New species for EU aquaculture

Deliverable Report

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Objective: The objective of the present deliverable was to recommend levels for protein, carotenoids, taurine and essential fatty acids for an improved diet to overcome the unreliable reproduction of greater amberjack. The deliverable is a report containing the results related to the feeding of broodstock with experimental diets on gonad maturation, frequency of spawns, fecundity, fertilization rates, hatching rates, larval survival and lipid composition of eggs and sperm. In addition, the deliverable includes the diet effect on (a) incidence of pathological episodes during and after the spawning season and (b) hematological and biochemical indicators of health and welfare.

Description: Wild-captive and F1 greater amberjack broodstock were fed with different diets, to determine the optimum essential fatty acid content in diets. Wild-captive fish fed with different protein, histidine and taurine diets were treated with injected hormonal therapy and spawns were obtained. 4 years old F1 greater amberjack broodstock were fed with an Experimental diet and compared with fish fed with a control commercial diet and mackerel diet. The young fish were implanted with an increased dose of GnRHa in consecutive treatments. The effects of the Experimental diet and spawning induction treatment were evaluated in terms of reproductive performance of F1 greater amberjack.

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1. Introduction

The greater amberjack (*Seriola dumerili*) has been considered as a good candidate for the species diversification of aquaculture production in the Mediterranean region (Mylonas *et al*., 2016), but the major constraint on its commercial production was the insufficient production of eggs and the poor larval quality (Lazzari *et al*., 2000; Mylonas *et al*., 2004; Papandroulakis *et al*., 2005).

Since egg quality largely depends on nutrient transfer from the female, a well balance and adapted diet to the specific nutritional requirements of greater amberjack broodstock, would contribute to optimize reproduction and spawning quality, as has been shown in other species (Brooks *et al*., 1997; Izquierdo *et al*., 2001).

There are a few studies that provide information on the nutritional requirements of the greater amberjack broodstock (Rodríguez-Barreto *et al*., 2012, 2014; Zupa *et al*., 2017b), but in general, the information is still scarce.

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), 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*., 2015; 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 (*Oncorhynchus nerka*) (Mommsen *et al*., 1980; Mommsen, 2004) and it is the main amino acid in gonads during spawning of certain species such as goldlined seabream (*Rhabdosargus sarba*) (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 defense, 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, 2012b).

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.

Few studies exist on greater amberjack female gonad biochemical composition (Rodríguez-Barreto *et al*., 2012, 2014; Zupa *et al*., 2017b), and information available on male specimens is even more scarce (Zupa *et al*., 2017b), even though a close relationship between gonad composition–and more generally fish nutritional state- and reproductive success has been demonstrated widely (Fernández-Palacios *et al*., 1995; Almansa *et al*., 1999; Bruce *et al*., 1999; Izquierdo *et al*., 2001; Sorbera *et al*., 2001; Pérez *et al*., 2007; Martín *et al.,* 2009). More specifically, dietary fatty acids have proven to be very important in the reproduction of several fish species, including greater amberjack, since they determine gonad composition, affecting not only sperm and egg quality (Izquierdo *et al*., 2001; Rodríguez-Barreto *et al*., 2012, 2014, 2017; Tocher, 2010), but also being involved in the synthesis of eicosanoids that are autocrine mediators in the reproductive process (Sorbera *et al*., 2001; Patiño *et al.,* 2003; Tocher, 2003).

Comparisons of proximate and fatty acid compositions from tissues of wild fish and their cultivated counterparts can provide a good estimation of the suitability of the diet offered to the broodstock (Cejas *et al*., 2004 Grigorakis *et al*., 2002; Pérez *et al*., 2007; Rodríguez-Barreto *et al*., 2012, 2014, 2015). The efficacy of dietary ingredients for broodstock has been ascribed to its superior protein quality, as well as its higher phospholipid and cholesterol content. In fact, two thirds of the lipid fraction in vitellogenin is made of phospahatidylcholine (PC) (Sargent, 1995) that is also the main phospholipid in mature ovaries and fertilised eggs (Watanabe & Vasallo-Agius, 2003). Similarly, sperm is particularly rich in phosphatidylserine (PS) and phosphatidylethanolamine (PE). Regarding nutrition, dietary fatty acids have also proven to be particularly important in the reproduction of several species, since they determine gonad composition and function, affecting not only sperm and egg quality (Izquierdo *et al*., 2001; Tocher, 2010), but also being involved in the synthesis of eicosanoids, autocrine mediators in the reproductive process (Henrotte *et al*., 2011; Mercure & Van Der Kraak, 1996; Patiño *et al*., 2003; Sorbera *et al*., 2001; Stacey & Sorensen, 2005). Considering the importance of lipids in breeder diet, several studies have been performed in greater amberjack by the research group involved in DIVERSIFY (P15. ULL), leading to the formulation of a broodstock diet that approximates the ovary lipid composition of cultured fish to that from wild specimens, with some improvement of reproduction success under captive conditions (Rodríguez-Barreto *et al*., 2012, 2014, 2017).

Carotenoids including astaxanthin, are widely present in fish gonads and eggs. They are precursors of vitamin A being involved in reproduction and embryonic development, as well as in the prevention of oxidative stress processes (Guerin *et al*., 2003; Miki, 1991). Carotenoids are actively mobilized into the gonads during sexual reproductive activity in aquatic animals. And their transport mechanisms are totally linked to the formation lipoproteins where lipids play again an essential role. There is evidence to suggest that the presence of carotenoids mitigates deleterious oxidative damage to the developing embryo and may be also present in the gonads to ensure larval visual function and adequate chromatophore responses. There is also evidence that carotenoids protection against oxidative damage is directly related to sperm functional fertility.

Deliverable 3.3 "Identification of possible reproductive dysfunction of gametogenesis of greater amberjack reared in captivity based on the comparative evaluation of fish sampled in the wild, in terms of proliferating and apoptotic germ cells, vitellogenin accumulation, yolk content in the oocytes and nutritional status", concluded that captive-reared greater amberjack suffer significant reproductive dysfunctions with impaired oogenesis and spermatogenesis. Females were affected by extensive atresia of vitellogenic oocytes and failed to undergo oocyte maturation and males displayed a precocious decrease of germ cell proliferation, an abnormal increase of apoptosis during the recrudescence period (early gametogenesis; EG) and the complete cessation of spermatogenic activity much earlier compared with the wild population. Additionally the proportions of total polar lipids, and specific lipid classes and EFA proportions particularly differed among EG wild and captive-reared fish gonads, with the latter displaying clearly lower contents of specific phospholipids, EFA and DHA/EPA and ARA/EPA ratios, all crucial factors for reproductive success and sperm and egg and larval quality. The later nutritional differences were presumably the result exclusively of differences in the diet between wild and captive fish (Zupa *et al.*, 2017b).

The objectives of the present deliverable were: 1.- To determine the optimum EFA content on broodstock diets. 2.- to examine the reproductive effects of an experimental diet with a potentially improved formula of lipids, on reproductive development of hatchery produced greater amberjack, in order to compare the egg and sperm quality and certain indicators of fish health and 3.- to evaluate the effects of supplemental protein, histidine, and taurine in broodstock diets, on egg quality of greater amberjack.

2. Determination of the optimum EFA content on broodstock diets

The optimum ARA, DHA and EPA levels as essential fatty acids for reproductive success of greater amberjack (*Seriola dumerili*) were studied. Groups of greater amberjack broodstock were fed diets containing different essential fatty acids levels (SARC) in order to determine the effect on reproduction reliability. The effects on gonad maturation, frequency of spawns, fecundity, fertilization rates, hatching rates and larval survival rates were determined. Proximate composition of diets and eggs were analysed.

Sixteen greater amberjacks, captured in May 2011 in the Municipality of Mogan in the Southwestern coast of Gran Canaria (Canary Islands, Spain) (**Fig. 9.4.1**), were used in the present experiment. Groups of fish with a mean ± SD weight of 3.41 ± 1.12 kg for females and 2.37 ± 1.07 kg for males, were acclimated in tanks of 10 m^3 (3 m x 3 m x 1.5 m depth) (**Fig. 9.4.2**), at the facilities of the Grupo de Investigación en Acuicultura (GIA), located in the Parque Cientifico Tecnologico Marino (PCTM), of the Universidad de Las Palmas de Gran Canaria (ULPGC). Fish were 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. 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) (**Fig. 9.4.3**). There were no mortalities during this acclimation period. Before starting the experiment (3 June 2016), in late March 2016, all fish were anesthetized with clove oil (Guinama SL, Valencia, Spain; 50 ppm), weighted and sized. Weight evolution of broodstock lot from 2011 to 2016 is shown in **Fig. 9.4.4**.

Figure 9.4.1. Municipality of Mogan (Gran Canaria, Canary Islands, Spain).

Figure 9.4.2. Experimental tanks of 10 m3 (3 m x 3 m x 1.5 m depth) at the facilities of the Grupo de Investigación en Acuicultura (GIA), located in the Parque Cientifico Tecnologico Marino (PCTM), of the Universidad de Las Palmas de Gran Canaria (ULPGC).

Figure 9.4.3. Experimental tanks of 40 m^3 (5 m x 2.35 m depth) at the facilities of the Grupo de Investigación en Acuicultura (GIA), located in the Parque Cientifico Tecnologico Marino (PCTM), of the Universidad de Las Palmas de Gran Canaria (ULPGC).

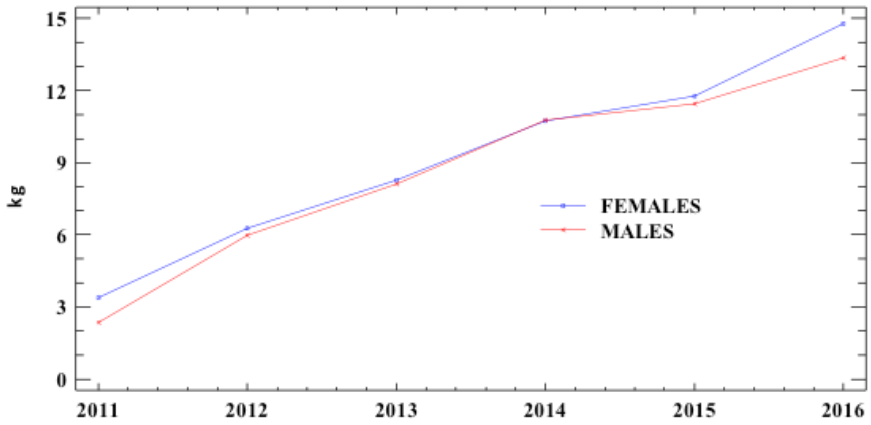


Figure 9.4.4. Weight evolution of broodstock lot from 2011 to 2016.

The selected fish were distributed (sex ratio 1:1) in four circular tanks of 40 m^3, 2♀ and 2♂ in each one. The tanks were filled with natural seawater of 37 ‰ salinity and natural photoperiod following the day extension in concordance with the geographical position (27º 59' 28" N; 15º 22'05" W). The flow rate set at 6 cycles per day and the temperature was determined continuously through a system of sensors monitored by computer (Miranda, Innovaqua, Sevilla, Spain), and was ranged from 21.58 ± 0.36ºC and 23.30 ± 0.17°C (June–October).

Given that all females used in the present experiment spawned the previous years and to avoid an excessive handling, they were not cannulated, neither the abdominal massage was performed on males. 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*., 2015) (**Fig. 9.4.5**). These hormonal treatments were weekly applied to avoid excessive stress for the broodstock, from June 3th to October 11th, 2016.

At the beginning of the spawning season, from June 3th to July 12th, broodfish were fed twice a week with commercial diet (13 mm, Vitalis CAL, Skretting, Burgos, Spain) at 1% of their estimated total biomass, and once a week with Atlantic mackerel (*Scomber scombrus*) at 2% (diet GIA), to ensure that there were no significant differences in the spawning quality of the different experimental groups. During this period, a total of 12 induction per tank were performed (6 per couple), in tank 1 (diet 1) 9 spawns were obtained, in tanks 2 and 3 (diet 2 and 3, respectively) 7 spawns, and in tank 4 (diet 4) 8 spawns.

