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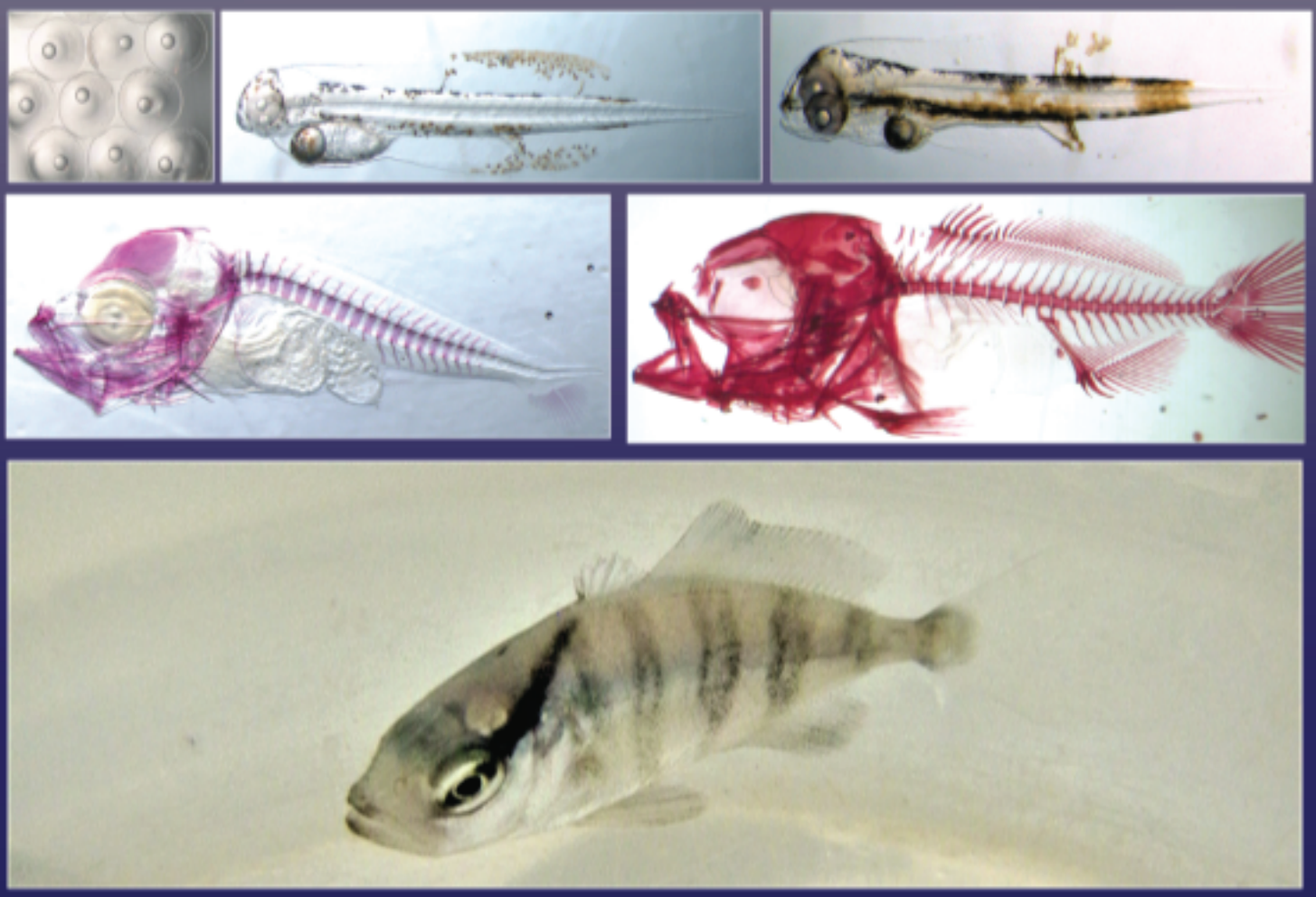

Longfin yellowtail (*Seriola rivoliana*) larval rearing. Skeletal development and effects of increasing dietary DHA levels at weaning phase.

Antonio Mesa Rodríguez

TESIS DOCTORAL

LAS PALMAS DE GRAN CANARIA
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"Longfin yellowtail (*Seriola rivoliana*) larval rearing. Skeletal development and effects of increasing dietary DHA levels at weaning phase"

Antonio Mesa Rodríguez

Doctorado en Acuicultura: Producción Controlada de Animales Acuáticos.

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Por ti, por mí...

Gracias Mamá

"Mi sinceridad es un regalo que NO todo el mundo se merece....

a partir de ahora procuraré NO ser tan Generoso"

"Nadie nace con ello... pero se cultiva y crece desde dentro.

Procura disfrutar de tu propia cosecha"

"La Felicidad es un estado excepcional

muy difícil de alcanzar debido a la inconformidad social

NO natural del ser humano"

" Y nos dejó....

dejando todo su amor en nosotros.

Somos fruto de su influencia en vida....

y siendo tal como somos,

estará siempre con nosotros...

Toda una Vida"

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Summary

Seriola rivoliana is considered as relevant species for aquaculture diversification and the information available is limited. The main objective of the present Thesis was to improve longfin yellowtail *(S. rivoliana)* larval production. In this sense, three specific objectives were established, in order to evaluate the most appropriate larval rearing technique, the obtention of bone development information and the effect of increasing docosahexaenoic acid (22:6n-3; DHA) levels in inert diets on biological performance.

During the study, the longfin yellowtail was reared for first time in Europe. Two different growing periods were identified, one slow daily growth in coincidence with the pre-flexion stages (0-15 days after hatching; dah), and one with accelerated daily growth post at notochord flexion (15-30 dah). As well as the growth, two peaks of mortality were identified, one observed from 8 to 12 dah, that could be associated to low success at first feeding and nutritional imbalances in broodstock; and a second period after 20 dah, with the appearance of aggressive behaviour and the presence of continuous mortality with weak larvae floating on the water surface. Throughout the larval rearing culture, *Brachionus sp.* (L-strain) seems to be an adequate live prey for first feeding of *S. rivoliana* larvae, whereas the apparition of larvae with faeces, including undigested and even live Artemia, demonstrated the low digestibility of this prey, which leads to larval malnutrition when Artemia is the main source of food (15–30 dah). Regarding the comparison between larval rearing techniques, the improved larval survival in the semi-intensive system in comparison with the intensive system would suggest that *S. rivoliana* larval rearing should be performed following the semi-intensive system regime.

The present Thesis reported for first time the skeletal development and mineralization of *S. rivoliana*. The first structures to mineralize were jaws and breathing structures at 3. 43 ± 0.15 and 3.75 ± 0.14 mm standard length (SL; respectively), supporting the importance of feeding and breathing at early stages. The identification of pre and post-flexion stages is a useful external indicator of the vertebral column, caudal complex and paired fins (5.12 ± 0.11 mm SL) mineralization. Meristically, *S. rivoliana* has a total of 24 vertebral structures and its mineralization follows an unidirectional pattern, differing from the bidirectional pattern common in most Perciforms.

Summary

According to the increasing DHA levels in inert diets, the inclusion of dietary DHA in feed up to a 3.17% (dw) improved larval resistance to air exposure. Also, DHA did not significantly affect fish final growth or final survival. On the contrary, high levels of DHA tend to increase the incidence of skeletal anomalies in *S. rivoliana* larvae, albeit no significant differences were observed. Furthermore, the occurrence of severe anomalies such as kyphosis and lordosis, was mainly associated to the larvae fed with the highest levels of dietary DHA.

Additionally, an adequate ratio of the omega 3 (n3) long chain polyunsaturated fatty acids (LC-PUFA) DHA and eicosapentaenoic acid (20:5n-3; EPA) should be above 3.1. This ratio and the role of n-3 LC-PUFA in bone formation in *S. rivoliana* should also be studied in order to understand the requirements of these emerging fast growth species. Moreover, further studies of EFA requirements should be studied to enhanced *S. rivoliana* larval production.

Acknowledgements

Several institutions supported the present Thesis. In these sense, I would like to thank the *Viceconsejería de Pesca del Gobierno de Canarias* through the programme “Proyecto piloto para el cultivo de especies de rápido crecimiento en Canarias (RAPCREC)"; the *Agencia Canaria de Investigación, Innovación y Sociedad de la Información (ACIISI)* and *Fondo Europeo de Desarrollo Regional (FEDER)* through the program “Mejora de las técnicas de cría de larvas de *(Seriola rivoliana):* Determinación de requerimientos de ácidos grasos esenciales en su etapa larvaria y optimización de la secuencia alimentaria (METCSER- ProID20100094)”.

I would also like to thank the Universidad de Las Palmas de Gran Canaria (ULPGC) for financing my PhD.

Many thanks to all IMARES group from Yerseke (Wageningen University & Research, Netherlands) for letting me the opportunity to work at their facilities with RAS systems, specially to Dr. Wout Abbink.

