Doctoral Thesis

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Influence of hormonal induction and brood-stock nutrition on reproductive performance and spawning quality of greater amberjack (*Seriola dumerili*, Risso, 1810)

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Influence of hormonal induction and broodstock nutrition on reproductive performance and spawning quality of greater amberjack (*Seriola dumerili*, Risso, 1810)

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El Director, La Directora, La Doctorando,

z

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This thesis is lovingly dedicated to my parents, their support, encouragement, and constant love have sustained me throughout my life.

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LIST OF ABBREVIATIONS

ANOVA	One-way Analysis of Variance
ARA	Arachidonic acid
BPG	Brain-pituitary-gonadal
CPE	Carp pituitary extract
C-t	C-terminal peptide
DA	Dopamine
DHA	Docosahexaenoic acid
dph	Days post-hatching
EAA	Essential amino acid
EPA	Eicosapentaenoic acid
EVAc	Ethylene and vinyl acetate
E2	17β-estradiol
FAMEs	Fatty acid methyl esters
FSH	Follicle-stimulating hormone
FSHR	Follicle-stimulating hormone receptor
GABA	Gamma-aminobutyric
GnRH	Gonadotropin-releasing hormone
GnRHa	Analogue of gonadotropin-releasing hormone
GSI	Gonadosomatic index
GtHs	Gonadotropins
hCG	Human chorionic gonadotropin
LC-PUFAs	Long-chain polyunsaturated fatty acids
LGD	Diameter of oil globule
LH	Luteinizing hormone
LHR	Luteinizing hormone receptor
Lv	Lipovitellin
LvH	Lipovitellin heavy chain
LvL	Lipovitellin light chain
MIS	Maturation-inducing steroids
NEAA	Non-essential amino acid
NPY	Neuropeptide Y
PGE3	Prostaglandin E3
PGE2	Prostaglandin E2
PGF2α	Prostaglandin F2α
PGF3	Prostaglandin F3
PGs	Prostaglandins
PIT	Passive Integrated Transponder
PITC	Phenyl isothiocyanate
PUFA	Polyunsaturated fatty acids

Pv	Phosvitin
SL	Standard length
T	Testosterone
TFA	Total fatty acids
TL	Total length
VLDL	Very low-density lipoprotein
YSV	Volume of the yolk sack
VTG	Vitellogenins
YSH	Yolk sack width
YSL	Yolk sack length
β'c	β'component
11-KT	11-ketotestosterone
17,20-P	17α,20β-dihydroxy-4-pregnen-3- one

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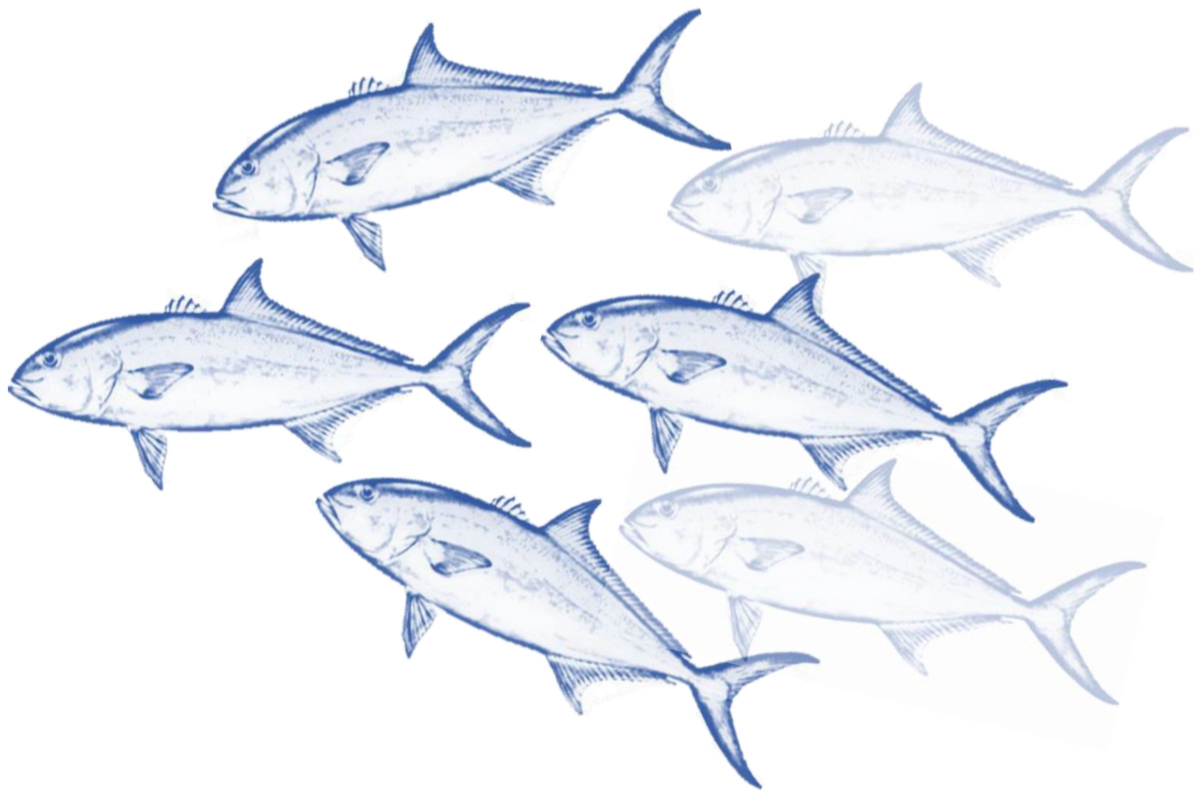
Chapter 1 General introduction



1.1. Ovarian development and yolk formation in fish

Fishes exhibit great diversity in reproductive strategies that result from adaptations to their respective environments. As examples: gonochoristic reproduction, where individuals have separate sexes; protandry, where individuals develop first as males and after one or more spawning seasons they may change to females; or protogyny where individuals develop first as females but have the possibility of changing later to males. True hermaphroditism and parthenogenesis also accur (Devlin and Nagahama, 2002). In addition, most adult fish possess the ability to produce eggs over multiple spawning seasons during their reproductive life, except for semelparous species, such as migratory salmonids that spawn only once in their lifetime and then die (Grier, 2012). Ovarian development in female teleosts can follow one of three main patterns: (1) synchronous, where all oocytes complete growth, maturation and ovulation at the same time and spawned simultaneously; (2) group synchronous, where two or more distinct populations or clutches of oocytes at different stages of growth or maturation are present in the ovary during the reproductive cycle and more than one batch of eggs are ovulated in succession during the spawning season; and (3) asynchronous, where oocytes in all stages of development are present at the same time in the ovary, without a dominant population at any specific stage, and oocytes are recruited into maturation and ovulation in many batches during the spawning season (Fernández-Palacios et al., 2011; Lubzens et al., 2017).

Despite the divergent reproductive strategies and ovarian development of teleost fish, there are common morphological and physiological changes that are characteristic of oocyte growth and maturation. Growth of ovarian follicles can be divided into previtellogenic and vitellogenic growth stages, during which the major lipid and protein nutrients required for embryonic and larval development are stored within the oocyte (Reading et al., 2018). Previtellogenic oocytes initiate meiosis (arresting at prophase I) and accumulate maternal gene transcripts, which function to direct early embryonic development shortly following fertilization, and begin to accumulate neutral lipids, which are stored as lipid droplets in the ooplasm (Lubzens et al., 2017; Reading et al., 2018). Vitellogenic stage oocytes involve a prodigious accumulation of materials in the ooplasm, accounting for most of the final mass of the ovulated egg. These include neutral lipids, apparent as oil droplets in the ooplasm, and phospholipid-rich yolk protein precursors called vitellogenins (Vtgs) (Lubzens et al., 2017). Vtgs are

synthesized in the liver under regulation by estrogen in maturing females, taken up from the maternal circulation by growing oocytes via receptor-mediated endocytosis and enzymatically processed into yolk proteins that are stored in the ooplasm (Hiramatsu et al., 2015). These yolk proteins always include lipovitellin (Lv), made up of a heavy chain (LvH) and light chain (LvL), and may also include phosvitin (Pv), β'component (β'c), a C-terminal peptide (C-t), and various Lv-Pv or other complexes depending on the Vtg type and species (Lubzens et al., 2017). Vtgs are rich in polyunsaturated fatty acids (PUFA, 50% of total) providing fluidity to cell membranes in developing embryos. In species spawning demersal eggs or floating eggs lacking prominent oil droplets, Vtgs are major carriers of lipids into oocytes. In species spawning pelagic eggs with large oil droplets, Vtg makes only a minor contribution to yolk lipids, and neutral lipids including triacylglycerol and wax- or steryl-esters, predominate (Sullivan and Yilmaz, 2018). Very low-density lipoprotein (VLDL) delivers triacylglycerol to the ovary, from which free fatty acids are cleaved by lipoprotein lipase to enter the ooplasm where they are used to synthesize the neutral lipids found in the oil droplets (Hiramatsu et al., 2015). Also, Vtgs are the primary source of amino acids for developing embryos and larvae (Finn and Fyhn, 2010). In addition to phosphate, carbohydrate and lipids, Vtgs are important carriers of iron, calcium, magnesium and other minerals, fat-soluble vitamins and related, and hormones, such as thyroid hormones and cortisol that are soluble in their lipid components (Sullivan and Yilmaz, 2018). When vitellogenesis ends, the ovary is filled with fully yolked oocytes that subsequently undergo maturation and ovulation. The entire period encompassed by previtellogenic and vitellogenic growth is typically a critical timeframe for captive broodstock management as appropriate conditioning and diet are required for the fish to produce good egg quality (Reading et al., 2018).

1.2. Fish reproductive endocrinology

In fish, maturation and reproduction are regulated by neuropeptides and sexual hormones from the reproductive endocrine system of the brain-pituitary-gonadal (BPG) axis (Figure 1-1) (Levavi-Sivan et al., 2010; Zohar et al., 2010). The regulation of this axis is very complex, and there are certain mechanisms in fish that are not fully understood. It is known that the central nervous system plays a key role in the integration of various environmental and hormonal signals regulating reproduction (Zohar et al., 2010; Biran and Levavi-Sivan, 2018). Among various brain areas

identified in the control of reproductive processes, the hypothalamus seems to be the most important (Zohar et al., 2010). Several factors involved in the hypothalamic control of reproduction have been identified: gonadotropin-releasing hormone (GnRH), dopamine (DA), neuropeptide Y (NPY), gamma-aminobutyric (GABA) and more recently kisspeptin (Zohar et al., 2010). Among these factors, the decapeptide GnRH was the first relating to the pituitary control during reproduction (Biran and Levavi-Sivan, 2018). The GnRH acts directly at the pituitary gland to stimulate the production of two gonadotropins (GtHs): follicle-stimulating hormone (FSH) and luteinizing hormone (LH), that are released into the circulation to act on the gonad, and stimulate the synthesis of gonad steroid hormones, which are the ultimate effectors of gonadal development (Cabrita et al., 2009; Mañanós et al., 2009; Zohar et al., 2010; Biran and Levavi-Sivan, 2018).

In male fish, FSH regulates spermatogenesis, while LH controls testicular maturation (Knapp and Carlisle, 2011; Hatef and Unniappan, 2019). These GtHs stimulate testicular interstitial Leydig cells to produce androgens predominantly testosterone (T) and 11-ketotestosterone (11-KT), which in turn promote spermatogenesis (Schulz et al., 2010). At spawning, spermatozoa maturation is regulated by LH, which induce the production of maturation-inducing steroids (MIS), promoting final maturation of spermatozoa and spermiation (Schulz et al., 2010; Knapp and Carlisle, 2011). In the case of female fish, the endocrine roles of gonadal steroids are more complex; alongside their roles in feedback regulation and gonadal development, gonadal estrogens induce the synthesis and release of Vtg, the primary storage protein in fish oocytes, by the female liver (Lubzens et al., 2017). FSH bind to their respective receptors in the ovary, promoting follicle growth and oocyte development by stimulation of the production of T, which is aromatized into estrogen, 17β-estradiol (E2) by the granulosa cells (Lubzens et al., 2010). These steroids regulate ovarian development through its control of Vtg synthesis in the liver during the oocyte growth period (Hara et al., 2016). During final oocyte maturation and ovulation, pituitary LH secretion induces a shift in the steroid biosynthetic activity of the ovary with a reduction in T and E2 production and elevation in MIS production, mainly 17α,20β-dihydroxy-4-pregnen-3- one (17,20-P) (Lubzens et al., 2010). DA is a neurotransmitter secreted also by the hypothalamus that in some teleost fish species exerts an inhibitory effect on the GnRH system and gonadotropin release, at the hypothalamic and pituitary levels, respectively (Zohar et al., 2010). In general terms, some species have been

reported to have strong dopaminergic inhibition such as in cyprinids, therefore spawning induction would require the use of DA antagonists, while in marine species a lack of DA inhibition has been reported (Levavi-Sivan et al., 2010). The success of reproductive maturation and viable gamete release depends on the correct functioning of all components of the BPG axis throughout the entire reproductive cycle, from gametogenesis to spawning. The synchronized secretion of GnRH, GtHs and steroids through the reproductive cycle and their coordinated action is essential for successful spawning (Mañanós et al., 2009). In addition to their role in regulating gonadal development, sex steroid hormones also exert both positive and negative feedback on the BPG axis, thus, regulating GtH release. A major negative action of these steroid hormones is exerted through the dopaminergic system, increasing DA turnover and thus enhancing the DA inhibitory tone over GtH secretion. In this way, the brain is constantly informed about the evolution of gonad development, through the action of the fluctuating circulating levels of steroids during the reproductive cycle (Dufour et al., 2010).

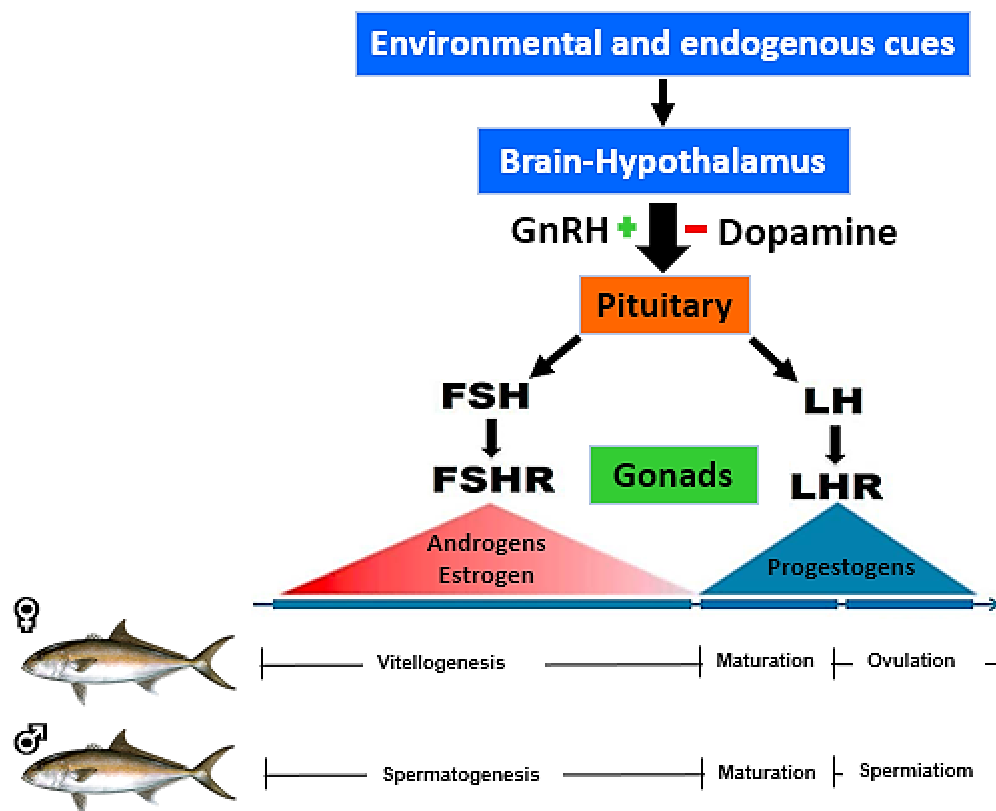


Figure 1-1. Schematic representation of the brain-pituitary-gonad (BPG) axis in fish, its major components and phases. FSH: follicle-stimulating hormone; FSHR: follicle-stimulating hormone receptor; GnRH: gonadotropin-releasing hormone; LH: luteinizing hormone; LHR: luteinizing hormone receptor.

1.3. Reproductive dysfunctions in captive fish

In their natural habitat, the BPG axis of the fish functions correctly and reproduction develops successfully, this taking place at the moment when the fish detect that the external conditions are the most appropriate for the survival of the offspring (Mañanós et al., 2009). Unfortunately, the situation changes drastically when fish are reared in captivity, the stress associated with captivity, the absence of appropriate spawning environment and/or nutritional deficiencies may act on the brain blocking the BPG axis, and causing reproductive dysfunctions (Mañanós et al., 2009; Mylonas et al., 2010; Duncan et al., 2013). These dysfunctions depend on the species and the females exhibit more serious reproductive problems than males (Gioacchini et al., 2014).

Three types of reproductive dysfunction have been described in females fish depending on which phase of the reproductive cycle is affected (Zohar and Mylonas, 2001). The first and most severe reproductive dysfunction, which is fortunately restricted to a limited number of species, affects the vitellogenic phase. This problem occurs in the European eel (*Anguilla anguilla*) and the grey mullet (*Mugil cephalus*) where reproduction is blocked at very early stages of development when maintained in captivity (Gioacchini et al., 2014). The second and most common type of reproductive dysfunction is the absence of oocyte maturation at the completion of vitellogenesis (Zohar and Mylonas, 2001; Mylonas et al., 2010). Vitellogenesis progresses normally, but once it is completed, oocytes fail to undergo oocyte maturation and ovulation and undergo atresia (Berlinsky et al.,1997; Larsson et al., 1997; Mylonas et al.,1997). For example, in greater amberjack (*Seriola dumerili*); severe impairment of oogenesis resulted in extensive atresia of late vitellogenic oocytes during the advanced vitellogenesis and spawning phases in captivity (Zupa et al., 2017a). Atresia of vitellogenic oocyte and failure to undergo oocyte maturation have been attributed to an insufficient pituitary LH release occurring in captive Atlantic bluefin tuna (*Thunnus thynnus*) (Rosenfeld et al., 2012). The last type of reproductive dysfunction of female broodstocks is the inhibition of spawning only (Zohar and Mylonas, 2001; Mañanos et al., 2009). Fish exhibiting this dysfunction undergo all phases of the reproductive cycle correctly, but spawning is blocked, and the ovulated oocytes remain in the ovarian or abdominal cavity (Mañanos et al., 2009). This dysfunction is generally solved by stripping the gametes from the broodstock and

applying pressure to the abdomen, as for salmonids, turbot (*Psetta maxima*) and Atlantic halibut (*Hippoglossus hippoglossus*) (Duncan et al., 2013). Reproductive dysfunctions of captive fish are not restricted only to females, males also have some alterations, although in most fish species males adapt better to captive conditions than females, and spermiate. In males, reproductive dysfunctions mainly involve diminished sperm volume and diminished milt fluidity, which can affect negatively the success spawning and production of fertilized eggs (Mañanos et al., 2009).

1.4. Hormonal therapies in fish reproduction

The use of hormonal treatments in cultured fish has permitted the reproduction in captivity of several fish species that do not do so spontaneously and the synchronization of gamete release in both sexes at a convenient time (Manañós et al., 2009; Mylonas et al., 2010). It was demonstrated in several fishes, that the inhibition of spontaneous spawning in captivity was clearly related to the inhibition and/or diminution of LH release from the pituitary (Mylonas et al., 2010). Methods to enhance oocyte maturation and spermiation have focused on the use of exogenous GtHs preparations or the use of GnRH with or without a DA (Mylonas et al., 2010). These two types of hormonal therapies act at different levels of the reproductive BPG axis, hormones pertaining to the first type act directly on the gonads, while hormones of the second type act on the pituitary and thus indirectly on the gonad, through stimulation of endogenous pituitary GtH release (Manañós et al., 2009).

GtH preparations include homogenates and purified extracts from the pituitary of mature fish during the reproductive season (commonly of carp and salmonids) and, purified human chorionic gonadotropin (hCG). Pituitary homogenates were the first type of exogenous hormonal treatments used for the induction of maturation and spawning (Houssay, 1930). Today, preparations of carp pituitary extract (CPE) and purified salmon GtH are available commercially and are more effective than the earlier pituitary homogenates, since they are purified to various extents and their activity is usually calibrated using bioassays (Yaron, 1995). Nevertheless, these treatments showed several problems, such as costs and low availability of the products, high risking pathogen transmissions and possible interference of the hormone administered with the endocrine pathways of the fish. Besides, they were active only on fish phylogenetically close to the donors. For this reason, these GtHs were replaced with hCG. hCG has also been used extensively in hormonal manipulation of reproduction

in fishes, that was characterised by a wide availability on the market and a higher chemical purity, ensuring a better efficacy (Manañós et al., 2009).

GnRH analogue (GnRHa) is one of the most commonly used hormones which stimulates the pituitary and released GtHs. The application of GnRH therapies has important advantages over the previous GtH preparations, due to the possibility of acting at a higher level of the BPG axis and thus promoting a more general and physiological stimulation of the whole reproductive process (Manañós et al., 2009). Another's important advantages are the high structural similarity of native GnRHs among fishes (Lethimonier et al., 2004), risk elimination of the transmission of infectious diseases and the possibility of applying exact doses of GnRH (Mylonas et al., 2010). In some species, GnRHa treatment is joined with the administration of DA antagonists, to neutralise the inhibiting effects of DA on the hypothalamus (Mylonas and Zohar, 2001).

The classical mode of hormone administration is the intra-peritoneal or intramuscular injection of saline-dissolved GnRHa, at the required dose. Depending on the fish species, a single GnRHa injection induces an LH increment that lasts 12 to 72 hours, before the effect disappears. In some cases, this short-lived effect is enough to induce spawning 2-3 days after treatment. However, in many cases, further injections are necessary to induce prolonged LH release and stimulate complete gonad maturation and spawning (Manañós et al., 2009). For asynchronous or multiple-batch group synchronous species and in species whose gonadal development is inhibited at early stages and need prolonged stimulation to affect the whole maturation process, multiple GnRHa injections are normally required (Carrillo et al., 1995; Fernández-Palacios et al., 2015 a, b; Setiawan et al., 2016). GnRHa treatments have been also administered in the form of sustained-release delivery systems (Mylonas et al., 2010). Many different types of delivery systems have been developed for GnRHa administration. The first delivery system was prepared using cholesterol. Cholesterol implants are prepared as solid, cylindrical pellets (3 mm in diameter) and are implanted intramuscularly using an implanter. The next type of GnRHa delivery system was fabricated in the form of biodegradable microspheres using copolymers of lactic acid and glycolic acid or a copolymer of fatty acid dimer and sebasic acid (Mylonas et al., 1995). The microspheres are suspended in a viscous vehicle and are injected into the muscle (Mylonas and Zohar, 2001). Another type of GnRHa-delivery system was

prepared in the form of a solid implant, using a non-degradable co-polymer of ethylene and vinyl acetate (EVAc) (Mylonas et al., 2004a). The EVAc implants are fabricated as disks 2 or 3 mm in diameter and are administered intramuscularly using an implanter (Mylonas et al., 2007).

The nature of the hormonal treatment and the method of its delivery depend on the species and the gonad developmental stage at which it is applied. In the multiple batch spawner Senegalese sole (*Solea senegalensis*), two different GnRHa sustained-release delivery systems were proven to be the most effective for spawning induction when compared with a single (Guzmán et al., 2009) or multiple injections of GnRHa (Agulleiro et al., 2006). In meagre, (*Argyrosomus regius*), multiple injections of GnRHa were considered more advantageous compared to GnRHa implants due to better egg production control and repeatability of response (Mylonas et al., 2015). In greater amberjack, spawning induction experiments have been done with both GnRHa implants (Mylonas et al., 2004a) and multiple GnRHa injections (Fernández-Palacios et al., 2015 a). In the Japanese yellowtail (*Seriola quinqueradiata*) a comparison among three different hormonal therapies was examined, using a single or double hCG injection, or a single cholesterol implant with GnRHa (Chuda et al., 2001). Even though the latter hormonal administration produced the highest fecundity, a single hCG injection was considered as the most efficient method to induce oocyte maturation and ovulation, based on the production of better-quality eggs (Chuda et al., 2001). However, when hCG administration is used repeatedly in subsequent years, the fish may develop an immune response and the injected preparation is immune-neutralized (Zohar and Mylonas, 2001).

1.5. Indicators of egg quality

The provisioning of high-quality eggs and larvae in high numbers is a prerequisite for the successful establishment of species in aquaculture. However, variation in egg quality, defined as the ability of an egg to be fertilized and subsequently develop into a normal embryo (Kjörsvik et al., 1990; Brooks et al., 1997; Bobe and Labbé, 2010), has been identified as one of the main factors limiting the expansion of both marine and freshwater aquaculture species (Migaud et al., 2013). An extensive research has been conducted during the past decade on the determinants of egg quality and several morphologic, biologic and biochemical criteria have been used (Brooks et al., 1997; Bobe and Labbé, 2010; Migaud et al., 2013), remembering that a

good practical criterium for the determination of egg quality should be both possible to identify early in development and be simple to use (Kjørsvik, 1990).

The most reliable indicators used by hatcheries are fertilization success, hatching rate and larval survival (Fernández-Palacios et al., 1995). Fertilization success is probably one of the earliest estimators that can be recorded to accurately estimate egg quality and it is the most integrative estimator of sperm quality (Kjörsvik et al., 1990; Bobe and Labbé, 2010). In many species, the assessments of egg quality include the evaluation of hatching success and larval survival at multiple time points during early larval life (Kjörsvik et al., 1990; Fernández-Palacios et al., 1995; Bobe and Labbé, 2010). A total number of eggs and percentage of morphologically normal eggs are also good indicators of egg quality (Fernández-Palacios et al., 1995). The size and appearance or morphology of unfertilized eggs have sometimes been used to evaluate or estimate the overall developmental potential of the eggs after fertilization (Da Silva et al., 2018). Kjorsvik et al. (1990) pointed out that variation in diameter seems to be one of the most important criteria in the determination of egg quality for fish. The use of lipid droplets distribution in the unfertilized eggs was shown to be reflective of embryo survival at the eyed stage (Mansour et al., 2007). However, the use of such parameters is limited under normal hatchery conditions and the lack of a consistent relationship between the distribution of lipid droplets and egg quality has been pointed out by other investigators (Ciereszko et al., 2009).

Egg quality can be also predicted using the shape of the first embryonic cells and the patterns of cell division to identify abnormal development during early embryogenesis, in species that produce transparent eggs (Shields et al., 1997; Kjørsvik et al., 2003). However, these indicators appear not consistently to correlate with egg quality in all species (Migaud et al., 2013). Other parameters may improve our understanding of egg quality, in particular, endogenous variables such as nutrient content (Da Silva et al., 2018) and maternal RNA content (Bonnet et al., 2007; Lubzens et al., 2017) have been shown to strongly influence eggs viability in fish species. During the endogenous feeding phase, larvae rely on their vitelline reserves for energy and growth, and the quantity and quality of vitellus are important factors influencing early development (Finn and Fyhn, 2010). Thus, evaluation of the quantity and composition of vitelline reserves, in particular, the content and balance of essential fatty and amino

acids, are important predictors of larval viability post-hatch (Finn and Fyhn, 2010; Mommens et al., 2015; Da Silva et al., 2018).

1.6. Broodstock nutrition

1.6.1. Nutrient requirements and their effects on egg quality

Broodstock nutrition plays a critical role in the reproduction of most organisms including fish. The availability of high-quality eggs, fry and juveniles have been identified as the main bottlenecks of fish culture. Hence, the improvement of broodstock management and its effects on egg and larval quality was considered a priority from both the scientific and technical perspectives (Mylonas and Robles, 2014). The nutritional status of broodstock has been reported to affect egg quality and spawning performance including the chemical composition of eggs, fertilization, hatching rates and larval survival rates (Izquierdo et al., 2001; Samaee et al., 2010; Henrotte et al., 2010; Stuart et al., 2018). It is therefore important that broodstock diets are optimized to ensure good larval survival and early development (Izquierdo et al., 2001). However, studies on the specific effects of broodstock diet on gamete quality are limited and comparison of their results is often inconclusive. Nutritional requirements vary between species and may not be characterized well enough, thus leading to a lower reproductive performance than that reported for breeders fed natural diets.

1.6.1.1. Protein and amino acids

Recently more attention has been paid to the level of different nutrients such as protein and amino acids in broodstock diets. Proteins and their constituent animo acids are the most abundant components of fish eggs (Finn and Fyhn, 2010), and act as a reservoir of materials used during many biosynthetic activities that are essential for the formation of some structures during embryogenesis and early larval development (Finn et al., 1996; Rønnestad et al., 1999; Liu et al., 2018). In the gut, proteins are degraded and absorbed, and the amino acids absorbed are used to either form tissues or to generate energy during organogenesis (Li et al., 2009; Finn and Fyhn, 2010). Furthermore, proteins and amino acid status of the broodstock can directly influence egg yolk composition and, therefore, impact egg quality (Lahnsteiner et al., 2009; Bobe and Labbe, 2010). The role of proteins and amino acids in egg development of fish is well established (Rønnestad et al., 1998, 1999; Finn, 2007; Moran et al., 2007), but

very little is known about the effect of dietary proteins and amino acids in reproduction or egg quality. Low protein levels in broodstock diets can alter the secretion of GnRH (Kah et al., 1994) and LH (Navas et al., 1996), affecting oocyte maturation, the regulation of ovulation and therefore egg production (Fernández-Palacios et al., 2011). The level of dietary protein influences the viability of offspring, with very low levels (10-20 %) resulting in low fertilization rates of eggs and a large percentage of deformed larvae (Gunasekera et al., 1996). Moreover, reduction of dietary protein levels from 51% to 34% together with an increase in dietary carbohydrate levels from 10 % to 32 % was reported to reduce egg viability in European sea bass (*Dicentrarchus labrax*) (Cerdá et al., 1994). In labeo roho (*Labeo rohita*) broodstock fecundity increased with an increase in dietary protein levels from 20 to 25 and 30 %, while an increase over 35 or 40% reduced fecundity (Khan et al., 2005). Also, in green catfish (*Hemibagrus nemurus*), dietary protein levels influence gonadal maturation, fecundity and larval production (Aryani and Suharman, 2015). Thus, fish fed 20% dietary protein, compared to 32 % and 37 %, displayed the longest time to obtain matured gonads and, the lowest relative fecundity and number of larvae (Aryani and Suharman, 2015).

The contribution of adequate amounts of protein with a good balance of essential and non-essential amino acids is important for the development of eggs and larvae (Moran et al., 2007; Conceição et al., 2010). The concentrations of essential and nonessential amino acids in eggs were proportional to their content in the diet of yellowfin seabream (*Acanthopagrus latus*) broodstock, where the amino acid profiles of eggs and larvae of 3 days post-hatching (dph) were affected by the amount of amino acids contained in their diets (Zakeri et al., 2014). Amino acids concentration in Atlantic halibut eggs is used as an indicator of good egg quality (Mommens et al., 2015). In this study, egg valine concentrations were correlated with fertilization and hatching rates, and aspartic acid and leucine were correlated with normal blastomere symmetry. Besides, a high level of leucine eggs is used as an indicator of good egg quality in channel catfish (*Ictalurus punctatus*) (Lochmann et al., 2007; Sink et al., 2010). Also, histidine seems to be particularly important for reproductive success, since histidine muscle concentration is substantially increased just before spawning in sockeye salmon (*Oncorhynchus nerka*) (Mommsen et al., 1980; Mommsen, 2004) and it is the main amino acids in gonads during spawning of certain species such as goldlined seabream (*Rhabdosargus sarba*) (Qari et al., 2013). Interestingly, in that species,

histidine levels were higher in testis than in ovaries, suggesting the importance of this amino acid to improve sperm functioning and fertilization rates (Qari et al., 2013). However, there are not studies that shows the importance of histidine levels in broodfish diets. Taurine (beta-sulfonic amino acid) has been found particularly important for broodstock and larval nutrition (Matsunari et al., 2006, 2013; Pinto et al., 2010, 2013; Al-Feky et al., 2016; Allon et al., 2016). In fish, this nutrient is involved in anti-oxidative defence, osmoregulation, neurotransmitter modulation, hormone release, bile salt synthesis (Chen et al., 2001; Takagi et al., 2006; Kato et al., 2014) and protection of spermatogonia from oxidative stress (Higuchi et al., 2012 a, b). Besides this, taurine also affects growth rate and improved maturation in premature females cobia (*Rachycentron canadum*) (Widiastuti et al., 2011). Taurine is also essential in broodstock diets for *Seriola spp*. (Matsunari et al., 2006). Few studies have investigated the role of taurine in egg quality. In Japanese yellowtail, egg quality, especially fertilization and hatching rates, was improved by the addition of at least 1 % of taurine to the broodstock diet (Matsunari et al., 2006). Supplementation of taurine in broodstock diets for Nile tilapia (*Oreochromis niloticus*) increases spawning frequency, fecundity, hatching rates and weight of larvae at hatching (Al-Feky et al., 2016). In addition, taurine requirements seem to be higher during early life stages (Pinto et al., 2010; Kim et al., 2016) and in particular, increase of taurine in greater amberjack larval diets has positive effects on growth (Matsunari et al., 2013).

1.6.1.2. Lipids and fatty acids

Dietary lipid and fatty acid composition have been identified as major factors determining successful reproduction and survival of offspring in fish (Izquierdo et al., 2001). It is widely known that long-chain polyunsaturated fatty acids (LC-PUFAs), such as docosahexaenoic acid (22:6n-3; DHA), eicosapentaenoic acid (20:5n-3; EPA) and arachidonic acid (20:4n-6; ARA), are essential for reproductive control as well as egg and larval development in many fish species (Izquierdo et al., 2001; Tocher, 2010). They are also a source of stored metabolic energy, integral components during organogenesis, and precursors of physiologically active molecules, such as prostaglandins and other eicosanoids (Sargent, 1995; Tocher, 2003). However, marine fish species have a limited ability to synthesize LC-PUFAs from their 18 carbon unit precursors (linoleic acid, 18:2n-6 and linolenic acid, 18:3n-3), due to a deficient activity of the Δ5 and Δ6 desaturases, enzymes involved in the conversion pathway from C18

precursors to LC-PUFA (Figure 1-2) (Sargent et al., 2002; Castro et al., 2012). Thus, it is vitally important that these LC-PUFA are provided to the broodstock in sufficient quantities to allow optimal transfer to the developing gonads (Izquierdo, 1996; NRC, 2011).

