even though the activity of this enzyme seems too low to convert enough EPA to DHA to compensate the dietary deficiency (Vagner and Santigosa, 2011).

Among the LC-PUFAs, DHA and EPA are the most abundant in marine fish eggs and tissues and the dietary requirements for these two fatty acids have been widely studied in comparison to ARA, though the latter plays a critical role in the development of the larvae (Sargent, Bell, et al., 1999; Bell, 2003). ARA is

specifically concentrated in fish eggs, attesting to its high biological importance during the early phases of larval development (Bell et al., 1997).

EPA and ARA compete for the enzymes involved in the production of eicosanoids, with eicosanoids produced from ARA being more biologically active, implying that, like DHA and EPA, the ratio of ARA to EPA might be of greater importance than the absolute quantity (Sargent, Mcevoy, et al., 1999; Tocher, 2003). Results from this experiment are consistent with these earlier observations. Both the Gemma diet and larvae presented a lower ARA and EPA content than for the other treatments. However, they presented the highest ARA:EPA ratio and displayed the fastest growth. These results reinforce the fact that LC-PUFA dietary requirements need to be considered as a whole, where the amount of each LC-PUFA influences the final ratios and the required metabolic pathways. An important difference in the fatty acid profile of the different diets tested in this trial was the amount of LA. Despite the higher levels of LA in the Gemma and LR803 diet, no increase in the content of the desaturation/elongation pathway products was observed and it is likely that this fatty acid was primarily used as a source of energy.

In addition to their impact on larvae development and fatty acid incorporation, microdiets can strongly influence the development of the digestive system (Cahu and Zambonino-Infante, 2001). Marine fish larvae have to switch from a primary mode of digestion to an adult mode of digestion; the maturational process is characterized by the acquisition of a progressive efficient secretion of pancreatic enzymes, and the transition from a cytosolic to a brush border membrane digestion at the intestinal level (Dabrowski, 1984; Ronnestad et al., 2013; Zambonino-Infante et al., 1997).

An increase in growth and survival has been reported in correlation with an increase of both pancreatic and intestinal enzyme activity in sea bass (Cahu and Zambonino-Infante, 1995b), sole (Ribeiro et al., 1999) and cod (Wold et al., 2007). Pancreatic enzymes specific activity (activity per gram of protein) follow the same pattern in temperate marine fish, with an increase during the first days/weeks of the life cycle depending on the species, followed by a decrease to a constant level, not due to a decline in the amount of digestive enzymes, but as a result of the increase of tissue proteins in the growing larvae (Zambonino-Infante and Cahu, 2001). Changes in the enzyme activity during ontogeny are genetically programmed, however the diet influences the plateau level of enzymes and can delay or stop the digestive system maturation process if inadequate (Cahu and Zambonino-Infante, 2001; Krogdahl and Sundby, 1999). In this trial, the composition of the different diets impacted the development of digestive functions of the larvae. Results from the amylase and trypsin analyses indicated that the pancreas is fully functional at 16 dph, which does not exclude that the pancreas may be functional at an earlier date. Amylase specific activity is high in young fish larvae then declines to a constant low level (Cahu and Zambonino-Infante, 1994; Péres et al., 1998; Ribeiro et al., 1999), a pattern comparable to the decline of lactase expression during the development of mammals (Freund et al., 1990). In the present study, the strong decline in amylase activity was observed in the pancreatic segment from 16 dph for all treatments. In the intestinal segment, amylase specific activity was significantly higher for larvae fed the Otohime diet indicating a stimulation in amylase secretion likely due to the potato starch contained in this diet. Indeed, starch content in the diet can influence amylase

expression as demonstrated in sea bass (Péres et al., 1998, 1996), red drum (Buchet et al., 2000) and yellow croaker *Pseudosciaena crocea* (Ma et al., 2005).