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Dr. Pipo, me "bautizaste" de la mejor manera posible durante mi primer congreso... mostrándome que siempre hay un momento para pasarlo bien y echarnos unas risas. Gracias por dedicarme en cualquier momento un minuto para debatir y compartir buenos consejos. Qué bueno se hace trabajar con alguien tan implicado, sincero y transparente... demostrando tu calidad humana. Sin duda, de la misma escuela que Carmen !! Mis más sincera admiración y respeto.

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Y para todo el que lo lea, procura ser Feliz... es lo único que nos llevamos.

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List of Abbreviations

A	Artificial
AA	Amino Acids
ABARE	Australian Base Aquaculture
AR	Alizarin Red
ARA	Araquidonic Acid
Art	Artemia
BW	Body Weight
CH	Cephalic Height
D	Dark
dah	Days after hatch
DHA	Docosahexaenoic Acid
dw	Dry weight
E	Absolute Ethanol
ED	Eye Diameter
EFA	Essential Fatty Acids
EPA	Eicosapentaenoic Acid
FA	Fatty Acids
FAMEs	Fatty Acid Methyl Esters
FAO	Fisheries and Aquaculture Organization
Fig.	Figure
GIA	Grupo de Investigación en Acuicultura
Gly	Glycerine
GnRHa	Gonadotropin Releasing Hormone analogue
HUFA	High Unsaturated Fatty Acid

List of Abbreviations

IGFA	International Game Fishing Association
IS	Intensive System
L	Light
l	litre
LC-PUFAs	Long Chain Polyunsaturated Fatty Acids
LGD	Lipid Globule Diameter
LSK	Lordosis-Scoliosis-Kyphosis
M	Mesocosms
m	Meter
ml	Mililitre
mt	Million tonnes
N	Natural
P	Paired structures
PAL	Pre-anal length
PL	Phospholipids
ppt	Parts per trillion
RAS	Recirculating Aquaculture System
rot	Rotifer
S	Single structures
SBI	Swim Bladder Inflation
SIS	Semi-Intensive System
SL	Standard Lenght
sp.	Specie
t	Tonnes

List of Abbreviations

TL	Total Lenght
TS	Trypsine Solution
ULPGC	University of Las Palmas de Gran Canaria
USA	United States of America
UV	Ultra Violet
YSL	Yolk Sac Length

Introduction

1. Introduction

1.1. Introduction to Aquaculture

According to FAO definition, the aquaculture is the farming of aquatic organisms that implicates the intervention in the rearing process to enhance production (FAO, 1988). Involving the ponds fish farming, known references establish the first primary production in China 4000 years ago. Nowadays, aquaculture implies more intervention at rearing and feeding process.

World fisheries total capture productions in 2014 was 93.4 million tonnes (mt), from which 81.5 mt were marine captures. Since 1950, global catches without anchoveta (*Engraulis ringens*) due to its highly variable production (influenced by El Niño episodes) rose until 1988, when exceeded 78 mt. Total catches remained relatively stable until 2014, reaching a new maximum of 78.4 mt (FAO, 2016a). In this way, as long as wild stocks are at their harvesting limits, aquaculture seems to be the only solution to supplement the constant increasing demand of fish products. As a relative recent industry, in the last decades, aquaculture has increased mostly due to the combination of population growth, expansion and improvement of fish production and more efficient distribution channels. The contribution of aquaculture had an average annual rate of 6.2% in the last decade (2000-2014), reaching up to a 44.1 % of the total world fish production with 73.8 mt (FAO, 2016a). Moreover, a total of 49.8 m are finfish aquaculture production, being mainly for human consumption.

In order to fulfil this fish increasing human demand, diversification of aquaculture is one of the main objectives to supply the needs of aquatics organisms market. The assessment of diversification may offer new species to enlarge the market and aquaculture products, and preferable, local species rather than introduced foreign species. In this sense, more than 20 fish species have been studied throughout the last years. Most of the new emerging species are fast growing and/or large finfishes that can be processed into a wide variety of valuated products (Abellán & Basurco, 1999). Species such as cobia (*Rachycentron canadum*) (Benetti *et al*., 2010), dolphin fish (*Coryphaena hippurus*) (Ostrowski, 1995), Florida pompano (*Trachinotus carolinus*) (Mc.Master, 2014), *Thunnus sp*. or *Seriola sp*. have special interest, but the main limiting factor is still the lack of biological knowledge and the control of production cycle, particularly reproduction control and larval rearing. As well as other fast growth species, the *Thunnus* genus has acquired special interest in the sushi and sashimi markets in Japan, particularly Atlantic bluefin tuna (*Thunnus tynnus*) (Mylonas *et al.*, 2014) , Pacific bluefin tuna (*Thunnus orientalis*) (Thomson *et al.*, 2010) and southern bluefin tuna (*Thunnus maccoyii*) (Hilder *et al*., 2015). Other species such as Atlantic halibut (*Hippoglossus hippoglossus*) and wreckfish (*Polyprion americanus*) have been cultured during the last years, but the remaining

bottlenecks for increased a stable production are related to and steady supply of juveniles in halibut and slow reproductive maturation in captivity of wreckfish (Mylonas & Robles, 2014).

The success of diversification is directly related to domestication level, which is, closing its life cycle in captivity and modifying its physiological and production characteristics to suit human needs (Telechea & Fotaine, 2014). These authors stated five domestication levels, in which levels 1,2 and 3 are a transitory production that depend on the wild source availability; whereas levels 4 and 5 are those species in which life cycle is closed in captivity without wild inputs and selective breeding programs (growth rate, flesh quality, etc). Well known marine finfish species such as gilthead sea bream (*Sparus aurata*), European seabass (*Dicentrarchus labrax*) or meagre (*Argyrosomus regius*) (domestication levels 5, 5 and 4, respectively) are well domesticated in captivity, but the high market demand requires a variety of species like the wild stocks offers, and fish that grow faster will achieve market size more quickly. In this sense, fast growth species become the main objective for aquaculture diversification.

Fast growth species such as *Seriola sp*. became a strong candidate for aquaculture diversification, due to its high market value and quality fillet (Mazzola *et al*., 2000; Nakada, 2002). These carangids are found in the Atlantic, Indian and Pacific oceans developing in tropical and subtropical waters. A few species have a worldwide distribution (such as greater amberjack, *S. dumerili*; longfin yellowtail, *S. rivoliana*; and yellowtail kingfish, *S. lalandi*) and others limited to regional distribution. Currently, 9 species of Seriola (Fishbase) have been described (Guinean amberjack, *S. carpenteri*; *S. dumerili*; lesser amberjack, *S. fasciata*; samson fish, *S. hippos*; *S. lalandi*; fortune jack, *S. peruana*; Japanese amberjack, *S. quinqueradiata*; *S. rivoliana* and banded rudderfish *S. zonata*), and very few are considered partially domesticated (Table I).

Table I. Domestication aquaculture levels (Telechea & Fotaine, 2014; modified from Bilio, 2007) for *Seriola sp*.

Scientific names	Common names	Domestication level	References
S. quinqueradiata	Yellowtail	2	Bilio, 2007.
S. lalandi	Yellowtail kingfish	2 - 3	Kolkovski & Sakakura,2004.
S. dumerili	Greater amberjack	4	Hong & Zhang, 2003.
S .rivoliana	Longfin yellowtail	2 - 3	Roo *et al*., 2012.
S. carpenteri	Guinean amberjack	0	-
S. fasciata	Lesser amberjack	0	-
S. hippos	Samson fish	0	-
S. peruana	Fortune jack	0	-
S. zonata	Banded rudderfish	0	-

1.1.1. Seriola Aquaculture Production

Considering its relative long farming history, the yellowtail or Japanese amberjack, *S. quinqueradiata* (Temminck & Schlegel, 1845) is the mayor relevant carangid species. The commercial farming of yellowtail started in Japan by the 1940's and expanded rapidly in the 1960's. Nowadays, the production peak is ranged between 140.000 and 160.000 tonnes (t) (Fig. 1), due to the consideration of fish farmer to maintain a significant level of wild juveniles in order to preserve their seasonally seed's supply captured from the natural environment (Mojako). Due to the markedly influence of Japanese gastronomy, *Seriola* culture (*S. quinqueradiata*, *S. dumerili* and *S. lalandi*) accounts with the 57% of all Japanese marine fish aquaculture (Ohara *et al*., 2005).