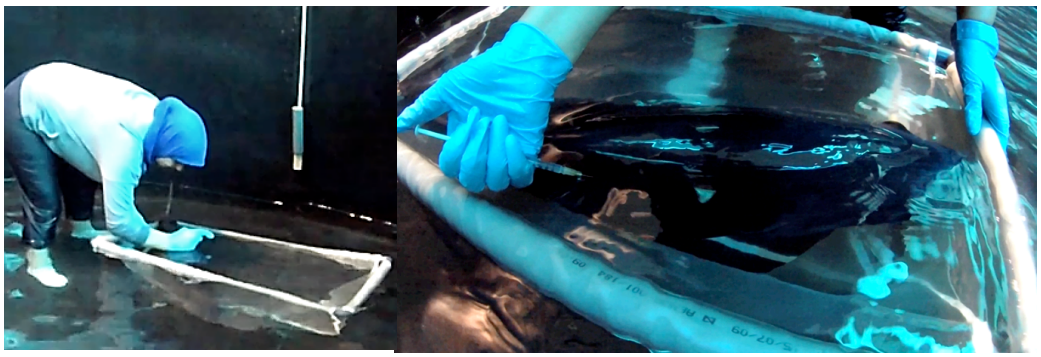


Figure 9.4.5. Hormonal induction of spawning.

Spawning quality was determined as: the percentage of fertilization, viable eggs at 24 hours (%), hatching and larval survival at 1, 3 and 5 days post-hatching (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 5 dph were calculated (Fernández-Palacios *et al*., 2011). Also, for each spawn the total number of eggs, the number of eggs per spawn and the number of eggs per spawn and weight of female (per 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 analyzed following standard procedures (AOAC, 2012). 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). All analyses were conducted in triplicate.

Total number of eggs, total number of eggs per spawn and the total number of eggs per spawn and kg female are shown in **Figs 9.4.6**, **9.4.7** and **9.4.8.** In **Table 9.4.1** and **Table 9.4.2**, the results corresponding to the egg quality and the composition of fatty acids in eggs are indicated, during the feeding period with the GIA diet.

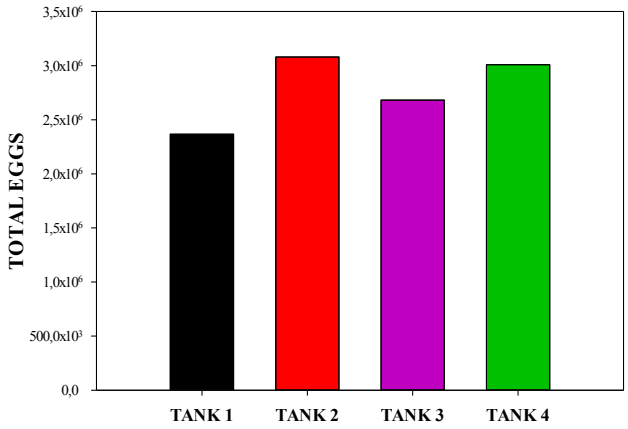


Figure 9.4.6. Number of eggs obtained during the feeding period with the GIA diet.

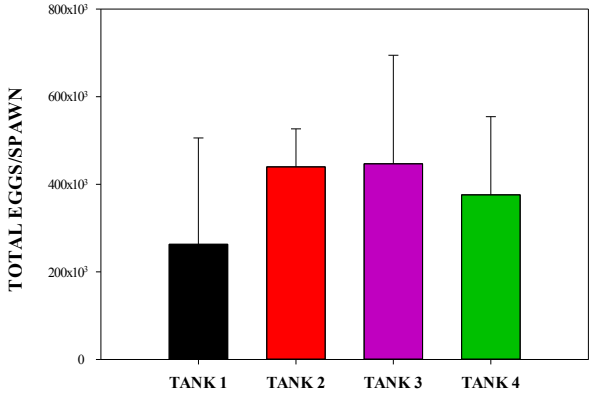


Fig. 9.4.7. Number of eggs per spawn obtained during the feeding period with the GIA diet.

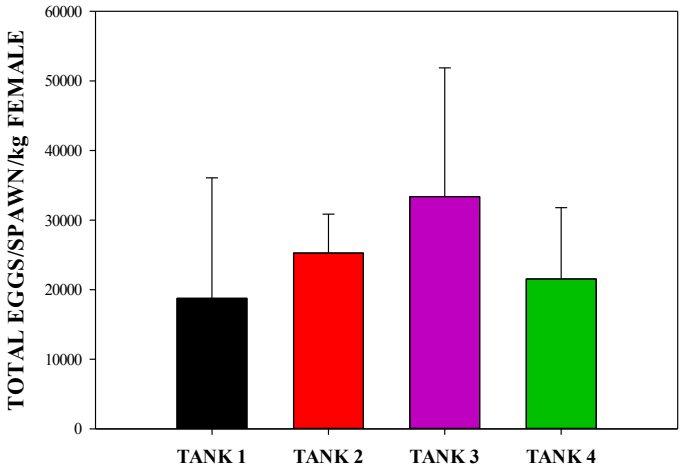


Figure 9.4.8. Number of eggs per spawn and kg female obtained during the feeding period with the GIA diet.

Table 9.4.1. Quality of egg and larvae obtained during the feeding period with the GIA diet.

Tank	% Fertilization	% Viable 24h	% Hatching
1	55.66±26.22	83.43±7.50	81.04±8.03
2	61.65±15.94	88.71±0.76	86.90±0.96
3	41.93±29.61	89.58±4.55	86.67±6.01
4	64.92±14.86	86.67±7.21	82.29±8.05
Tank	**% 1 dph survival**	**% 3 dph survival**	**% 5 dph survival**
1	63.68±14.93	39.81±14.13	2.84±1.63
2	74.34±9.70	28.32±7.35	4.27±1.66
3	70.45±9.33	29.78±8.25	3.17±2.27
4	72.04±11.08	37.39±12.95	3.21±2.27

Table 9.4.2. Fatty acid composition (% total fatty acids) of eggs obtained during the feeding period with the GIA diet.

Fatty acid	Tank 1	Tank 2	Tank 3	Tank 4
14:0	1.48 ± 0.21	1.59 ± 0.08	1.81 ± 0.34	1.74 ± 0.22
16:0	15.09 ± 0.65	15.87 ± 0.53	16.44 ± 0.56	16.33 ± 1.55
16:1n-7	4.31 ± 0.21	4.33 ± 0.22	4.76 ± 0.54	4.43 ± 0.45
16:1n-5	0.14 ± 0.01	0.13 ± 0.00	0.13 ± 0.01	0.14 ± 0.01
16:2n-4	0.22 ± 0.05	0.23 ± 0.03	0.24 ± 0.06	0.22 ± 0.06
17:0	0.17 ± 0.03	0.16 ± 0.03	0.18 ± 0.04	0.25 ± 0.15
16:3n-3	0.10 ± 0.00	0.10 ± 0.00	0.10 ± 0.01	0.13 ± 0.06
16:3n-1	0.13 ± 0.01	0.13 ± 0.03	0.10 ± 0.01	0.14 ± 0.05
16:4n-3	0.09 ± 0.03	0.09 ± 0.02	0.09 ± 0.03	0.06 ± 0.04
18:0	5.26 ± 0.57	5.31 ± 0.67	4.51 ± 0.26	5.98 ± 1.38
18:1n-9	23.00 ± 0.53	23.31 ± 1.57	24.27 ± 1.03	23.99 ± 0.68
18:1n-7	4.04 ± 0.06	3.94 ± 0.16	4.05 ± 0.08	4.10 ± 0.04
18:1n-5	0.15 ± 0.01	0.14 ± 0.01	0.15 ± 0.01	0.15 ± 0.01
18:2n-9	0.17 ± 0.01	0.33 ± 0.10	0.30 ± 0.10	0.20 ± 0.06
18:2n-6	10.15 ± 0.19	10.21 ± 0.45	9.77 ± 0.51	9.51 ± 0.32
18:3n-6	0.24 ± 0.01	0.36 ± 0.04	0.31 ± 0.07	0.25 ± 0.02
18:3n-3	1.31 ± 0.07	1.29 ± 0.11	1.21 ± 0.08	1.29 ± 0.13
18:4n-3	0.55 ± 0.09	0.59 ± 0.07	0.57 ± 0.07	0.53 ± 0.10
20:0	0.18 ± 0.04	0.16 ± 0.03	0.15 ± 0.03	0.16 ± 0.05
20:1n-9	0.15 ± 0.02	0.14 ± 0.01	0.15 ± 0.02	0.15 ± 0.02
20:1n-7	0.89 ± 0.13	0.81 ± 0.03	0.86 ± 0.05	0.93 ± 0.11
20:1n-5	0.15 ± 0.02	0.13 ± 0.00	0.14 ± 0.01	0.16 ± 0.02
20:2n-9	0.09 ± 0.01	0.11 ± 0.01	0.12 ± 0.01	0.11 ± 0.02
20:2n-6	0.35 ± 0.02	0.30 ± 0.00	0.29 ± 0.02	0.33 ± 0.03
20:4n-6 (ARA)	1.45 ± 0.07	1.34 ± 0.04	1.21 ± 0.10	1.56 ± 0.07
20:3n-3	0.18 ± 0.01	0.16 ± 0.01	0.16 ± 0.01	0.16 ± 0.02
20:4n-3	0.63 ± 0.02	0.57 ± 0.02	0.59 ± 0.02	0.59 ± 0.08
20:5n-3 (EPA)	5.99 ± 0.37	5.99 ± 0.28	5.65 ± 0.50	5.16 ± 0.45
22:1n-11	0.13 ± 0.03	0.10 ± 0.02	0.11 ± 0.03	0.12 ± 0.03
22:1n-9	0.07 ± 0.01	0.08 ± 0.02	0.07 ± 0.01	0.09 ± 0.07
22:4n-6	0.11 ± 0.01	0.08 ± 0.00	0.08 ± 0.01	0.11 ± 0.02
22:5n-6	0.43 ± 0.01	0.39 ± 0.01	0.37 ± 0.03	0.41 ± 0.02
22:5n-3	2.42 ± 0.06	2.11 ± 0.01	2.32 ± 0.19	2.13 ± 0.22
22:6n-3 (DHA)	18.63 ± 0.53	17.84 ± 0.80	17.09 ± 0.44	16.62 ± 1.66
Total saturates	27.56 ± 0.46	27.58 ± 0.68	27.79 ± 0.44	26.87 ± 1.03
Total monoenoic	22.43 ± 0.27	23.36 ± 1.16	23.37 ± 0.68	24.83 ± 2.97
Total n-3	33.10 ± 0.58	33.21 ± 1.53	34.81 ± 0.47	34.47 ± 0.50
Total n-6	29.91 ± 0.53	28.73 ± 1.25	27.78 ± 0.53	26.66 ± 2.11
Total n-9	12.93 ± 0.22	12.88 ± 0.41	12.24 ± 0.72	12.44 ± 0.28
Total n-3 HUFA	23.52 ± 0.50	24.00 ± 1.67	24.93 ± 1.08	24.67 ± 0.65
EPA/ARA	3.12 ± 0.24	2.98 ± 0.04	3.04 ± 0.33	3.23 ± 0.35
DHA/EPA	12.82 ± 0.35	13.36 ± 0.34	14.20 ± 1.20	10.67 ± 1.10
DHA/ARA	4.12 ± 0.32	4.48 ± 0.08	4.70 ± 0.69	3.32 ± 0.43
EPA+DHA	24.62 ± 0.49	23.83 ± 1.07	22.74 ± 0.35	21.77 ± 1.85
n-3/n-6	2.31 ± 0.05	2.23 ± 0.07	2.27 ± 0.18	2.14 ± 0.18
18:1n-9/n-3 HUFA	0.83 ± 0.03	0.88 ± 0.09	0.94 ± 0.06	0.97 ± 0.06

After checking that there were no significant differences (P <0.05), with GIA diet, in any of production parameters and egg quality, we started to feed broodstock with the four experimental diets. The formulation and proximate composition of the experimental diets are presented in **Table 9.4.3**. The fatty acid composition of the four diets is shown in **Table 9.4.4**. The analysed content of EPA+DHA in the diets were slightly lower than formulated values and amounted to 23.5, 17.7, 14.2 and 6.7 for diet 1 to 4, respectively. Fish were hand feed twice a day and 5 days a week (2% of biomass day^{-1}).

Table 9.4.3. Proportion of ingredients and proximate of the experimental diets (Skretting Aquaculture Research Center, Stavanger, Norway).

