Doctoral Thesis

Advances in greater amberjack (*Seriola dumerili*, Risso, 1810) larval husbandry: nutrition, rearing systems and weaning protocols

Adnane Djellata 2021

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Advances in greater amberjack (*Seriola dumerili*, Risso, 1810) larval husbandry: nutrition, rearing systems and weaning protocols

Adnane Djellata

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PhD Supervisor:

Dr. Francisco Javier Roo Filgueira

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INFORMA,

De que la Comisión Académica del Programa de Doctorado, en su sesión de fecha .... tomó el acuerdo de dar el consentimiento para su tramitación, a la tesis doctoral titulada "**Advances in greater amberjack (*Seriola dumerili*, Risso, 1810) larval husbandry: nutrition, rearing systems and weaning protocols**" presentada por el doctorando **D. Adnane Djellata** y dirigida por el Doctor **Francisco Javier Roo Filgueira.**

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protein (*crhbp*), **(f)** *thyrotropin releasing hormone* (*trh*), **(g)** *proopiomelanocortin a* (*pomca*), **(h)** *proopiomelanocortin b* (*pomcb*), and **(i)** *steroidogenic acute regulatory protein* (*star*). Different letters indicate significant differences among co-feeding protocols at the same sampling time (P < 0.05; one-way ANOVA; Post-hoc Tukey test), whereas asterisks indicate significant differences between 40 and 48 dph sampling times in the same co-feeding protocol (P < 0.05; Student's *t*-test). ***Page 102***

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CHAPTER 1

GENERAL INTRODUCTION

1.1. Greater amberjack (*Seriola dumerili*)

1.1.1. General aspects of biology and larval rearing

Greater amberjack (*Seriola dumerili*) (Risso, 1810) (Figure 1.1) is a perciform fish from the Carangidae family (Liu, 2001). It is a marine fish species with a cosmopolitan distribution, which includes the Indo-West Pacific Ocean, the Western Atlantic Ocean, the Eastern Atlantic Ocean (from the UK to Senegal), and the Mediterranean Sea (Smith-Vaniz et al., 2015). It tends to aggregate around reefs, rocky outcrops, and shipwrecks (Harris et al., 2007). It is a fast-swimming predator feeding with a diet subjected to ontogenetic variations. Zooplankton and nekton species represent the main food items until they reach 20 cm in length. After this period, juveniles move closer to coastal areas, where they switch their diet to fish and cephalopods (FAO, 2016-2021).

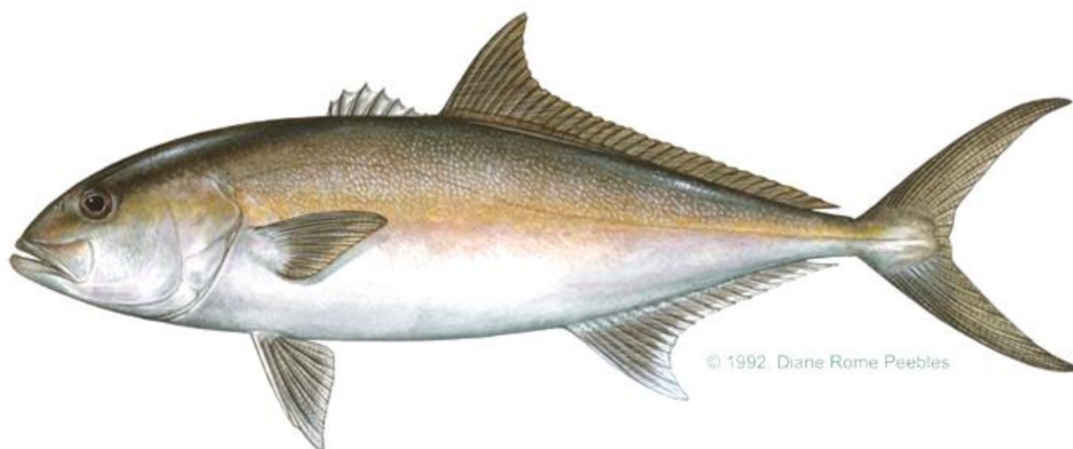


Figure 1.1. Greater amberjack (*Seriola dumerili*). Source: https://www.igfa.org

Greater amberjack is a gonochoric species, with group-synchronous ovarian development and a multiple spawning pattern, that achieves sexual maturity in males at 2 years of age (61 cm) and females at 3 years (80 cm) (Marino et al., 1995; Mandich et al., 2004). Greater amberjack eggs are spherical and transparent, with a diameter of slightly more than 1 mm and a single large oil droplet (Papandroulakis et al., 2005; Sarih et al., 2018, 2019). After hatching, greater amberjack larvae have a small size, ranging from 2.5 to 3.5 mm (Sarih et al., 2018; Pérez et al., 2020). Larvae rely on yolk reserves until 2 to 3 days post-hatching (dph) when mouth opening occurs (Pérez et al., 2020). The rearing of greater

amberjack larvae still relies largely on live feeds. The feeding protocol is based on the use of rotifers (*Brachionus sp.*) from the first feeding, followed by the use of brine shrimp (*Artemia sp.* nauplii and metanauplii) appropriately enriched when larvae increase in size and are weaned onto artificial diets at a later stage of development (Hamasaki et al., 2009; Matsunari et al., 2013; Yamamoto et al., 2013; Roo et al., 2019). Also, the nutritional requirements of the larvae have been studied to determine optimum levels of long-chain polyunsaturated fatty acids (LC-PUFAs) in enriched rotifer and *Artemia*, evaluating their effects on survival, growth, and skeleton anomalies occurrence (Matsunari et al., 2013; Roo et al., 2019). During the last years, most experiments carried out on greater amberjack larviculture followed a semi-intensive or mesocosms rearing system in indoor facilities and under controlled conditions (Lazzari et al., 2000; Papandroulakis et al., 2005; Hamasaki et al., 2009; Hirata, 2009; Miki et al., 2011; Matsunari et al., 2012a, b). The results obtained within the mesocosms system are very often better than those obtained with intensive or extensive systems (Papandroulakis et al., 2004). In the mesocosms system, weaning is completely achieved at 30 days, and post-weaned fry survival ranges between 40-90%, skeletal anomalies occurrence is around 5-10% with swim bladder inflation up to 95% and low size dispersion with low cannibalism incidence (Divanach and Kentouri, 2000). The use of mesocosms technology is very suitable for species diversification and is considered as a key tool to produce difficult marine species such as *Seriola*, Tuna, groupers, etc (Papandrolakis et al., 2005; Roo et al., 2010 a, 2014; Pérez et al., 2020).

1.1.2. Potential of greater amberjack aquaculture

Members of the genus *Seriola* have been notable species for the aquaculture production worldwide (Symonds et al., 2014; O'Neill et al., 2015; Sicuro and Luzzana, 2016), with the greater amberjack being a prominent new emerging species in the European aquaculture sector due to its excellent flesh quality, worldwide market potential, and high consumer acceptability (Nakada, 2000; Mylonas et al., 2017). The greater amberjack is one of the largest *Seriola* species, and its rapid growth and large size (i.e., the market size for greater amberjack is usually 3-5 kg and is achieved 24-36 months after hatching) (FAO, 2016-2021) make this species very suitable for product diversification and development of value-added seafood products (Jover et al., 1999). Moreover, this species shows a good acclimatation to captivity (Jerez et al., 2006; Fernández-Palacios et al., 2015; Sarih et al.,

2018). The greater amberjack could be positioned as a sustainable cultured alternative for wild tuna (Nijssen et al., 2019).

Although the aquaculture of *Seriola* species has sharply increased in recent decades, its production is constrained by the reduced number of hatcheries and grow-out installations (Yamamoto et al., 2008). Japan is the largest aquaculture producer, and most of the *Seriola* production is based on the fattening of wild-caught juveniles (FAO, 2016-2021). The farming of the greater amberjack in the Mediterranean Sea started in the 1980s, where major producers have been Spain, Italy, Malta, Croatia and Turkey, however, the commercial production of greater amberjack in Europe is still very limited (Figure 1.2) (FAO, 2016-2021). In 2018, 70 tons were produced in the United Arab Emirates and 48 tons in Spain (APROMAR, 2020). Significant greater amberjack production occurs in Japan, but the informed statistics include all Seriola spp. together. Even so, it is estimated that the Japanese production of greater amberjack is more than 30 % of the total *Seriola* spp. cultured (FAO, 2016-2021). Recently, Taiwan, Korea, China and Saudi Arabia started the cultivation. (Sicuro and Luzzana, 2016). Some studies have been published on various aspects to understand the optimal culture conditions, reproduction and nutrition of these emerging fast-growth species (Zupa et al., 2017; Navarro-Guillén et al., 2019; Roo et al., 2019; Sarih et al., 2019, 2020; Fernandez-Montero et al., 2020; Pérez et al., 2020; Barany et al., 2021). However, many parameters must be considered to achieve the implementation and accomplish greater amberjack culture.

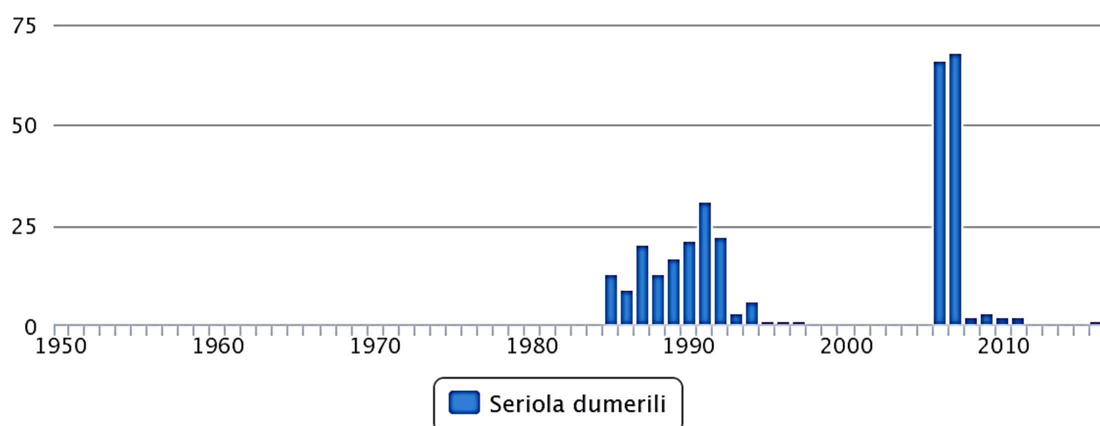


Figure 1.2. Global greater amberjack aquaculture production (tonnes) from 1950 to 2016. Source: www.FAO.com

1.1.3. Major bottlenecks in greater amberjack culture

Despite the great potential of greater amberjack for the expansion of the European Union aquaculture industry, the culture of this species is determined by several bottlenecks (Mylonas et al., 2017). In the first place, it has problems with reproduction in captivity, wild-caught greater amberjack rarely undergoes spontaneous oocyte maturation, ovulation and spawning (Mylonas et al., 2004; Zupa et al., 2017). This failure can be related to nutritional imbalances, and/or inappropriate management and culture conditions that could lead to a mismatch in the environmental factors required to promote complete gonad development or spawning (Carrillo et al., 1995; Sarih et al., 2018, 2019, 2020). Significant progress has been achieved by researchers in the last years, hormonal manipulation using exogenous gonadotropin-releasing hormone synthetic analogue has been shown to be effective in inducing maturation and multiple spawn (Mylonas et al., 2004; Fernández-Palacios et al., 2015; Sarih et al., 2018), while the occurrence of spontaneous spawning has been reported rarely (Jerez et al., 2006; Sarih et al., 2018). However, eggs and larval quality could be compromised by the hormonal induction methods and further research must be conducted to improve the spawning quality and the efficacy of the spawning induction protocols (Sarih et al., 2018). In addition, recent studies focused on enhancing the spawning quality of hormonally induced greater amberjacks by modifying broodstock nutrition, have obtained good spawning quality (Sarih et al., 2019, 2020).

Another important bottleneck related to the mass production of greater amberjack is unreliable juvenile supply due to low larval survival. Greater amberjack larvae are very vulnerable during the first stages of development and have strict requirements for biotic and abiotic conditions to survive, develop and grow properly (Mylonas et al., 2017). Larval mortality takes place a couple of days post-hatching with the start of the exogenous feeding and weaning stages, pointing to nutritional aspects as the main problem of the mortalities (Papandroulakis et al., 2005). Recent studies have shown that it is possible to obtain good eggs and larval quality in greater amberjack using adequate broodstock diets (Sarih et al, 2019, 2020). Thus, broodstock nutrition is an important variable to keep in mind for the larval quality regulating larval survival, larvae body size and growth (Izquierdo et al., 2001). For the recent development of greater culture, a more recent study has shown that adequate levels of n-3 highly unsaturated fatty acids (n-3 HUFAs) in live food can increase growth, survival and decrease skeletal anomalies occurrence in greater amberjack (Roo et al., 2019).

Also, early weaning seems to be a good solution to reduce mass mortality, however, this technique increased the aggressiveness of the larvae and led to cannibalism (Miki et al., 2011). Furthermore, the specific microdiets has not been developed yet.

The last bottleneck for the commercial production of greater amberjack is fish health. Bacterial pathogens cited in the literature as potential threats include *Photobacterium damsella* (Crespo et al., 1994) and E*pitheliocystis* (Rigos and Katharios, 2010), and *Cryptocaryon irritans* has caused severe losses in broodstock (Rigos et al., 2001). During grow out, monogenean ectoparasite, *Neobenedenia sp*. was identified in a major outbreak causing losses in both greater amberjack juveniles and breeders (Shirakashi et al., 2013). Fernández-Montero et al. (2020) manifest that *Neobenedenia girellae* can induce the important alterations in the greater amberjack epidermis independently of the skin region that explains the appearance of secondary infections and associated mortalities.

1.2. Effect of broodstock nutrition on fish larvae

Fish eggs and early larval stages rely on their nutritional reserves stored in the yolk sac and oil droplet for the supply of energy and essential nutrients, for growth, embryonic and early larval development (Sargent, 1995). It is well known that the yolk sac and oil droplet biochemical composition reflect the parental nutrient reserves, which in turn depends on the broodstock diet (Sargent, 1995; Rønnestad et al., 1998). Thus, the development of adequate diets for broodstock should be a priority in fish culture, especially in new emergent species since egg and larval quality are the basis for the successful culture of later stages of fish (Fernández-Palacios et al., 2011). However, studies on the specific effects of broodstock diet on eggs and larval quality are limited and only a few studies provide some data on nutrition during reproduction (Rodríguez-Barreto et al., 2012, 2014; Morais et al., 2014; Roo et al., 2014; Izquierdo et al., 2015; Sarih et al., 2019, 2020).

Lipid is the most studied nutrient in terms of broodstock nutrition, especially LC-PUFA. In fish larvae, these fatty acids have an important role in embryogenesis and larval development (Izquierdo et al., 2015). They are also a source of metabolic energy for the embryo and precursors of eicosanoids that regulate cell proliferation and differentiation (Izquierdo and Koven, 2011). Several studies have shown a close relationship between lipids and essential fatty acids composition of the broodstock diets and larvae quality produced by these breeders (Fernández-Palacios et al., 2011). For example, increasing dietary n-3 LC-

PUFA levels in greater amberjack broodstock diets can result in the production of large newly hatched larvae with an increased survival rate (Sarih et al., 2020). Yellowfin seabream (*Acanthopagrus latus*) broodstock fed a diet containing 6.6 % n-3 LC-PUFA, improves the percentage of buoyant eggs, hatchability and survival rate of 3 dph larvae in comparison to diets with 2.9-4.2 % n-3 LC-PUFA (Zakeri et al., 2011). Senegalese sole (*Solea senegalensis*) larvae from broodstock fed a diet fortified with LC-PUFAs and vitamins during spawning season were significantly smaller at hatching but grew faster, resulting in significantly larger juveniles than eggs produced by adults on the control diet (Morais et al., 2014). In addition, larvae from the supplemented diet had a lower incidence of caudal fin deformity (Morais et al., 2014). Replacement of ≥ 60 % fish oil in the broodstock diet with vegetable oils caused reduced growth in gilthead seabream (*Sparus aurata*) larvae 45 dph compared to common diet (Izquierdo et al., 2015).

Protein is the most abundant nutrient in fish eggs which serves as a principal energy source during the early developmental stages of fish (Fernández-Palacios et al., 2011). Furthermore, proteins and amino acids play important roles in larval growth (Hamre et al., 2013) and the contribution of adequate amounts of protein with a good balance of essential and non-essential amino acids is important for the development of eggs and larvae (Moran et al., 2007; Conceição et al., 2010). In most fish, the broodstock diet should contain at least 30-56% of protein level with a balanced composition of amino acids for improved quality of eggs and reduced rate of deformed larvae (Fernández-Palacios et al., 2011, Sarih et al., 2019). An increase in dietary protein contents from 51 to 56 % for greater amberjack broodstock lead to an increase in protein content in eggs, as well as a larger yolk sac volume (Sarih et al., 2019). A very low dietary protein level (10-20 %) results in a large percentage of deformed larvae (Gunasekera et al., 1996). Also, in green catfish (*Hemibagrus nemurus*), dietary protein levels influence gonadal maturation, fecundity and larval production (Aryani and Suharman, 2015). Thus, broodstock fed 20 % dietary protein, compared to 32 % and 37 %, displayed the lowest number of larvae (Aryani and Suharman, 2015).

Histidine and taurine are particularly important for broodstock nutrition. Sarih et al. (2019) reported that broodstock dietary supplementation of histidine improves fecundity, fertilization rates, and eggs and larval quality. Moreover, histidine is preferentially retained over other amino acids during early larval development (Costa et al., 2014). Taurine has been found particularly important for broodstock and larval nutrition (Matsunari et al., 2006, 2013; Al-Feky et al., 2016; Allon et al., 2016). Supplementation of taurine in broodstock

diets for Nile tilapia (*Oreochromis niloticus*) increases fecundity, hatching rates and weight of larvae at hatching (Al-Feky et al., 2016). The inclusion of taurine in greater amberjack broodstock diets improves spawn quality, particularly fecundity (Sarih et al., 2019). In yellowtail (*Seriola quinqueradiata*), fertilization and hatching rates were improved by the addition of at least 1 % of taurine to the broodstock diet (Matsunari et al., 2006).

1.3. Nutritional requirements of fish larvae

The determination of larval nutritional requirements is one of the most complicated aspects of fish nutrition and the lack of knowledge in these requirements is one of the main causes for high mortalities observed in marine fish larvae (Holt, 2011; Hamre et al., 2013). The requirements for a particular nutrient could be defined as the requirement for maximal growth and/or survival, where the relation fish-diet-feeding has an important effect in the determination of the quantitative needs (Izquierdo and Robaina, 2006). It is evident that better knowledge of the nutritional requirements of larvae contributes to the optimization of diets and feeding protocols and eventually improves growth rates and survival. Unlike juvenile and adult fish, the larval stage is characterized by rapid growth and different metamorphic stages, accompanied by changing nutritional requirements (Kolkovski et al., 2009; Holt, 2011; Hamre et al., 2013; Yúfera, 2018).

In the last decade, there has been significant progress in understanding the key issues in fish larval nutrition (Kolkovski et al., 2009, 2013; Holt, 2011; Hamre et al., 2013; Rønnestad et al., 2013; Yúfera, 2018). Several nutrients, such as macronutrients (proteins, lipids and/or carbohydrates) and micronutrients (vitamins and minerals) are required in the diets of fish larvae for their normal growth and development (Hamre et al., 2013). Most of the research on larval nutritional requirements has been focused on lipids requirements, particularly LC-PUFAs, such as docosahexaenoic acid (22:6n-3; DHA) and eicosapentaenoic acid (20:5n-3; EPA) due to its importance for the growth and development of larvae (Roo et al., 2009, 2019; Hamre et al., 2013; Mesa-Rodriguez et al., 2019). Inadequate content of those essential fatty acids in live prey or inert diets brings about several biological symptoms in larvae such as reduced appetite, growth, swimming activity, survival, and particularly increases skeleton anomalies occurrence (Izquierdo, 1996, 2005; Cahu et al., 2003; Roo et al., 2019). In greater amberjack, a recent study has shown that n-3 LC-PUFAs content in enriched *Artemia* should also be considered to satisfy the larval nutritional requirements (Roo et al., 2019). In that study, when larvae were fed *Artemia*, the

best growth, survival, and stress resistance were obtained with an n-3 LC-PUFAs range of 12-17 % total fatty acids, DHA content of 5-8.5 %, and EPA content of 4.3-5.5 % total fatty acids.

Dietary proteins and amino acids appear to influence the larval quality, but these components have received little attention, then lipid and fatty acids (Holt, 2011; Hamre et al., 2013; Rønnestad et al., 2013). Protein is one of the most important and expensive nutrients in feed formulations for fish larvae and inert diets designed for larval rearing containing between 50 and 70 % protein (Izquierdo and Robaina, 2006). The optimal dietary protein level for the maximum growth and survival of olive flounder larvae were suggested to be 50-55 % (Pérez et al., 1996; Ha et al., 2018). In Senegalese sole, the inert diet containing 62% crude protein supported larvae with higher growth, survival and better developmental status than the microdiet with 55% crude protein (Yúfera et al., 2005). The inclusion of hydrolyzed protein in weaning diets for European sea bass (*Dicentrarchus labrax*) and Atlantic cod (*Gadus morhua*), has been shown to improve survival (Cahu et al., 1999; Kvåle et al., 2009) and both survival and growth in common carp (*Cyprinus carpio*) (Carvalho et al., 2004).

Recent studies have shown that fish larvae require not only a certain quantitative amount of dietary protein in their feed, but they also depend on the quantity of some dietary amino acids (Hamre et al., 2013; Saavedra et al., 2015; Canada et al., 2016). In particular, histidine appears to be a limiting amino acid in enriched rotifers when they were fed to sharpsnout seabream (*Diplodus puntazzo*) larvae at 4 dph (Saavedra et al., 2007) or in live food and in inert diet tested for meagre (*Agryrosomus regius*) larvae (Saavedra et al., 2015). The amino acid that is not incorporated in proteins is often not considered in feed formulations. Taurine, a 2-aminoethanesulfonic acid that only exists in free form, is formed from methionine or cysteine via decarboxylation of cysteine sulphinic acid to hypotaurine with subsequent oxidation of the latter (Hamre et al., 2013; Salze and Davis, 2015). Larval stages of several marine fish appear to benefit from taurine supplementation through enrichment of the live prey or supplementation in inert diets (Salze and Davis, 2015). Taurine enrichment of rotifers improves growth and survival in marine fish larvae (Matsunari et al., 2013; Betancor et al., 2019). Matsunari et al. (2013) suggest that taurine enrichment of rotifers is an effective method of enhancing the growth of greater amberjack larvae. Taurine enrichment of rotifers was effective to improve the growth and survival of Atlantic bluefin tuna (*Thunnus thynnus*) larvae (Betancor et al., 2019). Also, the improvements were seen of

other species in growth and survival rates (Yamamoto et al., 2008; Xie et al., 2015; Rotman et al., 2017; Stuart et al., 2018), as well as in morphological development (Hawkyard et al., 2014), and metamorphosis and amino acid retention (Pinto et al., 2010).

1.4. Weaning strategies

Larvae of much marine fish have complete dependence on zooplankton live food during the first periods of their lives (Støttrup, 2003). Rotifers and *Artemia* are commonly used as live food in aquaculture, and standardized production procedures make them easy as larval feeds. However, a prolonged period of using live food is costly and may cause nutritional deficiency since it does not contain an adequate nutrition content for the growth and development of fish larvae (Callan et al., 2003; Ma et al., 2015). Consequently, considerable progress was done in formulated inert diet, a potential replacement of live food (Hamre et al., 2013; Estevéz et al., 2019). In this sense, it became important to adopt strategies to replace live food with inert diets, as well as to determine the ideal time to stop feeding live food.

Weaning is considered as a transition from live food to inert diets (Rosenlund et al., 1997). Currently, two different weaning strategies are used: replacement of live food with inert diets can be made abruptly, completely replacing the live food with inert diets, this method is called sudden weaning, or gradually, starting with a phase of adaptation where both types of food are provided for some time with a slow decrease in the administration of live food, usually *Artemia*, and a progressive increase of inert diets, this combined feeding method is called co-feeding strategy (Person Le Ruyet et al., 1993; Chèvre et al., 2011; Williot et al., 2011; Estévez et al., 2019). The co-feeding strategy of appropriate inert diets provides higher growth and survival than feeding solely live food or inert diets (Kolkovski, 2013), and can nutritionally precondition the larvae to accept the inert diet more easily when live food is gradually withdrawn during the weaning period (Rosenlund et al., 1997; Gisbert et al., 2018).

There is not a universal weaning strategy for fish larvae and different weaning strategies has been investigated in a variety of fishes, with a focus on the effects of the co-feeding method, duration, and start time of weaning on growth performance and survival of larvae (Faulk and Holt, 2009; Nguyen et al., 2011; Pradhan et al., 2014; Gisbert and Mozanzadeh, 2019). For example, in silver therapon (*Leiopotherapon plumbeus*), the abrupt

weaning to inert diet should be done at 26 dph to promote successful rearing of larvae in the hatchery, and the gradual weaning for 4 days from 20 dph improves the growth performance of larvae (Aya et al., 2021). Yellowtail amberjack (*Seriola lalandi)* larvae can be weaned from 21 dph (Hu et al., 2017) while meagre should be weaned at 12 dph (Campoverde et al., 2017). In addition, the success rate of weaning can be affected by the nutrient composition of formula feeds (Gisbert and Mozanzadeh, 2019) and/or the density of the live feed (Callan et al., 2003; Ballagh et al., 2010). Ballagh et al. (2010) showed that it was possible to co-feed the mulloway (*Argyrosomus japonicus*) larvae an inert diet along with a reduced amount of *Artemia* (up to 50 %) without compromising the growth, however, if *Artemia* was excluded from the diet, growth was reduced. Callan et al. (2003) indicated that similar results to live food controls could be achieved by weaning Atlantic cod larvae early while supplementing inert diet feed with reduced rations (25-50% of the live food) of *Artemia*.

Weaning is a complex physiological process that is inexorably associated with a critical period of mortality in marine fish larvae (Chen et al., 2007; Hamre et al., 2013). A good larval weaning strategy would enable the transition at inert diets at a particular time and stage when larvae can readily digest and utilize the feeds efficiently. This critical process varies among species as the gradual acceptance of food and digestive capability depend on the developmental progression of the gastrointestinal tract and its capacity to digest and absorb nutrients (Cahu and Zambonino-Infante, 2001, Gisbert and Mozanzadeh, 2019). Also, determining feeding frequency and quantity is important because the application of overfeeding can lead to water quality problems, stress, and disease outbreaks due to water cloudiness and the accumulation of uneaten feed on tank bottoms. Insufficient feed increases cannibalism and causes stress that also leads to disease outbreaks (Gisbert et al., 2018). Moreover, the best weaning strategy is based on a good balance of the combination of several parameters, such as high survival and larval growth, and low size dispersion and incidence of skeletal anomalies, having a direct impact on fingerlings quality during the on-growing phase (Gisbert et al., 2018). In addition to the above-mentioned biological variables, other parameters need also to be considered such as labour, production costs, and the use of facilities. Although weaning is still a bottleneck of most marine fish larvae, a better knowledge of nutrient requirements and digestive function have increased the growth and survival rate during this phase (Estévez et al., 2019).

1.5. Larval rearing techniques

Larval rearing is a crucial phase for the successful propagation of any marine fish species, and the range of available hatchery techniques is diverse (Roo et al., 2010a, 2014; Zouiten et al., 2011; Estévez et al., 2019). Larval rearing technologies have been classified by Divanach (1985), Divanach et al. (1998), and Van der Meeren and Naas (1997) considering factors such as larval stocking density, tank capacity, prey sources, phytoplankton addition, and water supply. Divanach (1985) was the first to classify larval rearing techniques, based on larval density and tank volume, in three main categories: extensive, intensive, and Mesocosms systems, whose larval density range from 0.1-1 larva/litre (extensive technique) to 150-200 larvae/litre (hyper-intensive technique) (Figure 1.3). In the present thesis, the Divanach (1985) terminology will be used.

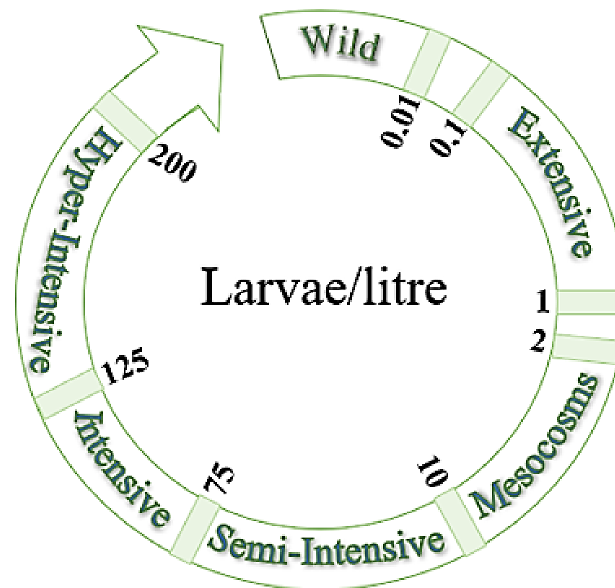


Figure 1.3. Larval rearing classification according to Divanach (1985).

1.5.1. Extensive systems

These systems are cheap and ecological based on the stimulated productivity system in which fish larvae are the upper level of the generated artificial food chain. Culture volumes are inoculated with phytoplankton and zooplankton, stocked with fish eggs or larvae at low densities (0.1-1 larva/litre). Larviculture is performed in big water volumes (hundreds or even thousands of cubic meters: ponds (Figure 1.4), coastal lagoons, or big plastic bags), and run under such natural conditions as to generate a trophic chain in order to provide the

necessary and enough flux of matter towards larvae until they reach a suitable size. Two main culture cycles are used: (i) the short one (30-60 days) providing ready-to-wean post-larvae for intensive aquaculture; and (ii) the longer one (60-180 days) providing juveniles for restocking or extensive aquaculture (Divanach and Kentouri, 1982; Houde and Lubbers, 1986).

The success of extensive techniques is usually higher than the intensive ones, providing larvae with natural biological patterns (Pitta et al., 1998) and good fry quality without skeletal deformities, swim bladder inflation abnormality, problem of pigmentation, or deviations in natural behaviour (Divanach et al., 1996).

Figure 1.4. Ancient Hawaiian fishpond (Alekoko Fishpond, USA). Source: https://www.gohawaii.com/islands/kauai/regions/lihuealekoko-menehune-fishpond

1.5.2. Intensive systems

Production in intensive systems (Figure 1.5) depends totally on man and technology which makes it expensive. They are sophisticated indoor-rearing techniques characterized by high larval densities in small well-shaped (often cylindroconical) tanks, under strict conditions such as hydrodynamics, temperature, oxygenation, photoperiod, and feeding regimes. They present three variants: semi-intensive = 25-50 larvae/litre, intensive= 75-125 larvae/litre and hyper-intensive =150-200 larvae/litre, whose complexity increases with the larval density (Divanach et al., 1998). This complexity results in consequences such as aggressive and cannibalistic behaviour (Hecht et al., 1996; Baras and Jobling, 2002), low

growth and survival rates under stressful conditions (Hernández-Cruz et al., 1999; Roo et al., 2007, 2010 a, b) as well as the occurrence of skeletal deformities (Andrades et al., 1996; Roo et al., 2005 a, b). Thus, the intensive systems are less suitable for the culture of new species due to their requirement of sophisticated facilities, high investments, and qualified manpower (Divanach et al., 2002).

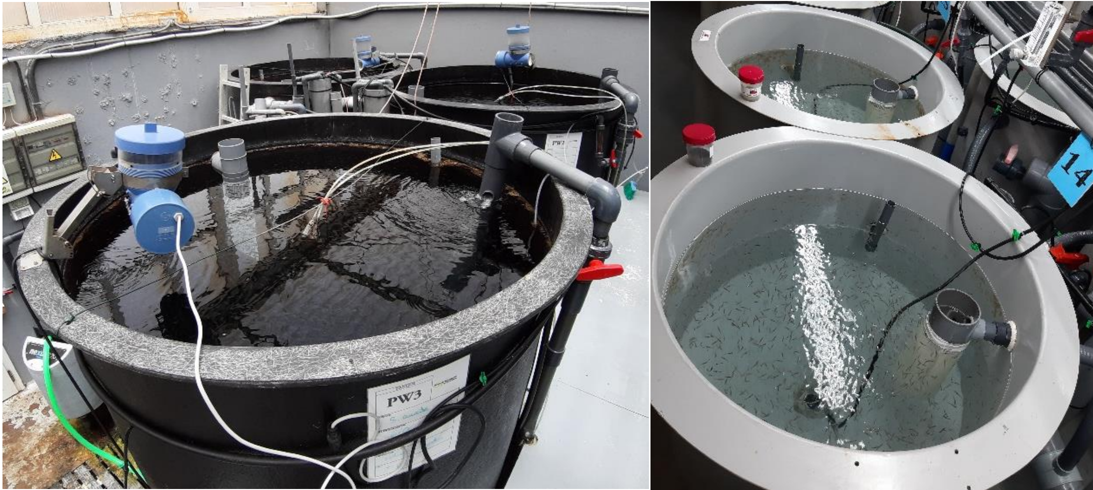


Figure 1.5. Intensive larval rearing tanks (right: 200 L; left: 2000 L) mainly used for research purposes (ECO-AQUA facility, ULPGC, Spain).

1.5.3. Semi-intensive systems

As an intermediate approach between the intensive and extensive hatchery techniques, semi-intensive system techniques, also called mesocosm technology (Figure 1.6), have been developed (Roo, 2009; Estévez et al., 2019). It requires indoor or semi-outdoor conditions. The most important characteristic is the volume of larval tanks, which should range between 30 and 100 m^3 with 1.5-2.5 m of depth. These tanks are located in well-organized aquaculture facilities, ensuring good quality and production of a high number of fry per tank (50,000 to 300,000 according to species) with high human productivity (>2 x 10^6 fry/man/year) (Roo, 2009). Both natural and artificial environmental conditions are combined during the rearing, making the method independent of any climatic seasonal changes (Roo, 2009; Estévez et al., 2019). There is a control of photoperiod and minimal control of the temperature (Estévez et al., 2019). The mesocosm methodology results in higher fry survival (between 40-90% from the initial eggs incubated after 50-70 days of rearing) and low percentage incidence of anomalies (less than 10%), while, in general, larval

growth performance is better than in the intensive Systems. This technology is applied for the rearing of several species, such as: greater amberjack, red porgy, longfin yellowtail (*Seriola rivoliana*) and European sea bass (Papandroulakis et al., 2005; Roo et al., 2010a, 2014; Zouiten et al., 2011).

Figure 1.6. Larval rearing in Mesocosms tanks (40 m^3) for research purposes (ECO-AQUA facility, ULPGC, Spain).

1.6. Objectives

Greater amberjack (*Seriola dumerili*) has been proposed as a prominent new emerging species in the European aquaculture sector due to its good biological characteristics. Although considerable progress has been made during the past years, the production of larva and juveniles is still one of the main bottlenecks for greater amberjack culture. It is evident that better knowledge of broodstock and larval feeding contributes to the optimization of diets and feeding protocols and eventually improves growth performance and survival. For that reason, the general objective of the present PhD dissertation was: Improving larval rearing and juveniles' production of greater amberjack based on nutritional optimization broodstock and larval feeds and husbandry.

To achieve this general objective, four specific objectives will be addressed:

Chapter 3: Effect of broodstock diet on the larval quality of greater amberjack (*Seriola dumerili*).

It has been well demonstrated that the composition of the broodstock diet has profound effects on the larval quality of marine fish, and many of the problems encountered by newly hatched larvae during the early rearing stages are directly related to the broodstock feeding

regime. In this sense, this study was aimed to examine the effects of supplemental taurine, histidine, and protein in broodstock diets, on larval biological performance and larval quality of greater amberjack during the first weeks of life.

Chapter 4: Comparison between intensive and semi-intensive larval rearing systems in greater amberjack (*Seriola dumerili*, Risso, 1810)

The best larval rearing protocol for commercial production of greater amberjack fingerlings is unknown. Thus, the comparison between the intensive and semi-intensive systems, for larval rearing of greater amberjack, will contribute to a better understanding of the husbandry needs of the species regarding future application in commercial production. For that reason, this study aimed to determine the most appropriate rearing technique (intensive versus semi-intensive systems) at the initial culture phases of greater amberjack to improve growth performance and survival rate.

Chapter 5: The effect of different co-feeding protocols in greater amberjack (*Seriola dumerili,* Risso 1810) larvae.

To improve larval feeding in early stages, it was proposed to minimize the use of live food trying to maximize survival in the greater amberjack larval during the weaning phase, this study had the following objective: to find out whether reducing the inclusion level of *Artemia sp.* in daily feeding protocol from 32 up to 48 dph affects growth, survival, fatty acids composition, skeleton anomalies occurrence, hepatocyte vacuolization in the liver and the expression of growth and stress-related genes. If the use of *Artemia sp.* in the larval diet can be minimized, then substantial savings in food cost, floor space, and labour can be achieved. The results obtained from this investigation will provide basic information that would be valuable in the development of hatchery techniques for greater amberjack production.

Chapter 6: Improving the larval performance of greater amberjack (*Seriola dumerili*) during the weaning phase with taurine supplementation on dry diets.

Taurine appears to be a crucial nutrient for fish, especially carnivorous marine fish such as greater amberjack. While dietary taurine supplementation has been highly recommended, there is a lack of studies on taurine for this species. In this sense, the overall objective of this study was to investigate the effect of graded levels of dietary taurine on

growth, survival rate, skeleton anomalies occurrence, and hepatocyte vacuolization of greater amberjack during the transition from live feed to microdiets.

CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1. Broodstock management and egg stocking

Rearing was undertaken in the facilities of the Grupo de Investigación en Acuicultura (GIA), located in the ECOAQUA Institute (Universidad de Las Palmas de Gran Canaria, ULPGC, Spain). All the experiments mentioned in this thesis were conducted according to the European Union Directive (2010/63/EU) on the protection of animals for scientific purposes at the ECOAQUA Institute.

Twelve greater amberjack broodstock (12.19 ± 1.35 kg and 11.79 ± 2.05 kg females and males body weight, respectively) were distributed in three 40 m^3 (5 m x 2.35 m) circular tanks (2♀ and 2♂ in each tank, sex ratio 1:1) (Figure 2.1 a). Tanks were filled with seawater (37 ‰ salinity) and kept under a natural photoperiod of approximately 13 h light. The temperature was continuously monitored (Miranda, Innovaqua, Sevilla, Spain) and ranged between 20.46 and 24.52 ºC. Gonad female maturation state was evaluated by ovarian biopsies obtained with cannulation of the genital pore using a catheter of 1.3 mm outside diameter (Figure 2.2 b) (Kruuse, Langeslov, Denmark). Oocytes were placed in Serra's solution (6:3:1, 60 % ethanol at 96 %, 30 % Formalin at 40 % and 10 % of glacial acetic acid at 96 %) to make the oocyte transparent to determine nuclear position. They were then observed in a profile projector (Mitutoyo PJ-3000A, Kanagawa, Japan) to estimate the diameter of 150 oocytes randomly selected. Females were considered eligible for spawning induction if they contained fully vitellogenic oocytes with a diameter > 600 µm. Maturation of the males was examined by the release of sperm upon application of gentle abdominal pressure. If this was not possible, a sperm sample was obtained by inserting a catheter at the opening of the genital pore. Broodstock were injected intramuscularly with gonadotrophin-releasing hormone analogue (LHRHa, des-Gly10, [D-Ala6]-; Sigma-Aldrich, St. Louis, MO, USA) at a dose of 20 µg/kg body weight, based on the reported dosage for greater amberjack (Fernández-Palacios et al., 2015). Eggs produced by broodstock groups were collected every morning and the quality of the eggs and larval survival were estimated following the methodology described by Fernández-Palacios et al. (1995) and Sarih et al. (2018). Eggs were directly stocked in the rearing tanks.

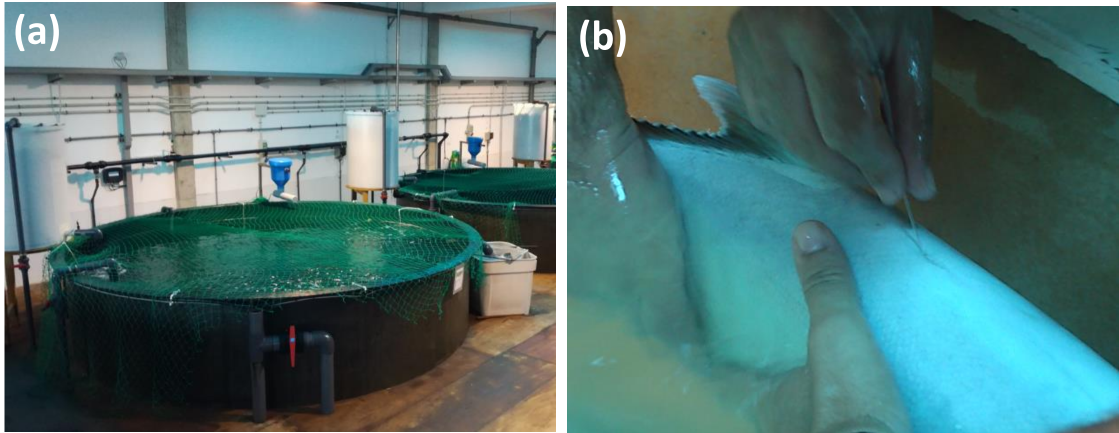


Figure 2.1. **(a)** 40 m^3 broodstock tanks used throughout the study. **(b)** Cannulation of the genital pore.

2.2. Diets and feeding

2.2.1. Broodstock diet

Broodstock diets were manufactured by Skretting ARC Feed Technology Plant (Stavanger, Norway). Three different diets, one rich in taurine, another in histidine and the third one in protein contents, were tested. The formulation and proximate composition of the diets were presented in Table 3.1, and the amino acid composition of the three diets was shown in Table 3.2 (Chapter 3).