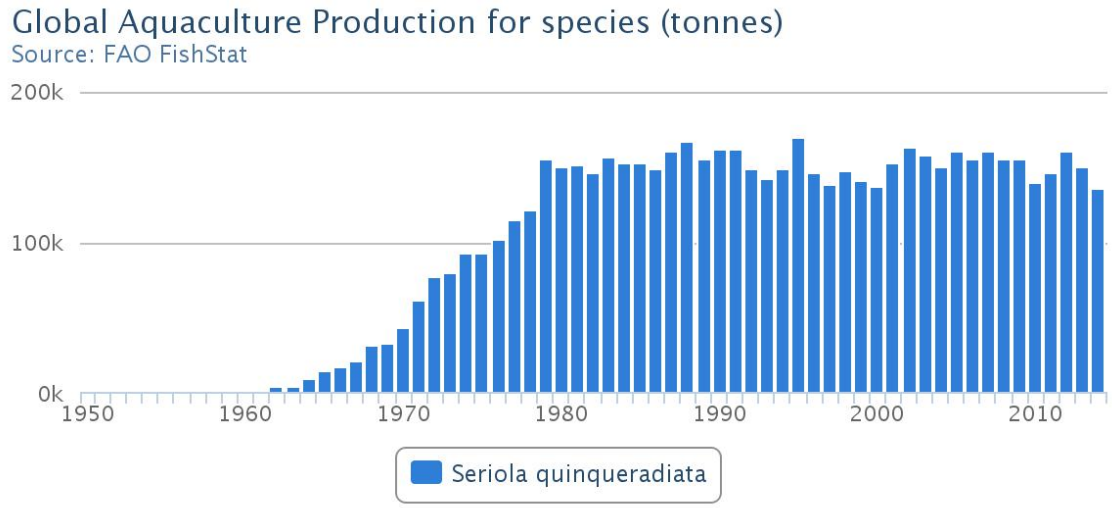


Figure 1. *S. quinqueradiata* (Temminck & Schlegel, 1845) Japanese production (FAO, 2014b).

With an estimated annual production of 13.200 t in China (FAO, 2014a), 4558 t in Japan (Benedetto Sicuro & Umberto Luzzana, 2016) and 3000-4000 t in Australia (ABARE, 2009), Yellowtail kingfish (*S. lalandi* Valenciennes, 1833) is the second highest production of the Seriola's family. Other countries such as New Zealand (Kolkovski & Sakakura, 2004; Symonds *et al*., 2014), USA (Stuart & Drawbridge, 2011; Buentello *et al*., 2015), Chile (Orellana *et al*., 2014), Netherlands (Abbink *et al*., 2011; Garcia *et al*., 2015), México (BAJASEAS, 2016), South Africa and Namibia (O'Neill *et al*., 2015) are promoting the Yellowtail kingfish culture, but still limited productions. As well as the yellowtail, the greater amberjack (*S. dumerili* Risso, 1810) farming begun late in the 1970's in Japan and continued in the Mediterranean Sea by the 1980's with the on-growing of wild caught juveniles (Fig.2).

Major Mediterranean greater amberjack producers have been Spain (Grau *et al*., 1999), Italy (Lazzari *et al*., 2000), Malta (FAO, 2016b), Croatia (Benovic, 1980) and Turkey (Yilmaz & Şereflişan, 2011), as well as China (Rongxing *et al*., 2008), Vietnam (Ottolengui *et al*., 2004) and Taiwan (Lu *et al*., 2012). However, most of this farming activity stopped due to the lack of seed supply and pathological disease. Nowadays, the main producer is Japan, with 46.000 t of wild on-growed juveniles (FAO, 2014c).

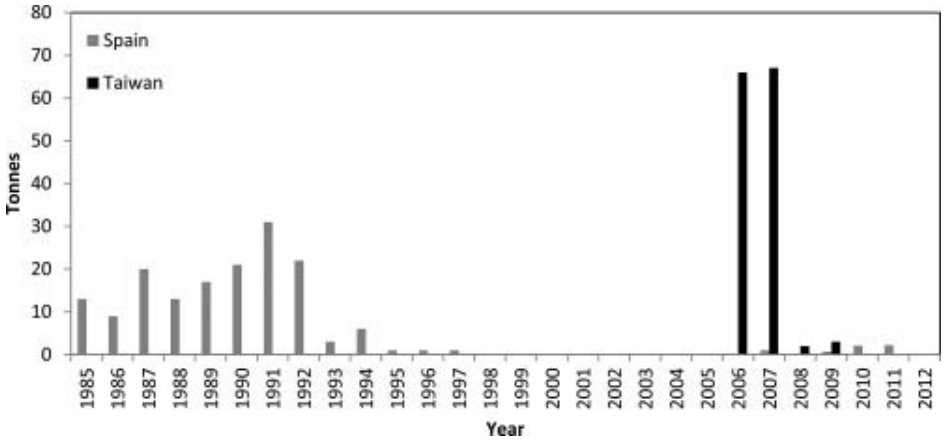


Figure 2. World production of farmed greater amberjack (*S. dumerili*). FAO, 2014c. FishStat.

Nowadays, pilot *S. dumerili* culture programs are being developed according to national (PNA-SERIOLA) and international (DIVERSIFY) aquaculture programs. In Spain, the production of seasonally fingerlings of greater amberjack is still uncertain (Table II). Further studies on larval rearing and nutritional requirements need to be carried out to obtain fries of good quality.

Table II. *Seriola dumerili* seed (units) production in Spain.

	2012	2013	2014	2015	Source Information
Andalucía	20.000	13.000	50.000	210.000	JACUMAR (2015)*[1]
Canary Islands	5.000	10.000	13.000	25.000	GIA Facilities (2016)*[2]

*[1]JACUMAR (2015).Ministerio de Agricultura, Alimentación y Medio Ambiente. Spain.

*[2]Grupo de Investigación en Acuicultura (GIA), IU-ECOAQUA, Universidad de Las Palmas de Gran Canaria (Unpublished data).

Longfin yellowtail (*S. rivoliana*, Valenciennes 1833) has a brief farming history, finding the first larvae rearing records at Ecuador (Blacio *et al*., 2003; Blacio, 2004) and Hawaii (Laidley *et al.*, 2004). In Hawaii, longfin yellowtail has been commercially cultured since 2005 (Sims & Key, 2011) with a total of 400-500 t/year FAO, 2014a (Fig.3). Moreover, in the Canary Islands (Spain), pilot production scale of *S. rivoliana* was developed according to regional funding for aquaculture diversification (Roo *et al*., 2011; 2012).

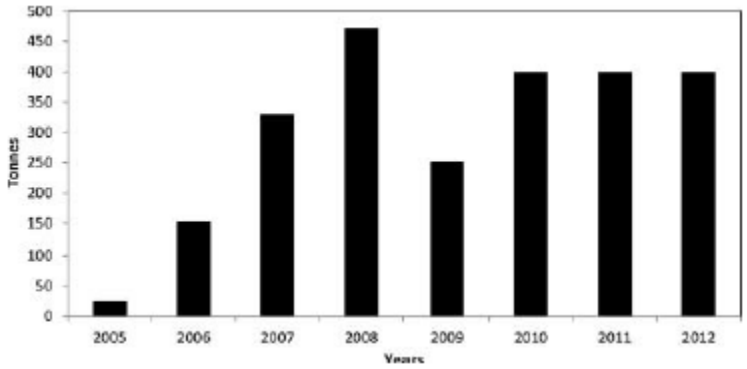


Figure 3. World production of farmed longfin Yellowtail (*S. rivoliana*). FAO, 2014a. FishStat

1.2. Marine Fish Larvae Production

In natural environment, larval fish mortality varies from 20% per day after hatching to >99% over the entire larval period (Bailey & Duffy-Anderson, 2001). On the contrary, even that predation risk and starvation conditions are absent, major mortality occurs at early developmental stages (Blaxter, 1986; Houde, 1989; Papandroulakis *et al*., 2005; Roo *et al*., 2010a, 2012). The obtention of a constant amount of larvae in emerging new species is still complicated. Several problems have been encountered in juvenile's production on *S. lalandi* (Benetti *et al*., 2005), *S. rivoliana* (Roo *et al*., 2012), *S. dumerili* (Papandroulakis *et al*., 2005). Whitmore, the dependence on the availability of seeds in *S. quinqueradiata* is one of the critical factors for the commercial success of Seriola production in Japan (Masumoto, 2002; Nakada, 2002). Moreover, the state of *Seriola sp*. seeds production is still at the initial phases, with the incessant needs of larvae and juvenile culture and nutritional requirements research.

Divanach (1985), established first a larval rearing classification based on larval density and tank volume. Thus, three main categories could be established: intensive, extensive and mesocosms systems (Fig. 4). Moreover, these systems can vary depending on the localization and the type of facilities, being opened, semi-opened or closed systems. In this sense, systems

such as extensive are majorly closed and exposed to environmental conditions, whereas other systems such as intensive or RAS can be open, semi-opened or closed, with high recirculation rates and highly controlled water conditions.

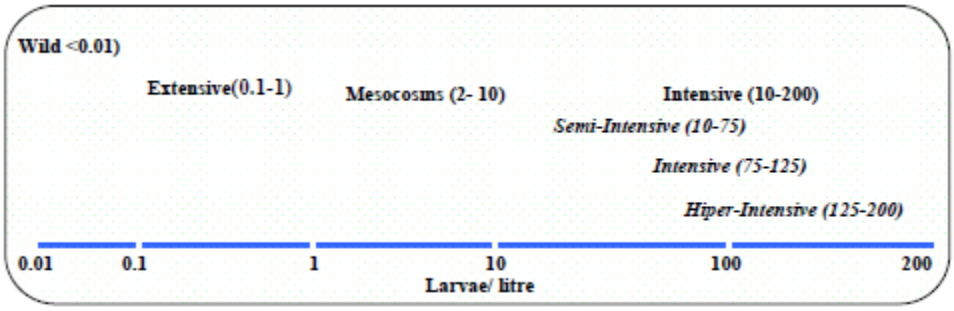


Figure 4. Larval rearing classification Divanach (1985).