Diet	**1**	**2**	**3**	**4**
Linseed oil	0.00	1.52	3.01	4.50
Wheat	19.09	19.13	19.13	19.13
Wheat gluten	13.62	14.99	14.99	14.99
Fish meal	44.97	43.46	43.46	43.46
Squid meal	10.00	10.00	10.00	10.00
Fish oil	10.93	7.48	4.04	0.61
Palm oil	0.00	2.03	3.98	5.93
Premix vit. Min.	0.64	0.64	0.64	0.64
EPA+DHA (% total fatty acids)	**28.0**	**21.7**	**15.7**	**9.6**
Proximate composition, analysed (%)				
Crude protein (dw)	58.50	58.91	58.91	59.06
Crude fat (dw)	24.25	24.89	24.35	25.61
Moisture	7.27	5.41	7.22	8.30
Ash (dw)	7.46	7.19	7.25	7.30

Table 9.4.4. Fatty acid composition (% total fatty acids) of the experimental diets.

Fatty acid	Diet 1	Diet 2	Diet 3	Diet 4
14:0	6.51	5.35	4.26	2.19
16:0	18.55	20.39	21.22	23.06
16:1n-7	6.73	5.29	4.08	1.78
16:1n-5	0.28	0.20	0.16	0.06
16:2n-4	0.82	0.64	0.50	0.20
17:0	0.80	0.62	0.47	0.16
16:3n-3	0.23	0.18	0.13	0.05
16:3n-1	0.11	0.08	0.06	0.03
16:4n-3	1.19	0.93	0.72	0.28
18:0	3.64	3.59	3.49	3.47
18:1n-9	11.03	15.32	17.80	23.69
18:1n-7	2.68	2.22	1.86	1.14
18:1n-5	0.17	0.14	0.12	0.08
18:2n-9	0.06	0.06	0.04	0.01
18:2n-6	4.58	6.72	8.41	11.26
18:3n-6	0.22	0.17	0.13	0.05
18:3n-3	1.73	6.50	9.89	17.11
18:4n-3	2.37	1.86	1.50	0.77
20:0	0.30	0.28	0.28	0.20
20:1n-9	0.34	0.29	0.26	0.21
20:1n-7	3.44	2.98	2.83	2.33
20:1n-5	0.31	0.24	0.18	0.07
20:2n-9	0.06	0.04	0.03	0.01
20:2n-6	0.18	0.13	0.18	0.14
20:3n-9	0.08	0.06	0.05	0.02
20:4n-6 (ARA)	0.86	0.65	0.52	0.24
20:3n-3	0.10	0.06	0.08	0.06
20:4n-3	0.59	0.43	0.34	0.15
20:5n-3 (EPA)	11.21	8.51	6.70	2.95
22:1n-11	4.84	4.07	3.96	3.28
22:1n-9	0.46	0.37	0.34	0.26
22:4n-6	0.10	0.05	0.05	0.02
22:5n-6	0.08	0.06	0.05	0.03
22:5n-3	1.21	0.88	0.68	0.31
22:6n-3 (DHA)	12.29	9.17	7.49	3.79
Total saturates	30.31	30.43	30.06	29.25
Total monoenoic	30.60	31.59	31.79	32.99
Total n-3	30.93	28.51	27.52	25.47
Total n-6	6.14	7.98	9.47	11.78
Total n-9	11.95	16.08	18.48	24.17
Total n-3 HUFA	25.41	19.05	15.28	7.25
EPA/ARA	13.03	13.00	13.00	12.39
DHA/EPA	1.10	1.08	1.12	1.29
DHA/ARA	14.29	14.01	14.54	15.93
EPA+DHA	23.51	17.67	14.18	6.74
n-3/n-6	5.04	3.57	2.90	2.16
18:1n-9/n-3 HUFA	0.43	0.80	1.16	3.27

Total number of eggs, total number of eggs per spawn and the total number of eggs per spawn and kg female are shown in **Figs 9.4.9**, **9.4.10** and **9.4.11**. In **Table 9.4.5**, the results corresponding to the egg quality, obtained starting from August 4th, after three weeks of feeding with experimental diets (Watanabe *et al*., 1985; Fernández-Palacios *et al*., 1995; Tandler *et al*., 1995). A total of 20 inductions were performed (10 per couple). In Tanks 1 and 2, 14 spawns were obtained, and in Tanks 3 and 4, 15 spawns.

The composition in fatty acids of the fertilized eggs from broodstocks fed the different experimental diets is shown in **Table 9.4.6.**

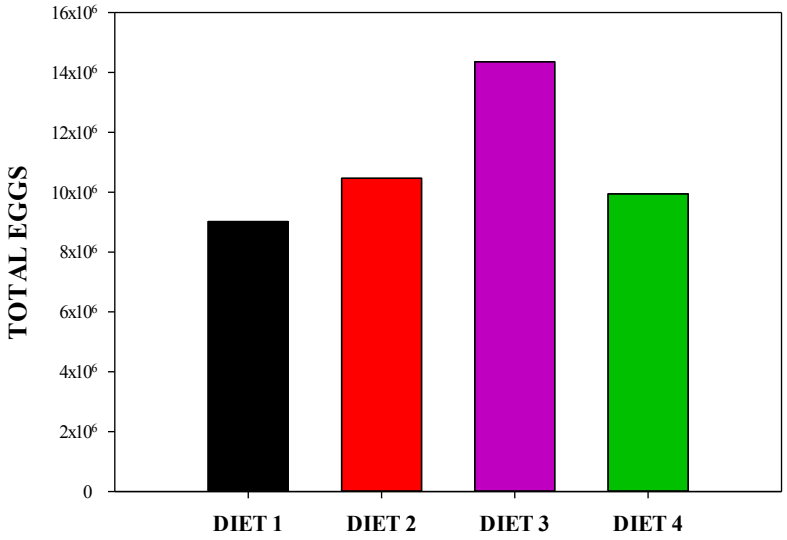
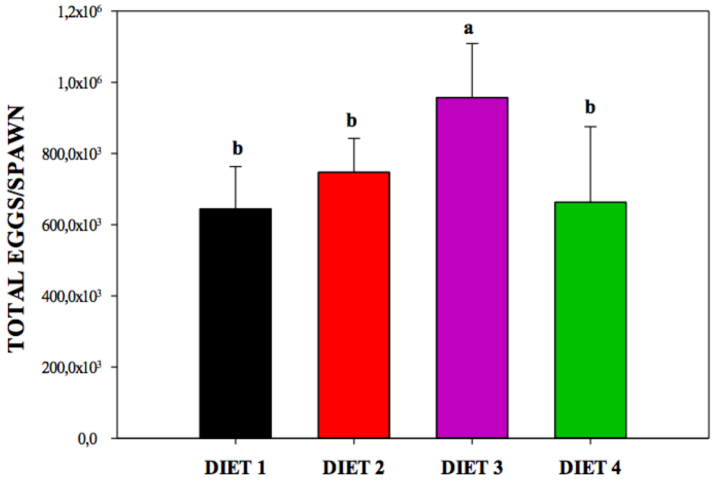


Figure 9.4.9. Number of eggs obtained after feeding with the experimental diets.



*Bars, of the same shade, with the same letter were not significantly different (P < 0.05).

Fig. 9.4.10. Number of eggs per spawn obtained after feeding with the experimental diets.

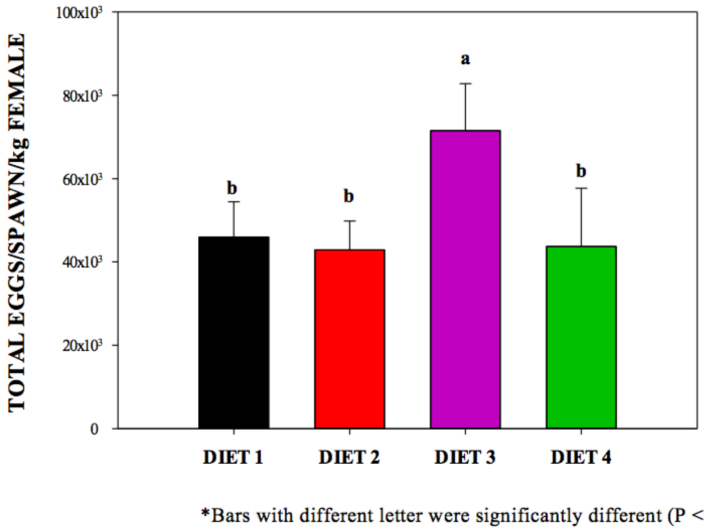


Figure 9.4.11. Number of eggs per spawn and kg female obtained after feeding with the experimental diets.

Table 9.4.5. Quality of egg and larvae obtained after feeding with the experimental diets.

Diet	% Fertilization	% Viable 24h	% Hatching
1	52.42±10.64^{c}	90.28±3.28^{c}	76.99±8.94^{b}
2	69.02±7.38^{b}	85.07±1.73^{b}	79.68±3.74^{b}
3	91.76±3.12^{a}	95.99±2.81^{a}	94.22±3.62^{a}
4	86.32±1.67^{a}	93.88±2.48^{a}	92.51±2.27^{a}
Diet	**% 1 dph survival**	**% 3 dph survival**	**% 5 dph survival**
1	57.44±3.08^{b}	16.15±4.96^{b}	1.56±1.04^{b}
2	59.85±2.94^{b}	11.59±2.22^{c}	2.95±1.98^{a}
3	85.25±9.97^{a}	28.33±8.01^{a}	3.98±1.52^{a}
4	87.04±2.92^{a}	28.12±2.05^{a}	3.73±1.08^{a}

* Means ± SD. Different superscripts in the same column indicate significant differences (P <0.05).

Table 9.4.6. Fatty acid composition (% total fatty acids) of eggs obtained after feeding with the experimental diets.