2.2.2. Live food

2.2.2.1. Rotifer culture and enrichment

Rotifers (*Brachionus plicatilis*, L-Strain) were cultured in 1700 L cylindrical-conical tanks filled with a mixture of fresh and seawater to attain a salinity of 25 ppt. Rotifers were fed with a mixture (1:1) of lyophilized baker yeast (*Saccharomyces cerevisiae*) and dry microalgae (*Tetraselmis sp.*) at a dose of 0.4 g/10^6 rotifers. Tanks were aerated with a porous stone and oxygen was continuously monitored. Each 4 days rotifers were filtered to clean the tank and resuspended in clean water at an optimum density. Prior to being supplied to greater amberjack larvae, rotifers were enriched. The 500 L troncoconical tanks used for the enrichment contained well-aerated, filtered and UV-treated seawater (37 ‰), and the rotifers were stocked at 400 rotifers mL^{-1} and enriched with commercial Ori-Green (Skretting,

Burgos, Spain) for 3 h (Chapter 3 and 4) or with an experimental emulsion (GIA, own formula) for 12 h (chapter 5).

2.2.2.2. *Artemia* culture and enrichment

Brine shrimp (*Artemia sp.*) cysts (EG type; INVE Aquaculture, Dendermonde, Belgium) were incubated for 24 h in 150 L cylindrical-conical tanks with vigorously aerated seawater (30 g L^{-1}) at 28 ºC. These *Artemia sp.* cysts have been treated with Sep-Art Technology provides a magnetic coating on the cyst which after passing through separator tube containing passive magnets attract the iron-coated shells and obtaining clean nauplii after hatching. Newly hatched *Artemia* nauplii were harvested, washed and transferred to 100 L tank filled with clean seawater and provided with moderated aeration, oxygen supply automatically controlled to maintain 6 ppm and 28 ºC temperature. *Artemia* nauplii were enriched with Ori-Green (Skretting, Burgos, Spain) for 13 h (Chapter 3, 4 and 6) or with an experimental emulsion (GIA, own formula) for 18 h (Chapter 5), and the density of *Artemia* was 500 *Artemia*/ml.

2.2.3. Microdiets

The experimental microdiets (pellet size of 500-750 µm) were formulated and prepared at the ECOAQUA-ULPGC facilities (Chapter 6). Additionally, a commercial inert microdiet (Gemma Micro 150 and 300 µm; Skretting, France) was also used (Chapter 4 and 5). In the experimental microdiets, krill meal was the source of protein. The desired lipid content was completed with krill oil, EPA oil and ARA (arachidonic acid, 20:4n-6) oil. *Schizochytrium* was obtained from Alltech (Kentucky, USA), and *Tetraselmis* powder from the Technological Institute of the Canary Islands (Gran Canaria, Spain). The liposoluble and hydrosoluble vitamins mixture (Table 2.1) and mineral mixture (Table 2.2) were prepared as described by Teshima et al. (1982) and the attractants mixture (Table 2.3) was like that described by Kanazawa et al. (1989). Ingredients, proximal composition and fatty acid content of the experimental microdiets are shown in Tables 6.1 and 6.2 (Chapter 6).

The microdiets were prepared according to the protocol established by Liu et al. (2002). Firstly, the krill meal was mixed with water-soluble ingredients (attractants, minerals, and water-soluble vitamins). Oils and fat-soluble vitamins were mixed and blended with the powder mixture. Finally, gelatin dissolved in warm water was added to the previously mixed ingredients to form a paste that was pelleted and dried at 38 ºC for 24-48

h. The final pellets were ground and sieved to obtain several particle sizes between 500 and 750 µm.

Table 2.1. Composition of vitamins mix used to prepare experimental microdiets.

Vitamin	mg/100 g DW
Liposoluble	
Retinol acetate (Vit. A)	0.18
Ergocalciferol (Vit. D)	3.65
α-Tocopherol acetate (Vit. E)	30.00
Menadione (Vit. K)	17.28
Hydrosoluble	
Cyanocobalmin (Vit. B12)	0.03
Folic Acid (Vit. B9)	5.44
Pyridoxine-HCl (Vit. B6)	17.28
Thiamine-HCl (Vit. B1)	21.77
Riboflavin (Vit. B2)	72.53
Calcium Pantothenate (Vit. B5)	101.59
p-aminobenzoic acid (Vit. B4)	145.00
Nicotinic acid (Vit. B3)	290.16
myo-Inositol (Vit. B8)	1450.90
Ascorbic polyphosphate (Vit. C) Rovimix 20%	1000.00
Choline chloride (Vit. B5)	2965.80

Table 2.2. Composition of minerals mix used to prepare experimental microdiets.

Minerals	**mg/100 g DW**
Fe- Bioplex (15g/100g)	150.00
Mn-Bioplex (15g/100g)	12.00
$ZnSO_4.7H_2O$	200.00
Se-Selplex (0.23 g/100g)	350.00
Iodine	27.50
$ZnSO_4.7H_2O$	200.00
$Al_2(SO_4)_3.6H_2O$	0.69
$CoSO_4.7H_2O$	10.71
$MgSO_4.7H_2O$	677.55
$NaH_2PO_4.H_2O$	381.45
K_2HPO_4	758.95
$Ca(H_2PO_4).2H_2O$	671.61
$C_3H_5O_3.1/2Ca$	1617.21
NaCl	215.13

Table 2.3. Composition of attractants mix used to prepare experimental microdiets.

Attractants	**mg/100 g DW**
Arginine	1000.00
Threonine	75.00
L-Valine	250.00
Histidine	1000.00
Lysine	1000.00
L-Phenylalanine	250.00
Methionine	500.00
Glycine	500.00
DL-Alanine	500.00
Aspartic acid-phosphoserine	330.00
L-Serine	170.00
L-Tyrosine	170.00
Cysteine	500.00
Betaine	660.00
Inosine-5- monophosphate	620.00

2.2.4. Feeding protocols

In the first experiment, three different broodstock diets, one high in histidine, another in taurine and the third one in protein contents, were tested. The feeding protocol used in this experiment is presented in Figure 2.2 (Chapter 3).

In the second experiment, two experimental rearing systems: semi-intensive (SIS) using tanks of 40 m^3 capacity stocked with 10 eggs L^{-1}, and intensive (IS) using tanks of 2 m^3 capacity stocked with 75 eggs L^{-1} during the first 30 days of life. The feeding protocol used in this experiment is presented in Figure 2.3 (Chapter 4).

In the third experiment, five co-feeding protocols named 1% A, 25% A, 50% A, 75% A and 100% A, according to different initial Artemia sp. densities provided (0.02, 0.5, 1, 1.5 and 2 Artemia sp. mL^{-1} day^{-1}, respectively) were tested from larvae at 32 to 48 dph. The feeding protocol used in this experiment is presented in Figure 2.4 (Chapter 5).

In the fourth experiment, four experimental microdiets (Tau 0.24, Tau 1.24, Tau 2.24 and Tau 4.24) containing 0.24 to 4.24 % taurine levels were tested from larvae at 30 to 44 dph. The feeding protocol used in this experiment is presented in Figure 2.5 (Chapter 6).

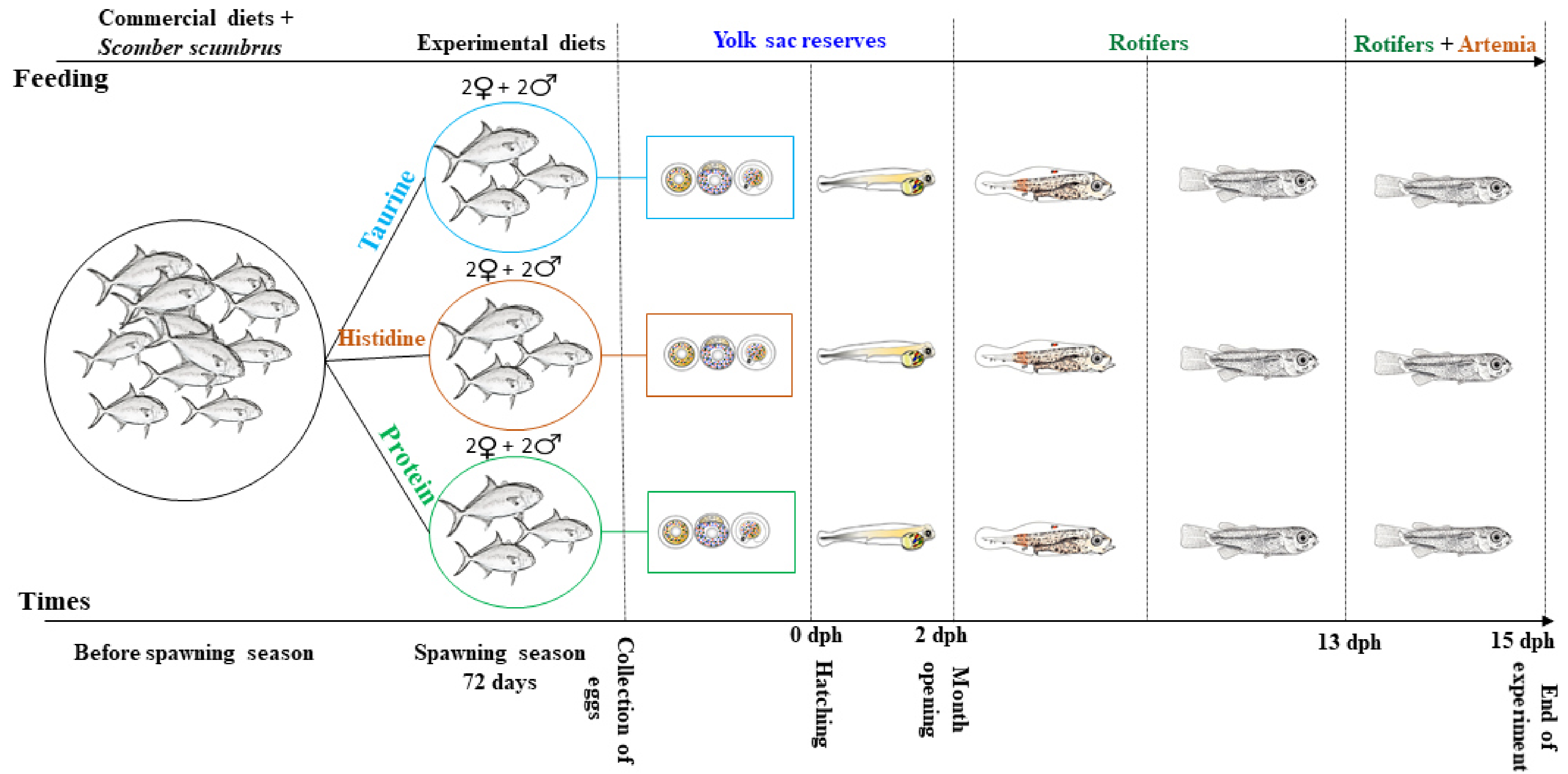


Figure 2.2. Scheme of the feeding protocol from chapter 3.

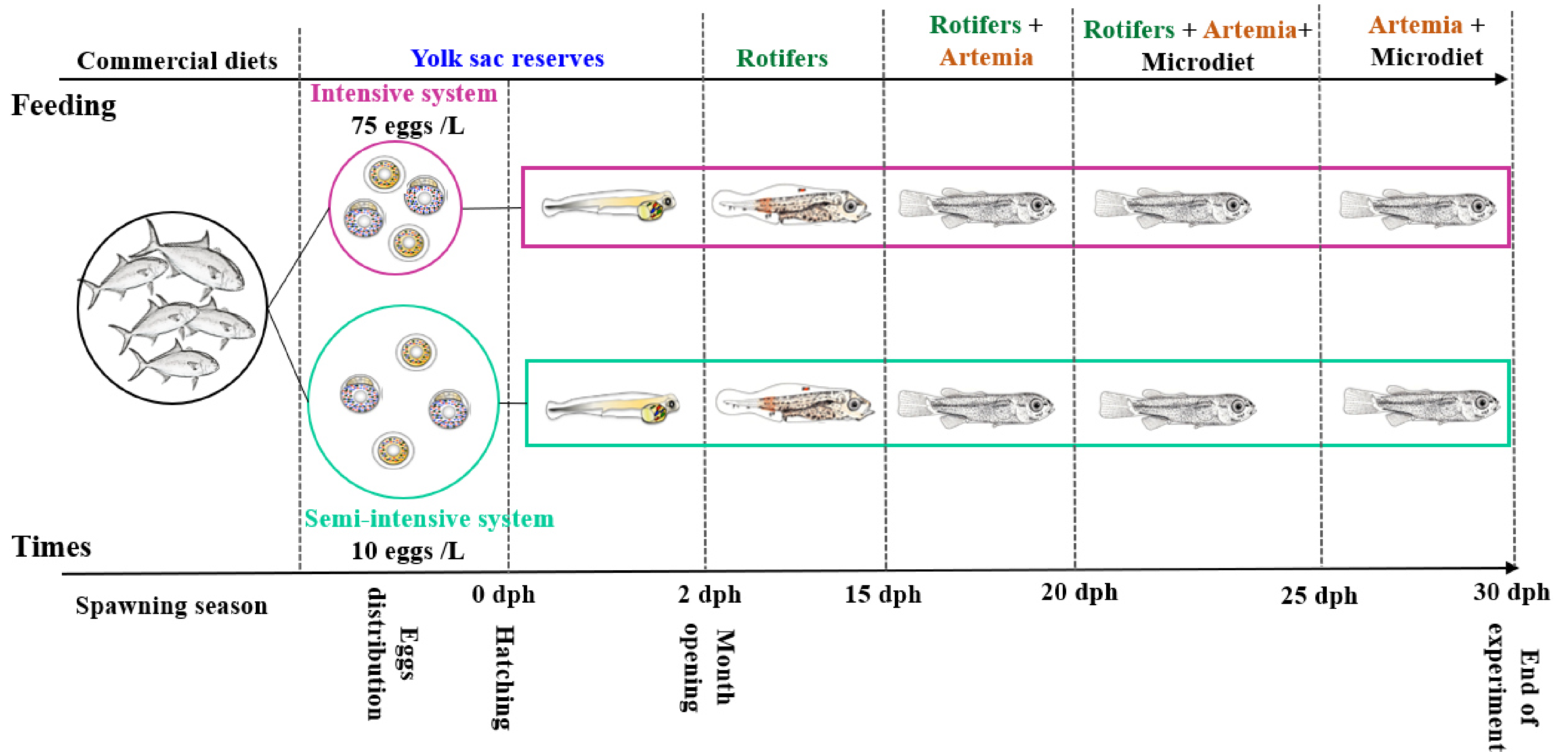


Figure 2.3. Scheme of the feeding protocol from chapter 4.

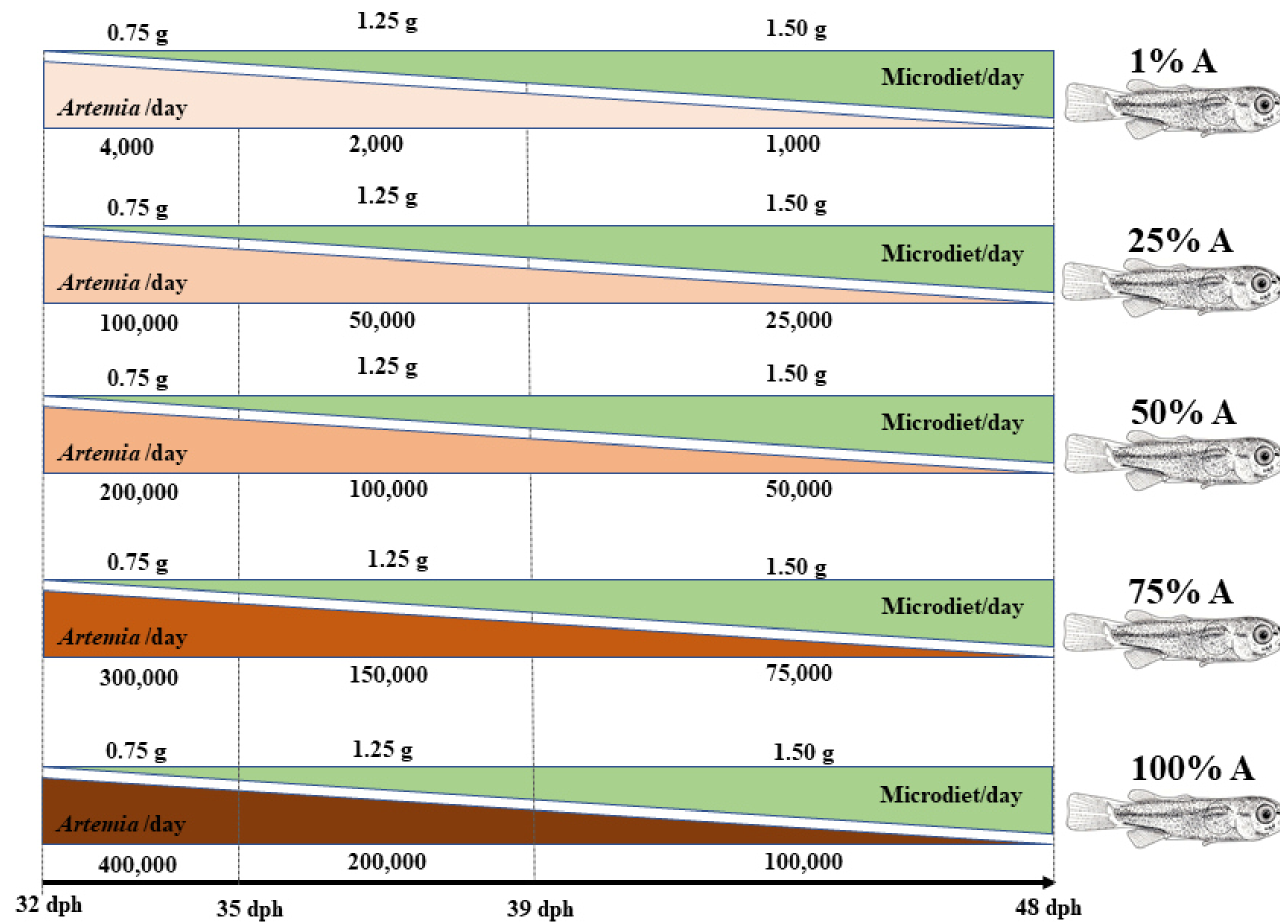


Figure 2.4. Scheme of the feeding protocol from chapter 5.

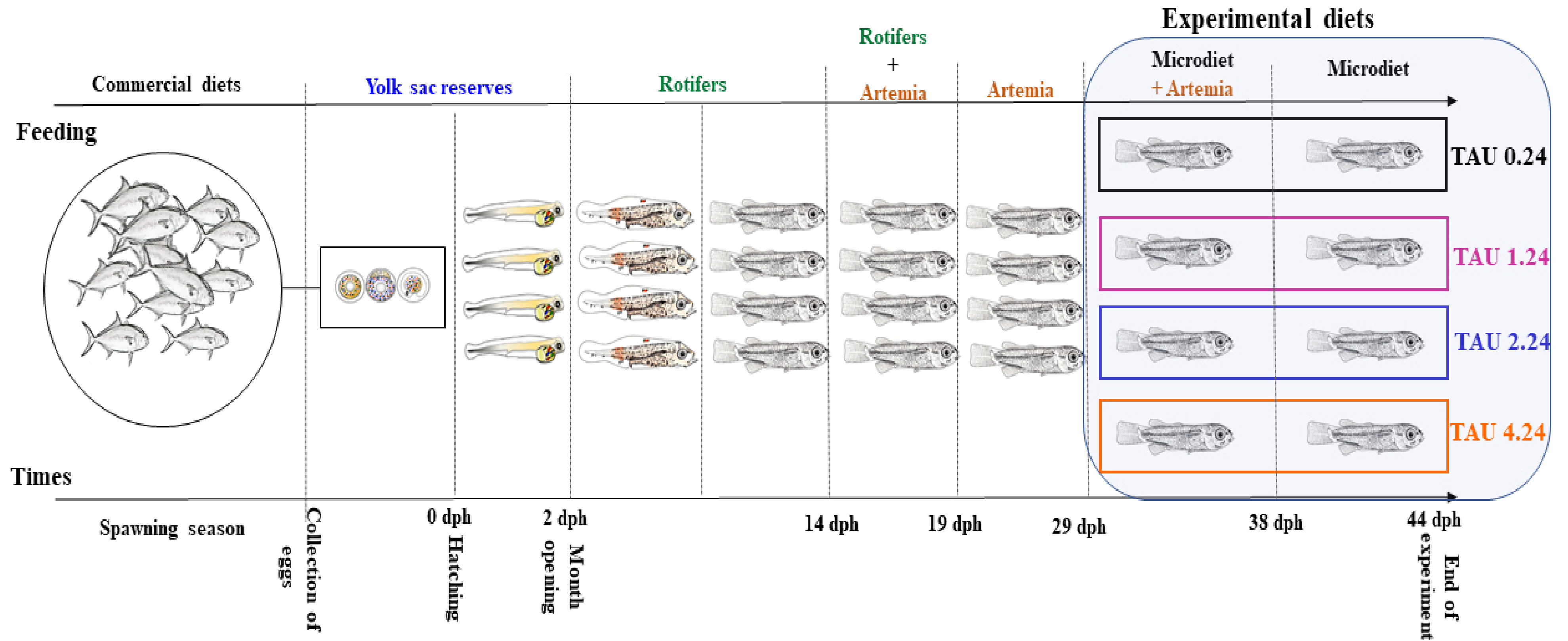


Figure 2.5. Scheme of the feeding protocol from chapter 6.

2.3. Measurements

2.3.1. Growth parameters

Egg and Oil droplet diameters were estimated from 150 eggs produced by broodstock (Chapter 3) (Figure 2.6). Larval growth was assessed by estimating the total length, dry body weight and wet body weight, in 30 randomly selected larvae per tank after the night starvation to avoid any influence of gut contents on the nutrient content of the larvae as much as possible. Larvae were previously anaesthetized with clove oil at 1% upon sampling. Individual larvae were photographed using a Leica DFC295 digital camera on a trinocular mount of a Leica M125 stereomicroscope and the images were used to measure total length employing the Leica Application Suite v.3.8.0. (Leica Microsystems, Wetzlar, Germany). All 30 larvae, previously washed with distilled water, were then weighed as a batch on an analytical balance (Gibertini Elettronia, E50 S/2, Milano, Italy). Growth parameters were calculated including specific growth rate (SGR), weight gain (WG), daily weight gain (DWG), thermal growth coefficient (TGC) and coefficient of variation (CV) of total length using the following formulae:

$$SGR = \frac{\ln(\text{Final length}) - \ln(\text{Initial length})}{\text{Duration of the experiment (days)}} \text{ x } 100$$

WG (mg) = Final weight (mg) – Initial weight (mg)

$$DWG \text{ (mg)} = \frac{\text{Final weight (mg)} - \text{Initial weight (mg)}}{\text{Duration of the experiment (days)}}$$

$$TGC = \frac{\text{Final weight}^{\frac{1}{3}}\text{(mg)} - \text{Initial weight}^{\frac{1}{3}}\text{ (mg)}}{\text{Mean water temperature (ºC)x Duration of the experiment (days)}} \text{ x } 1000$$

CV = 100*(standard deviation/mean)

Figure 2.6. Eggs greater amberjack measurement. Abbreviations: ED, Egg diameter; OD, Oil droplet diameter. Bar: 100 µm.

2.3.2. Survival and cannibalism rates

At the end of all experiments, the dead and live larvae count allowed us to calculate the survival and cannibalism rates per tank. Survival rate = 100* Nf/(Ni-Ns) and estimated cannibalism rate = 100* (Ni-Nc-Nf)/Ni, where Nf was the number of living larvae at the end of the experiment, Ns was the number of larvae sampled during the trial, Ni was the initial larvae numbers stocked in each tank, and Nc was the accumulated number of dead larvae during the trial (Cortay et al., 2019).

2.4. Biochemical analysis

Along the study, analysis of the proximate composition of feeds, fertilized eggs and larvae were performed following the methodologies described by AOAC (2016). Samples were frozen (-80 ºC) for later analysis. Amino acids profile of broodstock diets and fertilized eggs were made in the Skretting Aquaculture Research Centre (Stavanger, Norway) (Chapter 3). Taurine content and amino acids profile of the experimental microdiets were determined at the laboratory of the Eurofins Group (Santa Cruz de Tenerife, Spain) (Chapter 6). All other biochemical analyses were made at the GIA laboratories (ECOAQUA Institute, ULPGC, Spain). All the analyses were performed at least in triplicate.

2.4.1. Moisture

Moisture content was determined after drying the fresh known sample quantity (W_i) in an oven at 110 ºC until a constant weight was obtained (W_f). Before being weighted, the samples were introduced in a desiccator for 30 min to ambient temperature adaptation and finally, they were weighted to obtain final data. Moisture content was expressed as a percentage of the weight as follows:

$$Moisture\ (\%) = ((W_i - W_f) \times 100)/W_i$$

2.4.2. Ash

Ash content was determined by combustion of a well-known quantity of sample (W_m) in an oven at 600 ºC for 12 hours, remaining ashes quantity was recorded (W_c) and weighted until constant weight according to the AOAC (2016). Final ash content was performed as follows:

$$Ash\ (\%) = (100 \times W_m)/W_c$$

2.4.3. Crude protein content

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

2.4.3.1. Amino acids

Total amino acid profiles were determined according to the adapted method of the European Commission (Commission Directive 98/64/EC of 3 September 1998). The reproducibility of the results was approximately 3 %. Samples were hydrolysed with 6 N hydrochloric acid (HCl) for 24 h at 110 ºC. The hydrolysed samples were then analysed as outlined below: (a) Cystine and methionine: Oxidative hydrolysis, amino acid analyzer with ninhydrin (ISO 13903:2005; EU 152/2009). (b) Tryptophan: Alkaline hydrolysis, quantification by high-performance liquid chromatography (HPLC) techniques (ISO 13903: 2005; EU 152/2009). (c) Other Amino acids: Acid hydrolysis, amino acid analyzer with ninhydrin (ISO 13903: 2005; EU 152/2009).

2.4.4. Crude lipid content

Total crude lipids content from microdiet, live preys, whole larvae (Chapters 4 and 5), broodstock diets and fertilized eggs (Chapters 3) were extracted with a chloroform-methanol (2:1 v/v) mixture as described by Folch et al. (1957). A sample of 50-500 mg was homogenised in an Ultra Turrax (IKA-Werke, T25 BASIC, Staufen, Germany) at 11000 rpm during 5min in a solution of 5ml of Chloroform: Methanol (2:1) with 0.01% of butylated hydroxytoluene (BHT). The resulting solution was filtered by gravimetric pressure through glass wool and 0.88% KCl was added to increase the water phase polarity. After decantation, the solution was centrifuged for 5 min at 2000 rpm to separate the watery and organic phases. Once the watery phase was eliminated, the solvent was dried under a nitrogen atmosphere and subsequently, total crude lipids were weighed.

2.4.4.1. Fatty acid methyl esters

Fatty acid methyl esters (FAMEs) were obtained by transmethylation of total lipid with 1 % sulphuric acid in methanol (Christie and Han, 2010). The reaction was conducted in dark conditions under nitrogen atmosphere for 16 h at 50 ºC. Afterwards, FAMEs were extracted with hexane: diethyl ether (1:1, v/v) and purified by adsorption chromatography on NH_2 Sep-pack cartridges (Waters S.A., Massachusets, USA). FAMEs were separated by gas-liquid chromatography (GC-14A; Shimadzu, Tokyo, Japan) in a Supercolvax-10-fused silica capillary column (length:30 mm, internal diameter: 0.32 mm, Supelco, Bellefonte, USA) using helium as a carrier gas. Column temperature was 180 ºC for the first 10 min, increasing to 215 ºC at a rate of 2.5 ºC min^{-1} and then held at 215 ºC for 10 min, following the conditions described by Izquierdo et al. (1990). FAMEs were quantified by Flame ionization detector and identified by comparison to previously characterized standards and GLC-MS (Polaris QTRACETM Ultra; Thermo Fisher Scientific).

2.5. Histological analysis

Collected samples were fixed in 10 % neutral-buffered formalin until further analysis. Samples were dehydrated with an increased graded series of ethanol (70-96%), submerged in xylene, and embedded in paraffin blocks with a Histokinette 2000 tissue processor (Leica, Nussloch, Germany). Paraffin-embedded larvae were cut at 4 µm on a microtome (Mod. Jung Autocut 2055; Leica, Nussloch, Germany), fixed to the microscope slide, heated to eliminate the remaining paraffine, and finally stained with hematoxylin and

eosin (H&E) (Chapter 3, 5 and 6) or with periodic acid-Schiff (PAS) (Chapter 3) (Martoja and Martoja-Pierson, 1970). The mounted sections were examined under light microscopy using an Olympus CX41 binocular microscope (Olympus, Hamburg, Germany) connected to an Olympus XC30 camera (Olympus), which was linked to a computer using Image Capturing Software (CellB®; Olympus). Tissue morphology of hepatic was examined by two independent observers. Hepatocyte vacuolization was assessed by using a three-point scoring system where: Score 0 (normal hepatic vacuolization): The hepatocytes are small, with few small vacuoles in their cytoplasm, central positioning of their nucleus. Score 1 (moderate hepatic vacuolization): The hepatocytes are slightly enlarged, with vacuoles in their cytoplasm, or one big vacuole, and their nucleus is pushed towards the periphery in most hepatocytes. Score 2 (severe hepatic vacuolization): Almost all hepatocytes are significantly enlarged, the cytoplasm appears 'empty' due to the presence of big vacuoles and their nucleus is pushed towards the periphery. The incidence of intestinal injury was assessed by counting the number of larvae presenting any intestinal alteration (Betancor et al., 2012).

2.6. Analysis of skeletal anomalies

To identify and quantify larval skeletal anomalies, 150 larvae per tank (Chapter 4), 100 larvae per tank (Chapter 3 and 5) or 50 larvae per tank (Chapter 6) were randomly sampled, preserved in 10 % neutral-buffered formalin. The alcian blue - alizarin red double staining technique was used to stain cartilaginous and bony tissue structures (Vandewalle et al., 1998), and the larvae were examined under a Stereoscope (Leica, M125, Wetzlar, Germany) (Table 2.4).

Photos were taken using a Leica DFC295 digital camera (Leica, Wetzlar, Germany) and processed using the Leica application suite (LAS 32167, Leica, Wetzlar, Germany) to characterize skeletal anomalies occurrence by two independent observers. The incidence of skeletal anomalies was determined in the cranium, vertebral column, and caudal fin complex (Figure 2.7). Special attention was given to severe vertebral deformities, which included the fusion and compression of adjacent vertebral bodies, deformation of vertebral bodies, and changes in the anterior-posterior alignment of vertebrae (kyphosis and lordosis). The nomenclature of skeletal elements was conducted according to the method described by Boglione et al. (2014). The percentage of high-quality larvae produced per tank was

estimated as follows: Survival rate at the end of the experiment multiplied by the percentage of non-malformed larvae (Chapter 5).

Table 2.4. Cartilage-bone staining protocol.

Steps		**Time**	**Solution preparation**
Dehydration and cleaning	Ethanol 96 %	15 min	96 mL absolute ethanol 4 mL distilled water
Cartilage staining	Alcian-Blue	120 min	20 mg Alcian-blue BGX 80 mL de alcohol 95 % 20 mL glacial acetic acid
Hydration	Ethanol 95 % Ethanol 95 % Ethanol 95 %	60 min 60 min 60 min	95 mL absolute ethanol 5 mL distilled water
	Ethanol 75 %	60 min	75 mL absolute ethanol 25 mL distilled water
	Ethanol 40 %	60 min	40 mL absolute ethanol 60 mL distilled water
	Ethanol 15 %	60 min	15 mL absolute ethanol 85 mL distilled water
	Distilled Water	60 min or overnight	
Clarification	Trypsin Solution	60 min or overnight	90 mg tripsin 30 mL de $Na_2B_4O_7$-$10H_2O$ 70 mL distilled water
Bone staining	Alyzarin Red	60 min or overnight	1 g/L Alizarin Red in 0.5 % KOH
Clarification storage	Glycerin: KOH (1:3)	12-24 hours	25mL Glycerine 75mL KOH (0.5 %)
	Glycerin: KOH (1:1)	12-24 hours	50 mL Glycerine 50 mL KOH (0.5 %)
	Glycerin: KOH (3:1)	12-24 hours	75 mL Glycerine 25 mL KOH (0.5 %)
Storage	Glycerin	Storage	Pure Glycerin

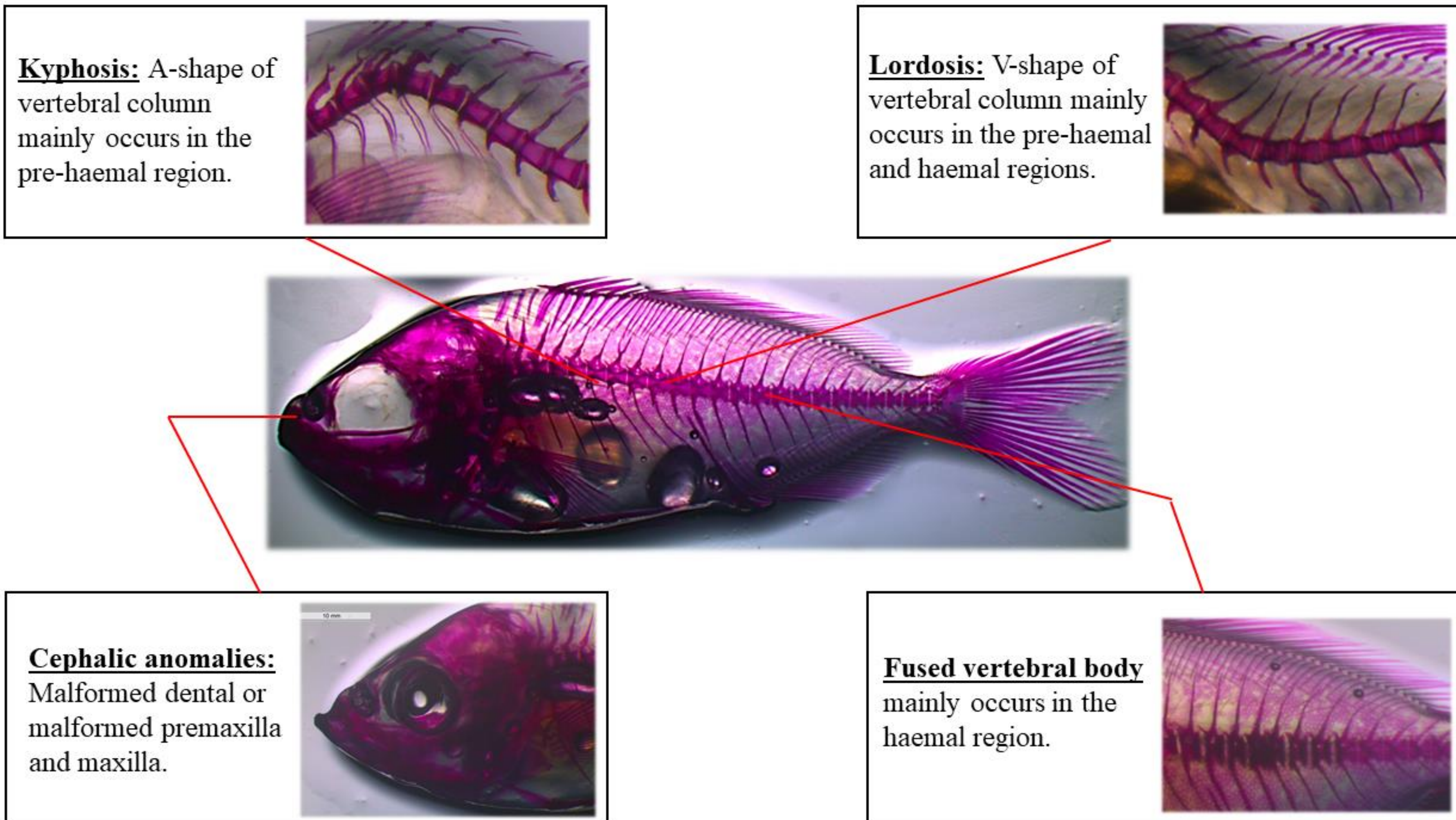


Figure 2.7. Different severe anomalies in larval greater amberjack.

2.7. Molecular analysis

2.7.1. Total RNA isolation

Whole greater amberjack larvae were taken and preserved directly in RNA Later (Sigma-Aldrich) at -80 ºC until RNA extraction and analyses. Total RNA was extracted from samples after RNAlater® (Ambion, Applied Biosystems) was eliminated using lint-free laboratory wipes, employing a Polytron PT 1200 E with a dispersing tool PT-DA 03/2EC-E050 (Kinematica AG), or an Ultra Turrax® T25 (IKA®-Werke) with a dispersing tool S25N-8G, and the NucleoSpin® kit (Macherey-Nagel). In all cases, a digestion step with RNase-free DNase was performed to eliminate or reduce the genomic DNA contamination and, finally, samples were stored at -80 °C. RNA concentration was measured with a Qubit® 2.0 fluorimeter and a Qubit™ RNA BR kit, while its quality was assessed with a Bioanalyzer 2100 and an RNA 6000 Nano kit (Agilent Technologies, LifeSciences).

2.7.2. Quantification of mRNA expression levels

Reverse transcription was performed with the qScript™ cDNA synthesis kit (Quanta BioSciences) using only samples that had an RNA integrity number (RIN) greater than 7.0 and 500 ng of total RNA. Each reaction was carried out in a volume of 20 µL, according to the manufacturer's instructions, and it was diluted 1/10th with 10 mM Tris-HCl, 0.1 mM EDTA (pH = 8) to obtain a final concentration of 2.5 ng µL^{-1}. The cDNA sequences used in this thesis were obtained from greater amberjack brain, hypophysis, liver, and kidney samples sent to Bioarray (Spain), where an RNAseq was performed by NGS mass sequencing, using the Ion Total RNA-Seq Kit v2 in the Ion Proton Sequencer from Life Technologies. The sequences were aligned with the Trinity software, annotating the transcripts with the Blast2GO software. Primers for real-time PCR were designed using Primer3 software v.0.4.0 (available at http://bioinfo.ut.ee/primer3/ (Table 1)). Two internal reference genes, *actin beta (actb)* and *eukaryotic elongation factor 1 alpha (eef1a)*, were used as internal reference genes, owing to their lower than 0.5 target stability M value and lower than 0.25 CVs. All reactions were performed in a CFX Connect™ and a CFX 96 Real-Time Detection System with BioRad CFX Maestro Software v1.1 (BIORAD Laboratories). A pool of cDNAs (CAL), derived from mixing the 5 RNA samples from the 32 dph group, was used to correct for inter-assay errors.

Before samples analyses, every primer was tested at final concentrations of 400 and 200 nM, and a temperature range of 55 to 60 ºC. Furthermore, 1:10 serial dilutions (from 10 ng to 100 fg) of cDNA were carried out to verify amplification efficiency and to produce a calibration curve. Those pairs of primers that showed an efficiency (E) between 90 and 110 %, a determination coefficient (R^2) higher than 0.980, and a calibration curve interpolating at least two points over six, were chosen for real-time PCR reactions (Table 2.5). Negative control with RNA was used to check for the presence of genomic DNA contamination, and negative control with water was used to determine the existence of artefacts such as primer-dimers. Each reaction mixture contained 0.5 µL of each specific forward and reverse primers at their best-tested concentration, 5 µL of iTaq™ Universal SYBR Green Supermix (BioRad), and 4 µL of cDNA (10 ng). Reactions were accomplished in a volume of 10 µL using Hard-Shell® Low-Profile Thin-Wall 96 White-Well Skirted PCR plates (BioRad) covered with Microseal® B Adhesive Seals (BioRad). PCRs were performed with an initial denaturation and polymerase activation at 95 ºC for 10 minutes, followed by 40 cycles of denaturation in 15 seconds at 95 ºC, annealing and extension at 60 ºC for 30 seconds and finishing with a melting curve from 60 to 95 ºC increasing 0.5 ºC every 5 seconds. Relative gene quantification was performed according to the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001), corrected for efficiencies (Pfaffl, 2001), and normalized by geometric average of the two internal control genes (Vandesompele et al., 2002).

Table 2.5. Specifications of the real-time PCR assays including forward (F) and reverse (R) primers, efficiencies (Eff) of PCR reactions, length of amplicon, and coefficient of determination (R^2).

Gene	Acronym	Eff (%)	Amplicon length (bp)	R^2	Primer sequence (5'–3')	GenBank Accession No.
actin beta	*actb*	94.2	145	0.999	F: CAGTGGTTGGCGCATACTTAT R: GAAGAGGTCACGATTGGGTTT	MW311085.1 XM_022757055.1
eukaryotic elongation factor 1 alpha	*ef1a α*	99.9	142	0.998	F: CCCTGGATCACCTTCTCTGA R: TAAGAGGCACCGTCATGTGA	MW311086.1 XM_022744048.1
corticotropin releasing hormone	*crh*	92.6	110	0.996	F: TCGGGAGATGATGGAGATGT R: TTGGCGGACTGGAAAGAG	MW311087.1 XM_022744291.1
corticotropin releasing hormone-binding protein	*crhbp*	104.2	103	0.996	F: ATGGTGGTGAGGATGGTGTC R: CGTTGTTGAGTTTGATGGTCTG	MW311088.1 XM_022769490.1
growth hormone	*gh*	100.3	105	0.998	F: AGGCGAAGAGTTGCTGAGAC R: GGAGAGAGCCGACATTTAGC	MW311089.1 XM_022769709.1
insulin-like growth factor I	*igf1*	92.7	169	0.998	F: GTCCCTCGGTGTATCTGGAA R: GCGGAGTCGTGAATGTCT	XM_022749759.1 MW311090.1
insulin-like growth factor II	*igf2*	100.1	187	0.997	F: TTTCCTCCTCCTCCATTGTG R: CACTGCCTGCTTTGTTGC	XM_022754221.1 MW311091.1
proopiomelanocortin A	*pomca*	92.5	129	0.996	F: CAAAGATGGACAGCAGCAGA R: GCTTCCCTGGCTAATGAGAA	MW311092.1 XM_022770028.1
proopiomelanocortin B	*pomcb*	98.4	147	0.996	F: CACTGCTCACGCTCTTCAAA R: AAGGACTTGTAGGCCGATCA	MW311093.1 XM_022757428.1
steroid acute regulatory protein	*star*	109.5	103	0.995	F: TCAGGCAAAGGATGGCTAAT R: TTTCTGCCACTGTTGAGTGC	MW311096.1 XM_022754985.1
thyrotropin-releasing hormone	*trh*	106.3	82	0.999	F: AGCACCCAGGTAAGCGGTAT R: GGCAGGTCTTCGTCTCCATC	XM_022739244.1 MW311097.1

2.8. Statistical analysis

The statistical analysis in this thesis was done using the IBM SPSS version 22.0 for Windows (IBM SPSS Inc. Chicago, IL, USA). A significance level of 5 % ($P < 0.05$) was used for all tests performed. Values presented were generally mean with the standard deviation (SD) if not otherwise stated. All figures were created using SigmaPlot 12.0 (Systat Software, San José, USA).