Intensive Systems: the complexity that implies the intensive systems denote in consequences such as cannibalism and aggressive behaviour (Hecht *et al*., 1996; Baras & Jobling, 2002), low survival and growth under stressful conditions (Hernández-Cruz *et al*., 1999; Roo *et al*., 2007, 2010a, b) as well as anomalies occurrence (Andrades *et al*., 1996; Roo *et al*., 2005a, b). In this way, the intensive systems (Fig.5a,b) techniques require sophisticated facilities and elevated investments, being less suitable for the culture of new species (Divanach *et al*., 2002).

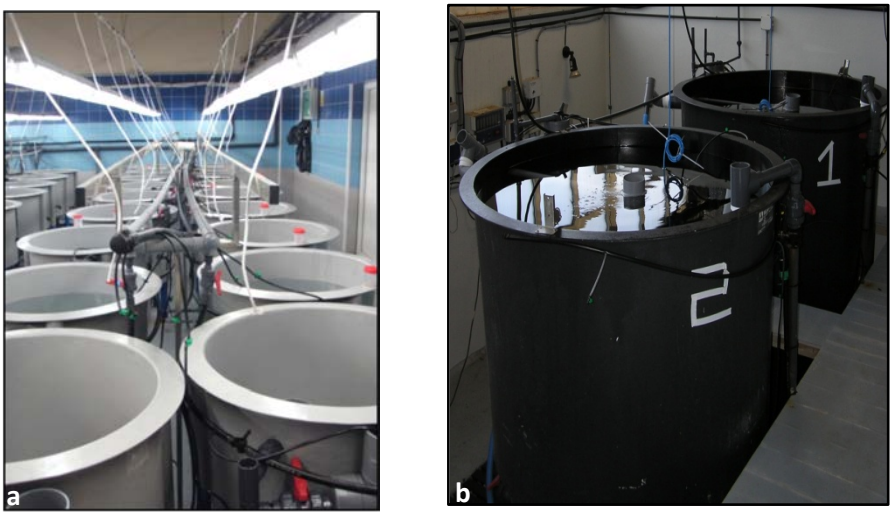


Figure 5 (a, b). Intensive larval rearing tanks mainly used at ULPGC-ECOAQUA facilities for research (a:0.2m^3 ;b: 2m^3).

Extensive Systems: the success of extensive systems (Fig.6) is usually higher than intensive systems, showing larvae with natural biological patterns (Pitta *et al.*, 1998) and excellent fry quality without problems such as abnormal swim bladder inflation, skeletal deformities, pigmentation anomalies or deviations in natural behaviour (Divanach *et al*., 1996).

Figure 6. Ancient Hawaii fishpond "Menehune", on the island of Kauai (Hawaii).

Mesocosms Systems: the results obtained with mesocosms system (Fig. 7a,b) are commonly better than intensive or extensive techniques (Papandroulakis *et al.*, 2004), with weaning totally achieved at one month, a generally fry survival ranging between 40-90% after weaning , deformities incidence around 5-10%, swim bladder inflation up to 95%, low size dispersion and low cannibalism incidence (Divanach & Kentouri, 2000). This culture system has been used favourably for more than 25 marine fish species and 5 hybrids for fry production (Divanach & Kentouri, 2000).

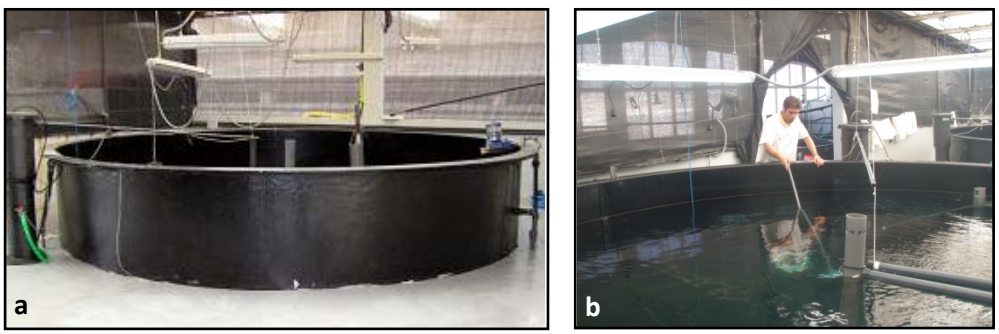


Figure 7 (a, b). Mesocosms tanks (40m^3) in the ULPGC-ECOAQUA facilities in Telde (Canary Islands, Spain).

Recirculating Aquaculture Systems (RAS)

The recirculation aquaculture systems (RAS) are systems in which water is (partially) re-used after undergoing treatment (Rosenthal *et al.*, 1986). Each treatment step reduces the system water exchange needs. This system has been developed to satisfy the increasing environmental regulations in countries with limited access to land and water (Fig. 8a,b).

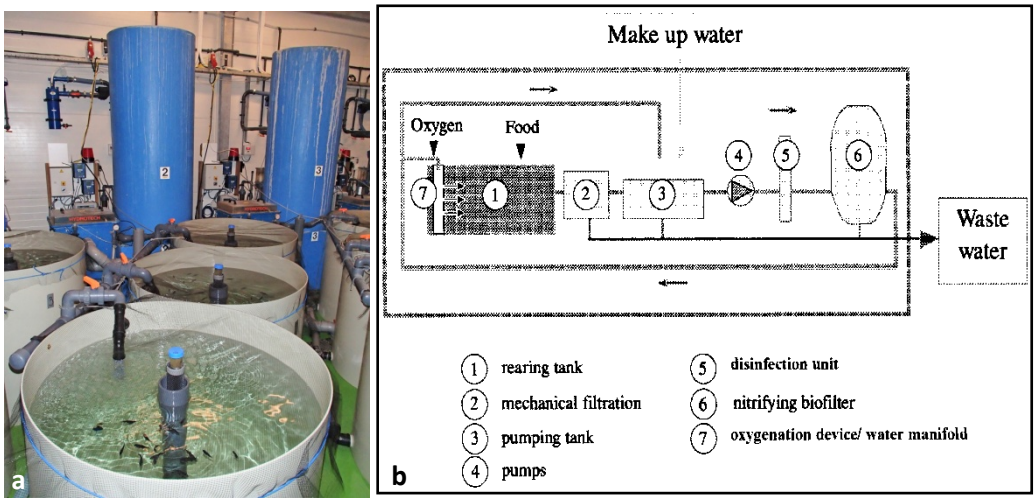


Figure 8 (a, b). a) *S. rivoliana* indoor RAS culture; b) Recirculating system used for larval rearing of Mediterranean species (Blancheton, 2000).

Compared to common aquaculture system flows, several positive effects are defined. In RAS, not only the reduction of water consumptions (Verdegem *et al.*, 2006) and consequently the reduction of aquaculture waste water impact, but also the conservation of heat (avoiding seasonal changes), the reduction of disease due to its environmental control and the optimization of growth rates and fish health by the surveillance of water quality (Blancheton *et al.*, 2009; Martins *et al.*, 2010).

Another important aspect of RAS is the establishment of microbial control in the cultivation tanks by stabilizing the substrate to an adequate bacteria ratio. Well-balanced concentrations of substrate induce growth of a stable, slow growing and more beneficent bacterial community (Vadstein *et al.*, 1993; Skjermo *et al.*, 1997; Salvesen *et al.*, 1999). In RAS conventional water treatments, solid particles larger than 40-60µm are removed to prevent the mineralization and production of smaller components (Chiam & Sarbatly, 2011). More sophisticated techniques in RAS systems utilize a biofilm membrane bioreactor (BF-MBR). This membrane enhances the reduction of fine suspended solids, colloidal particles and nutrient

from the system, with the significantly reduction of the turbidity, number of colloidal particles and bacterial concentration, as well as more stability in the water system (Holan *et al*., 2014).

Since the introduction of RAS, the production and diversity of species has significantly increased (Martins *et al*., 2005). In Europe, the most established RAS technology is found in The Netherlands and Denmark with indoors production of African catfish (*Clarias gariepinus*) and eel (*Anguilla anguilla*), and semi-closed and out-doors production of trout, respectively. Actually, more than 10 species are produced in RAS, being seabass and sole as the major marine species, and African catfish, trout and eel as major freshwater species cultured in Europe (Martins *et al*., 2010).

Fast growth species, such as *S. lalandi*, have been cultured in RAS on a research scale (Partridge *et al*., 2003). Due to its rapid growth rate to market size (3kg in 1 year), market value and life cycle closed in captivity, yellowtail kingfish was considered a species with great potential for RAS culture (Abbink *et al*., 2012). Recently, limited amounts of yellowtail kingfish are commercially produced with RAS in the Netherlands (Garcia *et al*., 2015), Chile (Orellana *et al*., 2014) and Mexico (BAJASEAS, 2016).