Fatty acid	Diet 1	Diet 2	Diet 3	Diet 4
14:0	2.61 ± 0.33	2.30 ± 0.12	2.05 ± 0.04	1.81 ± 0.37
16:0	18.73 ± 0.86	18.18 ± 0.62	18.52 ± 0.23	18.50 ± 0.45
16:1n-7	5.45 ± 0.33 ^{a}	4.63 ± 0.09 ^{b}	4.11 ± 0.02 ^{b}	4.17 ± 0.11 ^{b}
16:1n-5	0.15 ± 0.04	0.11 ± 0.01	0.08 ± 0.01	0.12 ± 0.02
16:2n-4	0.36 ± 0.09	0.35 ± 0.03	0.25 ± 0.02	0.23 ± 0.09
17:0	0.25 ± 0.06	0.24 ± 0.01	0.16 ± 0.01	0.17 ± 0.05
16:3n-3	0.14 ± 0.00	0.12 ± 0.00	0.10 ± 0.00	0.11 ± 0.00
16:3n-1	0.10 ± 0.00	0.11 ± 0.02	0.10 ± 0.01	0.11 ± 0.01
16:4n-3	0.11 ± 0.04	0.12 ± 0.00	0.07 ± 0.01	0.07 ± 0.04
18:0	5.78 ± 0.48	6.16 ± 0.22	4.90 ± 0.15	5.48 ± 0.66
18:1n-9	20.88 ± 2.43	20.85 ± 0.12	24.05 ± 0.57	24.43 ± 2.21
18:1n-7	4.09 ± 0.29	3.26 ± 0.17	3.04 ± 0.07	3.41 ± 0.48
18:1n-5	0.22 ± 0.00 ^{a}	0.20 ± 0.01 ^{ab}	0.17 ± 0.00 ^{b}	0.19 ± 0.01 ^{ab}
18:2n-9	0.15 ± 0.03	0.08 ± 0.01	0.09 ± 0.01	0.16 ± 0.02
18:2n-6	6.82 ± 0.45 ^{c}	9.02 ± 0.56 ^{ab}	10.39 ± 0.01 ^{a}	8.66 ± 0.21 ^{b}
18:3n-6	0.21 ± 0.01 ^{a}	0.17 ± 0.01 ^{b}	0.16 ± 0.01 ^{b}	0.19 ± 0.01 ^{ab}
18:3n-3	1.02 ± 0.06 ^{c}	4.67 ± 0.21 ^{b}	6.54 ± 0.40 ^{a}	4.07 ± 0.44 ^{b}
18:4n-3	0.89 ± 0.25	0.92 ± 0.03	0.65 ± 0.04	0.64 ± 0.25
20:0	0.15 ± 0.00	0.20 ± 0.02	0.18 ± 0.01	0.15 ± 0.01
20:1n-9	0.28 ± 0.04	0.28 ± 0.01	0.28 ± 0.01	0.26 ± 0.04
20:1n-7	1.30 ± 0.23	1.50 ± 0.05	1.39 ± 0.05	1.19 ± 0.27
20:1n-5	0.17 ± 0.02	0.16 ± 0.01	0.14 ± 0.01	0.13 ± 0.02
20:2n-9	0.06 ± 0.00	0.04 ± 0.00	0.05 ± 0.01	0.06 ± 0.01
20:2n-6	0.26 ± 0.01	0.24 ± 0.01	0.26 ± 0.01	0.24 ± 0.02
20:3n-9	0.04 ± 0.01	0.04 ± 0.01	0.03 ± 0.00	0.03 ± 0.01
20:4n-6	1.33 ± 0.01 ^{a}	1.16 ± 0.08 ^{ab}	0.93 ± 0.01 ^{c}	1.07 ± 0.07 ^{bc}
20:3n-3	0.11 ± 0.01 ^{b}	0.13 ± 0.01 ^{b}	0.15 ± 0.00 ^{a}	0.14 ± 0.01 ^{ab}
20:4n-3	0.69 ± 0.07 ^{a}	0.56 ± 0.01 ^{ab}	0.50 ± 0.01 ^{b}	0.56 ± 0.04 ^{ab}
20:5n-3 (EPA)	6.35 ± 1.10	6.22 ± 0.07	4.86 ± 0.11	4.98 ± 0.97
22:1n-11	0.38 ± 0.11	0.46 ± 0.04	0.39 ± 0.01	0.31 ± 0.14
22:1n-9	0.09 ± 0.02	0.10 ± 0.01	0.08 ± 0.01	0.07 ± 0.02
22:4n-6	0.10 ± 0.02	0.10 ± 0.04	0.07 ± 0.01	0.11 ± 0.07
22:5n-6	0.05 ± 0.01	0.05 ± 0.01	0.04 ± 0.00	0.05 ± 0.01
22:5n-3	2.23 ± 0.01 ^{a}	1.60 ± 0.07 ^{b}	1.70 ± 0.03 ^{b}	1.85 ± 0.23 ^{ab}
22:6n-3 (DHA)	16.88 ± 0.84 ^{a}	14.47 ± 0.35 ^{b}	12.43 ± 0.04 ^{c}	15.05 ± 0.28 ^{ab}
Total saturates	27.82 ± 0.00	27.32 ± 0.72	26.05 ± 0.02	26.33 ± 0.61
Total monoenoic	33.13 ± 2.00	31.66 ± 0.25	33.82 ± 0.52	34.37 ± 2.06
Total n-3	28.40 ± 2.36	28.80 ± 0.14	26.98 ± 0.54	27.46 ± 1.80
Total n-6	8.94 ± 0.40 ^{b}	10.84 ± 0.61 ^{a}	11.97 ± 0.01 ^{a}	10.48 ± 0.36 ^{ab}
Total n-9	21.50 ± 2.40	21.38 ± 0.13	24.56 ± 0.58	24.99 ± 2.16
Total n-3 HUFA	26.24 ± 2.01 ^{a}	22.97 ± 0.37 ^{ab}	19.63 ± 0.07 ^{b}	22.57 ± 1.06 ^{ab}
EPA/ARA	2.69 ± 0.34	2.33 ± 0.08	2.56 ± 0.06	3.08 ± 0.54
DHA/EPA	12.79 ± 0.71	12.57 ± 1.12	13.38 ± 0.13	14.08 ± 1.20
DHA/ARA	4.81 ± 0.86	5.39 ± 0.28	5.23 ± 0.08	4.68 ± 1.22
EPA+DHA	23.22 ± 1.94 ^{a}	20.69 ± 0.29 ^{ab}	17.29 ± 0.09 ^{b}	20.02 ± 1.24 ^{ab}
n-3/n-6	3.19 ± 0.41	2.66 ± 0.16	2.26 ± 0.05	2.63 ± 0.26
18:1n-9/n-3 HUFA	0.80 ± 0.16	0.91 ± 0.01	1.23 ± 0.04	1.09 ± 0.15

* Means ± SD. Different superscripts in the same column indicate significant differences (P <0.05).

Results from the present experiment showed that the best spawn quality and production parameters were obtained from broodstock fed the diet based on 14.2% EPA+DHA, and the diet based on 6.7% EPA+DHA of total fatty acid. The lowest fertilization and egg viability were obtained from broodstock fed diet based on 23.5% EPA+DHA.

3. Effects of experimental diet with optimized EFA content on reproductive performance of greater amberjack

3.1 *Materials and Methods*

The greater amberjack broodstock consisted of 50 PIT-tagged hatchery-produced 4 years old F1 fish, reared in the facilities of Centro Oceanográfico de Canarias (IEO, P8), in Tenerife, Spain, since 2014. Fish had been fed with a commercial pellet for turbot (Initial diet) (Skretting Ltd, Norway; composition in % dry weight was: 52% crude protein, 20% crude fat, 8.7% ash, 1.7% crude cellulose and 1.4% total phosphorus), with size, frequency and quantity of pellet adjusted accordingly to fish weight.

In February 2018, the fish were distributed in three groups which were fed on different diets: A Mackerel Group (7 ♀ 6.6±0.9 Kg and 8 ♂ 5.9±0.8 Kg), fed on frozen mackerel (*Scomber colias*) (composition in % dry weight: crude protein: 22.0%, crude fat: 9.6%, moisture 76.8%) supplemented with a vitamin premix, to contain: 500 ppm of vitamin C and 200 ppm of vitamin E for each kg of raw fish in dry matter. A Control Group (7 ♀ 7.6±1.2 Kg and 12 ♂ 6.0±1.1 Kg), which was fed with a commercial Control pellet manufactured by P20 (SARC). Finally, an Experimental Group (5 ♀ 6.8±1.1Kg and 10 ♂ 5.6±0.8 Kg), which was fed with a diet also manufactured by P20 (SARC), accordingly to certain pre-requisites established by ULL (P15)/IEO (P8), to contain higher proportions of polar lipids and a fatty acid profile, with particular emphasis to contain essential fatty acids (EFA; ARA, EPA, and DHA and ratios) mimicking those from the wild mature gonads (**Table 9.4.7).** It was intended to maximize the presence of specific marine-origin ingredients, including carotenoids. This formula was based on the comparison of wild and captive greater amberjack broodstock composition obtained by IEO/ULL in several previous studies performed the last few years (Rodriguez-Barreto *et al*., 2012, 2014) and also those performed within Diversify Project (Zupa *et al*., 2017b; WP3; D3.3).

According to these results and specially at early gametogenesis, captive-reared fish gonads display clearly lower contents of specific lipid classes (PC, PE and PI) and lower contents of ARA and DHA and, consequently DHA/EPA and ARA/EPA ratios, all crucial factors for reproductive success and sperm, egg and larval quality (Zupa *et al.,* 2017b). These previous studies also demonstrated that the captive animals display much higher fat contents and also of 18:1n-9, 18:2n-6 and 20:5n-3, in gonads, muscle and liver.

For this reason, after several email contacts, the involved partners (IEO, ULL and SARC) held a Skype meeting on March 2017 to agree on the ingredients to formulate an experimental diet with a similar proximate composition to that of the control diet (51% crude protein /18% crude fat) and marine based lipid sources. The experimental diet was then formulated with commercial available ingredients to contain comparable lower levels of EPA and to maximize ARA and DHA contents (**Table 9.4.7**).

To maximize the inclusion of DHA, algae meal was added and the level of ARA was increased by adding a ARA-rich oil (10 % ARA). However, since the diets mainly contained marine raw materials, EPA could not be so low, as shown in Table 9.4.8. Finally, as a source of phospholipids rapeseed lecithin phospholipids was used to minimize the contribution of n-6 fatty acids. The resultant analysis showed that the experimental diet was lower in 18:1n-9 and 18:2n-6 and higher than the control one in ARA and DHA, and was still higher in EPA.

Table 9.4.7 Raw material and proximate composition of control and experimental diets (Skretting Aquaculture Research Center, Stavanger, Norway).

Raw material	Control	Experimental
Rapeseed lecithin	1.00	1.00
Algae meal	0.00	2.50
krill meal	7.00	7.00
Wheat	18.49	17.46
Wheat gluten	17.00	18.00
Fish meal	43.17	43.17
Arachidonic acid, 10 %	0.26	0.60
Squid meal	3.00	3.00
Fish oil	9.09	6.27
Vitamin premix	0.50	0.50
Mineral premix	0.50	0.50
Proximate composition (analyzed)		
Total	100	100
Moisture	8.7	8.1
Crude protein	50.7	51.8
Crude fat	18.20	18.00
Ash	8.3	8.3

Table 9.4.8. Analyzed main fatty acid composition (% of total fatty acids) of diets and mackerel.

Fatty acid	Initial Diet	Control Diet	Experim. Diet	Mackerel
16:0	14.0	18.2	18.5	16.1
18:1n-9	26.0	13.3	11.3	5.5
18:2n-6	10.4	6.3	5.6	1.3
20:4n-6 (ARA)	0.5	1.1	1.6	2.8
20:5n-3 (EPA)	6.8	11.1	12.0	5.5
22:6n-3 (DHA)	7.1	10.2	14.5	6.5
EPA/ARA	13.6	10.1	7.5	2.5
DHA/EPA	1.0	0.9	1.2	1.2
DHA/ARA	14.2	9.3	9.1	2,3
ARA+EPA+DHA	14.4	22.4	28.1	14.8
Carotenoids		50ppm	50ppm	

Fish were maintained in three outdoor covered raceway tanks of 500 m^3 with continuous water supply (10 renewals day^{-1}) under natural photoperiod and seawater temperature (19.2±0.3ºC), and hand-feeding once a day and 3 days a week to apparent satiation. Measurements of temperature and water quality (Dissolved Oxygen, NH_3-N and NO_2-N) were conducted once per week throughout the year. At the expected onset of the

spawning season (May), a passive egg collector was placed in the outflow of the spawning tanks and daily checked, in order to collect the spawned eggs.

The fish were sampled monthly during 2018 spawning season (June, July, August, September, and October). Fish were starved for two days prior to sampling and were tranquilized initially in their tank with the use of chlorobutanol (0.1 ml l^{-1}) and then transferred to an anesthetic bath for complete sedation with a higher concentration of chlorobutanol (0.3 ml l^{-1}). Fish were individually identified with PIT tags and biometric parameters of length and body weight were measured. Ovarian biopsies for the evaluation of oocyte development were obtained by inserting a plastic cannula (Pipelle de Cornier). A wet mount of the biopsy was examined first under a compound microscope (40 and 100x) to evaluate the stage of oogenesis and measure the mean diameter of the largest, most advanced vitellogenic oocytes (n = 10). 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 plastic catheter into the genital pore. The collected sperm was stored on ice and then transferred to a 4°C refrigerator until evaluation.

Sperm quality parameters that were evaluated included (a) sperm density (number of spermatozoa ml^{-1} of sperm), (b) initial percentage of spermatozoa showing forward motility immediately after activation (sperm motility, %), (c) duration of forward sperm motility of ≥5% of the spermatozoa in the field of view (motility duration, min) and (d) survival of sperm during storage at 4°C (sperm survival, days). Sperm density was estimated after a 2211-fold dilution with 0.9% saline using a Neubauer hemocytometer under a compound light microscope at 400X magnification (in duplicate). Sperm motility and duration were evaluated on a microscope slide at 400X magnification after mixing 1 μl of sperm with a drop of seawater (~ 50 μl) in duplicate. Activated sperm samples were observed under a compound light microscope for the first time 10 sec after activation. Sperm motility was determined subjectively using increments of 10% and sperm was considered immotile when < 5% of the spermatozoa was exhibiting forward motility. Oocyte and sperm samples were stored at -80°C until biochemical analysis.

At each sampling, blood was collected from all fish from the caudal vessel using heparinized syringes, in order to measure the concentrations and blood biochemical parameters. Hematological parameters were estimated from fresh samples of blood. Total erythrocytes and leucocytes were determined by counting in 1/100 dilutions of blood in Natt and Herricks solution, using a Neubauer hemocytometer. Hematocrit count was carried out by capillary diffusion and centrifugation. Blood was centrifuged at 1400 rpm for 20 min and plasma was collected, frozen in liquid nitrogen and stored at -80°C until the biochemical analysis. Plasma levels of protein, triglycerides, cholesterol and glucose were measured in duplicates by enzymatic colorimetric assays (Biosystems, Spain).