Trypsin is responsible for the digestion of proteins during the early development of the larvae and its activity is influenced by both the source and quantity of protein in the diet (Guerreiro et al., 2010; Péres et al., 1996; Zambonino-Infante et al., 1996). In the present study, the higher specific activity of trypsin observed in the intestinal segment of the larvae fed Otohime suggests that the peak of specific activity in the pancreatic segment occurred before 16 dph and was likely a sign of a higher pancreatic secretory activity. The Gemma diet includes the highest amount of protein followed by the Otohime diet and then the LR803 diet. Therefore the increase of trypsin activity was not a result of the protein content of the diet but probably the result of differences in the source and molecular form of the protein in the diet. Indeed, each diet had a different main source of protein with the predominent ingredient being krill meal in Otohime, fish meal (native protein and protein hydrolysate) in Gemma and squid meal in LR803. Protein sources in microdiets have to provide an appropriate amino acid profile and also have to be highly digestible given the larvae's poor digestive capacities; in addition, they should have low water solubility due to the high surface to volume ratio of the diet particles (Nankervis and Southgate, 2006). To facilitate proteins digestion and assimilation by the larvae, pre-digested proteins (protein hydrolysates) are frequently incorporated into microdiets. Low to moderate levels of protein hydrolysates have proven beneficial in several species including gilthead sea bream *Sparus aurata* (Zambonino-Infante et al., 1997) and European sea bass (Cahu et al., 1999) while high levels were detrimental in these same two species (Cahu et al., 1999; Kolkovski and Tandler, 2000). In the present

study, the exact ingredient quantities and the presence or proportions of protein hydrolysates in the diets are not known. Therefore, it is difficult to characterize the effect of each source of protein on larvae development. However, the satisfactory results obtained with the Gemma larvae suggest that fish meal including a mix of native proteins and protein hydrolysates is an appropriate source of protein for pompano larvae.

AP and AN are intestinal enzymes mainly located in the brush border membrane of enterocytes while leu-ala peptidase is an intestinal enzyme mainly located in the cytosol of enterocytes (Cahu et al., 1998). As the enterocytes mature and proliferate, the brush border membrane (BBM) develops and the associated enzyme activities increase while cytosolic enzyme activities decline, indicating the establishment of the adult digestive system (Boglino et al., 2012; Zambonino-Infante and Cahu, 2001). In this trial, no decline in leu-ala activity was observed in Gemma and LR803 larvae, while a peak was observed at 22 dph for Otohime larvae but with an activity level at 28 dph superior to 16 dph. Cytosolic digestion in Florida pompano seems to remain elevated after the onset of BBM enzymes; in consequence, the maturation of the enterocytes should be evaluated primarily through the activity of the BBM enzymes (i.e. AP and AN). Very minor changes were observed in the specific activity of AP and AN in the LR803 larvae, suggesting a delay in the maturation of the enterocytes in this group. At 22 dph, highest AP and AN specific activities were observed in the Otohime larvae. In contrast, this positive sign of enterocyte maturation was not confirmed at 28dph, particularly for AP. In contrast, larvae fed Gemma exhibited an appropriate maturation of the BBM enzymes. This suggests that the Otohime diet was probably adequate for young larvae before 22dph, but failed to

sustain an appropriate development of Florida pompano larvae after this developmental date. Contrastingly, the larval development seemed to be more continuous and balanced with the Gemma diet, as demonstrated by the growth and enzymatic results.

In conclusion, this study brings new knowledge on Florida pompano dietary requirements and digestive development. Results first suggest that a diet including 20 % lipids, 55 % proteins, a DHA:EPA ratio greater than 1 and an ARA:EPA ratio of at least 0.08 is an adequate weaning diet for Florida pompano since the Gemma diet, with similar characteristics, promoted the best larvae performance in this experiment. Second, results from the enzyme analysis showed that the pancreas is fully functional by 16 dph suggesting that weaning onto a dry diet could occur earlier than in the present study. Additional research is required to determine more precisely larval nutritional requirements in Florida pompano. Indeed, even though lipids are one of the most important nutritional factors known to affect larvae growth and survival, numerous other macro and micro-nutrients can either enhance or inhibit larval development. This includes ingredients and supplementations present in low quantities in the diets tested in this study such as taurine (Pinto et al., 2013, 2010; Salze et al., 2012), yeast (Tovar-Ramírez et al., 2010, 2004) or vitamins and minerals (Hamre et al., 2013; Moren et al., 2011) which were beyond the scope of this study. However, the present results give a baseline of a suitable weaning diet, which can be used in future trials to determine the optimal weaning time for Florida pompano.