1.2.1 State of culture techniques for *Seriola.*

The establishment of adequate rearing conditions for new aquaculture species, mainly stated by the high market price and commercial demand, became complicated. *Seriola* culture, as an emerging fast growth genus, requires culture techniques that differ from the well-known commercial intensive techniques used for other marine finfish such as *Sparus aurata* or *Discentrarchus labrax*. Thus, the selection of an appropriated rearing technology depends on the species specific characteristics and its susceptibility to different parameters (larval density, tank volume, turbidity, temperature, photoperiod, light quality and intensity, live prey regimes, etc.) directly affecting the larval rearing success. Thereby, mesocosms systems seems to be an adequate technique for the initial phases of new candidate species, due to its similar conditions to the natural environment with large water volumes and low larval density (Papandroulakis *et al*., 2005; Roo, 2009a; Roo *et al*., 2012).

1.2.1.1. Larval rearing of *Seriola*.

Two critical periods have been identified in the culture of *Seriola*, with a high mortality from hatching to the first feeding period and the second being related to aggression behaviour and cannibalism. Thus, a considerable difference at larval hatching time of fast growth species, may produce disparity at first exogenous feeding and consequently, size heterogeneity, encouraging aggressive behaviour and cannibalism at later larval stages. The onset of cannibalism behaviour and its development in *Seriola* (Fig. 9a,b) was first described by

Sakakura & Tsukamoto (1996). These authors stated the onset of cannibalism after metamorphosis and it occurs until the apparition of schooling behaviour, outcome by distance to the nearest neighbour. The "J-posture" is determined as a precursor to aggressive behaviour (Sakakura & Tsukamoto, 1996) and is assumed to be some kind of intimidation behaviour after notochord flexion. Larvae that show this conduct frequently become dominants, existing a positive correlation between dominant larvae, "J-posture" and aggressive behaviour (Sakakura & Tsukamoto, 1999).

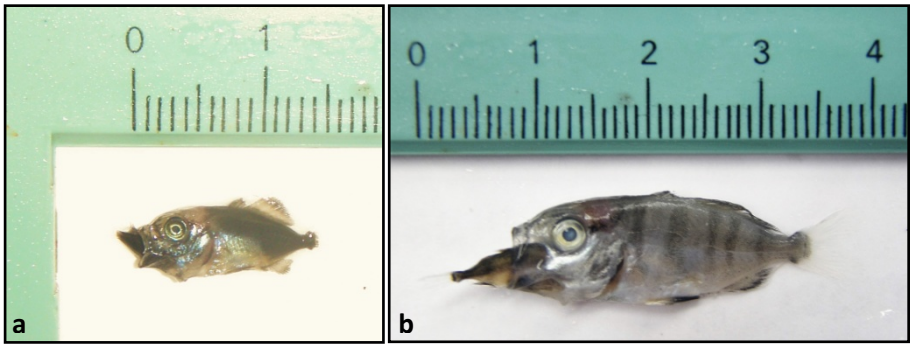


Figure 9 (a, b). Cannibalism in *S. rivoliana* larvae culture.

A high level of uncontrolled aggressive behaviour has been associated with the appearance of mass mortality in *S. quinqueradiata* (Sakakura & Tsukamoto, 1996; 1999; Sakakura *et al*., 1998), *S. lalandi* (Ebisu & Tachihara, 1993; Yamazaki *et al*., 2002; Moran, 2007; Stuart & Drawbridge, 2013), *S. mazatlana* (Benetti, 1997), *S. dumerili* (Shiozawa *et al*., 2003; Papandroulakis *et al*., 2005; Miki *et al.*, 2011; Mesa-Rodriguez, Unpublished data) and *S. rivoliana* (Blacio, 2004; Mesa-Rodriguez, Unpublished data) larvae, mainly the smallest specimens. These dead specimens are mostly subordinated larvae, which are continuously subjected to stressful situations being chased by the larger (total length) dominants larvae (Sakakura *et al*., 1998), causing food depravation or physical trauma (Moran, 2007).

Other factors, such as live prey regimes could indirectly affect the onset of cannibalism. Moran (2007) stated that the introduction of *Artemia sp*. as a food source was correlated with the increase of size heterogeneity and aiming behaviour in *S. lalandi* larvae, being a precursor of aggressive behaviour. Moreover, restricted live prey feeds amplified the size heterogeneity, thereby significantly exacerbating aggressive behaviour in *S. dumerili* larvae (Miki *et al*., 2011).

In the natural environment, the floating seaweed prevents the aggressive behaviour and works as a shelter from other predators. The utilization of shelter or floating objects to promote the schooling behaviour and avoid the cannibalism in culture tanks has been used in *S. quinqueradiata* (Sakakura & Tsukamoto, 1996; 1999) and *S. rivoliana* (Blacio, 2004; Mesa-Rodriguez, Unpublished data) culture.

In order to prevent size heterogeneity, with the consequently social rank and aggressive behaviour, the grading of fish larvae becomes an alternative (Fig. 10a,b). This method has previously reported to significantly improve the survival and production of *S. lalandi* (Ebisu & Tachihara, 1993; Moran, 2007), *S. quinqueradiata* (Yamazaki *et al*., 2002), *S. dumerili* (Shiozawa *et al*., 2003; Miki *et al*., 2011; Mesa-Rodriguez, Unpublished data) and *S. rivoliana* (Mesa-Rodriguez, Unpublished data) larvae. The early grading of fish larvae may reduce the size heterogeneity consequences.

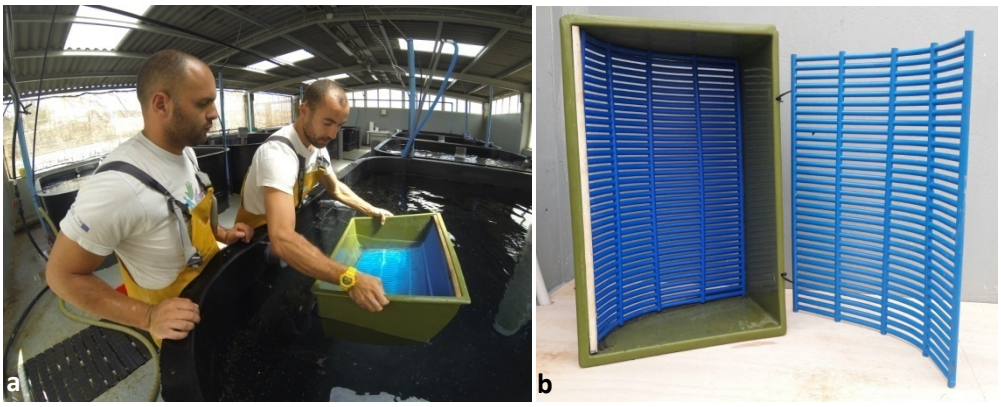


Figure 10 (a, b). a) Grading *S. rivoliana* larvae; b) larvae grader.

Moreover, larval quality is directly related to biotic and abiotic rearing factors.

1.2.1.2. Larval density and tank volume.

The larval stocking density as well as rearing systems (intensive and semi-intensive systems) of *Seriola* and emerging fast growth species is directly related to larval growth and survival. High larval densities employed in intensive and semi-intensive (up to 50 egg/l) systems may decrease the theoretical prey availability and increase larval interaction and competition for prey. Furthermore, the incoming size heterogeneity, together with the visual acuity development and increment of larval interaction may incite aggressive behaviour and cannibalism. The comparison between different larval densities has been previously studied. Roo and collaborators (2012), compared semi-intensive/mesocosms system (SIS-M: 4.5 egg/l, 40m^3) and intensive system (IS: 125 egg/l, 2m^3) in *S. rivoliana* larval rearing, showing better average survival under SIS. Moreover, this better survival could also be associated with the use of larger water volume tanks. In this sense, Stuart & Drawbridge (2013) stated that *S. lalandi* larvae should be moved into a large diameter (3.6 m), shallow (0.9 m), flat-bottom 6000 l fibreglass tank after 10 days after hatch (dah) reared in incubators tanks due to their very strong

surface orientation and the associated crowding that occurred over time in the constricted surface layer. For *S. dumerili* larvae culture, larval stocking density has also been tested, showing better results at 25 egg/l than 50 and 75 egg/l with significant differences at 30 dah final survival (La Barbera *et al.*, 2016). Generally, semi-intensive systems (10-75 egg/l) with low stocking densities (under 60egg/l) are used for *Seriola* culture (Table III).

Table III. *Seriola sp.* rearing conditions.