All data were tested for normal distribution with the one sample Kolmogorov–Smirnoff test, as well as for homogeneity of the variances with the Levene's test (Sokal and Rohlf, 2012). When the assumptions were correct, one-way Analysis of Variance (ANOVA) test was performed, followed by Tukey's post hoc test and/or Student's t-test for paired samples. 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.

CHAPTER 3

EFFECT OF BROODSTOCK DIET ON THE LARVAL QUALITY OF GREATER AMBERJACK (*Seriola dumerili*).

Adnane Djellata, Samira Sarih, Hipólito Fernández-Palacios, Carmen María Hernández-Cruz and Javier Roo

Abstract

Broodstock nutrition has been shown to influence the quality of eggs and larvae in marine fish. This study was carried out to evaluate the influence of supplemental taurine, histidine and protein in diets of greater amberjack broodstock on larval quality during the first 16 days of life. Broodstock were fed three different diets, one higher in taurine, another in histidine and the third one in protein contents. Egg diameter from broodstock fed higher histidine and protein levels were larger than those from broodstock fed higher taurine. At the end of the experiment, the total length and dry weight of larvae produced from broodstock fed higher taurine were greater than those fed the other diets. Also, SGR was improved in the 15 dph larvae from broodstock fed higher taurine. Nevertheless, the highest larval survival after 15 dph was obtained in larvae from broodstock fed higher histidine and protein contents. In addition, the larvae from these two experimental groups had a low incidence of skeletal anomalies. In conclusion, the results of this study have pointed out the importance of supplemental histidine and protein in broodstock diets to optimize the larval quality of greater amberjack. Besides, the study showed that taurine levels in broodstock diets increased growth larvae at 15 dph, but further studies must be conducted to determine the optimum taurine dietary levels for greater amberjack broodstock.

3.1. Introduction

The greater amberjack is one of the species selected to increase the farmed fish variety in Europe, for its fast growth rate and its high market value (Nakada, 2000). Along with its high consumer acceptance, it is currently recognized as a potential species for aquaculture diversification (Sicuro and Luzzana, 2016). The improvement in the production of greater amberjack larvae and juveniles is essential to the commercialization of this species. In recent years, considerable research effort has been undertaken in order to develop aquaculture of greater amberjack and some important improvements, mainly at larval rearing stages have been achieved in the last years (Hamasaki et al., 2009; Matsunari et al., 2013; Roo et al., 2019; Djellata et al., 2021). Different husbandry, management and feeding studies had deal with larval rearing methods in order to reduce the high mortalities occurring during the first days of life (Papandroulakis et al., 2005). However, inconsistent and relatively low larval survival remains to be a bottleneck for this species. Higher mortality observed within the

first days post-hatching when larvae are mostly dependent on yolk sac reserves and oil globule for nutrition, could be due to poor egg quality.

It has been well demonstrated that the composition of the broodstock diet has profound effects on the egg and larval quality of marine fish, and many of the problems encountered by newly hatched larvae during the early rearing stages are directly related to the broodstock feeding regime (Izquierdo et al., 2001). Also, broodstock nutrition was determinant of reproductive success in other *Seriola* species such as yellowtail amberjack (Tachihara et al., 1997) or yellowtail (Matsunari et al., 2006), where appropriate feeding during reproduction allows the production of a higher quality of eggs and good quality larvae. However, information on the nutritional requirements of greater amberjack broodstock is lacking and only very few studies provide some data on nutrition during reproduction (Rodríguez-Barreto et al., 2012, 2014; Sarih et al., 2019, 2020). These studies have demonstrated that the quality of eggs and the incorporation of essential nutrients into the eggs depends on the availability of these nutrients in the broodstock diets.

Among the different nutritional factors affecting eggs and larvae quality, dietary protein constitutes a crucial nutrient (Zakeri et al., 2014; Navarro-Guillén et al., 2019). Proteins and amino acids play important roles in larval growth both as-deposited protein and energy source (Hamre et al., 2013). Furthermore, the nutritional protein and amino acids status of the broodstock can influence the amount and the quality of the eggs (Lahnsteiner et al., 2009; Sarih et al., 2020). Histidine is an essential amino acid for fish (NRC, 2011) with several functions including tissue formation and repair as well as maintenance of osmoregulation and myelin sheaths, it also regulates the immune system and acts as an antioxidant in fish (Rhodes et al., 2010; Remo et al., 2011; Andersen et al., 2016; Ramos-Pinto et al., 2021). Sarih et al. (2019) reported that broodstock dietary supplementation of histidine improves fecundity, fertilization rates, and egg and larval quality. Moreover, histidine is preferentially retained over other amino acids during early larval development (Costa et al., 2014), and, since live preys are deficient in histidine (Aragao et al., 2004), particularly in the larval stage of Seriola species (Yamamoto et al., 2008), supplementation through the broodstock diet must be important to prevent deficiencies in the larvae. Taurine is a beta sulfonic amino acid found in high concentrations in animal tissues, including fish (Salze and Davis, 2015). It is involved in numerous important biological functions, including antioxidation, osmoregulation, immunoregulation, gamete and egg quality, and anti-stress capability (Katakawa et al., 2016; Li et al., 2016; Gao et al., 2017; Cheng et al., 2018; Salze et al., 2019; Sarih et al., 2019). The inclusion of taurine in greater amberjack broodstock

diets improves spawn quality, particularly fecundity (Sarih et al., 2019). Also, the increase of taurine in diets of greater amberjack larvae has positive effects on growth (Matsunari et al., 2013) and taurine requirements seem to be higher during early life stages (Pinto et al., 2010; Kim et al., 2016). In this sense, this study was aimed to examine the effects of supplemental taurine, histidine, and protein in broodstock diets, on larval biological performance and larval quality of greater amberjack during the first weeks of life.

3.2. Materials and methods

3.2.1. Broodstock conditions

Twelve greater amberjack broodstock (12.19 ± 1.35 kg and 11.79 ± 2.05 kg females and males body weight, respectively) were distributed in three 40 m^3 (5 m x 2.35 m) circular tanks (2♀ and 2♂ in each tank, sex ratio 1:1) (Sarih et al., 2018). The three tanks were located at the facilities of the Grupo de Investigación en Acuicultura (GIA), located in the ECOAQUA Institute (Universidad de Las Palmas de Gran Canaria, ULPGC, Spain), with equal illumination and noise conditions. Tanks were filled with seawater (37 ‰ salinity) and kept under a natural photoperiod of approximately 13 h light. The temperature was continuously monitored (Miranda, Innovaqua, Sevilla, Spain) and ranged between 20.46 and 24.52 ºC.

Broodstock 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), and they were fed with three different diets. The diets were formulated to be one rich in taurine, another in histidine and the third one in protein. The formulation and proximate composition of the experimental diets are presented in Table 3.1, and the amino acid composition of the three diets is shown in Table 3.2. The fish were hand-fed twice a day and 5 days a week (1 % of biomass/day), and the feeding period lasted for 72 days.

Table 3.1. Ingredients and proximate composition of the experimental diets for greater amberjack broodstock.

	Diets		
	Taurine	**Histidine**	**Protein**
Raw material (%)			
Wheat[a]	18.29	17.94	11.81
Wheat gluten[b]	13.00	13.00	17.00
Fish meal[c]	44.64	45.14	48.36
Squid meal[d]	10.00	10.00	10.00
Fish oil[e]	12.50	12.47	12.18
Premix incl. vitamins & minerals[f]	0.64	0.64	0.64
Taurine[g]	0.93	0.00	0.00
Histidine HCl[h]	0.00	0.81	0.00
Proximate composition (% Dry weight)[i]			
Moisture	7.00	7.60	5.90
Crude protein	51.50	51.30	56.10
Crude lipid	18.50	17.80	18.30
Ash	8.40	8.40	8.40

[a] Wheat: Skretting, Stavanger, Norway.
[b] Wheat gluten: Cargill Nordic, Charlottenlund, Denmark.
[c] Fish meal: South American fish meal, Skretting, Stavanger, Norway.
[d] Squid meal: Skretting France.
[e] Fish oil: Skretting, Stavanger, Norway.
[f] 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).
[g] Taurine: Trouw Nutrition, The Netherlands.
[h] Histidine HCl: Kyowa Hakko, Japan.
[i] Values are reported as mean of duplicate analyses.

Table 3.2. Taurine content and amino acids profile (g/100 g total amino acids) of experimental diets for greater amberjack broodstock.

	Diets		
	Taurine	**Histidine**	**Protein**
Taurine	1.13	0.30	0.36
EAA (g/100 g dry mass)			
Arginine	2.76	2.67	2.63
Histidine	1.02	1.50	0.95
Isoleucine	1.93	1.88	1.82
Leucine	3.55	3.45	3.36
Lysine	3.09	2.90	2.84
Methionine	1.28	1.25	1.19
Phenylalanine	2.02	1.91	1.85
Threonine	1.91	1.85	1.79
Valine	2.12	2.04	2.01
NEAA (g/100 g dry mass)			
Cysteine	0.58	0.55	0.54
Tyrosine	1.40	1.30	1.25
Alanine	2.56	2.48	2.42
Aspartic acid	4.08	3.98	3.84
Glutamic acid	9.20	8.92	8.65
Glycine	2.91	2.86	2.77
Proline	2.93	2.93	2.79
Serine	2.21	2.13	2.06
Total EAA	19.68	19.45	18.44
Total NEAA	25.87	25.15	24.68
TEAA/TNEAA	0.76	0.77	0.75

EAA, essential amino acids; NEAA, non-essential amino acids.
Values are reported as mean of duplicate analyses.

3.2.2. Larval rearing

Fertilized eggs produced by different broodstock groups were collected on the same day and volumetrically counted and set into twelve black cylindro-conical tanks of 2 m^3 capacity at a density of 32 eggs/L (3 tanks per group) with soft aeration for 16 days. The rearing tanks were supplied with UV treated filtered seawater and conducted in under flow-through system with an increasing daily water exchange from 5%/hour at hatching (0 dph) to 12 %/hour at 15 dph. During the period of experimentation, the water temperature was

kept at 23.95 ± 0.35 ºC, the dissolved oxygen level was maintained around 6.03 ± 0.44 g/L and salinity 37 ‰.

The larval culture was conducted under natural photoperiod. At mouth opening, 2 dph, larvae started being fed enriched rotifers (*Brachionus plicatilis*) at a density of 10-15/mL until 15 dph. From 13 to 15 dph, enriched *Artemia sp*. (EG type; INVE Aquaculture, Dendermonde, Belgium) was provided at a density of 0.5/mL (Figure 3.1). The feeding frequency of rotifers and *Artemia* during the trial was twice a day and both live preys were enriched using Ori-Green (Skretting, Stavanger, Norway), for 3 hours at 23 ºC in the case of rotifers and 13 hours at 28 ºC in the case of *Artemia*. Green water containing live phytoplankton (*Nannochloropsis sp.*) was supplied to the rearing tanks every morning to achieve an approximate concentration of 250 000 cells/mL.

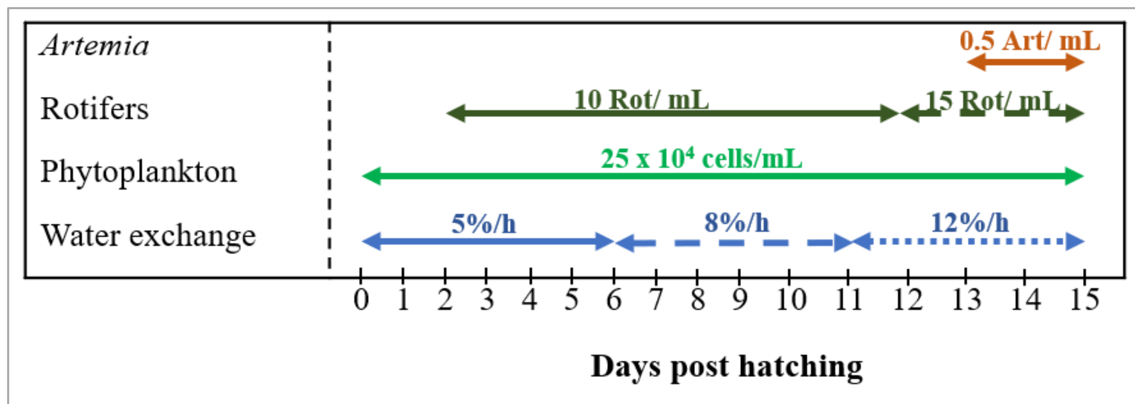


Figure 3.1. Feeding protocol used during the larval rearing.

3.2.3. Biometric analysis of eggs and larvae

Egg and oil droplet diameters were estimated from 150 eggs produced by broodstock fed different diets. Larval growth was assessed by estimating the total length and dry body weight at 0, 5, 10 and 15 dph, in 30 randomly selected larvae per tank after the night starvation. Larvae were previously anesthetized with clove oil at 1% upon sampling. Individual larvae were measured under a profile projector (Mitutoyo, PJ-A3000, Japan). All 30 larvae, previously washed with distilled water, and dried in a glass slide in an oven at 110 °C, for approximately 24 h, were then weighed as a batch on an analytical balance (Gibertini Elettronia, E50 S/2, Milano, Italy).

Specific growth rate (SGR) was calculated in relation to dry body weight using the following formula: SGR = 100*(ln (DWf) -ln (DWi))/Δt, where DWf was the final larval dry body weight (mg), DWi was the initial larval dry body weight (mg) and Δt was the time

between sampling days. The larval survival rate (expressed in percentage of larvae) was determined by hand counting of all the remaining alive larvae in each tank at the end of the experiment at 15dph.

3.2.4. Biochemical analysis

The proximate composition analysis of experimental diets, eggs, and larvae at 3 and 5 dph was performed according to the standard methods of the Association of Official Analytical Chemists (AOAC, 2016). Crude lipid was extracted following the Folch method (Folch et al., 1957) and crude protein content (N x 6.25) was determined using the Kjeldahl method. Moisture was determined after drying the samples in an oven-dried at 105 ºC. Ash content was determined by combustion at 600 ºC for 12 h. Taurine content was determined using the GB 5009.169-2016 First method and high-efficiency chromatography (EU 152/2009 (F).

Total amino acid profiles were determined according to the adapted method of the European Commission (Commission Directive 98/64/EC of 3 September 1998). The reproducibility of the results was approximately 3 %. Samples were hydrolysed with 6 N hydrochloric acid (HCl) for 24 h at 110 °C. The hydrolysed samples were then analysed as outlined below: (a) Cystine and methionine: Oxidative hydrolysis, amino acid analyzer with ninhydrin (ISO 13903:2005; EU 152/2009). (b) Tryptophan: Alkaline hydrolysis, quantification by high-performance liquid chromatography (HPLC) techniques (ISO 13903: 2005; EU 152/2009). (c) Other Amino acids: Acid hydrolysis, amino acid analyzer with ninhydrin (ISO 13903: 2005; EU 152/2009).

3.2.5. Histological analysis

For the histological study, a total of 20 larvae at 15 dph per tank were collected and fixed for 48 h in 10 % neutral-buffered formalin. Before embedding in paraffin, larvae were dehydrated in gradually increasing ethanol solutions (70 % - 100 %) and cleared with xylene. Serial sections of 4 µm were obtained with a microtome (Mod. Jung Autocut 2055; Leica, Nussloch, Germany). Sections were stained with hematoxylin and eosin (H&E) (Martoja and Martoja-Pierson, 1970) and periodic acid-Schiff (PAS).

3.2.6. Analysis of skeletal anomalies

To identify and quantify larval skeletal anomalies, 100 larvae per tank were randomly sampled at 15 dph, preserved in 10 % neutral-buffered formalin. The alcian blue-alizarin red

double staining technique was used to stain cartilaginous and bony tissue structures and the larvae were examined under a Stereoscope (Leica, M125, Wetzlar, Germany). Skeletal anomalies were determined according to the method described by Boglione et al. (2014).

3.2.7. Statistical analysis

Data are presented as mean ± standard deviation (SD). Prior to the statistical analysis, the data were checked for normality with the one-sample Kolmogorov-Smirnov test and homogeneity of variances with the Levene's test. A one-way analysis of variance (ANOVA) followed by a Tukey post hoc test. The level of significance was established at $P < 0.05$. 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).

3.3. Results

Egg diameter from broodstock fed higher histidine and protein levels were significantly larger ($P < 0.05$) than those from broodstock fed higher taurine (Table 3.3). However, oil droplet diameter was not significantly ($P < 0.05$) different among broodstock fed the different diets.

Table 3.3. Morphometric parameters of eggs from different experimental groups.

	Diets		
	Taurine	**Histidine**	**Protein**
Egg diameter (mm)	1.08 ± 0.02^{b}	1.09 ± 0.03^{a}	1.09 ± 0.02^{a}
Oil droplet diameter (mm)	0.28 ± 0.01	0.28 ± 0.02	0.28 ± 0.02

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

The results in terms of larval growth in total length and dry weight are shown in Figure 3.2. At hatch and 5 dph larvae, there was no significant difference in total length and dry weight among larvae from the different experimental groups. However, at 10 dph and 15 dph, larvae from broodstock fed higher taurine had significantly larger lengths than larvae from broodstock fed the other diets (Figure 3.2 b). Significant differences were observed in the dry weight of the larvae only at 15 dph, with the highest value in larvae from broodstock fed higher taurine (Figure 3.2 a). The same trend was observed in SGR, with the highest SGR observed in larvae from broodstock fed higher taurine (Figure 3.3).

In addition, marked mortality was observed in the first days of life, around 3-4 dph and 6-9 dph, in all experimental groups and a low larval survival rate was observed at the end of the experiment (Figure 3.4). The highest values were found in larvae from broodstock fed higher histidine (5.57 ± 0.72 %) and the lowest in larvae from broodstock fed higher taurine (3.83 ± 0.54 %). Significant differences ($P < 0.05$) were detected in larval survival between the taurine group and the other groups.

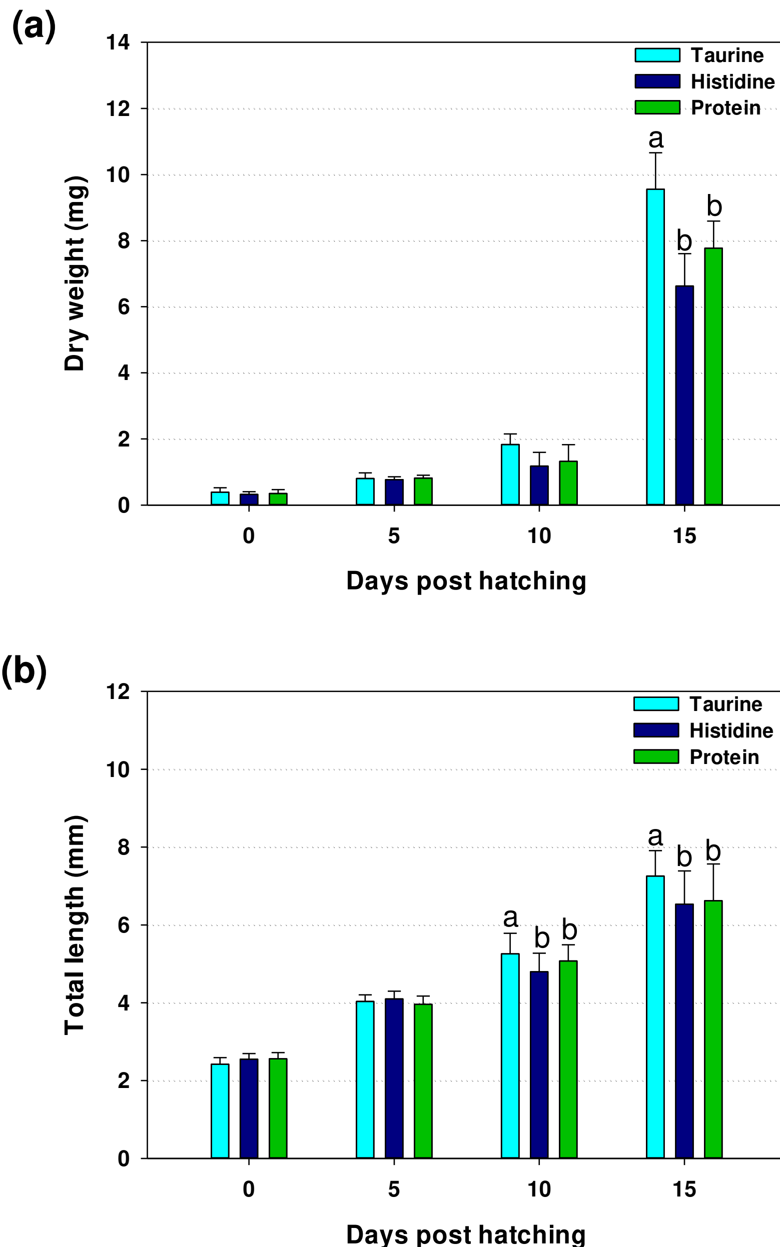


Figure 3.2. Growth parameters of greater amberjack larvae **(a)** Dry weight (mg), and **(b)** Total length (mm) from different experimental groups at 0, 5, 10 and 15 dph. Different letters denote significant differences among experimental groups ($P < 0.05$; one- way ANOVA; Post-hoc Tukey test).

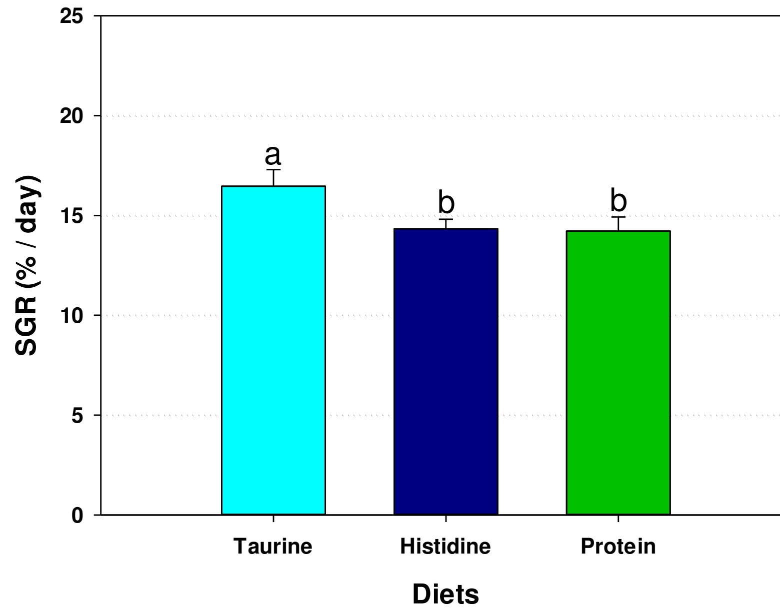


Figure 3.3. Specific growth rate (SGR, %/day) in 15 dph greater amberjack larvae from different experimental groups (means ± SD, n=3). Different letters denote significant differences among experimental groups ($P < 0.05$; one- way ANOVA; Post-hoc Tukey test).

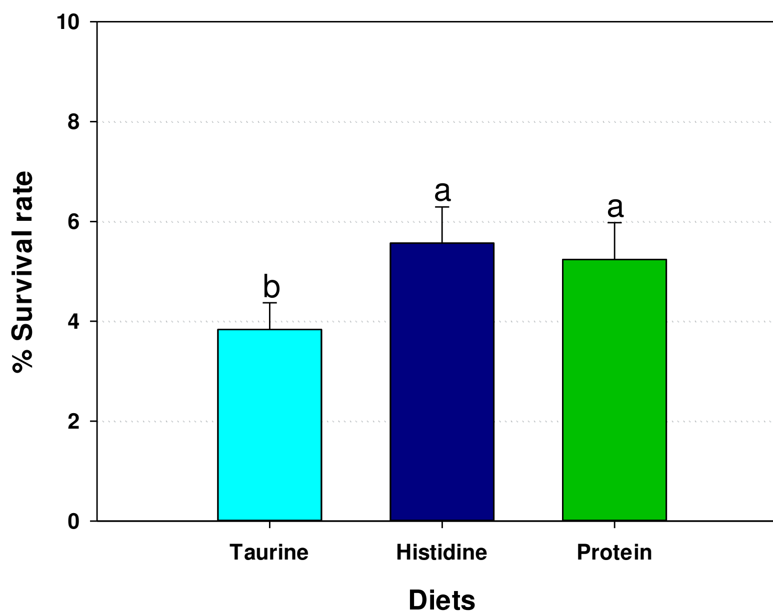


Figure 3.4. Survival rate at 15 dph greater amberjack larvae from different experimental groups (means ± SD, n=3). Different letters denote significant differences among experimental groups ($P < 0.05$; one- way ANOVA; Post-hoc Tukey test).

Crude lipid, moisture and ash contents of eggs were similar among the different experimental groups. The protein content of eggs from broodstock fed higher protein was significantly higher (P < 0.05) than eggs from broodstock fed higher histidine, which in turn showed no statistical differences in protein content than eggs from broodstock fed higher taurine (Table 3.4). There were no differences in all EAA and NEAA composition of eggs produced by broodstock fed any of the diets (Table 3.5). Regardless of the broodstock diet used, the most abundant EAA, in eggs and larvae, 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 EAA and NEAA was similar in all experimental groups. However, eggs from broodstock fed higher taurine diet contained more taurine than eggs from broodstock fed the other diets (Table 3.4). The histidine content in eggs from broodstock fed higher histidine was higher than those in eggs from the other treatments, although there were no significant differences among the treatments.

Table 3.4. Proximate composition (% dry weight) of eggs from different experimental groups.

Proximate composition	**Diets**		
	Taurine	**Histidine**	**Protein**
Moisture	83.39 ± 0.03	83.92 ± 0.04	83.98 ± 0.29
Crude protein	9.83 ± 0.55^{ab}	8.87 ± 0.76^{b}	10.18 ± 0.14^{a}
Crude lipid	4.56 ± 0.15	3.96 ± 1.26	3.74 ± 0.55
Ash	0.40 ± 0.07	0.42 ± 0.06	0.46 ± 0.03

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

The amino acid composition of the larvae at 3 dph and 5 dph from broodstock fed the different experimental diets were similar (Table 3.6). The taurine content in 3 dph and 5 dph larvae from broodstock fed a higher taurine diet were high than those from broodstock fed the other diets (Table 3.6), and they were lower than the respective taurine content in eggs (Table 3.5 and table 3.6). There is a clear decreasing trend in EAA and NEAA contents from eggs to 3 dph and 5 dph larvae.

Table 3.5. Taurine content and amino acids composition (% dry matter) of eggs from different experimental groups.

	Diets		
	Taurine	**Histidine**	**Protein**
Taurine	0.87 ± 0.01[a]	0.69 ± 0.07[b]	0.61 ± 0.09[b]
EAA (g/100 g dry mass)			
Arginine	3.71 ± 0.05	3.83 ± 0.15	3.45 ± 0.26
Histidine	1.94 ± 0.07	1.96 ± 0.03	1.85 ± 0.14
Isoleucine	3.50 ± 0.06	3.49 ± 0.07	3.28 ± 0.25
Leucine	5.25 ± 0.10	5.27 ± 0.12	4.97 ± 0.29
Lysine	4.57 ± 0.14	4.60 ± 0.04	4.24 ± 0.42
Methionine	1.71 ± 0.03	1.76 ± 0.03	1.62 ± 0.11
Phenylalanine	3.15 ± 0.06	3.09 ± 0.02	2.94 ± 0.15
Threonine	3.14 ± 0.07	3.16 ± 0.04	3.01 ± 0.16
Valine	3.78 ± 0.09	3.80 ± 0.05	3.60 ± 0.15
NEAA (g/100 g dry mass)			
Cysteine	1.08 ± 0.03	1.08 ± 0.03	1.06 ± 0.03
Tyrosine	2.19 ± 0.24	2.50 ± 0.07	1.97 ± 0.37
Alanine	4.80 ± 0.09	4.81 ± 0.03	4.51 ± 0.33
Aspartic acid	4.62 ± 0.13	4.62 ± 0.11	4.47 ± 0.20
Glutamic acid	7.93 ± 0.23	7.90 ± 0.15	7.65 ± 0.37
Glycine	2.26 ± 0.10	2.29 ± 0.12	2.19 ± 0.14
Proline	4.13 ± 0.12	4.23 ± 0.10	4.03 ± 0.09
Serine	3.45 ± 0.15	3.50 ± 0.01	3.25 ± 0.40
Total EAA	30.75 ± 0.56	30.95 ± 0.29	28.97 ± 1.91
Total NEAA	30.47 ± 0.59	30.93 ± 0.56	29.13 ± 1.82
TEAA/TNEAA	1.01 ± 0.00	1.00 ± 0.02	0.99 ± 0.01

EAA, essential amino acids; NEAA, non-essential amino acids. Values are reported as mean ± SD (n=3). Different superscripts in the same row indicate significant differences ($P < 0.05$).

Table 3.6. Taurine content and amino acids composition (% dry matter) of eggs from different experimental groups.

	Diets					
	Taurine		Histidine		Protein	
	3 dph	5 dph	3 dph	5 dph	3 dph	5 dph
Taurine	1.27[a]	1.25[a]	1.03[b]	0.90[b]	1.02[b]	0.97[b]
EAA (g/100 g dry mass)						
Arginine	3.60	3.38	3.34	2.32	3.38	2.75
Histidine	1.58	1.26	1.57	0.89	1.63	0.99
Isoleucine	2.30	2.18	2.38	1.71	2.34	2.12
Leucine	4.24	3.93	4.19	3.00	4.28	3.64
Lysine	4.31	3.90	4.19	2.78	4.39	3.20
Methionine	1.55	1.42	1.52	0.97	1.64	1.17
Phenylalanine	2.34	2.17	2.51	1.62	2.64	2.16
Threonine	2.48	2.30	2.41	1.76	2.58	2.22
Valine	2.62	2.43	2.58	1.90	2.70	2.44
NEAA (g/100 g dry mass)						
Cysteine	0.78	0.76	0.82	0.59	0.81	0.78
Tyrosine	1.76	1.47	1.34	0.58	2.03	1.33
Alanine	3.03	2.93	3.03	2.17	3.13	2.76
Aspartic acid	5.36	4.89	5.11	3.72	5.36	4.61
Glutamic acid	7.69	6.86	7.56	5.03	7.83	6.13
Glycine	2.93	2.92	2.84	2.15	2.84	2.66
Proline	2.10	2.07	2.10	1.52	2.29	2.13
Serine	2.49	2.38	2.35	1.76	2.47	2.16
Total EAA	25.02	22.97	24.69	16.95	25.58	20.69
Total NEAA	26.14	24.28	25.15	17.52	26.76	22.56
TEAA/TNEAA	0.96	0.95	0.98	0.97	0.96	0.92

EAA, essential amino acids; NEAA, non-essential amino acids. Values are reported as mean (n=3). Different superscripts in the same row indicate significant differences ($P < 0.05$).

Besides, no significant differences were observed in the hepatocyte vacuolization in the liver of larvae from broodstock fed the different experimental diets (Figure 3.5). However, in the liver of larvae from broodstock fed higher taurine, most hepatocytes are small, with few small vacuoles in their cytoplasm (Figure 3.6 a). In the other groups, the hepatocytes are enlarged, with big vacuoles in their cytoplasm (Figure 3.6 b and Figure 3.6 c). No lipid inclusions were observed in the enterocytes of the anterior intestine (not shown). However, acidophilic supranuclear inclusions were present in the enterocytes of the posterior intestine of all larvae (Figure 3.7). These acidophilic supranuclear inclusions were contained abundant proteins revealed with PAS stain (Figure 3.8).

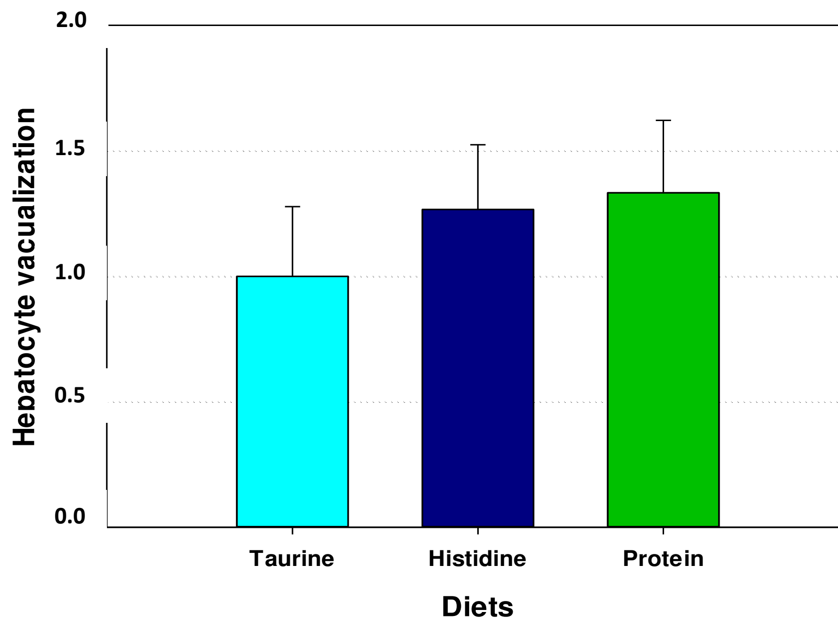


Figure 3.5. Hepatocyte vacuolization found at 15 dph greater amberjack larvae from different experimental groups (means ± SD, n=3).

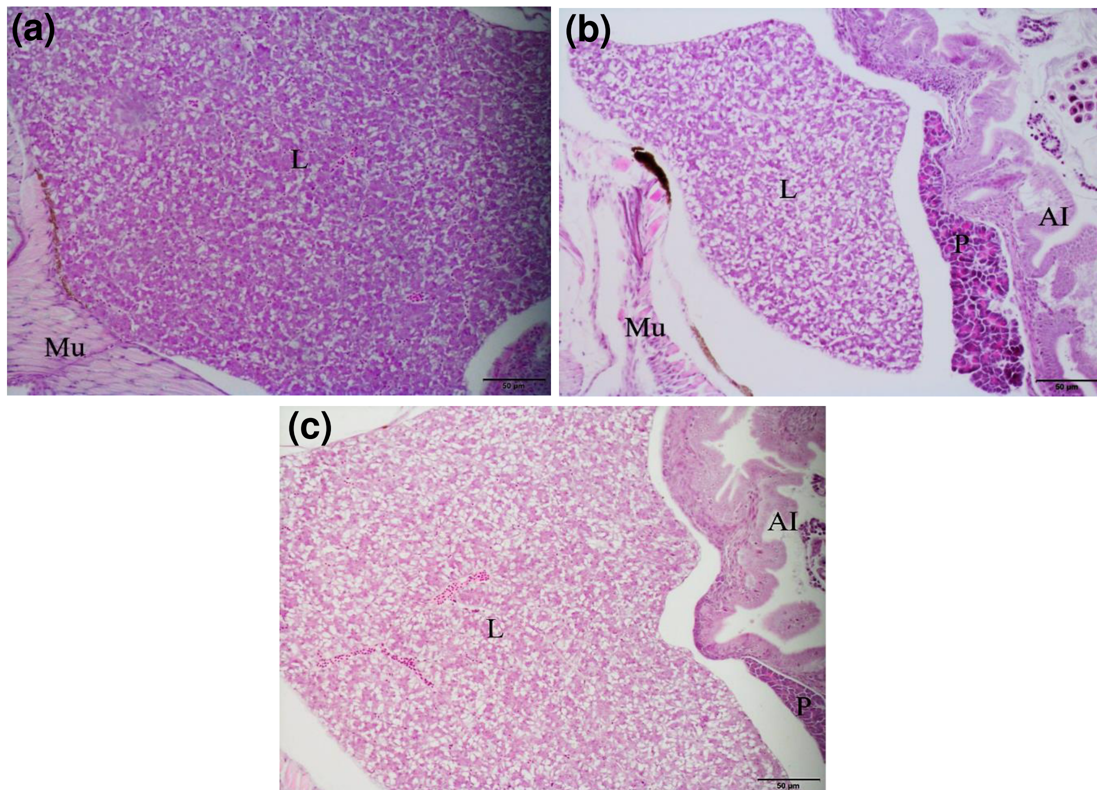


Figure 3.6. Liver sections of 15 dph greater amberjack larvae from broodstock fed: **(a)** high taurine diet. **(b)** high histidine diet. **(c)** high protein diet. Abbreviations: AI, anterior intestine; L, liver, Mu, musculature. Stain H&E.

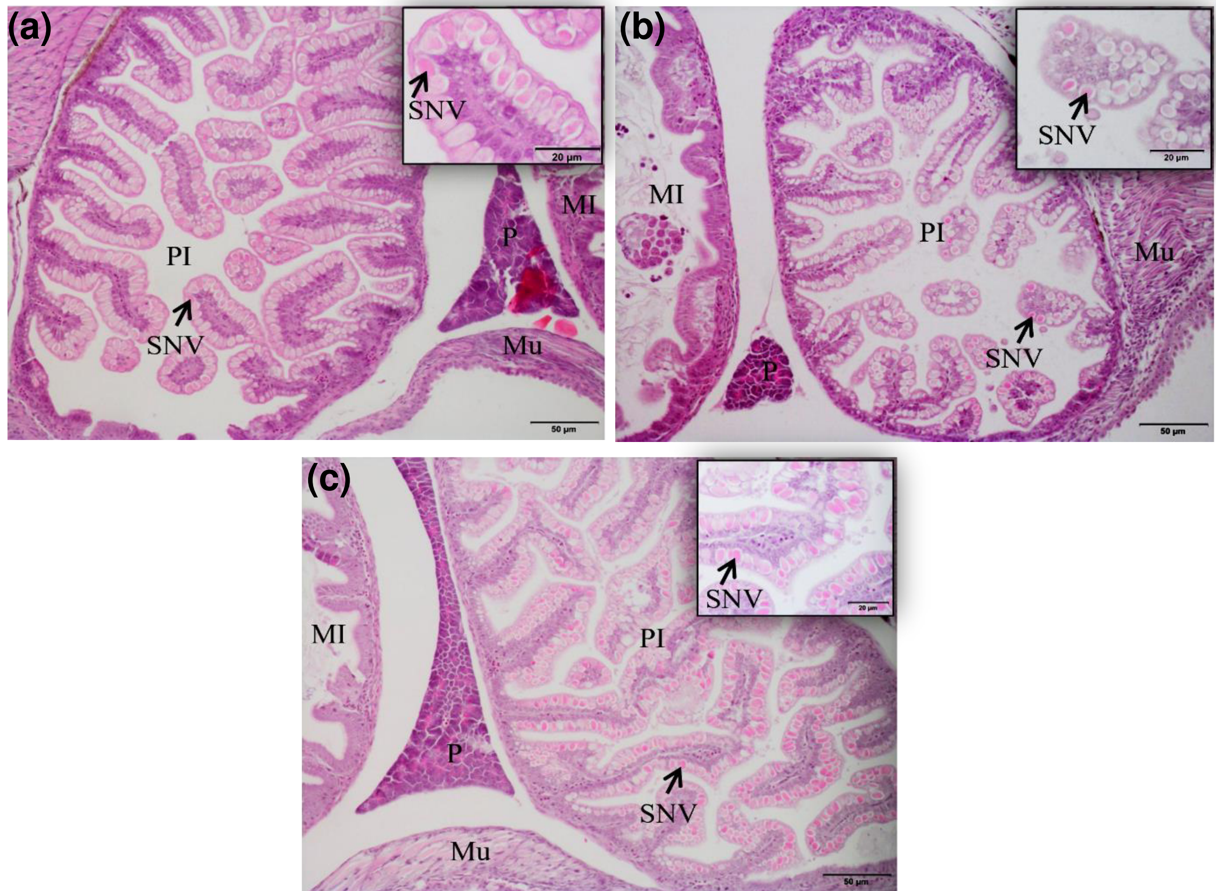


Figure 3.7. Intestine sections of 15 dph greater amberjack larvae broodstock fed: **(a)** high taurine diet. **(b)** high histidine diet. **(c)** high protein diet. Abbreviations: MI, mid-intestine; Mu, musculature; P, pancreas; PI, posterior intestine; SNV, supranuclear vacuole. Stain H&E.