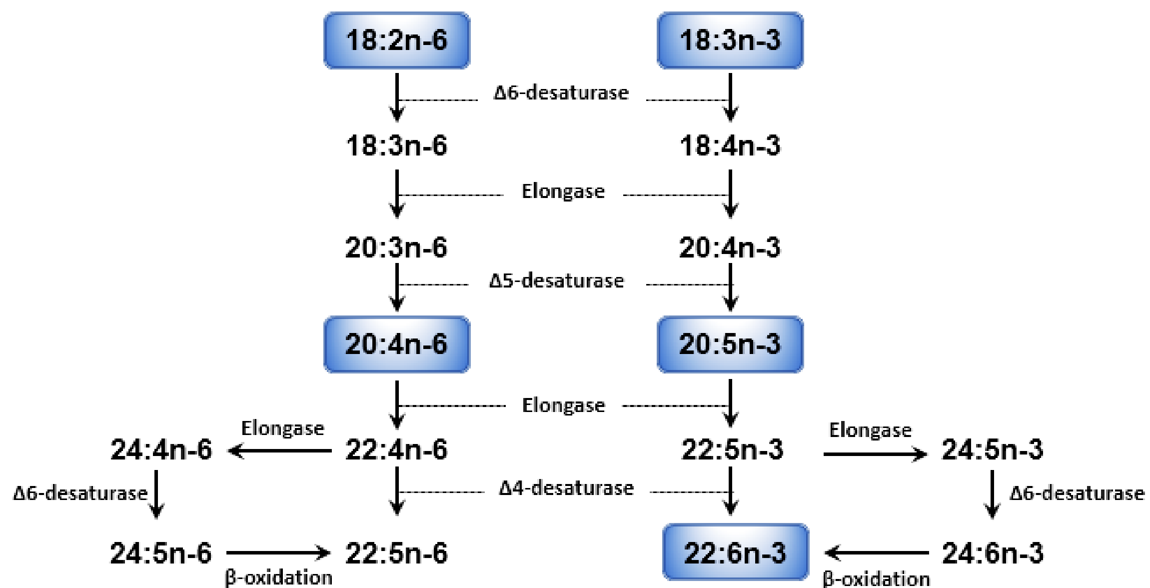


Figure 1-2. Elongation and desaturation pathways of LC-PUFA (Adapted from Monroig et al., 2011).

Generally, the eggs of marine species present a high content of n-3 LC-PUFA, particularly DHA, which indicates its high requirement during the early developmental stages of the eggs and larvae and reflects the high levels of n-3 LC-PUFA usually present in natural feed for broodstock (Fernandez-Palacios et al., 1995; Izquierdo et al., 2001; Tocher, 2003, 2010; Izquierdo and Koven, 2011). The n-3 LC-PUFA, especially DHA and EPA, have important structural and functional roles in all cell membranes (Tocher, 2010). The optimal broodstock dietary amounts of n-3 LC-PUFA vary among species, and both deficient or excessive levels of n-3 LC-PUFA have negative effects on egg and larval quality (Brooks et al., 1997; Bobe and Labbé, 2010). For instance, fecundity in gilthead sea bream (*Sparus aurata*) significantly increases with the elevation of dietary n-3 LC-PUFA levels up to 1.6 % (Fernández-Palacios et al., 1995). Feeding yellowfin seabream broodstock with a diet containing 6.6% n-3 LC-PUFA, improves relative fecundity, percentage of buoyant eggs, hatchability and survival rate 3 dph larvae in comparison to diets with 2.9-4.2 % n-3 LC-PUFA (Zakeri et al., 2011). Also, feeding flame angelfish (*Centropyge loriculus*) a diet containing 3.6

% n-3 LC-PUFA lead to increased fecundity, fertilization rates and embryo viability in comparison to those fed 1.8 and 2.9 % n-3 LC-PUFA diets (Callan et al., 2014). Apart from dietary n-3 LC-PUFA deficiencies causing detrimental effects in fish, the excess of n-3 LC-PUFA has been also reported to have a negative effect on reproductive performance of fish. Thus, excessive levels of dietary n-3 LC-PUFA (3.15% in comparison to 1.13%) reduced the total amount of eggs produced by gilthead sea bream broodstock (Fernández-Palacios et al., 1995). Similarly, egg buoyancy and larval survival are reduced in crescent sweetlips (*Plectorynchus cinctus*) fed a diet containing a high n-3 LC-PUFA level (5.85 % in comparison to 2.40% or 3.70%) (Li et al., 2005).

ARA levels in broodstock diets are also determinant of egg quality has been in certain species (Fernández-Palacios et al., 2011; Migaud et al., 2013). For instance, egg quality is enhanced in Japanese flounder (*Paralichthys olivaceus*), in terms of total egg production, percentage of buoyant eggs, hatching rate and larval survival by dietary increase of ARA from 0.1- 0.6 % (Furuita et al., 2003). Similarly, in Atlantic cod (*Gadus morhua* L.), eggs from broodstock fed a diet with a high ARA level (2.5 % of total fatty acid) show higher fertilization and hatching rates than those from broodstock fed lower ARA levels (0.8 % of total fatty acid) (Røjbek et al., 2014). Also, supplemental dietary ARA improves spawning performance in yellowtail, which shows higher viability, hatching rates, as well as larger egg diameter when dietary ARA is increased from 1.4 to 4.7 g ARA/100 g of total fatty acids (Stuart et al., 2018). However, in Japanese flounder fed ARA enriched diets (0.1 %, 0.6 % and 1.2 % of dry diet), the highest egg production is found in fish fed the 0.6% diet and lowest in the 1.2 % diet (Furuita et al., 2003).

EPA and ARA have both specific roles as precursors of eicosanoids, EPA being a precursor of PGs from 3-series prostaglandins (PGE3 and PGF3) and, ARA from 2-series prostaglandins (PGE2 and PGF2α). However, ARA seems to be the preferred substrate for eicosanoid production despite the presence of higher levels of EPA (Monroig et al., 2018). Also, EPA can also serve as a substrate for the eicosanoid-producing enzymes and exert a modulating influence on the production of ARA-derived eicosanoids (Bell et al., 1997; Tocher, 2003; Ganga et., 2005). Thus, eicosanoid actions are determined by the ratio of EPA/ARA in cellular membranes, this, in turn, is determined by the ratio of the fatty acids in the diet (Tocher, 2003). Several studies have shown the importance of the EPA/ARA ratio for influencing reproduction as well

as egg and sperm quality (Bell and Sargent, 2003; Salze et al., 2005; Henrotte et al., 2010). In Eurasian perch (*Perca fluviatilis*), the lowest EPA/ARA ratio yielded the highest spawning quality, especially in terms of hatching and larval survival rates than the control diet with highest EPA/ARA ratio (Henrotte et al., 2010). An improvement in yellowtail egg quality in relation to the amount of ARA in the eggs was also attributed to a lower EPA/ARA ratio in the higher-quality eggs (Stuart et al., 2018).

1.6.2. Feeding periods

During spawning, the composition of the broodstock diet and the duration of feeding are important (Bromage, 1995; Sargent, 1995). The feeding period needed to change the nutrient composition of the developing gonads and eggs varies among the different fish species (Fernández-Palacios et al., 2011). In gilthead seabream, the egg composition is quickly affected by the diet, even after only three weeks of feeding (Fernández-Palacios et al., 1995). Also, in red drum (*Sciaenops ocellatus*) and yellowtail, ARA is transferred from the diet to eggs very quickly, in only 4-16 days (Fuiman and Faulk, 2013; Stuart et al., 2018). Short-term supplementation of astaxanthin in diets for Atlantic cod broodstock, during a period of two months prior to peak spawning increases concentrations of carotenoids in the eggs by around 3-fold, indicating an efficient and rapid uptake (Sawanboonchun et al., 2008). These species are batch spawners with group synchronic ovaries and a short vitellogenesis periods, where it is possible to improve the spawning quality by modifying the nutritional quality of the diet even during the spawning season (Fernández-Palacios et al., 1995, 2005; Sawanboonchun et al., 2008; Stuart et al., 2018). Thus, the timing of the provision of dietary nutrients is important and, particularly, the period of vitellogenesis is a key moment for the incorporation of essential nutrients into the developing oocytes (Fernández-Palacios et al., 2011). Therefore, the vitellogenic period appears to be the most influential time during which these nutriments are incorporated most effectively into the developing oocytes (Navas et al., 1997). In European sea bass, with a longer vitellogenesis period (6 months), a longer feeding period is needed for obtaining appropriate levels of n-3 LC-PUFA in the eggs (Navas et al., 1997). In this species, the administration of a diet during vitellogenesis produces eggs of similar quality to those of broodstock fed a higher n-3 LC-PUFA diet throughout the whole year. In other studies to determine optimum protein or lipid levels in broodstock diets, broodstock has

been fed the experimental diets during 4 months before the reproductive season in yellowfin seabream (Zakeri et al., 2009) or 8 months in Atlantic halibut (Mazorra et al., 2003).

1.7. Greater amberjack *Seriola dumerili*

1.7.1. Importance of greater amberjack aquaculture

Diversification is an urgent need for aquaculture (Teletchea and Fontaine, 2014) and new candidate species must be highly appreciated by consumers to get good prices and large market size. *Seriola spp.*, belonging to the Carangidae family, are group of fish species with exceptional consumer acceptance (Sicuro and Luzzana, 2016). The greater amberjack has a great potential for the expansion of the European Union (EU) aquaculture industry (Nakada, 2000, 2008) and, therefore, is one of the species targeted by the EU Project DIVERSIFY (7FP-KBBE-2013-GA 602,131). Its rapid growth, excellent flesh quality and large size make this species very suitable for product diversification and development of value-added products (Jover et al., 1999). The greater amberjack could be positioned as a sustainable cultured alternative for wild tuna (Nijssen et al., 2019).

Of the total production reported to FAO (Figure 1-3), Spain was the only producer from 1992 to 1997, while in 2006 and 2007 Taiwan was the largest producer. Significant greater amberjack production occurs in Japan, but the informed statistics include all Seriola spp. together. Even so, it is estimated that the Japanese production of greater amberjack is more than 30 % of the total Seriola spp. cultured (FAO, 2019). Currently, there is also a production of 70 tons per year in the United Arab Emirates and 11 tons in Spain (APROMAR, 2019).

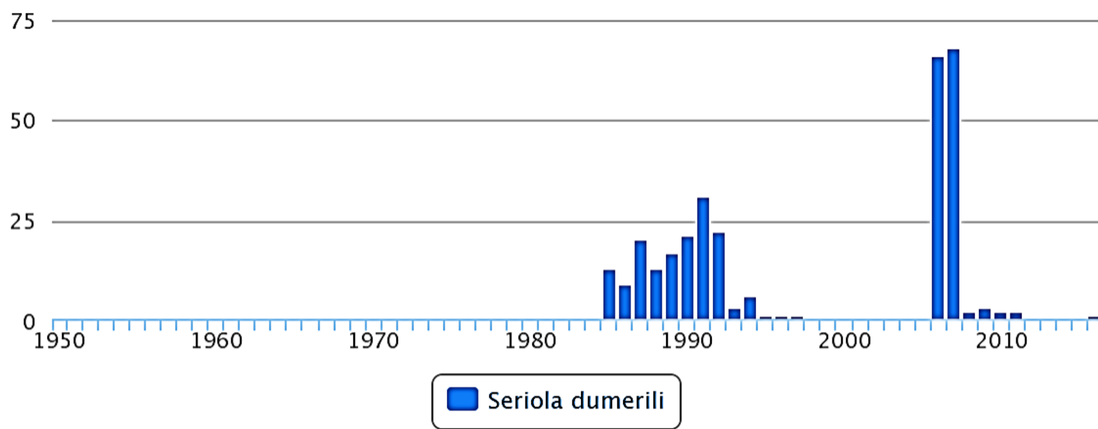


Figure1-3. Global aquaculture production greater amberjack (Source: FAO, 2019)

1.7.2. Biology and distribution

Greater amberjack is a large member of the family Carangidae, with a maximum reported size of 190 cm and 80.6 kg (Paxton et al., 1989). It is slender, with a fusiform body shape, a short and pointed head and relatively small eyes. Its pectoral fins are shorter than the head, the second dorsal fin is much longer than the anal fin, the caudal peduncle is deep and the caudal fin is lunate, or moon-shaped, efficient for fast swimming in pursuit of prey (Smith-Vaniz, 2002). It has a brownish or bluish grey dorsal side and a silvery white or yellow-golden ventral side (Figure 1-4). There is a dark amber stripe running from the nose to just in front of the dorsal fin. Some individuals may have a pale-yellow stripe along the sides (Smith-Vaniz, 2002).

This large fish is an epibenthic and pelagic species, usually in warm-temperate ocean waters (Manooch and Potts, 1997a). It is distributed along the eastern and western Atlantic coasts, in the Mediterranean Sea, and throughout much of the Indian and Pacific oceans (Smith-Vaniz, 2002; Smith-Vaniz et al., 2015). It tends to aggregate around reefs, rocky outcroppings, wrecks, and man-made structures such as oil platforms (Manooch and Potts, 1997b; Harris et al., 2007). Smaller fish (less than 3 kg) may be taken in shallow water (<10 m). Larger fish usually occur between 18-72 m and have been taken as deep as 360 m (Smith-Vaniz et al., 2015). A fast-swimming predator, greater amberjacks feed on squid, crustaceans and fish. Juveniles feed on planktonic decapod larvae and other small invertebrates (Pipitone and Andaloro, 1995; Andaloro and Pipitone, 1997).

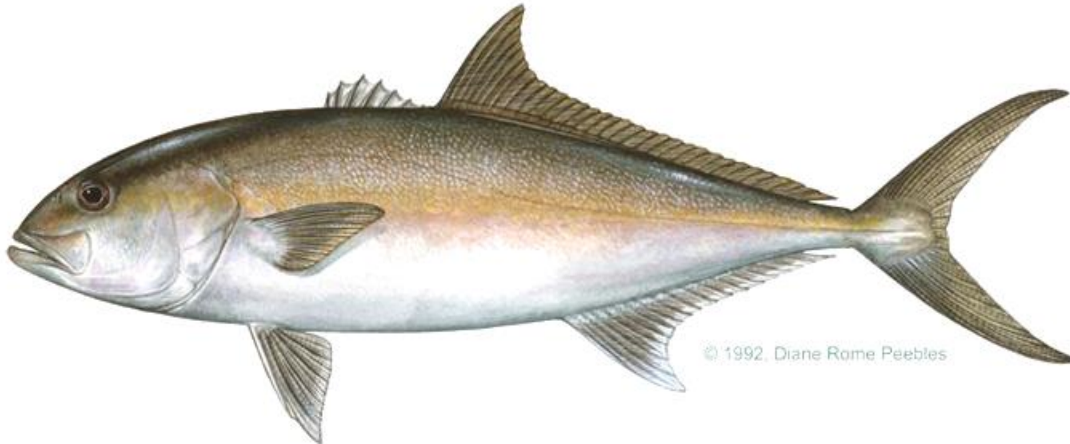


Figure1-4. Greater amberjack (Source: https://www.igfa.org).

1.7.3. Reproduction

Greater amberjack is a dioecious species, both sexes are separated, with a sex ratio of 1:1 (Lazzari and Barbera, 1989). The individuals can be sexually differentiated at 4 to 5 months old (Marino et al., 1995a), and sexually mature males and females are detected at the minimum standard length of 61 cm (24 months) and 80 cm (36 months), respectively (Marino et al., 1995b; Kozul et al., 2001). It is a gonochoric species with group-synchronous ovarian development and a multiple spawning pattern (Marino et al., 1995b; Mandich et al., 2004; Mylonas et al., 2004a). In the Mediterranean, the ovaries of wild-caught greater amberjack have batches of oocytes at distinct stages of development with diameters of 120-400 µm in early vitellogenesis (Marino et al., 1995b), 400-600 µm at the beginning of the spawning season in May (Lazzari et al., 2000) and the largest diameter (650-750 µm) in June (Kozul et al., 2001).

Important reproductive dysfunctions have been observed in captive-reared greater amberjack confined in sea cages or tanks (Diaz et al., 1997; Micale et al., 1999; Zupa et al., 2017a, b; Pousis et al., 2018). Recently, a comparative study of reproductive development in wild and captive-reared greater amberjack has shown the need for minimum handling of greater amberjack during the reproductive season, as this apparently induced significant long-term reductions in plasma T, E2 and 17,20β-P in the females (Zupa et al., 2017b). As a result, the gonadosomatic index (GSI) was significantly reduced at the peak of the reproductive season, and extensive follicular atresia was present in the ovaries. Similarly, significant reductions in plasma T, 11-KT

and 17,20β-P were observed in the males at the peak of spermatogenesis, again resulting in significant reductions in GSI (Zupa et al., 2017b), concomitant with elevations in plasma E2, reduction in spermatogonial mitosis and high level of apoptosis at the beginning of the reproductive season (Zupa et al., 2017a). It has been shown that handling greater amberjack during the reproductive season may induce these reductions in spermatogenesis and oogenesis (Zupa et al., 2017a, b; Pousis et al., 2018). Some of the reproductive dysfunctions of captive-reared greater amberjack have been overcome occasionally through the administration of exogenous reproductive hormones, such as hCG when females had vitellogenic oocytes of 550–600 µm in diameter (Marino et al., 1995b; Kozul et al., 2001) or GnRHa when oocytes were at 500 and 650 µm in diameter (Mylonas et al., 2004a; Fernández-Palacios et al., 2015a), while the occurrence of spontaneous spawning has been reported rarely (Kawabe et al., 1996, 1998; Jerez et al., 2006).

1.7.4. Nutritional requirements

The studies concerning nutritional requirements for greater amberjack are scarce, but there are several studies on the effect of different dietary formulations for greater amberjack juveniles, and most of them related to the optimization of dietary protein inclusion rates, or substitution partial or total of fishmeal with other animal or plant protein sources (Tomás et al., 2005; Uyan et al., 2009; Monge-Ortiz et al., 2018a, b). It is known that species of the genus *Seriola* need relatively high levels of protein (45-55%) in the diet for maximum growth, probably because these species are highly dependent on proteins to grow and obtain energy (Masumoto et al., 1998; Jover et al., 1999; Takakuwa et al., 2006; Tomás et al., 2008). Also, the nutritional requirements of the larvae have been studied to determine optimum levels of LC- PUFA in enriched rotifer and Artemia, evaluating their effects on survival, growth and skeleton anomalies occurrence (Matsunari et al., 2013; Roo et al., 2019).

In greater amberjack broodstock**,** an experiment was carried out to assess the effect of an experimental diet, with high levels of 18:1n-9 and low EPA/ARA ratio, on the fatty acid profile of ovary and eggs, in contrast to a non-specific commercial diet, taking wild fish lipid composition as a positive reference (Rodriguez-Barreto et al., 2014)**.** To obtain basic information for the formulation of an experimental diet that better suits greater amberjack broodstock fatty acid requirements, the lipid composition of several tissues (muscle, liver and ovary) of mature wild females and cultured females

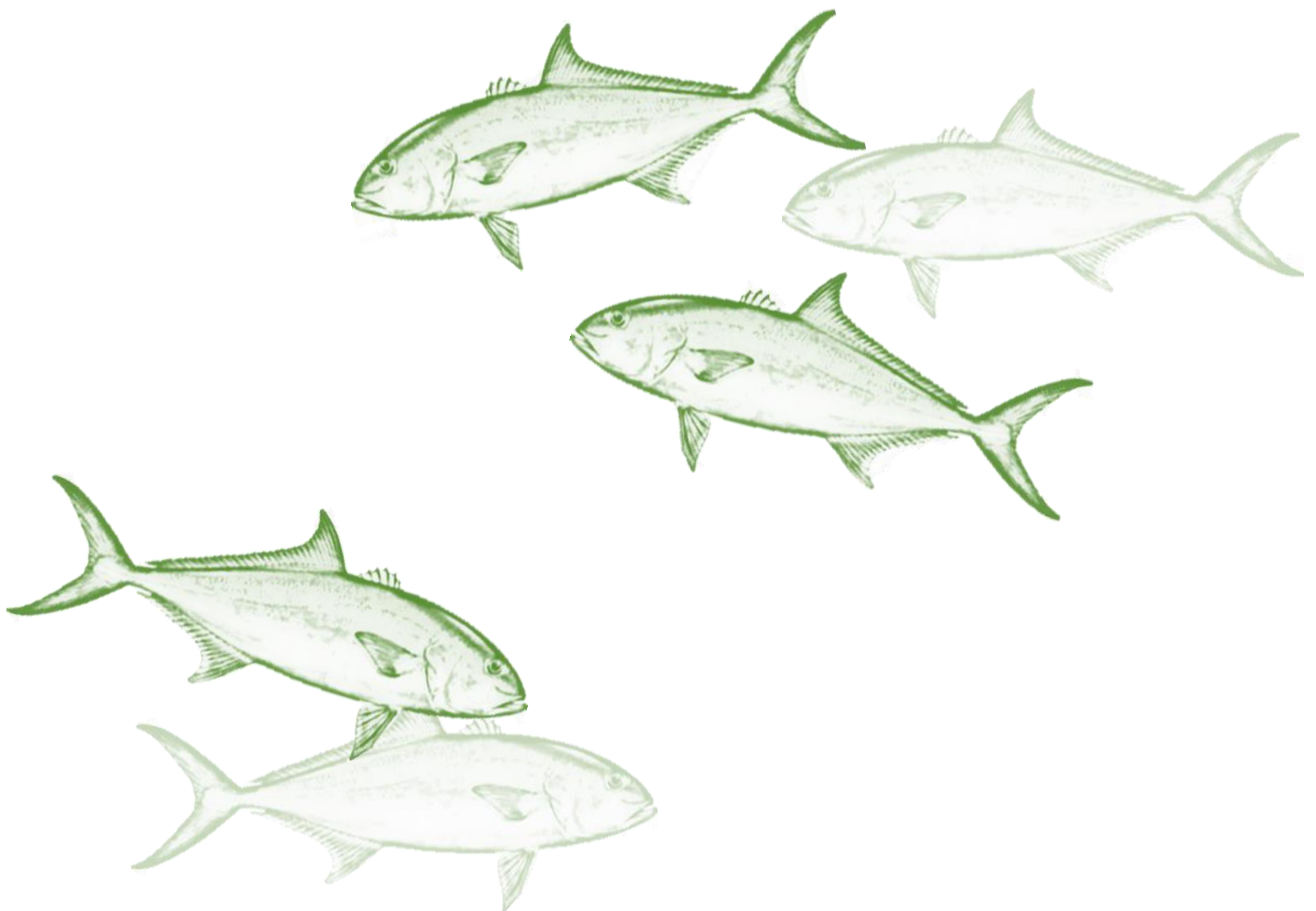
was compared, in order to identify possible nutritional deficiencies in fatty acids profiles (Rodriguez-Barreto et al., 2012). In captive-reared greater amberjack, a comparative study on the reproductive function showed the reduction of gonadal development and an early cessation of gametogenic activity was associated with differences in total lipids and essential fatty acid content in the ovaries and testes, particularly low ARA levels, at the early and advanced phases, and, as a consequence, a lower DHA/ EPA and ARA/EPA ratios (Zupa et al., 2017b; Pousis et al., 2018). Also, feeding greater amberjack broodstock with raw fish such as mackerel and squid are reported to have a positive effect on artificial spawn induction and fecundity (Fernández-Palacios et al., 2015 a).

1.8. Objectives

Greater amberjack is a leading candidate species for enhancing European aquaculture. Recently, interest for this species in the aquaculture industry is expanding, due to its high demand and market price, rapid growth, and its capacity to accept inert food adapting well to rearing conditions. However, the propagation of greater amberjack in European aquaculture is impeded by high variability in gamete quality associated with low fertilization success and low larval survival. There is a lack of information on the effects of broodstock management and broodstock nutrition potentially contributing to this variability. Thus, the main objectives of this thesis were to:

1. Evaluate and compare the egg quality of hormonal induction protocols with GnRHa injection or GnRHa implant with the spontaneous spawn (Chapter 3).
2. Examine the effects of supplemental histidine, taurine and protein in broodstock diets, on egg quality (Chapter 4).
3. Determine the effect of graded n-3 LC-PUFA levels in broodstock diets on spawning performance and egg quality (Chapter 5).
4. Investigate the effects of increased ARA levels in broodstock diets on spawning performance and, egg and larval quality (Chapter 6).

Chapter 2 General materials and methods



2.1. Broodstock maintenance

Rearing was undertaken in the facilities of the Grupo de Investigación en Acuicultura (GIA), located in the ECOAQUA Institute (Universidad de Las Palmas de Gran Canaria, ULPGC, Spain). The broodstock consisted of twenty-two greater amberjack captured in May 2011 in the south-western coast of Gran Canaria (Canary Islands, Spain), individually identified with Passive Integrated Transponder (PIT) tags. Fish with a mean and standard deviation (SD) weight of 3.41 ± 1.12 kg for females and 2.37 ± 1.07 kg for males, were conditioned in tanks of 10 m^3 volume (3 m x 3 m x 1.5 m depth) and kept under natural photoperiod using seawater at a temperature range 20.83 ± 0.32 °C in winter and 23.84 ± 0.18 °C in summer. Broodfish were fed twice a week with a commercial compound diet specific for broodstock maintenance (Vitalis Repro, Skretting, Burgos, Spain) at 1% of their estimated total biomass, and once a week with locally fished Atlantic mackerel (*Scomber scombrus*) at 2%. In January 2013, greater amberjack (8.27 ± 1.11 kg females body weight and 8.12 ± 1.82 kg males body weight) were transferred to three circular tanks of 40 m^3 (5 m x 2.35 m) (Figure 2-1). All the experiments mentioned in this thesis were conducted according to the European Union Directive (2010/63/EU) on the protection of animals for scientific purposes at Instituto Universitario ECOAQUA, University of Las Palmas de Gran Canaria (Canary Islands, Spain).

In all the experiments, standardized culture conditions were followed. Tanks were supplied with filtered seawater (37 ‰ salinity) in a water open system under natural photoperiod (27º 59' 28" N; 15º 22'05" W) of approximately 13 h light. Flow rate allowed 6 complete water tank renovations daily and the temperature was continuously monitored (Miranda, Innovaqua, Sevilla, Spain).

Figure 2-1. Tanks of 40 m^3 used along the studies of this thesis.

2.2. Evaluation of gonad maturation and broodstock selection

For sampling, the water level in the broodstock tanks was reduced to 50 cm of and clove oil (Guinama SL, Valencia, Spain) was added at a concentration of 0.01 ml l^{-1} for light sedation. When the fish were swimming slowly, they were taken one by one with a stretcher and transferred to a 500 l circular tank with clove oil at a concentration of 0.025 ml l^{-1} for complete anaesthesia. Fish were individually identified by their Passive Integrated Transponder (PIT) tag, and biometric parameters of length and body weight were measured. Gonad female maturation state was evaluated by ovarian biopsies obtained with cannulation of the genital pore using a catheter of 1.3 mm outside diameter (Figure 2-2) (Kruuse, Langeslov, Denmark). Serra solution (6:3:1, 60 % ethanol at 96 %, 30 % Formalin at 40 % and 10 % of glacial acetic acid at 96 %) was added to each ovary sample to disperse the oocytes and make them transparent to determine nuclear position. They were then observed in a profile projector (Mitutoyo PJ-3000A, Kanagawa, Japan) to estimate the diameter of 100 oocytes randomly selected. Females were considered eligible for spawning induction if they contained fully vitellogenic oocytes with a diameter > 600 µm. Maturation of the males was examined by the release of sperm upon application of gentle abdominal pressure. If this was not possible, a sperm sample was obtained by inserting a catheter at the opening of the genital pore.

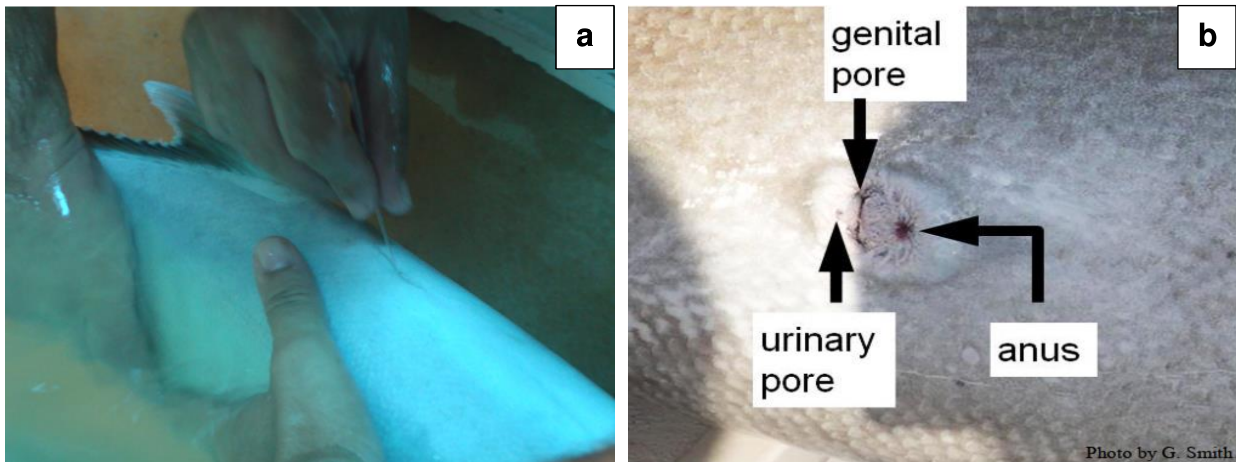


Figure 2-2. (a) Cannulation of the genital pore and (b) urogenital region of the greater amberjack (Smith et al., 2014).

2.3. Experimental diets

All experimental diets were manufactured by Skretting ARC Feed Technology Plant (Stavanger, Norway). In the first nutritional experiment, three different diets, one high in histidine, another in taurine and the third one in protein contents, were tested. The formulation of the diets was presented in Table 2-1. Broodstock groups were fed with these three diets and the effects of supplemental histidine, taurine and protein on spawning quality were evaluated.

In the second nutritional experiment, four diets containing different levels of n-3 LC-PUFA: diet D1 with 7.3 % of the total fatty acids (TFA), diet D2 with 15.3 %, diet D3 with 19.1 % and diet D4 with 25.4 %, were tested. The formulation of the diets was presented in Table 2-2. Broodstock groups were fed with these four diets and the effects of increasing dietary n-3 LC-PUFA levels on spawning performance and egg quality were examined.

In the third nutritional experiment, four formulated diets containing 0.8 (ARA-0.8), 1.6 (ARA-1.6), 2.4 (ARA-2.4) and 3.2 g ARA/kg feed (ARA-3.2) were fed to broodstock. The formulation of the diets was presented in Table 2-3. Broodstock groups were fed with these four diets and the effects of graded ARA levels in broodstock diets on spawning performance, the fatty acid composition of eggs, and egg and larval quality were tested.

Table 2-1. Main ingredients and proximate composition of the experimental diets used for examining the effects of supplemental histidine, taurine and protein in broodstock diets, on egg quality of greater amberjack.

	Diet		
	Histidine	**Taurine**	**Protein**
Ingredients, % of the diet			
Wheat[a]	17.94	18.29	11.81
Wheat gluten[b]	13.00	13.00	17.00
Fish meal[c]	45.14	44.64	48.36
Squid meal[d]	10.00	10.00	10.00
Fish oil[e]	12.47	12.50	12.18
Taurine[f]	0.00	0.93	0.00
Histidine HCl[g]	0.81	0.00	0.00
Premix incl. vitamins & minerals[h]	0.64	0.64	0.64
Proximate composition, %[i]			
Dry matter	92.40	93.00	94.10
Moisture	7.60	7.00	5.90
Crude protein	51.30	51.50	56.10
Crude fat	17.80	18.50	18.30
Ash	8.40	8.40	8.40

[a] Wheat: Skretting, Stavanger, Norway.
[b] Wheat gluten Cargill Nordic, Charlottenlund, Denmark.
[c] South American fish meal, Skretting, Stavanger, Norway.
[d] Squid meal: Skretting France.
[e] Fish oil: Skretting, Stavanger, Norway.
[f] Taurine: Trouw Nutrition, The Netherlands.
[g] Histidine Hcl: Kyowa Hakko, Japan.
[h] Include vitamins and minerals; Trouw Nutrition, Boxmeer, the Netherlands, proprietary composition Skretting ARC, vitamin and mineral supplementation as estimated to cover requirements according to NRC (2011).
[i] Values are reported as the mean of duplicate analyses.

Table 2-2. Ingredients and proximate composition of the experimental diets used to evaluate the effects of increasing dietary n-3 LC-PUFA levels on spawning performance and egg quality of greater amberjack.

	Broodstock group			
	D1	**D2**	**D3**	**D4**
Raw material (%)				
Linseed oil[a]	4.50	3.01	1.52	0.00
Wheat[b]	19.13	19.13	19.13	19.09
Wheat gluten[c]	14.99	14.99	14.99	13.62
Fish meal[d]	43.46	43.46	43.46	44.97
Squid meal[e]	10.00	10.00	10.00	10.00
Fish oil[f]	0.61	4.04	7.48	10.93
Palm oil[g]	5.93	3.98	2.03	0.00
Premix[h]	0.64	0.64	0.64	0.64
Total	100.00	100.00	100.00	100.00
Proximate composition (% dry weight)				
Crude protein	59.06	58.91	58.91	58.50
Crude lipid	25.61	24.35	24.89	24.25
Moisture	8.30	7.22	7.41	7.27
Ash	7.30	7.25	7.19	7.46

[a] Linseed oil: European Commodity Company S.A., Luxemburg.
[b] Wheat: Linas Agro AB, Lithuania.
[c] Wheat gluten: Roquette, France.
[d] Fish meal: FF Skagen AS, Denmark.
[e] Squid meal: Inproquisa SA, Spain.
[f] Fish oil: Norsildmeal AS, Norway.
[g] Palm oil: AAK AB, Sweeden.
[h] Include vitamins and minerals; Trouw Nutrition, Boxmeer, the Netherlands, proprietary composition Skretting ARC, vitamin and mineral supplementation as estimated to cover requirements according to NRC (2011).

Table 2-3. Experimental diets' main ingredients and proximate composition used to evaluate the effects of increasing dietary ARA levels on spawning performance and egg quality of greater amberjack.

	Diet			
	ARA-0.8	**ARA-1.6**	**ARA-2.4**	**ARA-3.2**
Raw material (%)				
Wheat[a]	20.78	20.78	20.78	20.78
Wheat gluten[b]	15.00	15.00	15.00	15.00
Fish meal[c]	43.04	43.04	43.04	43.04
Rapeseed oil[d]	5.71	5.49	5.26	5.03
Arachidonic acid[e]	0.00	0.23	0.45	0.68
Squid meal[f]	10.00	10.00	10.00	10.00
Fish oil[g]	4.52	4.52	4.52	4.52
Premix vit. Min[h]	0.64	0.64	0.64	0.64
Histidine HCl[i]	0.31	0.31	0.31	0.31
Proximate composition (% dry weight)				
Crude protein	53.76	53.69	53.45	53.61
Crude lipids	18.31	18.12	18.14	18.46
Ash	13.28	13.11	13.12	13.18
Moisture	8.58	8.79	8.45	8.47
Arachidonic acid (g kg^{-1} feed)	0.8	1.6	2.4	3.2

[a] Wheat: Linas Agro AB, Lithuania.
[b] Wheat gluten: Roquette, France.
[c] Fish meal: FF Skagen AS, Denmark.
[d] Rapeseed oil: Bunge Deutschland Gmbh.
[e] Arachidonic acid: Hua Xing Enterprises Co. LTD. China
[f] Squid meal: Inproquisa SA, Spain.
[g] Fish oil: Norsildmeal AS, Norway.
[h] Include vitamins and minerals; Trouw Nutrition, Boxmeer, the Netherlands, proprietary composition Skretting ARC, vitamin and mineral supplementation as estimated to cover requirements according to NRC (2011).
[i] Histidine: Kyowa Hakko, Japan
[j] Values are reported as the mean of triplicate analyses.