Specie	System	Spawning	Larval density	Tank volume	Green water	Light	Rotf (dah)	R. density	Art (dah)	A.density	Pellet	References
S. quinqueradiata	-	Induction	-	90m³ ponds	-	Natural	-	-	-	-	-	1996, Sakakura & Tsukamoto
S. quinqueradiata	SE	Induction	10 larvae/l	90m³ ponds	-	Natural	3 to 20	-	7 to 24	-	at 22	1999, Sakakura & Tsukamoto
S. rivoliana	SI	Natural	50 larvae/l	500 l	yes	-	MO to 12	3 rot/ml	9 to ??	1-2/ml	at 15	2004, Blacio
S. lalandi	SI	-	60 larvae/l	500 l	yes	A- 18L:6D (2.000Lux)	4 to ??	5-15 rot/ml	9 to ??	3/ml	-	2004, Cobcroft *et al.*
S. lalandi	-	Natural	10 larvae/l	-	-	-	-	6 rot/ml	-	-	-	2005, Carton
S. dumerili	M	Induction	9.800 larvae's	40m³	yes	A- 24L:0D*	2 to 27	2-3 rot/ml	11 to 40	0.1-0.5/ml	11 to 40	2005, Papandroulakis *et al.*
S. lalandi	SI	-	60 larvae/l	600 l	-	A- 13L:11D (3-6.000Lux)	3to 12	10 rot/ml	10 to 24	5 /ml	18 to 36	2007, Chen *et al*.
S. lalandi	SI	Natural	10-25 larvae/l	10m³	yes	N&A- 14L:10D (5-10.000Lux)	3 to 19	10 rot/ml	12 to 35	1/ml	at 16	2007, Moran
S. dumerili	SI	Induction	10-12 larvae/l	500 l	-	A- 12L:12D (1-2.400 Lux)	4 to 20	15 rot/ml	-	-	-	2009, Hamasaki *et al*.
S. dumerili	-		-	500 l	-	Best: A-18L:6D	-	-	-	-	-	2009, Hirata *et al*.
S. dumerili	-		-	50m³	-	A-12L:12D (1.000Lux)	-	-	-	-	-	2011, Miki *et al*.
S. lalandi	SI	-	50 larvae/l	320 l	yes	Best: A-14.850Lux	2 to ??	30 rot/ml	10 to 16 (5/ml)	5 /ml	-	2011, Stuart & Drawbridge
S. rivoliana	M	Induction	4.5 egg/l	40m³	yes	A&N- 12L:12D (1-3.000Lux)	2 to 25	4-5 rot/ml	15 to ?	-	at 20	2012, Roo *et al*.
S. rivoliana	I	Induction	125 egg/l	2m³	yes	A&N- 12L:12D (1-3.000Lux)	2 to 25	7.5-10rot/ml	15 to ?	-	at 20	2012, Roo *et al*.

A, Artificial; D, Dark; I, Intensive; L, Light; M, Mesocosms; MO, Mouth Opening; N, Natural; SE, Semi-extensive; SI, Semi-intensive.; *Day time (1.000-5.000Lux) and Night time (250Lux).

Table III. *Seriola sp.* rearing conditions. (Continued).

Specie	System	Spawning	Larval density	Tank volume	Green water	Light	Rotf (dah)	R. density	Art (dah)	A.density	Pellet	References
S. lalandi	SI	-	50 larvae/ l	320 l	yes	A- Best 24L:0D(1675Lux)	2 to 10	15-20rot/ml	6 to 10	3-5/ml	-	2012, Stuart & Drawbridge
S. lalandi	SI	-	45 larvae/ l	300 l	yes	A- 10L:14D (1150-1800Lux)	3 to 15	3-10rot/ml	Tested	-	-	2012, Woolley *et al.*
S. lalandi	SI - I	-	50-150 egg/ l	1.600 - 6.000 l ***	yes	A- 7.000-13.000 Lux	2 to 12	30 rot/ml	10 to 18	15-25/ml	at 15	2013, Stuart & Drawbridge
S. lalandi	SI - I	-	50-150 egg/ l	1.600 - 6.000 l ***	yes	A- 7.000-13.000 Lux	2 to 10	20 rot/ml	6 to 14	12-35/ml	at 15	2013, Stuart & Drawbridge
S. lalandi	SI - I	Natural	66 & 20 larve/ l	170 l	yes	A- 14L:10D (800-2.400Lux)	MO to 15	10-20rot/ml	15 to 22	0.8-9/ml**	-	2013, Ma *et al*.
S. lalandi	SI	Natural	45 larvae/ l	170 l	yes	A- 14L:10D (1.000Lux)	2 to 13	20 rot/ml	9 to 22	5/ml	at 18	2014, Ma.
S. lalandi	-	Natural	-	5-10m³	-	A- (1-3.000Lux)	3 to 18	18 rot/ml	13 to 30	0.3/ml	-	2014, Symonds *et al*.
S. lalandi	-	Natural	-	2m³	yes	Tested	2 to ??	10 rot/ml	12 to ??	-	-	2014, Woolley *et al*.
S. rivoliana	M	Induction	4.5 egg/ l	40m³	yes	A&N- 12L:12D (1-3.000Lux)	2 to 25	4-5 rot/ml	15 to ??	-	at 20	2014b, Mesa-Rodriguez *et al.*
S. lalandi	SI	Natural	10 larvae/ l	2.5m³	-	-	2 to ??	0.5-25rot/ml	-	-	-	2015, Hilder *et al*.
S. lalandi	I	Natural	170 larvae/ l	2.5m³	-	-	3 to ??	0.5-25rot/ml	-	-	-	2015, Hilder *et al*.
S. lalandi	-	Natural	60 larvae/ l	300l	yes	A-12L:12D (4.700 Lux)	3 to 12	10-30rot/ml 5-8rot/ml	-	-	-	2016, Woolley & Partridge
S. rivoliana	M	Induction	4.5 egg/ l	40m³	yes	A&N- 12L:12D (1-3.000Lux)	2 to 25	4-5 rot/ml	15 to ??	-	at 20	2016, Mesa-Rodriguez *et al.*
S. dumerili	SI	Natural	25, 50, 75 egg/ l	2m³	yes	Natural	-	-	-	-	-	2016, La Barbera *et al.*

A, Artificial; D, Dark; I, Intensive; L, Light; M, Mesocosms; MO, Mouth Opening; N, Natural; SE, Semi-extensive; SI, Semi-intensive;. **Increasing Artemia from 0.8/ml at 15dah to 9/ml at 22dah ;

*** 1.600 l from hatch to 10dah and 6.000 l after 10 dah.

1.2.1.3. Turbidity.

The positive effect of phytoplankton in the tank during larval development, such as bacterial control, water oxygenation, light diffusion and prey colour contrast, has being widely supported by many authors. As an important larvae rearing parameter, the use of live or paste phytoplankton is commonly added for *Seriola* culture (Table III). In *S. lalandi*, the greenwater treatment against clear water has being tested, developing bigger larvae, enhancing survival and more swimbladder inflation in larvae reared with green water treatment (Stuart & Drawbridge, 2011) as well as increasing feeding intake (Carton, 2005). In the other hand, excess in water column turbidity may have an adverse impact on the ability to capture free swimming prey during the first-feeding window (Carton, 2005). Moreover, the use of greenwater in *S. lalandi* culture may reduce the appearance of skeletal malformation (breakages of the lower jaw) due to larval "wall-nosing" behaviour (Cobcroft *et al*., 2004).

Another system to increase turbidity without introducing organic matter in the larval rearing tanks is the use of ceramic clay. In contrast to live microalgae or pastes, clay may aggregate and sediment organic matter and bacteria, facilitating its removal from the bottom of the larval fish tanks. Recently, studies with *S. lalandi* larvae (Stuart *et al.*, 2016), demonstrated that the use of clay as a turbidity source improved larval survival by reducing bacteria levels.

1.2.1.4. Temperature.

As many other factors, the water temperature has a direct influence in larval performance. The feeding, growth, survival and metabolism are significantly affected by temperature fluctuations (Blaxter, 1992). Indeed, high rearing temperatures may increase feed intake and metabolism, encouraging size heterogeneity, aggressive behaviour and cannibalism. In *Seriola* larvae culture, mid rearing temperatures (21-23ºC) seems to improve larval survival and first feeding intake, but growth was faster at higher temperatures after 10 dah in *S. lalandi* (Ma, 2014) whereas in *S. dumerili*, mid rearing temperature (22ºC) larval growth was improved without affecting the larval feeding and swim bladder inflation (Hirata *et al*., 2009).

Egg incubation of *Seriola* has been also tested. Moran (2007) tested egg incubation between 16º and 24ºC. These authors observed that in warmer temperatures, larvae hatch smaller but with a larger yolk sac and oil droplet. The relationship between a larger oil droplet at hatch and warmer temperatures appears to simply represent a lower biosynthetic requirement for lipids by smaller larvae. Whitmore, larvae reared at cooler temperatures conserve more endogenous energy reserves, being available during critical first feeding period.