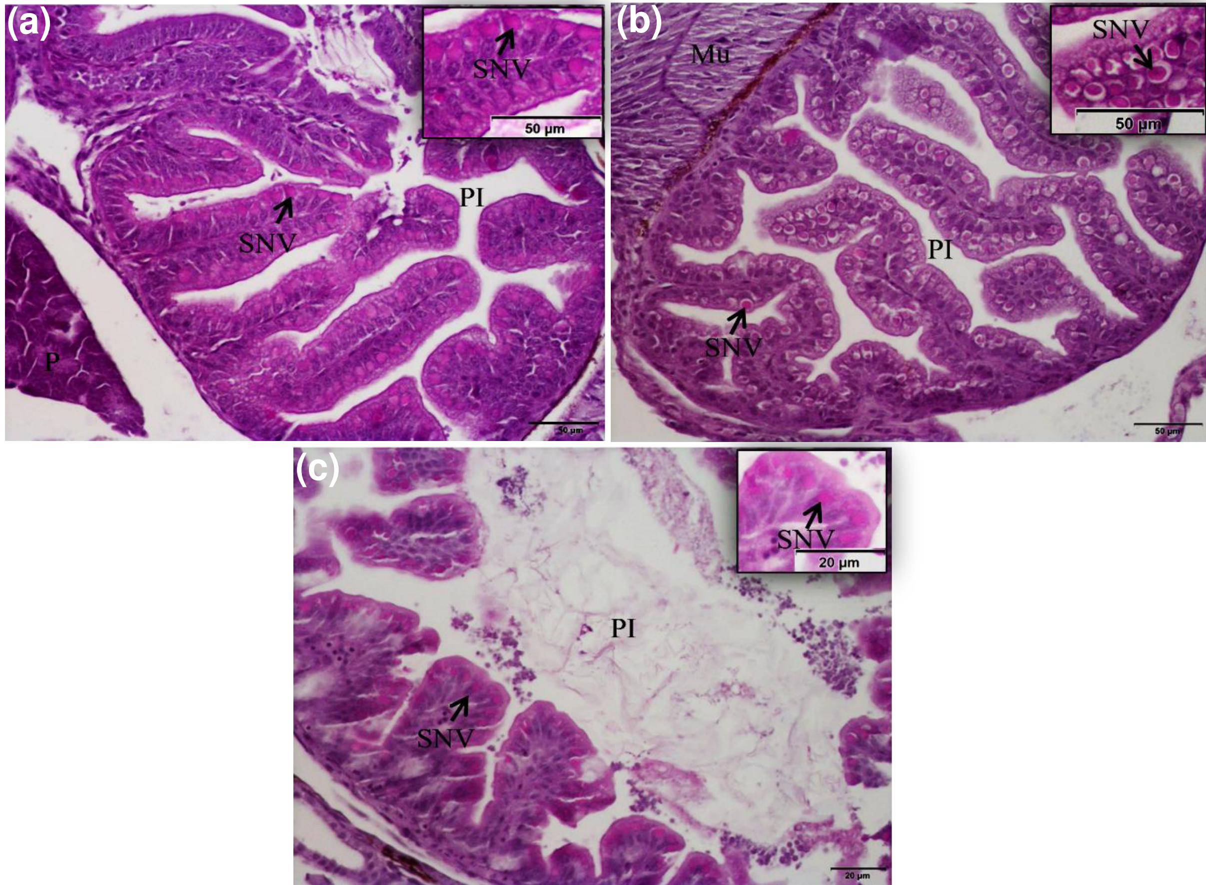


Figure 3.8. Intestine sections of 15 dph greater amberjack larvae broodstock fed: **(a)** high taurine diet. **(b)** high histidine diet. **(c)** high protein diet. Abbreviations: Mu, musculature; P, pancreas; PI, posterior intestine; SNV, supranuclear vacuole. Stain PAS.

In all the treatments, different types of deformities were observed in the vertebral body, being the malformations in the vertebrae and the neural and haemal spines the most frequent (Figure 3.9). Significant differences ($P < 0.05$) were found in the percentage of larvae with total malformations, between the larvae from broodstock fed the diet rich in taurine and the larvae from the other treatments. The same profile was found in the percentage of severe deformities, vertebral malformations, and neural and haemal spine malformations.

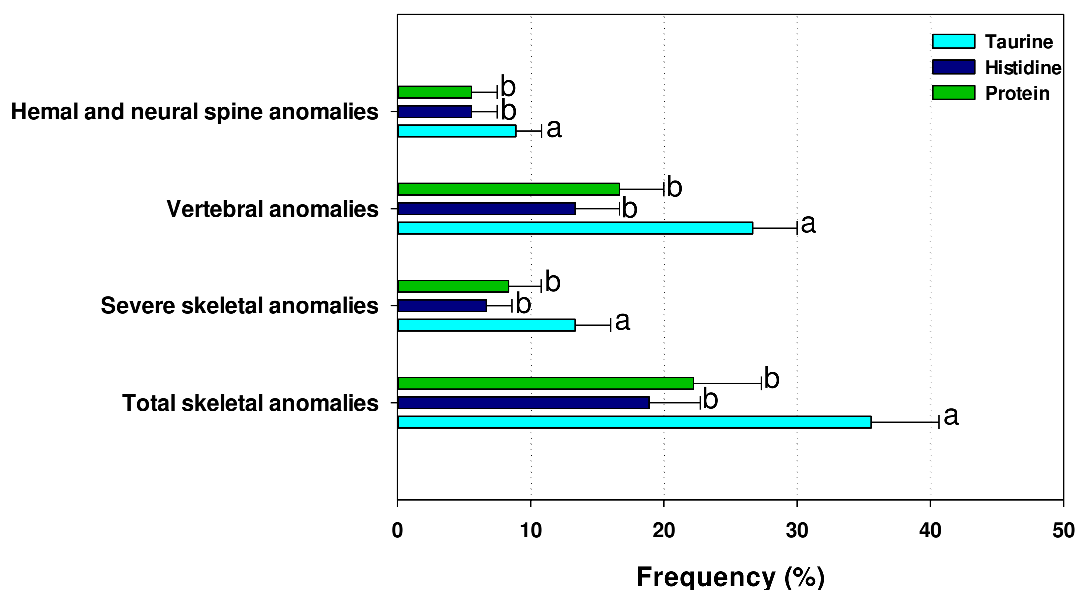


Figure 3.9. The incidence of skeletal anomalies on greater amberjack larvae from different experimental groups. Different letters denote significant differences among experimental groups (P < 0.05; one- way ANOVA; Post-hoc Tukey test).

3.4. Discussion

In the present study, the diameter of the eggs and the oil droplet from broodstock fed the different experimental diets were greater than those found in eggs of the same species, with a diameter of 1.03 ± 0.2 mm and 0.26 ± 0.01 mm for eggs and oil droplet, respectively (Papandroulakis et al., 2005). In a study on the red snapper (*Lutjanus campechanus*), they found that a larger diameter of the eggs and with higher lipid reserves is advantageous for the larvae at the beginning of exogenous feeding (Papanikos et al., 2003). One of the factors that influence the size of the eggs is the broodstock diet (Brooks et al., 1997). In our case, the eggs from the broodstock fed the diet rich in taurine had the smallest diameter. However, in yellowtail broodstock, there were no statistical differences in the diameter of eggs or oil droplet, by increasing the dietary taurine level from 0.5 to 1 % (Matsunari et al., 2006).

The best growth, at 15 dph, with respect to total length and dry weight, was observed in the larvae from broodstock fed the diet rich in taurine, this size is greater than that reported for this same species at 15 dph, with a total length of 5.5 ± 0.5 mm (Mylonas et al., 2004). In a study on the same species, the growth of larvae fed rotifers enriched with different concentrations of taurine was evaluated, and it was observed that, by increasing the proportion of taurine, the larvae grew more (Matsunari et al., 2013). In olive flounder

(*Paralichthys olivaceus*), Rotifers enriched with taurine also led to increased growth parameters (Chen et al., 2005). The higher growth observed in our study could also be attributed to the low survival rate at 15 dph, and therefore to the existence of more available food, as has been indicated in the red porgy (*Pagrus pagrus*) (Roo, 2009). Another factor to consider would be that these larvae, due to their larger size, their digestive system and metabolic capacity are more developed and functional (Izquierdo et al., 2000; Zambonino and Cahu, 2001).

Differences were observed among the different treatments, in larval survival at the end of the present experiment, the highest survival was obtained in larvae from broodstock fed higher histidine and protein. For this same species, the mean survival rates obtained by other authors were 3.6 % at 15 dph (Hamasaki et al., 2009), from 1.6 to 11.9 % at 30 dph (Yamamoto et al., 2008), 11.6 % (Shiozawa et al., 2003) and 3.5 % at 40 dph (Papandroulakis et al., 2005). Likewise, Sarih et al. (2020) observed that by increasing histidine and protein in the diets of greater amberjack broodstock, larval survival at 3 dph was increased. Marked mortality was observed in the first days of life, around 3-4 dph and 6-9 dph, in the larvae from all experimental groups, especially in the taurine group, coinciding with yolk sac absorption and the appearance of the swim bladder. During the larval culture of this species, two mortality peaks are often observed, the first one is linked to the transition from endogenous to exogenous feeding around 3-4 dph and the second one occurs at 20 dph, coinciding with the beginning of the aggressive or cannibalistic behaviour (Shiozawa et al., 2003; Yamamoto et al., 2008; Miki et al., 2011). The first peak of mortality is related to the quality of the eggs, the environmental conditions of culture, and the nutritional quality of live prey (Yamamoto et al., 2008). Taking into account that, in our experiment, the larval culture conditions were the same in all the experimental tanks, and that the larvae were fed with the same food, the marked mortality in the first days of life, of larvae from broodstock fed higher taurine, it may be related to the quality or size of their eggs, affected by broodstock feeding. In fact, in the common dentex (*Dentex dentex*), it was observed that larval survival at 3 and 5 dph is directly related to the amount of endogenous reserves that the egg contains (Giménez et al., 2006; Samaee et al., 2013). In this sense, in Atlantic cod, the size of the egg is correlated with the size of the larva, which favors its survival at the time of the first feeding (Kamler, 2008). Although in our case, the eggs from broodstock fed higher histidine or protein, which were larger in diameter, produced smaller larvae but with high survival at 15 dph. In fact, in brook trout (*Salvelinus fontinalis*), a

positive correlation was not found between egg size and survival, during embryonic development until hatching, however, it was positively correlated with larval survival during the first 50 days of exogenous feeding (Jónsson and Svavarsson, 2000). Also, in the pejerrey (*Odontesthes bonariensis*) a higher larval survival was found at 30 dph, when the eggs were larger (Chalde et al., 2014).

The larval survival rate is directly related to the quality of the yolk reserves, which in turn depends on broodstock feeding (Álvarez-Lajonchere, 2006). Thus, the lipid and protein contents in broodstock diet have been shown to play an important role in the quality of eggs and larvae (Fernández-Palacios et al., 1997; Izquierdo et al., 2001), since lipids and proteins are the main components of egg yolk (Zakeri et al., 2009). On the other hand, dietary lipids have a profound influence on the protein requirements of broodstock, and an adequate balance between dietary protein and lipids should be considered since an inadequate level of lipids lead to the use of proteins in the diet as a source of energy instead of using them for development and growth (Zakeri et al., 2009). Furthermore, when the broodstock diet contains high amounts of protein, a proportion of this latter can be used as an energy source (El-Sayed and Kawanna, 2008). In our study, significant differences were found in the protein content of the eggs, being higher in the eggs from broodstock fed higher protein. Similarly, the total protein amount in eggs is affected by the total protein content of the broodstock diet in yellowfin seabream (Zakeri et al., 2014) and Nile tilapia (El-Sayed et al., 2003; El-Sayed and Kawanna, 2008).

The essential amino acid profile of eggs is of great importance for the hatching and survival of marine fish larvae (Rønnestad et al., 1999; Moran et al., 2007). In the present study, the amino acid profiles of the eggs from all experimental groups were similar, and the main EAA of the eggs were leucine, lysine, valine, arginine, and isoleucine. The same results were found in fertilized eggs of yellowtail (Matsunari et al., 2003) and yellowtail kingfish (Moran et al., 2007). Similar results were found in other marine species such as yellowfin seabream (Zakeri et al., 2014), red snapper (Hastey et al., 2010), European sea bass (Rønnestad et al., 1998) and Atlantic halibut (*Hippoglossus hippoglossus*) (Finn et al., 2002).

The profiles of EAA and NEAA in eggs of the three experimental groups were proportional to their content in the diet. In yellowfin seabream, the concentrations of histidine, threonine, valine, and phenylalanine in the eggs showed an increasing trend, with the increase in their concentrations in the broodstock diet, and to improve the quality of

larvae, the proportion of EAA in the broodstock diet should be similar to that of eggs (Zakeri et al., 2014). The supply of adequate amounts of protein with a good balance of EAA and NEAA is important for the development of the egg and larvae (Finn, 2007; Moran et al., 2007), as observed in spawns of yellowfin seabream broodstock, where the amino acid profile of eggs and larvae of 3 dph were affected by the content of amino acids in their diets (Zakeri et al., 2014). In fact, the profile of lysine in yellowfin seabream eggs was negatively correlated with the percentage of abnormal larvae, and positively with the survival rate of 3 dph larvae (Zakeri et al., 2013). On the other hand, it has been reported in species such as turbot (*Psetta maxima*) and lemon sole *(Microstomus kitt*), that 80 % and 70 % respectively, of the free amino acids, are used in the energy metabolism of embryos and yolk-sac stage, being a key process in larval survival (Rønnestad and Fyhn, 1993).

Histidine content in eggs from broodstock fed the diet rich in histidine was higher than in eggs from the other groups, although there were no significant differences. The 10 and 15 dph larvae from these eggs were the smallest. In white seabream (*Diplodus sargus*), histidine appears to be a limiting amino acid in larval growth at 2 dph (Saavedra et al., 2006). Regarding taurine content in eggs, significant differences were observed between the group fed higher taurine and other groups. In addition, during the first stage of larval development of the different experimental groups, a decrease in taurine content in larvae was observed, similarly in the yellowtail (Matsunari et al., 2003). The role of taurine during embryonic and larval development is poorly understood, it is known that the increase in taurine in larval diets has positive effects on larval growth and survival in certain species (Salze and Davis, 2015). Its benefits on the morphological development of larvae have been reported in cobia (*Rachycentron canadum*) (Salze et al., 2011), and in Senegalese sole (Pinto et al., 2010). In this last species, an increase in the retention of amino acids in the body of the larvae was observed, when the content of taurine increased in the diets (Pinto et al., 2010). Similar results were observed in greater amberjack larvae, which improved larval growth (Matsunari et al., 2013).

An important aspect of larval growth is the development of the digestive tract, which will allow the larvae to use the food efficiently (Govoni et al., 1986). One of the biomarkers used in studies of the digestive physiology of larvae is protein and lipid inclusions in enterocytes and hepatocytes (Gisbert et al., 2008). In the present study, protein absorption was evidenced with the formation of eosinophilic supranuclear vacuoles, present in the epithelial cells of the posterior intestine of the larvae, originating from broodstock fed the

different experimental diets. In Atlantic halibut, the accumulation of large supranuclear vacuoles in the posterior intestine is due to greater absorption of proteins by enterocytes (Kvåle et al., 2007), as it happens in the common dentex (Santamaría et al., 2004).

The uptake of macromolecules by pinocytosis and their subsequent intracellular digestion in the posterior intestine is the main protein absorption mechanism in the absence of a functional stomach (Holt, 2011). Supranuclear vacuoles in the posterior intestine are seen for most of the larval period of many species (Sarasquete et al., 1995; Holt, 2011; Hernández et al., 2014), they are acidophilic and contain neutral substances, glycoproteins and proteins rich in cystine, arginine, lysine, tyrosine and cysteine (Hachero-Cruzado et al., 2009). The number and size of these vacuoles change with the growth of the larvae and are indicative of changes in the nutritional physiology of the larva, before the appearance of the gastric glands (Govoni et al., 1986; Holt, 2011). In our study, these vacuoles were very abundant in the posterior intestine of all larvae from broodstock fed the different experimental diets, suggesting that all larvae had a developed and functional digestive system (Zambonino and Cahu, 2001). Regarding lipid vacuoles in hepatocytes, a moderate presence was observed in the liver of the larvae from broodstock fed with different experimental diets. In red porgy, lipid vacuoles were present in 6-7 dph larval hepatocytes, reflecting the ability of fish larvae to store energy, and their number increased with larval growth (Andrade et al., 2015).

Different types of malformations were observed in the vertebral body in all experimental groups, with malformations in the vertebrae, and neural and haemal spines being the most frequent. In addition, larvae from broodstock fed higher taurine presented the highest percentage of severe skeletal anomalies. Greater amberjack is very susceptible to skeletal anomalies, especially in the larval stages (Roo et al., 2019; Djellata et al., 2021). The negative effects on ossification lead to abnormal bone development and malformations in the spinal column and fin rays have been observed in species fed inadequate amounts of unbalanced amino acid composition (Saavedra et al., 2009). In fact, in another study on greater amberjack larvae (Chapter 6), we observed that increasing the level of taurine to 2.24 g / 100 g of the dietary dry mass led to a low incidence of anomalies. However, the increase in dietary taurine up to 4.24 g / 100 g of the dietary dry mass led to an increase in the incidence of total severe anomalies.

In conclusion, the results of this study have pointed out the importance of supplementation histidine and protein in broodstock diets to optimize the larval performance of greater amberjack, particularly to improve survival and larval quality. Besides, the study showed that the supplementation of taurine in broodstock diets increase growth larvae and the incidence of total severe anomalies. Thus, further studies must be conducted to determine the optimum taurine dietary levels.

CHAPTER 4

COMPARISON BETWEEN INTENSIVE AND SEMI-INTENSIVE LARVAL REARING SYSTEMS IN GREATER AMBERJACK (*Seriola dumerili*, Risso, 1810)

Adnane Djellata, Samira Sarih, Carmen María Hernández-Cruz, Gonzalo Martínez-Rodríguez, Neda Gilannejad and Javier Roo

Abstract

Larviculture is a critical period during the production of greater amberjack, and it is associated with a high maortality and incidence of skeletal anomalies. Therefore, research on aspects related to this biological stage, such as the rearing systems, is very relevant to define and standardized zoothecnical conditions for this species. This study analyses the biological performance related to growth, survival, larval quality and the expression of stress and growth-related genes, in greater amberjack larvae under two different rearing systems: semi-intensive (SIS) using tanks of 40 m^3 capacity stocked with 10 eggs L^{-1}, and intensive (IS) using tanks of 2 m^3 capacity stocked with 75 eggs L^{-1}. Fertilized eggs and the corresponding larvae were reared in the two different systems until 30 days post hatching (dph). The results revealed that the survival rate and total length at 30 dph were higher in SIS system. Larvae quality under IS system was lower than in SIS system, since lower resistance to stress test and a higher incidence of skeletal anomalies was observed in IS. The expression of growth-related genes (*gh*, *igf1*and *igf2*) was increased in both rearing systems, which is consistent with the higher larval growth recorded during the trial. In addition, the expression of stress-related genes (*crh*, *crhbp* and *trh*) was upregulated in IS system. In summary, these findings revealed that rearing system and its zootechnical conditions are a crucial factor affecting greater amberjack biological performance, and suggesting SIS system as more advantageous than IS for larviculture of this species.

4.1. Introduction

Larval rearing is a crucial phase for the successful propagation of any marine fish species, and the range of available hatchery techniques is diverse (Roo et al., 2010a, 2014; Estévez et al., 2019). In marine fish species, the intensification of the rearing system appears to increase the larval mortality and the incidence of skeletal anomalies (Boglione et al., 2001; Roo et al., 2010a, 2014). The results obtained within the semi-intensive system are very often better than those obtained with the intensive system (Papandroulakis et al., 2004). Generally, in the semi-intensive system, weaning is completely achieved at 30 days, and post-weaned fry survival ranges between 40-90 %, skeletal anomalies occurrence is around 5-10 % with swim bladder inflation up to 95% and low size dispersion with low cannibalism incidence (Divanach and Kentouri, 2000). The comparison between different larval rearing systems has been previously studied in some marine species. Roo et al. (2014) compared semi-intensive systems and intensive systems in longfin yellowtail, showing better average

survival under semi-intensive systems. Also, in red porgy, improved larval growth and survival were observed in the semi-intensive system in comparison with the intensive system (Roo et al., 2010a). The use of semi-intensive technology is very suitable for species diversification and is considered a key tool to produce difficult marine species such as *Seriola* (Papandrolakis et al., 2005; Roo et al., 2010a, 2014; Pérez et al., 2020). However, there is no previous studies on greater amberjack which address this zootechnical and rearing system neither the biological and genetical aspect behind these results.

In vertebrates, including teleost fish, the growth hormone (Gh)/insulin-like growth factor (Igfs) axis plays a key role in the coordination of growth (Reinecke, 2010; Bertucci et al., 2019). Both Gh and Igfs promote growth, although Igf1 seems to be particularly responsible for body growth (Triantaphyllopoulos et al., 2019). However, the influence of the larval rearing system on the function of these growth factors has not been investigated. Another key aspect of the effect of the rearing system during early development is the potential for stress. The stress response in fish is based on the activation of the hypothalamic-pituitary-interrenal axis (HPI). Briefly, hypothalamic corticotropin-releasing hormone (Crh) levels are regulated by the (Crhbp) and Thyrotropin-releasing hormone (Trh) (Gorissen and Flik, 2016; Ruiz-Jarabo et al., 2018). Crh stimulates the proopiomelanocortin (Pomcs) production in the pituitary, which in turn activates the signalling pathway leading to cortisol synthesis (Faught et al., 2016). Consequently, activation of this pathway enhances the activity of enzymes such as the Steroidogenic acute regulatory protein (Star), producing cortisol as the end-product (Montero et al., 2015).

The greater amberjack (*Seriola dumerili*) is considered one of the best candidate species for diversification in the European aquaculture sector, due to its fast growth rate, excellent flesh quality and acceptance in the market and its large size lends itself to the processing and development of value-added products (Nakada, 2000; Fernàndez-Palacios et al., 2015; Mylonas et al., 2017). Nevertheless, larval rearing of greater amberjack is currently the major bottleneck for the successful culture of this species, due to the early mortality, cannibalism, variable size distribution and malformations observed during this period (Papandrolakis et al., 2005; Yamamoto et al., 2008; Miki et al., 2011; Hashimoto et al., 2015; Roo et al., 2019). Greater amberjack larvae are very vulnerable during the first stages of development and have strict requirements for biotic and abiotic conditions to survive, develop and grow properly, and the development of rearing protocols according to the specific requirements of the larvae during the early developmental stages is one of the main

scientific goals for the larval rearing for this species (Mylonas et al., 2017; Roo et al., 2019; Pérez et al., 2020; Djellata et al., 2021). During the last years, different larval rearing techniques from semi-intensive to intensive systems are used in this fish (Papandroulakis et al., 2005; Hamasaki et al., 2009; Pérez et al., 2020; Gamberoni et al., 2021). However, the best larval rearing protocol for commercial production of greater amberjack fingerlings is unknown. Thus, the comparison between the intensive and semi-intensive systems, for larval rearing of greater amberjack, will contribute to a better understanding of the husbandry needs of the species regarding future application in commercial production. For that reason, this study aimed to determine the most appropriate rearing technique (intensive versus semi-intensive systems) at the initial culture phases of greater amberjack to improve growth performance and survival rate.

4.2. Material and methods

4.2.1. Larval rearing

Fertilized greater amberjack eggs were obtained from the natural spawning of wild breeders kept in ECO-AQUA facilities (ULPGC, Spain). Greater amberjack larvae were reared under two different rearing systems, mainly differing in larval density and tank capacity according to Roo et al. (2010 a, 2014): a semi-intensive system (SIS) using black cylindro-conical tanks of 40 m^3 (5 m x 2.35 m) capacity, stocked with 10 eggs L^{-1}, and an intensive system (IS), using black cylindro-conical tanks of 2 m^3 (1.5 m x 2.1 m) capacity stocked with 75 eggs L^{-1}. Each rearing system was tested in duplicates for 30 days.

In both systems, larval rearing was conducted under a natural photoperiod (27º 59' 28" N; 15º 22'05" W) of approximately 13 h light. During the larval rearing, seawater temperature was varied from 22.8 to 24.9 ºC, and dissolved oxygen fluctuated from 6.0 to 8.1 ppm. All tanks were equipped with continuous aeration and supplied with filtered UV-sterilised seawater (salinity 37 psu), and the rate of water renewal was increased progressively from 15% of the tank volume per day at 2 dph for both rearing systems to 100% from 15 dph onwards in the INT and from 20 dph in the MESO one, to guarantee a good water quality during the trial. The pseudo-green water technique was used with live phytoplankton (*Nannochloropsis sp.*) kept at 2.5 x 10^5 cells/mL in both rearing systems.

Larvae were fed according to the following protocol: from 2 to 25 dph, rotifers, (*Brachionus plicatilis*) cultured on baker's yeast (*Saccharomyces cerevisiae*) and enriched

with Ori-Green (Skretting, Stavanger, Norway), for 3 hours at 23 °C, were added twice a day (at 08:00 and 14:00). Rotifers density was adjusted to 4-5 rotifers/mL in SIS rearing system and from 7.5 to 10 rotifers/ml in IS rearing system. From 15 dph, instar II *Artemia* nauplii enriched with Ori-Green for 13 hours at 28 ºC, were offered to the larvae twice a day (at 11:00 and 17:00) at a starting concentration of 0.05 to 0.35 nauplii/mL. The weaning microdiet (Gemma Micro, Skretting, Vervins, France) was supplied by hand four times a day for 5 days from 20 dph and by automatic feeders every hour from day 25 until 30 dph.

4.2.2. Growth and survival

Larval growth was assessed by estimating the total length at 10, 15, 20 and 30 dph, in 30 randomly selected larvae per tank after the night starvation. Larvae were previously anesthetized with clove oil at 1 % upon sampling. Individual larvae were photographed using a Leica DFC295 digital camera on a trinocular mount of a Leica M125 stereomicroscope and the images were used to measure total length employing the Leica Application Suite v.3.8.0. (Leica Microsystems, Wetzlar, Germany). Growth parameters were calculated including specific growth rate (SGR) and coefficient of variation (CV) of total length using the following formulae:

$$\text{SGR}= \frac{\ln(\text{Final length})-\ln\text{ (Initial length)}}{\text{Duration of the experiment (days)}} \text{ x } 100$$

$$\text{CV} = 100*(\text{standard deviation/mean})$$

On 5, 10, 15, 20 and 25 dph, 30 larvae per tank from each rearing tank, were sampled in the morning, approximately one hour and a half after live prey density adjustment. They were observed in vivo under Leica M125 stereomicroscope to evaluate the feeding incidence (= number of larvae with live prey in the gut/number of larvae examined×100) (Hamasaki et al., 2009; Roo et al., 2014). Survival rate (%) was calculated by counting live larvae at the beginning and the end of the trial using the following formula:

$$\text{Survival rate} = \frac{\text{Nf}}{\text{Ni}-\text{Ns}} \text{ x } 100$$

where Nf was the number of living larvae at the end of the experiment, Ns was the number of larvae sampled during the trial, Ni was the initial larvae numbers stocked in each tank.

4.2.3. Stress test

At 20 dph, an air exposure test was conducted by handling 30 larvae per tank (n=3) out of the water in a 500 µm nylon mesh screen for 5, 15 and 30 s and, subsequently, allocating them to a 10-L beaker supplied with clean seawater and aeration to determine larval survival at 24 h after the stress test (Roo et al., 2010a, 2014).

4.2.4. Analysis of skeletal anomalies

At 30 dph, 150 larvae per tank were randomly sampled, anesthetized without recovery using clove oil, and preserved in 10 % neutral-buffered formalin. The alcian blue - alizarin red double staining technique was used to stain cartilaginous and bony tissue structures (Vandewalle et al., 1998). Photos were taken using a Leica DFC295 digital camera (Leica, Wetzlar, Germany) and processed using the Leica application suite (LAS 32167, Leica, Wetzlar, Germany) to characterize skeletal anomalies occurrence by two independent observers. The incidence of skeletal anomalies was determined in the cranium, vertebral column, and caudal fin complex. The nomenclature of skeletal elements was conducted according to the method described by Boglione et al. (2014).

4.2.5. Molecular analysis

Whole greater amberjack larvae were processed for total RNA extraction using the NucleoSpin® RNA XS kits (Macherey-Nagel, Düren, Germany). A Polytron PT 1200 E with a dispersing tool PT-DA 03/2EC-E050 (Kinematica AG), or an Ultra Turrax® T25 (IKA®-Werke) with a dispersing tool S25N-8G, were used to homogenize them. In all cases, a digestion step with RNase-free DNase was performed to eliminate or reduce the genomic DNA contamination and, finally, samples were stored at -80 °C. The RNA concentration was measured with a Qubit® 2.0 fluorimeter and a Qubit™ RNA BR assay kit (Invitrogene, Thermo Fisher Scientific, Walthem, MA, USA). RNA quality was tested with a Bioanalyzer 2100 and an RNA 6000 Nano kit (Agilent Technologies, Inc.,Santa Clara, CA, USA). Reverse transcription was performed with the qScript™ cDNA synthesis kit (Quanta Bio, Beverly, MA, USA) using only samples that had an RNA integrity number (RIN) greater than 7.0 and 500 ng of total RNA. Each reaction was carried out in a volume of 20 µL, according to the manufacturer's instructions. Reactions were diluted 10-fold with 10 mM Tris-HCl, 0.1 mM EDTA (pH = 8), to obtain a final nominal concentration of 2.5 ng μL^{-1}. The cDNA sequences used in this work were obtained from greater amberjack brain,

hypophysis, liver, and kidney samples sent to Bioarray (Spain), where an RNAseq was performed by NGS mass sequencing, using the Ion Total RNA-Seq Kit v2 in the Ion Proton Sequencer from Life Technologies. The sequences were aligned with the Trinity software, annotating the transcripts with the Blast2GO software. Primers for real-time PCR were designed using Primer3 software v.0.4.0 (available at http://bioinfo.ut.ee/primer3/ (Table 1). Two internal reference genes, *actin beta (actb)* and *eukaryotic elongation factor 1 alpha (eef1a)*, were used as internal reference genes, owing to their lower than 0.5 target stability M value and lower than 0.25 CVs. All reactions were performed in a CFX Connect™ and a CFX 96 Real-Time Detection System with BioRad CFX Maestro Software v1.1 (BIORAD Laboratories).

Previously, every primer was tested at final concentrations of 400 and 200 nM, and a temperature range of 55 to 60 °C. Furthermore, 1:10 serial dilutions (from 10 ng to 100 fg) of cDNA were carried out to verify amplification efficiency and to produce a calibration curve. Those pairs of primers that showed an efficiency (E) between 90 and 110 %, a determination coefficient (R^2) higher than 0.980, and a calibration curve interpolating at least two points over six, were chosen for real-time PCR reactions (Table 2.5). Negative control with RNA was used to check for the presence of genomic DNA contamination, and negative control with water was used to determine the existence of artefacts such as primer-dimers. Each reaction mixture contained 0.5 µL of each specific forward and reverse primers at their best-tested concentration, 5 µL of iTaq™ Universal SYBR Green Supermix (BioRad), and 4 µL of cDNA (10 ng). Reactions were performed in a final volume of 10 µL using Hard-Shell® Low-Profile Thin-Wall 96 White-Well Skirted PCR plates covered with Microseal® B Adhesive Seals (BioRad, Hercules, CA, USA). The thermocycling procedures were carried out with an initial denaturation and polymerase activation at 95 °C for 10 minutes, followed by 40 cycles of denaturation in 15 seconds at 95 °C, annealing and extension at 60 °C for 30 seconds and finishing with a melting curve from 60 to 95 °C increasing 0.5 °C every 5 seconds. Relative gene quantification was calculated by the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001), corrected for efficiencies (Pfaffl, 2001), and normalized by geometric averaging of two references genes (Vandesompele et al., 2002).

4.2.6. Statistical analysis

All the data were statistically treated using SPSS Statistical Software System (version 22.0 for Windows; Inc, IBM, Chicago, IL, USA) and visualized using SigmaPlot 12.0 (Systat Software, San José, USA). The data were tested for normality with the one-sample

Kolmogorov-Smirnoff test and homogeneity of the variances with Levene's test (Sokal and Rohlf, 2012). A one-way analysis of variance (ANOVA) followed by a Tukey post hoc test was performed to determine significant differences among developmental stages in the same rearing system. A Student t-test was applied for comparisons between both rearing systems (MESO versus INT). The level of significance was set at $P < 0.05$. Results are presented as mean values ± SD.

4.3. Results

4.3.1. Larvae growth

The growth performance of 10-30 dph greater amberjack larvae was dependent on the rearing system (Figure 4.1). Although the total length of greater amberjack larvae at 10 dph was not significantly different between rearing systems, at 15, 20 and 30 dph the total length of the larvae reared in the SIS system was significantly higher ($P < 0.05$) than that of the IS system (Figure 4.1). During the whole rearing period, SIS system larvae had a higher SGR, although without statistically significant differences between rearing systems (Figure 4.2). Two different growth periods in terms of SGR were observed: an early rapid growth period (10-20 dph) (6.2 and 5.7% SGR in the SIS and IS systems respectively) when prey sources were restricted to live prey, a second period with a strong reduction in SGR (20-30 dph) (4.4 and 3.7% SGR in the SIS and IS systems respectively) when microdiet was incorporated. Furthermore, at the end of the rearing trials, larvae reared in the IS system showed the highest CV of total length (11.82 ± 0.16 %), whereas larvae reared in the SIS system showed the lowest CV (9.46 ± 0.30 %) ($P < 0.05$).

Enriched rotifers were supplied from 2 to 25 dph, and regardless of the rearing system utilized, the feeding incidence was 91,7% at 5 dph, 92,5% at 10 dph, 96,4 % at 15 dph and 100% by 20 and 25 dph. The transfer to *Artemia* feeding occurred at 15-20 dph was successful, and all larvae were recorded with *Artemia* inside the gut lumen.

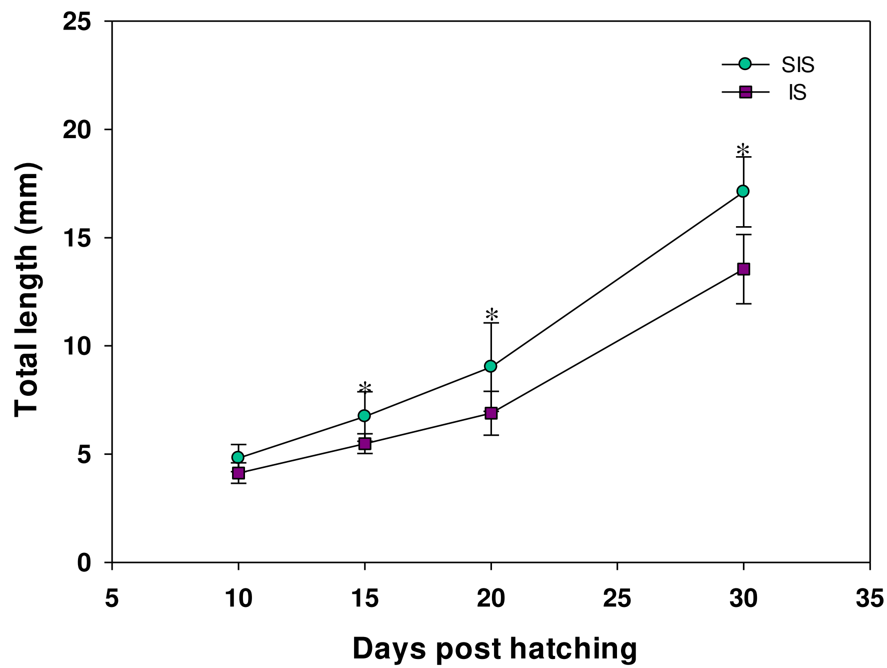


Figure 4.1. Comparison of total length (mm) of greater amberjack larvae between semi-intensive (SIS) and intensive (IS) rearing systems at 10, 15, 20 and 30 dph. Asterisks (*) indicate significant differences between rearing systems in the same larval age (P < 0.05; Student's *t*-test).

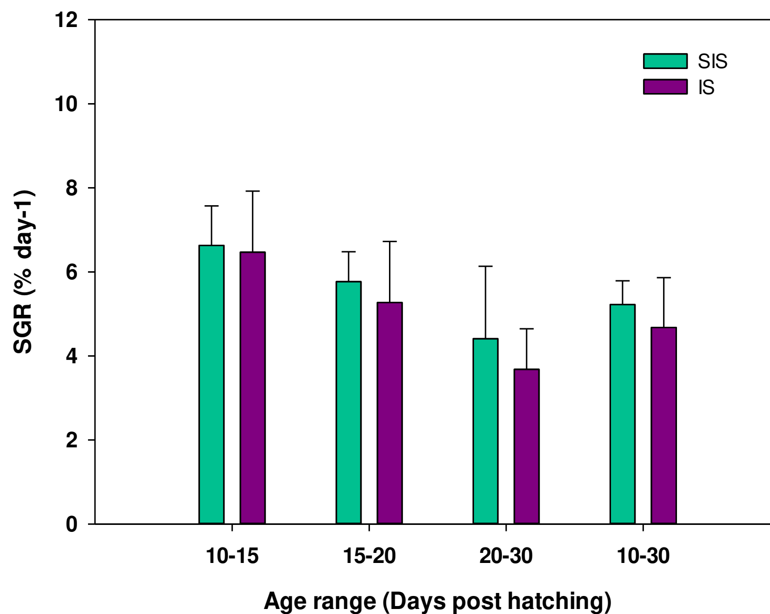


Figure 4.2. Specific growth rate evolution (SGR, % day^{-1}) of greater amberjack larvae according to the rearing system.

4.3.2. Survival rate

At the end of the rearing trials, the larval survival rate was 27.84 ± 7.11 % and 7.05 ± 1.42 % in SIS and IS systems respectively, and it was significantly different between rearing systems. The highest larval mortality period was from 7 to11 dph in the two rearing systems. In the following days, continuous larval mortality was recorded until 20 dph in IS system. At this point, IS system larvae showed very high sensitivity to the air exposure stress test; even the lowest air exposure (5 s) was associated with an almost immediate total larval mortality (Figure 4.3). However, the larvae of the SIS system showed high resistance to the activity test.

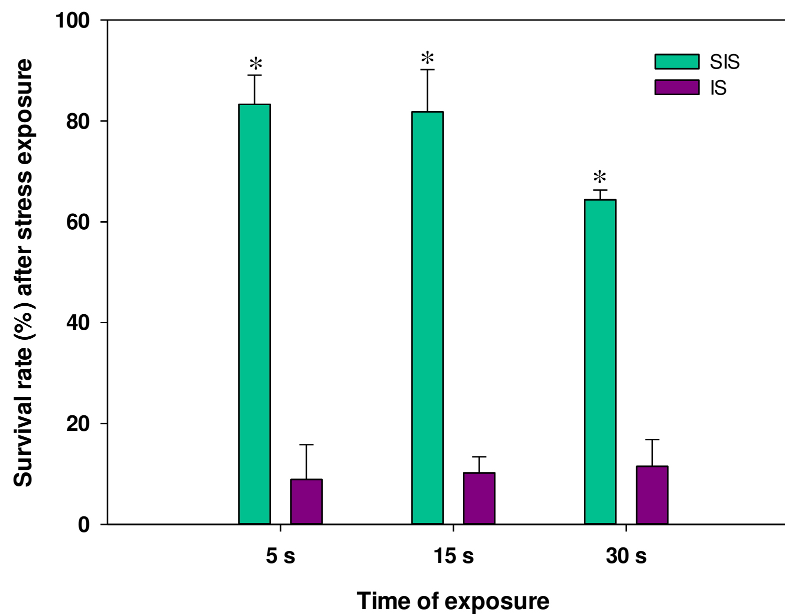


Figure 4.3. Survival rate (%) after 24h of stress exposure of greater amberjack larvae at 20 dph cultured under semi-intensive (SIS) or intensive (IS) rearing systems. Asterisks (*) indicate significant differences between culture systems at the same time of exposure ($P < 0.05$; Student's *t*-test).

4.3.3. Analysis of skeletal anomalies

In both rearing systems, different types of skeletal anomalies were observed, and lordosis was the most prevalent skeletal anomaly among the different types recorded, followed by kyphosis and cranial anomalies. The comparison between rearing systems showed a significant reduction ($P < 0.05$) of these anomalies in SIS system (Figure 4.4). However, no significant differences were observed between rearing systems in the vertebral anomaly.

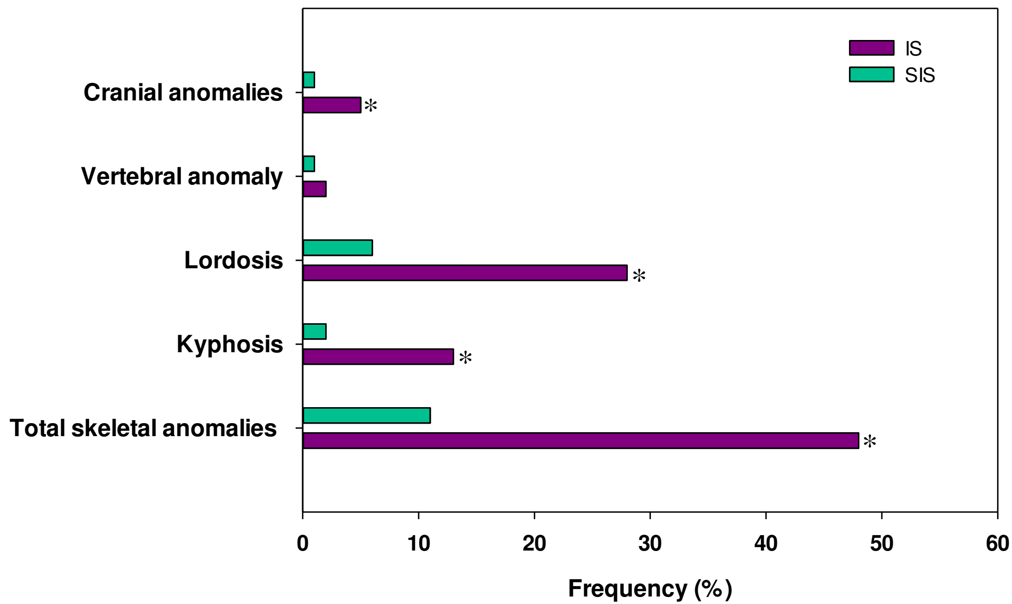
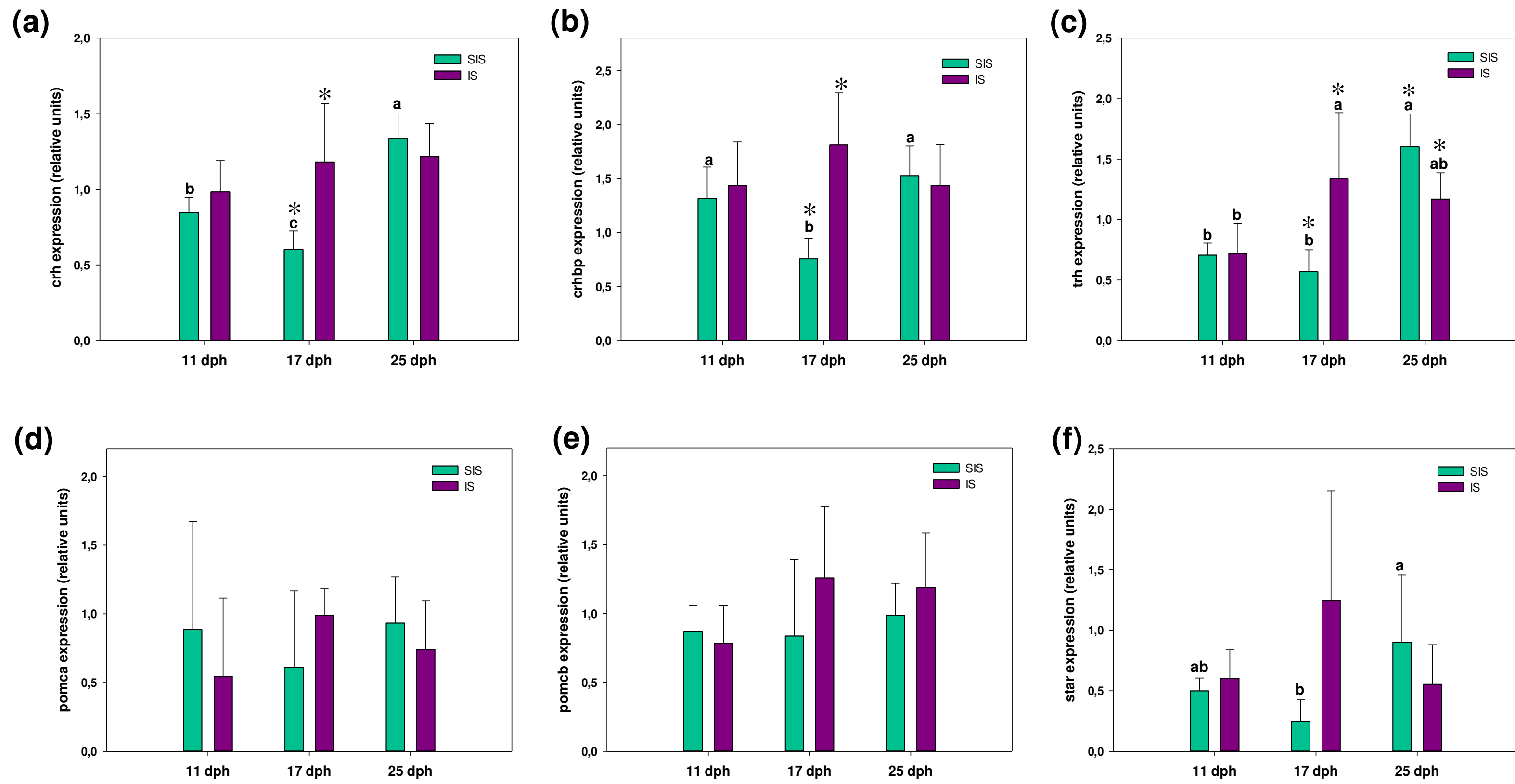


Figure 4.4. The incidence of skeletal anomalies on 30 dph greater amberjack larvae cultured under semi-intensive (SIS) or intensive (IS) rearing systems. Asterisks (*) indicate significant differences between culture systems (P < 0.05; Student's *t*-test).