2.4. Spawning induction therapies

In the first experiment, for the spawning induction trial, fish were selected and divided in three groups: In group one, the fish were not induced hormonally or handled and spawned spontaneously, being considered the control group. In group two, the fish were injected intramuscularly with gonadotrophin-releasing hormone analogue (LHRHa, des-Gly10, [D-Ala6]-; Sigma-Aldrich, St. Louis, MO, USA) at a dose of 20 µg/kg body weight, based on the reported dosage for greater amberjack (Fernández-Palacios et al., 2015a). The fish selected of group three were induced using 500-µg GnRHa implants (Mylonas and Zohar, 2001). One implant (500 µg) was used for each female (Mylonas et al., 2004a) and half the dose for males. Implants were given subcutaneously, at about three scale rows down from the posterior end of the dorsal fin. In the others three experiments, spawning was hormonally induced with intramuscular injections of gonadotropin-releasing hormone analogue at a dose of 20 µg/kg body weight (Fernández-Palacios et al*.,* 2015 a).

2.5. Evaluation of egg and larval quality

At the onset of the spawning season, a passive egg collector was placed in the outflow of the spawning tank and examined daily. All spawned eggs were collected every morning and put in a 10 l bucket, filled with seawater. In order to evaluate fecundity and fertilization rate, a sample of 10 ml was taken, put in a 17 ml Falcon tube and then placed in a Bogorov chamber. The stage of the eggs was determined, and the number of live (L) and dead (D) eggs were counted under a stereoscope (Leica S6E, Wetzlar, Germany). The fecundity (total number of eggs per female), the number of eggs per spawn and the relative fecundity (total number of eggs per kg female) were determined. Fertilization success was calculated at the same time as [L/ (L + D)] %.

To estimate the quality of the eggs and larval survival, eggs from each spawn were placed individually in 96-well microtiter plates (in duplicates) according to the procedure of Panini et al. (2001), with some modifications. Briefly, floating fertilized eggs were taken in a 300 µm mesh filter and were rinsed with sterilized seawater and poured in a 1 l beaker. Only fertilized eggs were taken one by one with a micropipette set to 200 µl, and they were transferred to the wells of the microtiter plates (one egg per well). The microtiter plates were then covered with a plastic lid to avoid any evaporation or contamination, placed in a controlled-temperature incubator set at the

same temperature as the water spawning tanks. Once per day, embryonic and early larval development were evaluated with the stereoscope, and the number of live and dead embryos/larvae was recorded every day for 7 days. With this data the other egg quality parameters were evaluated: (a) Percentage of viable eggs at 24 hours was calculated as the number of eggs having live embryos/the number of fertilized eggs initially loaded in the microtiter plates, (b) hatching success was calculated as the number of hatched larvae/the number of viable eggs at 24 hours, (c) larval survival at 1dph was calculated as the number of live larvae 1 dph/the number of hatched larvae and (d) larval survival at 3 dph was calculated as the number of live larvae 3 dph/the number of hatched larvae.

2.6. Egg and larvae sizes

Egg diameter was estimated from 150 eggs from 10 different spawns. Eggs from the same spawns were also stocked in 500 l incubators (50 eggs/l) supplied with the same water as the broodstock tanks. From these spawns, 30 newly hatched larvae and 30 larvae at the end of yolk sack absorption were measured for total length (TL), standard length (SL), diameter of oil globule (LGD), yolk sack length (YSL) and width (YSH), using a profile projector as mentioned before for the oocyte diameter (Figure 2-3). All the measurements were made in live larvae anaesthetized with clove oil at 1% to avoid deformities produced upon dying. The volume of the yolk sack (YSV) was calculated using the formula proposed by Blaxter and Hempel (1963)**:** $YSV = \pi / 6 \; YSL \times YSH^2$.

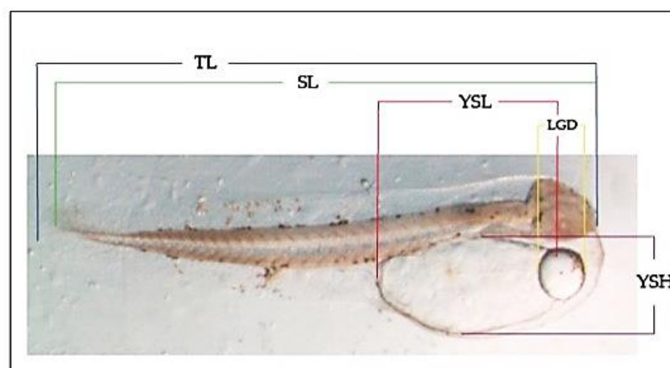


Figure 2-3. Morphometric measurement procedures on the larvae of greater amberjack (Sarih et al., 2018).

2.7. Biochemical analysis

Along the study, feed and fertilized eggs biochemical composition were conducted following standard procedures (AOAC, 2016). Samples were frozen (-80 ºC) for its later analysis. Amino acid separation and quantification were made in the Skretting Aquaculture Research Centre (Stavanger, Norway) and all others biochemical analyses were made in the GIA laboratories SABE (ECOAQUA Institute, ULPGC, Spain). All the analyses were performed at least in triplicate.

2.7.1. Moisture content

Dry matter content was determined after drying the fresh known sample quantity (P_i) in an oven at 110 ºC until constant weight was obtained (P_f). Before being weighted, the samples were introduced in a desiccator for 30 min to ambient temperature adaptation and finally they were weighted to obtain final data. The dry matter content was obtained by the following expression.

$$\%DM = ((P_i - P_f) \times 100)/P_i$$

2.7.2. Ash content

The ash content was determined by combustion a well-known amount of sample (P_m) in a Muffla furnace, at 600ºC for 12 hours, remaining ashes amount was recorded (P_c) and weight until constant weight according to the AOAC (2016). Final ash content was obtained applying the following expression:

$$\%Ash = (100 \times P_m)/P_c$$

2.7.3. Crude lipid content

Total crude lipids content was extracted following the method of (Folch et al., 1957), by homogenising a sample amount between 50-500 mg in an Ultra Turrax (IKA-Werke, T25 BASIC, Staufen, Germany) at 11,000 rpm during 5min in a solution of 5ml of Chloroform: Methanol (2:1) with 0.01% of butylated hydroxytoluene (BHT). The resulting solution was filtered by gravimetric pressure through glass wool and 0.88% KCl added to increase the water phase polarity. After decantation and centrifugation at 2000rpm during 5min, the watery and organic phases were separated. Once watery phase was eliminated, the solvent was dried under nitrogen atmosphere and subsequently total crude lipids weighed.

2.7.4. Fatty acid methyl esters preparation and quantification

Fatty acid methyl esters (FAMEs) were obtained by transmethylation of total lipid with 1% sulphuric acid in methanol (Christie and Han, 2010). The reaction was conducted in dark conditions under nitrogen atmosphere for 16 h at 50 ºC. Afterwards, FAMEs were extracted with hexane: diethyl ether (1:1, v/v) and purified by adsorption chromatography on NH_2 Sep-pack cartridges (Waters S.A., Massachussets, USA). FAMEs were separated by Gas-Liquid Chromatography (Agilent 7820A, CA, USA) in a Supercolvax-10-fused silica capillary column (length:30 mm, internal diameter: 0.32 mm, Supelco, Bellefonte, USA) using helium as a carrier gas. Column temperature was 180 ºC for the first 10 min, increasing to 215 ºC at a rate of 2.5 ºC min^{-1} and then held at 215 ºC for 10 min, following the conditions described in (Izquierdo et al., 1990). FAMEs were quantified by Flame ionization detector and identified by comparison with external standards and well-characterized fish oils (EPA 28, Nippai, Ltd. Tokyo, Japan).

2.7.5. Protein content

Protein content was estimated from analysis of the total nitrogen present in the samples, using the Kjeldhal method (AOAC, 2016). Briefly, after the digestion of the sample (between 200 - 500 mg) with concentrated sulphuric acid at a temperature of 400 ºC. Then total nitrogen content was determined and converted to total crude protein value by multiplying by the empirical factor 6.25.

2.7.6. Amino acid separation and quantification

Samples were hydrolysed with 6 N hydrochloric acid (HCl) in a closed test tube, shaken for 20 min and then kept in the oven for 22 h at 110 °C. Amino acids separated and quantified by gradient exchange chromatography using an HPLC, according to the Pico-Tag method after prederivatization with phenyl isothiocyanate (PITC) (Cohen et al.,1989), using norleucine as an internal standard.

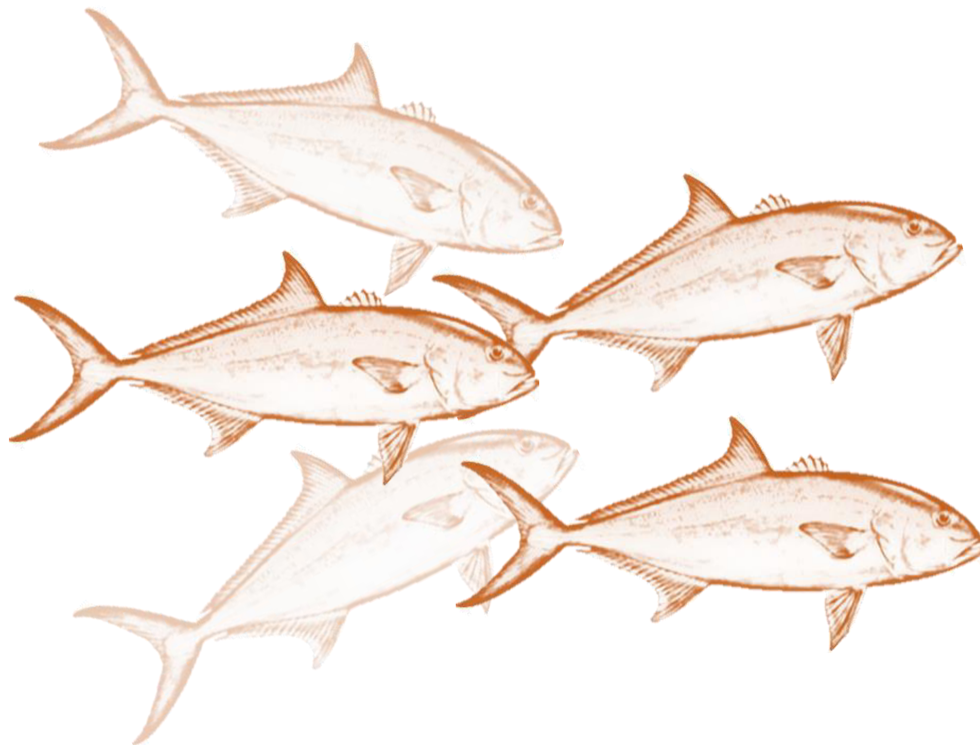
2.8. Statistical analysis

Results are presented as means and standard deviation (SD) and the significant level for all analyses was set at 1 and/or 5%. All data were tested for normal distribution with the one sample Kolmogorov–Smirnoff test, as well as for homogeneity of the variances with the Levene test (Sokal and Rohlf, 2012). When the assumptions were

correct, one-way Analysis of Variance (ANOVA) test was performed, followed by Tukey's post hoc test. When the heterogeneity of the variances was not correct and/or data were not normally distributed, Kruskal-Wallis test was applied and differences between treatments were graphed with a box and whisker plot. Pearson's correlation coefficients were used to assess the relationships between egg quality variables. Analyses were conducted by SPSS statistics (version 22.0 for Windows; Inc, IBM, Chicago, IL, USA) and visualized using SigmaPlot 12.0 (Systat software, San José, USA).

Chapter 3

High-quality spontaneous spawning in greater amberjack (*Seriola dumerili*, Risso 1810) and its comparison with GnRHa implants or injections



High-quality spontaneous spawning in greater amberjack (*Seriola dumerili*, Risso 1810) and its comparison with GnRHa implants or injections

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Abstract:

Production of sufficient high-quality eggs of greater amberjack still constitutes the main bottleneck for commercial production of this species. The main objective of this study was to compare the quality of spontaneous spawn of greater amberjack with those obtained by either GnRHa injection or GnRHa implant protocols. Captive amberjack broodstock were distributed in 3 circular tanks of 40 m^3. Broodstock from tank 1 were not hormonally induced and spawned spontaneously, whereas those of tank 2 were intramuscularly injected with GnRHa (20 µg.kg^{-1} body weight) and those of tank 3 were given EV-500 µg GnRHa implants. The number of eggs per spawn obtained in the broodstock without hormonal treatment was larger than in those obtained with injections or implants. Egg quality was best in broodstock with spontaneous spawn, followed by GnRHa injected fish and then GnRHa implants. Besides, size of larvae from control and injected broodstock were similar between them and significantly higher ($P < 0.01$) than those from GnRHa implant spawn. Overall, this study showed that it is possible to obtain very high quality spontaneous spawn in greater amberjack, providing the adequate conditions. Furthermore, GnRHa weekly injections lead to similar egg viability and hatching rates than spontaneous spawn and higher fertilization rates than GnRHa hormonal implants, which is better than in previous studies.

Keywords: *Seriola dumerili*, induction, spawning, GnRHa, egg quality.

3.1. Introduction

Diversification is an urgent need for aquaculture (Teletchea and Fontaine, 2014) and new candidate species must be highly appreciated by consumers so to get high prices on the market. The first prerequisite for the culture and industrial production of a new species is the control of its reproduction. To have a sustainable production of high quality eggs, it is necessary to have good knowledge of a species' reproductive physiology and control of reproductive function in captivity. However, in most new aquaculture species, reproduction in captivity is the main bottleneck, as many species exhibit reproductive dysfunctions (Mylonas and Zohar, 2007; Mylonas et al., 2010).

Greater amberjack has a great potential for the expansion of the European Union (EU) aquaculture industry and, therefore was one of the species targeted by the EU Project DIVERSIFY (7FP-KBBE-2013-GA 602131). However, in captivity, wild-caught greater amberjack rarely undergo spontaneous oocyte maturation, ovulation and spawning (Micale et al., 1999; Mandich et al., 2004; Mylonas et al., 2004a). This failure can be related to a maladaptation of the wild fish to captive conditions, or to a mismatch in the environmental factors required to promote complete gonad development until spawning (Carrillo et al., 1995). Thus, controlled reproduction still constitutes one of the main bottlenecks for commercial production of this species. Hormonal manipulation using exogenous gonadotropin-releasing hormone synthetic analogue (GnRHa) has been shown to be effective in inducing maturation and multiple spawn (Kožul et al., 2001; Mylonas et al., 2004a; Fernández-Palacios et al., 2015a), while the occurrence of spontaneous spawning has been reported rarely (Kawabe et al., 1996, 1998; Jerez et al., 2006). But the spawning quality is relatively low, with highly variable percentages of fertilization (10-61.75 %) and hatching (4.17-20.88 %), and low larval survival at 40 days post-hatching (3.5 %) (Mylonas et al., 2004a; Papandroulakis et al., 2005; Jerez et al., 2006).

Greater amberjack has a rapid growth rate, reaching a weight of 6 kg in 2.5 years in culture (Jover et al.,1999). Maturity occurs at the age of 3 years, but in captive conditions functional breeders are 4 and 5 years old for males and females, respectively (Herrera and Smith-Vaniz, 2015), and exhibits a group-synchronous oocyte development and a multiple spawning pattern (Marino et al., 1995a; Díaz et al., 1997) with a reproductive season extending from late spring to early autumn, depending on geographic location (Jerez, 2013). In meagre, which is also a species

with group-synchronous ovarian development, multiple GnRHa injections resulted in more consistent spawning results and better control of egg production in comparison with GnRHa implants, and this method may offer significant advantages to commercial aquaculture production (Mylonas et al., 2015). Similarly, in European sea bass, the fertilization and hatching rates were improved by using repeated injections of GnRHa (Forniés et al., 2001). This species, which also shows a group-synchronous type of ovarian development, needs high levels of gonadotropins in a short period of time to induce oocyte maturation and spawning (Asturiano et al., 2000; Carrillo et al., 2015). On the other hand, the quality of eggs, in terms of fertilization, hatching and survival rates, obtained with hormonal induction may be often lower than from spontaneously spawning broodstocks in species as southern flounder (*Paralichthys lethostigma*) (Watanabe et al., 2001) or red snapper (*Lutjanus campechanus*) (Papanikos et al., 2003; Phelps et al., 2009). Nevertheless, the percentage of egg viability is not different between spontaneous or induced spawn in gilthead seabream (Barbaro et al., 1997), and the fertilization and hatching rates of eggs from induced spawn are better than those from spontaneous spawn in brill (*Scoptlhalmus rhombus*) (Basaran et al., 2008) and cobia (Nhu et al., 2011).

Therefore, there is a need to improve broodstock management methods for greater amberjack, to optimize the reproductive function of greater amberjack maintained in captivity and to promote spontaneous spawning with a potential better egg quality than hormonal therapies. The present study evaluated and compared the egg quality of hormonal induction protocols with injections or implants with the spontaneous spawn of greater amberjack broodstock.

3.2. Material and methods

3.2.1. Broodstock selection

Before starting the experiment (3 June 2014), in late May 2014, all fish were anesthetized with clove oil (Guinama SL, Valencia, Spain; 50 ppm), weighted and sized (Table 3-1). Gonad maturation state was also evaluated by ovarian biopsies obtained with cannulation of the genital pore. All examined females (♀, n=10) had oocytes of more than 500 µm in diameter, and all males (♂, n=12) emitted sperm upon abdominal massage. Broodfish were fed twice a week with a commercial compound diet specific

for broodstock maintenance (Vitalis Repro™, Skretting, Burgos, Spain) at 1% of their estimated total biomass, and once a week with locally fished Atlantic mackerel at 2%.

Table 3-1. Biometric data of greater amberjack broodstock with spontaneous spawns (Control) and injected or implanted GnRHa*.

	Treatments		
	Control	**Injected**	**Implanted**
Weight (kg)			
Female	9.81 ± 1.09	11.83 ± 1.10	10.72 ± 0.98
Male	9.48 ± 1.76	11.78 ± 1.77	10.19 ± 1.02
Total length (cm)			
Female	90.00 ± 2.83	94.50 ± 5.56	96.66 ± 2.25
Male	87.20 ± 4.81	95.66 ± 3.78	92.17 ± 4.54
Fork length (cm)			
Female	78.50 ± 2.12	83.83 ± 4.72	82.33 ± 1.52
Male	77.20 ± 4.08	85.00 ± 3.46	82.00 ± 4.77
Biomass (kg/m^3)	1.67**	2.39	1.83

*Means ± SD, n=3, exception in the control treatment (n=2 females).
**14 June a male jumped out of the tank and died, biomass changed to 1.44 kg/m^3.

At the end of May 2014, two females from the monitored population had oocytes >800 µm. Since Fernández-Palacios et al. (2015a) and Roo et al. (2009) in Las Palmas, Kawabe et al. (1996, 1998) in Japan, and Jerez *et al.* (2006) in Tenerife, obtained spontaneous spawn without hormonal induction with wild-caught females having oocytes of only ~600 µm in diameter, these two females were allowed to spawn spontaneously, and therefore were not induced. From the other eight females, those six with the highest oocyte diameter (672 ± 87 µm) were selected for the experiment. Tanks were filled with seawater (37 ‰ salinity) and kept under a natural photoperiod (27º 59’ 28” N; 15º 22’05” W) of approximately 13 h light. Flow rate allowed 6 complete water tank renovations daily and the temperature was continuously monitored (Miranda, Innovaqua, Sevilla, Spain). Fish were fed twice a week with commercial feeds (13 mm, Vitalis CAL, Skretting, Burgos, Spain) at 1% of their estimated total biomass, and once a week with Atlantic mackerel at 2% of their total biomass.

3.2.2. Spawning induction

The selected fish were distributed in three 40 m^3 (5 m x 2.35 m) circular tanks, as follows. In tank 1 (2 ♀ and 5 ♂), the fish were not induced hormonally or handled, and spawned spontaneously, being considered the control group. In tank 2 (3 ♀ and 3 ♂), the fish were injected intramuscularly with gonadotropin-releasing hormone analogue (LHRHa, des-Gly10, [D-Ala6]-; Sigma-Aldrich, St. Louis, MO, USA) at a dose of 20 µg. kg^{-1} body weight, based on the reported dosage for greater amberjack (Fernández-Palacios et al., 2015a). These hormonal treatments were applied twice a week alternating the broodstock (1 ♀ and 1 ♂). The three males and three selected females of tank 3 (3 ♀ and 3 ♂), were induced using 500-µg GnRHa implants (Mylonas and Zohar, 2001). One implant (500 µg) was used for each female (Mylonas et al., 2004a) and half the dose for males. Implants were given subcutaneously, at about three scale rows down from the posterior end of the dorsal fin. The frequency of GnRHa injection was once every 11.8 ± 3.0 days, while the frequency of GnRHa implantation was once every 26.9 ± 7.6 days.

3.2.3. Evaluation of egg and larval quality

To test the spawning induction efficiency (Fernández-Palacios et al., 2015a) the following parameters were determined: number of spawning females that responded to hormonal treatment, number of spawn, spawn obtained per induction and latency period (time from the hormonal treatment and the first spawn, based on the presence of eggs in the outflow egg collectors, which were monitored during the day every 15-20 minutes). Only in the case of spontaneous spawning, the number of spawning females and the number of spawn were determined. Also, for each treatment, the total number of eggs, the number of eggs per female, the number of eggs per spawn, and the number of eggs per spawn and weight of female were determined. For induced spawn, the number of eggs per kg of females and per induction was also determined.

For each spawn, the fertilization rate was determined to evaluate egg quality. Eggs were placed in a 10-l bucket provided with strong aeration, from where a random sample of 10-ml was taken using a plastic pipet and placed into a 17-ml Falcon tube. From there, all the eggs in the sample were placed in a Bogorov chamber and identified as live (fertilized L) or dead (D) under a binocular microscope (Leica- S6E, Wetzlar, Germany). The fertility of the spawn was calculated as [(L + D) x 1000] and fertilization

success was calculated as [L / (L + D)] %. Fertilized eggs were then placed in 96-well microtiter plates in replicates (Greiner Bio-One, Kremsmunster, Australia) (Panini *et al.*, 2001) and were incubated in a controlled temperature incubator at 22.1 ± 0.4 ºC, in order to estimate the percentage of viable eggs at 24 hours, hatching and larval survival at 4 days just before the yolk sac was almost completely absorbed.

3.2.4. Egg and larval measurements

Egg diameter was estimated from 150 fertilized eggs from 10 different spawn for each treatment. From these spawn, 30 newly hatched larvae and 30 larvae at the end of yolk sac absorption were measured for total length (TL), standard length (SL), diameter of oil globule (LGD), yolk sac length (YSL) and width (YSH). The volume of the yolk sac (YSV) was calculated using the formula: $YSV = \pi / 6\ YSL \times YSH^2$.

3.2.5. Statistical analysis

The data were analysed using the statistical program SPSS statistics (version 22.0 for Windows; Inc, IBM, Chicago, IL, USA) and visualized using SigmaPlot 12.0 (Systat software, San José, USA). The data were expressed as a mean ± SD. Normality and homogeneity of the variance of all the variables were evaluated using the Kolmogorov-Smirnov test and Levene tests respectively (Sokal and Rolf, 2012). When the assumptions were correct, one-way ANOVA tests were performed, followed by Duncan's New Multiple Range Test. When an ANOVA was not possible due to the heterogeneity of the variances and/or the data were not distributed normally, a Kruskal-Wallis test was applied. The differences between treatments were graphed with a box and whisker plot.

3.3. Results

Biometric studies of initial broodstock denoted no significant differences among fish with the different broodstock management protocols, and a low biomass rate was kept in all the tanks (Table 3.1). At the beginning of the trial, the mean oocyte diameter of non-hormonally treated females (837 ± 167 µm) was statistically higher ($P < 0.01$) than those of hormonally induced (690 ± 100 µm injected females and 648 ± 59 µm implanted females).

All GnRHa induced females spawned during the study. First spontaneous spawn was obtained in control broodstock on June 1st, 2014 and GnRHa injection and implants were given on June 3rd. Last spontaneous spawn was obtained on October 18th, whereas spawn from GnRHa injected and implanted broodstock were respectively obtained on October 21st and 14th. Three further hormonal inductions after this period did not result in any spawn.

The number of spawn produced after each GnRHa induction was significantly higher ($P < 0.01$) for the implanted broodstock (2.23 ± 1.85) than for the injected one (0.78 ± 0.53). No significant differences were recorded between the latency periods of induced spawn (43.06 ± 2.49 h in hormonal injected and 44.19 ± 7.44 h in hormonal implanted broodstock).

The number of eggs per spawn and number of eggs per kg of female and spawn were significantly ($P < 0.01$) higher in spontaneously spawning broodstock, whereas the number of eggs per kg of female and per induction was higher ($P < 0.05$) in implanted fish than in injected fish (Table 3-2).

Table 3-2. Number of eggs obtained from greater amberjack broodstock after treatment with GnRHa injections or implants, in comparison with spontaneously spawning fish (Control)*.

	Treatments		
	Control	**Injected**	**Implanted**
Number of eggs (x 10^6)	25.60	12.90	10.53
Number of eggs per female (x 10^6)	12.80	4.30	3.51
Number of eggs per spawns (x10^6)	1.11 ± 0.32^a	0.44 ± 0.27^b	0.28 ± 0.29^b
Number of eggs per kg female and spawns (x 10^4)	5.67 ± 1.66^a	3.72 ± 2.30^b	2.52 ± 2.73^b

* Mean ± SD. Different superscripts in the same row indicate significant differences ($P < 0.01$). Total period of treatments: control from June 1st to October 18th; injection from June 3rd to October 21st; implant from June 3rd to October 14th.

Overall, the spawning quality of the spontaneous spawn was extremely good and significantly higher than the GnRHa induced spawn (Table 3-3). Thus, the highest ($P < 0.01$) fertilization rates were found in spontaneous spawn, followed by those of injected fish and, then, implanted fish. Percentage of viable eggs at 24 hours ($P < 0.05$) and hatching rates ($P < 0.01$) were significantly higher in the control fish in comparison to the implanted fish, whereas injected fish showed intermediate values. Highest larval survival ($P < 0.01$) was also found in spontaneous spawn.

Table 3-3. Quality of egg and larvae obtained from greater amberjack broodstock after treatment with GnRHa injections or implants, in comparison with spontaneously spawning fish (Control)*

	Treatments		
	Control	**Injected**	**Implanted**
Fertilization rate (%)	84.37 ± 21.57[a]	58.82 ± 26.79[b]	32.15 ± 34.60[c]
Viable eggs rate (%)	92.21 ± 9.43[A]	86.37 ± 25.39[AB]	77.60 ± 34.01[B]
Hatching rate (%)	96.60 ± 6.56[a]	91.13 ± 25.44[ab]	77.96 ± 34.93[b]
Larval survival rate at 4 days (%)	69.91 ± 16.51[a]	58.09 ± 23.66[b]	49.45 ± 27.36[b]

[*] Mean ± SD. Different superscripts in the same row indicate significant differences (Lower letters P < 0.01; Capital letters P < 0.05).

A total number of eggs produced per kg of female and per spawn in spontaneously spawning broodstock was significantly higher (P < 0.01) than in injected or implanted fish (Figure 3.1). Also, the number of fertilized and viable eggs, as well as the number of larvae produced at the different stages was highest in spontaneous spawn, followed by injected fish, whereas the significantly (P < 0.01) lowest values were obtained in implanted fish (Figure 3-1).

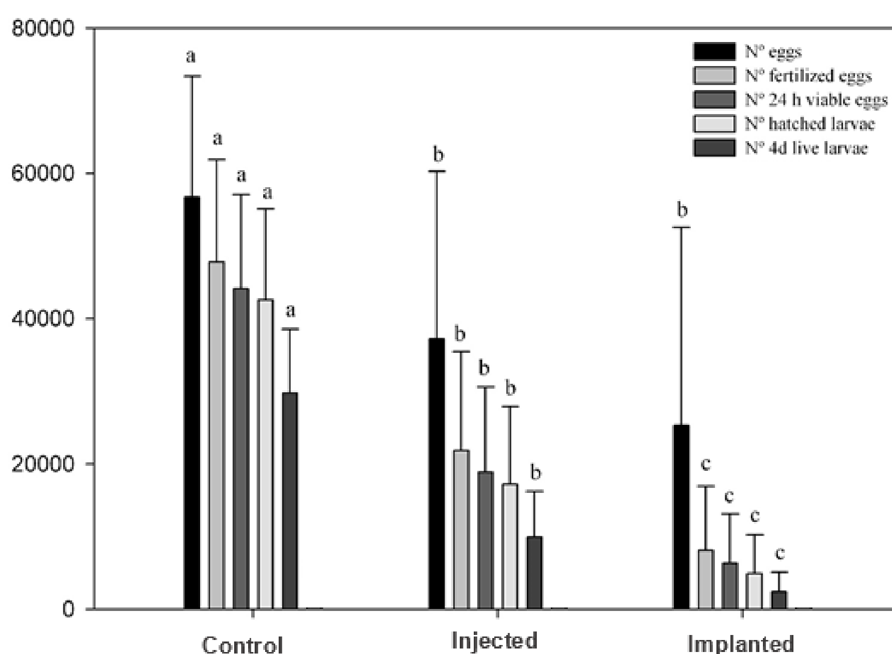

Figure 3.1. Production rates (per kg female and spawn) in greater amberjack with spontaneous spawns (Control) and injected or implanted GnRHa. Bars, of the same shade, with the same letter were not significantly different (P < 0.01).

Eggs from spontaneous spawn were significantly larger than those of induced spawn (Table 3-4). In newly hatched larvae from spontaneous spawn and injected broodstock, the total and standard length were similar and were significantly higher ($P < 0.01$) than larvae from GnRHa implant fish. The yolk sac volume was significantly greater ($P < 0.01$) in larvae from spontaneously spawning broodstock, compared to eggs from the other two treatments. The diameter of the oil droplet of larvae obtained in spontaneous spawn was significantly higher ($P < 0.01$) than in the hormonally induced spawn. The total and standard length in larvae with absorbed yolk sac, as well as the diameter of oil droplet, were significantly larger ($P < 0.01$) in larvae obtained from spontaneous spawn and GnRHa injected broodstock, compared to the GnRHa implanted fish.

Table 3-4. Egg and larvae sizes from greater amberjack broodstock with spontaneous spawns (Control) and injected or implanted GnRHa*

	Treatments		
	Control	Injected	Implanted
Egg diameter (mm, n=4500)	1.13 ± 0.03^a	1.10 ± 0.03^b	1.10 ± 0.03^b
Newly hatched larvae (n=450)			
Total length (mm)	2.59 ± 0.10^a	2.59 ± 0.13^a	2.45 ± 0.13^b
Standard length (mm)	2.49 ± 0.10^a	2.49 ± 0.13^a	2.36 ± 0.12^b
Yolk-sac volume (mm^3)	0.44 ± 0.11^a	0.34 ± 0.09^c	0.40 ± 0.12^b
Oil droplet diameter (mm)	0.30 ± 0.02^a	0.28 ± 0.02^b	0.28 ± 0.03^b
Larvae with yolk-sac absorbed (n=450)			
Total length (mm)	3.85 ± 0.13^a	3.82 ± 0.13^a	3.53 ± 0.26^b
Standard length (mm)	3.68 ± 0.13^a	3.67 ± 0.13^a	3.42 ± 0.24^b
Oil droplet diameter (mm)	0.11 ± 0.03^a	0.11 ± 0.02^a	0.14 ± 0.03^b

[*] Mean ± SD. Different superscripts in the same row indicate significant differences ($P < 0.01$).

3.4. Discussion

The good growth performance and survival of captured greater amberjack individuals, underline that this species may acclimatized well to captivity conditions, in agreement with previous studies (Fernández-Palacios et al., 2015a; Jerez et al., 2006). Despite vitellogenesis of this species may be inhibited in captivity (Micale et al., 1999), in other stocks only oocyte maturation fails to be completed (Lazzari et al., 2000; Mandich et al., 2004; Marino et al., 1995a; Mylonas et al., 2004a) due to unsuitable

environmental (Mylonas et al., 2010) or stress inducing conditions (Schreck, 2010). However, in the present study, greater amberjack caught in a single location of the Atlantic Ocean in a single day, therefore coming from the same fish school and having probably the same age (2-3 kg), acclimatized very well to captive conditions (40 m^3 tank) and completed gonadal development. Thus, at the beginning of the spawning season, oocytes reached a mean diameter of ~670 µm and up to >800 µm, larger than previously described for this species in captivity (Kawabe et al., 1996, 1998; Jerez et al., 2006; Roo et al., 2009; Fernández-Palacios et al., 2015a). These latter females with larger oocytes proceeded with very successful spontaneous spawning without any hormonal therapy. Despite induced spawn of the *Seriola* genus have been obtained in 10 m^3 tanks (Fernández-Palacios et al., 2015a, b), large volume tanks (50-80 m^3) have been recommended for these species (Benneti, 2008) and spontaneous spawn have been obtained in tank volumes ranging from 56 to 500 m^3 (Kawabe et al., 1996, 1998; Jerez et al., 2006).

Spontaneous spawn were obtained between June and October 2014, with a mean frequency of spawn every 6 days, in agreement with previous studies (Marino et al., 1995a; Jerez et al., 2006). Annual changes in photoperiod are responsible for gonadal maturation, activating the endocrine reproductive axis of fish, as well as temperature, which is also a synchronizing factor to indicate the appropriate environmental conditions for spawning (Carrillo et al., 1989; Falcón et al., 2003; Maitra et al., 2006). Spontaneous spawning of greater amberjack in captivity without any exogenous hormonal induction in individuals captured from the wild have been only obtained in the Canary Islands (Spain) or Ogasawara Archipelago (Japan), which are in the same geographical latitude and, therefore, similar photoperiod (Figure 3-2). The fact that both locations had the same geographical latitude explains the occurrence of these spontaneous spawnings, since the photoperiod triggers the spawning season (Carrillo et al., 1995). In Ogasawara Archipelago, the spawning season lasts 41-55 days within a range of temperatures of 21.8-25.9ºC (Kawabe et al., 1996, 1998), whereas in the Canary Islands the spawning season lasts 140-156 days, with temperatures between 19.7 and 24.5ºC (Jerez et al., 2006 and present study). Thus, spawning season is 3 times longer in the Canary Islands than in Ogasawara, what could be due to a longer period of water temperature between 19 and 26ºC (Figure 3-3), since water temperature controls the duration of the spawning (Carrillo et al., 1989).