1.2.1.5. Light intensity and photoperiod.

Light is another crucial physical parameter affecting growth and larval survival. Most marine fish larvae are visual feeders (Blaxter & Staines, 1970) and therefore require light to search and capture their prey. It seems that *Seriola* larvae reared under natural light conditions show a natural rhythm activity, being positively phototactic to the surface during daylight period (Carton, 2005; Mesa-Rodriguez, Unpublished data). Some studies with *S. lalandi* reveal that high light intensities may reduce the incidence of "Wall-nosing" behaviour (Cobcroft *et al*., 2004), improves feeding intake, larval growth and survival (Carton, 2005; Stuart & Drawbridge, 2011; Woolley *et al*., 2014), as well as longer photoperiod conditions (12-24 hours light) improves larval growth and survival for *S. lalandi* (Stuart & Drawbridge, 2012; Woolley *et al*., 2014) and *S. dumerili* (Hirata *et al.*, 2009). Contrarily, high light intensities directly affect the apparition of aggressive behaviour in *S. lalandi* (Sakakura & Tsykamoto, 1997), possibly related to better visual acuity in the water column between the specimens.

Moreover, in many marine finfish species, light acts as cue for fish to swim to the surface and gulp air (Woolley & Qin, 2010), and it seems that light intensity has an important impact on swim bladder inflation (SBI) in *Seriola* larvae. Poor SBI leads to the appearance of spinal deformities and consequently, inability to feed and poor larval performance. Thus, several studies have revealed that SBI was improved when *S. lalandi* larvae were reared at high light intensity (Stuart & Drawbridge, 2011; Woolley *et al*., 2014) but no differences were observed in *S. dumerili* and *S. lalandi* larvae under different photoperiod regimens (Hirata *et al*., 2009; Stuart & Drawbridge, 2012).

1.2.1.6. Live preys and feeding regimes.

Fish mortality at early stages is often related to first feeding success, optimum prey density and adequate prey sequence. Therefore, inadequate prey supply at first feeding may result in poor growth, reduction of the digestive capacity and enzymes activity, and detriment of digestive system in *S. lalandi* larvae (Chen *et al*., 2007). Due to the importance of first feeding, Hamasaki and collaborators (2009) studied the different body sizes of rotifer (*Brachionus sp*.). This authors suggested that SS-type rotifers (lorica length: 0.135 mm) are not appropriate prey for *S. dumerili* larvae during their early ontogeny, in agreement with the results obtained for *S. rivoliana* larvae (Roo *et al*., 2012), in which L-type rotifers (lorica length: 0.211 mm) seems to be an adequate prey size at first feeding.

Live food density varies greatly between species and management protocols. Controversially, several studies with *S. lalandi* larvae suggests that the optimal rotifer density to ensure available feed at first feeding should be at 20-40 rot./ml.(Ma *et al*., 2013; Stuart &

Drawbridge, 2013; Hilder *et al.*, 2015) whereas Woolley & Partridge (2016) stated that *S. lalandi* larvae are efficient hunters from first feeding, with no significant differences between 5 to 30 rot./ml., and the consequently possibility to reduce costs in rotifer production. Moreover, elevated densities to ensure feed availability lead to poor live prey quality due to long exposure period in the water column (Woolley & Partridge, 2016).

Transition to *Artemia sp.* feedings is a critical stage for *S. lalandi* larvae. The use of an adaptive feeding regime avoids the apparition of mass mortality by overfed larvae. These larvae seem to lose their neutral buoyancy and sinking during the night period, associated with mortality peak (Woolley *et al*., 2014). Mortality during this critical phase has been attributed to bacterial contamination of the larvae when they sink to the bottom (Yamazaki *et al*., 2002). Overfed larvae have also been observed in *S. rivoliana* larvae culture (Roo *et al*., 2012), observed as long faeces packets of undigested *Artemia sp.* (Fig. 11a,b).

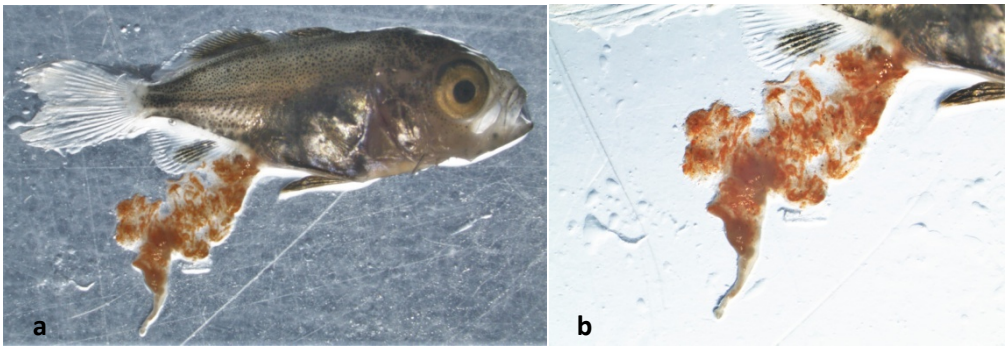


Figure 11(a, b). Faeces packets of undigested *Artemia* in 30 dah *S. rivoliana* larvae.

Occasionally, other spontaneous zooplankton could appear in the rearing tanks, such as copepods. Marine copepods produced endogenously in the SIS tanks during *Seriola sp.* larval rearing seems to be an alternative and additional prey (Papandroulakis *et al*., 2005; Roo *et al*., 2012). Generally, copepods are rich in essential nutrients that would promote *Seriola* larval survival such as digestible phospholipids, polyunsaturated fatty acids (PUFA), free amino acids, pigments or vitamins (Van der Meeren *et al*., 2008). Overall, elaboration of an adequate larval feeding species specific regime is complicated, and a large variety of feeding protocols are performed due to the difficulties to establish an adequate larval feeding regime specific to each specie and developmental stage.

1.3. Importance of Skeletal Development

One of the bottlenecks of finfish aquaculture production is the presence of morphological deformities. In this sense, the knowledge and the study of skeletal developmental pattern of reared species represent an important contribution for the optimization of larval rearing.

The occurrence of skeletal deformities directly depends on the rearing methodology applied, obtaining great differences between intensive and semi-intensive conditions which also affect the external shape of the fish (Boglione *et al*., 2001). These authors observed that in *S. aurata* culture, a range between 15-50% of the juvenile's present different deformities which result in important economic losses due to mortality, reduction in growth and market rejection by the final consumer. The occurrence of abnormalities in cultured marine finfish has been widely reported (European sea bass, *Dicentrarchus labrax*: Koumoundouros *et al*., 2002; barramundi, *Lates calcarifer*: Fraser *et al*., 2004;Atlantic halibut, *Hippoglossus hippoglossus*: Hamre *et al*., 2005; cod, *Gadus morhua*: Grotmol *et al*., 2005; red porgy, *Pagrus pagrus*: Roo *et al*., 2005b; *Diplodus sargus*, Saavedra *et al*., 2009; Atlantic salmon, *Salmo salar*: Witten *et al*., 2009).

1.3.1. Generalities of skeleton development.

The finfish skeletal system has multiple functions. Bones and cartilages provide support for the body, structural integrity, protection for major organs, provide attachment for ligaments and muscles, and serve as a mineral reservoir (Hall, 2005). Four classes of mineralized tissues (and related cells) can be identified: bone (osteoblast, osteocytes and osteoclast), cartilage (chondroblast, condrocytes and chondroclasts), dentine (odontoblast, odontocytes and odontoclasts) and enamel (ameloblats) (Hall & Witten, 2007). Moreover, Teleost fish display a large range of intermediate skeletal tissues.

- *Bone* is a specialized mesenchymal vascularized tissue formed by cells (osteoblasts, osteocytes and osteoclast), a mineral phase (mainly composed of calcium phosphate forming hydroxyapatite crystals) and an organic mineralized extracellular matrix. The major organic component of bone is collagen type I, and the degree of matrix mineralization is variable and seems to depend on type of bone (cellular o acellular bone), life style or fish habits (Meunier & Huysseune, 1992; Danos & Staab, 2010; Sfakianakis *et al.*, 2011; Dean & Shahar, 2012).

- *Cartilage* is mainly composed of chondrocytes, proteoglycans and collagen type II forming an extracellular matrix (Witten *et al.*, 2010).

In fish, according to the species and skeletal elements, there are three bone formation mechanisms: endochondral, perichondral and intramembranous ossification.

- *Endochondral ossification*: most of bones that ossify endochondrally originate from embryonic mesoderm. This involves a cartilaginous template, which is replaced or remodelled into bone (Hall, 2005).

- *Perichondral ossification*: is the most common in fish. Larval fish essentially only have perichondral bone formation. Perichondral ossification produces the bone that surrounds the cartilage (Benjamin, 1989). Witten & Huysseune (2007) reviewed that, perichondral bone formation is a basic process of ossification of the endoskeleton fin.

- *Intramembranous ossification*: this type of bone development has been described in many teleosts. Mesenchymal cells differentiate into osteoblasts and form bone without a cartilaginous template (Franz-Odendaal *et al*., 2006). Bones formed in this way are defined as dermal or membrane bones.

Taking into account the different mineralized tissues and the bone formation mechanisms, skeleton can be divided into two systems: i) dermal skeleton, that usually develops intramembranously, being mesenchymal precursor cells developing directly into bone (teeth, scales, fin rays, dermal skull bones, etc.); ii) endoskeletal, these are bones developed by endochondral ossification, where cartilaginous elements are replaced or remodelled by bone (branchial arches, axial and appendicular skeleton, etc.).