4.3.4. Expression of growth and stress-related genes

The expression of growth (*gh*, *igf1*, *igf2*) and stress-related (*crh*, *crhbp*, *trh*, *pomca*, *pomcb*, *star*) genes in whole greater amberjack larvae reared in the SIS or IS rearing systems at 11, 17 and 25 dph are shown in Figure 4.5. The rearing systems appeared to affect the mRNA expression levels of *crh, crhbp* and *trh* at 17 dph in both rearing systems, being higher in the larvae reared in the IS system (P < 0.05; Figure 4.5 a,b,c). Additionally, the expression pattern observed for *crh* and *crhbp* were not consistent among the developmental sampling points, from 11 dph until 17 dph the levels decrease but at 25 dph, larvae reared in the SIS system showed a significant increase (P < 0.05), whereas *crh* and *crhbp* levels in larvae reared in the IS system remained high and stable (Figure 4.5 a,b). Also, the rearing system appeared to affect the mRNA expression levels of *trh* at 25 dph with larvae reared in the SIS system exhibiting the highest levels of expression (P < 0.05, Figure 4.5 c). The mRNA expression levels of *pomca* and *pomcb* were not altered based on the rearing system or developmental sampling points (Figure 4.5 d,e). In the case of *star*, the expression level was decreased at 17 dph whereas it increased again at 25 dph in larvae reared in the SIS system (P < 0.05, Figure 4.5 f). In both rearing systems, the expression of *gh*, *igf1* and *igf2* tended to significantly increase at 17 and 25 dph compared to 11 dph (P < 0.05; Figure 4.5

g, h, i). In addition, significantly lower *gh* gene expression was evident in SIS larvae compared to IS larvae at 17 dph. On the other hand, *igf1* was significantly higher at 11 dph in larvae reared in the SIS system than IS system ($P < 0.05$; Figure 4.5 h). However, no significant differences were found in the expression of *igf2* between rearing systems.


(a)
SIS
IS
crh expression (relative units)
11 dph
17 dph
25 dph
(b)
crhbp expression (relative units)
(c)
trh expression (relative units)
(d)
pomca expression (relative units)
(e)
pomcb expression (relative units)
(f)
star expression (relative units)

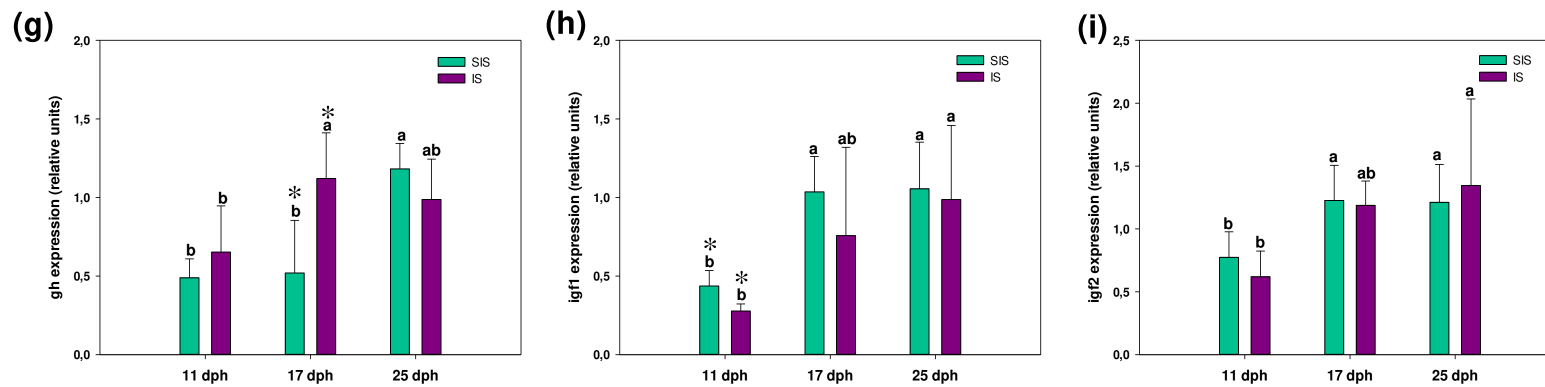


Figure 4.5. mRNA relative expression levels (means ± SD; n = 10) determined in whole greater amberjack larvae between the different culture systems at 11, 17 and 25 dph; **(a)** *corticotropin releasing hormone* (*crh*), **(b)** *corticotropin releasing hormone binding protein* (*crhbp*), **(c)** *thyrotropin releasing hormone* (*trh*), **(d)** *proopiomelanocortin a* (*pomca*), **(e)** *proopiomelanocortin b* (*pomcb*), and **(f)** *steroidogenic acute regulatory protein* (*star*), **(g)** *growth hormone* (*gh*), **(h)** *insulin-like growth factor I* (*igf1*), and **(i)** *insulin-like growth factor II* (*igf2*). Different letters indicate differences among 11, 17 and 25 dph in the same culture system (P < 0.05; one- way ANOVA; Post-hoc Tukey test), whereas asterisks (*) indicate significant differences between culture systems in the same larval age (P < 0.05; Student's *t*-test).

4.4. Discussion

The evaluation of the effect of the rearing system and zootechnical conditions in greater amberjack larviculture enables defining one of the most important parameters for adequate growth and survival in this critical stage of fish culture. In the present trial, the larvae rearing system affected the growth performance of greater amberjack, larvae reared in SIS system exhibited the highest growth rate at 10 dph and growth remained higher until the end of the trial. Different results in this respect have been found for marine species, such as European sea bass (Zouiten et al., 2011), red porgy (Roo et al., 2010a) and longfin yellowtail (Roo et al., 2014). In European sea bass, improved larval growth was observed also in the semi-intensive system in comparison with the intensive system (Zouiten et al., 2011). Similar results have been observed in red porgy, in which the larvae grew faster in the semi-intensive system (Roo et al., 2010a). However, in longfin yellowtail, the intensification of the rearing system appears not to affect the larval growth (Roo et al., 2014). On the other hand, the improved larval growth in the SIS system and lower stress levels (Roo et al., 2010a) in comparison with IS system could be associated with the use of larger water volumes, with a low larval density in the SIS system. Generally, low larval density is associated with high growth in some marine species, such as meagre (Hernández-Cruz et al., 1999), red porgy (Roo et al., 2010a) or fine flounder (*Paralichthys adspersus*) (Castro et al., 2019).

The difference in growth, observed in our trial, was detectable as early at 10 dph and was maintained until the end of the trial. This observation suggests that the effect of the rearing system takes places from the very earlier stages and should be considered to select the most suitable rearing system for this species. Similar results were observed in European sea bass, where after only 16 dph, larvae in the semi-intensive system grew fast compared to the intensive system (Zouiten et al., 2011). However, this effect has been observed even up to 50 dph in red porgy (Roo et al., 2010a). In both rearing systems, the expression of *gh*, *igf1* and *igf2* in our trial tended to significantly increase at 17 and 25 dph compared to 11 dph. In other fish, high g*h* expression had been attributed to the impressive fish growth during the first weeks of development (Ferraresso et al., 2013; Gilannejad et al., 2020). Also, the high correlation between fast somatic growth and elevated *gh* expression levels has been reported in cobia during early larval development (Ibarra-Castro et al., 2016). In our other experiment, we observed that the *gh* expressions were higher in smaller larvae than in larger

ones (Djellata et al., 2021). Similar results were obtained in the present trial, in which larvae reared in IS system exhibited lower growth with higher *gh* gene expression at 17 dph. Also, the expression of *gh* in the larvae body of zebrafish (*Danio rerio*) was lower in bigger larvae and suggests that the lower growth rate observed in these larvae was associated with the poor nutritional status (Opazo et al., 2017).

The main difference between semi-intensive and intensive methods, in addition to the lower stocking density of the larvae and the larger rearing volumes in the semi-intensive method, is related to the food availability in terms of quantity (Divanach and Kentouri, 2000; Roo et al, 2010). In the present trial, even though the prey quantity provided for the larvae had been calculated as being sufficient for all of them, this did not ensure that every larva would eat the prey quantity that had been estimated. Due to this inability to reliably predict larval fish feeding rates, an alternative approach is to evaluate the feeding incidence of fish larvae as an indicator of feeding success (Hamasaki et al., 2009; Roo et al., 2014). In this case, the feeding incidence in early stages was more than 91% during the trial in both rearing systems, suggesting that the amount of preys provided for each systems was enough to ensure a successfull early feeding in greater amberjack larvae. Similar data were previously reported by Hamasaki et al. (2009) in which the feeding incidence was more than 70% at 4 dph.

Regardless of the rearing system used, two different growth periods in terms of SGR were observed: a first one (10-20 dph) with high growth rates (5.7-6.2 %) when food sources were restricted to live prey, a second period (20-30 dph) with a strong reduction in growth rates (3.7-4.4 %) when microdiet was incorporated. This difference in growth periods could be related to the food delivered to the larvae, both in terms of quality and quantity. At the beginning of the rearing period, larvae grew well, and they consumed the delivered food when feeding was based on enriched rotifers and *Artemia*. In one study of the impact of intensive and semi-intensive rearing procedures on European sea bass larval development and quality, the authors suggested that the presence of enriched live preys (rotifers and *Artemia* nauplii) had a key effect on promoting larval development of this species (Zouiten et al., 2011). During the first period of development of red porgy (5-15 dph), high growth rates were also observed in semi-intensive and intensive systems, however, this larval performance was attributed to the success after the onset of exogenous feeding and the yolk reserves' depletion (Roo et al., 2010a). In the second period (20-30 dph), apart from the important changes occurring in zootechnical conditions such as water renewal, light

conditions and feeding sequences offered to greater amberjack larvae including the incorporation of inert diets. Additionally, during this period, relevant changes in the ontogeny are taking places including among others (skeletogenesis, digestive capacity and visual system) (Grossi-Dopico, 2010). In fact, the reduction in growth rates observed during this period could be related to the poor digestion of microdiets due to low development of greater amberjack gut at this stage, where, despite having a rudimentary stomach and gastric glands at 17 dph, acid digestion is not completely functional at 20 dph (Pérez et al., 2020). In marine fish larvae, delays in adaptation to the microdiet can lead to growth retardation and even death (Gisbert et al., 2004). In this sense, in our other experiment, we observed that the greater amberjack larvae grew slowly and suffered higher daily mortality during the first days of early weaning, mainly since most of the larvae that did not accept well the microdiet became vulnerable to predation by bigger larvae (Djellata et al., 2021).

Larvae reared in the SIS system exhibited the highest survival rates (27.84 ± 7.11 %) in contrast to the larvae reared in the IS system (7.05 ± 1.42 %). The results achieved with the SIS system for the greater amberjack larval rearing were higher than those reported by other authors for the same species cultivated in the SIS system from embryonated eggs to 40 dph (3.5%, Papandroulakis et al., 2005). Moreover, these results were better than those obtained for other fast-growing species like yellowtail amberjack (10.08 ± 1.17%; Hu et al., 2017 or 2.0 % to 5.8%; Stuart and Drawbridge, 2013) or meagre (2.8 ± 0.6%; Campoverde et al., 2017). In some marine fish, the use of intensive technology for larval rearing results in a lower survival rate than in semi-intensive larval rearing techniques (Papandroulakis et al., 2004; Roo et al., 2010a, 2014). In greater amberjack, high density tends to induce higher CV increasing competition for food, cannibalism rate and mortality (Shiozawa et al., 2003; Papandroulakis et al., 2005; Miki et al., 2011). In addition, comparing the restricted-feeding factor with the CV, the frequency of cannibalism and mortality in the diverse-size groups were higher than that found in the restricted-feeding groups in greater amberjack at 19-46 dph (Miki et al., 2011). Moreover, the consequences of a wider size-diversity during the larval stage are usually more drastic than in the adult stage, as larval size-diversity favors cannibalism behavior and mortality. In the present trial, the intensification of the rearing system affects the CV in larvae reared in the IS system showed the highest CV of total length in comparison with larvae reared in the SIS system. Therefore, the low survival rate in IS system was probably affected also by this factor and its consequences mentioned above. Similar findings have been reported for other fast-growing species such as meagre, where a

high dispersion in larvae size resulted in a reduction in survival rates, and cannibalism behavior during the larval stages (Campoverde et al., 2017).

Regarding to the skeletal anomalies observed in this study are in line to previous results reported in the larval culture of *Seriola* species with levels that can be varied from 10-50% of the total population showing some kind of skeletal anomalies (Mesa-Rodriguez et al., 2019; Roo et al., 2019; Sawada et al., 2020; Djellata et al., 2021). Different types of skeletal anomalies were observed in the greater amberjack vertebral body, and the most common deformities are those that affect the vertebral column and haemal or pre-haemal regions (Roo et al., 2019; Djellata et al., 2021). In the present trial, the incidence of the different types of deformities differed between treatments, larvae reared in SIS system displayed a significant reduction compared to IS system larvae of lordosis (6% vs. 28%), kyphosis (2% vs. 13%) and cranial anomalies (1% vs. 5%). This agrees with the results of Zouiten et al. (2011), who reported that 41.5% of European sea bass larvae reared in the intensive system exhibited severe body skeletal anomalies, in addition to 1.9% with fused caudal vertebrae while SIS larvae showed less than 7.7% severe deformitie. Additionally, other sparid species such as red porgy, sharpsnout seabream and white seabream reared in SIS results in a low percentage of individuals with skeletal deformities (Papandroulakis et al., 2004, Roo et al, 2010a).

Results of the mRNA expression levels of *crh, crhbp* and *trh,* obtained in this study showed higher levels and overexpression of those genes related to stress in IS in comparison to SIS one, particularly at 17dph. Differences in the expression of stress-related genes are probably related to more stressful rearing conditions in SI than in SIS consequences of factors such as crowding or feed competition. On this regard, Pérez et al. (2020) reported that the antioxidant defense system of semi-intensive greater amberjack larvae had less oxidative assaults, which may be attributed to lower stressful and more stable culture conditions however this authors did not compared rearing systems effect on their study. Considering the upregulation of *crh, crhbp* and *trh* gene expression in greater amberjack larvae reared in IS system in comparison to SIS, the higher stressful conditions of the IS would be confirmed by a lower biological performances of the larvae reared in SI with reduced growth and survival in IS larvae but also in poorer larval quality in SI obtained in this study, with lower resistance to activity test and higher skeletal anomalies incidence.

4.5. Conclusion

Based on our results, SIS utilized in our study offers more adequate and less stressful culture conditions with higher environmental stability than IS, that could be related to zootechnical differences between the two systems used, among others: larval density, water quality, or available water volume per larvae. These differences were reported as a higher growth and survival rates and a lower percentage of larvae with skeletal abnormalities (less than 12%) in SIS but also confirmed by the molecular study which demonstrated that growth related genes are more expressed in SIS and this related to high expression of stress-related genes in IS.

Based on our findings, we can conclude that the most suitable larval rearing protocol to sustain a regular and predictable greater amberjack fingerling demand would be the SIS during the time window evaluated.

CHAPTER 5

THE EFFECT OF DIFFERENT CO-FEEDING PROTOCOLS IN GREATER AMBERJACK (*Seriola dumerili*, RISSO 1810) LARVAE

Adnane Djellata, Samira Sarih, Carmen María Hernández-Cruz, Gonzalo Martínez-Rodríguez, Neda Gilannejad and Javier Roo

Abstract

Optimizing the growth and survival of marine fish larvae, while reducing the cost of production, is important for the development of viable hatcheries on an industrial scale. This study was aimed to determine whether *Artemia sp.* use could be minimized while maintaining high growth and survival, and low skeleton anomalies occurrence in greater amberjack during the weaning period. Five co-feeding protocols, named as 1%A, 25%A, 50%A, 75%A and 100%A according to different initial *Artemia sp*. densities provided (0.02, 0.5, 1, 1.5 and 2 *Artemia sp.* mL^{-1}/day, respectively) were tested from 32 to 48 days post-hatching (dph). Growth of larvae fed with protocols 1%A, 25%A and 50%A were significantly higher than larvae fed with protocols 75%A and 100%A. Survival increased as the effect of *Artemia sp.* increased. The incidence of total skeletal severe anomalies was high in larvae fed protocols 1%A and 25%A. Besides, the expression of growth and stress-related genes were higher at 40 dph and then decreased significantly at 48 dph in all the co-feeding protocols. The results obtained from this study suggest that the amount of *Artemia sp.* utilized can be significantly reduced during the weaning phase for this species.

5.1. Introduction

Greater amberjack is a high-value candidate for marine fish aquaculture production. This cosmopolitan fish species is mainly produced in Japan, Spain, Italy, and recently in Vietnam (Matsunari et al., 2013; Sicuro and Luzzana, 2016; Nijssen et al., 2019). It is of great interest to the European aquaculture sector due to its excellent flesh quality, high economic value, and high consumer acceptance (Mazzola et al., 2000; Sicuro and Luzzana, 2016). Its rapid growth and large size make this species very suitable for diversified marine products and the development of value-added products (Nijssen et al., 2019). As in many marine fish species, the mortality, cannibalism, and variable size distribution in greater amberjack larvae constitute the main bottleneck for efficient commercial production of fingerlings (Miki et al., 2011; Hashimoto et al., 2015). Previous studies have investigated the optimization of abiotic factors such as photoperiod or temperature to increase larval survival (Hirata et al., 2009). Likewise, it is well understood that nutrition is a critical factor for proper larval development, and some studies have been carried out on determining the nutritional requirements of greater amberjack during the live food stages to boost survival rates (Yamamoto et al., 2008; Matsunari et al., 2013). A more recent study has shown that

levels of n-3 highly unsaturated fatty acids (n-3 HUFAs) in live food can also affect growth, survival, and skeletal anomalies occurrence in greater amberjack (Roo et al., 2019). The feeding protocol adapted to greater amberjack is based on the use of rotifers (*Brachionus sp.*) from the first feeding, followed by the use of brine shrimp (*Artemia sp.* nauplii and metanauplii) appropriately enriched when larvae increase in size and are weaned onto artificial diets at a later stage of development (Hamasaki et al., 2009; Matsunari et al., 2013; Yamamoto et al., 2013; Roo et al., 2019). Significant progress on weaning has also been achieved for greater amberjack (Papandroulakis et al. 2005; Navarro-Guillén et al., 2019). However, even though greater amberjack larval development and larval rearing techniques have been studied, weaning to dry diets remains to be an important bottleneck for this species.

Weaning is a process to replace live feeds with artificial diets at a very critical moment during larval development (Rosenlund et al., 1997). It is not simply a change from one food to another but is a phase of adaptation where both types of food are provided for some time with a slow decrease in the administration of live prey and a progressive increase of microdiet (Chèvre et al., 2011; Williot et al., 2011). Moreover, weaning is considered a critical period and it is very stressful to fish. In this context, the best weaning protocol is based on a good balance of the combination of several parameters, such as high survival and larval growth, and low size dispersion and incidence of skeletal anomalies, having a direct impact on fingerlings quality during the on-growing phase (Gisbert et al., 2018). In addition to the above-mentioned biological variables, other parameters need also to be considered such as labour, production costs, and the use of facilities. A prolonged period of using live foods is costly and may cause nutritional deficiency since it does not contain an adequate nutrition content for the growth and development of fish larvae (Callan et al., 2003; Ma et al., 2015). Moreover, live food is a potential vector for protozoan parasites as well as infectious viruses and bacteria (Makridis et al., 2000; Bonaldo et al., 2011). Thus, reducing the reliance on live feeds will reduce material and labour costs, further increasing profitability margins.

In this sense, to minimize the use of live foods by using microdiet and maximize survival in the greater amberjack larval weaning, this study had the following objective: to find out whether reducing the inclusion level of *Artemia sp.* in daily feeding protocol from 32 up to 48 dph affects growth, survival, fatty acids composition, skeleton anomalies occurrence, hepatocyte vacuolization in the liver and the expression of growth and stress-

related genes. If the use of *Artemia sp.* in the larval diet can be minimized, then substantial savings in food cost, floor space, and labour can be achieved. The results obtained from this investigation will provide basic information that would be valuable in the development of hatchery techniques for greater amberjack production.

5.2. Material and methods

All the experiment mentioned below was conducted at the Marine Scientific and Technological Park of ECOAQUA University Institute of the University of Las Palmas de Gran Canaria (Las Palmas, Canary Islands, Spain), with an official aquaculture facility register number (REGA): ES350260026567. All animal experiments described in this manuscript fully comply with the recommendations in the Guide for Care and Use of Laboratory Animals of the European Union Council (2010/63/EU), with the general guidelines approved by the project "DIVERSIFICACIÓN DE LA ACUICULTURA ESPAÑOLA MEDIANTE LA OPTIMIZACIÓN DEL CULTIVO DE SERIOLA (*Seriola dumerili*) – SERIOLA, within the National Agricultural Aquaculture Plans 2016-2018 (Ref. 23.17.415A.741).

5.2.1. Larval rearing

Greater amberjack larvae of 32 dph (N = 6000; total length 11.54 ± 1.15 mm; fresh mass 23.57 ± 2.83 mg (mean ± standard deviation) were randomly distributed into 15 light grey colour cylindrical fibreglass tanks (five triplicate treatments) of 200 L. All tanks were equipped with continuous aeration and supplied with filtered UV-sterilised seawater (salinity 37 psu). Continuous water flow was maintained at a 25% per hour exchange rate from 32 to 35 dph and then increased to achieve a 50% per hour exchange rate at the end of the experiment, to guarantee a good water quality during the trial. Water entered the tanks at the bottom and exited at the surface. During the experimental period, tanks were siphoned daily to remove dead larvae, uneaten food, and faeces. Dissolved oxygen (6.1 ± 0.4 g/L) and temperature (22.3 ± 0.6 ºC) were monitored daily. An artificial fluorescent light above each tank provided a surface light intensity ranging between 1,000 and 1,500 lux (digital Lux Tester YF-1065; Powertech Rentals, Osborne, Australia) at the centre of each rearing tank for a photoperiod of 12 h light/12 h darkness cycle with lights on from 07:30 a.m. to 07:30 p.m., local time.

5.2.2. Feeding trial

Five different co-feeding protocols named 1% A, 25% A, 50% A, 75% A and 100% A according to different initial *Artemia sp*. densities provided 0.02, 0.5, 1, 1.5 and 2 *Artemia sp.* mL^{-1}/day, respectively, and the same amount of microdiet for all treatments, were tested from 32 to 48 dph (Table 5.1 and 5.2). *Artemia sp.* (EG type; INVE Aquaculture, Dendermonde, Belgium) were enriched for 18 hours in 100 L tanks (250,000 individuals L^{-1}) maintaining 28 ºC seawater, with an experimental emulsion (GIA, own formula), under vigorous aeration and oxygen supply. During the experimental period, *Artemia sp.* feeding frequency was gradually reduced from four to one fed per day (Table 5.1). Additionally, microdiet (Gemma Micro; Skretting, France) was used by mixing (1:1) two different particle sizes, 150 µm and 300 µm. The microdiet was manually fed to the larvae 7-10 times a day (approximately every hour). The amount of microdiet was always offered to assure enough feed, based on estimated consumption and growth, and periodically adjusted based on visual inspection to avoid a large excess of uneaten food. The feeding quantity of the microdiet for each tank was 0.75 g day^{-1} at the first co-feeding days (32 to 35 dph), increased to 1.25 g day^{-1} at 36 to 39 dph, and reached to 1.50 g day^{-1} at 40 dph until the end of the experiment (Table 5.1). Proximate analysis and fatty acids composition of *Artemia sp.* and microdiet are shown in Table 5.2.

Table 5.1. Co-feeding protocols using *Artemia sp.* and microdiet for larval greater amberjack.

		Treatment				
		1 % A	**25 % A**	**50 % A**	**75 % A**	**100 % A**
From 32 to 35 dph	*Artemia sp.*/day***	4,000	100,000	200,000	300,000	400,000
	Microdiet g/day	0.75	0.75	0.75	0.75	0.75
	Energy content (KJ)/day	16.92	18.03	19.18	20.33	21.49
From 36 to 39 dph	*Artemia sp.*/day**	2,000	50,000	100,000	150,000	200,000
	Microdiet g/day	1.25	1.25	1.25	1.25	1.25
	Energy content (KJ)/day	28.15	28.70	29.28	29.85	30.43
From 40 to 48 dph	*Artemia sp.*/day*	1,000	25,000	50,000	75,000	100,000
	Microdiet g/day	1.50	1.50	1.50	1.50	1.50
	Energy content (KJ)/day	33.76	34.04	34.33	34.61	34.90

Note: *** *Artemia sp.* was offered to the larvae four times per day per tank; ** twice/day; *once/day.

Table 5.2. Proximate (% dry matter) and fatty acids composition (% total fatty acids, TFA) of *Artemia sp.* and microdiet.

	Artemia sp.	Microdiet
	Proximate analysis (% dry matter)	
Lipids	20.77 ± 1.00	18.78
Ash	10.00 ± 1.55	13.73
Proteins	63.29 ± 0.78	63.91
Moisture	91.62 ± 0.16	14.20
Energy content (KJ/g)*	23.06	22.50
	Fatty acid content (% TFA)	
Total SFA	19.85 ± 2.02	19.46
Total MUFA	28.38 ± 1.52	38.21
Total n-3	38.63 ± 3.27	33.41
Total n-6	11.23 ± 0.84	7.80
Total n-9	18.74 ± 0.87	19.57
Total n-3 PUFA	12.90 ± 2.00	29.62
14:0	0.33 ± 0.11	1.10
16:0	11.29 ± 1.43	14.17
16:1 n-7	1.57 ± 0.15	4.47
18:0	7.64 ± 0.64	3.73
Oleic (18:1 n-9)	17.65 ± 0.86	15.14
18:1 n-7	6.34 ± 0.39	5.57
18:2 n-6	4.85 ± 0.13	6.17
18:3 n-3	22.62 ± 1.33	1.25
20:1 n-9	0.10 ± 0.02	3.16
ARA (20:4n-6)	2.52 ± 0.35	0.81
EPA (20:5n-3)	3.04 ± 0.38	14.36
DHA (22:6n-3)	6.77 ± 1.53	13.27
DPA (22:5n-6)	2.04 ± 0.32	0.18
DHA/DPA	3.31 ± 0.33	72.07
ARA/EPA	0.83 ± 0.05	0.06
DHA/EPA	2.23 ± 0.42	0.92
DHA/ARA	2.72 ± 0.71	16.29
Oleic/DHA	2.72 ± 0.74	1.14
Oleic/n-3 PUFA	1.40 ± 0.28	0.51
n-3/n-6	3.45 ± 0.35	4.28

Note: Proximate analysis and fatty acid content data of *Artemia sp.* represent means ± SD, n=3.
* Energy content was estimated for: Lipid × 39.5 KJ/g; Protein × 23.6 KJ/g (New, 1987).
Abbreviations: ARA, arachidonic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; MUFA, monounsaturated fatty acids; PUFA, long-chain polyunsaturated fatty acids; SFA, saturated fatty acids.

5.2.3. Growth and survival

Larval growth was assessed by estimating the total length (TL) and wet body mass (WW) at 32, 40, and 48 dph, in 30 randomly selected larvae per tank, one hour before the first feeding at 32 and 40 dph and after the night starvation at 48 dph, to avoid any influence of gut contents on the nutrient content of the larvae as much as possible. Larvae were previously anaesthetized with clove oil at 1% and TL was measured under a profile projector (Mitutoyo, PJ-A3000, Japan). The same larvae were used to estimate the wet body mass, previously washed with distilled water and weighed in an analytical balance (Gibertini Elettronica, E50 S/2, Milano, Italy). Specific growth rate (SGR) was calculated in relation to total length at the end of the co-feeding period using the following formula: $SGR = 100*(\ln(TLf) - \ln(TLi))/\Delta t$, where TLf was the final larval total length (mm), TLi was the initial larval total length (mm) and Δt was the time between sampling days (Hopkins, 1992; Lugert et al., 2014). The coefficient of variation (CV) of total length was calculated according to the formula: $CV = 100*(\text{standard deviation}/\text{mean})$.

At the end of the experiment, the dead and live larvae count allowed us to calculate the survival and cannibalism rates per tank. Survival rate $= 100* Nf/(Ni-Ns)$ and estimated cannibalism rate $= 100* (Ni-Nc-Nf)/Ni$, where Nf was the number of living larvae at the end of the experiment, Ns was the number of larvae sampled during the trial, Ni was the initial larvae numbers stocked in each tank, and Nc was the accumulated number of dead larvae during the trial (Cortay et al., 2019). The total yield from each treatment was determined by average larval weight multiplied by average live larvae.

5.2.4. Biochemical analysis

To analyse the proximate and fatty acid composition, a sample of 48 dph larvae from each tank was washed with distilled water and kept at -80 ºC. Proximate composition was conducted following standard procedures (Association of Official Analytical Chemists [AOAC], 2016). Moisture was determined by thermal dehydration until constant mass at 105 ºC. Ash content was determined by combustion at 600 ºC for 12 h. Crude protein content (N x 6.25) was determined by the Kjeldahl method, and crude lipid was extracted following the Folch method (Folch et al., 1957). Fatty acid methyl esters profiles were obtained by transmethylation of total lipids (Christie and Han, 2010), and separated by gas-liquid chromatography (GC-14A; Shimadzu, Tokyo, Japan) following the conditions described by Izquierdo et al. (1990) and identified by comparison to previously characterized standards

and GLC-MS (Polaris QTRACETM Ultra; Thermo Fisher Scientific). All analyses were conducted in triplicate.

5.2.5. Analysis of skeletal anomalies

To identify and quantify larval skeletal anomalies, 100 larvae per tank (300 larvae per treatment) were randomly sampled at 48 dph, preserved in 10 % neutral-buffered formalin, stained with alizarin red, and examined under a Stereoscope (Leica, M125, Wetzlar, Germany). Photos were taken using a Leica DFC295 digital camera (Leica, Wetzlar, Germany) and processed using the Leica application suite (LAS 32167, Leica, Wetzlar, Germany) to characterise skeletal anomalies occurrence (Vandewalle et al., 1998) by two independent observers. Several skeletal anomalies were determined in the different regions of the axial column according to the method described by Boglione et al. (2014). The percentage of high-quality larvae produced per tank was estimated as follows: Survival rate at the end of the experiment multiplied by the percentage of non-malformed larvae.

5.2.6. Histological analysis

At the end of the experiment, ten greater amberjack larvae per tank were sampled and fixed in 10 % neutral-buffered formalin. After 48 h, tissues were dehydrated with an increased graded series of ethanol, submerged in xylene, and embedded in paraffin blocks. Paraffin-embedded larvae were cut at 4 µm on a microtome (Mod. Jung Autocut 2055; Leica, Nussloch, Germany) and stained with haematoxylin and eosin (H&E) (Martoja and Martoja-Pierson, 1970). The mounted sections were examined under light microscopy using an Olympus CX41 binocular microscope (Olympus, Hamburg, Germany) connected to an Olympus XC30 camera (Olympus), which was linked to a computer using Image Capturing Software (CellB®; Olympus). Tissue morphology of hepatic was examined by two independent observers. Hepatocyte vacuolization was assessed by using a three-point scoring system where 1, 2, and 3 represented no, mild, and severe hepatic vacuolization, respectively (Betancor et al., 2012).

5.2.7. Molecular analysis

Total RNA was extracted from whole greater amberjack larvae after RNAlater® (Ambion, Applied Biosystems) was eliminated using lint-free laboratory wipes, employing a Polytron PT 1200 E with a dispersing tool PT-DA 03/2EC-E050 (Kinematica AG), or an

Ultra Turrax® T25 (IKA®-Werke) with a dispersing tool S25N-8G, and the NucleoSpin® kit (Macherey-Nagel). In all cases, a digestion step with RNase-free DNase was performed to eliminate or reduce the genomic DNA contamination and, finally, samples were stored at -80 °C. RNA concentration was measured with a Qubit® 2.0 fluorimeter and a Qubit™ RNA BR kit, while its quality was assessed with a Bioanalyzer 2100 and an RNA 6000 Nano kit (Agilent Technologies, LifeSciences). Reverse transcription was performed with the qScript™ cDNA synthesis kit (Quanta BioSciences) using only samples which had an RNA integrity number (RIN) greater than 7.0 and 500 ng of total RNA. Each reaction was carried out in a volume of 20 µL, according to manufacturer's instructions, and it was diluted 1/10th with 10 mM Tris-HCl, 0.1 mM EDTA (pH = 8) to obtain a final concentration of 2.5 ng μL^{-1}. The cDNA sequences used in this work were obtained from greater amberjack brain, hypophysis, liver, and kidney samples sent to Bioarray (Spain), where an RNAseq was performed by NGS mass sequencing, using the Ion Total RNA-Seq Kit v2 in the Ion Proton Sequencer from Life Technologies. The sequences were aligned with the Trinity software, annotating the transcripts with the Blast2GO software. Primers for real-time PCR were designed using Primer3 software v.0.4.0 (available at http://bioinfo.ut.ee/primer3/ (Table 1)). Two internal reference genes, *actin beta (actb)* and *eukaryotic elongation factor 1 alpha (eef1a)*, were used as internal reference genes, owing to their lower than 0.5 target stability M value and lower than 0.25 CVs. All reactions were performed in a CFX Connect™ and a CFX 96 Real-Time Detection System with BioRad CFX Maestro Software v1.1 (BIORAD Laboratories). A pool of cDNAs (CAL), derived from mixing the 5 RNA samples from the 32 dph group, was used to correct for inter-assay errors.

Before samples analyses, every primer was tested at final concentrations of 400 and 200 nM, and a temperature range of 55 to 60 ºC. Furthermore, 1:10 serial dilutions (from 10 ng to 100 fg) of cDNA were carried out to verify amplification efficiency and to produce a calibration curve. Those pairs of primers that showed an efficiency (E) between 90 and 110 %, a determination coefficient (R^2) higher than 0.980, and a calibration curve interpolating at least two points over six, were chosen for real-time PCR reactions (Table 2.5). Negative control with RNA was used to check for the presence of genomic DNA contamination, and negative control with water was used to determine the existence of artefacts such as primer-dimers. Each reaction mixture contained 0.5 µL of each specific forward and reverse primers at their best-tested concentration, 5 µL of iTaq™ Universal SYBR Green Supermix (BioRad), and 4 µL of cDNA (10 ng). Reactions were accomplished in a volume of 10 µL

using Hard-Shell® Low-Profile Thin-Wall 96 White-Well Skirted PCR plates (BioRad) covered with Microseal® B Adhesive Seals (BioRad). PCRs were performed with an initial denaturation and polymerase activation at 95 ºC for 10 minutes, followed by 40 cycles of denaturation in 15 seconds at 95 ºC, annealing and extension at 60 ºC for 30 seconds and finishing with a melting curve from 60 to 95 °C increasing 0.5 ºC every 5 seconds. Relative gene quantification was performed according to the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001), corrected for efficiencies (Pfaffl, 2001), and normalized by geometric average of the two internal control genes (Vandesompele et al., 2002).

5.2.8. Statistical analysis

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). The significant level for all analyses was set at 5 %. All data were tested for normal distribution with the one-sample Kolmogorov–Smirnoff test, as well as for homogeneity of the variances with the Levene test (Sokal and Rohlf, 2012). When the assumptions were passed, the one-way Analysis of Variance (ANOVA) test was performed, followed by Tukey’s post-hoc test and Student’s t-test for paired samples. When heterogeneity of variances and/or normality of distribution were not complied, the Kruskal-Wallis test was applied and differences among treatments were graphed with a box and whisker plot.

5.3. Results

5.3.1. Growth, survival and cannibalism

Co-feeding protocols did not have a significant ($P < 0.05$) impact on greater amberjack wet body mass and total length during the first period, from 32 dph until 40 dph (Figure 5.1). Nevertheless, at the end of the experiment (48 dph), larvae in 1% A, 25% A and 50% A co-feeding protocols were significantly ($P < 0.05$) heavier and longer than larvae in 75% A and 100% A co-feeding protocol, while there were no significant differences ($P < 0.05$) among 1% A, 25% A and 50% A co-feeding protocols (Figure 5.1).

The highest SGR was found in 1% A co-feeding protocol (3.57 ± 0.48% day^{-1}), whereas the lowest in the 100% A group (2.56 ± 0.17% day^{-1}) at the end of the experimental period (Figure 5.2). Furthermore, no significant differences in CV of total length were observed among co-feeding protocols ($P < 0.05$) (Figure 5.3).

Larval survival varied from 21.53 ± 4.04 % in 1% A co-feeding protocol, as the lowest, to 50.72 ± 4.87 % in 100% A co-feeding protocol, as the highest (Figure 5.4 a). Survival increased as an effect of *Artemia sp.* amount provided and was significantly higher (P < 0.05) in the 75% A and 100% A co-feeding protocols (Figure 5.4 a). Besides, cannibalistic behaviour was most frequently observed in tanks that received reduced amounts of *Artemia sp.* (1% A and 25% A), compared to the treatments receiving higher amounts (50% A, 75% A and 100% A) (Figure 5.4 b). In addition, the highest Total yield was observed in 75% A co-feeding protocol while the lowest in 1% A (Figure 5.5).

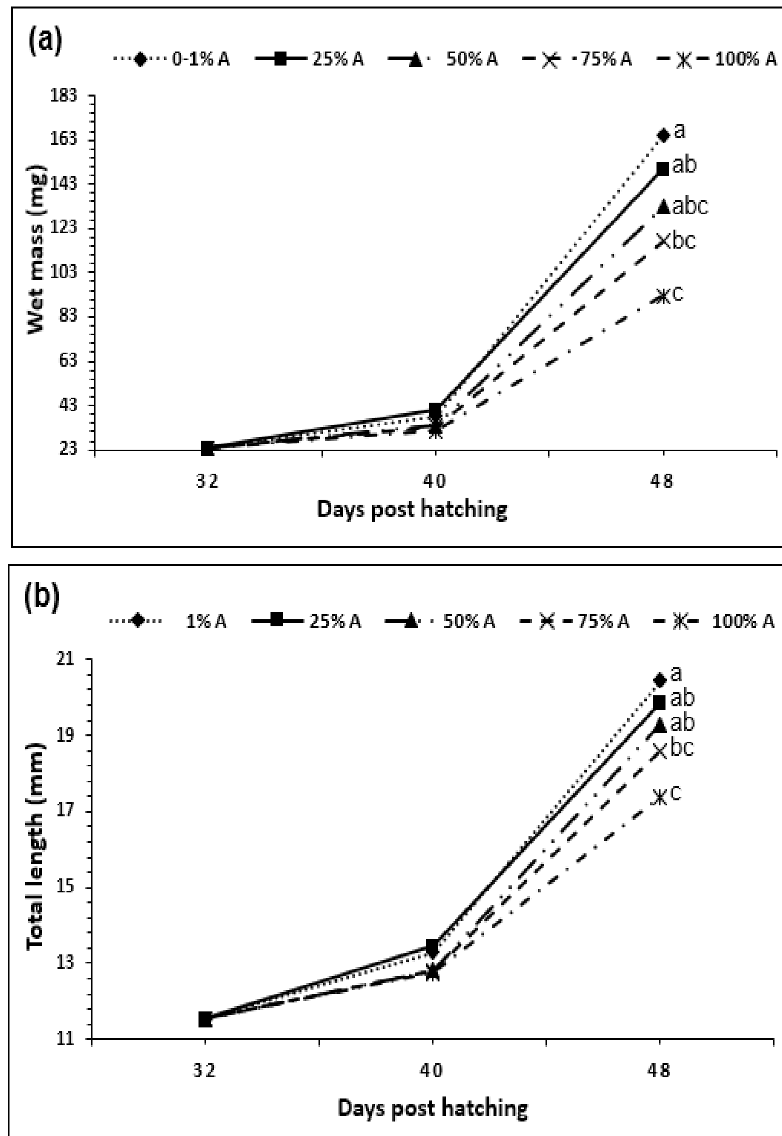


Figure 5.1. Growth parameters of greater amberjack larvae **(a)** wet mass (mg), and **(b)** total length (mm) from different co-feeding protocols at 32, 40 and 48 dph. Different letters denote significant differences among protocols (P < 0.05; one- way ANOVA; Post-hoc Tukey test).