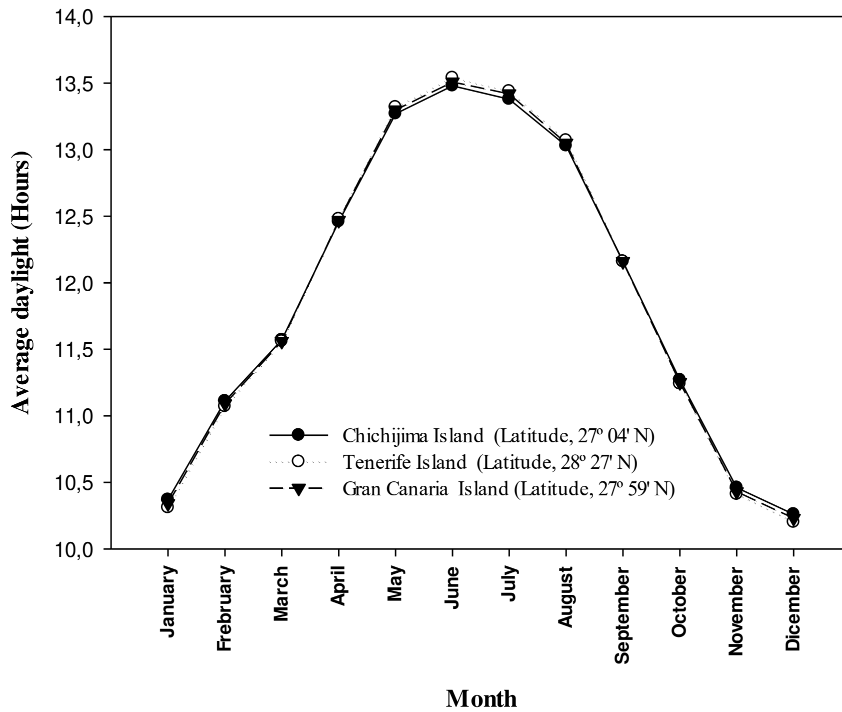

Figure 3-2. Mean monthly light hours in the island of Chichijima (Osagawara, Japan), Tenerife and Gran Canary (Canaries, Spain) (www.climatemps.com).

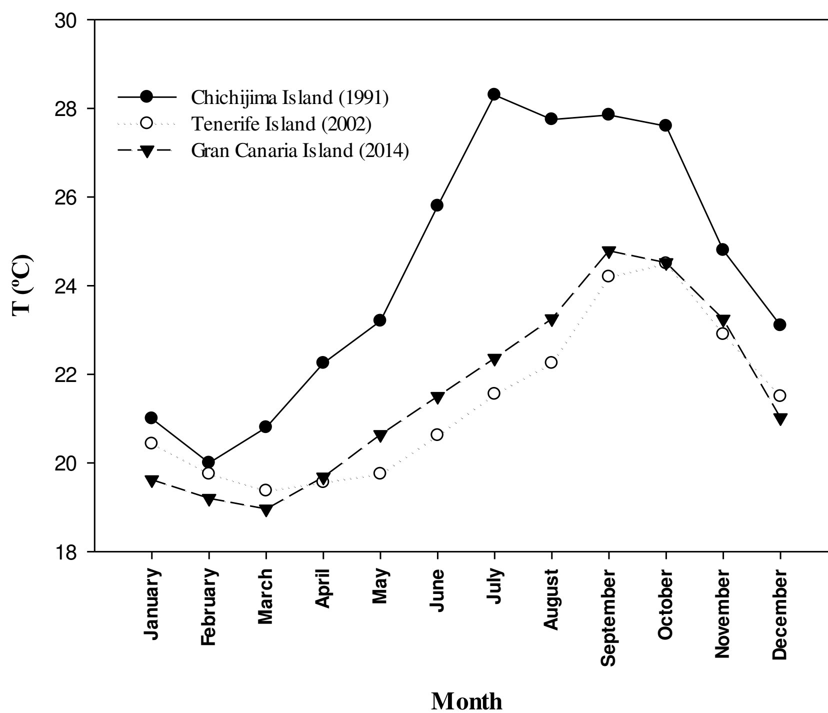

Figure 3-3. Mean monthly temperature in the island of Chichijima, Ogasawara, Japan (Kawabe et al., 1996), and Tenerife (Jerez et al., 2006) and Gran Canaria, Spain (this study).

Quality of spontaneous spawn was very high, in terms of total number of eggs per kg female/spawn, fertilization and larval survival rates in comparison to previous studies. For instance, total number of eggs per female (12.8 million eggs/female/spawn) was higher than in other studies with amberjack of similar weight (3.1 and 2.86 million eggs/female/spawn in Kawabe et al., 1996 and Jerez et al., 2006, respectively, assuming a ratio female: male of 1:1). Besides, the number of eggs per kg female per spawn in the present study was 5 times higher than in previous studies (Jerez et al., 2006). Moreover, the fertilization rates were between 11 and 35% higher than those obtained in previous studies (Kawabe et al., 1996, 1998; Jerez et al., 2006) where broodstock were fed fish. In addition, hatching rates were also 80% and 17% higher than in those studies in Japan (Kawabe et al., 1996, 1998) and Canary Islands (Jerez et al., 2006), respectively. This improved egg quality in the present study could be related to the larger oocyte size previously discussed, in relation to more favourable temperature, nutrition or fish welfare conditions. The hatched larvae had a similar yolk sac volume to those of other fast-growing species: 0.4-0.5 mm^3 for yellowtail amberjack (Moran, 2007), 0.43 mm^3 for meagre (Klimogianni et al., 2013) and higher than other marine species with pelagic eggs of similar size 0.36 mm^3 for European sea bass (Pope et al., 2014).

The hormonal induction by GnRHa injections or implants successfully induced spawn in >500 µm eggs bearing females, in agreement with previous studies (Kožul et al., 2001; De la Gándara et al., 2004; Mylonas et al., 2004a; Fernández-Palacios et al., 2015a, b). GnRHa has been used to induce spawning either through injections (Fernández-Palacios et al., 2015a) or through controlled release implants (Mylonas et al., 2004a). However, none of these studies compares spontaneous spawn with hormonal induced ones. In the present study, hormonal induction with either injections or implants lead to a lower number of eggs produced per kg of female per spawn in comparison to the spontaneously spawning fish, what could be related to the potential stress caused by the manipulation during hormonal induction. Indeed, handling stress has been shown to affect fish reproduction in different ways depending on the species, sex, gonad maturation and stress tolerance (Schreck et al., 2001). Besides, hormonal induction also caused a reduction in larval survival rates in comparison to spontaneously spawning amberjack, which is in agreement with studies on other species (Papanikos et al., 2003). During endogenous nutrition, larvae depend on their

yolk sac reserves, including the oil droplet (Sanderson and Kupferberg, 1999), which are correlated with egg size (Dabroswki and Luczynski, 1984) and higher larval survival (Brooks et al., 1997). Thus, in the present study, the lower survival rate in larvae from hormonally induced fish could be related to the lower egg and oil droplet diameter, frequently used as indicators of spawning quality in fish (Faulk and Holt, 2008), suggesting an impaired vitellogenesis in comparison to the spontaneous spawned eggs. Indeed, hormonally induced females produced more spawn episodes than the spontaneously spawning females, suggesting a faster vitellogenetic period in the former that could be related to the difference in the hormone's levels. Egg diameter has been also related to the number of spawn during the spawning season in other species (Brooks et al., 1997). Still, in the present study, egg diameters obtained by hormonal induction (1.1-1.13 mm) were similar to those obtained from GnRHa implanted greater amberjack (1.02 ± 0.01 mm) (Mylonas et al., 2004a) and from broodstock injected with hCG (1.12-1.14 mm) (Kožul et al., 2001).

Hormonal injection with GnRHa was particularly successful, leading to higher fertilization rates than hormonal implants, and equal egg viability and hatching rates than spontaneous spawning. Indeed, egg viability in GnRHa injected fish was 30% higher than the 55.5% previously reported for this species (Fernández-Palacios et al., 2015b). On the contrary, GnRHa implants lead to the lowest fertilization, egg viability and hatching rates. As a consequence, the lowest number of eggs and larvae were obtained with GnRHa implants in comparison to GnRHa injected or spontaneous spawning greater amberjack. These results are in agreement with the low fertilization rates previously obtained in GnRHa implanted fish that ranged between 22-50% (Mylonas et al., 2004a), whereas in GnRHa injected fish fertilization rates reached up to 80%. Similarly, in yellowtail kingfish (*Seriola lalandi*), spawning induction through GnRHa implantation was reported to have a negative effect on egg quality due to lower fertilization rates, decreased numbers of viable eggs and smaller oil globule diameters (Setiawan et al., 2016). Moreover, in the present study, the length of newly hatched and yolk sac reabsorbed larvae from implanted fish were significantly lower than from injected fish, which showed equal size than larvae from spontaneous spawn. Interestingly, together with smaller length, newly hatched larvae from implanted fish showed larger yolk sac denoting the lower utilization of nutrient reserves in comparison to injected or spontaneously spawning amberjack.

Considering that in a close related species of the same genus, the Japanese amberjack, there were no differences in fertilization rates between GnRHa implanted or injected fish (Chuda et al., 2002), the protocol for GnRHa implants for greater amberjack must be still improved in order to obtain good quality eggs as with GnRHa injections or spontaneous spawn. For instance, the interval between successive hormonal injections (12 ± 3 days) was closer to the spawning interval of the spontaneous spawn (6 ± 3 days) than between GnRHa implants (27 ± 8 days). In previous studies, hormonal treatment of this species with GnRHa implants produced a single spawn after the first induction and 3 spawn in the second induction, 15 days after (Mylonas et al., 2004a). In another study, it was observed that an interval of 10 days between hormonal injections was sufficient to have a spawn of good quality (Fernández-Palacios et al., 2015b). On the contrary, with the shi drum (*Umbrina cirrosa*) when 3 injections of GnRHa were administered within 10 days, females did not respond to the second or third injection but did respond to 20 days intervals (Mylonas et al., 2004b).

As expected, the mean number of spawn per injection was significantly higher in the implanted broodstock than in the injected ones, in relation to the long-term release of GnRHa in implants, and therefore a long-term stimulation of reproductive function, resulting in more consecutive spawn (Mylonas and Zohar, 2007). Similar latency periods were obtained between both hormonal treatments and in comparison, to other studies (Fernández-Palacios et al., 2015b). Latency periods of 36-52 h have been described depending on water temperature (Tachihara et al., 1993) and a negative correlation between temperature and latency period has been shown in longfin yellowtail (*Seriola rivoliana*) (Fernández-Palacios et al., 2015a). In greater amberjack, shorter latency periods of 36 h were obtained using implants with a dose of 40 $\mu g.kg^{-1}$ GnRHa (Mylonas et al., 2004a) or 30 h when injecting broodstock with 50 $\mu g.kg^{-1}$ GnRHa (García et al., 2001). Other authors have obtained a latency period between 46 and 66 h when inducing wild broodstock with 1000 $UI.kg^{-1}$ hCG (Kožul et al., 2001).

In conclusion, this study showed for the first time that it is possible to obtain very high quality spontaneous spawn in greater amberjack, in relation to adequate tank size, environmental conditions, particularly temperature between 19-26ºC, and possibly broodstock management and nutrition. Besides, the GnRHa weekly injections protocol

used lead to spawn with higher fertilization rates than GnRHa hormonal implants, similar egg viability and hatching rates than spontaneous spawning and better than in previous studies. Finally, GnRHa implants also produced successful spawn, although the protocol for GnRHa implants for greater amberjack must be still improved in order to improve spawn, eggs and larval quality to reach similar quality of spontaneous spawn and even GnRHa injections.

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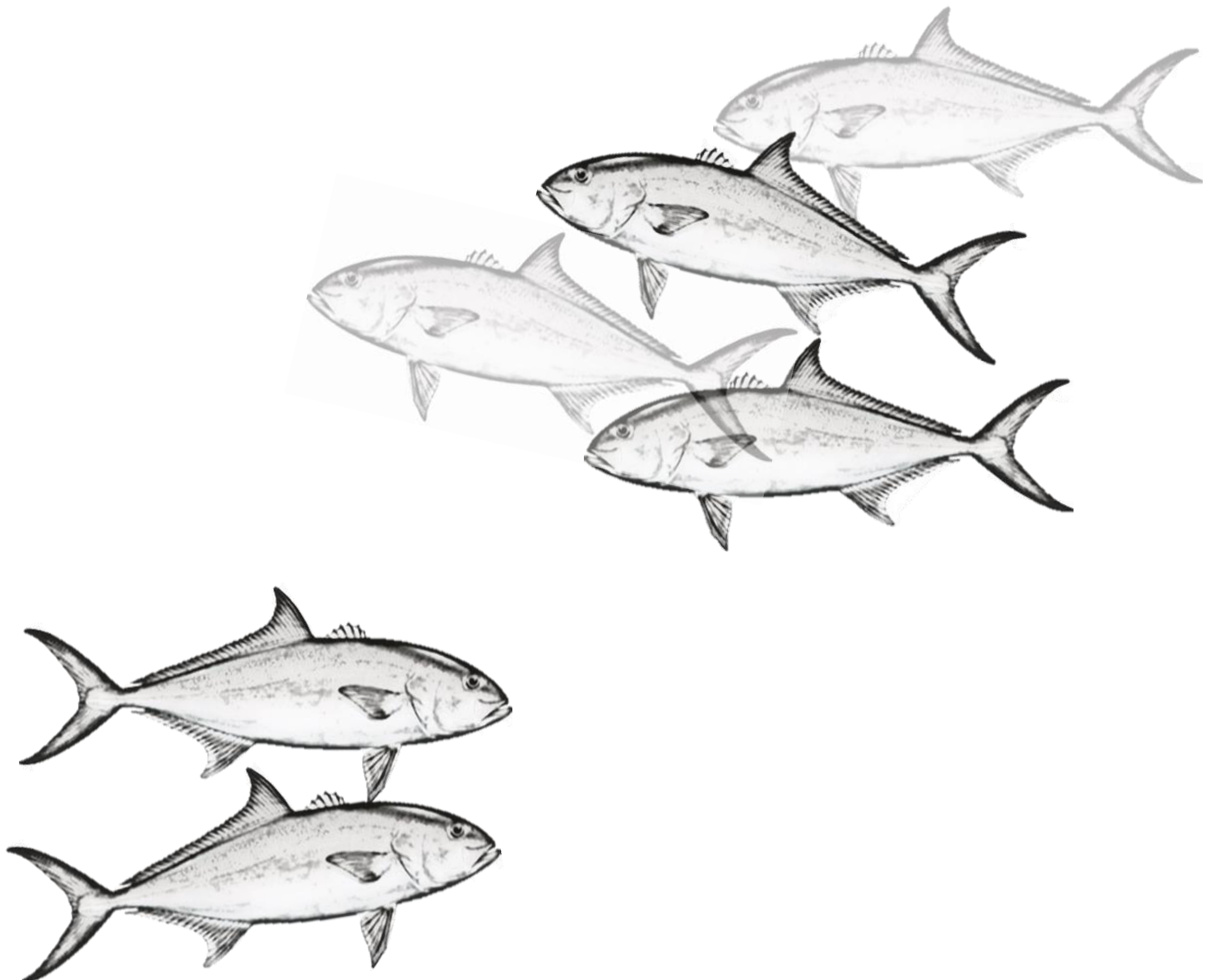
Chapter 4

Effects of increased protein, histidine and taurine dietary levels on egg quality of greater amberjack (*Seriola dumerili*, Risso, 1810)



Effects of increased protein, histidine and taurine dietary levels on egg quality of greater amberjack (*Seriola dumerili*, Risso, 1810)

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Abstract:

A well-balanced diet adapted to fulfil the specific nutritional requirements of greater amberjack would contribute to optimize reproduction and spawning quality. The main objective of the present study was to examine the effects of supplemental histidine, taurine and protein in broodstock diets, on egg quality of greater amberjack. Twelve broodstocks were distributed in three 40 m^3 circular tanks and fed three different diets, one high in histidine, another in taurine and the third one in protein contents. Overall the best spawning quality was obtained histidine levels were elevated from 1 to 1.5%, increasing the relative fecundity, fertilization rates, egg viability, hatching rates, larval survival, egg protein contents, and egg and larval size, denoting the importance of this amino acid for embryo and larval development. The increase of taurine from 0.3 to 1.1% in diets for greater amberjack increased the relative fecundity in comparison to fish fed higher protein levels. Fertilization rates tend to increase with the elevation of dietary taurine and were not significantly different from those of broodstock fed higher histidine levels. Besides, dietary taurine increased egg diameter and taurine contents. Increase in dietary protein contents from 51 to 56% lead to an increase protein content in egg, as well as a larger yolk sac volume, but did not improve any of the spawning quality parameters. In conclusion, the results of this study have pointed out the importance of raising histidine contents in broodstock diets from 1 to 1.5% to optimize the reproductive performance of greater amberjack. Besides, the study showed that taurine levels in broodstock diets increased fecundity, maintaining good fertilization rates, but further studies must be conducted to determine the optimum taurine dietary levels.

Keywords: Broodstock diet, protein, histidine, taurine, egg quality, *Seriola dumerili*.

4.1. Introduction

Greater amberjack has an excellent aquaculture potential due to its adaptability to captive conditions, fast growth and high market value (García and Díaz, 1995; Thompson et al., 1999; Harris et al., 2007; Fernández-Palacios et al., 2015a). However, the major constraint on its culture development is the insufficient production of eggs and the poor larval quality (Lazzari et al., 2000; Mylonas et al., 2004a; Papandroulakis et al., 2005). Since egg quality largely depends on nutrient transfer from the female, broodstock nutrition must be optimized to ensure good larval development and survival (Brooks et al., 1997; Izquierdo et al., 2001). Greater amberjack is a gonochoric species with group-synchronous ovarian development and a multiple spawning pattern (Marino et al., 1995a; Mandich et al., 2004; Mylonas et al., 2004a). Thus, as occurs in other species with short vitellogenetic periods, it is possible to boost spawning quality by improving the nutritional quality of broodstock diets even during the spawning season (Watanabe et al., 1985; Fernández-Palacios et al., 1995, 1997; Izquierdo et al., 2001). In particular, broodstock nutrition is determinant of reproductive success in other species of the same genus, such as goldstriped amberjack (yellowtail kingfish) or Japanese yellowtail (Tachihara et al., 1997; Matsunari et al., 2006), where appropriate feeding during reproduction allows the production of a higher quality of eggs and good quality larvae. However, information on nutritional requirements of greater amberjack broodstock is completely lacking and only very few studies provide some data on nutrition during reproduction (Rodríguez-Barreto et al., 2012, 2014; Zupa et al., 2017b). Among the different nutritional factors affecting fish reproduction, dietary protein constitutes a crucial nutrient for successful spawning (Coldebella et al., 2011; Zakeri et al., 2014; Aryani and Suharman, 2015). Thus, during reproduction, proteins and amino acids play important roles in fertilization, embryonic growth both as-deposited protein and energy source (Moran et al., 2007; Lochmann et al., 2007; Samaee et al., 2010; Fernández-Palacios et al., 2011; Lanes et al., 2012). In particular, fish egg proteins are determinant for fertilization success, in relation to different aspects including the formation of the cortical granules material (Hart, 1990).

Histidine is an essential amino acid for fish (NRC, 2011) and a major component of Japanese yellowtail (Tanahashi et al., 2014) that, directly or through its derivative

compounds, plays important roles in homeostasis maintenance, buffering and osmoregulation, anti-oxidation, anti-glycation of proteins and immune system regulation (Nagasawa et al., 2001; Li et al., 2009; Rhodes et al., 2010; Remo et al., 2011; Andersen et al., 2016; Ramos-Pinto et al., 2017). Histidine could be particularly important for reproductive success, since histidine muscle concentration is substantially increased just before spawning in sockeye salmon (Mommsen et al., 1980; Mommsen, 2004) and it is the main amino acid in gonads during spawning of certain species such as goldlined seabream (Qari et al., 2013). Moreover, histidine is preferentially retained over other amino acids during early larval development (Costa et al., 2014), suggesting the importance of adequate levels in fish eggs. However, there is no information about the effects of dietary histidine on reproductive performance.

Taurine (beta-amino sulfonic acid) has been found particularly important for broodstock and larval nutrition (Pinto et al., 2010, 2013; Matsunari et al., 2006, 2013; Al-Feky et al., 2016; Allon et al., 2016). In fish, this nutrient is involved in anti-oxidative defence, osmoregulation, neurotransmitter modulation, hormone release, bile salt synthesis (Chen et al., 2001; Takagi et al., 2006; Kato et al., 2014) and protection of spermatogonia from oxidative stress (Higuchi et al., 2012a, b). It has been also identified as an essential component in broodstock diets for Japanese yellowtail necessary to improve fecundity, egg viability and fertilization rates (Matsunari et al., 2006). Despite the importance of these nutrients for reliable reproduction of other *Seriola* species, their supplementation in diets for greater amberjack broodstock have not been yet studied. Therefore, the present study aimed at examining the effects of supplemental protein, histidine, and taurine in broodstock diets, on egg quality of greater amberjack.

4.2. Material and methods

Greater amberjack broodfish (12.19 ± 1.35 kg and 11.79 ± 2.05 kg females and males body weight, respectively) were distributed in three 40 m^3 (5 m x 2.35 m) circular tanks (2♀ and 2♂ in each tank, sex ratio 1:1) (Rodríguez-Barreto et al., 2014; Sarih et al., 2018), in order to achieve a similar initial biomass in all tanks (1.29 kg/m^3, 1.29 kg/m^3 and 1.24 kg/m^3). To ensure homogeneous environmental conditions the three tanks were located in a triangular location at the facilities of the Grupo de Investigación en Acuicultura (GIA), located in the ECOAQUA Institute (Universidad de Las Palmas

de Gran Canaria, ULPGC, Spain), with equal illumination and noise conditions. Tanks were filled with seawater (37 ‰ salinity) and kept under a natural photoperiod (27º 59' 28" N; 15º 22'05" W) of approximately 13 h light. Flow rate allowed 6 complete water tank renovations daily and the temperature was continuously monitored (Miranda, Innovaqua, Sevilla, Spain) and ranged between 20.46 and 24.52 ºC (Mai–October). Spawning was hormonally induced, and all females had an oocyte diameter higher than 600 µm and all males were spermiating. The selected broodfish were intramuscularly injected with gonadotropin-releasing hormone analogue (LHRHa, des-Gly10, [D-Ala6]-; Sigma-Aldrich, St. Louis, MO, USA) at a dose of 20 $\mu g.kg^{-1}$ (Fernández-Palacios et al., 2015a). These weekly inductions with intramuscular injections have proven to induce significantly better spawning quality than hormonal implants in greater amberjack (Sarih et al., 2018). These hormonal treatments were weekly applied from June 2nd to October 27th following the protocols previously described (Sarih et al., 2018).

Before starting the feeding trial, at the beginning of the spawning season, from May 18th to June 27th, broodfish were fed twice a week with a commercial diet (13 mm, Vitalis CAL, Skretting, Burgos, Spain) at 1% of their estimated total biomass, and once a week with Atlantic mackerel at 2%. After collection and study of 6 spawns from each couple, we statistically determined ($P<0.01$) that there were no differences among different couples inside the same tank or among tanks.

From June 29th to October 31st, broodfish were fed with three different diets. Diets were formulated and produced in Norway by Skretting (Table 4-1). Three diets were formulated to be higher in either histidine, taurine or protein. The amino acid composition of the three diets is shown in Table 4-2. Fish were hand feed twice a day and 5 days a week (1% of biomass day^{-1}). After 24 days of feeding each experimental diet, spawning quality was separately studied for each of the 2 couples for each diet during 10 consecutive spawns. No significant differences were found between couples being fed the same diet. Therefore, each diet was tested in duplicate couples whose 10 spawns were separately studied.

Table 4-1. Ingredients and proximate composition of the experimental diets for greater amberjack broodstock

	Diet		
	Histidine	**Taurine**	**Protein**
Ingredients, % of the diet			
Wheat[a]	17.94	18.29	11.81
Wheat gluten[b]	13.00	13.00	17.00
Fish meal[c]	45.14	44.64	48.36
Squid meal[d]	10.00	10.00	10.00
Fish oil[e]	12.47	12.50	12.18
Taurine[f]	0.00	0.93	0.00
Histidine HCl[g]	0.81	0.00	0.00
Premix incl. vitamins & minerals[h]	0.64	0.64	0.64
Proximate composition, %[i]			
Dry matter	92.40	93.00	94.10
Moisture	7.60	7.00	5.90
Crude protein	51.30	51.50	56.10
Crude fat	17.80	18.50	18.30
Ash	8.40	8.40	8.40

[a] Wheat: Skretting, Stavanger, Norway.
[b] Wheat gluten Cargill Nordic, Charlottenlund, Denmark.
[c] South American fish meal, Skretting, Stavanger, Norway.
[d] Squid meal: Skretting France.
[e] Fish oil: Skretting, Stavanger, Norway.
[f] Taurine: Trouw Nutrition, The Netherlands.
[g] Histidine Hcl: Kyowa Hakko, Japan.
[h] Include vitamins and minerals; Trouw Nutrition, Boxmeer, the Netherlands, proprietary composition Skretting ARC, vitamin and mineral supplementation as estimated to cover requirements according to NRC (2011).
[i] Values are reported as mean of duplicate analyses.

Table 4-2. Taurine content and amino acids composition (% dry matter) of experimental diets

	Diet		
Amino acids	**Histidine**	**Taurine**	**Protein**
Essential amino acid (EAA)			
Arginine	2.67	2.76	2.98
Histidine	1.50	1.02	1.09
Isoleucine	1.88	1.93	2.11
Leucine	3.45	3.55	3.85
Lysine	2.90	3.09	3.21
Methionine	1.25	1.28	1.33
Phenylalanine	1.91	2.02	2.10
Threonine	1.85	1.91	2.04
Valine	2.04	2.12	2.27
Non-essential amino acid (NEAA)			
Cysteine	0.55	0.58	0.62
Tyrosine	1.30	1.40	1.42
Alanine	2.48	2.56	2.74
Aspartic acid	3.98	4.08	4.28
Glutamic acid	8.92	9.20	10.07
Glycine	2.86	2.91	3.11
Proline	2.93	2.93	3.15
Serine	2.13	2.21	2.36
Taurine	0.30	1.13	0.36
Total EAA	19.45	19.68	20.98
Total NEAA	25.15	25.87	27.75
TEAA/TNEAA	0.77	0.76	0.76

*Values are reported as mean of duplicate analyses.

Spawning quality was determined as: fertilization rate (%), egg viability rate (%), hatching and larval survival at 1dph and 3 dph, using two replicates of 96-well microtiter plates according to the protocol described by Panini et al. (2001) and Fernández-Palacios (2005). With these percentages, the total numbers of fertilized, 24h viable and hatched eggs and larvae produced at 1 and 3 dph were calculated (Fernández-Palacios et al., 2011). Also, for each spawn the female fecundity (egg/female), the number of eggs per spawn and the relative fecundity (eggs/female kg) were determined.

Egg samples of all spawns per tank were collected during the experimental period and immediately frozen at −80 °C for biochemical analysis. Proximate composition of eggs from each treatment was conducted following standard

procedures (AOAC, 2016). Dry matter content was determined after drying the sample in an oven at 105 ºC until reaching constant weight, ash by combustion in a muffle furnace at 600 ºC for 12 h, protein content (N x 6.25) was determined by Kjeldahl method, and crude lipid was extracted following the Folch method (Folch et al., 1957). For amino acid analysis samples were hydrolysed in 6 N HCl for 22 h at 110 °C and amino acids separated and quantified by HPLC analyser system (Pico Tag) after prederivatization with phenyl isothiocyanate (Cohen et al.,1989), using norleucine as an internal standard. All analyses were conducted in triplicate.

Egg diameter was estimated from 150 eggs of 10 different spawns for each treatment. From these spawns, 30 newly hatched larvae and 30 larvae at the end of yolk sack absorption (3dph) were measured for total length (TL), standard length (SL), diameter of oil globule (LGD), yolk sack length (YSL) and width (YSH). The volume of the yolk sack (YSV) was calculated using the formula: $YSV = \pi / 6\ YSL \times YSH^2$.

Normality and homogeneity of the variance of all the variables were evaluated using the Kolmogorov-Smirnov test and Levene tests respectively (Sokal and Rolf, 2012). When the assumptions were correct, one-way ANOVA tests were performed, followed by Duncan's New Multiple Range Test. When the heterogeneity of the variances was not correct and or data were not normally distributed, Kruskal-Wallis test was applied and differences between treatments were graphed with a box and whisker plot. Data were expressed as mean ± standard deviation (SD). All statistical analyses were conducted by SPSS statistics (version 22.0 for Windows; Inc, IBM, Chicago, IL, USA) and visualized using SigmaPlot 12.0 (Systat software, San José, USA).

4.3. Results

Despite there were no significant differences in spawning quality among different broodstock while they were fed the same commercial diet, feeding the experimental diets markedly affected spawning quality (Table 4-3). Particularly, fertilization rates were significantly ($P < 0.01$) higher when broodstock were fed a high histidine diet in comparison with the higher protein diet (Table 4-3). Broodstock fed higher histidine also produced higher ($P < 0.01$) percentages of viable eggs than those fed higher protein, which in turn were higher ($P < 0.01$) than those fed higher taurine. The same trend was found in hatching and larval survival rates, where the highest

values (P < 0.01) were found when broodstock were fed higher histidine, followed by higher protein and, finally, higher taurine (Table 4-3).

Table 4-3. Quality of eggs and larvae obtained from greater amberjack after feeding with the experimental diets.

	Diet		
	Histidine	**Taurine**	**Protein**
% Fertilization	77.85 ± 14.19[a]	65.81 ± 16.49[ab]	56.21 ± 14.58[b]
% Viability	97.07 ± 3.57[a]	81.98 ± 6.59[c]	90.00 ± 4.71[b]
% Hatching	96.12 ± 4.35[a]	77.92 ± 8.20[c]	87.60 ± 5.57[b]
% 1 dph survival	87.72 ± 9.07[a]	53.23 ± 8.67[c]	68.03 ± 8.93[b]
% 3 dph survival	50.03 ± 15.88[a]	31.27 ± 18.29[b]	46.85 ± 11.61[a]

*Values are reported as mean ± SD. Different superscripts in the same row indicate significant differences (P < 0.01).

The female fecundity along the spawning period was highest in spawns from broodstock fed the diet rich in histidine, being over 4 and 5 times higher than in broodstock fed higher taurine or higher protein levels (Table 4-4). In agreement, the average number of eggs per spawn was significantly (P < 0.01) higher in broodstock fed higher histidine levels than in broodstock fed the other two diets (Table 4-4). Similarly, the relative fecundity was significantly (P < 0.01) highest in broodstock fed higher histidine, followed by those fed higher taurine and, finally, higher protein (Table 4-4).

Table 4-4. Number of eggs obtained from greater amberjack after feeding with the experimental diets.

	Diet		
	Histidine	**Taurine**	**Protein**
Female fecundity (x 10^6)	17.42	4.24	3.72
Number of eggs per spawns (x10^6)	1.45 ± 0.27[a]	0.38 ± 0.09[b]	0.34 ± 0.08[b]
Relative fecundity (x 10^4)	12.99 ± 2.40[a]	6.18 ± 1.39[b]	2.56 ± 0.60[c]

*Values are reported as mean ± SD. Different superscripts in the same row indicate significant differences (P < 0.01).

As a consequence of the higher spawning quality rates and total egg production, total number of fertilized and viable eggs, and total number of larvae produced were

significantly (P < 0.01) higher for broodstock fed higher histidine, followed by those fed higher taurine and, finally, higher protein (P < 0.01) (Figure 4-1).

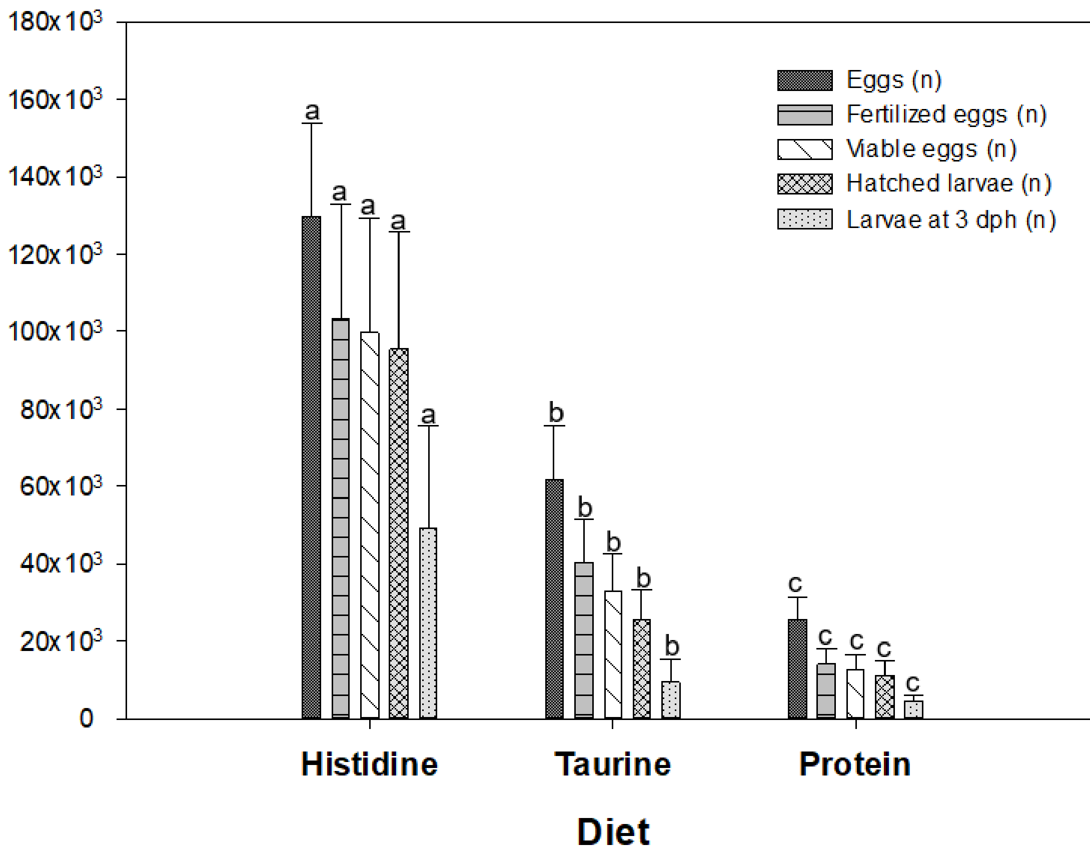

Figure 4-1. Production rates (per kg female and spawn) in greater amberjack after feeding with the experimental diets. Bars, of the same shade, with the different letter, were significantly different (P < 0.01).

Lipid, moisture and ash content of the fertilized eggs were similar among the different experimental groups. The protein content of fertilized eggs from broodstock fed higher protein was significantly higher (P < 0.05) than eggs from broodstock fed higher histidine, which in turn showed a higher protein content than eggs from broodstock fed higher taurine (Table 4-5).