Fish were treated with an Ethylene–Vinyl acetate (EVAc) GnRHa implant (Mylonas & Zohar, 2001) loaded with Des-Gly^{10}, D-Ala^{6}-Pro-NEth^{9}-mGnRHa (H-4070, Bachem, Switzerland) at the sampling times of July, August, September and October. There were variations in the effective GnRHa dose applied to each fish due to the fact that implants are loaded with fixed amounts of GnRHa. Moreover, the dose of GnRHa applied was increased in the September and October sampling. At the time of GnRHa implantation, selected females were in advanced vitellogenesis and intratesticular sperm was obtained from males.

To evaluate broodstock nutritional status, available samples of oocytes and sperm from the three treatments, taken and immediately frozen and kept at -80ºC, in August and September were analyzed. Samples of mackerel, the commercial control diet and the experimental one were also analyzed. Dry matter and protein contents were calculated using the methods of analysis of the Association of Official Analytical Chemists (AOAC, 2012). Moisture contents were determined in approximately 500 mg samples by thermal drying in an oven at 110°C, until constant weight. Protein was determined by sample digestion according to Kjeldahl method. Total lipid (TL) was extracted from the tissues and diet by homogenization in chloroform/methanol (2:1, v/v) according to the method of Folch *et al*. (1957). The organic solvent was evaporated under a stream of nitrogen and the lipid content was determined gravimetrically (Christie, 1982) and stored in chloroform/methanol (2:1), containing 0.01% butylated hydroxytoluene (BHT). Analysis of lipid class (LC) composition was performed by one-dimensional double development high performance thin layer chromatography (HPTLC; Merk, Darmstadt, Germany), and methyl acetate /isopropanol /chloroform

/methanol/0.25% (w/v) KCl (5: 5: 5: 2: 1.8, by volume) as developing solvent system for the polar lipid classes and isohexane/diethyl ether/acetic acid (22.5: 2.5: 0.25, by volume), for the neutral lipid separation. Lipid classes were visualized by charring at 160°C for 15min after spraying with 3% (w/v) aqueous cupric acetate containing 8% (v/v) phosphoric acid, and quantified by scanning densitometry using a dual-wavelength flying spot scanner Shimadzu CS-9001PC (Shimadzu, Duisburg, Germany) (Olsen & Henderson, 1989). To determine the fatty acid profiles, TL extracts were subjected to acid-catalysed transmethylation with 1% sulphuric acid (v/v) in methanol. The resultant fatty acid methyl esters (FAME) were extracted using isohexane: diethylether (1:1 by volume) and purified by TLC using isohexane/diethyl ether/acetic acid (90:10: 1, by volume) as developing system (Christie, 1982). Fatty acid methyl esters were separated and quantified using a TRACE-GC Ultra gas chromatograph (Thermo Electron Corp., Waltham, MA, USA) equipped with an on-column injector, a flame ionization detector and a fused silica capillary column, Supelcowax TM 10 (30 m 9 0.32 mm I.D. 9 0.25 lm; Sigma-Aldrich, Madrid, Spain). Helium was used as carrier gas and temperature programming was 50–50°C at 40°C min^{-1} slope, then from 150 to 200°C at 2°C min^{-1}, to 214°C at 1°C min^{-1} and, finally, to 230°C at 40°C min^{-1}. Individual FAME and DMA were identified by reference to authentic standards, and further confirmation of FAMEs and DMAs identity was carried out by GC-MS (DSQ II; Thermo Electron Corp). Due to the small size of the biological samples, carotenoids were measured only in the diets, according to the method of Barua *et al*. (1993), using ethyl acetate/ethanol (10mL, 1:1 v/v), ethyl acetate (5 ml) and hexane (10 ml) as extracting solvents. Afterwards, carotenoids contents were quantified by spectrophotometry at 470 nm.

Statistical analysis

Differences in egg and sperm quality and blood parameters were tested using one-way Analysis of Variance (ANOVA) followed by Tukey's post hoc test. The data were checked for normal distribution with the one sample Kolmogorov–Smirnoff test, as well as for homogeneity of the variances with the Levene test, and percentage data were Arcsine transformed prior to statistical analysis to normalize variances.

Pearson's correlation coefficients were used to assess the relationships between some egg quality variables. Percentage data were Arcsine transformed prior to statistical analyses to normalize variances. Results are presented as mean ± standard deviation (SD), unless mentioned otherwise. In all statistical tests used, differences with a *P* value of less than 0.05 were considered statistically significant. Analyses were performed with the IBM SPSS statistics package (version 20.0 for Windows).

3.2 *Results*

Fish conditions and reproduction performance

The feed intake (Food dry weight per body weight %) increased from March to May and declined thereafter with values in the overall period of 0.8±0.7 % for Mackerel Group, and 1.2±0.8 and 1.4±0.5 % for Control and Experimental Groups, respectively (**Fig. 9.4.12**).

The temperature from March to the first sampling (June) was stable (19.2±0.3ºC) and one degree below the usual in the month of June. From June to the end of the study (October), temperature increased by 4.4ºC with an average temperature in the period of 22.0±1.2ºC. The temperature was 1ºC lower than normally recorded from June to August.

The feed intake decreased significantly in all groups with the increase of temperature. However, experimental fish group showed a less marked decrease in intake (lineal slope = -0.043) than fish fed with control feed (lineal slope= -0.062) and mackerel fish (lineal slope= -0.053).

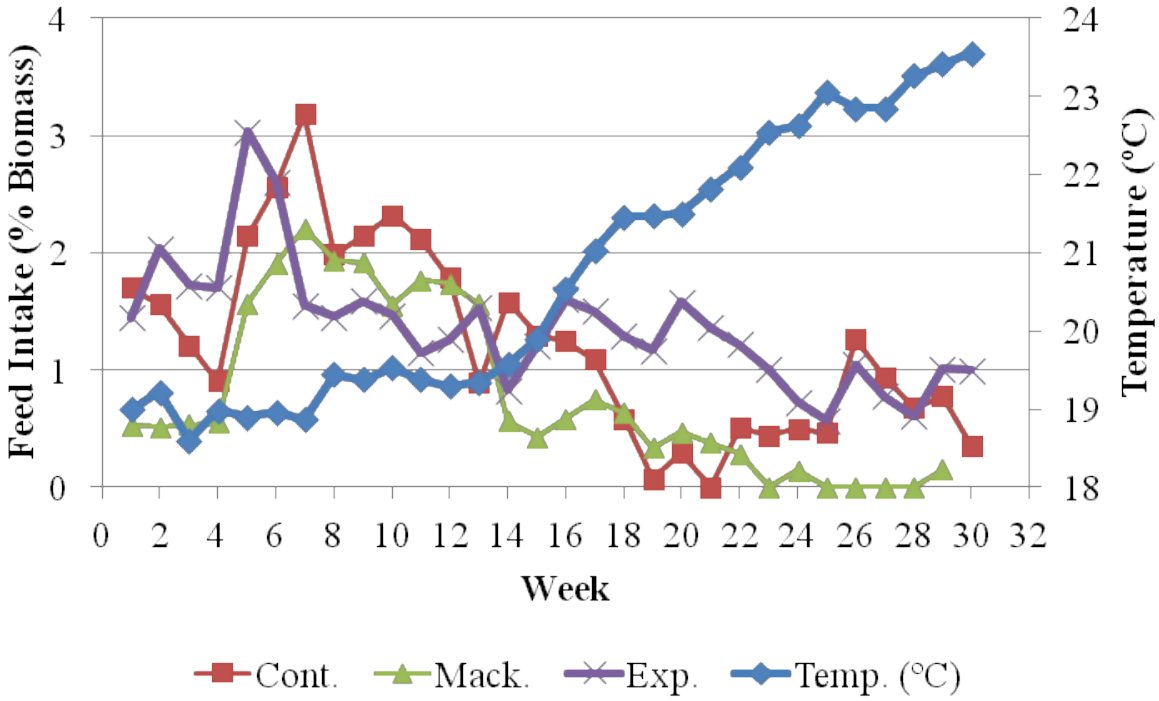


Figure 9.4.12. Weekly means of temperature (blue line) and food intake (dry weight) of Control (red line), Mackerel (green line), and Experimental (purple line) broodstock groups during the experimental period.

The weight of males and females of experimental groups in each sampling is shown in **Table 9.4.9**. The weights within each group were homogeneous in all sampling periods.

Table 9.4.9. Number of sampled fish and mean weight (± SD; kg) of the fish of each sex from the groups of breeders fed with commercial pellet (Control), frozen mackerel (Mackerel) and experimental diet (Experimental) at each sampling time. ns=not sampled.

	Treatment	**Experimental**		**Control**		**Mackerel**	
Month	**Sex**	n	Weight (kg)	n	Weight (kg)	n	Weight (kg)
June	**Females**	5	6.8 ± 1.1	6	7.7 ± 1.3	7	6.6 ± 0.9
	Males	10	5.6 ± 0.8	12	6.0 ± 1.1	8	5.9 ± 0.8
July	**Females**	5	6.6 ± 1.1	6	7.3 ± 1.3	7	6.2 ± 0.9
	Males	10	5.3 ± 0.8	12	6.1 ± 1.3	8	5.7 ± 0.8
Aug.	**Females**	5	6.6 ± 1.1		NS	6	6.1 ± 1.0
	Males	10	5.1 ± 0.8		NS	8	5.6 ± 0.9
Sep.	**Females**	5	6.1 ± 1.0	2	7.2 ± 0.4	4	5.3 ± 1.1
	Males	9	4.8 ± 0.8	5	5.8 ± 1.4	8	4.9 ± 0.8
Oct.	**Females**	5	5.8 ± 1.0	2	7.4 ± 0.6	3	5.1 ± 1.2
	Males	7	4.6 ± 0.7	5	6.0 ± 1.5	5	4.8 ± 0.9

During June, no spawns were registered and selected males and females (largest vitellogenic oocytes > 600 µm) were treated with a GnRHa implant at a dose of ~50 µg GnRHa kg^{-1} body weight (in the form of EVAc implant) in July. Because no spawns were obtained after the 1st treatment, the fish were treated with a higher dose (~100 µg GnRHa kg^{-1} body weight) in the successive spawning induction treatments (**Table 9.4.10**).

Table 9.4.10. Number of sampled fish, mean weight (± SD) and dose of GnRHa (µg kg^{-1} body weight) (mean ± SD) of implanted greater amberjack breeders fed with commercial pellet (Control), frozen mackerel (Mackerel) and experimental diet (Experimental) at each treatment/sampling time. All treated fish were given a GnRHa implant, and slight variations in the effective GnRHa dose were due to the fact that implants were loaded with fixed amounts of GnRHa. NS indicate fish no sampled.

Treatment		Experimental							Control							Mackerel					
Month	Sex	n	Weight (kg)			Dose (µg kg^{-1})			n	Weight (kg)			Dose (µg kg^{-1})			n	Weight (kg)			Dose (µg kg^{-1})		
Jul.	♀	2	6.4	±	0.5	55.1	±	4.5	2	7.9	±	1.3	64.3	±	10.7	2	5.8	±	0.4	63.4	±	25.6
	♂	2	5.4	±	0.0	46.5	±	0.2	2	7.1	±	1.3	35.6	±	6.5	2	6.5	±	1.3	56.4	±	16.4
Aug.	♀	3	6.8	±	1.0	111.6	±	15.8			NS			NS		3	5.7	±	1.4	137.3	±	32.4
	♂	3	5.4	±	0.6	92.6	±	10.8			NS			NS		3	5.4	±	0.4	93.3	±	7.3
Sep.	♀	3	6.6	±	1.0	115.3	±	17.8	2	7.2	±	0.4	103.9	±	6.3	3	5.1	±	1.3	152.6	±	36.5
	♂	3	5.1	±	0.6	98.1	±	11.0	2	5.7	±	1.0	89.3	±	15.1	2	5.1	±	0.1	98.4	±	2.2
Oct.	♀	2	5.9	±	0.7	105.8	±	17.2	2	7.5	±	0.5	83.3	±	18.2	1	6.1	±	-	81.4	±	-
	♂	2	4.8	±	0.5	104.8	±	11.1	3	5.3	±	1.8	101.0	±	34.0	1	4.8	±	-	103.3	±	-

The Control group was not treated in August, because there was a high mortality (50 %) probably caused by high incidence of parasitization in the group.

The percentage of females with oocytes larger than 500 µm was greater in the Experimental group during the first months (June-August) of the studied period (**Fig. 9.4.13)**.