5.6 ACKNOWLEDGEMENTS

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Chapter 6 General Discussion and Conclusion

6.1 MAIN FINDINGS

6.1.1 Chapter 2

- Captive snook incorporated significantly more lipid than their wild counterparts.
- CHOL and ARA levels were significantly lower in captive fish compared to wild fish.
- Eggs obtained from captive broodstock presented high DHA and EPA levels, associated with low ARA contents and as a result, ARA/EPA ratio in captive eggs was less than half of that in wild eggs.
- Wild snook survey identified the presence of hydrocarbons in the liver
- Overall, large differences were noticed between wild and captive broodstock that most likely contribute to the reproductive dysfunctions observed in captive snook broodstock (e.g. incomplete oocyte maturation, low milt production and poor egg and larval quality).

6.1.2 Chapter 3

- In both pompano and snook larvae, fatty acids were utilized as an energy source after hatching.
- Mono-unsaturated fatty acids were catabolized, while saturated and poly-unsaturated fatty acids were conserved.
- High levels of ARA in pompano and snook eggs, as well as selective retention in the unfed larvae suggest a high dietary requirement for this fatty acid during the early stages of larval development.

- A dietary ARA supplementation in snook successfully influenced the larvae FA profile to match that of wild eggs; however, no significant improvement in growth or survival was observed.

6.1.3 Chapter 4

- A *Bacillus* sp. probiotic supplementation did not affect snook, pompano or red drum larval survival.
- At the end of the pompano and snook trial, standard lengths of larvae receiving the probiotic supplementation were significantly greater than for the control larvae.
- For both pompano and snook, trypsin and alkaline phosphatase specific activities were higher in the larvae receiving the probiotic supplementation compared to the control larvae.
- No significant differences were observed in the activities of the enzymes tested for red drum at the end of the trial.
- A mix of *Bacillus* sp. may promote growth through an early maturation of the digestive system during the early larval stages of pompano and snook.

6.1.4 Chapter 5

- Survival was not different between 11-day-old pompano larvae co-fed micro-diets (Otohime, Gemma, or Larval Reference Diet 803 LR803) and live food from 11 dph to 17 dph then micro-diets only until 28 dph.
- At the end of the trial, the Gemma larvae were significantly longer and heavier than larvae fed the other diets.

- The Gemma larvae incorporated the lowest amount of EPA, DHA and ARA, however, they had the highest DHA/EPA and ARA/EPA ratios.
- Proportions of polyunsaturated fatty acids could be of greater importance than their absolute amount.
- Enzyme activities revealed the full functionality of the pancreas at 16 days post hatch, opening possibilities of an earlier weaning time.

6.2 DISCUSSION

The general aim of this research was to improve the larviculture of three of Florida's high value food and stock enhancement finfish, common snook, Florida pompano and red drum, by gaining knowledge on their nutritional requirements at different life stages depending on the bottleneck for each species.

The main obstacle in common snook culture is the very poor and inconsistent larval survival and this project studied the broodstock nutritional requirements and the impact of imbalances on eggs quality. In an effort to better understand fatty acid requirements during the early larval stages of snook and pompano, the mobilization of fatty acids was studied in unfed larvae of both species and different live food enrichment formulations were evaluated in snook larvae. Another strategy focused on testing a probiotic mix on larvae of all three species to investigate potential effects on tank bacteriology and digestive system development. Finally, the weaning stage was studied in pompano to gain the information necessary to improve rearing protocols.