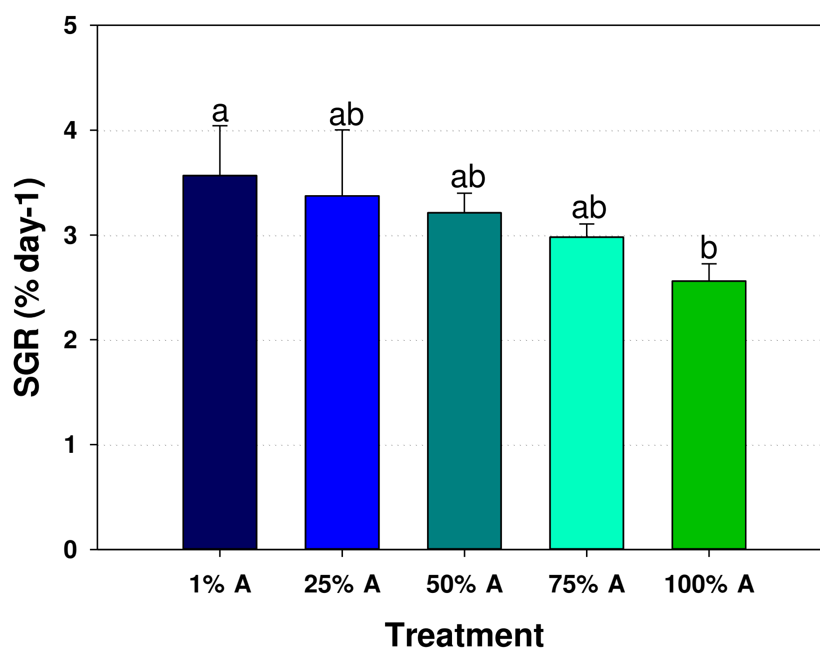


Figure 5.2. Specific growth rate (SGR, % day^{-1}) in 48 dph greater amberjack larvae fed different co-feeding protocols (means ± SD, n=3). Different letters above the bars indicate significant differences *a*mong protocols (P < 0.05; one- way ANOVA; Post-hoc Tukey test).

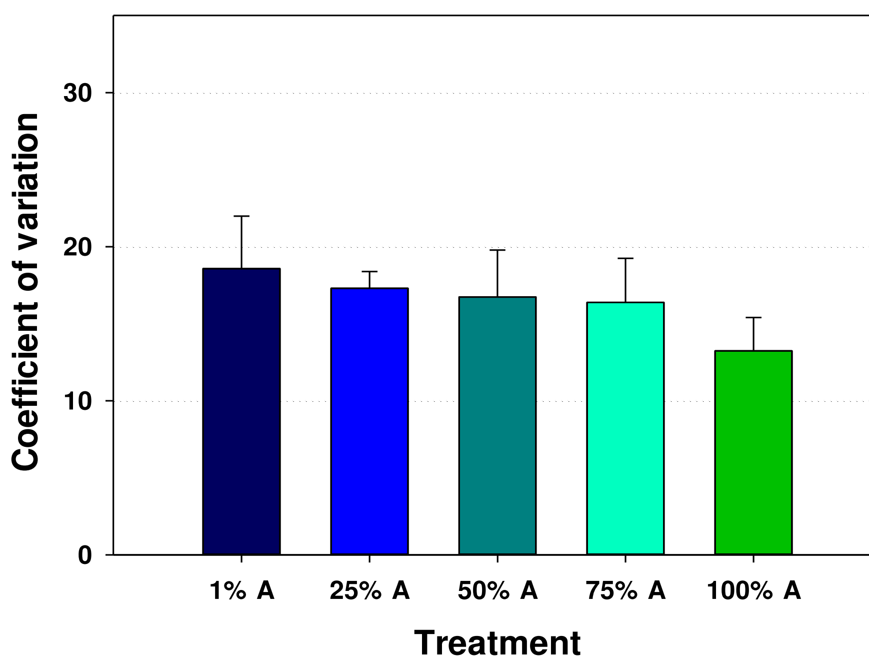


Figure 5.3. Coefficient of variation of total length in 48 dph greater amberjack larvae fed different co-feeding protocols (means ± SD, n=3).

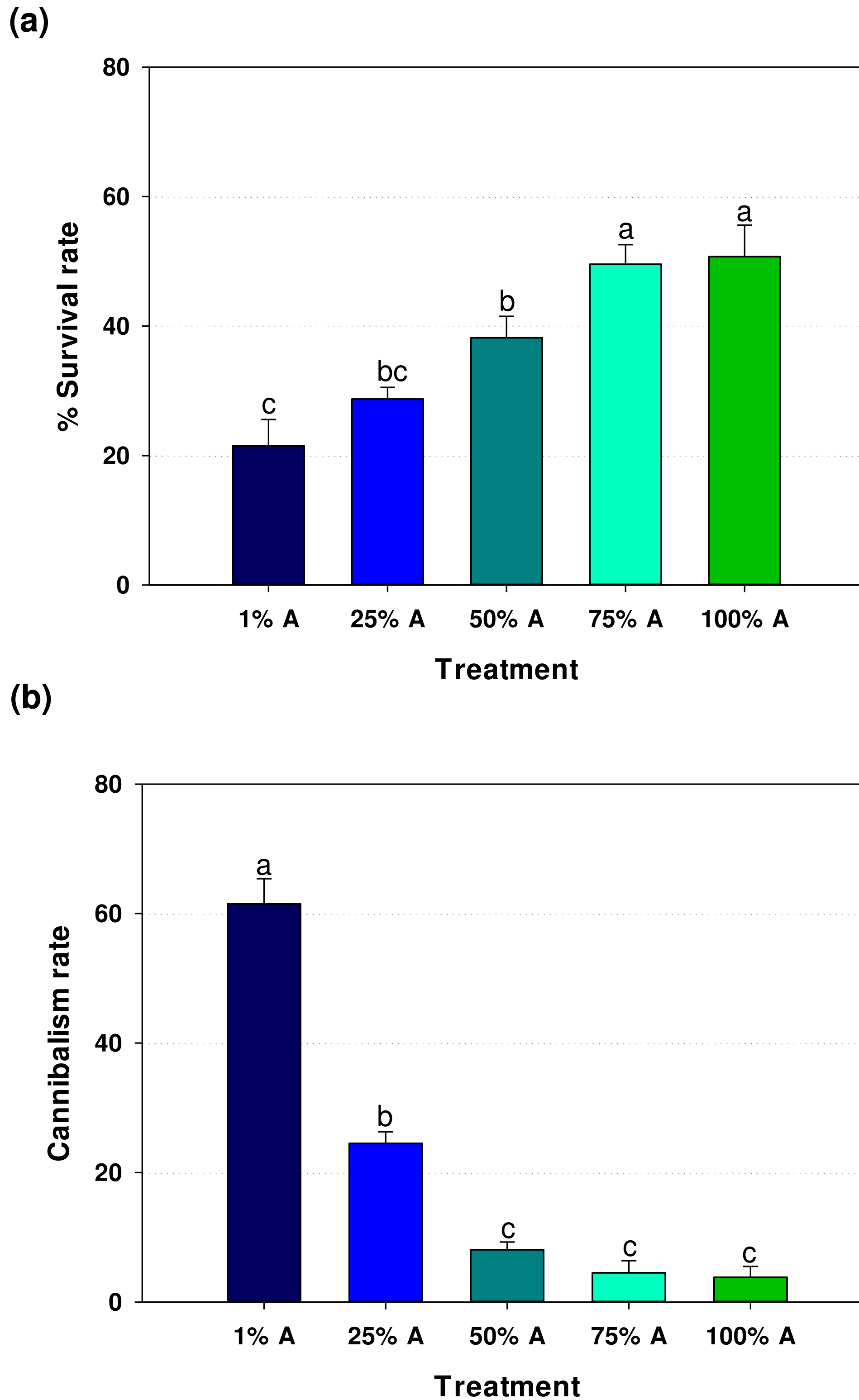


Figure 5.4. **(a)** Final survival rate and **(b)** Cannibalism rate in 48 dph greater amberjack larvae fed different co-feeding protocols (means ± SD, n=3). Different letters above the bars indicate significant differences among protocols ($P < 0.05$; one- way ANOVA; Post-hoc Tukey test).

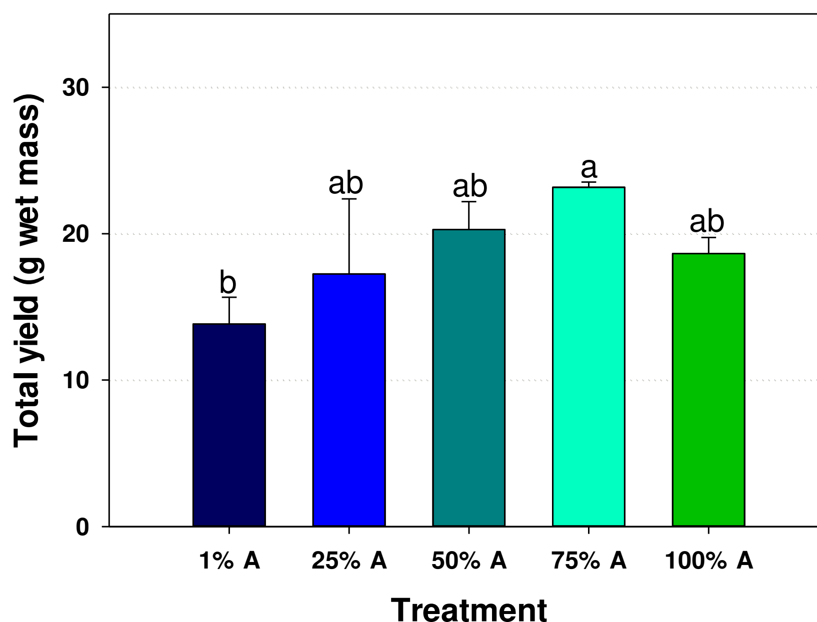


Figure 5.5. Total yield (g wet mass) in 48 dph greater amberjack larvae fed different co-feeding protocols (means ± SD, n=3). Different letters above the bars indicate significant differences among protocols ($P < 0.05$; one- way ANOVA; Post-hoc Tukey test).

5.3.2. Biochemical analysis

At the end of the co-feeding trial, larval total lipids, proteins and moisture were significantly different ($P < 0.05$) among co-feeding protocols (Table 5.3). Regardless of the treatments, palmitic acid (16:0) accounted for the bulk of the saturated fatty acids (SFA), oleic acid (18:1n-9) for most of the monounsaturated fatty acids (MUFA), and docosahexaenoic acid (DHA, 22:6n-3) and eicosapentaenoic acid (EPA, 20:5n-3) for most of the n-3 long-chain polyunsaturated fatty acids (PUFA) present in the larvae (Table 5.3). The fatty acid composition of total lipids from whole larval body lipids reflected the *Artemia sp.* and microdiet fatty acid profiles (Table 5.2). The increased amount of *Artemia sp.* was followed by increased arachidonic acid (ARA, 20:4n-6), α-linolenic acid (18:3n-3), and docosapentaenoic acid (DPA, 22:5n-6), together with reduced MUFA and n-9 fatty acids, particularly oleic acid (18:1n-9), in the larvae. The proportions of DHA and EPA in the total fatty acids of the greater amberjack larvae were similar in all the experimental groups (Table 5.3). However, an increase in *Artemia sp.* density from 0.02 (1% A) to 2 mL^{-1}/day (100% A) significantly ($P < 0.05$) increased the ARA/EPA ratio and decreased the DHA/ARA and DHA/DPA ratios (Table 5.3).

Table 5.3. Proximate (% dry matter) and fatty acids composition (% total fatty acids, TFA) of 48 dph greater amberjack larvae fed different co-feeding protocols (means ± SD, n=3).

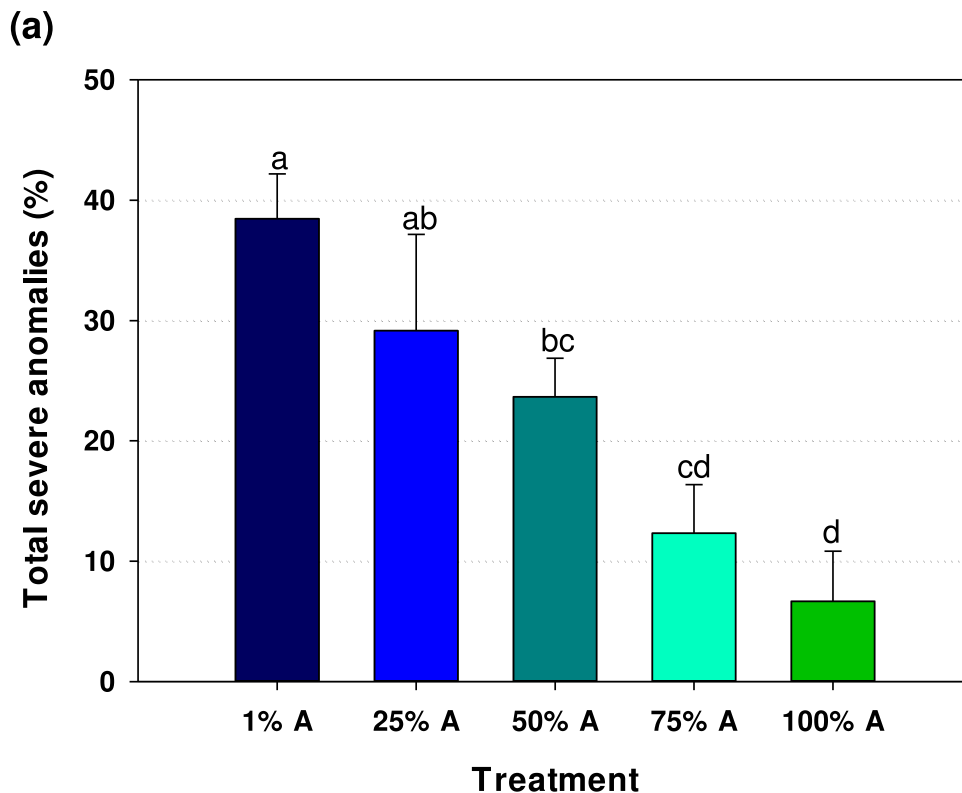
	Treatment				
	1% A	25 % A	50 % A	75 % A	100 % A
	Proximate analysis (% dry matter)				
Lipids	10.47 ± 1.04[b]	11.89 ± 0.82[b]	12.99 ± 1.29[b]	17.34 ± 0.70[a]	17.91 ± 0.84[a]
Ash	13.04 ± 2.68	14.77 ± 1.92	15.66 ± 1.54	14.58 ± 1.72	13.81 ± 1.48
Proteins	71.71 ± 0.62[ab]	77.90 ± 2.63[a]	77.02 ± 2.72[a]	77.89 ± 2.75[a]	69.60 ± 2.42[b]
Moisture	79.46 ± 1.29[b]	81.46 ± 2.21[ab]	81.30 ± 0.15[ab]	82.87 ± 0.68[ab]	83.74 ± 1.62[a]
	Fatty acid content (% TFA)				
Total SFA	23.27 ± 0.32	24.81 ± 1.90	26.59 ± 2.20	25.05 ± 1.83	23.15 ± 7.29
Total MUFA	26.72 ± 0.86[a]	27.19 ± 0.50[a]	23.97 ± 0.65[b]	22.60 ± 1.66[b]	22.86 ± 2.01[b]
Total n-3	38.72 ± 0.56	36.09 ± 2.29	36.80 ± 2.91	40.16 ± 2.89	41.38 ± 8.10
Total n-6	9.56 ± 0.45	9.91 ± 0.20	10.44 ± 0.59	9.89 ± 0.56	10.34 ± 0.82
Total n-9	15.14 ± 0.41[a]	15.86 ± 0.18[a]	14.47 ± 0.30[ab]	13.52 ± 0.96[b]	13.81 ± 1.09[b]
Total n-3 PUFA	36.31 ± 0.57	32.29 ± 2.34	33.08 ± 2.94	36.54 ± 3.28	37.21 ± 8.27
14:0	1.10 ± 0.28	1.11 ± 0.64	0.90 ± 0.20	0.81 ± 0.22	0.74 ± 0.49
16:0	14.71 ± 0.30	15.42 ± 1.68	16.36 ± 1.89	14.81 ± 1.66	13.06 ± 6.74
16:1n-7	2.90 ± 0.29	2.98 ± 0.59	2.36 ± 0.21	1.99 ± 0.28	1.78 ± 1.03
18:0	6.98 ± 0.31[c]	7.75 ± 0.73[bc]	8.79 ± 0.17[ab]	8.90 ± 0.04[a]	8.82 ± 0.29[a]
Oleic (18:1n-9)	13.23 ± 0.26[a]	13.11 ± 0.29[a]	12.37 ± 0.64[ab]	11.37 ± 0.87[b]	11.56 ± 0.84[b]
18:1n-7	4.55 ± 0.39	4.70 ± 0.15	4.46 ± 0.22	4.32 ± 0.29	4.46 ± 0.20
18:2n-6	6.57 ± 0.38	6.65 ± 0.17	6.69 ± 0.79	5.42 ± 0.61	5.41 ± 0.32
18:3n-3	1.09 ± 0.03[c]	2.36 ± 0.18[b]	2.45 ± 0.09[ab]	2.43 ± 0.34[ab]	2.93 ± 0.15[a]
20:1n-9	2.05 ± 0.30[a]	1.77 ± 0.16[ab]	1.24 ± 0.20[c]	1.29 ± 0.13[bc]	1.26 ± 0.14b[c]
ARA (20:4n-6)	1.60 ± 0.12[b]	1.73 ± 0.18[b]	2.03 ± 0.12[ab]	2.39 ± 0.07[a]	2.56 ± 0.51[a]
EPA (20:5n-3)	9.27 ± 0.31	8.34 ± 0.43	8.15 ± 0.63	8.20 ± 0.43	8.34 ± 0.87
DHA (22:6n-3)	21.91 ± 0.64	19.37 ± 1.82	20.60 ± 2.08	23.67 ± 2.55	24.18 ± 6.43
DPA (22:5n-6)	0.56 ± 0.07[b]	0.66 ± 0.09[b]	0.80 ± 0.13[ab]	1.13 ± 0.08[ab]	1.33 ± 0.43[a]
DHA/DPA	39.40 ± 3.97[a]	29.75 ± 3.03[b]	26.03 ± 1.62[bc]	21.01 ± 0.80[cd]	18.46 ± 1.66[d]
ARA/EPA	0.17 ± 0.01[c]	0.21 ± 0.02[bc]	0.25 ± 0.01[b]	0.29 ± 0.01[a]	0.30 ± 0.03[a]
DHA/EPA	2.37 ± 0.12	2.32 ± 0.15	2.52 ± 0.13	2.89 ± 0.29	2.87 ± 0.45
DHA/ARA	13.71 ± 0.86[a]	11.23 ± 0.70[b]	10.13 ± 0.52[b]	9.88 ± 0.83[b]	9.39 ± 0.86[b]
Oleic/DHA	0.56 ± 0.03	0.68 ± 0.08	0.61 ± 0.09	0.49 ± 0.08	0.50 ± 0.15
Oleic/n-3 PUFA	0.34 ± 0.02	0.41 ± 0.04	0.38 ± 0.05	0.31 ± 0.05	0.32 ± 0.08
n-3/n-6	4.06 ± 0.19	3.64 ± 0.22	3.54 ± 0.49	4.07 ± 0.41	3.98 ± 0.45

Note: Different superscripts within each row indicate significant differences among protocols ($P < 0.05$; one- way ANOVA; Post-hoc Tukey test).

Abbreviations: ARA, arachidonic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; MUFA, monounsaturated fatty acids; PUFA, long-chain polyunsaturated fatty acids; SFA, saturated fatty acids.

5.3.3. Analysis of skeletal anomalies

The highest incidence (P < 0.05) of total skeletal severe anomalies (lordosis, kyphosis, scoliosis, deformed vertebrae) was observed in 1% A and 25% A co-feeding protocols (Figure 5.6 a). Most of the acute skeletal anomalies were alterations in the pre-haemal region, related mainly to the lordosis appearance, while in the haemal region, the most important affection was recorded as kyphosis and vertebral anomalies, particularly identified as shape anomaly and ossification ridges (Figure 5.6 b, c). Furthermore, the co-feeding protocol had a significant (P < 0.05) effect on the percentage of high-quality larvae. The 1% A, 25% A and 50% A co-feeding protocols exhibited a lower rate of high-quality larvae (13.25 %, 20.36 %, and 29.16 %, respectively) while 75% A and 100 % A co-feeding protocols showed higher rates of high-quality larvae with 43.44 % and 47.34 %, respectively.



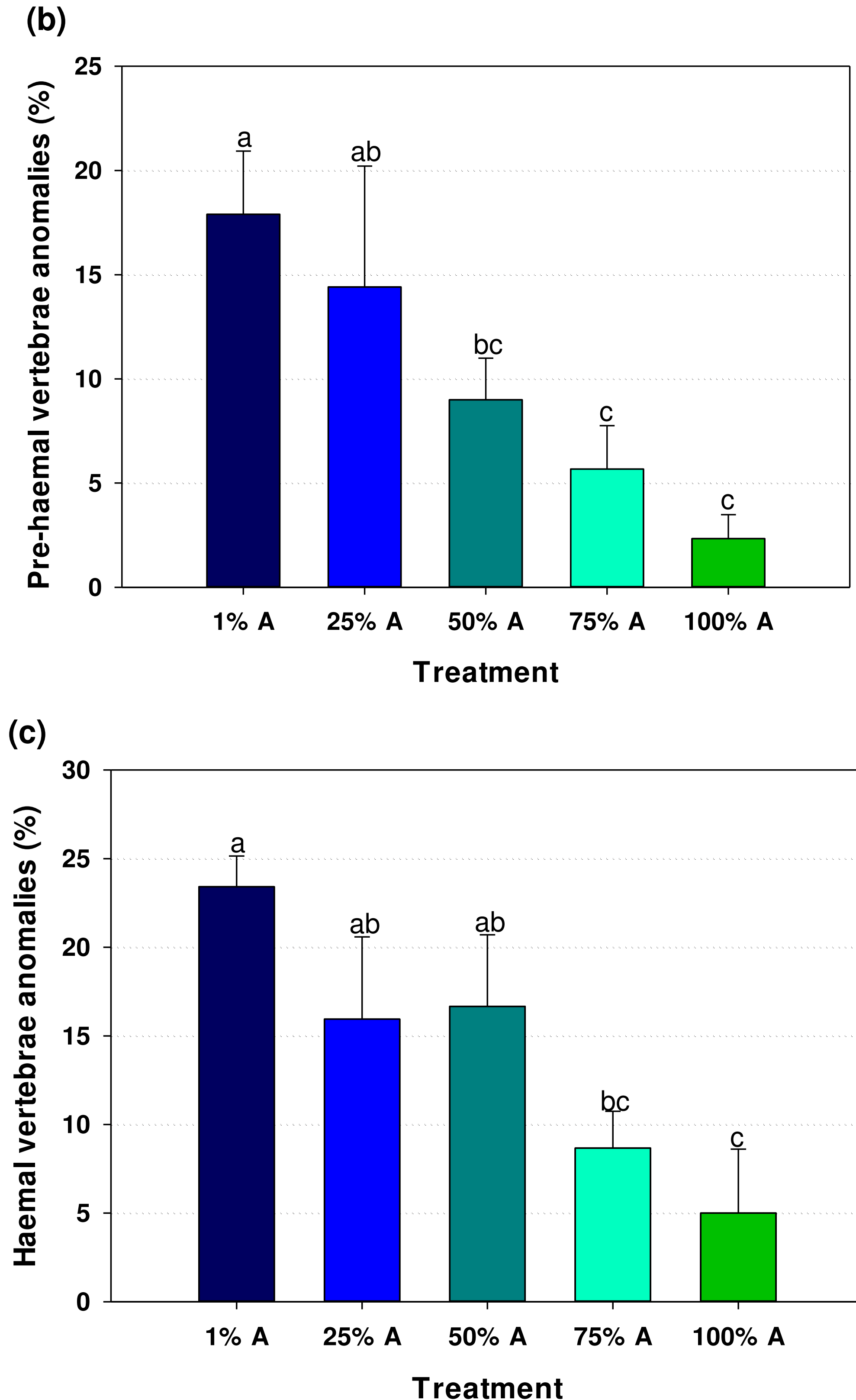


Figure 5.6. (a) Total skeleton severe anomalies (%), **(b)** pre-haemal vertebrae anomalies (%), **(c)** haemal vertebral anomalies (%) in larval greater amberjack at 48 dph fed different co-feeding protocols (means ± SD, n=3). Different letters above the bars indicate significant differences among protocols ($P < 0.05$; one- way ANOVA; Post-hoc Tukey test).

5.3.4. Histological analysis

Significant differences were determined in the hepatocyte vacuolization in the liver of larvae receiving different co-feeding protocols (Figure 5.7). The hepatocyte vacuolization decreased as an effect of *Artemia sp.* amount elevation and was significantly lower (P < 0.05) in the 75% A and 100% A co-feeding protocols (Figure 5.7). Furthermore, in the hepatocytes of greater amberjack larvae in 1% A, 25% A and 50% A co-feeding protocols, the infiltration of lipid vacuolization displaced the nuclei of cells from a central position to the periphery (Figure 5.8a). Contrarily, liver of larvae in 75% A and 100% A co-feeding protocols showed smaller hepatocytes, with spherical nuclei and mostly located at a central position in the cell (Figure 5.8b).

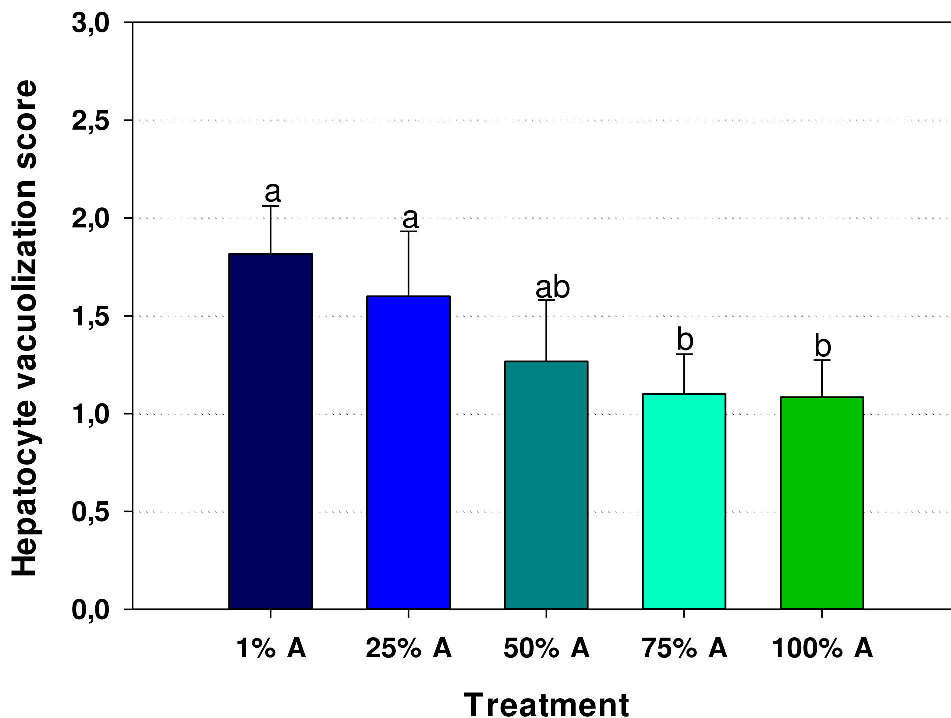


Figure 5.7. Hepatocyte vacuolization in 48 dph greater amberjack larvae fed different co-feeding treatments recorded on a 0–3 score (means ± SD, n=3). Different letters above the bars indicate significant differences among protocols (P < 0.05; one- way ANOVA; Post-hoc Tukey test).

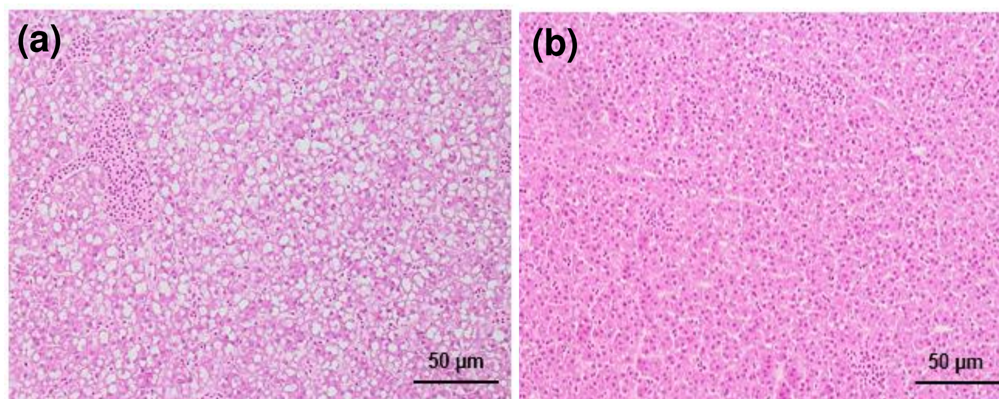
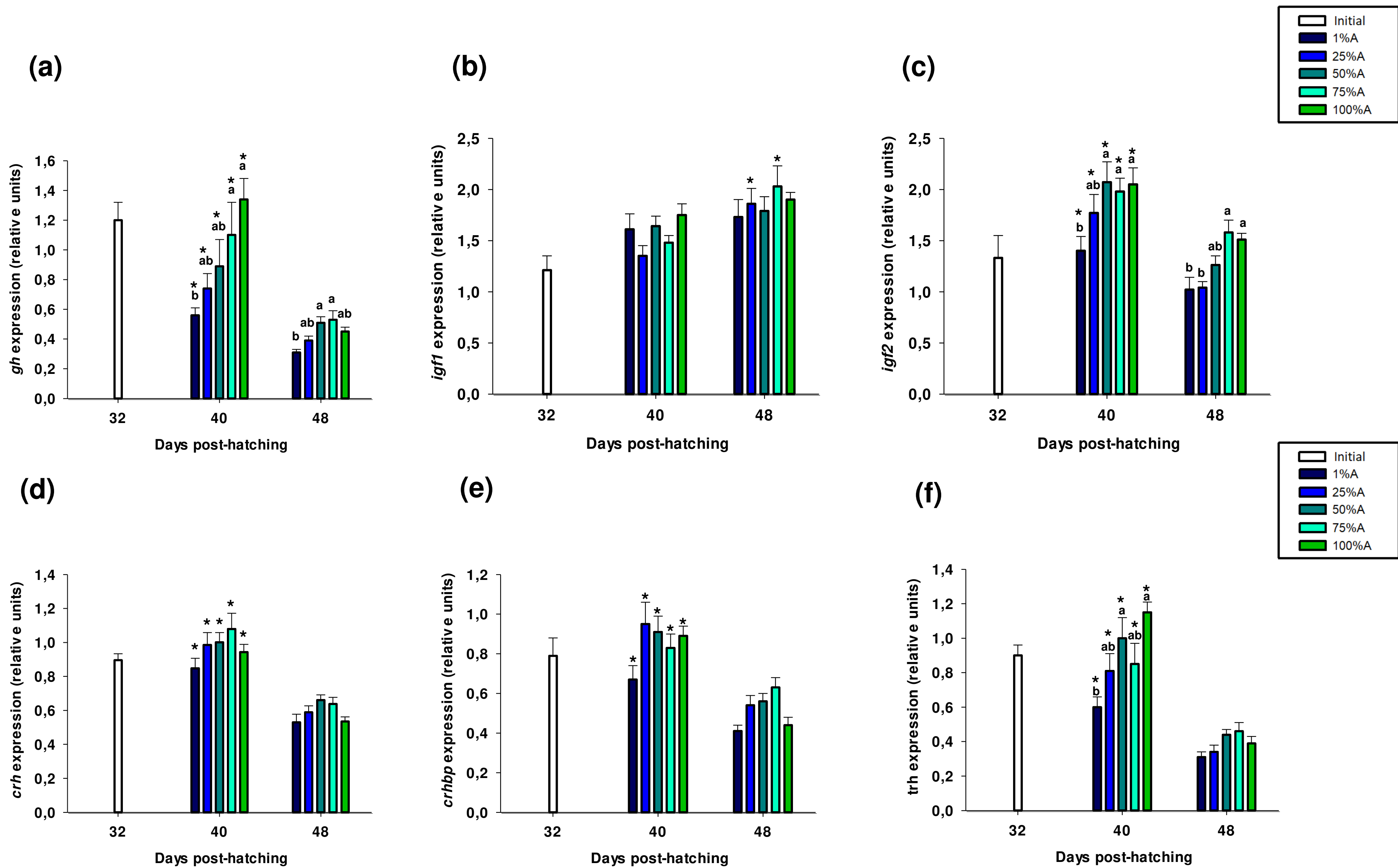


Figure 5.8. Different hepatocyte vacuolization found in 48 dph greater amberjack larvae stained with haematoxylin and eosin, **(a)** 1% A treatment **(b)** 100% A treatment.

4.3.5. Molecular analysis

The expression of growth (*gh*, *igf1*, *igf2*) and stress-related (*crh*, *crhbp*, *trh*, *pomca*, *pomcb*, *star*) genes in whole greater amberjack larvae from different co-feeding protocols at 32, 40 and 48 dph are shown in Figure 5.9. High *gh* and *igf2* expressions at 40 dph were followed by a significant decline at 48 dph in all co-feeding protocols (Figure 5.9 a, c). Significant differences ($P < 0.05$) were also found in the expression of *igf1*, but only in the 25% A and 75% A co-feeding protocols, where higher values were obtained at 48 dph (Figure 5.9 b). Within 40 and 48 dph, *gh* and *igf2* expression were significantly higher ($P < 0.05$) in 75% A and 100% A co-feeding protocols, while the lowest expression was observed in the 1% A co-feeding protocol (Figure 5.9 a, c). However, no significant differences were found in the expression of *igf1* among co-feeding protocols, within 40 or 48 dph (Figure 5.9 b). Expression of *crh* and *crhbp* at 40 dph was higher in all co-feeding protocols, compared to those at 48 dph ($P < 0.05$), however, no significant differences were found among co-feeding protocols, within both 40 and 48 dph (Figure 5.9 d, e). Similarly, the expression of *trh* was significantly higher in larvae at 40 dph compared to 48 dph in all co-feeding protocols, but significant differences were found among co-feeding protocols at 40 dph, with the lowest expression in 1% A (Figure 5.9 e, f). Concerning *pomca* and *pomcb*, they showed significant differences among co-feeding protocols at different times. At 40 dph, *pomca* expression was significantly higher ($P < 0.05$) in 100% A, while 75% A co-feeding protocol showed the lowest levels (Figure 5.9 g). At 48 dph, lower mRNA expression of *pomcb* was observed in the 1% A co-feeding protocol compared to the other protocols (Figure 5.9 h). Similar to *pomca*, *star* was also affected by the co-feeding protocols at 40 dph, and the larvae of 100% A co-feeding protocol showed a marked increase in gene expression (Figure 5.9 i).


(a)
(b)
(c)
(d)
(e)
(f)
Initial
1%A
25%A
50%A
75%A
100%A
gh expression (relative units)
igf1 expression (relative units)
igf2 expression (relative units)
crh expression (relative units)
crhbp expression (relative units)
trh expression (relative units)
Days post-hatching
32
40
48

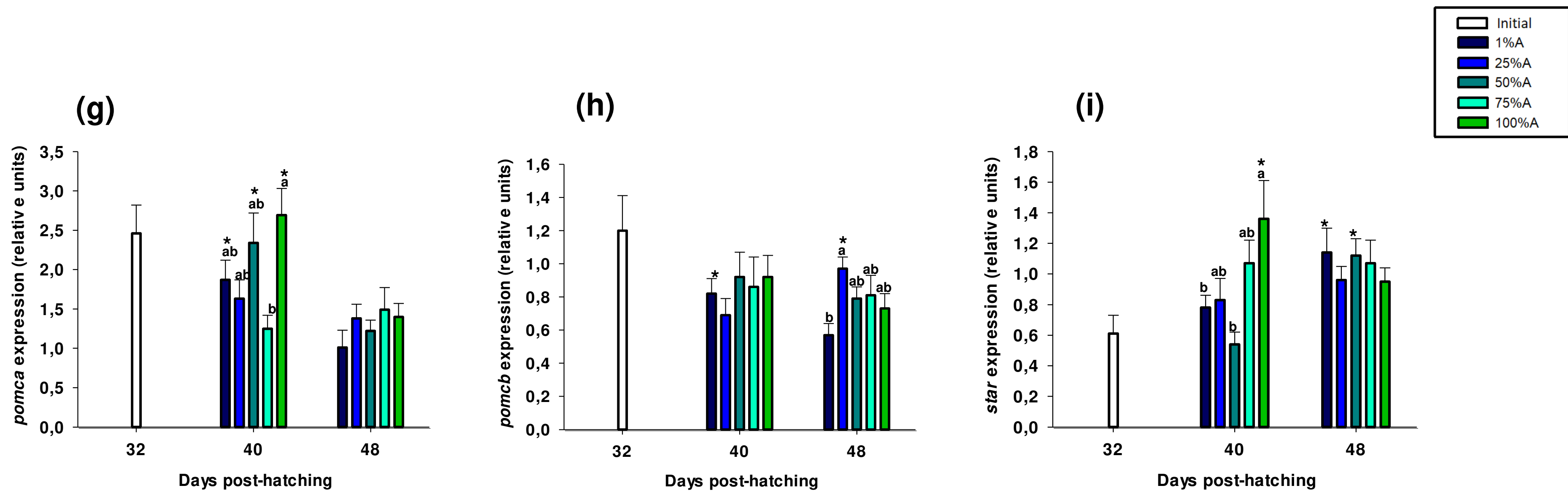


Figure 5.9. Relative gene expression levels determined in whole greater amberjack larvae from different co-feeding protocols, at 32, 40 and 48 dph (mean ± SEM, n = 15); **(a)** *growth hormone* (*gh*), **(b)** *insulin-like growth factor I* (*igf1*), **(c)** *insulin-like growth factor II* (*igf2*), **(d)** *corticotropin releasing hormone* (*crh*), **(e)** *corticotropin releasing hormone binding protein* (*crhbp*), **(f)** *thyrotropin releasing hormone* (*trh*), **(g)** *proopiomelanocortin a* (*pomca*), **(h)** *proopiomelanocortin b* (*pomcb*), and **(i)** *steroidogenic acute regulatory protein* (*star*). Different letters indicate significant differences among co-feeding protocols at the same sampling time (P < 0.05; one- way ANOVA; Post-hoc Tukey test), whereas asterisks indicate significant differences between 40 and 48 dph sampling times in the same co-feeding protocol (P < 0.05; Student's *t*-test).

5.4. Discussion

Weaning the marine fish larvae at first feeding directly onto microdiet is quite challenging, being a species-specific process. However, good results could be obtained when used in combination with live food (Curnow et al., 2006 a, b). The evaluation of five co-feeding protocols for greater amberjack made in this study revealed that the amount of *Artemia sp.* is an important factor affecting the growth performance, survival, and nutritional condition of the larvae. In the current study, weaning was carried out by a gradual replacement of *Artemia sp.* with a commercial diet over a minimum of 16 days, and its conditions influenced the growth of the greater amberjack larvae. The reduction of *Artemia sp.*, in 1-50% A protocols, was reflected in higher larval growth, in terms of total length, wet body mass, and SGR, especially during the second feeding period, from 40 to 48 dph. Normally, under low live food provision, fish larvae spend more time to capture and ingest the prey, leading to slow growth (Shaw et al., 2006; Ma et al., 2013). In yellowtail amberjack, the growth of fish larvae was improved by increasing the *Artemia* co-feeding (Ma et al., 2013). Similar results have been observed in mulloway, in which the larvae grew faster at high *Artemia* concentrations (Ballagh et al., 2010). Therefore, the increased growth observed in 1 % A and 25% A at the end of the present experiment is more likely linked to the significantly lower survival and higher cannibalism rates observed in low *Artemia sp.* co-feeding treatments. The higher growth observed in 1 % A and 25% A larvae could also be attributed to the enhanced nutrition of the large larvae that were consuming the smaller ones, which are presumably very good food for the larger ones. Successful weaning of fish larvae is largely dependent on the larvae's ability to catch, select and digest the microdiet, which is also related to larval capacity to adapt to the new feed characteristics (no mobility, size, form or hardness, palatability among others). Delays in adaptation to the microdiet can lead to starvation and nutrient deficiency, resulting in growth retardation and even death (Gisbert et al., 2004). In this sense, in the present experiment, the larvae of the treatments that received lower amounts of *Artemia* suffered higher daily mortality during the first days of early weaning, mainly due to the fact that most of the larvae that did not accept well the microdiet, grew slowly and became vulnerable to predation by bigger larvae.

In fact, in low *Artemia* co-feeding treatments, the high cannibalism and low survival rates led to a lower larval density, especially from 40 to 48 dph. Generally, low larval density is associated with high growth in some marine species, such as meagre and red porgy (Hernández-Cruz et al., 1999; Roo et al., 2010a). Another aspect to consider is that the

increase in larval density leads to high competition for food and space, a higher expenditure of energy deriving high metabolic rates, and therefore a decrease in growth (Ellis et al., 2002; Thorarensen and Farrell, 2010). In the present study, lowering the amount of *Artemia sp.* from 2 Artemia mL $^{-1}$/day (100% A) to 1.5 Artemia mL $^{-1}$/day (75% A), did not affect the growth, survival, or cannibalism incidence. Similarly, Ballagh et al. (2010) showed that it was possible to co-feed the mulloway larvae a microdiet along with a reduced amount of *Artemia* (up to 50%) without compromising the growth. However, if *Artemia* was excluded from the diet, growth was reduced. Callan et al. (2003) indicated that similar results to live food controls could be achieved by weaning Atlantic cod larvae early, while supplementing microdiet feed with reduced rations (25-50% of the live food) of *Artemia*.