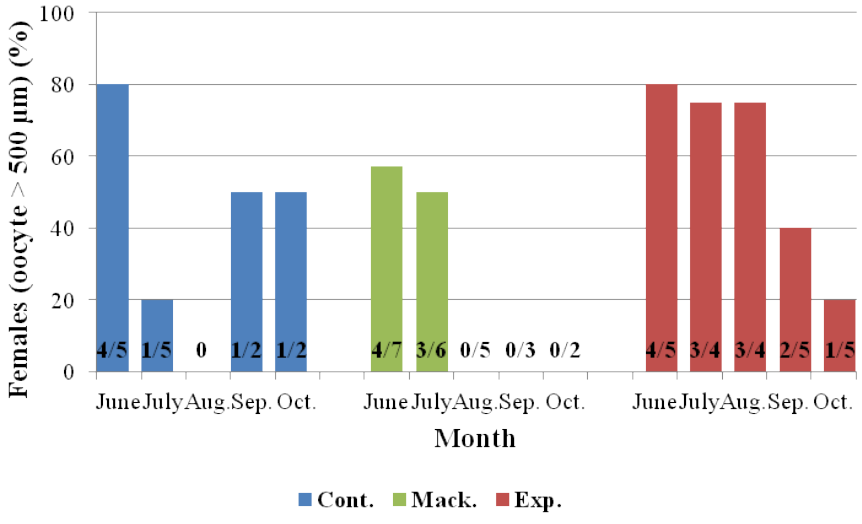


Figure 9.4.13. Percentage of Females with oocytes larger than 500 µm with respect to the total number of biopsied females in control (Cont.), frozen mackerel (Mack.) and experimental (Exp.) fed groups during the period of study. Numbers in bars indicate the number of females (oocytes > 500 µm) / number of females biopsied.

The mean diameter of the largest vitellogenic oocytes of the females biopsied are shown in the **Figure 9.4.14**. Of the females biopsied, all those fed with Mackerel showed decrease in oocyte diameter with time, and value higher than 500 µm only in June and July. In the Control group, all females, except one, showed a decline in the diameter of the oocyte with time, and with the highest general values (> 500 µm) in June. In the Experimental group, two of the five females showed an increase in the diameter of the oocytes with time, while the oocyte diameter of the other females decreased.

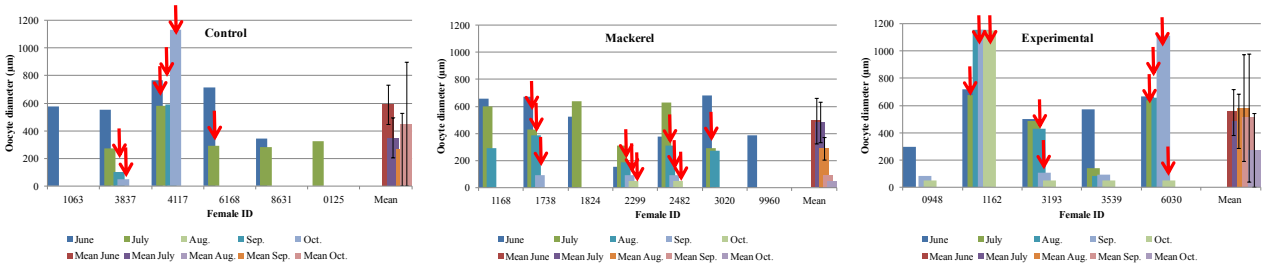
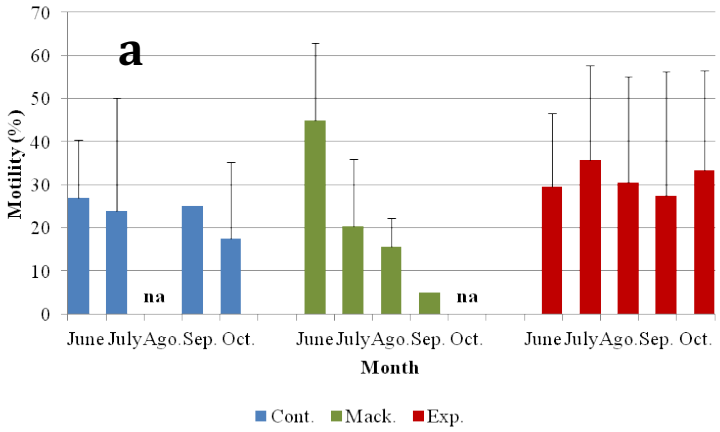


Figure 9.4.14. Oocyte size of individual females (ID) biopsied in each group at different samplings/treatment during the reproductive season. The red arrows indicate the female implanted in each group at each sampling time.

Sperm quality parameters of sperm motility (%) and sperm motility duration (sec.) tended to decrease along experimental period except in the Experimental Group which maintained similar values in all sampling points **(Fig. 9.4.15 a b**).

In the experimental group, mean sperm motility percentage (~ 30%) (**Fig. 9.4.15a**) and the duration of sperm motility (~ 120 sec.) (**Fig. 9.4.15b)** remained unchanged throughout the monitored period, while in the Mackerel group, and less evident in the Control group, the mean sperm density (**Fig. 9.4.15c**) and duration of sperm motility decreased at each sampling time, except for motility duration in control group in October that increased with respect to September.

The Mackerel group mean sperm density was slightly higher ($17.7 \pm 14.5 \times 10^9$ spermatozoa ml^{-1}) than Control and Experimental groups in June (**Fig. 9.4.15c**). However, the Experimental group showed the highest sperm density in September and October (> 25×10^9 spermatozoa ml^{-1}), although with higher individual variability.



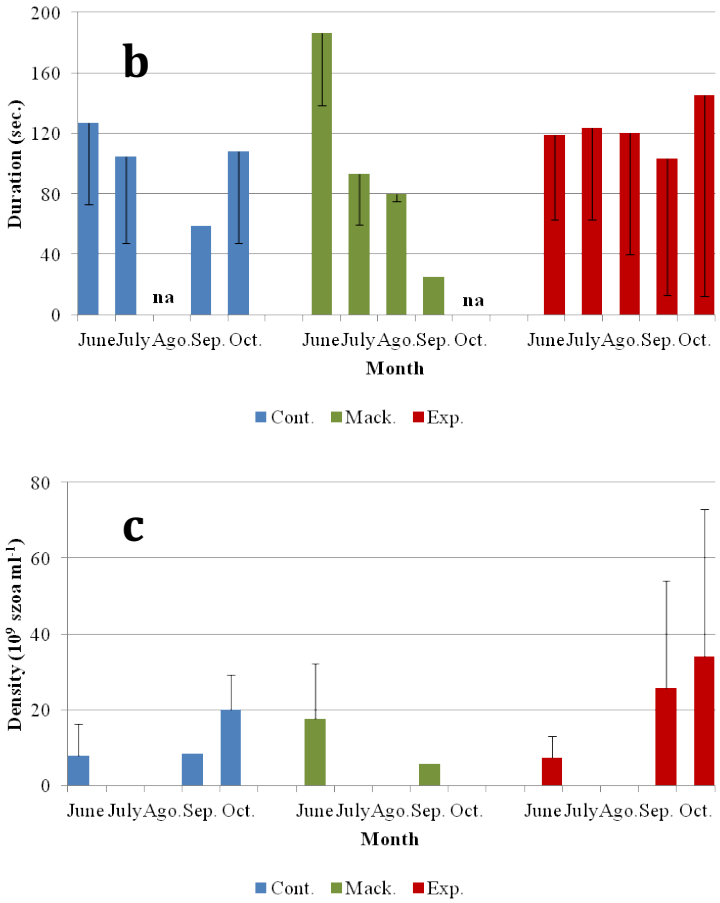


Figure 9.4.15. Mean (± SD) sperm quality parameters of greater amberjack in control (Cont.), frozen mackerel (Mack.) and experimental (Exp.) fed groups at different times during the reproductive season of 2018 (spermatozoa forward motility (%) (**a**), duration of motility (sec.) (**b**) and density (10^9 spermatozoa ml^{-1}) (**c**)). na = not available.

In general, the motility percentage of treated or not treated males of Control and Mackerel groups decreased during the repetitive samplings **(Fig. 9.4.16)**. However, in the Experimental group, both the implanted and non-implanted males showed an increase in the percentage of motility.

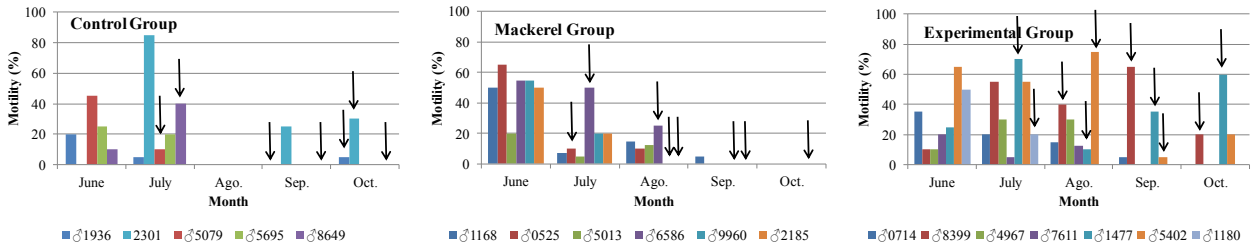


Figure 9.4.16. Motility percentage (%) of individual greater amberjack males in Control, frozen Mackerel and Experimental fed groups at different times during the reproductive season of 2018.

Regarding to the motility duration, Control and Mackerel groups showed a similar trend to that described for the motility percentage, with an important decrease during the consecutive samplings (**Fig. 9.4.17**). However, in the Experimental group, both the implanted and non-implanted males showed an increase in the motility duration.

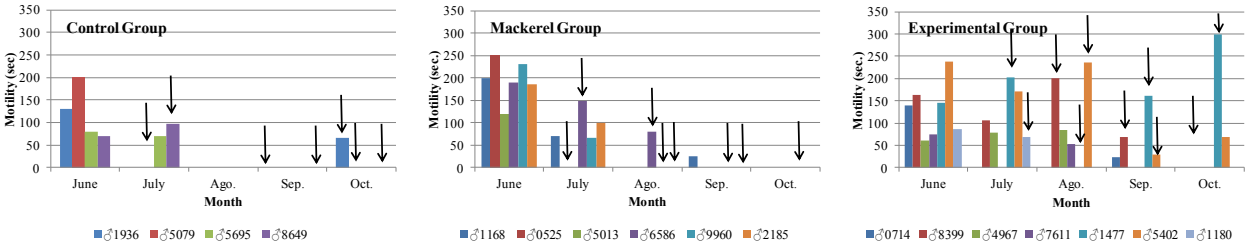


Figure 9.4.17. Motility duration (sec.) of individual greater amberjack males in Control, frozen Mackerel and Experimental fed groups at different times during the reproductive season of 2018.

The three groups of breeders did not release eggs naturally or induced, after the successive hormonal treatments, during the study period, although the biopsy of one female from the Control group and two females from the Experimental group showed mature eggs (>1100 µm) in October. In addition, one of the females of the Experimental group (ID 1162) showed mature eggs since August in the repetitive monthly samplings (**Fig. 9.4.18**).

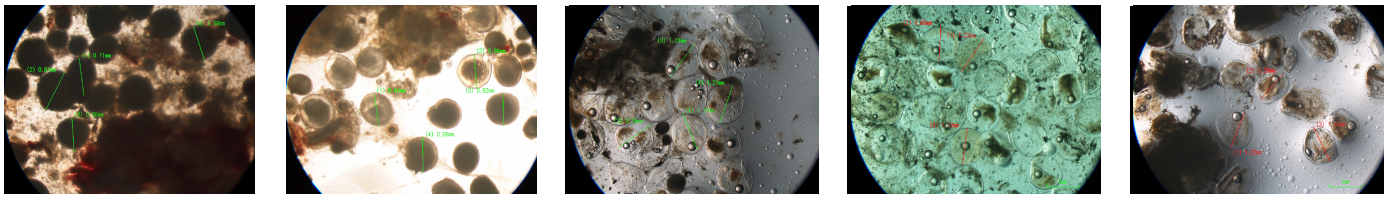


Figure 9.4.18. Oocyte diameter in the successive samplings of the greater amberjack female (ID 1162) of the Experimental group.