6.2.1 Snook broodstock nutritional requirements and egg quality

The comparison of flesh, liver and egg samples from wild broodstock and broodstock held in captivity for three years highlighted major differences. First, flesh and liver samples showed that captive fish incorporated significantly more lipid than wild fish. However, cholesterol levels and arachidonic acid (ARA) levels were significantly lower in captive fish. In addition, the presence of hydrocarbons was observed in liver samples from wild fish only. Despite the higher lipid content in captive fish, captive eggs contained less total fatty acids (FA) than wild eggs. As in captive broodstock, ARA levels were lower in captive eggs and high levels of eicosapentaenoic acid (EPA) lead to an ARA/EPA ratio more than half of that in wild eggs.

These results revealed strong imbalances in the fatty acid metabolism of captive broodstock, which are most likely involved, in the reproductive failure and poor egg quality and larval survival, observed in captivity. An ARA supplementation appears recommended to meet requirements, however, more research is required to determine the adequate level, as an excess of ARA can also lead to negative consequences (Furuita et al., 2002; Yan et al., 2013). To conduct this research, a switch from the current fresh diet to a range of compound pelleted diets would be necessary to run replicated studies. However, because common snook require very large tanks to spawn in captivity, the housing of several populations is difficult. Common snook and red drum are both batch spawners, sharing similar spawning patterns, sites and diet. Therefore, a rapid diet-egg transfer of ARA may occur in snook as well which would facilitate the research by potentially reducing study length.

Fatty acid levels fluctuated in wild fish depending on the month of capture, though no clear trend was detected. The relatively low number of fish samples at each time point probably explained part of the variability and additional data would allow for a better understanding of seasonal changes and provide more specific nutritional targets. More data is also needed to further investigate the presence of hydrocarbon in the liver of wild fish. Indeed, identifying the source of contamination is of critical importance to support the protection of the species and its environment. Despite the limited number of samples, differences between wild and captive broodstock were consistent and extensive, providing clear information for the improvement of captive snook broodstock diet.

6.2.2 Early fatty acid requirements

The study of snook and pompano fatty acid mobilization during the endogenous feeding period and subsequent starvation revealed clear patterns of conservation and utilization. First, results showed that both species catabolized fatty acids for energy after hatching. More specifically, mono-unsaturated fatty acids were catabolized while saturated and poly-unsaturated fatty acids were conserved. High levels of ARA in pompano and snook eggs followed by selective retention in unfed larvae suggested a high requirement for this fatty acid during the early larval stages. Therefore, the effect of an ARA supplementation was studied on snook larvae in an attempt to increase survival during the first feeding period. The larvae fed the ARA supplementation did incorporate higher levels of ARA, however survival was not increased as a result of higher ARA. ARA is involved in inflammatory processes and has been shown to improve stress resistance in European sea bass *Dicentrarchus*

labrax (Atalah et al., 2011a) and gilthead sea bream *Sparus aurata* larvae (W. Koven et al., 2001). Future research should therefore include this aspect and investigate a potential increase in stress resistance with a possible long-term effect on survival. In addition, more studies are required to develop a first feeding diet for snook larvae as the very low and inconsistent survival rates obtained so far are most likely related to a nutritional deficiency. This deficiency may be qualitative with an inadequacy in the enriched live food supplied, or quantitative with a difficulty in capturing and ingesting the proposed prey. The sampling of planktonic atricial larvae is very challenging and a strategy to study marine fish larvae wild diet is to offer wild plankton, in a laboratory setting, to identify and study the preys selected by the larvae. Nonetheless, the introduction of wild plankton in close recirculating systems presents a risk of contamination with bacteria, viruses or parasites. Therefore, the culture of small live prey such as copepods nauplii is a safer approach that should be investigated.