Optimization of prey density and its early replacement with microdiet are of great importance to improve the survival of marine fish larvae in general. Such optimization is of particular importance at the weaning period, as inadequate regimes during this critical transition, could lead to high rates of mortality (Chen et al., 2007; Hamre et al., 2013). Low amounts of *Artemia* negatively affected the survival rate in our study, 1% A and 100% A co-feeding protocol representing the minimum (21.53 ± 4.04 %) and maximum rates (50.72 ± 4.87 %), respectively. The present results showed an improvement in the survival rate compared to the previous reports by Roo et al. (2019), with 24%, and Papandroulakis et al. (2005), with 3.5%, for the same species. Moreover, these results were better than what has been obtained for other fast-growing species like yellowtail amberjack (10.08 ± 1.17%; Hu et al., 2017) or meagre (2.8 ± 0.6%; Campoverde et al., 2017). Additionally, during the experiment, we observed that the activity of the larvae became more vigorous, increasing their predation and cannibalism activity, especially under low *Artemia sp.* co-feeding protocols. When resources are limited in natural environments, cannibalism is considered as an alternative feeding strategy that is adopted mainly by piscivorous larvae (Hecht and Pienaar, 1993). Cannibalism affects directly the survival or indirectly the larval growth, as small individuals are typically exposed to more potential predators and the ability to evade predators generally increases with the body size (Bailey and Houde, 1989). This could explain why lowering the *Artemia* provision in 1 % A and 25% A treatments affected survival. In fact, cannibalism was also higher in treatments that received smaller amounts of *Artemia sp.*. Generally, there are two peaks of mortality in greater amberjack larval rearing (Miki et al., 2011). The first one is linked to the transition from endogenous to exogenous feeding and the swim bladder inflation. The second mortality peak, on the other hand, is

caused by cannibalism during the weaning. In the present study, significantly increased mortality as an effect of *Artemia sp.* reduction implies the enhancement of cannibalistic behaviour. Similar findings have been reported for other fast-growing species such as meagre, where high cannibalism resulted in a reduction in survival rates and dispersion in larvae size during the period of reducing the *Artemia* density (Roo et al., 2010b; Campoverde et al., 2017). Cannibalism occurs in other *Seriola* larvae such as yellowtail (Sakakura and Tsukamoto, 1999) or yellowtail amberjack (Moran, 2007; Stuart and Drawbridge, 2013). The authors attributed the cannibalistic aggressions to high size differences within the cohorts and suggested that the frequency of such behaviours increases when the size differences between prey and predator are larger. Although not statistically significant, low amounts of *Artemia* tended to increase the size deviation in the present experiment. Besides, other factors, such as live prey regimes could also affect the onset of cannibalism. Miki et al. (2011) indicated that restricted live prey feeds entailed aggressive behaviour in greater amberjack larvae. Similarly, in yellowtail amberjack, the introduction of *Artemia sp.* as a food source was correlated with the increase in size heterogeneity and cannibalism behaviour (Moran, 2007).

Increasing *Artemia sp.* replacement levels by microdiet (1% A, 25% A and 50% A) led to a higher incidence of skeleton anomalies appearance. In many fish species, skeletal anomalies have been frequently reported in larval culture, being often associated with a low survival of the fish larvae (Roo et al., 2009; Izquierdo et al., 2010; Boglione et al., 2013a, b; Mesa-Rodriguez et al., 2019; Roo et al., 2019). Greater amberjack larvae are particularly susceptible to the incidence of skeletal anomalies (Roo et al., 2019; Sawada et al., 2020). Despite the predominant number of anomalies in the pre-haemal and haemal vertebrae in all larvae, greater amberjack fed low *Artemia sp.* amount showed a significantly higher prevalence of skeletal anomalies compared to larvae fed high *Artemia sp.* amount. Various physiological and behavioural performances of fish larvae such as swimming, feed intake, and feeding efficiency may be affected by anomalies in the pre-haemal and haemal vertebrae (Powell et al., 2009). The cause of skeleton anomalies during the weaning phase depends on several factors including genetic, nutrition, and culture conditions. Nutrition has been considered an important factor affecting skeleton anomalies (Cahu et al., 2003; Hu et al., 2017; Roo et al., 2019). Feed supply with a reduction in *Artemia sp.* amount had an important nutritional implication related to lipids and fatty profile. In this regard, high substitution levels of this microdiet may be insufficient to support normal bone development in a fast-

growing species like greater amberjack. Roo et al. (2019) found that the occurrence of skeletal anomalies was associated with n-3 highly unsaturated fatty acids (HUFA) content in *Artemia*, and the occurrence of cranial anomalies was correlated to increased dietary n-3 HUFA levels. Likewise, previous studies with similar species such as longfin yellowtail suggest that the use of microdiet with high levels of n-3 HUFA content could induce the appearance of skeleton anomalies (Mesa-Rodriguez et al., 2019).

Most of the research on larval lipid nutrition has been focused on essential fatty acid requirements, particularly HUFA, due to its importance for larval growth and quality (Hamre et al., 2013). Inadequate content of those essential fatty acids in live prey or microdiet brings about several biological symptoms in larvae such as reduced appetite, growth, swimming activity, survival, and particularly skeleton anomalies (Izquierdo, 1996, 2005; Cahu et al., 2003; Roo et al., 2019). In greater amberjack, a recent study has shown that n-3 HUFA content in enriched *Artemia* should also be considered to satisfy the larval nutritional requirements (Roo et al., 2019). In that study, when larvae were fed *Artemia*, the best growth, survival, and resistance to stress were obtained with an n-3 HUFA range of 12-17% TFA, DHA content of 5-8.5%, and EPA content of 4.3-5.5% TFA. In the present study, the n-3 HUFA level in *Artemia sp.* (12.9 ± 2% TFA) was in the range reported by these authors. However, the n-3 HUFA level in microdiet (29.62 %) was markedly higher, probably being related to the high incidence of severe anomalies. Roo et al. (2019) reported a higher incidence of total deformities when n-3 HUFA were higher than 17% in the diet. Regardless of the co-feeding treatment, n-3 HUFA, DHA, and EPA contents in greater amberjack larvae were similar. Considering that n-3 HUFA content of greater amberjack larvae was more similar to the n-3 HUFA profile in the microdiet compared to the *Artemia sp.*, these results suggest that either the larvae assimilated the microdiet efficiently or such similar profile was due to the high cannibalistic behaviour of the larvae at the end of the experiment.

The liver is a good biomarker for nutritional effects of different diet composition and feeding regimes, as the hepatic energy stores respond sensitively and rapidly to the nutritional changes in fish larvae (Lazo et al., 2011). Fat deposition in hepatocytes reflects a physiological disorder originated from unsuitable feeding conditions or a nutritionally unbalanced diet (Gisbert et al., 2008; Lazo et al., 2011). In the present study, the co-feeding protocols with lower *Artemia* levels tended to increase the lipid content in the liver. The greater lipid accumulation might be due to higher digestive efficiency for this nutrient that results in greater lipid absorption from the intestine and storage in the liver (Boglino et al.,

2012). Alternatively, such high hepatic lipid accumulation might be due to a poor nutritional condition of the larvae fed smaller amounts of *Artemia*. Similarly, in Senegalese sole larvae reared with *Artemia* showed a normal histological pattern, while histological alterations were detected in those fed microdiet (Fernández-Díaz et al., 2006).

As in most vertebrates, growth endocrine regulation in fish is regulated by the growth hormone (Gh)/insulin-like growth-factor (Igf) axis (Reinecke, 2010; Bertucci et al., 2019). In this study, the expressions of some of the key genes from this axis, i.e. *gh*, *igf1* and *igf2,* were analyzed. Both Gh and Igfs promote growth, although Igf1 seems to be particularly responsible for body growth (Triantaphyllopoulos et al., 2019). In the present study, high *gh and igf2* expressions at 40 dph were followed by a significant decline at 48 dph in all the co-feeding protocols. Evidence at the nutrition level has shown that prolonged starvation or fasting generally results in suppressed body growth, with a concomitant reduction in the levels of circulating *igfs* (Triantaphyllopoulos et al., 2019). Many studies have shown that fasting or poor nutritional status in larvae fish alters the mRNA expression of components of the Gh/Igf axis (Opazo et al., 2017; Piccinetti et al., 2017). But the most suitable explanation for this variation along ages could be the normal ontogenic variation of these genes in this species, as it has been already published in others, like grey mullet (*Chelon labrosus*) (Gilannejad et al., 2020).

The *gh* expressions at 40 and 48 dph were higher in smaller larvae than in the larger ones. Similarly, the mean of *gh* mRNA levels in larvae body of zebrafish was higher in smaller larvae and suggests that the lower growth rate observed in these larvae was associated with the poor nutritional status (Opazo et al., 2017). Comparing the different co-feeding protocols at 48 dph, it was observed that *gh* expression was higher in 50%A and 75% A protocols. Nevertheless, *igf2* expression was higher in the 75% A and 100% A protocols, which also showed the lowest specific growth rate and highest survival rate, leading to higher larval density. A recent study has found that the higher expression of *igfs* was associated with high densities in Siberian sturgeon (*Acipenser baerii*) larvae. Highest stocking densities could provoke crowding stress with a subsequent increase in energy demand and utilization of energy reserves (Aidos et al., 2020). Moreover, the Gh/Igf axis is influenced by stress conditions, and any conditions that induce stress require the larvae to spend energy to maintain the homeostasis (Reindl and Sheridan, 2012**;** Sadoul and Vijayan, 2016). Stress responses associated with co-feeding protocols have been described in some aquaculture species (Liu et al., 2012; Piccinetti et al., 2012). In fish, in response to stressor

exposure, the hypothalamic-pituitary-interrenal (HPI) axis is activated leading to the production of Corticotropin-releasing hormone (Crh) in the hypothalamus, whose levels are regulated by the Crh-binding protein (Crhbp) and Thyrotropin-releasing hormone (Trh) (Gorissen and Flik, 2016; Ruiz-Jarabo et al., 2018). Crh stimulates the proopiomelanocortin (Pomcs) production in the pituitary, which in turn stimulates the cortisol synthesis in the steroidogenic cells located predominantly in the head kidney region in fish (Faught et al., 2016). Consequently, activation of this pathway enhances the activity of enzymes such as the Steroidogenic acute regulatory protein (Star), producing cortisol as the end-product (Montero et al., 2015). In this study, the changes in expression levels of hypothalamic factors (*crh, crhbp*, and *trh*), pituitary hormonal precursors (*pomca* and *pomcb*), and the first enzyme involved in cortisol synthesis (*star*), showed that some components that play a role in the HPI axis are affected by the weaning period. High expression of genes related to the stress response in the mid-weaning period might indicate that forcing the larvae to consume artificial compound feed may have a stressful effect. Differences in the expression of stress-related genes are probably explained because greater amberjack has poor adaptability to stress and can be easily affected by the weaning conditions. Fish larvae are especially sensitive to non-optimal feeding conditions or nutritional stress factors because most tissues and organs are under progressive differentiation and development (Gisbert et al. 2008; Holt, 2011). It has been shown that fish can become habituated to mild repetitive and predictable stressors (Madaro et al., 2016; Sánchez-Nuño et al., 2019). Therefore, a process of adaptation could explain the highest levels of stress genes in the mid-weaning period, being attenuated at the end of the experiment.

5.5. Conclusion

In summary, considering our results as a whole, we can suggest that the density of *Artemia sp.* in greater amberjack larval co-feeding could be reduced at least to 25% of the standard protocol, 1.5 *Artemia sp.* mL^{-1}/day being the optimum concentration under such co-feeding protocols. These co-feeding protocols are expected to ensure high growth and survival rates while reducing the costs, stress response, and occurrence of skeletal anomalies.

CHAPTER 6

Improving the larval performance of greater amberjack (*Seriola dumerili*) during the weaning phase with taurine supplementation on dry diets

Adnane Djellata, Samira Sarih, Carmen María Hernández-Cruz and Javier Roo

Abstract

Taurine has been identified as a required nutrient in several Seriola species, but no available data on dietary taurine supplementation during the weaning period for greater amberjack. Therefore, the taurine requirement in microdiets was determined for larval greater amberjack from 30 to 44 days post-hatching (dph). Four granulated microdiets containing 0.24 to 4.24 % taurine levels were formulated. Growth parameters, survival, histology, and skeletal anomalies characterization were determined. The total length, weight gain and daily weight gain of the greater amberjack larvae fed Tau 1.24 were greater than those fed the other microdiets. Larvae fed Tau 0.24, Tau 1.24 and Tau 2.24 were shown to have a healthy liver, and no signs of intestinal injury were observed along different intestinal regions in larvae fed different microdiets. Besides, the incidence of total skeletal severe anomalies was high in larvae fed Tau 0.24 and Tau 4.24. These findings suggest that dietary taurine supplementation is likely essential for greater amberjack during the weaning stage since this supplementation does result in apparent benefits for larval growth.

6.1. Introduction

The greater amberjack has a great potential for the expansion of the European Union aquaculture industry. Its rapid growth, excellent flesh quality and large size make this species very suitable for product diversification and development of value-added products (Sicuro and Luzzana, 2016). Transitioning from live feeds to microdiets is a critical period in greater amberjack culture. This transition often utilizes a co-feeding strategy where larvae are simultaneously offered both live feeds and formulated microdiets for some time (Djellata et al., 2021). In greater amberjack, while many nutritional studies have focused on either the larval (Hamasaki et al., 2009; Matsunari et al., 2013; Roo et al., 2019) or juvenile stages, after weaning onto formulated diets (Yokoyama et al., 2020), very little attention has been focused on this transition phases (Yamamoto et al., 2008; Djellata et al., 2021). It is therefore important to develop a formulated diet sustaining the growth of greater amberjack larvae during the substitution of live feed.

Taurine, or 2-aminoethanesulfonic acid, is a beta sulfonic amino acid found in high concentrations in animal tissues, including fish (Salze and Davis, 2015). It is involved in numerous important biological functions, including osmoregulation (Gao et al., 2017), antioxidation (Katakawa et al., 2016), neuromodulation (Mezzomo et al., 2017), immunoregulation (Li et al., 2016), gamete and egg quality (Salze et al., 2019, Sarih et al.,

2019), visual function (Markwell and Earle, 1995), detoxification (Chen et al., 2019) and anti-stress capability (Cheng et al., 2018). The role of cysteine sulfinate decarboxylase is crucial for taurine synthesis (Goto et al., 2001). In many fish, the activity of this enzyme in the liver is high and they are capable of synthesizing an adequate quantity of taurine to support normal physiological functions (Salze and Davies, 2015). However, some fish species, such as the *Seriola* genus, have a limited ability to synthesize taurine and its dietary supplementation is suggested in these fish species (Yokoyama et al., 2001; Takagi et al., 2008), notably in larval and early juvenile developmental stages (Salze and Davis, 2015). Larval stages of several carnivorous marine fish appear to benefit from taurine supplementation through enrichment of the live prey or inclusion in microdiets (Salze and Davis, 2015). Enrichment of live food with exogenous taurine for fish larvae has been investigated and revealed that taurine is essential for larval development and growth (Hawkyard et al., 2014). Thus, Matsunari et al. (2013) suggest that taurine enrichment of rotifers is an effective method of enhancing the growth of greater amberjack larvae. Taurine enrichment of rotifers was demonstrated to be effective to improve the growth and survival of Atlantic bluefin tuna larvae (Betancor et al., 2019). Also, the improvements were seen of other species in growth and survival rates (Yamamoto et al., 2008; Xie et al., 2015; Rotman et al., 2017; Stuart et al., 2018), as well as in morphological development (Hawkyard et al., 2014), in metamorphosis and amino acid retention (Pinto et al., 2010), in the activity of digestive enzymes (Salze et al., 2012) and anti-oxidative enzymes, taurine deficiency may cause a high requirement of vitamin C and vitamin E in microdiets of marine fish larvae (Izquierdo et al., 2019). Moreover, taurine supplementation in broodstock diets was proved to have a positive effect on the reproductive performance of yellowtail (Matsunari et al., 2006) and greater amberjack (Sarih et al., 2019).

Taurine appears to be a crucial nutrient for fish, especially carnivorous marine fish such as greater amberjack. While dietary taurine supplementation has been highly recommended, there is a lack of studies on taurine for this species in the larval stage. In this sense, our overall objective was to investigate the effect of graded levels of dietary taurine on growth, survival rate, skeleton anomalies occurrence, and hepatocyte vacuolization of greater amberjack during the transition from live feed to microdiets.

6.2. Material and methods

All the experiment mentioned below was conducted at the Marine Scientific and Technological Park of ECOAQUA University Institute of the University of Las Palmas de Gran Canaria (Las Palmas, Canary Islands, Spain), with an official aquaculture facility register number (REGA): ES350260026567. All animal experiments described in this manuscript fully comply with the recommendations in the Guide for Care and Use of Laboratory Animals of the European Union Council (2010/63/EU), with the general guidelines approved by the project "DIVERSIFICACIÓN DE LA ACUICULTURA ESPAÑOLA MEDIANTE LA OPTIMIZACIÓN DEL CULTIVO DE SERIOLA (*Seriola dumerili*) – SERIOLA, within the National Agricultural Aquaculture Plans 2016-2018 (Ref. 23.17.415A.741).

6.2.1. Larval rearing conditions

The entire experiment was conducted at ECOAQUA Institute (University of Las Palmas de Gran Canaria, Canary Islands, Spain). Greater amberjack eggs from natural spawns were volumetrically counted and set into one black cylindro-conical tank of 40 m^3 capacity at a density of 10 eggs L^{-1}. From 2 days post-hatch (dph) until 19 dph, larvae were fed enriched rotifers (*Brachionus sp*) at a density of 10 rotifers/ml in a green water system containing fresh microalgae *Nannochloropsis sp.* at a density of 0.25×10^6 cells ml^{-1}. From 14 to 29 dph, *Artemia sp.* (EG type; INVE Aquaculture, Dendermonde, Belgium) was provided, starting with 0.1 Artemia metanauplii/ml and increasing the density up to 0.5 Artemia metanauplii/ml at 29 dph. Both live preys were enriched using Ori-Green (Skretting, Stavanger, Norway), for 3 hours at 23 °C in the case of rotifers and 13 hours at 28 °C in the case of Artemia. Live prey was administered by hand every morning at 9:00 a.m. and using automatic feeders in the evening. Every day, the bottom of the tank was siphoned to remove dead fish, uneaten food, and fæces.

At 30 dph, 2700 larvae (initial total length 8.70 ± 0.51 mm, dry body weight 3.63 ± 0.42 mg, mean ±standard deviation) were transferred to 12 experimental cylindrical fibreglass tanks (200 L volume), from this point larvae were fed one of the four experimental microdiets (Tau 0.24, Tau 1.24, Tau 2.24 and Tau 4.24) containing 0.24 to 4.24 % taurine levels for 15 days (Table 6.1), each diet was tested in 3 replicates. The taurine contents and amino acid profiles of the experimental microdiets are shown in Table 6.2. Larvae were co-

fed enriched *Artemia sp.* and experimental diet from 30 to 38 dph. From 39 to 44 dph, larvae were fed exclusively with experimental microdiets. The experimental microdiets have a pellet size of 500-750 µm. To guarantee adequate feeding, the daily dry microdiet supply was initially 1 g and was increased up to 3 g throughout the feeding trial. Microdiets were manually supplied every 45 minutes from 8:00 a.m. to 7:00 p.m. The water temperature for the larval rearing was 23.02 ± 0.21 ºC, dissolved oxygen level was maintained around 6.16 ± 0.24 g L^{-1} and salinity 37 g L^{-1}. All parameters were monitored daily. The flow rate was increased from 0.83 L min^{-1} at 30 dph to 2.5 L min^{-1} at 44 dph, to guarantee good water quality during the trial. A flow trough system was used with a water flow entering from the tank bottom and exited by overflow near de water surface promoting an upwelling movement. An artificial fluorescent light above each tank provided a surface light intensity ranging between 1,000 and 1,500 lux (digital Lux Tester YF-1065; Powertech Rentals, Osborne, Australia) at the centre of each rearing tank for a fixed photoperiod of 12 h light/12 h dark cycle with lights on from 07:30 a.m. to 07:30 p.m., local time.

Table 6.1. Ingredients and proximate composition of the experimental microdiets supplemented with different taurine levels.

	Microdiet			
	Tau 0.24	**Tau 1.24**	**Tau 2.24**	**Tau 4.24**
Ingredients (g/100g Diet)				
Squid meal[a]	60.0	60.0	60.0	60.0
Krill oil (PL0)[b]	4.0	4.0	4.0	4.0
EPA50 Oil[c]	0.2	0.2	0.2	0.2
ARA Oil[d]	0.5	0.5	0.5	0.5
Yeast extract (CMOS)	0.4	0.4	0.4	0.4
Schizochytrium [e]	4.0	4.0	4.0	4.0
Tetraselmis powder[f]	6.0	5.0	4.0	2.0
Gelatine	3.0	3.0	3.0	3.0
Carboximethyl cellulose	3.0	3.0	3.0	3.0
Mineral premix[g]	5.1	5.1	5.1	5.1
Vitamin premix[h]	3.4	3.4	3.4	3.4
Attractants premix[i]	7.5	7.5	7.5	7.5
Taurine	0.0	1.0	2.0	4.0
Proximate analysis (% Dry matter)				
Crude lipid	13.56 ± 0.25	14.59 ± 0.46	13.76 ± 0.90	13.65 ± 1.07
Ash	8.86 ± 0.06	8.73 ± 0.03	8.04 ± 0.03	8.05 ± 0.03
Crude protein	69.59 ± 0.33	70.72 ± 0.16	67.96 ± 0.23	70.73 ± 0.39
Moisture	14.00 ± 0.03	14.46 ± 0.09	14.60 ± 0.08	13.91 ± 0.32

[a] Rieber and son, Bergen, Norway.
[b] Krill, Aker BioMarine, Fjordalleen, Norway.
[c] Croda Chemicals, East Yorkshire, UK.
[d] VEVODAR ®, DSM Food Specialties, Delft, the Netherlands.
[e] Alltech, Inc., Kentucky, USA.
[f] Technological Institute of the Canary Islands, Spain.
[g] Minerals premix supplied per 100 g diet: Fe-Bioplex, 150.00 mg; Mn-Bioplex, 12.00 mg; Se-Selplex, 350.00 mg; ZnSO4.7H2O, 200.00 mg; Al2(SO4)3.6H2O, 0.69 mg; CoSO4.7H2O, 10.71 mg; MgSO4.7H2O, 677.55 mg; NaH2PO4.H2O, 381.45 mg; K2HPO4, 758.95 mg; Ca(H2PO4).2H2O, 671.61 mg; C3H5O3.1/2Ca, 1617.21 mg; NaCl, 215.13 mg.
[h] Vitamins premix supplied per 100 g diet: cyanocobalamin, 0.03 mg; folic acid, 5.40 mg; pyridoxine-HCI, 17.30 mg; thiamine-HCl, 21.80 mg; riboflavin, 72.50 mg; calcium-pantothenate, 101.60 mg; p- aminobenzoic acid, 145.00 mg; nicotinic acid, 290.20 mg; myo-inositol, 1450.90 mg; retinol acetate, 0.20 mg; ergocalcipherol, 3.70 mg; α-tocopherol, 300.00 mg; menadione, 17.30 mg; L-ascorbic acid, 1000.00 mg.
[i] Attractant premix supplied per 100 g diet: inosine-5-monophosphate 620.00 mg, betaine 660.00 mg, L -serine 170.00 mg, L-phenylalanine 250.00 mg, DL-alanine 500.00 mg, L-sodium aspartate 330.00 mg, L-valine 250.00 mg, glycine 500.00 mg; L-arginine HCL, 1000.00 mg; L-threonine, 75.00 mg; L-histidine, 1000.00 mg; L-lysine, 1000.00 mg; L-methionine, 500.00 mg; L-tyrosine, 170.00 mg; L-cysteine, 500.00 mg; Sigma-Aldrich, Madrid, Spain.

Table 6.2. Taurine content and amino acids profiles (g/100 g dry mass) of the experimental microdiets.

	Microdiet			
	Tau 0.24	**Tau 1.24**	**Tau 2.24**	**Tau 4.24**
Taurine	0.32 ± 0.03	1.39 ± 0.13	2.46 ± 0.24	4.16 ± 0.41
EAA				
Arginine	4.48 ± 0.63	4.11 ± 0.56	3.74 ± 0.52	3.41 ± 0.48
Histidine	2.38 ± 0.33	2.16 ± 0.31	1.95 ± 0.27	1.88 ± 0.26
Isoleucine	2.05 ± 0.29	1.88 ± 0.26	1.72 ± 0.24	1.68 ± 0.24
Leucine	3.65 ± 0.51	3.36 ± 0.49	3.07 ± 0.43	2.90 ± 0.41
Lysine	4.03 ± 0.56	3.70 ± 0.51	3.38 ± 0.47	3.20 ± 0.45
Methionin	1.79 ± 0.25	1.81 ± 0.25	1.83 ± 0.26	1.72 ± 0.24
Phenylalanine	2.40 ± 0.34	2.18 ± 0.30	1.96 ± 0.27	1.79 ± 0.25
Threonine	2.26 ± 0.32	2.11 ± 0.30	1.94 ± 0.27	1.84 ± 0.26
Valine	2.51 ± 0.35	2.30 ± 0.32	2.10 ± 0.29	2.02 ± 0.28
NEAA				
Cystein + Cystine	1.02 ± 0.14	1.02 ± 0.14	1.02 ± 0.14	0.95 ± 0.13
Tyrosine	2.37 ± 0.33	2.16 ± 0.30	1.95 ± 0.27	1.89 ± 0.26
Alanine	3.75 ± 0.52	3.46 ± 0.48	3.16 ± 0.44	2.97 ± 0.42
Aspartic acid	5.09 ± 0.71	4.78 ± 0.67	4.49 ± 0.63	4.12 ± 0.58
Glutamic acid	6.58 ± 0.92	5.97 ± 0.83	5.37 ± 0.75	5.04 ± 0.71
Glycine	5.14 ± 0.72	4.75 ± 0.65	4.36 ± 0.61	4.17 ± 0.58
Proline	2.94 ± 0.41	2.66 ± 0.37	2.39 ± 0.33	2.39 ± 0.33
Serine	2.35 ± 0.33	2.20 ± 0.31	2.06 ± 0.29	1.97 ± 0.28

Data are means ± SD (n = 3). EAA, essential amino acids; NEAA, non-essential amino acids.

6.2.2. Growth and survival

Larval growth was assessed by estimating the total length and wet body weight at 44 dph, in 30 randomly selected larvae per tank after the night starvation. Larvae were previously anaesthetized with clove oil at 1% upon sampling. Individual larvae were photographed using a Leica DFC295 digital camera on a trinocular mount of a Leica M125 stereo-microscope and the images were used to measure total length employing the Leica Application Suite v.3.8.0. (Leica Microsystems, Wetzlar, Germany). All 30 larvae, previously washed with distilled water, were then weighed as a batch on an analytical balance (Gibertini Elettronia, E50 S/2, Milano, Italy). Growth parameters were calculated including specific growth rate (SGR), weight gain (WG), daily weight gain (DWG) and thermal growth coefficient (TGC) using the following formulae:

$$\text{SGR} = \frac{\ln(\text{Final length}) - \ln(\text{Initial length})}{\text{Duration of the experiment (days)}} \times 100$$

WG (mg) = Final weight (mg) – Initial weight (mg)

$$\text{DWG (mg)} = \frac{\text{Final weight (mg)} - \text{Initial weight (mg)}}{\text{Duration of the experiment (days)}}$$

$$\text{TGC} = \frac{\text{Final weight}^{\frac{1}{3}}\text{(mg)} - \text{Initial weight}^{\frac{1}{3}}\text{(mg)}}{\text{Mean water temperature (ºC)} \times \text{Duration of the experiment (days)}} \times 1000$$

Survival rate (%) was calculated by counting individual live larva at the beginning and the end of the trial (n=3 per treatment) using the following formula:

$$\text{Survival rate} = \frac{\text{Nf}}{\text{Ni} - \text{Ns}} \times 100$$

where Nf was the number of living larvae at the end of the experiment, Ns was the number of larvae sampled during the trial, Ni was the initial larvae numbers stocked in each tank.

6.2.3. Biochemical analysis

The proximate composition analysis of experimental microdiets (triplicate assays for each microdiet) and 44 dph larvae (10 larvae per tank) were performed according to the standard methods of the Association of Official Analytical Chemists (AOAC, 2016). 50 g of each diet was stored (-20 ºC) at the beginning of the experimental trial to conduct all analyses. Moisture was determined after drying the samples in an oven-dried at 105 ºC until reaching constant weight. Ash content was determined by combustion at 600 ºC for 12 h. Crude lipid was extracted following the Folch method (Folch et al., 1957) and crude protein content (N x 6.25) was determined by Kjeldahl method. Taurine content and amino acids profile of the experimental microdiets were determined in triplicate at the laboratory of the Eurofins Group (Santa Cruz de Tenerife, Spain) using the GB 5009.169-2016 First method and high-efficiency chromatography (EU 152/2009 (F), Standards EN ISO 13904, ISO 13903:2005), respectively. All analyses were conducted in triplicate.

6.2.4. Histological analysis

For histological analysis, ten 44 dph larvae per tank were randomly sampled and fixed for 48 h in 10% neutral-buffered formalin. Fixed larvae were dehydrated with an increased graded series of ethanol, submerged in xylene, then individually embedded in paraffin blocks and sectioned in serial sagittal sections (4 µm), using a rotary microtome

(Mod. Jung Autocut 2055; Leica, Nussloch, Germany). The slides were stained with hematoxylin and eosin (H&E) (Martoja and Martoja-Pierson, 1970). The sections were examined under light microscopy using an Olympus CX41 binocular microscope (Olympus, Hamburg, Germany) connected to an Olympus XC30 camera (Olympus), which was linked to a computer using Image Capturing Software (CellB®; Olympus). Tissue morphology of hepatic and intestine integrity were examined by two independent observers. Hepatocyte vacuolization was assessed by using a three-point scoring system where: Score 0 (normal hepatic vacuolization): The hepatocytes are small, with few small vacuoles in their cytoplasm, central positioning of their nucleus. Score 1 (moderate hepatic vacuolization): The hepatocytes are slightly enlarged, with vacuoles in their cytoplasm, or one big vacuole and their nucleus are pushed towards the periphery in most hepatocytes. Score 2 (severe hepatic vacuolization): Almost all hepatocytes are significantly enlarged, the cytoplasm appears ‘empty’ due to the presence of big vacuoles and their nucleus is pushed towards the periphery. The incidence of intestinal injury was assessed by counting the number of larvae presenting any intestinal alteration (Betancor et al., 2012).

6.2.5. Analysis of skeletal anomalies

To evaluate the impact of feeding with different microdiets on the incidence of skeletal anomalies in greater amberjack, 50 larvae aged 44 dph per tank were randomly sampled at the end of the trial. Larvae were preserved in 4% formalin buffered with phosphate (0.13 M, pH 7.2) and stored until staining. Larvae were stained with alizarin red (Sigma-Aldrich, Barcelona, Spain) to detect bony tissues. Once stained, individual larvae were photographed using a Leica DFC295 digital camera on a trinocular mount of a Leica M125 stereomicroscope and processed using Leica Application Suite v.3.8.0. (Leica Microsystems, Wetzlar, Germany) to characterize skeletal deformities (Vandewalle et al., 1998) by two independent observers. The incidence of skeletal anomalies was determined in the cranium, vertebral column and caudal fin complex. Special attention was given to severe vertebral deformities, which included the fusion and compression of adjacent vertebral bodies, deformation of vertebral bodies and changes in the anterior-posterior alignment of vertebrae (kyphosis and lordosis). The nomenclature of skeletal elements was conducted according to the method described by Boglione et al. (2014).

6.2.6. Statistical analysis

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). Data were expressed as mean ± standard deviation (SD) and tested by one-way ANOVA test. When a significant difference was found between treatments, a Tukey's post-hoc test was performed for multiple range comparisons with the level of significant difference set at $p < 0.05$. All the data were tested for normality, homogeneity, and independence to satisfy the assumptions of ANOVA, whereas data expressed as percentages were previously arcsine-transformed (Sokal and Rohlf, 2012).

6.3. Results

6.3.1. Larval growth and survival

The total length and wet weight of greater amberjack larvae at 44 dph fed microdiets with different levels of taurine is shown in Figure 6.1. Total length and weight were significantly ($P < 0.05$) highest when larvae were fed Tau 1.24, and significantly ($P < 0.05$) lowest in those fed Tau 4.24. The other growth parameters of larvae are shown in Table 6.3. WG and DWG were significantly ($P < 0.05$) higher in larvae fed Tau 1.24 compared to larvae fed other microdiets. TGC was significantly ($P < 0.05$) higher in larvae fed Tau 0.24 and Tau 1.24, while there were no significant differences ($P < 0.05$) among Tau 0.24, Tau 2.24 and Tau 4.24. SGR did not show any differences among larvae fed the different microdiets. Furthermore, the final survival rate ranged between 28.9 to 30% and was not affected by the taurine level in the microdiets (Figure 6.2).

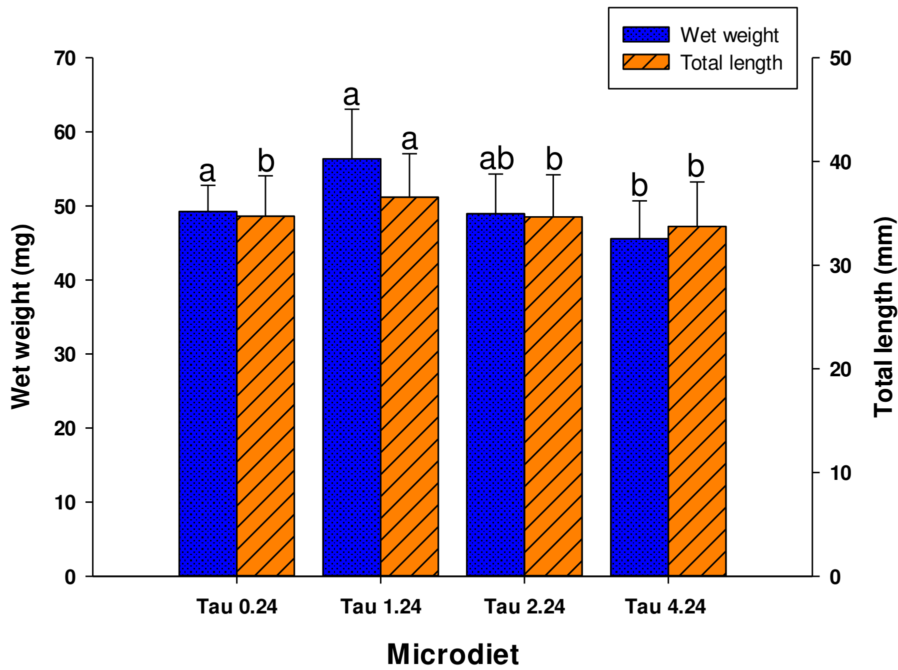


Figure 6.1. Wet weight (mg, left y-axis) and total length (mm, right y-axis) in larval greater amberjack at 44 dph fed microdiets containing different levels of taurine (means ± SD, n=3). Different letters above the bars indicate a significant difference among treatments ($P < 0.05$; one-way ANOVA; Post hoc test Tukey).

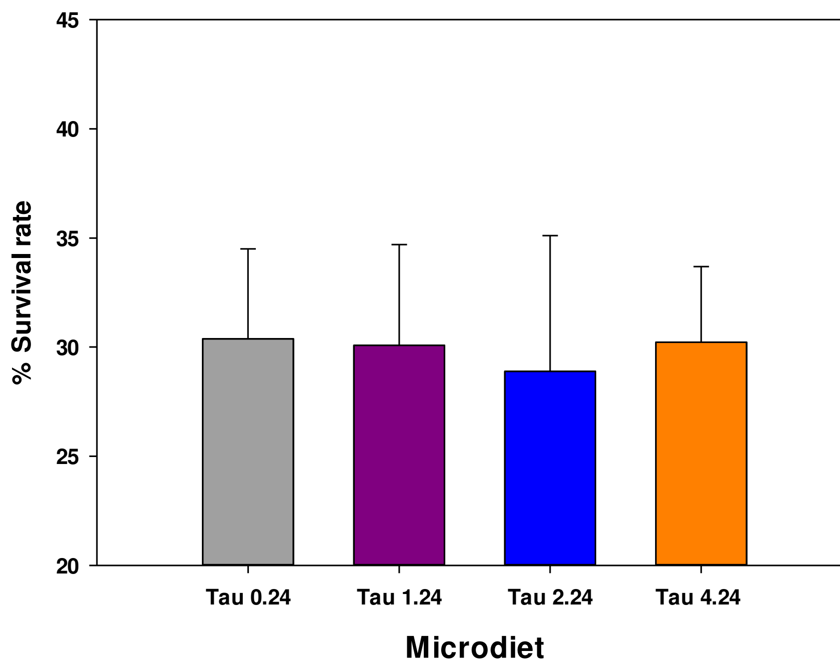


Figure 6.2. Survival rate at 44 dph greater amberjack larvae fed microdiets containing different levels of taurine (means ± SD, n=3).

Table 6.3. The growth performance of greater amberjack larvae at 44 dph fed microdiets containing different levels of taurine (means ± SD, n=3). Different superscripts within each row indicate significant differences among protocols (P < 0.05; one-way ANOVA; Post-hoc Tukey test).

	Microdiet			
	Tau 0.24	**Tau 1.24**	**Tau 2.24**	**Tau 4.24**
Weight gain (mg)	42.68 ± 3.52^{b}	49.80 ± 6.68^{a}	42.39 ± 5.36^{b}	39.01 ± 5.11^{b}
Daily weight gain (mg)	2.85 ± 0.24^{b}	3.32 ± 0.45^{a}	2.82 ± 0.36^{b}	2.60 ± 0.34^{c}
Thermal growth coefficient	5.19 ± 0.25^{ab}	5.67 ± 0.43^{a}	5.16 ± 0.38^{b}	4.91 ± 0.38^{b}
Specific growth rate (% day^{-1})	10.29 ± 0.08	10.65 ± 0.30	10.28 ± 0.06	10.09 ± 0.22

6.3.2. Biochemical analysis

The proximate composition of the whole body of greater amberjack larvae at 44 dph fed different microdiets is shown in Table 6.4. Despite no differences in the crude protein and crude lipid contents of the diets (Table 6.1), larval crude protein content tended to increase gradually while the crude lipid content tended to decrease with the increase in dietary taurine. The larval crude protein content of the Tau 4.24 group was significantly (P < 0.05) higher than those of the Tau 0.24 and Tau 1.24 groups, yet there was no difference when compared with that of the Tau 2.24 group (P < 0.05). On the contrary, the larval crude lipid content of the Tau 4.24 groups was significantly (P < 0.05) lower than those of the Tau 0.24. The moisture content of the whole body from groups Tau 0.24, Tau 1.24 and Tau 2.24 were significantly (P < 0.05) lower than that in the Tau 4.24 group. The ash content was not significantly different between treatments (P < 0.05).

Table 6.4. Proximate composition (% dry matter) on the whole body of greater amberjack larvae at 44 dph fed microdiets containing different levels of taurine (means ± SD, n=3). Different superscripts within each row indicate significant differences among protocols (P < 0.05; one-way ANOVA; Post-hoc Tukey test).

	Microdiet			
	Tau 0.24	**Tau 1.24**	**Tau 2.24**	**Tau 4.24**
Crude lipid	12.81 ± 0.57^{a}	10.83 ± 0.29^{ab}	10.83 ± 0.18^{ab}	9.87 ± 0.88^{b}
Ash	12.20 ± 0.60	13.56 ± 1.75	13.42 ± 0.47	13.71 ± 1.52
Crude protein	75.26 ± 0.14^{c}	76.87 ± 0.23^{bc}	78.53 ± 0.38^{ab}	79.66 ± 1.20^{a}
Moisture	80.18 ± 0.26^{b}	80.38 ± 0.04^{b}	80.57 ± 0.01^{b}	81.82 ± 0.03^{a}

6.3.3. Histological analysis

The hepatocyte vacuolization increases gradually with the increase in dietary taurine. Significant differences were observed in the hepatocyte vacuolization in the liver of larvae receiving different microdiets, and hepatocyte vacuolization was significantly ($P < 0.05$) higher in Tau 4.24 group compared to Tau 0.24 group, while there were no significant differences ($P < 0.05$) among Tau 0.24, Tau 1.24 and Tau 2.24 groups or Tau 1.24, Tau 2.24 and Tau 4.24 groups (Figure 6.3). The histological changes of hepatocytes can be seen clearly in Figure 6.4. In Tau 0.24 group, most hepatocytes are small, with few small vacuoles in their cytoplasm, central positioning of their nucleus (Figure 6.4 a). In Tau 1.24 and Tau 2.24 groups, the hepatocytes are enlarged, with vacuoles in their cytoplasm, or one big vacuole and their nucleus are pushed towards the periphery in most hepatocytes (Figure 6.4 b). However, in Tau 4.24 group, almost all hepatocytes are enlarged, the cytoplasm appears empty due to the presence of big vacuoles and their nucleus is pushed towards the periphery (Figure 6.4 c). On the other hand, intestinal injuries were observed along different intestinal regions in larvae fed different microdiets with no significant differences ($P < 0.05$) between treatments and the average incidence of injury was 3.3-13.3% (Figure 6.5).