Lipid composition of oocytes and sperm

In the absence of spawns, the biopsies of females and males of each group, sampled in August and September were also subjected to analysis of lipid composition (**Tables 9.4.11-9.4.14**). Although samples taken the last month (October) are kept at -80ºC, they could not be analyzed on time for the present Deliverable.

Tables 9.4.11 and 9.4.12 respectively show the female and male samples contents of total lipids and their corresponding lipid classes.

According to the standard deviation of data, it can be highlighted that de composition of oocytes was more variable for the control group. That means that some of the samples were very high and other very low in terms of total lipid contents, a condition that is reflected in each single lipid class. In addition, no samples from this group were available for comparisons in August. The most consistent results at both sampling points were obtained for the experimental group, where total lipid contents ranged between 2.1-2.2, with PC as the main representative phospholipid and the sterol esters as the most abundant neutral lipid class. In spite of the high deviation of the data and that oocytes in the control group had a higher content of total fat, overall the lipid class profile was similar to that of the experimental group. Oocytes from the mackerel group were more different between August and September and generally displayed some higher contents of cholesterol and lower of PC than oocytes from those of the two the extruded diets-fed groups.

Table 9.4.11. Total lipid content (% wet weight) and main lipid class composition (% total lipid) of oocytes from greater amberjack broodstock fed different diets

	Experimental		Control	Mackerel	
	August	September	September	August	September
TL (%ww)	2.29±1.11	2.12±1.01	3.85±3.60	2.74±1.51	1.19±0.40
SM	2.00±0.94	1.59±0.92	2.22±2.08	2.68±0.66	3.01±0.49
PC	12.35±1.14	11.85±3.52	15.39±9.14	9.68±2.52	10.08±2.37
PS	1.04±0.14	0.94±0.64	1.97±1.90	1.25±1.32	2.03±1.06
PI	0.73±0.60	1.59±0.93	2.12±1.62	1.18±0.40	1.25±0.70
PE	2.17±1.65	3.47±4.34	4.68±4.48	4.13±2.91	6.05±2.45
TPL	**21.68±6.78**	**20.59±9.53**	**27.34±20.3**	**19.84±7.65**	**23.13±7.16**
PAG	5.07±1.57	2.69±0.91	4.47±2.71	5.50±1.69	6.94±4.13
Chol	13.88±6.45	14.27±8.95	13.75±5.74	16.08±3.30	20.87±4.12
TG	23.14±5.81	22.75±5.99	19.03±12.8	22.56±1.38	21.37±5.46
SE	35.94±9.68	39.03±9.73	34.86±16.6	35.13±9.94	27.41±14.6
TNL	**78.32±6.78**	**79.41±9.53**	**72.66±20.3**	**80.16±7.65**	**76.87±7.16**

Data are means ± SD (n=3, 4). SM, sphingomyelin; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; PE phosphatidylethanolamine; TPL, total polar lipids; PAG, partial acyglycerols; Chol, Cholesterol; TG, triacylglicerides; SE, sterol esters; TNL, total neutral lipids.

Table 9.4.12. Total lipid content (% wet weight) and main lipid class composition (% total lipid) of sperm from greater amberjack broodstock fed different diets sampled in September.

	Experimental	Control	Mackerel
TL (%ww)	1.45±0.76	2.33	9.38
SM	1.25±0.46	0.48	1.99
PC	20.75±2.14	21.42	13.47
PS	5.79±2.52	3.94	4.14
PI	2.87±1.85	2.04	1.73
PE	15.21±2.87	15.68	2.43
TPL	**48.06±5.90**	**46.17**	**26.87**
PAG	7.12±1.83	10.24	36.17
Chol	33.13±2.87	36.08	25.06
TG	8.18±6.23	5.72	10.59
SE	2.40±1.18	0.59	nd
TNL	**51.94±5.90**	**53.83**	**73.13**

Data of the experimental diet are means ± SD (n=2). SM, sphingomyelin; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; PE phosphatidylethanolamine; TPL, total polar lipids; PAG, partial acyglycerols; Chol, Cholesterol; TG, triacylglicerides; SE, sterol esters; TNL, total neutral lipids. Nd, not detected.

As shown in **Table 9.4.12**, and although only one sample was available for the control group, one more time the composition is quite similar between the sperm from the experimental and the control fed groups. In both cases PC was the most abundant phospholipid, followed by PE and cholesterol the most prominent lipid class. This situation completely changes however with regard to the sperm sampled from the mackerel fed fish which contained a very high level of lipids, with much less proportions of polar lipids.

Main fatty acid composition of total lipids from the above mentioned samples are given in **Tables 9.4.13 and 9.4.14**. As shown in **Table 9.4.13**, composition of oocytes from the experimental group was quite stable, with 16-17% of DHA, 5-6% of EPA and 2-3% of ARA in % of total fatty acids. Although much more variable, particularly for the ARA contents, overall, the fatty acid profile was also similar for the control group oocytes. However, oocytes from the mackerel fed fish displayed higher contents of DHA and ARA at both sampling points, and also lower levels of 18:2n-6, and EPA, compared to the oocytes from the two extruded diet fed fish.

Table 9.4.13. Main fatty acid composition (% total fatty acids) of oocytes from greater amberjack broodstock fed different diets.

	Experimental		Control	Mackerel	
	August	September	September	August	September
16:0	16.53±0.75	10.03±2.34	15.52±2.86	15.89±1.78	17.52±0.37
18:1[1]	23.24±2.09	26.17±3.29	26.34±6.64	21.21±2.16	21.65±2.00
18:2n-6	9.20±2.41	10.03±2.34	10.00±4.26	2.77±0.36	4.41±1.19
20:4n-6	2.57±1.21	2.16±0.31	3.30±2.81	5.78±1.52	7.13±1.46
20:5n-3	6.13±0.84	5.43±0.89	4.97±0.57	2.85±0.42	2.91±1.01
22:6n-3	17.31±1.91	15.77±1.71	17.13±2.34	28.29±2.04	22.52±4.60
DHA/EPA	2.87±0.53	2.98±0.70	3.44±0.08	9.17±2.03	8.67±4.00
ARA/EPA	0.40±0.20	0.37±0.07	0.64±0.49	1.89±0.95	2.75±1.36

Data are means ± SD (n=3, 4). [1], mainly n-9 isomer. DHA, docosahexaenoic acid, 22:6n-3; EPA, eicosapentaenoic acid, 20:5n-3; ARA, arachidonic acid, 20:4n-6.

Table 9.4.14. Main fatty acid composition (% total fatty acids) of sperm from greater amberjack broodstock fed different diets sampled in September.

	Experimental	Control	Mackerel
16:0	23.16±0.96	23.15	12.86
18:1[1]	19.20±2.60	24.00	36.36
18:2n-6	4.68±0.73	5.92	5.74
20:4n-6	4.37±0.74	2.56	1.97
20:5n-3	5.49±0.89	4.63	1.38
22:6n-3	23.90±3.15	18.69	11.76
DHA/EPA	4.39±0.38	4.03	8.53
ARA/EPA	0.80±0.03	0.55	1.43

Data of the experimental diet are means ± SD (n=2). [1], mainly n-9 isomer. DHA, docosahexaenoic acid, 22:6n-3; EPA, eicosapentaenoic acid, 20:5n-3; ARA, arachidonic acid, 20:4n-6.

Haematological and plasma biochemical parameters

Hematological and plasma biochemical parameters of fish from experimental, control and mackerel groups are shown in **Tables 9.4.15** and **Table 9.4.16**. In general, the number of erythrocytes tended to decrease along the experimental period in all treatments while the number of leucocytes tended to increase. When we compare between treatments, most of studied parameters were similar although the number of erythrocytes was lower (P<0.05) and leucocytes higher (P<0.05) in blood from mackerel group compared to other groups in July. No significant differences were observed in hematocrit.

Regarding biochemical parameters, triglycerides diminished significantly (P<0.05) along de experimental period in all treatments groups. A similar trend was observed for cholesterol but only significant for Mackerel group. In addition, fish from mackerel group presented lower levels of triglycerides compared to the other groups in all periods. Several differences were detected in glucose levels between treatment groups in July and October.

Table 9.4.15. Erythrocytes (10^4 mm^{-3}), leucocytes (10^3 mm^{-3}) and hematocrit (%) in blood from greater amberjack of Experimental (Exp,), Control (Cont.) and Mackerel (Mack.) groups during experimental spawning period. Values are means ± SD. Different letters indicate significant differences between month in each group (ANOVA, $P<0.05$). Different capital letters indicate significant differences between treatments (ANOVA, $P<0.05$).

Parameter		Erythrocytes					Leucocytes					Hematocrit			
Treat.	Month	Mean ± SD					Mean ± SD					Mean ± SD			
Exp.	Jun.	232.99	±	110.75	ab		31.77	±	19.33	b	B	44.01	±	9.00	
	Jul.	330.42	±	56.92	a	A	76.33	±	47.01	ab	B	46.75	±	5.93	
	Ago.	240.94	±	45.39	ab		95.21	±	60.24	ab		40.67	±	8.94	
	Sep.	138.88	±	55.04	bc		107.90	±	21.38	a		45.14	±	12.11	
	Oct.	89.75	±	34.79	c		154.54	±	26.29	a		40.69	±	6.85	
Cont.	Jun.	210.63	±	20.37	b		79.18	±	33.47		A	55.10	±	7.11	a
	Jul.	289.64	±	31.84	a	A	111.31	±	69.08		B	47.94	±	3.41	ab
	Sep.	138.33	±	73.36	c		71.85	±	11.65			45.54	±	4.55	c
	Oct.	122.50	±	21.64	c		151.98	±	45.96			35.56	±	4.07	bc
Mack.	Jun.	246.88	±	30.32	a		27.23	±	8.50	b	B	50.63	±	1.96	
	Jul.	216.25	±	49.27	a	B	171.00	±	78.02	ab	A	52.08	±	7.36	
	Ago.	139.79	±	33.01	b		121.46	±	39.98	ab		42.75	±	12.47	
	Sep.	62.00	±	17.96	c		110.03	±	28.31	ab		37.74	±	10.60	
	Oct.	134.06	±	55.20	bc		212.20	±	63.70	a		37.51	±	13.29	

Table 9.4.16. Triglycerides (mg dl^{-1}), cholesterol (mg dl^{-1}), protein (g l^{-1}) and glucose (mg dl^{-1}), in blood from greater amberjack of Experimental (Exp,), Control (Cont.) and Mackerel (Mack.) groups during experimental spawning period. Values are means ± SD. Different letters indicate significant differences between month in each group (ANOVA, $P<0.05$). Different capital letters indicate significant differences between treatments (ANOVA, $P<0.05$).

Parameter		Protein				Glucose					Triglycerides					Cholesterol			
Treat.	Month	Mean ± SD				Mean ± SD					Mean ± SD					Mean ± SD			
Exp.	Jun.	43.76	±	8.06		133.75	±	25.67	a	A	430.37	±	78.49	a	AB	290.57	±	59.34	
	Jul.	52.27	±	3.38		78.80	±	27.80	b		161.01	±	124.80	bc		276.84	±	19.24	
	Ago.	43.74	±	4.01		102.68	±	32.92	ab		168.71	±	28.52	bc	*	256.10	±	34.93	
	Sep.	42.47	±	8.39		81.11	±	31.00	b		340.66	±	221.64	ab	B	239.52	±	47.93	
	Oct.	43.12	±	5.16		98.18	±	23.24	ab	A	108.68	±	60.53	c		226.33	±	23.20	
Cont.	Jun.	49.71	±	5.92	ab	116.31	±	32.62		AB	566.98	±	161.59	a	A	301.74	±	26.96	
	Jul.	52.34	±	7.87	a	111.96	±	50.84			78.68	±	20.82	b		282.88	±	42.39	
	Sep.	47.87	±	8.41	ab	118.73	±	34.19			611.45	±	267.70	a	A	114.34	±	39.40	
	Oct.	38.06	±	5.43	b	65.87	±	13.71		B	170.68	±	8.21	b		201.58	±	35.53	
Mack.	Jun.	45.51	±	9.29		74.06	±	22.65		B	339.72	±	84.13	a	B	299.04	±	68.22	a
	Jul.	48.92	±	5.79		119.31	±	49.14			75.61	±	41.46	b		258.83	±	24.24	ab
	Ago.	43.17	±	7.28		106.69	±	23.72			28.39	±	4.61	b		254.67	±	47.24	ab
	Sep.	40.89	±	5.18		111.29	±	43.10			31.67	±	15.41	b	B	203.85	±	23.01	b
	Oct.	36.94	±	7.17		107.64	±	19.01		A	44.41	±	15.48	b		189.75	±	28.02	b

4. Effects of increased protein, histidine and taurine dietary levels on egg quality of greater amberjack

4.1 *Materials 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 (5m×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 (27° 59′ 28” N; 15° 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 20.5 and 24.5 °C (May-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 µg kg^{-1} (Fernández-Palacios *et al.*, 2015). These weekly inductions with intramuscular injections have proven to induce significantly better spawning quality than hormonal implants in greater amberjack. 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 (*Scomber scombrus*) 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 9.4.17**). 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 9.4.18**. 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.