The amino acid composition of the fertilized eggs from broodstocks fed the different experimental diets was similar (Table 4-6). Regardless of the diet used, the most abundant essential amino acids were leucine, lysine, valine, arginine and

isoleucine, and the non-essential ones were glutamic acid, aspartic acid and alanine. Further, it was observed that the proportion of essential and non-essential amino acids was similar in all treatments. However, taurine content of eggs from broodstock fed diet rich in taurine was significantly higher ($P < 0.05$) than those from broodstocks fed the other diets.

Egg diameter from broodstock fed higher histidine and protein levels were significantly larger ($P < 0.01$) than those from broodstock fed higher taurine. The same results were obtained regarding the total and standard length of the newly hatched larvae and the larvae with absorbed yolk-sac (Table 4-7). Yolk sac volume was significantly larger ($P < 0.01$) in larvae from broodstock fed higher protein levels, followed by those from broodstock fed higher histidine and, finally, higher taurine. However, the oil droplet diameter of both newly hatched larvae and yolk-sac absorbed larvae from broodstock fed diet rich in taurine were significantly higher ($P < 0.01$).

Table 4-5. Proximate composition of eggs obtained from greater amberjack after feeding with the experimental diets

Proximate composition	Diet		
	Histidine	Taurine	Protein
Crude protein	10.98 ± 0.24^{b}	5.51 ± 0.30^{c}	12.30 ± 0.33^{a}
Crude fat	3.91 ± 0.14	4.29 ± 0.07	3.57 ± 0.36
Moisture	83.98 ± 0.29	83.92 ± 0.04	83.39 ± 0.03
Ash	0.46 ± 0.03	0.42 ± 0.06	0.43 ± 0.07

* Values are reported as mean ± SD of triplicate analyses. Different superscripts in the same row indicate significant differences ($P < 0.05$).

Table 4-6. Taurine content and amino acids composition (% dry matter) of eggs from broodstock fed experimental diets

Amino acids	Diet		
	Histidine	Taurine	Protein
Essential amino acid (EAA)			
Arginine	3.87 ± 0.18	3.68 ± 0.03	3.34 ± 0.24
Histidine	1.98 ± 0.04	1.91 ± 0.01	1.83 ± 0.19
Isoleucine	3.51 ± 0.09	3.48 ± 0.05	3.22 ± 0.33
Leucine	5.30 ± 0.16	5.23 ± 0.13	4.90 ± 0.36
Lysine	4.60 ± 0.05	4.50 ± 0.07	4.13 ± 0.52
Methionine	1.77 ± 0.03	1.70 ± 0.03	1.60 ± 0.15
Phenylalanine	3.10 ± 0.01	3.12 ± 0.06	2.92 ± 0.21
Threonine	3.18 ± 0.01	3.11 ± 0.02	2.98 ± 0.21
Valine	3.82 ± 0.03	3.73 ± 0.01	3.58 ± 0.21
Non-essential amino acid (NEAA)			
Cysteine	1.07 ± 0.04	1.07 ± 0.01	1.06 ± 0.04
Tyrosine	2.31 ± 0.19	2.38 ± 0.06	1.78 ± 0.25
Alanine	4.82 ± 0.04	4.76 ± 0.05	4.40 ± 0.38
Aspartic acid	4.65 ± 0.14	4.55 ± 0.02	4.48 ± 0.28
Glutamic acid	7.92 ± 0.21	7.81 ± 0.08	7.58 ± 0.49
Glycine	2.31 ± 0.17	2.22 ± 0.08	2.17 ± 0.19
Proline	4.24 ± 0.13	4.06 ± 0.04	3.99 ± 0.06
Serine	3.50 ± 0.01	3.38 ± 0.10	3.15 ± 0.51
Taurine	0.69 ± 0.07^{b}	0.77 ± 0.01^{a}	0.61 ± 0.09^{b}
Total EAA	31.12 ± 0.02	30.44 ± 0.13	28.48 ± 2.41
Total NEAA	31.04 ± 0.75	30.13 ± 0.11	28.59 ± 2.21
TEAA/TNEAA	1.00 ± 0.02	1.01 ± 0.00	1.00 ± 0.01

[*] Values are reported as mean ± SD (n=3). Different superscripts in the same row indicate significant differences ($P < 0.05$).

Table 4.7. Morphometric analysis of egg and larvae of greater amberjack after feeding with the experimental diets

	Diet		
	Histidine	**Taurine**	**Protein**
Egg diameter (mm, n=4500)	1.091 ± 0.035^{a}	1.083 ± 0.021^{b}	1.094 ± 0.022^{a}
Newly hatched larvae (n=450)			
Total length (mm)	2.55 ± 0.15^{a}	2.42 ± 0.15^{b}	2.56 ± 0.10^{a}
Standard length (mm)	2.44 ± 0.14^{a}	2.34 ± 0.13^{b}	2.45 ± 0.10^{a}
Yolk-sac volume (mm^3)	0.43 ± 0.13^{b}	0.42 ± 0.13^{c}	0.47 ± 0.14^{a}
Oil droplet diameter (mm)	0.28 ± 0.03^{c}	0.31 ± 0.02^{a}	0.30 ± 0.03^{b}
Larvae with yolk-sac absorbed, 3 dph (n=450)			
Total length (mm)	3.79 ± 0.14^{a}	3.61 ± 0.30^{b}	3.85 ± 0.15^{a}
Standard length (mm)	3.63 ± 0.14^{a}	3.47 ± 0.28^{b}	3.67 ± 0.14^{a}
Oil droplet diameter (mm)	0.11 ± 0.02^{c}	0.14 ± 0.04^{a}	0.13 ± 0.04^{b}

[*] Values are reported as mean ± SD. Different superscripts in the same row indicate significant differences ($P < 0.01$).

4.4. Discussion

In the present study, spawning quality was good in comparison to previous studies on the same species. For instance, the highest fertilization rates in the present study (77.85%) were better than those obtained in hormonally induced (22-50%, Mylonas et al., 2004a) or natural spawns (61.75%, Jerez et al., 2006; 65.8-76.0%, Kawabe et al., 1996; 70.7%, Kawabe et al., 1998). However, other previous studies in this species in our facilities showed better fertilization rates for induced (92.28%, Fernández-Palacios et al., 2015a) or natural spawns (84.37%, Sarih et al., 2018). Nevertheless, the total number of eggs per female in the present study (17.42 million) was more than four times higher (4.30 million) (Sarih et al., 2018) for amberjack fed a commercial diet, and higher than previously reported for this species (Mylonas et al., 2004a; Jerez et al., 2006; Fernández-Palacios et al., 2011). Besides, egg diameter was also larger than that obtained by GnRHa injections (1.03 ± 0.02 mm, Papandroulakis et al., 2005) or GnRHa implants (1.02 ± 0.01 mm) (Mylonas et al., 2004a), but smaller than that obtained from fish injected hCG (1.12-1.14 mm, Kozul et al., 2001) or LHRHa (1.15 mm, Lazzari et al., 2000).

Regardless of the diet used, the main essential amino acids contained in eggs were leucine, lysine, valine, arginine and isoleucine. The same results were found in fertilized eggs of several species such as the Japanese yellowtail (Matsunari et al., 2003), goldstriped amberjack (Moran et al., 2007), European sea bass (Rønnestad et al., 1998), red snapper (Hastey et al., 2010) and yellowfin seabream (Zakeri et al., 2014). The contribution of adequate amounts of protein with a good balance of essential and non-essential amino acids is important for the development of eggs and larvae due to the fast growth rates (Moran et al., 2007; Conceição et al., 2010). The concentrations of essential and non-essential amino acids in eggs of the three experimental groups, were proportional to their content in diet, as observed in spawns of yellowfin seabream broodstock, where the amino acid profile of eggs and larvae of 3 dph were affected by the amount of amino acids contained in their diets (Zakeri et al., 2014).

In the present study, overall the best spawning quality was obtained when broodstock were fed histidine levels increased from 1 to 1.5%. Histidine must play important roles in *Seriola* species since it is the predominant free amino acid in the muscle of the adult Japanese amberjack (Endo et al., 1974; Matsunari et al., 2005; Thakur et al., 2009; Tanahashi et al., 2014). Histidine must also play a specific role during reproduction since intramuscular histidine concentration increases substantially before the spawning migration in other species such as sockeye salmon (Mommsen et al., 1980; Mommsen, 2004). However, this is the first study that shows the importance of histidine levels in broodfish diets. Feeding higher histidine levels in broodstock diets for greater amberjack improved the relative fecundity. Histidine is the most abundant amino acid in ovaries of other fish species such as goldlined seabream (Qari et al., 2013). Interestingly, in that species, histidine levels were higher in testis than in ovaries (Qari et al., 2013), suggesting the importance of this amino acid for sperm functioning and activity, in agreement with the improved fertilization rates found in greater amberjack in the present study. The importance of histidine for sperm functioning and improved fertilization rates could be related to the histidine functioning as an energy source. Histidine increase in broodstock diets improved egg viability, hatching rates and larval survival, suggesting the importance of this amino acid for embryo and larval development. Improved egg and larval quality would be in agreement with the increase in egg protein content, and egg and larval size since the

content on endogenous reserves is directly related to larval survival rates (Giménez et al., 2006; Samaee et al., 2013). Deficiency in other essential nutrients in broodstock diets also affects larval growth, development and survival (Izquierdo and Fernández-Palacios, 1997; Fernández-Palacios et al., 2011). In agreement with the importance of histidine for greater amberjack embryo and larval development, histidine contents change along development in spotted rose snapper (*Lutjanus guttatus*) (Abdo-de la Parra et al., 2017) or pacu (*Piaractus mesopotamicus*) (Portella et al., 2013). Histidine contents increase with fish growth (Ng and Hung, 1994) and are reduced during fasting (Kaushik et al., 1991). Moreover, histidine seems to be a first limiting amino acid during early larval development (Saavedra et al., 2006; Hamre et al., 2013) and, since live preys are deficient in this amino acid (Aragao et al., 2004), particularly for larvae of Seriola species (Yamamoto et al., 2008), supplementation through the broodstock diet must be important to prevent deficiencies in the larvae.

Increase of taurine from 0.3 to 1.1% in diets for greater amberjack increased the relative fecundity in comparison to fish fed higher protein levels in the present study, as well as compared to previous studies where greater amberjack was fed with commercial diets generally designed for marine fish broodstock (Mylonas et al., 2004a; Jerez et al., 2006; Fernández-Palacios et al., 2011; Sarih et al., 2018). These results are in agreement with the higher number of eggs obtained in Japanese yellowtail fed increased taurine levels (Matsunari et al., 2006). Fertilization rates tend to increase with the elevation of dietary taurine and were not significantly different from those of broodstock fed higher histidine levels. In agreement, in Japanese amberjack, which has a deficiency of cysteine sulphinate decarboxylase, the key enzyme in the synthesis of taurine (Yokoyama et al., 2001), inclusion of taurine up to 1.23% in broodstock diets improves spawn quality, particularly fertilization rates (Matsunari et al., 2006). In addition, in this same species, taurine content in the ovary decreased as the gonado-somatic index increased, while that of the testes was constant, indicating that probably the testes require more taurine than the ovary (Khaoian et al., 2014). In the Japanese eel (*Anguilla japonica*), the role of taurine synthesis was evaluated in the testis (Higuchi et al., 2012a, b), and it was observed that taurine has an important role in spermatogenesis, increasing the effects of sex steroids in the promotion of spermatogonial proliferation and meiosis (Higuchi et al., 2012b), as well as in the protection of germ cells from oxidative stress (Higuchi et al., 2012a). Besides, egg

diameter and taurine contents in eggs were increased by the elevation of taurine in the broodstock diets. The amino acid profile of eggs may be also associated with fertilization rates (Kwasek et al., 2009; Zakeri et al., 2013; Mommens et al., 2015). Taurine requirements seem to be higher during early life stages (Pinto et al., 2010; Kim et al., 2016) and in particular, increase of taurine in greater amberjack larval diets has positive effects on growth (Matsunari et al., 2013).

Despite the increase in dietary taurine raised the taurine contents in the egg, it did not elevate the egg protein contents. On the contrary, an increase in egg protein content was found when taurine was increased in Nile tilapia broodstock diets up to 10 g/kg when plant ingredients were used as the main source of protein (Al-Feky et al., 2016). Moreover, excessive taurine supplementation may reduce free amino acids and the efficiency of their utilization, reducing body protein deposition (Matsunari et al., 2008; Qi et al., 2012; Zhou et al., 2015). Therefore, the lack of an increase in egg protein contents could be a sign of an excessive supplementation of taurine in broodstock diets for greater amberjack and further studies must be conducted to determine the optimum dietary levels.

Increase in dietary protein contents from 51 to 56% lead to an increase in protein content in egg, as well as a larger yolk sac volume. These results are in agreement with the higher egg size found in broodstock fed increased protein levels in other species such North African catfish (*Clarias gariepinus*) (Sotolu, 2010) or giant gourami (*Osphronemus goramy*) (Masrizal et al., 2015). Proteins are the most abundant nutrients contained in fish eggs (Watanabe and Kiron, 1994) and a main source of energy during the embryonic and larval development of many species (Rønnestad et al., 1992; Finn et al., 1996; Lochmann et al., 2007). Therefore, increased yolk sac volume and egg protein content could be related to the higher egg viability, hatching rate and larval survival than that of fish fed higher taurine levels. Previous studies have shown that greater amberjack broodstock bred in captivity have a lower content of proteins in the ovaries than wild ones, what could be related to the reproductive dysfunction that this species presents in captivity (Zupa et al., 2017b). Indeed, low protein levels in broodstock diets can alter the secretion of GnRH (Kah et al., 1994) and LH (Navas et al., 1996), affecting oocyte maturation, the regulation of ovulation and therefore egg production (Fernández-Palacios et al., 2011). In the present study,

increase in dietary protein contents did not increase egg production, suggesting that 51% protein is enough to cover greater amberjack requirements for this nutrient. These results are in agreement with previous studies on European bass (Cerdá et al., 1994; Navas et al., 1998), roho labeo (*Labeo rohita*) (Khan et al., 2005), silver catfish (*Rhamdia quelen*) (Coldebella et al., 2011) or in grass carp (*Ctenopharyngodon idella*) (Khan et al., 2004), where an increase in dietary protein did not increase egg production. However, other studies indicate that the production of eggs and larvae are higher from spawns of females fed high protein levels (Khan et al., 2005; Aryani and Suharman, 2015).

One of the main problems in broodstock nutrition studies is the large tank size required, particularly for fast growing species such as *Seriola* spp, as well as the large fish size and number of individuals. In any nutrition study 4-5 diets in triplicate tanks are used, implying 15 tanks of 40,000 m^3 and around 150 fish of over 10 kg adapted to captive conditions for 4-5 years. The cost of such a trial in infrastructure, energy, feeds and man-power is completely unbearable for any scientific facility. For this reason, it is common in *Seriola* broodstock studies to use single tanks or cages per treatment (Kawabe et al., 1996; Watanabe et al., 1996; Mushiake et al., 1994; Kozul et al., 2001; Moran et al., 2007; Stuart and Drawbridge, 2013; Setiawan et al., 2016). In our study, despite the size and cost of the trial did not allowed to use triplicate tanks to determine a potential tank effect, the fact that while feeding the commercial diet for one and a half months at the beginning of the trial the spawn quality was equal for the all the broodstock stocked in different tanks confirms the lack of any negative tank effect or even differences among different brood fish. Therefore, the differences in spawning quality obtained during feeding the different experimental diets should be related to the diet's differences.

In conclusion, the results of this study have pointed out the importance of raising histidine contents in broodstock diets from 1 to 1.5% to optimize the reproductive performance of greater amberjack, particularly to improve fecundity, fertilization rates, and egg and larval quality. Besides, the study showed that taurine levels in broodstock diets increase fecundity, maintaining good fertilization rates, but further studies must be conducted to determine the optimum taurine dietary levels.

Chapter 5

Adequate n-3 LC-PUFA levels in broodstock diets optimize reproductive performance in GnRH injected greater amberjack (*Seriola dumerili*) equaling to spontaneously spawning broodstock

Adequate n-3 LC-PUFA levels in broodstock diets optimize reproductive performance in GnRH injected greater amberjack (*Seriola dumerili*) equaling to spontaneously spawning broodstock

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Abstract

Dietary lipids and fatty acids composition play an important role in the reproductive processes, embryo development and larval survival in marine fish. Long-chain polyunsaturated fatty acids (LC-PUFA) are especially important for egg and larval quality. The main objective of the present study was to evaluate the effects of increasing dietary n-3 LC-PUFA levels on spawning performance and egg quality of greater amberjack. Sixteen mature broodfish were distributed in four 40 m^3 circular tanks and fed four diets containing different levels of n-3 LC-PUFA: diet D1 with 1 % of the dry weight basis (dw), diet D2 with 1.7 % dw, diet D3 with 2.3 % dw and diet D4 with 3 % dw. The fecundity was significantly largest ($P < 0.05$) in broodstock fed D2. Moreover, a higher fertilization rate ($P < 0.05$) was obtained from broodstock fed D2 (91.8 ± 3.1 %) and D1 (86.3 ± 1.7 %) in comparison to those fed D3 (69.0 ± 7.4 %) and D4 (52.4 ± 10.6 %). The same trend was found in egg viability at 24h, hatching and larval survival rates, where the highest values ($P < 0.05$) were found when broodstock were fed D1 and D2. Besides, fertilization, hatching rates and larval survival at 1- and 3-days post-hatching showed a negative correlation with dietary n-3 LC-PUFA. The fatty acid composition of eggs showed marked differences, reflecting the influence of fatty acid profiles in the broodstock diets. Based on the overall results the recommended dietary n-3 LC-PUFA level for broodstock greater amberjack was suggested between 1-1.7 % dw.

Keywords: Greater amberjack, broodstock diet, n-3 LC-PUFA, spawning performance, egg quality.

5.1. Introduction

Successful mass production of aquaculture species requires the provision of sufficient numbers of high-quality gametes (Brooks et al., 1997; Bobe and Labbé, 2010; Migaud et al., 2013). Breeders nutrition markedly affects egg and larval quality and, frequently, their biochemical composition (Izquierdo et al., 2001). Lipids and highly unsaturated fatty acids with 20 or more carbon atoms (LC-PUFAs) are known as nutritional components that greatly affect spawning performance and egg quality (Fernández-Palacios et al., 1997; 2011). These nutrients influence both the physiological processes occurring during gametogenesis and the biochemical composition of eggs. Being the main energy source for embryos and larvae before the starting of exogenous feeding, lipids are particularly important constituents of eggs (Izquierdo et al., 2001; Bobe and Labbé, 2010; Tocher, 2010; Fernández-Palacios et al., 2011). In particular, n-3 LC-PUFA, docosahexaenoic acid (DHA, 22:6n-3), and eicosapentaenoic acid (EPA, 20:5 n-3) are essential for the optimal functioning of cells, brains, tissues, and organs in the developing embryo and larvae (Izquierdo et al., 2000; Migaud et al., 2013). However, marine fish have limited ability to convert α-linolenic acid (18:3n-3) into EPA and DHA, and therefore these compounds must be provided in the diet to ensure normal growth and development (Izquierdo, 1996; NRC, 2011). Yolk fatty acid composition directly affects the optimal development of the embryo and yolk-sac larvae by providing DHA, essential for structure and functioning of all membranes, especially in neural tissues, such as brain and eye (Feller, 2008; Izquierdo and Koven, 2011). EPA is also important as a precursor of the eicosanoids involved in the modulation of neural, hypothalamic and immune functions (Sargent et al., 2002; Tocher, 2003; Ganga et al., 2005).

The optimal dietary amounts of n-3 LC-PUFA vary among species and both deficiency or excessive levels of n-3 LC-PUFA have negative effects on egg and larval quality (Brooks et al., 1997; Bobe and Labbé, 2010). In yellowfin sea bream and flame angelfish fecundity, fertilization, hatching and larval survival rates are improved when broodstock are fed high n-3 LC-PUFA level (Zakeri et al., 2011; Callan et al., 2014). European sea bass fed a diet deficient in n-3 LC-PUFA had a lower fecundity and egg viability (Valdebenito et al., 2015). Similar results are obtained for gilthead seabream broodstock, but also an excessive level of n-3 LC-PUFA in the broodstock diets

reduces larval survival (Fernández-Palacios et al., 1995). Thus, in order to determine the optimum requirements of dietary n-3 LC-PUFA for broodstock fish, it is important to understand how egg quality is affected by dietary n-3 LC-PUFA levels.

The greater amberjack (Risso, 1810) is an excellent candidate marine fish species for European aquaculture due to its high consumer acceptability worldwide, high market value and excellent flesh quality (Sicuro and Luzzana, 2016). To date, the aquaculture production of this species has been very limited (FAO, 2018), due to the reproductive dysfunctions in captivity and the poor eggs and larval quality (Sarih et al., 2018). In captive-reared greater amberjack, a comparative study on the reproductive function showed the reduction of gonadal development and an early cessation of gametogenic activity (Zupa et al., 2017a). This gametogenesis impairment was associated with important changes in gonad fatty acid composition and sex steroid plasma concentrations (Zupa et al., 2017b; Pousis et al., 2018). The n-3 LC-PUFA, including DHA and EPA, can modulate steroids levels (Dabrowski et al., 2015). For instance, in tongue sole (*Cynoglossus semilaevis*), excessive EPA levels in broodstock diets have an inhibitory effect on gonadal steroidogenesis (Xu et al., 2017). In greater amberjack, recent study has found marked differences in the n-3 LC-PUFA contents, particularly, EPA and DHA, of captive-reared stocks compared with wild ones (Zupa et al., 2017a). However, the optimum levels of these fatty acids in broodstock diets to boost reproductive success in this species have not been yet determined.

Therefore, the present study aimed to determine the effect of graded n-3 LC-PUFA levels on broodstock diets on spawning performance and egg quality of greater amberjack. For that purpose, a captive-reared greater amberjack broodstock was divided into four groups of the same spawning quality and fed for over 3 months with four graded levels of n-3 LC-PUFA during the spawning season to determine the dietary effect on reproductive performance.

5.2. Material and methods

All the experiment mentioned below was conducted according to the European Union Directive (2010/63/EU) on the protection of animals for scientific purposes and held at Instituto Ecoaqua, University of Las Palmas de Gran Canaria (Canary Islands, Spain).

5.2.1. Broodstocks and rearing system

The experiment lasted for over 5 months (151 days). Sixteen greater amberjacks (14.79 ± 2.17 kg and 13.55 ± 3.35 kg body weight, respectively for females and males) were randomly selected and distributed in four 40 m^3 (5 m x 2.35 m) circular tanks (2♀ and 2♂ in each tank, sex ratio 1:1) (Sarih et al., 2018), in order to achieve a similar initial biomass in all tanks (1.42 kg/m^3, 1.43 kg/m^3, 1.44 kg/m^3 and 1.44 kg/m^3). The four experimental tanks were located at the facilities of the Grupo de Investigación en Acuicultura (GIA), located in the ECOAQUA Institute (Universidad de Las Palmas de Gran Canaria, ULPGC, Spain), with equal illumination and noise conditions. Tanks were supplied with running seawater (37 ± 0.5 salinity) and kept under a natural photoperiod (27º 59'28" N; 15º 22'05" W) of approximately 13 h light. Flow rate allowed 6 water tank renovations daily and the temperature was continuously monitored (Miranda, Innovaqua, Sevilla, Spain) and ranged between 21.58 ± 0.36 to 23.30 ± 0.17 °C in June-October 2016.

When all females had an oocyte diameter higher than 600 µm and all males were spermiating, spawning was hormonally induced with intramuscular injections of gonadotropin-releasing hormone analogue (LHRHa, des-Gly10, [D-Ala6]-; Sigma-Aldrich, St. Louis, MO, USA) at a dose of 20 $\mu g.kg^{-1}$ (Fernández-Palacios et al., 2015a; Sarih et al., 2018). Only one of the two couples of each broodstock tank was hormonally induced each time to determine the spawning quality of that particular couple. The second couple was hormonally induced 4 days after injection of the first one and, therefore, spawning was alternated with each couple from a given tank being injected every 9 days along the whole spawning season.

5.2.2. Experimental diets

Before starting the feeding trial, at the beginning of the spawning season, from June 3rd to July 12th, broodfish were fed twice a week with a commercial diet for broodstock (Vitalis CAL, Skretting, Burgos, Spain) (1 % of biomass day^{-1}), and once a week with Atlantic mackerel (2 % of biomass day^{-1}). After collection and study of 5 spawns from each couple, there were no significant ($P < 0.05$) differences in spawning quality among different couples inside the same tank or among tanks.

From July 13th to October 27th, broodfish were fed with four different diets. Diets were formulated to contain four different proportions of n-3 LC-PUFA, 1 % (D1), 1.7 %

(D2), 2.3 % (D3) and 3 % dw (D4) by changing the contents of fish oil and vegetable oils (linseed and palm oils). Diet composition and proximate analysis are shown in Table 5-1 and dietary fatty acid composition in Table 5-2. The experimental diets were manufactured by Skretting ARC Feed Technology Plant (Stavanger, Norway) with a pellet size of 13 mm, analysed for proximate and fatty acid composition at GIA laboratories (ECOAQUA Institute, ULPGC, Spain) and kept in a cold room at 10 ºC until daily broodstock feeding. Fish were hand-fed twice a day and 5 days a week (2 % of biomass day^{-1}). After 24 days of feeding each experimental diet (August 4th), spawning quality was separately monitored for each of the 2 couples from each tank during 10 consecutive spawns.

Table 5-1. Ingredients and proximate composition of the experimental diets for greater amberjack broodstock.

	Broodstock group			
	D1	**D2**	**D3**	**D4**
Raw material (%)				
Linseed oil[a]	4.50	3.01	1.52	0.00
Wheat[b]	19.13	19.13	19.13	19.09
Wheat gluten[c]	14.99	14.99	14.99	13.62
Fish meal[d]	43.46	43.46	43.46	44.97
Squid meal[e]	10.00	10.00	10.00	10.00
Fish oil[f]	0.61	4.04	7.48	10.93
Palm oil[g]	5.93	3.98	2.03	0.00
Premix[h]	0.64	0.64	0.64	0.64
Total	100.00	100.00	100.00	100.00
Proximate composition (% dry weight)				
Crude protein	59.06	58.91	58.91	58.50
Crude lipid	25.61	24.35	24.89	24.25
Moisture	8.30	7.22	7.41	7.27
Ash	7.30	7.25	7.19	7.46
n-3 LC-PUFA	1.03	1.70	2.34	3.03

[a] Linseed oil: European Commodity Company S.A., Luxemburg.
[b] Wheat: Linas Agro AB, Lithuania.
[c] Wheat gluten: Roquette, France.
[d] Fish meal: FF Skagen AS, Denmark.
[e] Squid meal: Inproquisa SA, Spain.
[f] Fish oil: Norsildmeal AS, Norway.
[g] Palm oil: AAK AB, Sweeden.
[h] Include vitamins and minerals; Trouw Nutrition, Boxmeer, the Netherlands, proprietary composition Skretting ARC, vitamin and mineral supplementation as estimated to cover requirements according to NRC (2011).

Table 5-2. Main fatty acids contents of the four experimental diets for greater amberjack broodstock (% of the total fatty acids identified).

	Broodstock group			
Fatty acid	**D1**	**D2**	**D3**	**D4**
14:0	2.19	4.26	5.35	6.51
16:0	23.06	21.22	20.39	18.55
16:1n-7	1.78	4.08	5.29	6.73
16:1n-5	0.06	0.16	0.20	0.28
16:2n-4	0.20	0.50	0.64	0.82
17:0	0.16	0.47	0.62	0.80
16:3n-4	0.08	0.14	0.18	0.22
18:0	3.47	3.49	3.59	3.64
18:1n-9	23.69	17.80	15.32	11.03
18:1n-7	1.14	1.86	2.22	2.68
18:1n-5	0.08	0.12	0.14	0.17
18:2n-6	11.26	8.41	6.72	4.58
18:2n-4	0.06	0.15	0.20	0.26
18:3n-6	0.05	0.13	0.17	0.22
18:3n-3	17.11	9.89	6.50	1.73
18:4n-3	0.77	1.50	1.86	2.37
20:0	0.20	0.28	0.28	0.30
20:1n-9	0.21	0.26	0.29	0.34
20:1n-7	2.33	2.83	2.98	3.44
20:1n-5	0.07	0.18	0.24	0.31
20:2n-6	0.14	0.18	0.13	0.18
20:4n-6 (ARA)	0.24	0.52	0.65	0.86
20:3n-3	0.06	0.08	0.06	0.10
20:4n-3	0.15	0.34	0.43	0.59
20:5n-3 (EPA)	2.95	6.70	8.51	11.21
22:1n-11	3.28	3.96	4.07	4.84
22:5n-3	0.31	0.68	0.88	1.21
22:6n-3 (DHA)	3.79	7.49	9.17	12.29
Total saturates	29.25	30.06	30.43	30.31
Total MUFA	32.99	31.79	31.59	30.60
Total n-3	25.47	27.52	28.51	30.93
Total n-6	11.76	9.40	7.87	6.14
Total n-9	24.20	18.52	16.14	12.03
Total n-3 LC-PUFA	7.25	15.28	19.05	25.41
DHA/EPA	1.29	1.12	1.08	1.10
EPA/ARA	12.39	13.00	13.00	13.03
EPA+DHA	6.74	14.18	17.67	23.51
n-3/n-6	2.17	2.93	3.62	5.04
18:1n-9/n-3 LC-PUFA	3.27	1.16	0.80	0.43

*MUFA, monounsaturated fatty acid; LC-PUFA, Long-chain highly unsaturated fatty acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; ARA, arachidonic acid.

5.2.3. Spawning and egg quality

Spawning and egg quality was determined by quantifying the total number of eggs produced (eggs/ kg female /spawn), and the percentages of fertilized, viable and hatched eggs and larval survival (Fernández-Palacios et al., 2005). Briefly, collected eggs were placed in 10 L beakers provided with aeration, from where five randomized 10 ml samples were placed in a Bogorov chamber, counted and observed under a binocular microscope (Leica S6E, Wetzlar, Germany) to calculate the percentage of fertilization (Sarih et al., 2018). Fertilized eggs were then placed in 96-well microtiter plates in replicates (Greiner Bio-One, Kremsmunster, Australia) and incubated in a controlled temperature incubator at 21.9 ± 0.4 °C, to determine the percentages of viable eggs at 24 hours (%), hatching and larval survival at 1- and 3-days post-hatching (dph) just before the yolk sac was almost completely absorbed.

5.2.4. Egg and larval morphometry

Egg and oil droplet diameter were determined from 150 fertilized eggs of 10 different spawns for each treatment. From these spawns, 30 newly hatched larvae and 30 larvae at the end of yolk sac absorption (3dph) were anesthetized with clove oil at 1% to determine total length (TL), standard length (SL), diameter of oil globule (LGD), yolk sac length (YSL) and height (YSH). The volume of the yolk sack (YSV) was calculated as $YSV = \pi / 6\ YSL \times YSH^2$.

5.2.5. Biochemical analysis

Diets and fertilized eggs collected during the experimental period were analysed for proximate and fatty acid composition. Eggs were thoroughly rinsed in distilled water and then stored at -80 ºC. Proximate composition was conducted following standard procedures (AOAC, 2016). Moisture was determined by thermal dehydration to constant weight at 105 °C. Ash content was determined by combustion in a muffle furnace at 600 °C for 12 h. Crude protein content was calculated by Kjeldahl method (N x 6.25). Crude lipids were extracted with chloroform: methanol 2:1 (Folch et al., 1957) and fatty acid methyl esters obtained by transmethylation (Christie and Han, 2010). Fatty acid methyl esters were then separated by gas-liquid chromatography (GC-14A; Shimadzu, Tokyo, Japan) following the conditions described previously (Izquierdo et al., 1990) quantified by FID and identified by comparison to previously

characterized standards and GLC-MS (Polaris QTRACETM Ultra; Thermo Fisher Scientific).

5.2.6. Statistical analysis

The results were expressed as mean ± standard deviation (SD). Normality and homogeneity of the variance of all the variables were evaluated using the Kolmogorov-Smirnoff test and Levene test respectively (Sokal and Rohlf, 2012). When the assumptions were correct, one-way Analysis of Variance (ANOVA) test was performed, followed by Tukey's post hoc test. When the heterogeneity of the variances was not correct and or data were not normally distributed, Kruskal-Wallis test was applied and differences between treatments were graphed with a box and whisker plot. Simple linear and non-linear regressions were performed to correlate the results obtained. All statistical analyses were conducted by SPSS statistics (version 22.0 for Windows; Inc, IBM, Chicago, IL, USA).

5.3. Results

Before feeding the experimental diets, there were no significant ($P < 0.05$) differences in fecundity or indicators of egg and larval quality (Table 5-3) among the broodfish groups. On the contrary, after feeding the experimental diets, the fecundity was significantly ($P < 0.05$) increased over 60 % by the elevation of n-3 LC-PUFA from 1 (D1) up to 1.7 % dw (D2). However, further increase in n-3 LC-PUFA up to 2.3 and 3 % dw (D3 and D4) significantly ($P < 0.05$) reduced fecundity (Table 5-3). Similarly, the highest fertilization, viability and hatching rates were also obtained in broodstock fed D2, although without significant differences with fish fed D1 (Table 5-3). Again, broodstock fed D3 and D4 diets, high in n-3 LC-PUFA, showed significantly ($P < 0.05$) lower values for these egg-quality indicators. Moreover, these D3 and D4 broodstock produced larvae with a significantly ($P < 0.05$) lower survival at 1 and 3 dph, in comparison to broodstock fed diets D1 and D2 (Table 5-3).

Table 5-3. Fecundity and egg and larval quality parameters obtained from greater amberjack before and after feeding the experimental diets.

	Broodstock group			
	D1	**D2**	**D3**	**D4**
Before feeding the experimental diets				
Fecundity ((eggs/kg female/spawn) x10^4)	2.9 ± 0.5	2.5 ± 0.7	2.0 ± 1.2	3.4 ± 0.2
Fertilization (%)	62.7 ± 7.9	54.7 ± 12.7	67.5 ± 6.9	65.8 ± 11.0
Viability (%)	86.3 ± 9.4	84.9 ± 2.9	84.9 ± 5.3	84.5 ± 9.3
Hatching (%)	80.6 ± 9.8	80.9 ± 4.9	83.2 ± 5.6	78.0 ± 8.7
1 dph larval survival (%)	67.8 ± 4.8	63.3 ± 9.8	70.0 ± 1.4	62.2 ± 8.3
3 dph larval survival (%)	33.3 ± 5.1	29.3 ± 4.0	35.0 ± 3.9	32.8 ± 7.8
After feeding the experimental diets				
Fecundity ((eggs/kg female/spawn) x10^4)	4.4 ± 1.40^b	7.1 ± 1.1^a	4.3 ± 0.7^b	4.6 ± 0.8^b
Fertilization (%)	86.3 ± 1.7^a	91.8 ± 3.1^a	69.0 ± 7.4^b	52.4 ± 10.6^c
Viability (%)	93.9 ± 2.5^a	95.9 ± 2.8^a	85.1 ± 1.7^b	90.3 ± 3.3^{ab}
Hatching (%)	92.5 ± 2.3^a	94.2 ± 3.6^a	79.7 ± 3.7^b	77.0 ± 8.9^b
1 dph larval survival (%)	87.0 ± 2.9^a	85.2 ± 9.9^a	59.8 ± 2.9^b	57.4 ± 3.1^b
3 dph larval survival (%)	38.1 ± 2.1^a	38.3 ± 8.0^a	11.6 ± 2.2^c	16.1 ± 4.9^b

*Values are reported as mean ± SD. Different superscripts in the same row indicate significant differences (P < 0.05).