6.2.3 Probiotic supplementation

A *Bacillus* sp. supplementation was tested on tank hygiene, survival, growth and digestive activities of pompano, snook and red drum larvae. No impact on survival was observed for the three species. During the pompano trial, microbiological analyses were performed and results indicated that the number of presumptive *Vibrio* sp. were not a concern in the experimental system as numbers were low and not statistically different between treatments. Impact on growth and digestive enzyme activities were contrasted, with enhanced growth through an early maturation of the digestive system in snook and pompano larvae, while no significant effect was

observed in red drum larvae. However, no detrimental effect were noticed in red drum larvae development and the study of additional factors such as disease and stress resistance might have revealed an impact of the supplementation. Moreover, a longer trial period may have been necessary to observe significant changes. Along with the improved growth, the lack of increase in survival of the snook and pompano larvae receiving the probiotic supplementation is unexpected, particularly in snook larvae where survival is very low. Numerous factors are involved in the survival of young marine fish larvae, and many aspects of snook larval rearing still require improvement, as it is a new species in aquaculture. Still, the promotion of growth and early maturation of the digestive system is likely to strengthen the larvae and a difference in survival might be observed after stressful events of larval life history such as metamorphosis and weaning. Further studies should focus on gaining more information on the mode of action of probiotics and explore additional ways through which probiotics influence larval physiology. Present results revealed the impact of the mix of probiotics on the maturation of the digestive system but other studies have shown various modes of action (Cutting, 2011; Newaj-Fyzul et al., 2013) and recent research on juvenile pompano *Trachinotus ovatus* demonstrated non-specific immune response and disease resistance in addition to growth enhancement, by a mix of Bacillus sp and fructo-oligosaccharide probiotics (Zhang et al., 2014).

6.2.4 Transition to a dry diet

Three microdiets were tested in Florida pompano larvae: Otohime, Gemma and a reference diet LR803. Survival was similar for all treatments, but at the end of the trial the Gemma larvae were significantly longer and heavier than the larvae fed the

other diets. Significant differences were also observed in fatty acid composition of the diets and larvae, with the Gemma larvae incorporating the lowest amount of EPA, DHA and ARA but presenting the highest DHA/EPA and ARA/EPA ratios. This supports the concept that relative proportions of polyunsaturated fatty acids are of greater importance than their absolute amounts. Results from the enzyme analysis showed that fishmeal is suitable as the main protein source for Florida pompano larvae and demonstrated the full functionality of the pancreas at 16 days post hatch. These findings identified a suitable weaning diet for Florida pompano larvae and enzyme analyses opened opportunities of early weaning. Future studies should further investigate the ontogeny of the Florida pompano digestive system to better identify the timing of digestive enzyme development. In addition, best weaning time should be determined by testing various weaning and co-feeding protocols using the Gemma diet. This research will allow for the determination of the most cost-efficient and reliable weaning protocol, an essential step in the development of Florida pompano mass production.

6.3 RESEARCH PRIORITIES

The present work stresses the necessity to focus future research on broodstock nutrition, as improvements in live feed quality alone do not seem sufficient to effectively increase larval survival. The production of high quality gametes appears to be essential in providing the developing larvae with the tools required to efficiently exploit their dietary intake and successfully undergo dramatic morphological and physiological changes. Superior quality eggs and newly hatched larvae will then facilitate further studies on larval nutritional requirements.

6.4 CONCLUSION

The present work highlights the importance of nutrition at all stages of marine fish production cycle. Results suggest that nutrition should no be neglected when hormonal therapies fail to insure reproductive success, as broodstock diet appears to not only affect egg quality but also most likely impacts steroid and prostaglandin production, reproductive behavior and gametogenesis. This research project also described for the first time to the author's knowledge the pattern of conservation and utilization of fatty acids in atricial common snook and Florida pompano larvae, providing fundamental information for the development of suitable first feeding diet. In addition, this work demonstrated the interest of a probiotic supplementation in snook and pompano larval rearing, providing an additional tool in the development of successful and reliable rearing protocols. Finally, the present findings provide valuable insight into pompano nutritional requirements and digestive capacities during the transition from larvae to juveniles, allowing for the improvement of commercial hatcheries protocol. Overall, this doctoral work brings new insight on the nutritional requirements of each species studied, providing new keys to improve larval rearing protocols

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