Table 9.4.17. Ingredients and proximate composition of the experimental diets for greater amberjack broodstock.

	Diet		
	Histidine	Taurine	Protein
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 9.4.18. 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.

Therefore, each diet was tested in duplicate couples whose 10 spawns were separately studied.

Spawning quality was determined as: fertilization rate (%), egg viability rate (%), hatching and larval survival at 1 and 3 days posthatching (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, 24 h 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^{-1}) 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, 2012). Dry matter content was determined after drying the sample in an oven at 105°C until reaching constant weight, ash by combustion in a muffle furnace at 600°C 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 ºC and amino acids separated and quantified by HPLC analyser system (Pico Tag) after prederivatization with phenyl isothiocyanate (PITC) (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, using a profile projector (Mitutoyo, PJ-A3000, Japan). Eggs from each spawn were also separately incubated in 500-l tanks (50 eggs l

[1]) 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 (3 dph) 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 proposed by Blaxter and Hempel (1963): YSV=π/6 YSL x YSH2.

Normality and homogeneity of the variance of all the variables were evaluated using the Kolmogorov-Smirnov test and Levene tests respectively (Sokal and Rolf, 1996). 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).

4.2 *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 9.4.19**). Particularly, fertilization rates were significantly (P <0.01) higher when broodstock were fed a high histidine diet in comparison with the higher protein diet. 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 9.4.19. 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 9.4.20**). 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. 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 9.4.20. 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) (**Fig.9.4.19**).

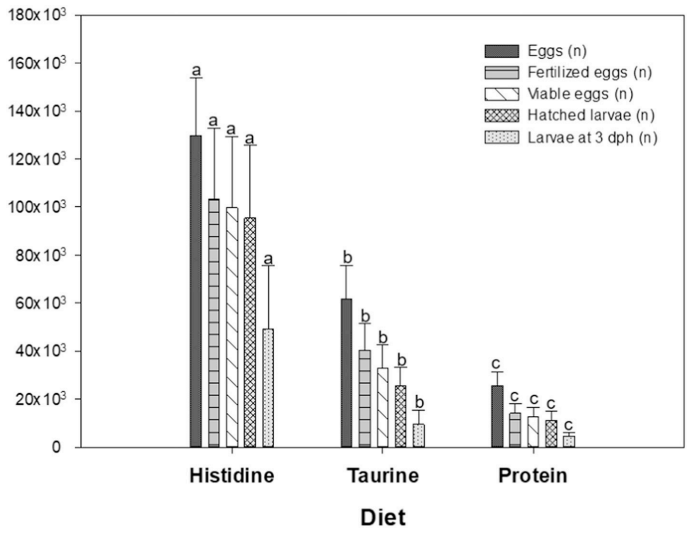


Figure 9.4.19. Production rates (per kg female and spawn) in greater amberjack after feeding with the experimental diets. Bars, of the same shade, with the different letter, were significantly different (P <0.01).

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 9.4.21**).

The amino acid composition of the fertilized eggs from broodstocks fed the different experimental diets was similar (**Table 9.4.22**). 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 9.4.23**). 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 9.4.21. 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 9.4.22. 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 9.4.23. 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).

5. Discussion

Effects of experimental diet with optimized EFA content on reproductive performance of greater amberjack

In greater amberjack, wild-caught and F1 fish, have been shown to complete gametogenesis in captivity, and spawning have been induced using different hormonal therapies (Fernández-Palacios, *et al*., 2015a; García, *et al*., 2001; Jerez *et al*., 2018; Kozul, *et al*., 2001; Lazzari, *et al*., 2000; Mylonas, *et al*., 2004).

The absence of natural spawns in F1 greater amberjack broodstock is frequent. Even after completing vitellogenesis and reaching the appropriate oocyte sizes, a considerable number of females fail to mature and ovulate, and hormonal treatments are necessary to induce spawning in captivity. The efficiency of the hormonal treatments depends on the stage of ovarian development at the time of treatment, the hormone type, the dose and the method of application and probably also on the age of fish. Captive-reared wild greater amberjack have spawned successfully after being induced with human Chorionic Gonadotropin (hCG) injections when females had vitellogenic oocytes of 550-600 µm in diameter (Díaz, *et al*., 1997; Kozul, *et al*., 2001), and after GnRHa injection (Fernández-Palacios, *et al*., 2015a) or implantation (Mylonas, *et al*., 2004) when oocytes were at 500 and 650 µm in diameter, respectively.

In assay using the 4 years old F1 fish, some of the females underwent full vitellogenesis and had larger oocytes that were appropriate to be induced for spawning during the reproductive period. In June, a high percentage of females showed oocytes larger than 500 µm in all groups. However, only a month later, the number of females that could be implanted decreased in the groups fed with the control diet (Control) and with mackerel (Mackerel) but this did not happen with the Experimental diet group. In fact, in July, only one female in the Control group had oocytes with an average size of 580 µm, and showed positive response to the successive implants, while the oocyte size of the females of Mackerel group was much lower than necessary for a positive response to the implants. In the experimental group, the diameter of the oocytes increased, after hormonal treatment, when the females showed oocytes close to 600 µm. In comparison, the study carried using wild fish in Las Palmas testing different dietary levels of EPA+DHA, showed good spawning performance during the full experimental period. Several parameters may explain this difference like fish size (the fish in the wild fish-trial were at least twice as big), origin of the fish (wild *vs* F1), effect of feeding prior to study and the hormonal treatment.

In F1 assay, both extruded diets, compared to the frozen mackerel, supplied much higher contents of fuel and nutrients, through the higher levels of proteins and fats, also providing an extra antioxidant protection through the supplementation of astaxanthin and a balance mixture of minerals and vitamins. In absolute terms both the Control and the Experimental diet also displayed higher contents of specific lipid classes and fatty acids to the developing eggs and sperm. Although the results must be taken with much caution from the youth of the fish and the very short experimental period (only 5 months) and until more analysis from the samples taken in October are performed, the analysis of the lipid profiles, either total lipid contents, lipid classes and fatty acids, confirm a more stable and consistent composition which may denote a more homogenous transfer of phospholipids, cholesterol and sterol esters but also of essential fatty acids (EFA) to the developing oocyte in the experimental fed fish, both in August and September. Even though, this finding may also be related to the number of fish with larger oocytes. Compared to the experimental and control oocytes, those from de mackerel fed fish clearly decreased the fat contents in September, also decreasing the supply of sterol esters to the oocyte. In addition, PC proportions were lower and those of PE very unstable in the mackerel oocytes. This variation of the fat contents and lipid class profiles was also evident in the control oocytes compared to those from the experimental fish. If the sampled oocytes are not at a same stage, the lipid contents and lipid classes can greatly vary which could be the case in this study. Our previous results (Zupa *et al*., 2017b) demonstrate that at early gametogenesis not only the lipid contents but also the sterol esters are lower than those of the oocytes at advanced gametogenesis or at spawning, whereas the contents of phospholipids follow the opposite trend. When compared to our previous results of wild gonads at spawning (see D3.3 and Zupa *et al*., 2017b), it can be also confirmed that the average composition of mackerel oocytes presented an excess of cholesterol and poorer levels of PC. A particularly lower content of sterol esters was also evident in September in many of the mackerel samples. Regarding fatty acid profiles, the mackerel oocytes seem to resemble the Mediterranean and Atlantic wild female gonad profiles (Rodríguez Barreto *et al*., 2012, 2014, 2017; Zupa *et al*., 2017b), at least until September, although the oocytes from the extruded diets fed-fish were also rich in DHA and ARA.

In previous trials performed with older F1 greater amberjack in Canary Islands the hormonal treatment with implants of GnRHa was successful and maintained their vitellogenic production for an extended period of time (Jerez *et al*., 2018), as it is customary for this species in the subtropical area of the Canary Islands (Fernández-Palacios, *et al*., 2015a; Jerez, *et al*., 2006). However, smaller fish in this study seemed not having sufficient reserves to maintain an optimal reproductive condition for a long period.

In this F1 assay, sperm could not be collected by abdominal pressure at any sampling time, even though the fish were in spermiation condition and sperm samples were taken using a catheter introduced into the genital pore. This is a common situation in captive male greater amberjack broodstocks (Fakriadis, *et al*., 2017; Mylonas, *et al*., 2017b; Zupa, *et al*., 2017a; Zupa, *et al*., 2017b). In addition, in captive-reared fish is very common that males produce lower amounts of sperm, often of high sperm density (Mylonas & Zohar, 2001; Zohar & Mylonas, 2001), making it difficult to obtain sperm with abdominal pressure. In this study, the mean sperm density was lower than cited previously for F1 greater amberjack broodstock (Jerez *et al*., 2018). Moreover, the mean sperm motility and motility duration increased in response to the GnRHa treatment over the course of the monitoring period only in one male of Experimental group (ID 1477) that showed an increase in both parameters after implant with a dose about 100 µg kg^{-1} body weight. The low quality of the semen in this study, compared with the previously referenced for F1 males (Jerez *et al*., 2018) could be due to the lower age and size of the fish.

Analysis of haematological and biochemical parameters in blood is a valuable tool that can be used as an effective index to monitor fish health and pathological changes. Both haematological and biochemical parameters obtained in the F1 assay were within the normal range for greater amberjack, compared to previous studies (Uyan *et al*., 2009; Dawood *et al*., 2015; Hossain *et al*., 2017). The absence of significant changes in haematological and biochemical parameters between treatment groups suggests that the physiological condition of greater amberjack broodstock seems unaffected by assayed diets. The changes detected in triglycerides and cholesterol along the spawning period are related to the mobilization of lipid reserves towards the gonad in formation (Sargent, 1995; Izquierdo *et al*., 2001). The decrease in plasma triglycerides from mackerel fed fish is in line with the diminution of triglycerides in oocytes from this group.

The use of consecutive GnRHa-delivery systems over a long reproductive period resulted in multiple spawns of fertilized and viable eggs in F1 greater amberjack fed mackerel (Jerez *et al*., 2018). However, 4 year old F1 broodstock of greater amberjack fed with different diets, including mackerel, did not spawn. The fact that the temperature has not increased in the usual way and that the fish are still young (4 years old) could be related to the absence of spawns. Therefore, this reproductive failure could be related to the age-size of the broodstock.

Effects of increased protein, histidine and taurine dietary levels on egg quality of greater amberjack

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*., 2004) 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*., 2015) 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) than for amberjack fed a commercial diet, and higher than previously reported for this species (Mylonas *et al*., 2004; 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*., 2004), 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 (*Dicentrarchus labrax*) (Rønnestad *et al*., 1998), red snapper (*Lutjanus campechanus*) (Hastey *et al*., 2010) and yellowfin seabream (*Acanthopagrus latus*) (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*., 2004; 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, 2012b), 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 (*Oreochromis niloticus*) broodstock diets up to 10 g kg^{-1} 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*., 2017). 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 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 (Mushiake *et al*., 1994; Kawabe *et al*., 1996; Watanabe *et al*., 1996; 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 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 diets 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.

6. Recommendations for greater amberjack broodstock diets

A diet containing 14-15 % EPA+DHA of total fatty acids (corresponding to 2.5-3 % in a dry diet) resulted in best spawning performance in greater amberjack broodstock obtained as wild caught juveniles. Increasing dietary EPA+DHA contents did not improve spawning performance. In contrast, there seems to be some advantages on the reproductive status of young F1 greater amberjack broodstock fed a diet with higher contents of EPA+DHA (26.5 % of total fatty acids) and ARA and lower levels of 18:2n-6 and 18:1n-9. However these findings must be tested for a longer period since no spawning was obtained.

Histidine contents in broodstock diets from 1 to 1.5%

Taurine in broodstock diets increase the reproductive performance of greater amberjack.

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8. Deviations

No deviations from the DOW

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