Before feeding the experimental diets, there were no significant (P < 0.05) differences in morphometric parameters of egg and larvae among the broodfish groups. Mean egg diameter was 1.08 ± 0.07 mm and oil droplet diameter 0.29 ± 0.02 mm. Total length newly hatched larvae, yolk-sac volume, and oil droplet diameter were 2.52 ± 0.19, 0.31 ± 0.13 and 0.27 ± 0.03 mm, respectively. Egg diameter was not significantly (P < 0.05) different among broodstock fed the different diets, however, an increase in dietary n-3 LC-PUFA levels tends to increase the oil droplet diameter (Table 5-4). Total and standard length of the newly hatched larvae were highest in larvae from broodstock fed D2, being significantly (P < 0.05) higher than in D3 larvae, which was higher than in D4. The length of D1 larvae was intermediate and not significantly

different from D2 and D3. Yolk sac volume and oil droplet diameter were significantly larger ($P < 0.05$) in larvae from D3 and D4 broodfish than those from D1 and D2 broodfish. Besides, dietary n-3 LC-PUFA content was directly correlated with yolk sac volume ($R = 0.897$, $P = 0.001$) and oil droplet diameter ($R = 0.923$, $P < 0.001$) of the newly hatched larvae.

Table 5-4. Morphometric parameters of egg and larvae obtained from greater amberjack after feeding with the experimental diets.

	Broodstock group			
	D1	**D2**	**D3**	**D4**
Egg				
Egg diameter (mm)	1.10 ± 0.01	1.10 ± 0.03	1.10 ± 0.03	1.11 ± 0.02
Oil droplet diameter (mm)	0.28 ± 0.02	0.29 ± 0.02	0.30 ± 0.02	0.30 ± 0.02
Newly hatched larvae				
Total length (mm)	2.56 ± 0.14^{a}	2.58 ± 0.10^{a}	2.53 ± 0.14^{a}	2.44 ± 0.14^{b}
Standard length (mm)	2.47 ± 0.15^{a}	2.48 ± 0.11^{a}	2.43 ± 0.14^{a}	2.35 ± 0.12^{b}
Yolk-sac volume (mm^{3})	0.34 ± 0.10^{b}	0.36 ± 0.11^{b}	0.44 ± 0.12^{a}	0.47 ± 0.10^{a}
Oil droplet diameter (mm)	0.27 ± 0.02^{c}	0.28 ± 0.02^{a}	0.28 ± 0.03^{a}	0.30 ± 0.03^{a}

*Values are reported as mean ± SD. Different superscripts in the same row indicate significant differences ($P < 0.05$).

Along the feeding trial, total lipids, ash and moisture contents in eggs were not significantly different ($P < 0.05$) among groups (Table 5-5). Fatty acid composition of the eggs obtained from the broodfish during the four weeks before feeding the experimental diets showed that there were no significant differences among eggs from different broodfish (Table 5-6). Regardless of the diet fed, the main fatty acids in eggs were 18:1n-9, 16:0, DHA, linoleic acid (18:2n-6), 18:0 and EPA (Tables 5-6 and 5-7). Saturated fatty acids contents in the eggs were not significantly ($P < 0.05$) affected by the different diets, despite a 10% reduction in the dietary contents of 16:0. The sum of monounsaturated fatty acids was neither affected by the diet. Despite a 3-fold increase in dietary 16:1n-7 when n-3 LC-PUFA were increased in the diet, this fatty acid increased only about 40 % in the eggs. Similarly, 18:1n-9 was only reduced in a 20 % despite a 50 % reduction in the diet. On the contrary, n-6 fatty acids were highly correlated to their respective dietary levels (linoleic acid ($R = 0.899$, $P < 0.001$) and ARA ($R = 0.818$, $P = 0.001$)), as well as EPA ($R = 0.761$, $P = 0.004$), DHA ($R = 0.960$,

$P < 0.001$) and n-3 LC-PUFA ($R = 0.953$, $P < 0.001$). Additionally, eggs n-3/n-6 ratio was increased as n-3 LC-PUFA dietary content increased, whereas 18:1n-9/ n-3 LC-PUFA ratio decreased ($P < 0.05$) (Table 5-7).

Table 5-5. Proximate composition of eggs obtained from greater amberjack before and after feeding the experimental diets.

	Broodstock group			
Proximate composition	**D1**	**D2**	**D3**	**D4**
Before feeding the experimental diets				
Crude lipid	15.3 ± 1.6	15.6 ± 0.6	15.7 ± 1.0	15.0 ± 0.5
Crude protein	71.8 ± 3.1	73.8 ± 4.0	78.4 ± 4.0	76.1 ± 0.4
Ash	3.1 ± 0.1	3.3 ± 0.4	4.3 ± 0.9	3.7 ± 0.2
After feeding the experimental diets				
Crude lipid	25.6 ± 1.0	25.8 ± 0.0	25.20 ± 0.4	24.9 ± 0.4
Crude protein	66.7 ± 0.9	67.0 ± 1.2	65.1 ± 0.8	65.3 ± 0.5
Ash	5.2 ± 0.5	4.4 ± 0.2	5.1 ± 0.6	4.7 ± 0.7

*Values are reported as mean ± SD. n=3.

Table 5-6. Fatty acid composition (% total fatty acids identified) of eggs obtained before feeding with the experimental diets.

	Broodstock group			
Fatty acid	**D1**	**D2**	**D3**	**D4**
14:0	1.74 ± 0.22	1.81 ± 0.34	1.59 ± 0.08	1.48 ± 0.21
16:0	16.33 ± 1.55	16.44 ± 0.56	15.87 ± 0.53	15.09 ± 0.65
16:1n-7	4.43 ± 0.45	4.76 ± 0.54	4.33 ± 0.22	4.31 ± 0.21
16:1n-5	0.14 ± 0.01	0.13 ± 0.01	0.13 ± 0.00	0.14 ± 0.01
16:2n-4	0.22 ± 0.06	0.24 ± 0.06	0.23 ± 0.03	0.22 ± 0.05
17:0	0.25 ± 0.15	0.18 ± 0.04	0.16 ± 0.03	0.17 ± 0.03
16:3n-4	0.42 ± 0.18	0.43 ± 0.02	0.42 ± 0.02	0.42 ± 0.02
18:0	5.98 ± 1.38	4.51 ± 0.26	5.31 ± 0.67	5.26 ± 0.57
18:1n-9	23.99 ± 0.68	24.27 ± 1.03	23.31 ± 1.57	23.00 ± 0.53
18:1n-7	4.10 ± 0.04	4.05 ± 0.08	3.94 ± 0.16	4.04 ± 0.06
18:1n-5	0.15 ± 0.01	0.15 ± 0.01	0.14 ± 0.01	0.15 ± 0.01
18:2n-6	9.51 ± 0.32	9.77 ± 0.51	10.21 ± 0.45	10.15 ± 0.19
18:2n-4	0.25 ± 0.03	0.25 ± 0.01	0.23 ± 0.02	0.25 ± 0.01
18:3n-6	0.25 ± 0.02	0.31 ± 0.07	0.36 ± 0.04	0.24 ± 0.01
18:3n-3	1.29 ± 0.13	1.21 ± 0.08	1.29 ± 0.11	1.31 ± 0.07
18:4n-3	0.53 ± 0.10	0.57 ± 0.07	0.59 ± 0.07	0.55 ± 0.09
20:0	0.16 ± 0.05	0.15 ± 0.03	0.16 ± 0.03	0.18 ± 0.04
20:1n-9	0.15 ± 0.02	0.15 ± 0.02	0.14 ± 0.01	0.15 ± 0.02
20:1n-7	0.93 ± 0.11	0.86 ± 0.05	0.81 ± 0.03	0.89 ± 0.13
20:1n-5	0.16 ± 0.02	0.14 ± 0.01	0.13 ± 0.00	0.15 ± 0.02
20:2n-6	0.33 ± 0.03	0.29 ± 0.02	0.30 ± 0.00	0.35 ± 0.02
20:4n-6 (ARA)	1.56 ± 0.07	1.21 ± 0.10	1.34 ± 0.04	1.45 ± 0.07
20:3n-3	0.16 ± 0.02	0.16 ± 0.01	0.16 ± 0.01	0.18 ± 0.01
20:4n-3	0.59 ± 0.08	0.59 ± 0.02	0.57 ± 0.02	0.63 ± 0.02
20:5n-3 (EPA)	5.16 ± 0.45	5.65 ± 0.50	5.99 ± 0.28	5.99 ± 0.37
22:1n-11	0.12 ± 0.03	0.11 ± 0.03	0.10 ± 0.02	0.13 ± 0.03
22:5n-3	2.13 ± 0.22	2.32 ± 0.19	2.11 ± 0.01	2.42 ± 0.06
22:6n-3 (DHA)	16.62 ± 1.66	17.09 ± 0.44	17.84 ± 0.80	18.63 ± 0.53
Total saturates	26.87 ± 1.03	27.79 ± 0.44	27.58 ± 0.68	27.56 ± 0.46
Total MUFA	24.83 ± 2.97	23.37 ± 0.68	23.36 ± 1.16	22.43 ± 0.27
Total n-3	34.47 ± 0.50	34.81 ± 0.47	33.21 ± 1.53	33.10 ± 0.58
Total n-6	26.66 ± 2.11	27.78 ± 0.53	28.73 ± 1.25	29.91 ± 0.53
Total n-9	12.44 ± 0.28	12.24 ± 0.72	12.88 ± 0.41	12.93 ± 0.22
Total n-3 LC-PUFA	24.67 ± 0.65	24.93 ± 1.08	24.00 ± 1.67	23.52 ± 0.50
DHA/EPA	3.23 ± 0.35	3.04 ± 0.33	2.98 ± 0.04	3.12 ± 0.24
EPA/ARA	3.32 ± 0.43	4.70 ± 0.69	4.48 ± 0.08	4.12 ± 0.32
EPA+DHA	21.77 ± 1.85	22.74 ± 0.35	23.83 ± 1.07	24.62 ± 0.49
n-3/n-6	2.14 ± 0.18	2.27 ± 0.18	2.23 ± 0.07	2.31 ± 0.05
18:1n-9/n-3 LC-PUFA	0.97 ± 0.06	0.94 ± 0.06	0.88 ± 0.09	0.83 ± 0.03

*Values are reported as mean ± SD. n=3. MUFA. monounsaturated fatty acid; LC-PUFA Long-chain highly unsaturated fatty acid; EPA. eicosapentaenoic acid; DHA. docosahexaenoic acid; ARA. arachidonic acid.

Table 5-7. Fatty acid composition (% total fatty acids identified) of eggs obtained after feeding with the experimental diets.

	Broodstock group			
Fatty acid	**D1**	**D2**	**D3**	**D4**
14:0	1.89 ± 0.28	1.97 ± 0.42	2.17 ± 0.09	2.67 ± 0.26
16:0	18.89 ± 0.68	18.71 ± 0.93	18.07 ± 0.61	18.85 ± 0.64
16:1n-7	4.04 ± 0.11[b]	4.44 ± 0.31[b]	4.36 ± 0.17[b]	5.57 ± 0.32[a]
16:1n-5	0.08 ± 0.01	0.10 ± 0.02	0.10 ± 0.03	0.14 ± 0.03
16:2n-4	0.22 ± 0.05	0.26 ± 0.11	0.31 ± 0.02	0.37 ± 0.07
17:0	0.15 ± 0.02	0.18 ± 0.06	0.21 ± 0.02	0.25 ± 0.04
16:3n-4	0.30 ± 0.04	0.33 ± 0.06	0.29 ± 0.03	0.40 ± 0.03
18:0	5.01 ± 0.23	5.64 ± 0.55	5.75 ± 0.67	5.62 ± 0.44
18:1n-9	24.86 ± 1.46[a]	23.43 ± 2.62[a]	21.83 ± 0.98[a]	20.63 ± 1.77[b]
18:1n-7	3.20 ± 0.28[b]	3.59 ± 0.19[ab]	3.09 ± 0.05[b]	4.07 ± 0.21[a]
18:1n-5	0.17 ± 0.01[b]	0.20 ± 0.00[ab]	0.18 ± 0.02[b]	0.22 ± 0.00[a]
18:2n-6	10.24 ± 0.26[a]	9.08 ± 0.30[a]	8.98 ± 0.73[a]	6.79 ± 0.32[b]
18:2n-4	0.20 ± 0.00[c]	0.24 ± 0.02[b]	0.22 ± 0.01[bc]	0.29 ± 0.01[a]
18:3n-6	0.15 ± 0.01[b]	0.16 ± 0.02[ab]	0.17 ± 0.02[ab]	0.21 ± 0.01[a]
18:3n-3	5.63 ± 1.59[a]	5.09 ± 0.68[a]	4.04 ± 1.10[b]	4.02 ± 0.04[b]
18:4n-3	0.59 ± 0.11	0.68 ± 0.24	0.82 ± 0.09	0.92 ± 0.18
20:0	0.19 ± 0.02	0.18 ± 0.02	0.17 ± 0.04	0.16 ± 0.01
20:1n-9	0.27 ± 0.01	0.26 ± 0.03	0.27 ± 0.03	0.29 ± 0.03
20:1n-7	1.28 ± 0.18	1.26 ± 0.27	1.35 ± 0.13	1.34 ± 0.17
20:1n-5	0.13 ± 0.01	0.14 ± 0.03	0.14 ± 0.01	0.17 ± 0.02
20:2n-6	0.26 ± 0.01	0.25 ± 0.01	0.24 ± 0.03	0.25 ± 0.01
20:4n-6 (ARA)	0.98 ± 0.10[b]	1.16 ± 0.05[ab]	1.08 ± 0.05[b]	1.31 ± 0.03[a]
20:3n-3	0.14 ± 0.01	0.13 ± 0.00	0.14 ± 0.02	0.11 ± 0.01
20:4n-3	0.48 ± 0.04[b]	0.53 ± 0.02[b]	0.58 ± 0.01[ab]	0.68 ± 0.05[a]
20:5n-3 (EPA)	4.60 ± 0.45	5.17 ± 1.01	5.92 ± 0.26	6.40 ± 0.78
22:1n-11	0.34 ± 0.09	0.35 ± 0.14	0.39 ± 0.05	0.39 ± 0.08
22:5n-3	1.74 ± 0.07	1.76 ± 0.23	1.74 ± 0.12	2.20 ± 0.05
22:6n-3 (DHA)	12.65 ± 0.38[c]	14.26 ± 0.58[bc]	14.87 ± 0.33[b]	16.88 ± 0.60[a]
Total saturates	26.37 ± 0.57	26.94 ± 1.11	26.62 ± 1.28	27.87 ± 0.08
Total MUFA	34.56 ± 1.34	33.98 ± 2.01	31.91 ± 0.87	33.05 ± 1.42
Total n-3	25.98 ± 1.78	26.76 ± 1.73	29.36 ± 0.95	28.46 ± 1.67
Total n-6	11.87 ± 0.16[a]	10.94 ± 0.29[a]	10.76 ± 0.78[a]	8.88 ± 0.30[b]
Total n-9	25.39 ± 1.49	23.97 ± 2.62	22.36 ± 1.01	21.25 ± 1.75
Total n-3 LC-PUFA	19.61 ± 0.06[c]	21.85 ± 0.84[bc]	23.24 ± 0.08[b]	26.27 ± 1.42[a]
DHA/EPA	2.77 ± 0.38	2.83 ± 0.60	2.52 ± 0.15	2.66 ± 0.25
EPA/ARA	4.73 ± 0.86	4.45 ± 0.69	5.47 ± 0.16	4.91 ± 0.63
EPA+DHA	17.25 ± 0.09[b]	19.43 ± 0.94[ab]	20.79 ± 0.19[ab]	23.28 ± 1.37[a]
n-3/n-6	2.19 ± 0.12[b]	2.44 ± 0.11[b]	2.73 ± 0.11[ab]	3.21 ± 0.29[a]
18:1n-9/n-3 LC-PUFA	1.27 ± 0.08[a]	1.07 ± 0.15[ab]	0.94 ± 0.04[bc]	0.79 ± 0.11[c]

*Values are reported as mean ± SD. n=3. Different superscripts in the same row indicate significant differences ($P < 0.01$). MUFA. monounsaturated fatty acid; LC-PUFA Long-chain highly unsaturated fatty acid; EPA. eicosapentaenoic acid; DHA. docosahexaenoic acid; ARA. arachidonic acid.

5.4. Discussion

N-3 LC-PUFA contents in broodstock diets markedly affect reproductive performance of marine fish species (Izquierdo et al., 2015) and broodfish requirements for these essential fatty acids may differ among species (Tocher, 2010; Fernández-Palacios et al., 2011). Despite reproductive success is one of the main constrains for the mass production of greater amberjack, the n-3 LC-PUFA requirements for the broodstock of this species (FAO, 2018) have not been yet determined. The present study has shown for the first time in greater amberjack that fecundity markedly increases by the elevation of dietary n-3 LC-PUFA levels in the broodstock diets from 1 to 1.7 % dw, leading to high-quality eggs and larvae. Indeed, the high fecundity values (71500 eggs/kg female/spawn) obtained when hormonally induced broodstock were fed 1.7 % n-3 LC-PUFA were as good as those obtained by spontaneous spawns in previous studies (56000 eggs/kg female/spawn, Sarih et al., 2018). Besides, egg quality was also highest in broodstock fed 1.7 % n-3 LC-PUFA, in terms of fertilization (91.76 %) and viability (95.99 %), and as good as those obtained from spontaneous spawning in previous studies (84.37 % and 92.21 %, respectively, Sarih et al., 2018). These results imply that adequate concentrations of n-3 HUFA in broodstock diets are required to optimize egg production and quality in greater amberjack.

The fertilization rate is a good indicator of spawning success and a major factor influenced by broodstock diets (Fernández-Palacios et al., 1995, 2011). Poor fertilization rates are among the problems for mass production of greater amberjack and may be affected by the hormonal induction method used and the quality of the broodstock diet (Fernández-Palacios et al., 2015a; Sarih et al., 2018, 2019). Therefore, despite in previous studies we found reduced fertilization rates in fish injected with GnRHa and even lower in fish with GnRHa implants in comparison to spontaneous spawning fish (Sarih et al., 2018), diets with adequate n-3 LC-PUFA levels may allow the optimization of egg quality when fish is injected with GnRHa as in the present study. Fertilization rates are also improved by an increase in dietary n-3 LC-PUFA contents in other species such as gilthead seabream (Fernández-Palacios et al., 1995), Japanese yellowtail broodstock (Verakunpiriya et al., 1996) or striped jack (*Pseudocaranx dentex)* (Vassallo-Agius et al., 1998). Improvement in fertilization rates by increased LC-PUFA may be related to the important role of EPA and ARA as

precursors and modulators of eicosanoids. ARA is the main precursor of 2-series prostaglandins (PGE2 and PGF2α), which are involved in the reproductive processes such as pheromonal attraction, oocytes maturation and gonadal steroidogenesis (Mercure and Van der Kraak, 1996; Henrotte et al., 2011). EPA competitively modulates the production of prostaglandins producing the 3-series prostaglandins (PGE3 and PGF3), biologically less potent (Henrotte et al., 2010, 2011) but the major PGs in marine fish (Ganga et al., 2005). Fertilization rate could be also affected by sperm quality (Bobe and Labbé, 2010). Indeed, sperm fatty acids composition and fatty acids ratios may affect sperm motility and viability (Vassallo-Agius et al., 2001; Lahnsteiner et al., 2009; Henrotte et al., 2010; Beirão et al., 2012; Hajiahmadian et al., 2016).

On the contrary, an excess of n-3 LC-PUFA (2.3 and 3 % dw) in the broodstock diet caused a decrease in fecundity, in agreement with the reduced fecundity found in gilthead sea bream (Fernández-Palacios et al., 1995) or brill (Hachero- Cruzado et al., 2012) when broodstock were fed diets with too high levels of n-3 LC-PUFA. Increased dietary n-3 LC-PUFA levels in broodstock diets up to 2.3 and 3 % dw also progressively reduced fertilization and hatching rates, in agreement with previous results in cobia (Nguyen et al., 2010). This negative effect of excessive n-3 LC-PUFA levels in greater amberjack broodstock diets could be related to an excess in dietary EPA levels, since broodstock of this species reared in captivity show a higher content of EPA (9.30 ± 0.60 % TFA) in the gonads than wild specimens (3.00 ± 0.79 % TFA) (Rodríguez-Barreto et al., 2012), and lower concentrations of 17β-estradiol (E2) and testosterone (T) in the plasma (Zupa et al., 2017a). The dysfunctions of spermatogenesis can lead to a qualitative and quantitative decrease in sperm production (Bobe and Labbé, 2010) and, therefore, to the unsuccessful production of fertilized eggs. In this sense, excessive levels of LC-PUFA, particularly EPA, in greater amberjack broodstock diet could negatively affect gonadal steroidogenesis, oocyte maturation and sperm quality, reducing fecundity and fertilization rates. In agreement, in gilthead sea bream there is a negative correlation between the DHA/EPA ratio in the sperm head plasma membrane and the sperm quality (Beirão et al., 2012). Besides, the reduction in fertilization rates by excessive LC-PUFA levels could be also related to an increased peroxidation risk (Izquierdo et al., 2001), since the high metabolic rate of spermatozoa can generate a significant production of reactive oxygen species that may negatively

affect sperm quality (Cabrita et al., 2014). Thus, increased DHA levels in diets for senegalese sole males, caused a decrease in the sperm velocity in relation to an increase in lipid peroxidation (Beirão et al., 2015). In this same study, the addition of antioxidants such as selenium and vitamin E (α-tocopherol) improved sperm quality (Beirão et al., 2015).

Although diet n-3 LC-PUFA levels did not affect the egg or the oil droplet diameter, it did significantly affect egg quality. Excessive n-3 LC-PUFA levels reduced eggs viability and hatching rates, as well as larval survival. These results are in agreement with those obtained in other species, in which an excess of n-3 LC-PUFA in the diet reduced egg quality and larval survival in gilthead sea bream (Fernández-Palacios et al., 1995), Japanese flounder (Furuita et al., 2002), crescent sweetlips (Li et al., 2005), Japanese eel (Furuita et al., 2007), cobia (Nguyen et al., 2010) or brill (Hachero-Cruzado et al., 2012). These negative effects of excessive n-3 LC-PUFA would be also related to an increased oxidative risk, since increased supplementation of broodstock diets with vitamin E, a potent antioxidant to protect polyunsaturated fatty acids, prevented these negative effects in egg and larval quality caused by excessive LC-PUFA levels in gilthead seabream (Izquierdo et al., 2001). Exceptionally, in yellowfin sea bream, fecundity, egg hatching rates and larval survival were improved when broodstock were fed a diet containing higher n-3 LC-PUFA level, 6.6 % dw (Zakeri et al, 2011). These differences could be attributed to the difference in the species studied and in the different n-3 LC-PUFA levels used in the experimental diets.

In spite of these negative effects in egg quality, lipid and proximate composition of the eggs were not affected by the high LC-PUFA levels in the broodstock diets. Similar results were also found in other species, such as sweetlips (Li et al., 2005), brill (Hachero-Cruzado et al., 2012) or yellowfin sea bream (Zakeri et al., 2011). Generally, the fatty acid composition of eggs is more conserved and relatively less influenced by broodstock diet than other fish tissues (Izquierdo et al., 2001; Sargent et al., 2002). Nevertheless, extreme changes in the dietary fatty acid composition of broodstock diets may alter the fatty acid profiles of eggs (Fernández-Palacios et al., 1995, Izquierdo et al., 2001; Fuiman and Faulk, 2013; Stuart et al., 2018). The required feeding period for broodstock to have an effect on egg composition is variable, depending on the reproductive strategy of the studied species (Izquierdo et al., 2001).

Greater amberjack is a gonochoric species with group-synchronous ovarian development and a multiple spawning pattern (Marino et al., 1995; Mandich et al., 2004; Zupa et al., 2017a). As occurs in other species with short vitellogenetic periods, it is possible to boost egg composition of greater amberjack by improving the nutritional quality of broodstock diets even during the spawning season (Fernández-Palacios et al., 1995; Stuart et al., 2018; Sarih et al., 2019). Indeed, in the present study, the fatty acid composition of the eggs was affected by the level of n-3 LC-PUFA in the broodstock diet administered during the spawning season. Similarly, red drum and yellowtail (*Seriola dorsalis)* appear to rapidly transfer dietary LC-PUFAs to egg lipids (Fuiman and Faulk, 2013; Stuart et al., 2018). Generally, the eggs of marine species present a high level of n-3 LC-PUFA (Tocher, 2003), which indicates its high requirement during embryonic development and early larval stages (Tocher, 2010; Izquierdo and Koven, 2011) and reflects the high levels of n-3 LC-PUFA usually present in natural feed for broodstock.

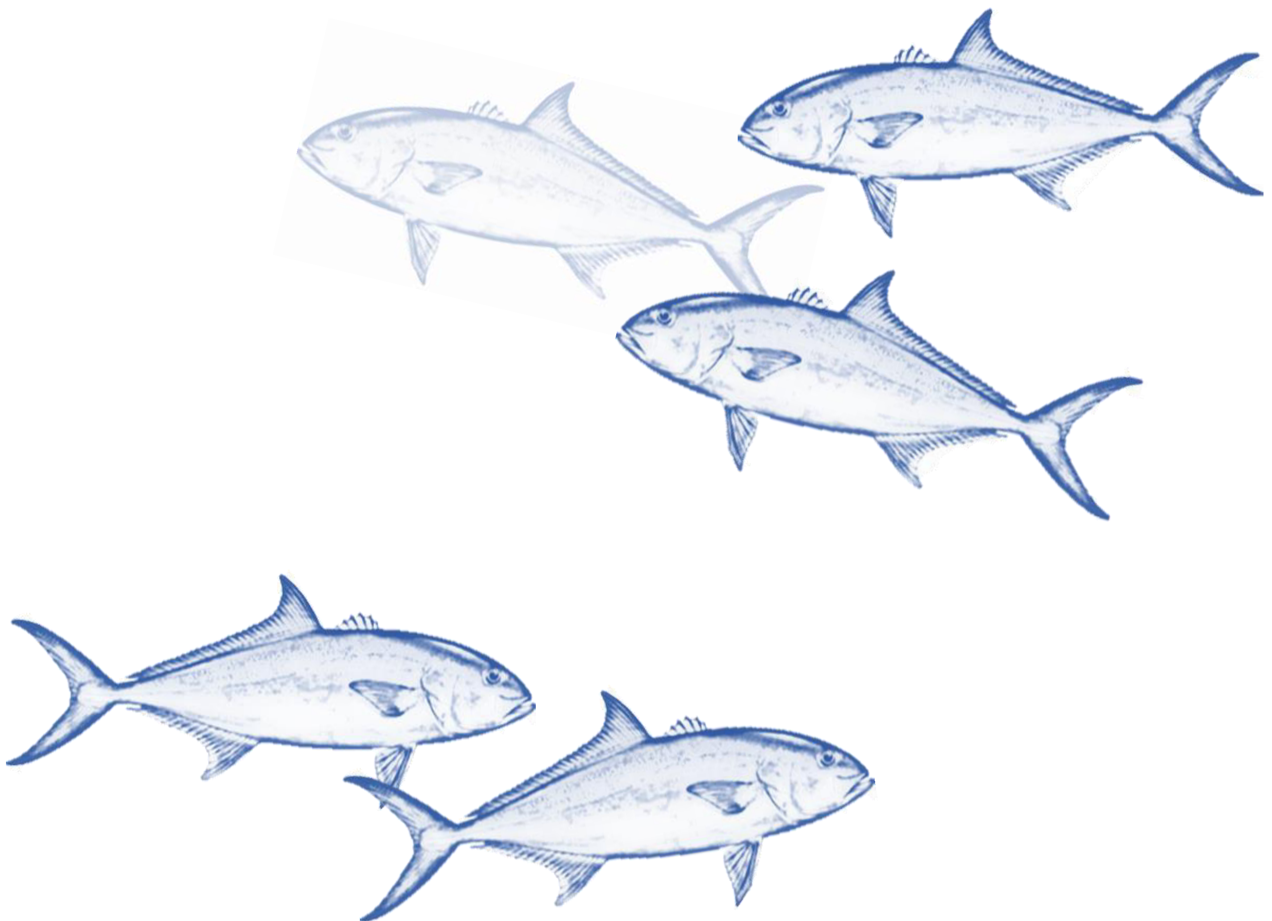
Regardless of the diet used, the fatty acid profile of fertilized eggs is characterized by presenting 16:0 as the main saturated fatty acid, followed by 18:0, 18:1n-9 as the largest representative of monounsaturated fatty acids (MUFA), and DHA and EPA as the most representative abundant n-3 LC-PUFA, which is in line with previous studies on non-fertilized eggs of this species (Rodríguez-Barreto et al., 2014). Also, these results coincide with those obtained in other marine species (Fernández-Palacios et al., 1995; Tocher, 2010; Izquierdo et al., 2015). The biochemical composition of greater amberjack eggs was significantly affected by broodstock diet composition, with egg n-3 LC-PUFA levels increasing in response to the increase in n-3 LC-PUFA levels in the diet. In particular, DHA appears to have been accumulated into developing eggs, while egg EPA level was not changed in the respective treatment diets. In addition, the eggs that contained a lower concentration of DHA were of better quality. In some species, an excessively high level of DHA in eggs has a negative effect on the quality of eggs and larvae (Liang et al., 2014; Morais et al., 2014; Parma et al., 2015). On the contrary, in common snook (*Centropomus undecimalis*), a significant correlation was found between the DHA concentrations of eggs and the quality of the obtained spawns (Yanes-Roca et al., 2009). The content of 18:1n-9 was significantly higher in eggs from broodstocks fed a diet containing lower n-3 LC-PUFA levels and

represents more than 24 % of total fatty acids. In fact, its proportion in the ovaries of wild specimens is much higher than in cultured ones, and also represented 24.79 % of total fatty acids, which is consistent with its potential role as a source of metabolic energy during reproduction (Rodríguez-Barreto et al., 2012). Nevertheless, in eggs of other species, this fatty acid is found in much lower relative proportions, around 15% of total fatty acids (Fernández-Palacios et al., 1995; Furuita et al., 2002; Wilson, 2009; Callan et al., 2014; Liang et al., 2014), and only a few species like cobia (Nguyen et al., 2010), Japanese yellowtail (Vassallo-Aguis et al., 2001) or yellowtail (Stuart et al., 2018) present values above 20 %. 18:1n-9 is an important source of energy during early development and, along with 16:0, generally makes up a significant proportion of muscle and liver phospholipid (Van der Meeren et al., 1991). Therefore, the high accumulation of 18:1n-9 in the eggs from broodstocks fed diets content 1-1.7 % n-3 LC-PUFA of dw, should be a preparation for the energy demand in the development of eggs and larvae, and would be related to the high rates of egg viability and hatching obtained after feeding the broodstock with these two diets. In fact, significant positive correlations have been observed between 18:1n-9 content in eggs, and the viability of eggs and hatching rates in gilthead sea bream (Fernández-Palacios et al., 1997). Since 18:2n-6 remains at low levels in the eggs of wild marine fish, the high content of linoleic acid in eggs from broodstocks fed diets D1, D2 and D3 is probably due to the use of raw materials of vegetable origin (linseed and palm oil) in the elaboration of these diets. High levels of 18:2n-6 in commercial diets have been described in various fish species as a result of the use of sources of plant origin as alternative ingredients in feed formulation (Turchini et al., 2009; Glencross and Turchini, 2011). Besides, it was found that the relative proportions of 18:2n-6 were significantly higher in gonads and eggs of cultured greater amberjack fed commercial diets compared to wild specimens (Rodríguez-Barreto et al., 2012, 2014; Zupa et al., 2017a).

In conclusion, the present study showed that the spawning quality of greater amberjack could be improved in terms of fertilization rate, relative fecundity, and egg and larval quality parameters to equal those obtained in spontaneous spawns by supplying an adequate level of n-3 LC-PUFA into the broodstock diet. The recommended n-3 LC-PUFA dietary level for greater amberjack broodstock was suggested to be within the range of 1-1.7 % dw.

Chapter 6

Arachidonic acid in the diet of greater amberjack (*Seriola dumerili*) and its effects on egg and larval quality



Arachidonic acid in the diet of greater amberjack (*Seriola dumerili*) and its effects on egg and larval quality

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Abstract

This study was conducted to determine the effects of graded arachidonic acid (ARA) levels in broodstock diets on spawning performance, fatty acid composition of eggs, and egg and larval quality of greater amberjack. Formulated diets containing 0.8, 1.6, 2.4 and 3.2 g ARA/kg feed were fed to greater amberjack broodstock for over 3 months. Greater amberjack fed the diet containing 3.2 g ARA/kg feed showed significantly increased fecundity, fertilization, egg viability, hatching, larval survival rates and larval size than fish fed the other three dietary ARA levels. However, egg diameter and egg oil droplet diameter, from the broodstock fed 3.2 g ARA/kg feed were not significantly different from those of broodstock fed other ARA levels. Egg ARA content reflected dietary ARA levels, whereas egg EPA was reduced as ARA dietary content increased. Consequently, elevation of dietary ARA contents increased DHA/EPA, and reduced EPA/ARA and DHA/ARA ratios in eggs. These results showed that the increase in dietary ARA content up to 3.2 g/kg feed improved broodstock reproductive performance and eggs and larval quality.

Keywords: *Seriola dumerili*, broodstock diet, arachidonic acid, larval quality, egg quality.