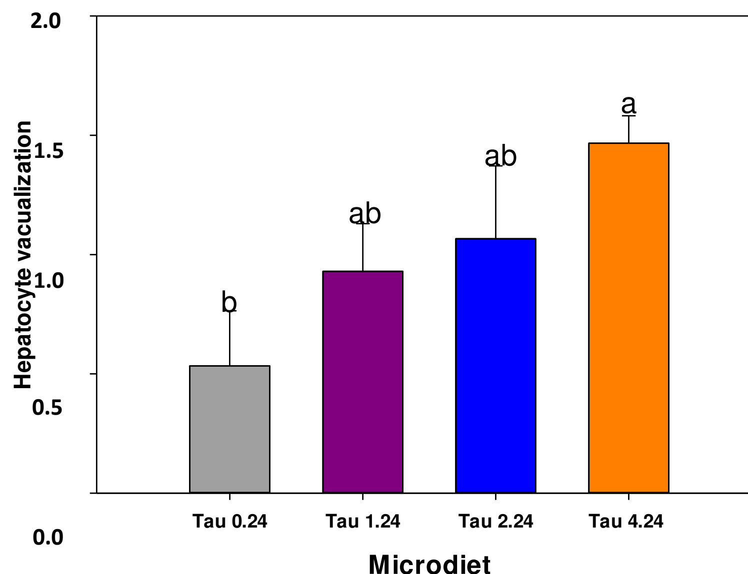


Figure 6.3. Hepatocyte vacuolization* found at 44 dph greater amberjack larvae fed microdiets containing different levels of taurine (means ± SD, n=3). Different letters above the bars indicate a significant difference among treatments ($P < 0.05$; one-way ANOVA; Post hoc test Tukey). * Normal vacuolization (0); Moderate vacuolization (1); Severe vacuolization (2).

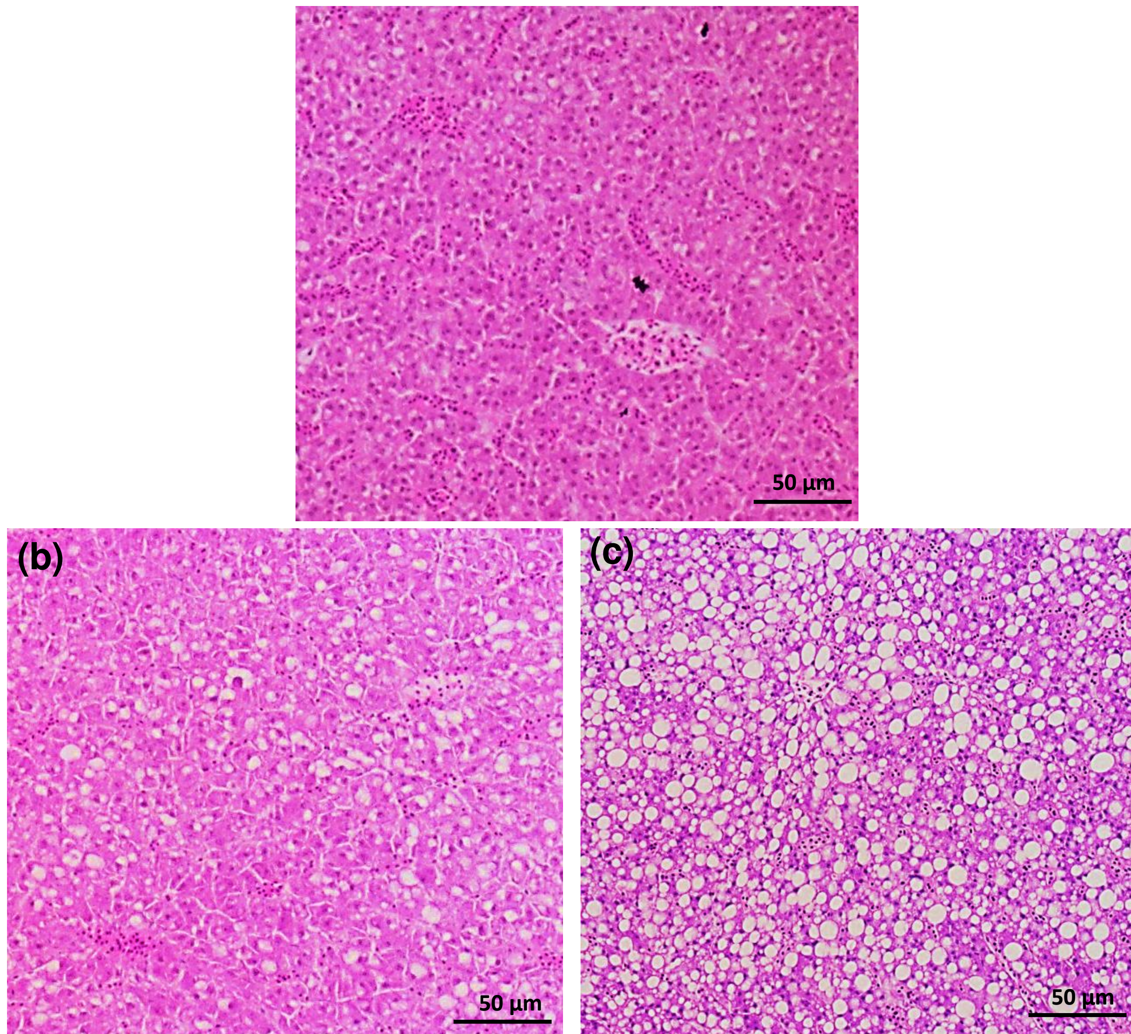


Figure 6.4. Greater amberjack liver tissue sections. **(a)** Sample collected from Tau 0.24 group exhibiting score 0 (normal hepatic vacuolization): The hepatocytes are small, with few small vacuoles in their cytoplasm, central positioning of their nucleus. **(b)** Sample from Tau 1.24 group, exhibiting score 1 (moderate hepatic vacuolization): The hepatocytes are slightly enlarged, with vacuoles in their cytoplasm, or one big vacuole and their nucleus is pushed towards the periphery in most hepatocytes. **(c)** Sample from Tau 4.24 group, exhibiting score 2 (severe hepatic vacuolization): Almost all hepatocytes are enlarged, the cytoplasm appears 'empty' due to the presence of big vacuoles and their nucleus is pushed towards the periphery. Stain H&E.

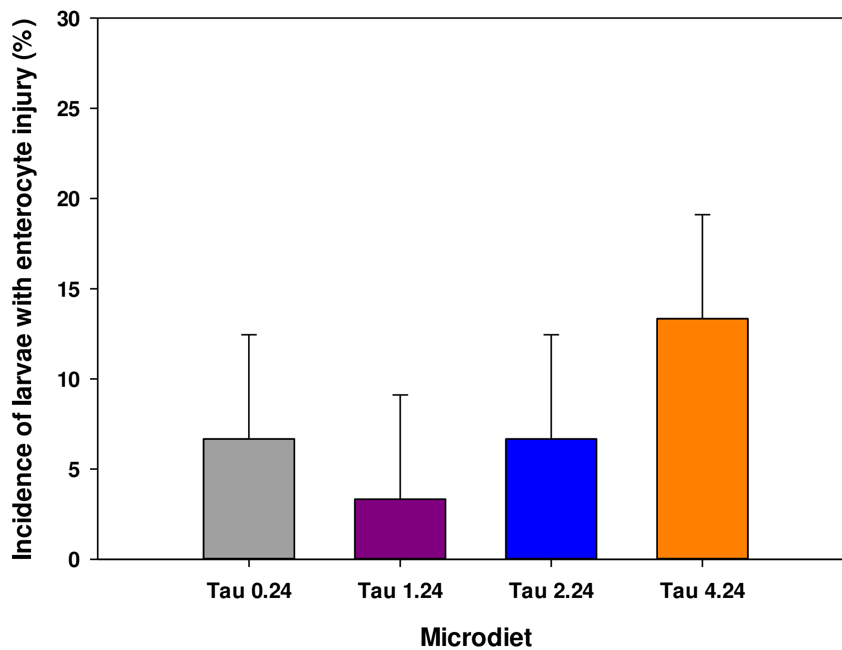
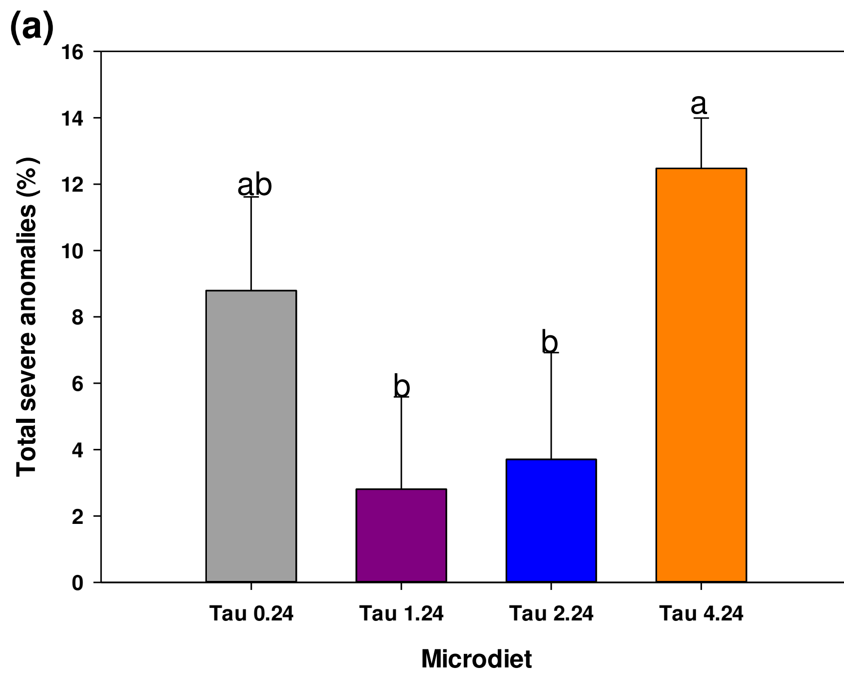
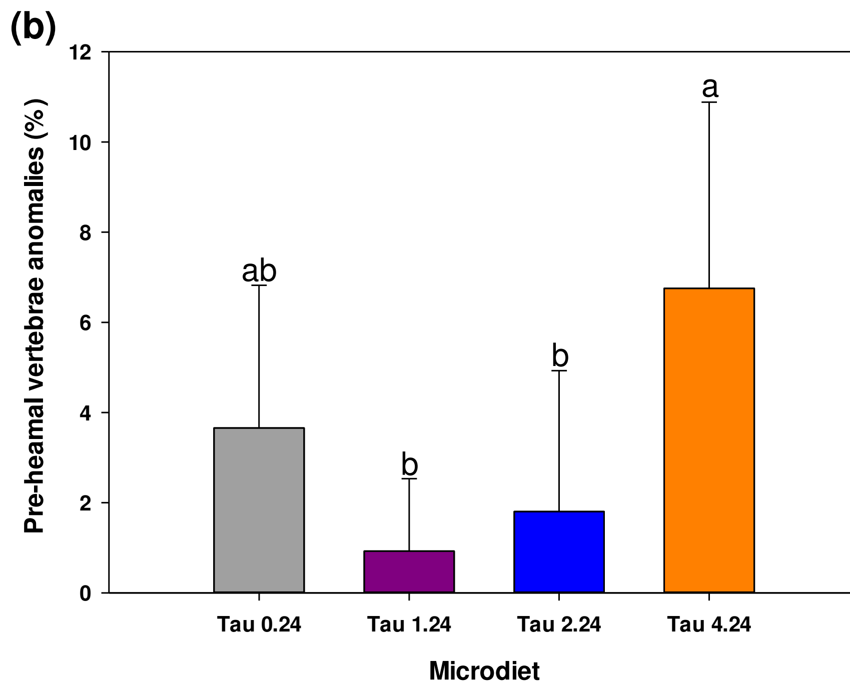


Figure 6.5. Incidence of larvae with enterocyte injury (%) was found in 44 dph greater amberjack larvae fed microdiets containing different levels of taurine (means ± SD, n=3).

6.3.4. Analysis of skeletal anomalies

The highest incidence ($P < 0.05$) of total skeletal severe anomalies was found in larvae fed Tau 4.24 (Figure 6.6 a). Most of the acute skeletal anomalies were alterations in the pre-haemal and haemal regions, related mainly to the fusion and compression of adjacent vertebral bodies, deformation of vertebral bodies and kyphosis. Decrease in dietary taurine from 4.24 g to 2.24-1.24 g in 100g diet, significantly ($P < 0.05$) reduced the total skeletal severe anomalies and pre-haemal vertebrae anomalies (Figure 6.6 a, b). On the contrary, further decreasing taurine level to 0.24 g in 100g diet significantly ($P < 0.05$) raised the total skeletal severe anomalies (Figure 6.6 a, b). No significant ($P < 0.05$) difference was observed in the vertebral anomalies and haemal vertebrae anomalies among dietary groups (Figure 6.6 c, d).


(a)
Total severe anomalies (%)
16
14
12
10
8
6
4
2
0
ab
b
b
a
Tau 0.24
Tau 1.24
Tau 2.24
Tau 4.24
Microdiet


(b)
Pre-heamal vertebrae anomalies (%)
12
10
8
6
4
2
0
ab
b
b
a
Tau 0.24
Tau 1.24
Tau 2.24
Tau 4.24
Microdiet

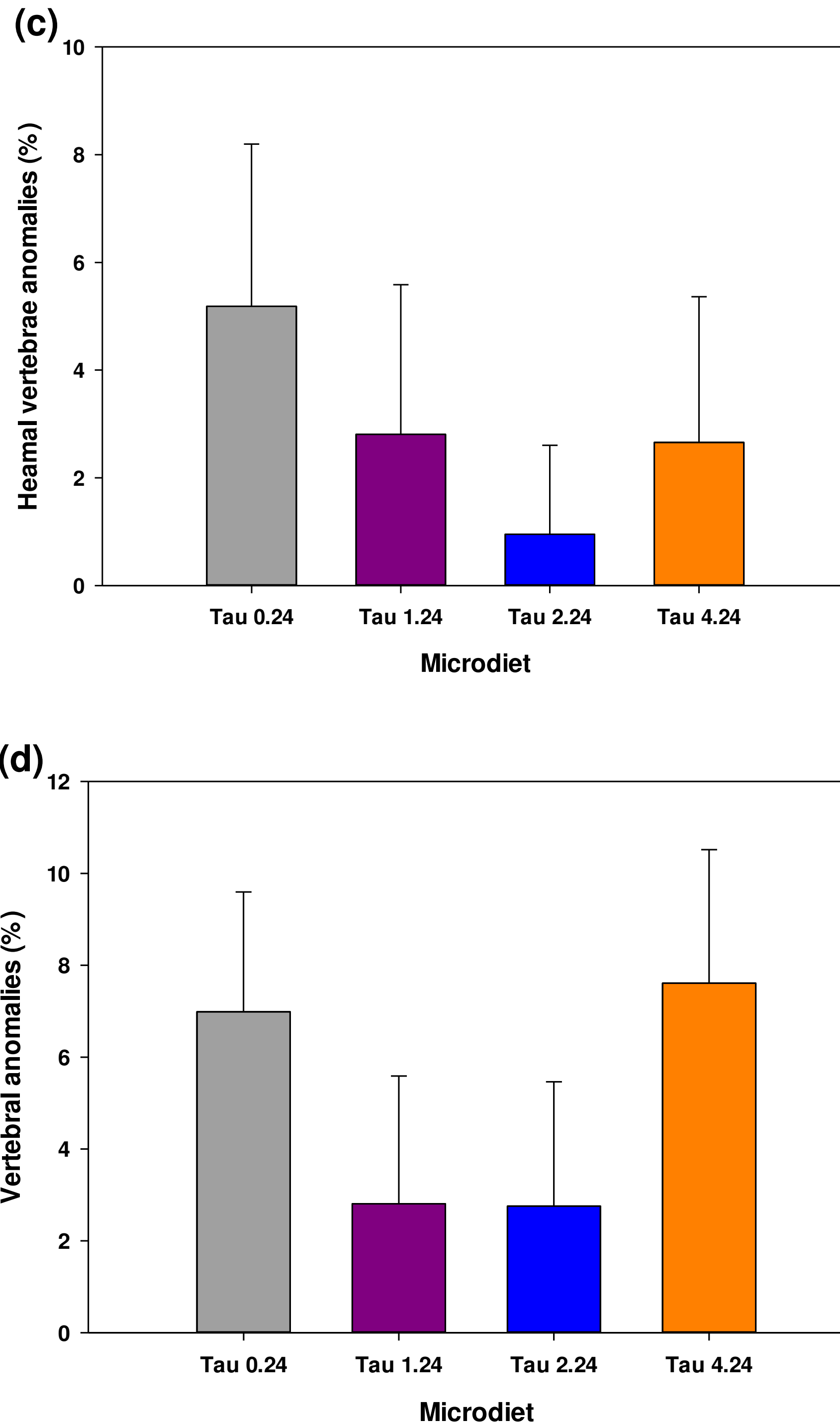


Figure 6.6. (a) Total severe anomalies (%), **(b)** Pre-haemal vertebrae anomalies (%), **(c)** Haemal vertebral anomalies (%), **(d)** Vertebral anomalies (%) in larval greater amberjack at 44 dph fed microdiets containing different levels of taurine (means ± SD, n=3). Different letters above the bars indicate a significant difference among treatments ($P < 0.05$; one-way ANOVA; Post hoc test Tukey).

6.4. Discussion

Taurine is a critical ingredient in fish nutrition, especially when feeding with plant protein-based diets (Salze and Davis, 2015). However, in the current study, we found that greater amberjack larvae can be benefited from dietary additional taurine supplementation even when microdiets were prepared with marine animal protein sources up to certain levels (krill and squid meal). Similar findings were observed in California yellowtail, where larvae benefited from the supplementation of taurine in the diets prepared with marine animal protein sources (Stuart et al., 2018).

In the present study, the results showed that dietary taurine supplementation brings apparent growth benefits for greater amberjack larvae. Total length and wet weight of greater amberjack larvae were highest when larvae were fed increasing levels of taurine (Tau 1.24) while further increased (Tau 4.24) results in growth impairments. Supplementation of dietary taurine (3% of total composition) was found to increase Senegalese sole larval growth at 32 dph (Pinto et al., 2010), whereas in gilthead seabream the effect of taurine supplementation seems to be controversial. Pinto et al. (2013) reported no enhanced growth whereas Izquierdo et al. (2019) found that elevated levels of taurine in dry diet enhanced growth and pointed out that 0.71% of dietary taurine significantly increased the growth of gilthead seabream larvae at 31 dph. In Nile tilapia, 1% of dietary taurine significantly increased the growth of larvae at 8-11 dph (Al-Feky et al., 2016). The wet weight of yellow drum (*Nibea albiflora*) larvae fed with supplemental taurine at 2% level was higher than that of larvae fed without supplemental taurine. Also, up to 2% level supplementation of dietary taurine significantly increased the total length of yellow drum larvae at 45 dph (Xie et al., 2015).

A positive correlation between dietary taurine level and growth rate has been found for marine fish larvae such as Pacific cod (*Gadus macrocephalus*) (Matsunari et al., 2005) and turbot (Conceiçao et al., 1997) larvae. In the present study, a significant improvement in amberjack larval growth was observed in fish fed microdiet with intermediate taurine level supplementation, Tau 1.24. Thus, the lower growth of the amberjack larvae fed Tau 4.24 microdiet can be attributed to the excessive taurine contents of the microdiet. Likewise, olive flounder larvae were fed three levels of taurine found an increase in growth when taurine was increased from 0.5 to1.7 g kg^{-1} dry weight of rotifers, a further increase to 3.0 g kg^{-1} did not increase the growth (Chen et al., 2005). Similarly, a study in Atlantic bluefin tuna, where increasing the levels from 3.7 to 9.0 mg taurine g^{-1} rotifer did not lead to increased larval

growth (Betancor et al., 2019). Ours findings would confirm those reported by Partridge and Woolley (2017) in yellowtail amberjack, where it was observed that the larvae have a lower requirement and a reduced tolerance to excess taurine in the diet. These results indicate that levels of taurine of around 1.3 g/100 g diet dry mass based on the measured taurine content of the microdiet may satisfy the requirements of greater amberjack larvae for this nutrient.

Dietary taurine supplementation has been reported to greatly improve survival rates in certain fish species (Salze et al., 2012; Xie et al., 2015; Rotman et al., 2017). In the present study, the survival rate of greater amberjack larvae was not significantly affected by the taurine level in the microdiets which agreed with Matsunari et al. (2013) who found that taurine enrichment of larval diets had no significant effect on the survivability of greater amberjack. On the contrary the survival rate of larvae fed taurine-supplemented rotifers was significantly higher than the control group (20.1% vs. 9.1%) in yellowtail kingfish larvae (Rotman et al., 2017), however, the effects of taurine supplementation on live feed in early stage can be also masked by other factors affecting larval survival in early stages. On this regard, the poor or no effects of dietary taurine on survival rate has also been reported in other species such as Atlantic bluefin tuna (Betancor et al., 2019), white seabass (*Atractoscion nobilis*) (Rotman et al., 2017) and Senegalese sole (Pinto et al., 2010). So, our findings indicate that taurine supplementation of live feeds and microdiets has no significant impact on larval survival of greater amberjack.

On the other hand, different studies have shown that taurine improves lipid digestion, absorption and metabolism (Richard et al., 2017; Xu et al., 2020), and digestive enzymes activities (Zheng et al., 2016), especially during early larval development, thus enhancing nutrient availability of diet (Salze et al., 2012). In the present study, an increase in dietary taurine caused a reduction in larval crude lipid content, which could be related to the improved utilization of dietary lipids. In agreement with these results, the elevation of dietary taurine, in seabream larvae, reduced whole body lipids and improve the utilization of dietary lipids (Izquierdo et al., 2019). Besides, an increase in dietary taurine significantly increased the crude protein content of greater amberjack, indicating that taurine promoted protein retention and deposition in the larval body. This was consistent with the results of previous studies in Senegalese sole larvae (Pinto et al., 2010), Pacific bluefin tuna (*Thunnus orientalis*) and yellowfin tuna (*Thunnus albacares*) larvae (Katagiri et al., 2017). Indeed, Katagiri et al. (2017) demonstrated that feeding the larvae with rotifers enriched with taurine promoted larval growth and total protein content, suggesting that taurine is an important

nutrient for the early stages of rapidly growing teleost species. It remains unclear if taurine improves growth through mechanisms that increase protein retention or decrease protein catabolism, which may result from more effective use of lipids as energy substrates. However, it was reported in larval Nile tilapia and red sea bream that the process of optimizing energy utilization leads to the acceleration of growth rates through dietary taurine supplementation (Al-Feky et al., 2016; Kim et al., 2016). On the other hand, the liver and the intestine are considered reliable nutritional and physiological biomarkers of fish larvae because their histological organization is very sensitive to dietary changes and reflects any physiological disorder originating from a nutritionally unbalanced diet or unsuitable feeding conditions (Gisbert et al., 2008). Larvae fed Tau 0.24, Tau 1.24 and Tau 2.24 experimental diets were shown to have a healthy liver with a moderate hepatocytes vacuolization and moderated signs of hepatic steatosis, however, larvae fed Tau 4.24 tended to accumulate fat and showed a higher impact on liver steatosis. This decrease in hepatic fat observed in larvae fed Tau 0.24, Tau 1.24 and Tau 2.24 experimental diets might be a consequence of higher growth and more advanced development of these larvae compared to those from Tau 4.24 treatment. Increased hepatic lipids mobilization could be another explanation for the reduction in hepatocytes vacuolization. In fact, a lower lipid deposition in seabream larvae hepatocytes suggests an enhanced mobilization of hepatic lipids (Betancor et al., 2012). No moderated signs of intestinal injury were observed along different intestinal regions in larvae fed different microdiets. These results indicated that dietary taurine levels did not compromise intestinal functions.

Skeletal anomalies have been studied in numerous marine fish, especially during the weaning phase, and literature data clearly suggests that unfavourable abiotic conditions, inappropriate nutrition and genetic factors are the most possible causative factors of skeletal anomalies in fish (Boglione et al., 2013; Hamre et al., 2013). Greater amberjack larvae are particularly susceptible to the incidence of skeletal anomalies (Roo et al., 2019; Sawada et al., 2020; Djellata et al., 2021), and given that taurine plays a role in bone growth and development (Salze et al., 2011), we suggested that taurine supplementation may reduce the incidence of skeleton anomalies in this species up to certain levels while further increased could impair larval quality. In this sense, increasing taurine level to 2.24 g/100 g diet dry mass led to a low incidence of skeleton anomalies appearance, which demonstrates the beneficial effects of taurine supplementation. However, the increased dietary taurine up to 4.24 g/100 g diet dry mass, led to the increment in the incidence of total severe anomalies

and pre-heamal vertebrae anomalies. It has also been shown in gilthead seabream larvae that taurine supplementation leads to reduced total bone anomalies, kyphosis and maxillary bones anomalies (Izquierdo et al. 2019).

6.5. Conclusion

The results of the present study indicate that moderated level of taurine supplementation (1.24 g/100 g diet dry mass) is necessary for microdiet for greater amberjack larvae despite including marine protein sources in its manufacture to support maximal growth parameters and minimal incidence of skeletal anomalies during the weaning stages. However, an excessive level of dietary taurine (4.24 g/100 g diet dry mass) caused impairment in larval growth and quality.

CHAPTER 7

GENERAL CONCLUSIONS

Chapter 3: Effect of broodstock diet on the larval quality of greater amberjack (*Seriola dumerili*).

1. Broodstock nutrition has a considerable effect on greater amberjack larval quality.

2. The supplementation of histidine and protein in broodstock diets optimize the larval performance of greater amberjack, particularly improving survival and larval quality. These results are very useful to advance the knowledge of the nutritional requirements of this species.

3. The elevation of taurine levels from 0.3 to 1.1% in broodstock diets increases larval growth but also increases the occurrence of total severe anomalies. Thus, further studies must be conducted to determine the optimum taurine dietary levels.

Chapter 4: Comparison between intensive and semi-intensive larval rearing systems in greater amberjack (*Seriola dumerili*, Risso, 1810).

4. SIS system utilized in our study offers more adequate and less stressful culture conditions with higher environmental stability than IS system, which could be related to zootechnical differences between the two systems used, among others: larval density, water quality, or available water volume per larvae.

5. Larvae reared in SIS system have higher growth and survival rates and a lower percentage of larvae with skeletal abnormalities (less than 12%).

6. Larvae reared in the IS system showed higher stress-related genes expression in comparison with larvae reared in the SIS system, which may be attributed to more stable and favourable rearing conditions in the SIS system.

7. The most suitable larval rearing protocol to sustain a regular and predictable greater amberjack fingerling demand would be the SIS.

Chapter 5: The effect of different co-feeding protocols in greater amberjack (*Seriola dumerili,* Risso 1810) larvae.

8. Survival, growth rates, the incidence of skeletal anomalies, and expression of growth and stress-related genes of greater amberjack larvae are influenced by co-feeding protocols.

9. Increasing *Artemia sp*. replacement levels by microdiet in greater amberjack larval co-feeding affected the survival rate and lead to a higher incidence of skeleton anomalies.

10. The activity of greater amberjack larvae became more vigorous, increasing their predation and cannibalism activity, especially under low *Artemia sp.* co-feeding protocols.

11. The density of *Artemia sp.* in greater amberjack larval co-feeding could be reduced at least to 25% of the standard protocol, 1.5 *Artemia sp.* mL^{-1}/day being the optimum concentration under such co-feeding protocols. This co-feeding protocol is expected to ensure high growth and survival rates while reducing the costs, stress response, and occurrence of skeletal anomalies.

Chapter 6: Improving the larval performance of greater amberjack (*Seriola dumerili*) during the weaning phase with taurine supplementation on dry diets.

12. Moderated level of taurine supplementation (1.24 g/100 g diet dry mass) is necessary for microdiet for greater amberjack larvae despite including marine protein sources in its manufacture to support maximal growth parameters and minimal incidence of skeletal anomalies during the weaning stages.

13. An excessive level of taurine (4.24 g/100 g diet dry mass) in microdiet for greater amberjack larvae may cause impairment in larval growth and quality.

Finally, the achievements of this thesis would provide valuable information for feed producers and hatcheries interested in greater amberjack aquaculture operations in the European aquaculture industry.

CHAPTER 8

Resumen en español

8.1. Objetivos

La Seriola o el medregal es una de las principales especies candidatas para mejorar la acuicultura europea, relacionado con las excelentes características biológicas para su cultivo. En los últimos años se han logrado avances considerables, sin embargo, la producción de un número adecuado de larvas y juveniles sigue siendo uno de los principales cuellos de botella para el cultivo de esta especie. Es evidente que un mejor conocimiento de la alimentación de los reproductores y de larvas contribuirá a la optimización de las dietas y los protocolos de alimentación, mejorando el rendimiento biológico tanto en crecimiento y supervivencia como en calidad de los juveniles producidos. Por ello, el objetivo general de la presente tesis es: mejorar la cría de larvas y la producción de juveniles de medregal (*Seriola dumerili*) basándose en la optimización de dietas de reproductores y larvas, y protocolos de cría larvaria.

Para lograr este objetivo general, se plantearon cuatro objetivos específicos que se relacionan con los diferentes capítulos del trabajo:

Capítulo 3: Efecto de la dieta de los reproductores sobre la calidad de las larvas de medregal (*Seriola dumerili*).

Se ha demostrado que la composición de la dieta de los reproductores tiene importantes efectos sobre la calidad larvaria de los peces marinos, y muchos de los problemas que enfrentan las larvas recién nacidas durante las primeras etapas de cría están directamente relacionados con el régimen de alimentación de los reproductores. En este sentido, este estudio tuvo como objetivo examinar los efectos de la suplementación nutrientes específicos: de taurina, histidina y proteína en las dietas de los reproductores, y como estos afectan al desarrollo biológico y la calidad de las larvas de medregal durante las primeras semanas de vida.

Capítulo 4: Comparación entre los sistemas intensivos y semi-intensivos para la cría de larvas del medregal (*Seriola dumerili*, Risso, 1810).

Se desconoce el mejor protocolo de cría de larvas para la producción comercial de alevines de medregal. Así, la comparación entre los sistemas intensivo y semi-intensivo contribuirá a una mejor comprensión de las necesidades de cría de la especie con respecto a su futura aplicación en la producción comercial. Por esta razón, este estudio tuvo como

objetivo determinar la técnica de cría más adecuada (sistemas intensivos versus semi-intensivos) en las fases iniciales de cultivo del medregal para mejorar el rendimiento del crecimiento, la tasa de supervivencia y la calidad de las larvas.

Capítulo 5: Efecto de diferentes protocolos de destete en larvas del medregal (*Seriola dumerili*, Risso 1810).

Para mejorar la alimentación de las larvas en estadios tempranos, se propuso minimizar el uso de alimentos vivos tratando de maximizar la supervivencia larvaria del medregal durante la fase de destete, este estudio tuvo como objetivo: averiguar si reducir el nivel de inclusión de alimento vivo (Artemia sp.) en el protocolo de alimentación diaria de 32 a 48 días después de eclosión (dde) afecta el crecimiento, la supervivencia, así como la composición bioquímica y la calidad de las larvas. Esta reducción en el uso de *Artemia sp.*, permitirá además ahorros sustanciales en el coste de los alimentos, en el espacio de las instalaciones y en la mano de obra. Los resultados obtenidos de esta investigación proporcionarán información básica que será valiosa en el desarrollo de técnicas de cría para una mayor producción de medregal.

Capítulo 6: Desarrollo de mejoras en el cultivo larvario del medregal (*Seriola dumerili*) durante la fase de destete mediante la suplementación de taurina en las dietas.

La taurina es un nutriente que parece ser importante en la alimentación de peces, especialmente en los peces carnívoros marinos como el medregal. Si bien la suplementación de taurina en la dieta fue recomendada para otras especies, no hay estudios previos sobre el efecto de este nutriente sobre el cultivo larvario del medregal en la fase de destete. En este sentido, el objetivo general de este estudio fue investigar el efecto de los niveles graduados de taurina en la dieta sobre el crecimiento, la tasa de supervivencia, así como aspectos histológicos durante la transición de alimento vivo a microdietas.

8.2. Resumen de los experimentos

8.2.1. Capítulo 3: Efecto de la dieta de los reproductores sobre la calidad larvaria del medregal (*Seriola dumerili*)

La nutrición de los reproductores influye en la calidad de los huevos y las larvas de los peces marinos. Por ello, este estudio se llevó a cabo para evaluar la influencia de la suplementación de taurina, histidina y proteína en las dietas de los reproductores del medregal sobre el

comportamiento biológico de las larvas y la calidad larvaria durante las primeras semanas de vida. Los reproductores fueron alimentados con tres dietas experimentales, una más rica en taurina, otra en histidina y la tercera rica en proteínas. El diámetro de los huevos provenientes de los reproductores alimentados con niveles más altos de histidina y proteínas fueron mayores que los provenientes de los reproductores alimentados con niveles más altos de taurina. Al final del experimento, la longitud total y el peso seco de las larvas producidas a partir de reproductores alimentados con dieta rica en taurina fueron mayores que los alimentados con las otras dos dietas. Además, se mejoró el SGR de las larvas de 15 dde de los reproductores alimentados con alto contenido de taurina. Sin embargo, la mayor supervivencia larvaria a 15 dde se obtuvo en larvas provenientes de reproductores alimentados dietas de alto contenido de histidina y proteína. Por otro lado, las larvas de estos dos grupos experimentales tenían una baja incidencia de anomalías esqueléticas. En conclusión, los resultados de este estudio han señalado la importancia de la suplementación de histidina y proteínas en las dietas de los reproductores para optimizar la calidad larvaria del medregal. Igualmente, el estudio mostró que el nivel de taurina suplementado en las dietas de reproductores aumentaron el crecimiento de las larvas a 15 dde, si bien se deberían realizar más estudios para determinar los niveles dietéticos óptimos de taurina para dietas de reproductores de medregal especialmente en dietas con reducidos niveles de proteína marina.

8.2.2. Capítulo 4: Comparación entre los sistemas intensivos y semi-intensivos para la cría de larvas del medregal (*Seriola dumerili*, Risso, 1810)

Uno de los periodos màs criticos en el ciclo de producción del medregal, es sin duda, la fase de larvicultura. Esta etapa biologica está asociada con una alta mortalidad y una alta incidencia de anomalías esqueléticas. Por tanto, la investigación sobre aspectos relacionados con esta etapa, como los sistemas de cría, es muy relevante para definir y estandarizar las condiciones zootécnicas de esta especie. Por ello, este estudio analiza el comportamiento biológico relacionado con el crecimiento, la supervivencia, la calidad larvaria y la expresión de genes asociados al estrés y al crecimiento, en larvas de medregal bajo dos sistemas de cría diferentes: semi-intensivo (SIS) utilizando tanques de 40 m^3 de capacidad y con una densidad de huevos de 10 huevos L^{-1}, e intensivo (IS) utilizando tanques de 2 m^3 de capacidad y con una densidad de huevos de 75 huevos L^{-1}. Los huevos fertilizados y las larvas correspondientes se criaron en los diferentes sistemas diferentes hasta 30 dde. Los

resultados revelaron que la tasa de supervivencia y la longitud total de las larvas a 30 dde fueron más altas en el sistema SIS. La calidad de las larvas bajo el sistema IS fue menor que en el sistema SIS, ya que en IS se observó una menor resistencia al "test de estrés" y una mayor incidencia de anomalías esqueléticas. La expresión de genes relacionados con el crecimiento (*gh*, *igf1* e *igf2*) aumentó en ambos sistemas de cría, lo que es consistente con el mayor crecimiento larvario registrado durante el estudio. Además, la expresión de genes relacionados con el estrés (*crh*, *crhbp* y *trh*) se incrementó en el sistema IS. En resumen, estos hallazgos revelaron que el sistema de cría y sus condiciones zootécnicas son un factor crucial que afecta un mayor rendimiento biológico del medregal y sugieren que el sistema SIS es más ventajoso que el IS para la larvicultura de esta especie.

8.2.3. Capítulo 5: Efecto de diferentes protocolos de destete en larvas del medregal (*Seriola dumerili*, Risso 1810)

Optimizar el crecimiento y la supervivencia de las larvas de peces marinos, mientras que se reduce el coste de producción, es importante para el desarrollo de criaderos viables a escala industrial. En ese sentido, este estudio tuvo como objetivo averiguar si reducir el nivel de inclusión de Artemia sp . en el protocolo de alimentación diaria durante el destete afecta el crecimiento, la supervivencia, la composición de ácidos grasos, la aparición de anomalías en el esqueleto, la vacuolización de hepatocitos y la expresión de genes relacionados con el crecimiento y el estrés. Se evaluaron cinco protocolos de destete, denominados 1% A, 25% A, 50% A, 75% A y 100% A de acuerdo con las diferentes densidades iniciales de *Artemia sp.* proporcionadas (0.02, 0.5, 1, 1.5 y 2 *Artemia sp.* mL^{-1} / día, respectivamente) y se probaron de 32 a 48 dde. El crecimiento de las larvas de los protocolos 1% A, 25% A y 50% A fue significativamente mayor que el de las larvas de los protocolos 75% A y 100% A. La supervivencia aumentó a medida que la densidad de *Artemia sp.* aumentaba en los protocolos. La incidencia de anomalías esqueléticas severas totales fue alta en larvas de protocolos 1% A y 25% A. Además, la expresión de genes relacionados con el crecimiento y el estrés fue mayor a 40 dde y luego disminuyó significativamente a 48 dde en todos los protocolos de destete. Los resultados obtenidos de este estudio sugieren que la cantidad de *Artemia sp.* utilizada se puede reducirse significativamente durante la fase de destete para esta especie.

8.2.4. Capítulo 6: Desarrollo de mejoras en el cultivo larvario del medregal (*Seriola dumerili*) durante la fase de destete mediante la suplementación de taurina en las dietas

La taurina es un nutriente importante en la alimentación de varias especies del género *Seriola*, pero no hay datos disponibles sobre la suplementación de taurina en las dietas para larvas de medregal durante el período de destete. Por lo tanto, en el presente estudio se determinó el requerimiento de taurina en las microdietas de destete para las larvas de medregal de entre 30 y 44 dph. Se formularon cuatro microdietas granuladas que contenían niveles de taurina de 0,24 a 4,24%. Se determinaron los parámetros de crecimiento, supervivencia, histología y incidencia de anomalías esqueléticas. La longitud total, la ganancia de peso y la ganancia diaria de peso de las larvas de medregal alimentadas con Tau 1.24 fueron mayores que las alimentadas con las otras microdietas. Se demostró que las larvas alimentadas con Tau 0.24, Tau 1.24 y Tau 2.24 tenían un hígado sano, y no se observaron signos de daño intestinal. Además, la incidencia de anomalías esqueléticas severas totales fue alta en larvas alimentadas con Tau 0.24 y Tau 4.24. Estos hallazgos sugieren que la suplementación de taurina en la dieta es probablemente esencial para el medregal durante la etapa de destete, ya que esta suplementación resulta en aparentes beneficios para el crecimiento larvario.

8.3. Conclusiones

Capítulo 3: Efecto de la dieta de los reproductores sobre la calidad larvaria del medregal (*Seriola dumerili*).

1. La nutrición de los reproductores tiene un considerable efecto sobre la calidad de las larvas del medregal.

2. La suplementación de histidina y proteínas en las dietas de los reproductores optimizó el desempeño larvario del medregal, particularmente mejoró la supervivencia y la calidad de las larvas. Estos resultados son muy útiles para avanzar en el conocimiento de los requerimientos nutricionales de esta especie.

3. La elevación de los niveles de taurina de 0,3 al 1,1% en las dietas de los reproductores aumentó el crecimiento de las larvas, pero también aumentó la aparición

de anomalías graves totales. Por lo tanto, se deben realizar más estudios para determinar los niveles dietéticos óptimos de taurina.

Capítulo 4: Comparación entre los sistemas intensivos y semi-intensivos para la cría de larvas del medregal (*Seriola dumerili*, Risso, 1810).

4. El sistema SIS utilizado en nuestro estudio ofrece condiciones de cultivo más adecuadas y menos estresantes con mayor estabilidad ambiental que el IS, lo que podría estar relacionado con diferencias zootécnicas entre los dos sistemas utilizados como la densidad larvaria, la calidad del agua o el volumen de agua disponible por las larvas.

5. Las larvas criadas en el sistema SIS tienen una mayor tasa de crecimiento y supervivencia y un menor porcentaje de larvas con anomalías esqueléticas (menos del 12%).

6. Las larvas criadas en el sistema IS mostraron una mayor expresión de genes relacionados con el estrés en comparación con las larvas criadas en el sistema SIS, lo que puede atribuirse a condiciones de cría más estables y favorables en el sistema SIS.

7. El sistema de cría de larvas más adecuado para mantener una demanda regular y predecible de alevines de medregal sería el sistema SIS.

Capítulo 5: Efecto de diferentes protocolos de destete en larvas del medregal (*Seriola dumerili*, Risso 1810).

8. La supervivencia, las tasas de crecimiento, la incidencia de anomalías esqueléticas y la expresión de genes relacionados con el crecimiento y el estrés de las larvas de medregal están influenciadas por los protocolos de destete.

9. El aumento de los neveles de remplazo de *Artemia sp.* con la microdieta en la alimentación de las larvas de medregal afectaron la tasa de supervivencia y condujeron a una mayor incidencia de anomalías esqueléticas.

10. La actividad de las larvas de medregal se hizo más vigorosa, aumentando la actividad de depredación y canibalismo de las larvas, especialmente en los protocolos de destete temprano.

11. La densidad de *Artemia sp.* en protocolo de destete de larvas de medregal podría reducirse al menos al 25% del protocolo estándar, 1,5 Artemia sp. mL-1 / día es la concentración óptima. Se espera que este protocolo de destete garantice altas tasas de crecimiento y supervivencia al tiempo que reduce los costos, la respuesta al estrés y la aparición de anomalías esqueléticas.

Capítulo 6: Desarrollo de mejoras en el cultivo larvario del medregal (*Seriola dumerili*) durante la fase de destete mediante la suplementación de taurina en las dietas.

12. A pesar de incluir ingredientes de origen marino como fuentes de proteína en la fabricación de microdietas para larvas de medregal, es necesario una suplementación moderada de taurina (1,24 g / 100 g de masa seca de dieta) en la microdieta para un crecimiento máximo y una incidencia de anomalías esqueléticas mínima

13. Un excesivo nivel de taurina (4,24 g / 100 g de masa seca de dieta) en la microdieta para larvas de medregal podría causar un deterioro en el crecimiento y la calidad de las larvas.

Finalmente, los resultados de este trabajo aportan información útil que pude ser de referencia para futuras aplicaciones industriales tanto por parte de la industria relacionado con la nutrición acuícola como por los criaderos interesados en la producción de *Seriola* en el entorno europeo o internacional.

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