6.1. Introduction

Evaluating the effects of broodstock nutrition on reproductive performance of farmed fish is complex due to the need for large facilities, high costs, complicated management, etc. (Izquierdo et al., 2001). All these factors contribute to the lack of sufficient information on the nutritional requirements of broodstock in many fish species. Some essential fatty acids (EFA) of the n-3 and n-6 series are particularly important in broodstock nutrition, their levels in broodstock diet determining the success of reproduction (Izquierdo et al., 2001; Tocher, 2003). Especially, the long-chain polyunsaturated fatty acids (LC-PUFAs) docosahexaenoic acid (22:6n-3; DHA), eicosapentaenoic acid (20:5n-3; EPA) and arachidonic acid (20:4n-6; ARA) are important for maturation and egg quality, and affect fecundity, hatching success and larval survival (Fernández-Palacios et al., 1997, 2011; Tocher, 2003). The n-3 LC-PUFA levels present in broodstock diets markedly affect egg and larval quality (Izquierdo et al., 2001). However, less attention has been paid to the effects of n-6 LC-PUFA levels in diets, in spite of its potential importance in reproduction. Particularly, ARA is a precursor for synthesis of eicosanoids of prostaglandins (PGs) from series II, such as PGE2 and PGF2α, which are involved in different reproductive processes including oocyte maturation and steroidogenesis (Mercure and Van der Kraak, 1996; Henrotte et al., 2011; Norambuena et al., 2013), embryogenesis (Bruce et al., 1999) and larval development (Izquierdo and Koven, 2011). In addition, ARA and EPA compete for the common enzymatic complex to generate different series of PGs with different biological activities (Sargent, 1995; Tocher, 1995). Moreover, ARA is an important substrate for eicosanoid synthesis despite the preponderance of EPA in fish tissues (Monroig et al., 2018). Therefore, an adequate EPA/ARA ratio in broodstock diets must be essential for egg and larval development (Tocher, 2003). Indeed, ARA levels in broodstock diets affect egg quality in different species (Fernández-Palacios et al., 2011; Migaud et al., 2013). For instance, ARA content in broodstock diets is directly related to fertilization rates and larval survival in gilthead seabream (Fernández-Palacios et al., 1995, 1997, 2005). Also, in Japanese flounder (*Paralichthys olivaceus*) total egg production, percentage of buoyant eggs, hatching rate and larval survival are promoted by the elevation of dietary ARA from 0.1- 0.6 g ARA/100 g diet (Furuita et al., 2003).

Information on nutritional requirements of broodstock from emerging species, such as greater amberjack, are scarcer (Rodríguez-Barreto et al., 2012, 2014; Sarih et al., 2019). In this species, n-3 LC-PUFA levels in broodstock diets markedly improve fertilization rates, relative fecundity and egg and larval quality (Sarih et al., 2019 under review at Aquaculture). Despite there is no information on the effect of dietary ARA levels in broodstock performance in this species, certain evidences suggest their importance. For instance, in the close yellowtail species, the increase in ARA contents in broodstock diets from 1.4 to 4.7 g ARA/100 g of total fatty acids (TFA) improves hatching rates and egg diameter (Stuart et al., 2018). Besides, comparison of ovaries and testes from culture and wild greater amberjack denoted low levels of ARA in the former (Rodríguez-Barreto et al., 2012; Zupa et al., 2017b). Therefore, the main objective of this study was to investigate the effects of increased ARA levels in broodstock diets on reproductive performance of greater amberjack. To achieve that aim, greater amberjack broodstock were fed four graded levels of ARA during the spawning season to determine their effect on egg and larval quality.

6.2. Material and methods

This study was conducted according to the European Union Directive (2010/63/EU) on the protection of animals for scientific purposes at Fundación Canaria Parque Científico Tecnológico (FCPCT), University of Las Palmas de Gran Canaria (Canary Islands, Spain).

6.2.1. Broodstock selection and experimental conditions

Rearing was undertaken in the facilities of the Grupo de Investigación en Acuicultura (GIA) in the ECOAQUA Institute (Universidad de Las Palmas de Gran Canaria, ULPGC, Spain). Greater amberjacks were captured in the south western coast of Gran Canaria (Canary Islands, Spain), individually identified with Passive Integrated Transponder tags and maintained for five years in 40 m^3 tanks. One month before the beginning of the experiment, all brood fish were anesthetized with clove oil (0.1 mL L^{-1}, Guinama SL, Valencia, Spain) to evaluate gonad maturation. Females were considered eligible for spawning induction if they contained fully vitellogenic oocytes with a diameter > 500 µm. Males were selected based on spermiation, which was confirmed with the collection of a sample of milt using a catheter.

In total, 8 females (15.3 ± 2.39 kg (mean ± SD)) and 8 males (14.7 ± 3.3 kg) were selected and distributed into four 40 m^3 (5 m x 2.35 m) circular tanks (2♀ and 2♂ per tank and sex ratio 1:1) (Sarih et al., 2018), to obtain the same initial biomass (1.49 kg/m^3, 1.49 kg/m^3, 1.50 kg/m^3 and 1.51 kg/m^3). Physico-chemical conditions were identical in all tanks. Tanks were supplied with running seawater (37.1 ± 0.3 salinity) and kept under a natural photoperiod (27º 59'28" N; 15º 22'05" W) of approximately 13 h light. Flow rate allowed 6 complete water tank renovations daily and the temperature was continuously monitored (Miranda, Innovaqua, Sevilla, Spain) and ranged between 20.98 and 23.21°C from June to October 2017.

6.2.2. Hormonal induction

Spawning was induced hormonally from June to October 2017. Fish were injected intramuscularly with gonadotropin releasing hormone analogue (LHRHa, des-Gly10, [D-Ala6]-; Sigma-Aldrich, St. Louis, MO, USA) at a dose of 20 µg kg^{-1} (Fernández-Palacios et al., 2015; Sarih et al., 2018). Only one of the two couples of each broodstock tank was hormonally induced each time to determine the spawning quality of that particular couple. The second couple was hormonally induced 4 days after injection of the first one and, therefore, spawning was alternated with each couple from a given tank being injected every 9 days along the whole spawning season.

6.2.3. Experimental diets

Until July 16th, broodfish were fed twice a week with the same commercial diet (Vitalis CAL, Skretting, Burgos, Spain) (1% of biomass day^{-1}), and once a week with Atlantic mackerel (2% of biomass day^{-1}). After 7 weeks of feeding and collection and study of 5 spawns from each couple, no significant ($P < 0.05$) differences were found in egg and larval quality among different couples inside the same tank or among tanks. Therefore, feeding of the experimental diets started from July 17th and lasted until October 30th. The experimental diets contained graded levels of arachidonic acid: 0.8 (ARA-0.8), 1.6 (ARA-1.6), 2.4 (ARA-2.4) and 3.2 g ARA/kg feed (ARA-3.2), while keeping the optimum levels of n-3 LC-PUFA determined in previous studies (Sarih et al., submitted). The desired ARA content was completed with a commercially available ARA rich oil obtained from Hua Xing Enterprises (Co. LTD, China). Diet ingredients, fatty acid profiles and proximate compositions are detailed in Tables 6-1 and 6-2. The experimental diets were manufactured by Skretting ARC Feed Technology Plant

(Stavanger, Norway) with a pellet size of 13 mm, analysed for proximate and fatty acid composition at GIA laboratories (ECOAQUA Institute, ULPGC, Spain) and kept in a cold room at 10 ºC until daily broodstock feeding. Fish were hand-fed twice a day and 5 days a week (2% of biomass day^{-1}). After 24 days of feeding each experimental diet (August 7th), spawning quality was monitored separately for each of the 2 couples from each tank during 10 consecutive spawns.

Table 6-1. Experimental diets' main ingredients and proximate composition for greater amberjack broodstock.

	Diet			
	ARA-0.8	**ARA-1.6**	**ARA-2.4**	**ARA-3.2**
Raw material (%)				
Wheat[a]	20.78	20.78	20.78	20.78
Wheat gluten[b]	15.00	15.00	15.00	15.00
Fish meal[c]	43.04	43.04	43.04	43.04
Rapeseed oil[d]	5.71	5.49	5.26	5.03
Arachidonic acid[e]	0.00	0.23	0.45	0.68
Squid meal[f]	10.00	10.00	10.00	10.00
Fish oil[g]	4.52	4.52	4.52	4.52
Premix vit. Min[h]	0.64	0.64	0.64	0.64
Histidine HCl[i]	0.31	0.31	0.31	0.31
Proximate composition (% dry weight)				
Crude protein	53.76	53.69	53.45	53.61
Crude lipids	18.31	18.12	18.14	18.46
Ash	13.28	13.11	13.12	13.18
Moisture	8.58	8.79	8.45	8.47
Arachidonic acid (g kg^{-1} feed)	0.8	1.6	2.4	3.2

[a] Wheat: Linas Agro AB, Lithuania.
[b] Wheat gluten: Roquette, France.
[c] Fish meal: FF Skagen AS, Denmark.
[d] Rapeseed oil: Bunge Deutschland Gmbh.
[e] Arachidonic acid: Hua Xing Enterprises Co. LTD. China
[f] Squid meal: Inproquisa SA, Spain.
[g] Fish oil: Norsildmeal AS, Norway.
[h] Include vitamins and minerals; Trouw Nutrition, Boxmeer, the Netherlands, proprietary composition Skretting ARC, vitamin and mineral supplementation as estimated to cover requirements according to NRC (2011).
[i] Histidine: Kyowa Hakko, Japan
[j] Values are reported as mean of triplicate analyses.

Table 6-2. Main fatty acids contents of the four experimental diets for greater amberjack broodstock (% of the total fatty acids identified).

	Diet			
Fatty acid	**ARA-0.8**	**ARA-1.6**	**ARA-2.4**	**ARA-3.2**
14:0	3.23	3.44	3.31	3.37
15:0	0.32	0.34	0.33	0.34
16:0	13.47	14.07	14.07	14.38
16:1n-7	3.84	4.01	3.89	3.94
16:1n-5	0.14	0.17	0.17	0.14
16:2n-4	0.39	0.39	0.38	0.37
17:0	0.41	0.42	0.40	0.39
16:3n-4	0.15	0.15	0.15	0.17
16:3n-3	0.13	0.11	0.10	0.11
16:4n-3	0.51	0.50	0.46	0.46
18:0	3.44	3.62	3.68	3.87
18:1n-9	28.70	27.88	28.84	28.86
18:1n-7	2.71	2.69	2.70	2.74
18:1n-5	0.11	0.12	0.12	0.11
18:2n-9	0.03	0.03	0.03	0.03
18:2n-6	11.74	11.35	11.60	11.32
18:2n-4	0.11	0.12	0.11	0.11
18:3n-6	0.12	0.14	0.17	0.20
18:3n-4	0.12	0.10	0.08	0.09
18:3n-3	3.96	3.71	3.69	3.56
18:4n-3	1.28	1.27	1.18	1.14
18:4n-1	0.06	0.06	0.06	0.06
20:0	0.51	0.52	0.53	0.54
20:1n-9	0.18	0.19	0.18	0.19
20:1n-7	3.17	3.25	3.24	3.26
20:1n-5	0.16	0.19	0.16	0.17
20:2n-9	0.03	0.03	0.03	0.03
20:2n-6	0.16	0.17	0.17	0.18
20:3n-9	0.03	0.03	0.03	0.03
20:3n-6	0.08	0.10	0.13	0.17
20:4n-6 (ARA)	0.90	1.09	1.48	1.94
20:3n-3	0.08	0.08	0.08	0.08
20:4n-3	0.30	0.30	0.29	0.27
20:5n-3 (EPA)	6.56	6.49	5.99	5.69
22:1n-11	3.40	3.55	3.46	3.50
22:1n-9	0.52	0.52	0.52	0.51
22:4n-6	0.10	0.10	0.10	0.10
22:5n-6	0.22	0.22	0.20	0.20
22:5n-3	0.92	0.92	0.85	0.81
22:6n-3 (DHA)	7.46	7.28	6.78	6.31
Total Saturates	21.38	22.41	22.31	22.89
MUFA	43.09	42.74	43.44	43.58
Total n-3	21.19	20.66	19.42	18.42
Total n-6	13.32	13.17	13.86	14.12
Total n-9	29.48	28.68	29.63	29.65
Total n-3 LC-PUFA	15.31	15.07	13.98	13.16
EPA/ARA	7.32	5.94	4.04	2.93
DHA/EPA	1.14	1.12	1.13	1.11
DHA/ARA	8.32	6.66	4.57	3.24
n-3/n-6	1.59	1.57	1.40	1.30
18:1n-9/n-3 LC-PUFA	1.87	1.85	2.06	2.19

*MUFA, monounsaturated fatty acid; LC-PUFA, Long chain highly unsaturated fatty acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; ARA, arachidonic acid.

6.2.4. Evaluation of egg and larval quality

Spawning and egg quality was determined by quantifying the total number of eggs produced (eggs/kg female/spawn), and the percentages of fertilized, viable and hatched eggs and larval survival (Fernández-Palacios et al., 2005). Collected eggs were placed in 10 L beakers provided with vigorous aeration, from where five randomized 10 ml sub-samples were placed in a Bogorov chamber, counted and observed under a binocular microscope (Leica S6E, Wetzlar, Germany) to estimate the total number of eggs. The percentage of fertilization was evaluated at the same time by examining all the eggs in this sub-sample for the presence of a viable embryo. Fertilized eggs were then placed individually in 96-well microtiter plates in duplicates (Greiner Bio-One, Kremsmunster, Australia) and incubated in a controlled temperature incubator at 21.9 ± 0.4°C. Using a binocular microscope, embryonic and early larval development was evaluated once a day for four days, to determine the percentages of viable eggs at 24 hours (%), hatching and larval survival at 1- and 3-days post-hatching (dph) just before the yolk sac was almost completely absorbed (Sarih et al., 2018; 2019).

6.2.5. Egg and larval measurements

From 100 fertilized eggs of five different spawns for each treatment, egg and oil droplet diameter were estimated. From these spawns, 30 newly hatched larvae and 30 larvae at the end of yolk sack absorption (3dph) were measured, after being anesthetized with clove oil at 1%, for total length (TL), standard length (SL), diameter of oil globule (LGD), yolk sack length (YSL) and width (YSH). The volume of the yolk sack (YSV) was calculated using the formula: $YSV = \pi / 6\ YSL \times YSH^2$.

6.2.6. Biochemical analysis

To analyse proximate and fatty acid composition, the diets and fertilized eggs of all spawns per tank were collected during the experimental period. Eggs were thoroughly rinsed in distilled water and then stored at -80ºC prior to biochemical analysis. Proximate composition was conducted following standard procedures (AOAC, 2016). Moisture was determined by thermal dehydration until constant weight at 105 °C. Ash content was determined by combustion at 600 °C for 12 h. Crude protein content (N x 6.25) was determined by Kjeldahl method, and crude lipid was extracted

following the Folch method (Folch et al., 1957). Fatty acid methyl esters profiles were obtained by transmethylation of total lipids (Christie and Han, 2010), and separated by gas-liquid chromatography (GC-14A; Shimadzu, Tokyo, Japan) following the conditions described by Izquierdo et al. (1990) and identified by comparison to previously characterized standards and GLC-MS (Polaris QTRACETM Ultra; Thermo Fisher Scientific). All analyses were conducted in triplicate.

6.2.7. Statistical analysis

Results are presented as means and standard deviations (SD) and the significant level for all analyses was set at 5%. All data were tested for normal distribution with the one sample Kolmogorov–Smirnoff test, as well as for homogeneity of the variances with the Levene test (Sokal and Rohlf, 2012). When the assumptions were correct, one-way Analysis of Variance (ANOVA) test was performed, followed by Tukey's post hoc test. When the heterogeneity of the variances was not correct and/or data were not normally distributed, Kruskal-Wallis test was applied and differences between treatments were graphed with a box and whisker plot. Pearson's correlation coefficients were used to assess the relationships between egg quality variables. Analyses were conducted by SPSS statistics (version 22.0 for Windows; Inc, IBM, Chicago, IL, USA) and visualized using SigmaPlot 12.0 (Systat software, San José, USA).

6.3. Results

At the beginning of the spawning season, before feeding the experimental diets, there were no significant differences in fecundity and the indicators of egg and larval quality among the different groups of broodstock (Table 6-3). However, after feeding the experimental diets, increase in ARA levels from 0.8 up to 3.2 g/kg feed increased fecundity in terms of total number of eggs produced, being significantly ($P < 0.05$) highest in broodstock fed ARA-3.2 diet (Table 6-3). Similarly, fertilization, viability and hatching rates were significantly ($P < 0.05$) improved by an increase in dietary ARA, being highest in broodstock fed ARA-3.2 diet, followed by those fed ARA-2.4 and ARA-1.6 diet and, finally, fish fed ARA-0.8 diet, which showed the lowest values for these parameters (Table 6-3). These spawning quality parameters were positively correlated with dietary ARA content (Figure 6-1). Besides, increase in dietary ARA levels also led

to significantly ($P < 0.05$) increased larval survival at 1 and 3 dph, being highest for broodstock fed ARA-3.2 diet, intermediate for those fed ARA-2.4 and ARA-1.6 diet and lowest for fish fed ARA-0.8 diet (Table 6-3). Neither before nor after feeding the experimental diets egg or oil droplet diameter differed among broodstock groups (Table 6-4). However, total and standard length of the newly hatched larvae after feeding the experimental diets were significantly ($P < 0.05$) highest in larvae from broodstock fed ARA-3.2 (Table 6-4).

Table 6-3. Fecundity and egg and larval quality parameters obtained from greater amberjack before and after feeding the experimental diets.

	Diet			
	ARA-0.8	**ARA-1.6**	**ARA-2.4**	**ARA-3.2**
Before feeding the experimental diets				
Fecundity ((eggs/kg female/spawn) x10^4)	2.2 ± 0.3	2.3 ± 0.9	2.6 ± 1.0	2.0 ± 1.1
Fertilization (%)	57.5 ± 4.2	58.2 ± 5.7	58.6 ± 8.1	58.4 ± 5.2
Viability (%)	70.5 ± 5.3	66.0 ± 4.7	70.1 ± 5.0	67.5 ± 1.0
Hatching (%)	61.6 ± 6.1	56.1 ± 6.5	64.6 ± 3.9	62.2 ± 4.3
1 dph larval survival (%)	57.7 ± 8.3	58.4 ± 6.0	60.3 ± 1.9	59.5 ± 7.8
3 dph larval survival (%)	32.7 ± 5.0	32.0 ± 3.1	33.0 ± 4.4	30.1 ± 4.8
After feeding the experimental diets				
Fecundity ((eggs/kg female/spawn) x10^4)	3.2 ± 1.1^{b}	3.9 ± 0.6^{b}	4.3 ± 0.8^{b}	7.8 ± 0.8^{a}
Fertilization (%)	50.3 ± 5.6^{c}	61.1 ± 2.7^{b}	61.8 ± 4.9^{b}	86.7 ± 3.0^{a}
Viability (%)	63.8 ± 8.8^{c}	75.2 ± 7.0^{b}	75.3 ± 7.8^{b}	94.6 ± 2.7^{a}
Hatching (%)	57.2 ± 11.1^{c}	67.6 ± 8.0^{b}	70.7 ± 8.2^{b}	92.1 ± 2.5^{a}
1 dph larval survival (%)	42.8 ± 8.3^{c}	49.1 ± 7.9^{bc}	51.5 ± 8.8^{b}	88.1 ± 3.3^{a}
3 dph larval survival (%)	18.9 ± 6.8^{c}	36.3 ± 5.3^{b}	38.3 ± 3.9^{b}	50.9 ± 2.6^{a}

*Values are reported as mean ± SD. Different superscripts in the same row indicate significant differences ($P < 0.05$).

Table 6-4. Morphometric parameters of egg and larvae obtained from greater amberjack after feeding with the experimental diets.

	Diet			
	ARA-0.8	**ARA-1.6**	**ARA-2.4**	**ARA-3.2**
Egg				
Egg diameter (mm)	1.10 ± 0.03	1.10 ± 0.02	1.10 ± 0.03	1.10 ± 0.03
Oil droplet diameter (mm)	0.28 ± 0.02	0.29 ± 0.02	0.29 ± 0.02	0.29 ± 0.01
Newly hatched larvae				
Total length (mm)	2.49 ± 0.10[b]	2.52 ± 0.18[b]	2.52 ± 0.15[b]	2.61 ± 0.10[a]
Standard length (mm)	2.40 ± 0.10[b]	2.42 ± 0.16[b]	2.43 ± 0.15[b]	2.52 ± 0.09[a]
Yolk-sac volume (mm^3)	0.40 ± 0.11	0.38 ± 0.13	0.38 ± 0.13	0.38 ± 0.09
Oil droplet diameter (mm)	0.28 ± 0.01	0.28 ± 0.02	0.28 ± 0.02	0.28 ± 0.03

*Values are reported as mean ± SD. Different superscripts in the same row indicate significant differences ($P < 0.05$).

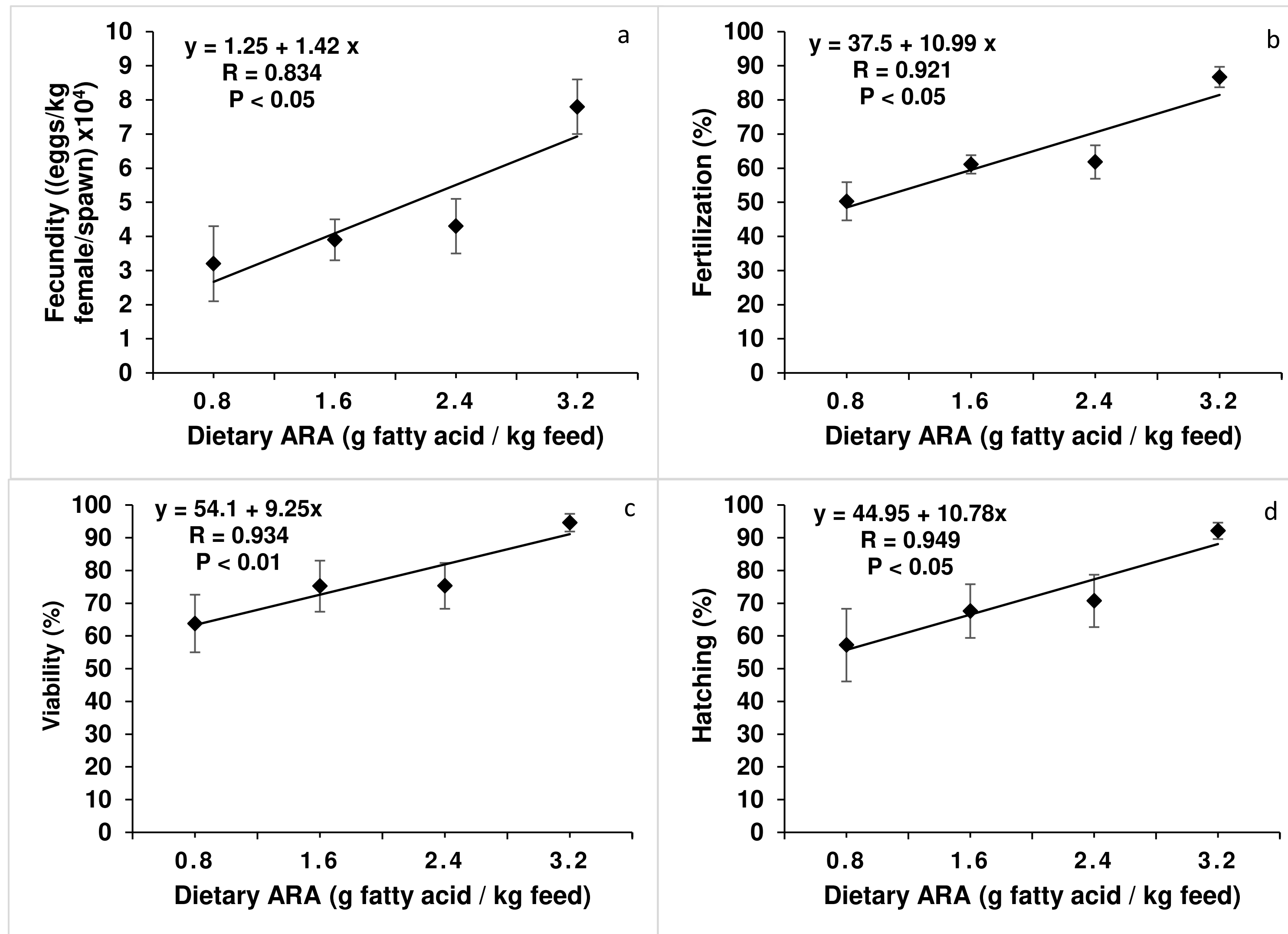


Figure 6-1. Relationship between (a) fecundity, (b) fertilization rate, (c) viability rate, or (d) hatching rate, and dietary ARA level (g fatty acid/kg feed).

There were no significant differences in crude protein, crude lipid or ash contents of the egg from the different broodstock groups before or after feeding the experimental diets (Table 6-5). Regarding the fatty acid composition of the eggs, no significant ($P < 0.05$) differences were found among eggs from different broodfish groups when they were fed the same commercial diet (Table 6-6). However, after feeding the experimental diets, ARA content in eggs was significantly ($P < 0.05$) increased and EPA content reduced with the elevation of ARA levels in the diet (Table 6-7). On the contrary, DHA levels were kept at constant levels in the eggs regardless the experimental diet fed to the broodstock. Accordingly, EPA/ARA, DHA/ARA and n-3/n-6 ratios were significantly reduced by the increase in dietary ARA, whereas DHA/EPA was increased.

Table 6-5. Proximate composition of eggs obtained from greater amberjack before and after feeding the experimental diets.

	Diet			
Proximate composition	**ARA0.8**	**ARA1.6**	**ARA2.4**	**ARA3.2**
Before feeding the experimental diets				
Crude lipid	21.1 ± 1.8	21.9 ± 1.8	21.9 ± 1.4	21.5 ± 1.6
Crude protein	68.3 ± 3.1	68.5 ± 5.5	68.7 ± 3.4	68.7 ± 3.3
Ash	5.2 ± 0.3	5.1 ± 0.8	5.0 ± 0.7	4.8 ± 0.6
After feeding the experimental diets				
Crude lipid	24.7 ± 0.9	24.9 ± 0.3	24.7 ± 0.8	24.8 ± 1.3
Crude protein	60.7 ± 0.9	62.2 ± 0.7	60.8 ± 1.4	61.1 ± 0.6
Ash	5.1 ± 0.4	4.6 ± 0.5	5.0 ± 0.5	4.9 ± 0.5

*Values are reported as mean ± SD. n=3.

Table 6-6. Fatty acid composition (% total fatty acids identified) of eggs obtained before feeding the experimental diets.

	Diet			
Fatty acid	**ARA-0.8**	**ARA-1.6**	**ARA-2.4**	**ARA-3.2**
14:0	1.68 ± 0.17	1.57 ± 0.17	1.90 ± 0.47	2.11 ± 0.42
15:0	0.27 ± 0.03	0.26 ± 0.02	0.28 ± 0.03	0.29 ± 0.03
16:0	15.95 ± 0.67	16.05 ± 1.97	16.08 ± 0.64	17.28 ± 1.58
16:1n-7	4.31 ± 0.29	4.35 ± 0.16	4.66 ± 0.56	4.77 ± 0.38
16:1n-5	0.12 ± 0.02	0.12 ± 0.03	0.13 ± 0.01	0.13 ± 0.02
16:2n-4	0.22 ± 0.03	0.21 ± 0.06	0.25 ± 0.06	0.25 ± 0.09
17:0	0.17 ± 0.02	0.16 ± 0.03	0.20 ± 0.04	0.24 ± 0.11
16:3n-4	0.39 ± 0.09	0.41 ± 0.01	0.39 ± 0.04	0.38 ± 0.16
16:3n-3	0.19 ± 0.14	0.10 ± 0.01	0.13 ± 0.06	0.10 ± 0.10
16:4n-3	0.07 ± 0.01	0.08 ± 0.04	0.08 ± 0.04	0.11 ± 0.10
18:0	4.68 ± 0.12	5.10 ± 0.76	4.95 ± 1.21	5.29 ± 1.71
18:1n-9	24.70 ± 0.54	24.22 ± 2.14	23.43 ± 1.76	23.37 ± 0.61
18:1n-7	3.86 ± 0.38	3.97 ± 0.12	3.67 ± 0.34	3.94 ± 0.29
18:1n-5	0.15 ± 0.02	0.16 ± 0.02	0.16 ± 0.05	0.17 ± 0.01
18:2n-9	0.25 ± 0.16	0.18 ± 0.03	0.25 ± 0.07	0.18 ± 0.07
18:2n-6	10.03 ± 0.28	9.97 ± 0.21	10.29 ± 0.46	9.25 ± 0.67
18:2n-4	0.24 ± 0.02	0.23 ± 0.02	0.25 ± 0.03	0.28 ± 0.03
18:3n-6	0.28 ± 0.11	0.22 ± 0.06	0.29 ± 0.10	0.19 ± 0.12
18:3n-4	0.20 ± 0.01	0.14 ± 0.01	0.21 ± 0.03	0.17 ± 0.03
18:3n-3	1.52 ± 0.47	1.24 ± 0.15	1.48 ± 0.34	1.35 ± 0.15
18:4n-3	0.58 ± 0.10	0.53 ± 0.12	0.71 ± 0.17	0.64 ± 0.18
18:4n-1	0.07 ± 0.02	0.07 ± 0.02	0.08 ± 0.02	0.08 ± 0.02
20:0	0.16 ± 0.05	0.18 ± 0.04	0.18 ± 0.03	0.23 ± 0.09
20:1n-9	0.15 ± 0.02	0.17 ± 0.03	0.18 ± 0.06	0.17 ± 0.02
20:1n-7	0.96 ± 0.22	0.96 ± 0.11	0.99 ± 0.28	1.08 ± 0.15
20:1n-5	0.13 ± 0.01	0.15 ± 0.02	0.14 ± 0.02	0.17 ± 0.03
20:2n-9	0.10 ± 0.03	0.10 ± 0.01	0.11 ± 0.01	0.11 ± 0.02
20:2n-6	0.29 ± 0.02	0.33 ± 0.03	0.32 ± 0.03	0.33 ± 0.03
20:3n-9	0.02 ± 0.01	0.02 ± 0.01	0.03 ± 0.01	0.03 ± 0.01
20:3n-6	0.20 ± 0.02	0.20 ± 0.01	0.20 ± 0.02	0.19 ± 0.02
20:4n-6 (ARA)	1.36 ± 0.20	1.43 ± 0.04	1.28 ± 0.14	1.47 ± 0.12
20:3n-3	0.15 ± 0.01	0.16 ± 0.02	0.17 ± 0.03	0.16 ± 0.01
20:4n-3	0.58 ± 0.02	0.57 ± 0.11	0.61 ± 0.07	0.62 ± 0.07
20:5n-3 (EPA)	5.47 ± 0.24	5.48 ± 1.04	6.00 ± 0.25	5.55 ± 0.74
22:1n-11	0.14 ± 0.08	0.14 ± 0.03	0.16 ± 0.10	0.17 ± 0.07
22:1n-9	0.07 ± 0.01	0.07 ± 0.01	0.09 ± 0.02	0.15 ± 0.09
22:4n-6	0.09 ± 0.01	0.10 ± 0.02	0.08 ± 0.01	0.10 ± 0.03
22:5n-6	0.36 ± 0.04	0.38 ± 0.08	0.35 ± 0.06	0.37 ± 0.06
22:5n-3	2.30 ± 0.18	2.36 ± 0.11	2.10 ± 0.01	2.23 ± 0.26
22:6n-3 (DHA)	17.30 ± 0.44	17.57 ± 1.32	16.78 ± 1.47	16.88 ± 0.85
Total Saturates	22.88 ± 0.63	23.32 ± 1.29	23.61 ± 0.99	24.50 ± 1.82
MUFA	34.68 ± 0.62	34.40 ± 1.94	33.73 ± 2.42	34.24 ± 0.61
Total n-3	28.17 ± 1.11	28.11 ± 2.79	28.07 ± 2.22	26.65 ± 2.11
Total n-6	12.61 ± 0.40	12.63 ± 0.34	12.81 ± 0.43	11.98 ± 1.04
Total n-9	25.29 ± 0.67	24.77 ± 2.17	24.08 ± 1.80	24.01 ± 0.69
Total n-3 LC-PUFA	25.81 ± 0.54	26.14 ± 2.53	25.67 ± 2.64	24.44 ± 1.80
EPA/ARA	4.06 ± 0.42	3.84 ± 0.78	4.72 ± 0.36	3.81 ± 0.82
DHA/EPA	3.17 ± 0.19	3.26 ± 0.42	2.79 ± 0.31	2.88 ± 0.28
DHA/ARA	12.89 ± 1.84	12.27 ± 1.22	13.07 ± 0.60	10.84 ± 1.29
n-3/n-6	2.23 ± 0.13	2.22 ± 0.17	2.19 ± 0.13	2.24 ± 0.32
18:1n-9/n-3 LC-PUFA	0.96 ± 0.04	0.94 ± 0.18	0.93 ± 0.17	0.96 ± 0.08

*Values are reported as mean ± SD. n=3. MUFA. monounsaturated fatty acid; LC-PUFA Long chain highly unsaturated fatty acid; EPA. eicosapentaenoic acid; DHA. docosahexaenoic acid; ARA. arachidonic acid.

Table 6-7. Fatty acid composition (% total fatty acids identified) of eggs obtained after feeding the experimental diets.

	Diet			
Fatty acid	**ARA-0.8**	**ARA-1.6**	**ARA-2.4**	**ARA-3.2**
14:0	1.77 ± 0.26	1.63 ± 0.03	1.51 ± 0.21	1.76 ± 0.10
15:0	0.28 ± 0.02	0.27 ± 0.02	0.27 ± 0.02	0.25 ± 0.01
16:0	17.08 ± 1.53	17.65 ± 0.69	17.65 ± 0.75	15.70 ± 1.28
16:1n-7	4.25 ± 0.60	4.24 ± 0.23	3.90 ± 0.27	3.96 ± 0.22
16:1n-5	0.12 ± 0.01	0.10 ± 0.02	0.13 ± 0.01	0.10 ± 0.02
16:2n-4	0.17 ± 0.06	0.15 ± 0.03	0.12 ± 0.04	0.22 ± 0.06
17:0	0.16 ± 0.07	0.12 ± 0.02	0.12 ± 0.01	0.17 ± 0.04
16:3n-4	0.35 ± 0.04	0.40 ± 0.03	0.38 ± 0.07	0.30 ± 0.03
16:3n-3	0.17 ± 0.03	0.17 ± 0.17	0.38 ± 0.29	0.21 ± 0.13
16:4n-3	0.04 ± 0.02	0.05 ± 0.00	0.04 ± 0.02	0.05 ± 0.02
18:0	3.65 ± 0.22	3.82 ± 0.25	3.64 ± 0.12	4.24 ± 0.63
18:1n-9	28.76 ± 0.69	29.16 ± 0.56	29.82 ± 1.09	28.92 ± 0.76
18:1n-7	3.38 ± 0.27	3.55 ± 0.47	3.68 ± 0.07	3.52 ± 0.19
18:1n-5	0.19 ± 0.02	0.18 ± 0.01	0.17 ± 0.01	0.17 ± 0.02
18:2n-9	0.16 ± 0.03	0.19 ± 0.03	0.20 ± 0.06	0.11 ± 0.04
18:2n-6	10.13 ± 0.96	10.43 ± 0.50	10.24 ± 0.63	11.23 ± 0.99
18:2n-4	0.22 ± 0.04	0.20 ± 0.03	0.19 ± 0.02	0.19 ± 0.03
18:3n-6	0.12 ± 0.06	0.15 ± 0.01	0.15 ± 0.01	0.17 ± 0.01
18:3n-4	0.18 ± 0.03	0.17 ± 0.03	0.20 ± 0.00	0.20 ± 0.02
18:3n-3	1.73 ± 0.33	1.47 ± 0.32	1.47 ± 0.58	1.36 ± 0.54
18:4n-3	0.53 ± 0.29	0.38 ± 0.06	0.31 ± 0.12	0.60 ± 0.15
18:4n-1	0.06 ± 0.03	0.05 ± 0.01	0.04 ± 0.01	0.07 ± 0.02
20:0	0.15 ± 0.05	0.14 ± 0.04	0.12 ± 0.04	0.11 ± 0.03
20:1n-9	0.21 ± 0.02	0.21 ± 0.01	0.20 ± 0.03	0.19 ± 0.03
20:1n-7	1.14 ± 0.12	1.06 ± 0.04	0.98 ± 0.14	1.28 ± 0.09
20:1n-5	0.13 ± 0.02	0.13 ± 0.01	0.11 ± 0.01	0.12 ± 0.01
20:2n-9	0.09 ± 0.02	0.10 ± 0.01	0.10 ± 0.03	0.06 ± 0.02
20:2n-6	0.29 ± 0.03	0.30 ± 0.01	0.28 ± 0.01	0.26 ± 0.02
20:3n-9	0.02 ± 0.01	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.01
20:3n-6	0.17 ± 0.01	0.18 ± 0.01	0.19 ± 0.02	0.19 ± 0.01
20:4n-6	1.02 ± 0.09[c]	1.23 ± 0.00[bc]	1.31 ± 0.02[b]	1.69 ± 0.18[a]
20:3n-3	0.14 ± 0.03	0.13 ± 0.01	0.13 ± 0.02	0.13 ± 0.01
20:4n-3	0.51 ± 0.12	0.46 ± 0.03	0.46 ± 0.05	0.51 ± 0.09
20:5n-3	5.02 ± 0.47[a]	4.05 ± 0.03[b]	2.82 ± 0.10[c]	2.43 ± 0.04[c]
22:1n-11	0.21 ± 0.06	0.16 ± 0.01	0.14 ± 0.06	0.25 ± 0.04
22:1n-9	0.07 ± 0.01	0.06 ± 0.01	0.06 ± 0.01	0.08 ± 0.01
22:4n-6	0.07 ± 0.01	0.07 ± 0.00	0.07 ± 0.01	0.09 ± 0.01
22:5n-6	0.27 ± 0.01	0.28 ± 0.02	0.28 ± 0.04	0.30 ± 0.03
22:5n-3	2.21 ± 0.30	2.30 ± 0.19	2.51 ± 0.19	2.26 ± 0.31
22:6n-3	14.54 ± 0.49	14.31 ± 0.54	15.40 ± 0.65	15.28 ± 0.36
Total Saturates	23.10 ± 1.33	23.64 ± 0.98	23.29 ± 0.97	22.24 ± 1.26
MUFA	38.55 ± 1.18	38.96 ± 0.66	39.28 ± 0.68	38.68 ± 0.77
Total n-3	24.87 ± 0.51	23.32 ± 0.91	23.52 ± 0.34	23.82 ± 0.75
Total n-6	12.07 ± 0.94	12.63 ± 0.51	12.53 ± 0.58	13.94 ± 1.14
Total n-9	29.31 ± 0.75	29.75 ± 0.54	30.39 ± 1.14	29.39 ± 0.81
Total n-3 LC-PUFA	22.42 ± 0.89	21.25 ± 0.72	21.32 ± 0.83	20.61 ± 0.27
EPA/ARA	4.98 ± 0.85[a]	3.30 ± 0.03[b]	2.15 ± 0.10[c]	1.45 ± 0.13[c]
DHA/EPA	2.92 ± 0.30[d]	3.53 ± 0.13[c]	5.47 ± 0.09[b]	6.28 ± 0.17[a]
DHA/ARA	14.36 ± 1.00[a]	11.65 ± 0.43[b]	11.75 ± 0.57[b]	9.08 ± 0.71[c]
n-3/n-6	2.07 ± 0.19[a]	1.85 ± 0.11[ab]	1.88 ± 0.07[ab]	1.71 ± 0.12[b]
18:1n-9/n-3 LC-PUFA	1.28 ± 0.05	1.37 ± 0.06	1.40 ± 0.00	1.40 ± 0.05

*Values are reported as mean ± SD. n=3. Different superscripts in the same row indicate significant differences ($P<0.05$). MUFA. monounsaturated fatty acid; LC-PUFA Long chain highly unsaturated fatty acid; EPA. eicosapentaenoic acid; DHA. docosahexaenoic acid; ARA. arachidonic acid.

6.4. Discussion

ARA levels in broodstock diets are important for the reproductive success of different fish species (Fernández-Palacios et al., 2011). Also, in greater amberjack the dietary increase up to 3.2 g ARA/kg feed, maintaining the optimum dietary n-3 LC-PUFA level of 1.7 % of the dry weight of diet determined in previous studies (Sarih et al., 2019 under review at Aquaculture), markedly improved fecundity. Thus, the total number of eggs produced per kg female in fish fed 3.2 g ARA/kg feed was even double than the values found in broodstock fed the 0.8 g ARA/kg feed. These results are in agreement with the improved fecundity found in other species such as gilthead seabream (Fernández-Palacios et al., 1995, 2005) or European sea bass (Bruce et al., 1999; Navas et al., 2001) when dietary ARA is increased. Interestingly, the high fecundity values (78000 eggs/kg female/spawn) obtained in this study were even better than those obtained in previous studies (71500-56000 eggs/kg female/spawn, Sarih et al.,2019 under review at Aquaculture; Sarih et al., 2018). Moreover, these values were similar to those obtained in spontaneous spawnings, despite in the present study broodstock were hormonally induced and in previous studies the spawning quality of hormonally induced greater amberjack was lower than that of fish with spontaneous spawns (Sarih et al., 2018). ARA is a preferred substrate for cycloxygenases in fish (Bell et al., 1995; Monroig et al., 2018), which renders PGE_2. This eicosanoid stimulates steroid hormones synthesis and ovulation, and, indirectly, oocyte formation and maturation (Fernández-Palacios et al., 2011), what could at least in part explain the increased production of eggs in greater amberjack broodstock fed higher levels ARA.

Increase in dietary ARA contents up to 3.2 g/kg feed also markedly improved fertilization rates by 75% in comparison to eggs from fish fed the 0.8 g/kg feed. Fertilization was also improved in gilthead seabream, Atlantic halibut or Atlantic cod (Fernández-Palacios et al., 1995, 1997, 2005; Mazorra et al., 2003; Røjbek et al., 2014). It has been shown that ARA contents in broodstock diets markedly affect male gonad and sperm production (Leray and Pelletier, 1985; Ferosekhan et al., submitted). In goldfish (*Carassius auratus*) testis, ARA stimulates testosterone release (Wade et al., 1994, Fernández-Palacios et al., 2011). Besides, PGF_2, another eicosanoid derived from ARA has a pheromone role in other fish species (Mustafa and Srivastava, 1989;

Sorensen and Goetz, 1993; Rosenblum et al., 1995), stimulating male sexual behaviour and synchronizing spawning (Sorensen et al., 1988). Therefore, the improved fertilization rates found in greater amberjack in the present study could be related to an increase in sperm production by stimulation of testosterone release, or to a better synchronization of male and female spawns.

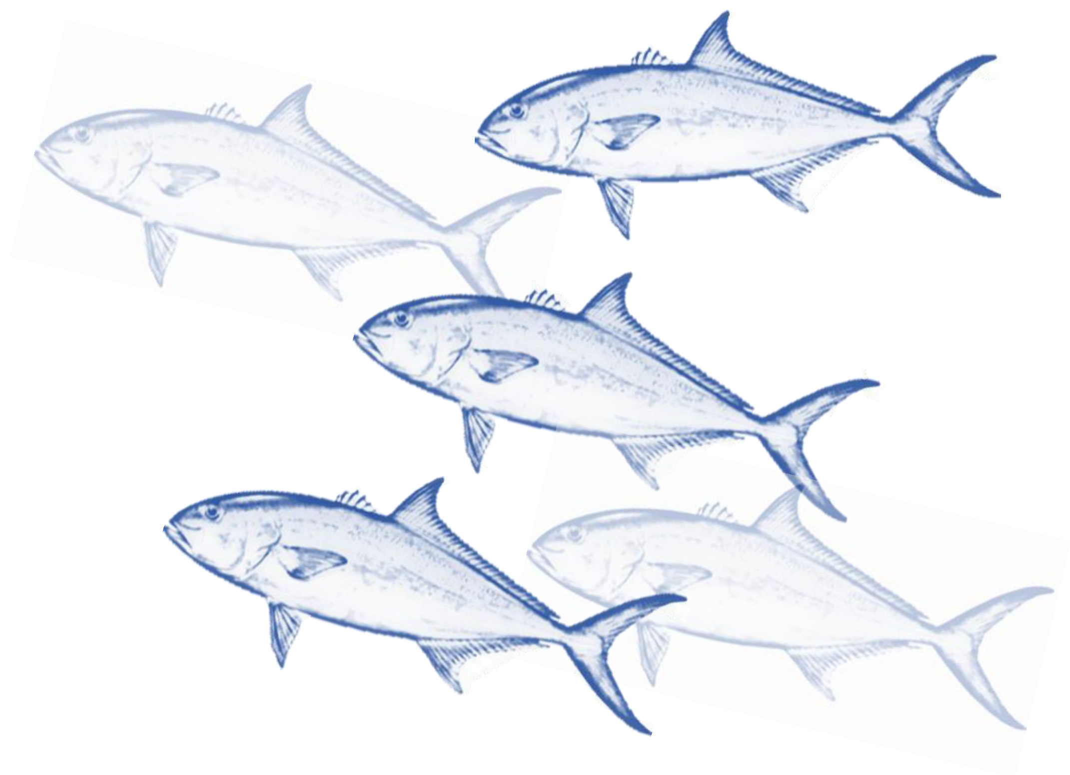
A previous study in greater amberjack showed that changes in LC-PUFA composition of the diet quickly produce changes in the EFA content of their eggs (Sarih et al., 2019 under review at Aquaculture). Similarly, the biochemical composition of greater amberjack eggs in the present study was also significantly affected by broodstock diet after 24 days of feeding. In particular, DHA and ARA contents in egg were less affected than EPA, in agreement with results obtained in other species, suggesting that DHA and ARA are accumulated or retained in the ovaries during gonad maturation (Mazorra et al., 2003; Lund et al., 2008; Callan et al., 2014). In red drum and yellowtail, ARA is readily transferred from the diet to the eggs, changing their fatty acid profile in only 4-16 days (Fuiman and Faulk, 2013; Stuart et al., 2018). Changes in the fatty acid composition of gilthead seabream eggs are also achieved within 15 days, following a dietary shift in n-3 LC-PUFA (Fernández-Palacios et al., 1995). These results suggest a direct pathway for LC-PUFA from diet to eggs, or at least a very short residence time in maternal body reserves (Fuiman and Faulk, 2014). Greater amberjack and gilthead seabream actively feed during gonadal maturation and spawning, and this may explain the rapid transfer from diet to eggs.

Dietary ARA levels proportionally increased ARA contents in greater amberjack eggs, in agreement with the high correlation between ARA contents in diet and ovarian tissue found in Atlantic cod (Norberg et al., 2017). In turn, the increase in ARA contents in the egg was directly related to an improvement in egg viability, hatching rates and newly hatched larvae length in greater amberjack, denoting the importance of this fatty acid for embryo and development. Improved egg viability and hatching rates with increased ARA contents in diets have been also found in other fish species (Bruce et al., 1999; Navas et al., 2001). Indeed, ARA derived eicosanoids modulate tissue and organ development and dietary ARA is required for larval growth of marine fish (Bessonart et al., 1999; Atalah et al., 2011; Izquierdo and Koven, 2011). Besides, an increase in dietary ARA also reduced the content in EPA in agreement with previous studies (Fernández-Palacios et al., 2011), affecting the EPA/ARA ratio in the egg. ARA

is incorporated preferentially into the sn-2 position of phosphatidylinositol (PI), displacing EPA from this minor, but functionally important, membrane glycerophospholipid (Izquierdo and Koven, 2011). In turn, EPA can also serve as a substrate for cycloxigenases and lipooxygenases, eicosanoid-producing enzymes, and exert a modulating influence on the production of ARA-derived eicosanoids (Bell et al., 1997; Tocher, 2003). Thus, EPA/ARA ratios in the eggs, and particularly in PI, will determine the proportion of PGs from II and III series, derived from ARA and EPA, respectively. These PGs have similar functions but different activity and affect cell differentiation and proliferation along embryo and larval development. The optimum EPA/ARA ratio in diets for marine fish broodstock has been scarcely studied (Henrotte et al., 2010; Fernández-Palacios et al., 2011). For instance, in Eurasian perch broodstock, the diet containing the lowest EPA/ARA ratio yielded the highest hatching and larval survival rates (Henrotte et al., 2010). In agreement, greater amberjack fed the highest dietary ARA contents in the present study, showed the highest ARA and the lowest EPA/ARA, as well as the highest percentage of eggs completing embryo development until hatching and the largest larval growth. Nevertheless, EPA also plays important roles in fish metabolism, and extreme reductions in EPA/ARA ratio in larval diets may also reduce growth (Atalah et al., 2011; Izquierdo and Koven, 2011). Therefore, a balance between both fatty acids may be necessary also in broodstock diets.

In conclusion, the results of this study have pointed out the importance of raising ARA levels in broodstock diets from 0.8 to 3.2 g/kg feed to optimize the reproductive performance of greater amberjack. Besides, this confirms our previous studies showing that it is possible to change in the EFA content of the eggs through the modification of the fatty acid profiles of broodstock diet to optimize fecundity, fertilization rates, and egg and larval quality.

Chapter 7 General conclusions



1. It is possible to obtain very high-quality spontaneous spawn in greater amberjack using adequate tank sizes, environmental conditions and, particularly, temperature between 19-26ºC, together with a good broodstock management.

2. Weekly treatments with the GnRHa injections lead to spawns with similar egg viability and hatching rates than spontaneous spawning and better than in previous studies.

3. Treatments with GnRHa injections at the dose of 20 µg kg^{-1} body weight were more effective than implants in promoting the proper endocrine pathways leading to multiple cycles of oocyte maturation, ovulation and spawning and allowed the production of good quality egg in terms of fertilization, hatching and larval survival rates.

4. Raising histidine contents in broodstock diets from 1 to 1.5% optimizes the reproductive performance of greater amberjack, particularly to improve fecundity, fertilization rates, and egg and larval quality.

5. The elevation of taurine levels from 0.3 to 1.1% in broodstock diets increased the fecundity, maintaining good fertilization rates, but further studies must be conducted to determine the optimum taurine dietary levels.

6. Increase in dietary protein contents from 51 to 56% led to an increase of protein content in egg, and to a larger yolk sac volume of larvae, but did not improve any of the spawning quality parameters studied.

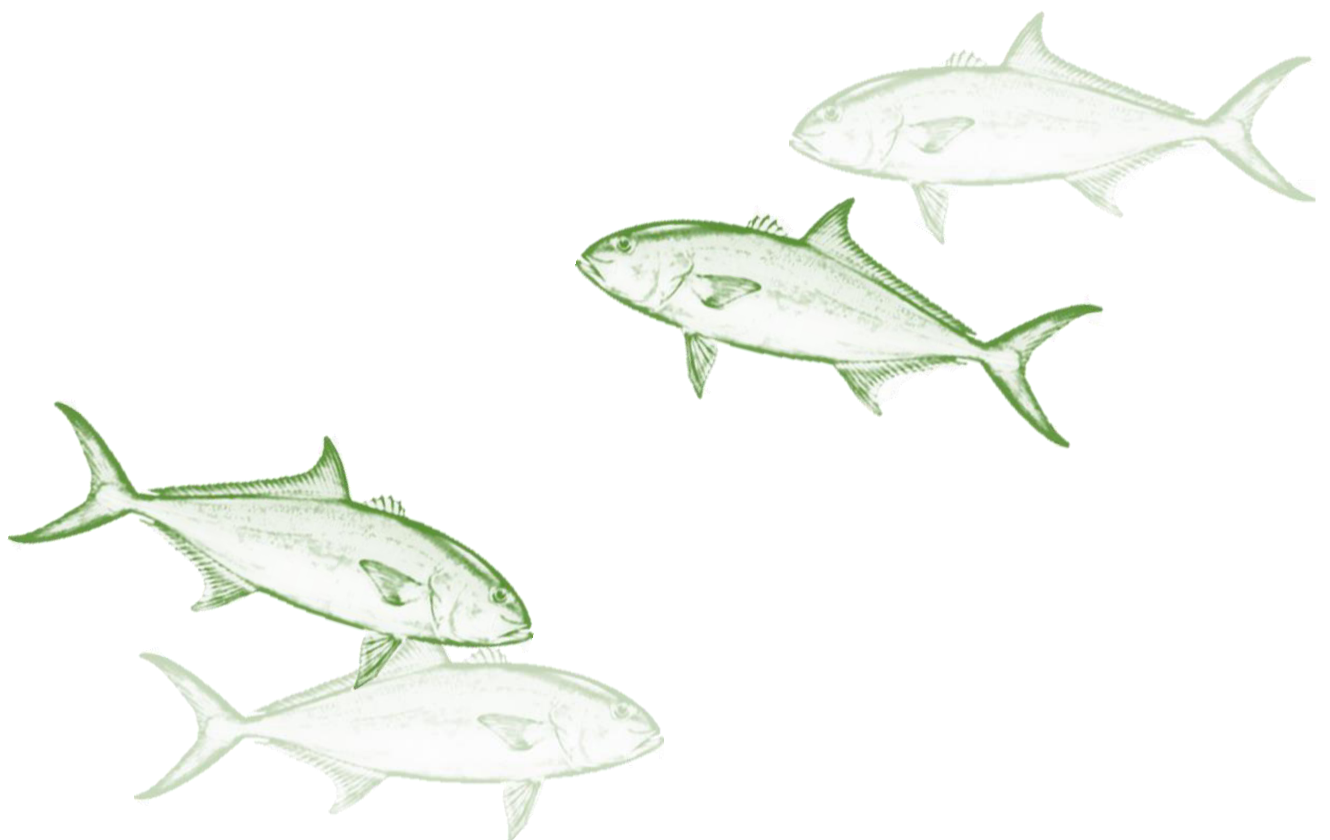
7. The spawning quality of greater amberjack and the biochemical composition of the eggs were affected by the levels of essential fatty acids in the experimental diets even after only three weeks of feeding.

8. Increase in n-3 LC-PUFA levels up to 1.7% in broodstock diet led to an increase of LC-PUFA content in eggs, especially DHA, and improved the spawning quality in terms of fecundity, fertilization rate, egg viability at 24 h, hatching and larval survival rates.

9. An excess of dietary n-3 LC-PUFA, above 2.3%, reduced the spawning quality in terms of fecundity, fertilization rate, hatching and larval survival rates.

10. Increase in ARA dietary level up to 3.2 g / kg of feed increased the DHA/EPA ratio and reduced the EPA/ARA and DHA/ARA ratios in eggs and improved the reproductive performance of the broodfish as well as egg and larval quality.

Chapter 8 Resumen en español



8.1. Objetivos

El pez limón es una de las principales especies candidatas para mejorar la acuicultura europea. Recientemente, el interés por esta especie en la industria de la acuicultura se está expandiendo, debido a su alta demanda y precio de mercado, su rápido crecimiento y su capacidad para aceptar alimentos inertes y adaptarse al cautiverio. Sin embargo, la incorporación del pez limón en la industria de la acuicultura europea se ve obstaculizada por la alta variabilidad en la calidad de las puestas, asociada a las bajas tasas de fertilización y de supervivencia larvaria. Teniendo en cuenta la influencia del manejo y de la nutrición de los reproductores sobre el proceso reproductivo y la calidad de las puestas, nos proponemos de avaluar los protocolos de inducción con GnRHa mediante diferentes modos de administración, además de formular una dieta que se adecue más a los requerimientos en algunos aminoácidos y ácidos grasos de los reproductores del pez de limón. Por lo tanto, los objetivos principales de esta tesis fueron:

1. Evaluar y comparar la calidad de las puestas obtenidas mediante inducción hormonal con inyección o implantes de GnRHa, con las puestas espontáneas (Capítulo 3).

2. Examinar los efectos de la suplementación de la histidina, la taurina y las proteínas en las dietas de reproductores, sobre la calidad de las puestas (Capítulo 4).

3. Determinar el efecto de diferentes niveles de LC-PUFA n-3 en las dietas de reproductores sobre el rendimiento reproductivo y la calidad de las puestas (Capítulo 5).

4. Investigar los efectos del aumento de los niveles de ARA en las dietas de reproductores sobre el rendimiento reproductivo y la calidad de los huevos y las larvas (Capítulo 6).

8.2. Resumen de los experimentos

8.2.1. Capítulo 3. La alta calidad de las puestas naturales del pez limón (*Seriola dumerili*, Risso 1810) y su comparación con las puestas inducidas mediante implantes o inyecciones de GnRHa

El principal obstáculo que impide la producción comercial a gran escala del pez limón es la producción de huevos de alta calidad. Por ello, el objetivo principal de este estudio fue comparar la calidad de las puestas espontáneas con las puestas obtenidas mediante inyecciones o implantes de GnRHa. Reproductores del pez limón adaptados al cautiverio se distribuyeron en 3 tanques circulares de 40 m^3. Los reproductores del tanque 1 no fueron inducidos hormonalmente y pusieron espontáneamente, mientras que los del tanque 2 fueron inyectados intramuscularmente con GnRHa (20 µg.kg-1 de peso corporal) y los del tanque 3 recibieron implantes de GnRHa. El número total de huevos por puesta obtenidos en los reproductores sin el tratamiento hormonal fue mayor del obtenido con inyecciones o implantes. También, la calidad de los huevos fue mejor en las puestas espontaneas, seguida de las puestas procedentes de los reproductores inyectados con GnRHa y luego de las puestas procedentes de los reproductores que recibieron implantes de GnRHa. El tamaño de las larvas procedentes de los reproductores sin el tratamiento hormonal e inyectados fue similar y significativamente mayor ($P < 0,01$) que las larvas procedentes de reproductores que recibieron implantes. En general, este estudio demostró que es posible obtener puestas espontáneas del pez limón de muy alta calidad proporcionando las condiciones de cultivo adecuadas. Además, el uso las inyecciones semanales de GnRHa conduce a unas tasas de viabilidad del huevo y de eclosión similares a las de las puestas espontaneas y una tasa de fertilización más altas que el uso de implantes de GnRHa, aunque el uso de este último dio resultados mejores que en estudios anteriores sobre esta especie.

8.2.2. Capítulo 4. Efecto del aumento de los niveles dietéticos de proteína, histidina y taurina en la calidad de las puestas del pez limón (*Seriola dumerili*, Risso 1810)

Una dieta bien equilibrada y adaptada a los requerimientos nutricionales específicos del pez limón contribuiría a optimizar el proceso reproductivo y la calidad de las puestas. El objetivo principal del presente estudio fue examinar los efectos de la suplementación de la histidina, la taurina y las proteínas en las dietas de reproductores, sobre la calidad de las puestas del pez limón. Doce reproductores se distribuyeron en tres tanques circulares de 40 m^3 y se alimentaron con tres diferentes dietas, una, alta en histidina, otra alta en taurina y la tercera alta en el contenido de proteínas. En general, la elevación los niveles de histidina de 1 a 1,5%, mejoró en la calidad de las puestas, aumentando la fecundidad relativa, la viabilidad del huevo, las tasas de fertilización, de eclosión y de supervivencia larvaria, el contenido de proteínas en el huevo y el tamaño del huevo y las larvas, lo que demuestra la importancia de este aminoácido en el desarrollo de los huevos y de las larvas de esta especie. El aumento del nivel de la taurina de 0,3 a 1,1% en las dietas de reproductores, aumentó la fecundidad relativa en comparación con los peces alimentados con niveles más altos de proteínas. La tasa de fertilización tiende a aumentar con la elevación de la taurina en la dieta y no fue significativamente diferente de la tasa de fertilización de los reproductores alimentados con niveles más altos de histidina. Además, la elevación de la taurina en la dieta aumentó el diámetro del huevo y el contenido de taurina en los huevos. El aumento en el contenido de proteínas en la dieta del 51 al 56% condujo a un mayor contenido de proteínas en el huevo, así como a un mayor volumen del saco vitelino, pero no mejoró ninguno de los parámetros de calidad de puestas. En conclusión, los resultados de este estudio han señalado la importancia de elevar los contenidos de histidina en las dietas de reproductores de 1 a 1,5% para optimizar el rendimiento reproductivo del pez limón. Además, este estudio mostró que el aumento de los niveles de taurina en las dietas de reproductores aumentó la fecundidad, manteniendo unas buenas tasas de fertilización, pero se deben realizar más estudios para determinar los niveles dietéticos óptimos de taurina.

8.2.3. Capítulo 5. Los niveles adecuados de n-3 LC-PUFA en las dietas de reproductores, inyectados con GnRH, optimizan el rendimiento reproductivo del pez limón (*Seriola dumerili*, Risso 1810) que es similar a las puestas espontaneas

La composición de lípidos y ácidos grasos en la dieta de los reproductores juega un papel importante en los procesos reproductivos, el desarrollo embrionario y la

supervivencia de las larvas de los peces marinos. Los ácidos grasos poliinsaturados de cadena larga (LC-PUFA) son especialmente importantes para la calidad de los huevos y de las larvas. El principal objetivo del presente estudio fue evaluar los efectos del aumento de los niveles de n-3 LC-PUFA en la dieta de los reproductores sobre el rendimiento reproductivo y la calidad de las puestas del pez limón. Dieciséis reproductores maduros se distribuyeron en cuatro tanques circulares de 40 m^3 y se alimentaron con cuatro dietas que contenían diferentes niveles de n-3 LC-PUFA: dieta D1 con 1% n-3 LC-PUFA en peso seco, dieta D2 con 1,7%, dieta D3 con 2,3% y dieta D4 con 3%. La fecundidad fue significativamente mayor ($P < 0,05$) en los reproductores alimentados con D2. Además, se obtuvo una mayor tasa de fertilización ($P < 0,05$) en las puestas procedentes de los reproductores alimentados con D2 (91,8 ± 3,1%) y D1 (86,3 ± 1,7%) en comparación con aquellas procedentes de los reproductores alimentados con D3 (69,0 ± 7,4%) y D4 (52,4 ± 10,6%). Se encontró la misma tendencia en la viabilidad del huevo a las 24 h, las tasas de eclosión y de supervivencia larvaria, donde los valores más altos ($P < 0,05$) se encontraron cuando los reproductores fueron alimentados con D1 y D2. Además, la fecundidad, las tasas de eclosión y de supervivencia larvaria a los 1 y 3 días después de eclosión mostraron una correlación negativa con la elevación de los niveles de n-3 LC-PUFA en la dieta. La composición de ácidos grasos de los huevos mostró unas marcadas diferencias entre los diferentes tratamientos, lo que refleja la influencia de los perfiles de ácidos grasos en las dietas de reproductores sobre su perfil en los huevos. En base a estos resultados, se recomienda un nivel entre 1-1,7% n-3 LC-PUFA en peso seco en la dieta de los reproductores del pez limón.

8.2.4. Capítulo 6. Ácido araquidónico en la dieta de reproductores del pez limón (*Seriola dumerili*) y su efecto sobre la calidad del huevo y de las larvas

Este estudio se realizó para determinar el efecto del aumento de los niveles del ácido araquidónico en las dietas de reproductores sobre el rendimiento reproductivo, la composición de ácidos grasos de los huevos y la calidad de las puestas del pez limón. Las dietas formuladas que contenían 0,8; 1,6; 2,4 y 3,2 g de ARA / kg de alimento fueron alimentadas a reproductores del pez limón durante más de 3 meses. Los peces alimentados con la dieta que contenía 3,2 g de ARA / kg mostraron un aumento significativo en la fecundidad, la tasa de fertilización, la viabilidad del huevo, la tasa de eclosión, la supervivencia larvaria y el tamaño de las larvas, en comparación

con que los peces alimentados con las otras tres dietas. Sin embargo, el diámetro del huevo y de la gota lipídica de los reproductores alimentados con 3,2 g de ARA / kg de alimento no fueron significativamente diferentes de los reproductores alimentados con las otras dietas. El contenido de ARA en los huevos reflejó los niveles de ARA en la dieta, mientras que el contenido de EPA se redujo a medida que aumentaba el contenido de ARA en la dieta. En consecuencia, la elevación del contenido de ARA en la dieta aumentó la proporción DHA / EPA y redujo las proporciones EPA / ARA y DHA / ARA en los huevos. Estos resultados mostraron que el aumento en el contenido de ARA en la dieta hasta 3,2 g / kg de alimento mejoró el rendimiento reproductivo de los reproductores y la calidad de los huevos y de las larvas.

8.3. Conclusiones

1. En el pez limón es posible obtener puestas espontáneas de muy alta calidad bajo condiciones de cultivo adecuadas, como el uso de tanques de 40m^3 y el manejo adecuado de los reproductores, además de las condiciones ambientales como la temperatura entre 19-26ºC.

2. Los tratamientos semanales con inyecciones de GnRHa producen puestas con tasas de viabilidad del huevo y de eclosión similares a las de las puestas espontáneas y mejores que las obtenidas en previos estudios.

3. Los tratamientos con inyecciones de GnRHa a una dosis de 20 $\mu g.kg^{-1}$ de peso corporal fueron más efectivos que los implantes para promover la vía endocrina del BPG que condujo a múltiples ciclos de maduración de ovocitos, ovulación y desove y permitió la producción de huevos de alta calidad en términos de tasas de fertilización, de eclosión y de supervivencia larvaria.

4. El aumento del contenido de histidina de 1 a 1,5% en las dietas de los reproductores optimiza el rendimiento reproductivo del pez limón, mejorando las tasas de fecundidad y de fertilización, y la calidad de los huevos y de las larvas.

5. La elevación de los niveles de taurina de 0,3 a 1,1% en las dietas de los reproductores aumentó la fecundidad y mantuvo buenas tasas de fertilización, pero se deben realizar más estudios para determinar los niveles dietéticos óptimos de taurina.

6. El aumento del contenido de proteínas del 51 al 56% en las dietas de los reproductores condujo a un aumento en el contenido de proteínas en los huevos, así como a un mayor volumen del saco vitelino de las larvas, pero no mejoró ninguno de los parámetros de calidad de las puestas.

7. Tanto la calidad de las puestas, como la composición bioquímica de los huevos fueron afectadas por los niveles de ácidos grasos esenciales en las dietas experimentales después de tan solo tres semanas de alimentación con dichas dietas.

8. El incremento de n-3 LC-PUFA dietéticos hasta el 1.7% TFA aumentó el contenido de n-3 LC-PUFA en los huevos, especialmente los niveles de DHA, mejorando la calidad de puesta en términos de fecundidad, tasa de fertilización, viabilidad del huevo a las 24 h y tasas de eclosión y de supervivencia larvaria a 1 y 3 días después de éclosion.

9. Un exceso de n-3 LC-PUFA, por encima del 2,3%, en la dieta de los reproductores redujo la calidad de las puestas en términos de fecundidad, tasa de fertilización y tasas de eclosión y de supervivencia larvaria a 1 y 3 días después de éclosion.

10. El incremento dietético de ARA hasta 3,2 g / kg de alimento, aumentó la proporción DHA/EPA y redujo las proporciones EPA/ARA y DHA/ARA en los huevos y mejoró el rendimiento reproductivo de los reproductores y la calidad de los huevos y de las larvas.

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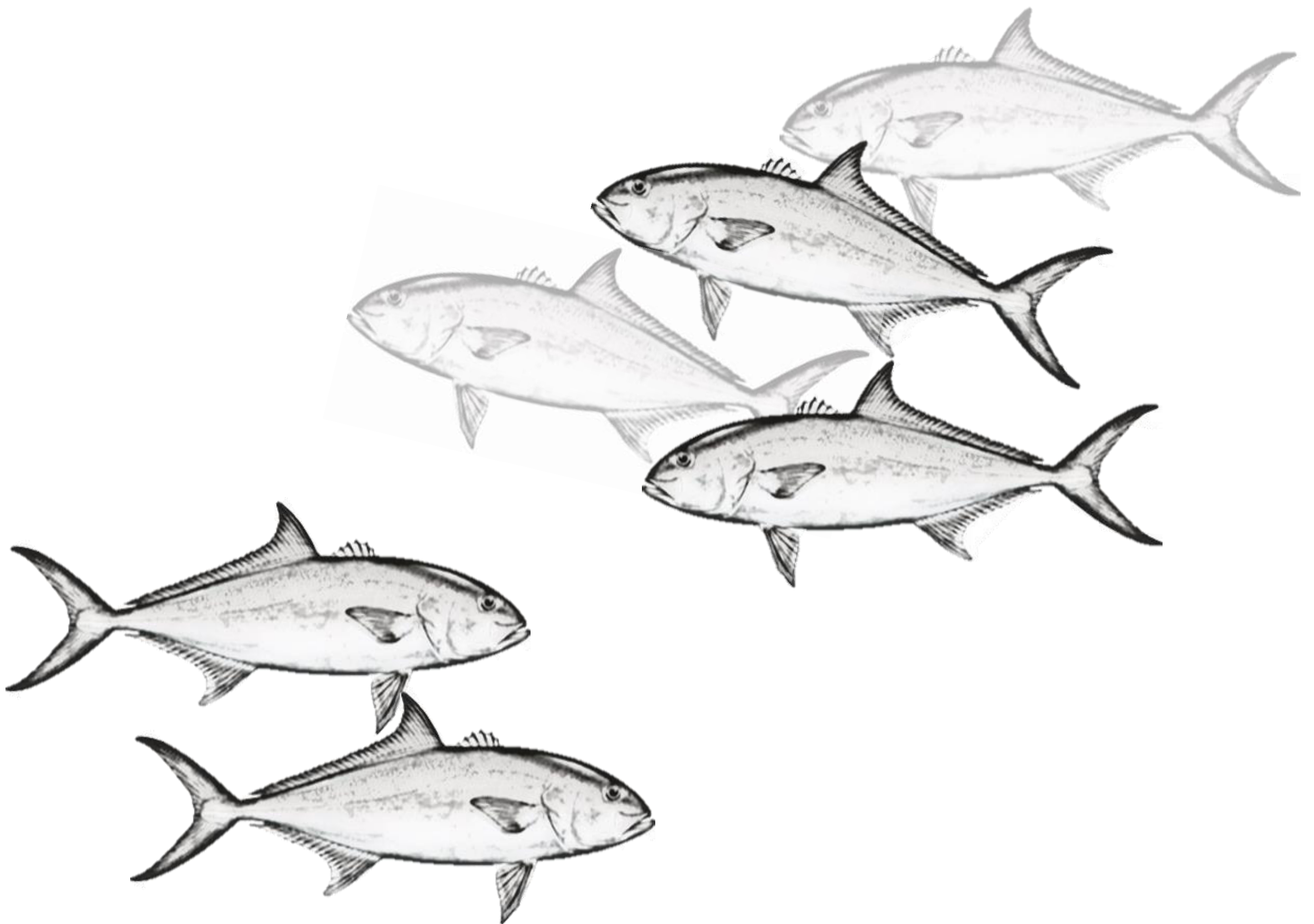

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