1.3.2. Finfish common skeletal deformities.

Skeletal anomalies in reared fish can affect all type of skeletal tissues, but alterations of the notochord, cartilage and bone are the most relevant for the aquaculture industry. The skeletal anomalies can appear throughout the culture as long as the skeletal elements are in incessant bone remodelling under the influence of rearing and/or nutritional factors. In this sense, skeletal anomalies can be ranged from slight internal anomalies (not affecting morphological shape) to severe internal abnormalities that directly affect the external body shape. Thus, externally detectable deformations such as lordosis, kyphosis, scoliosis (vertebral column abnormalities), shortened opercula and cranial deformity (head abnormalities) have a great economic impact in the final production.

1.3.2.1. Vertebral column abnormalities.

Vertebral column is composed by the vertebral body (centrum), neural and haemal arches. In teleost's, the mineralization of the notochord sheath establishes the identity of vertebral bodies and species, and is initiated by direct mineralization first, and intramembranous

mineral deposition after around the notochord sheath (Inohaya *et al.*, 2007). Vertebral column abnormalities have been widely reported in several species (*S. aurata*, Afonso *et al*., 2000; *D. labrax*, Koumoundouros *et al*., 2002; red sea bream, *Pagrus major*: Matsuoka, 2003; common pandora, *Pagellus erythrinus*: Sfakianakis *et al*., 2004; *L. calcarifer*, Fraser *et al.*, 2004; *P. pagrus*, Roo *et al*., 2009a; *S. salar*, Witten *et al*., 2009; *Pseudocaranx dentex*, Mesa-Rodriguez *et al.*, 2012). Vertebral column deformities involve lordosis (V-shaped dorsoventral curvature of vertebral column), kyphosis (ventraldorsal curvature), scoliosis (lateral curvature) (Fig.12a) and LSK syndrome (combination of Lordosis-Scoliosis-Kyphosis, Fig. 12b). Moreover, vertebral dislocation, fusion, shortening, deformation, compression or supernumerally vertebral bodies can take place.

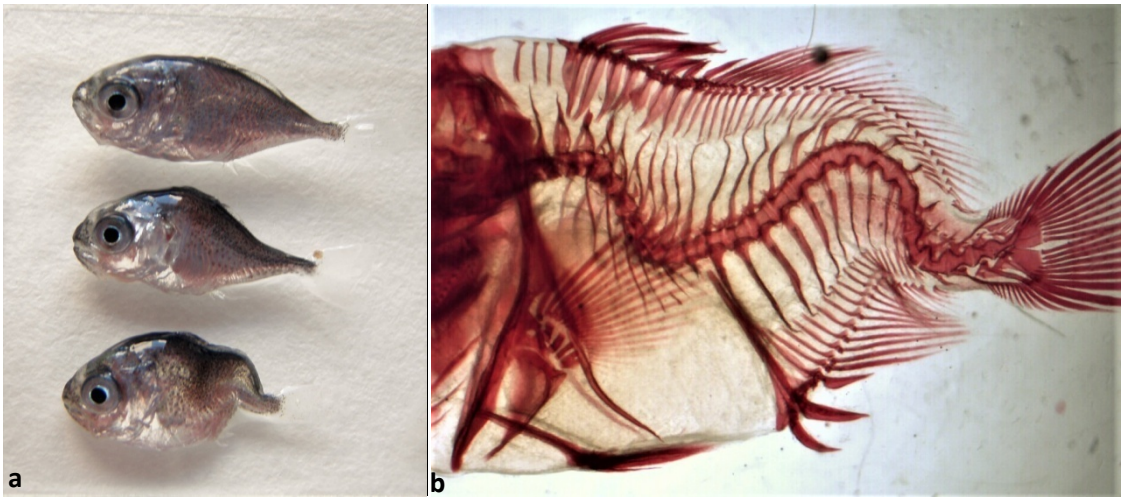


Figure 12(a, b). a) *P. dentex* normal larvae (upper) with kyphosis (mid) and scoliosis (lower); b) *S. rivoliana* larvae with LSK syndrome.

1.3.2.1. Head abnormalities

Cephalic deformities are frequently found in hatcheries. Several forms of mouth abnormalities have been reported, such as crossbite (Fig. 13a), pugheaded, sucker mouthed, prognatism, or reduction of dentale (Boglione *et al*., 2001, 2003) and reduction or twisting of the operculum (Fig. 13b).

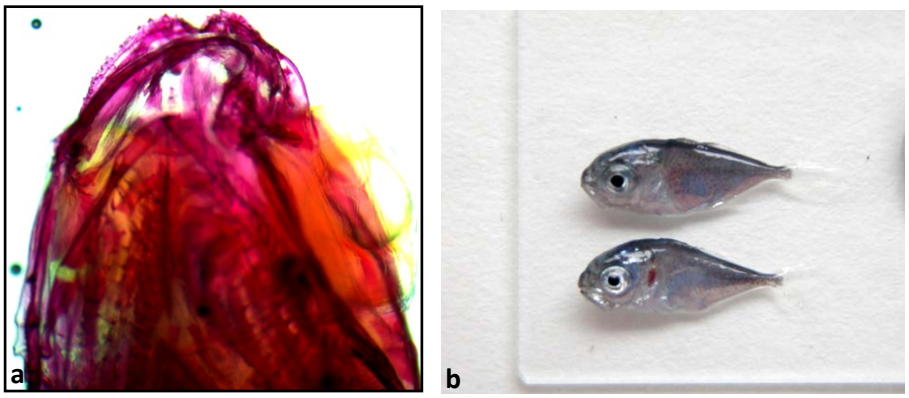


Figure 13 (a, b). a) Crossbite in *S. rivoliana* larvae; b) operculum reduction in *P. dentex* larvae.

1.3.3. Abnormalities in *Seriola*.

Even that some Seriola species are commercially produced, limited information is available about incidence of skeletal anomalies incidences. Cobcroft and collaborators (2004) reported several jaw malformations during *S. lalandi* larval rearing in New Zealand. Ma *et al*. (2014) compared the occurrence of jaw deformities from two sets of different broodstocks, suggesting the need for selective breeding to reduce occurrence of jaw deformity in *S. lalandi* larvae. At ULPGC-ECOAQUA facilities (Canary Islands, Spain), *S. rivoliana* and *S. dumerili* larvae reared under semi-intensive and intensive systems showed some jaw and operculum abnormalities (Mesa-Rodriguez, unpublished data).

Other studies found that infections with *Myxobolus buri* affect several regions of the brain, including the olfactory and optic lobes, cerebellum and the 4th ventricle, inducing disturbances in the central nervous system and leads to severe scoliosis in cultured *S. quinqueradiata* in Japan (Egusa, 1985). These findings suggest that lesions in the brain are related to abnormal swimming behaviour and deformity in the vertebral column of the diseased fish (Egusa, 1985; Sakaguchi *et al.*, 1987; Maeno & Sorimachi, 1992; Maeno *et al*., 1995).

Recently, and due to the high incidence of jaw malformations, heritability of lower jaw malformation, nasal erosion and deformed operculum has been also studied (Nguyen *et al.*, 2016).

1.4. Marine Fish Larvae Nutrition

In the natural environment, marine fish larvae are able to hunt and feed on a wide range of prey and sizes. Nevertheless, the difficulties for mass production of wild marine zooplankton have augmented the interest in controlled production of rotifer and *Artemia sp*. Even though rotifer and *Artemia sp.* are the main live preys used as larval feed, enrichment become necessary due to their poor nutritional quality. The *Seriola* larvae grow extremely rapid, being the content of essential fatty acids (EFA), amino acids (AA), minerals and vitamins an important issue in the live prey's enrichment in order to fulfil its nutritional requirements. Nevertheless, the live feed post-enrichment biochemical composition seems to be unstable, mainly when they are stored or left at rearing tank for long time, losing their nutritional value. According to this, adequate *Artemia sp.* enrichment with long chain polyunsaturated fatty acids (LC-PUFAs) such as docosahexaenoic acid (DHA; 22:6n-3), becomes difficult due its rapid catabolism (Danielsen *et al*., 1995; Evjemo *et al*., 2001; Naz, 2008). On the other hand, Naz (2008) stated that rotifers preferentially utilized other fatty acids rather than DHA as energy sources during starvation periods, being rotifers enriched with DHA a suitable live prey for marine fin fish requirements as they can conserve more efficiently the enrichment.

As long as endogenous feeding is undergoing, the yolk sac and oil droplet have an essential role in larvae performance by covering their primarily nutritional requirements. At these early stages, broodstock nutrition plays an important role in adequate larvae performance at first stages (Watanabe *et al*., 1984), as it is directly related to poor hatching quality when broodstock feeds are imbalanced. Indeed, the parental EFA reserve supplemented to the egg is essential for larval survival as well as development of body organs and structures (Izquierdo *et al*., 2000; Izquierdo & Koven, 2011). Other dietary compounds such as protein, vitamins or carotenoid levels directly affect the spawning, hatching and larval quality. Roo and collaborators (2015) used mackerel (*Scomber japonicus*) as feeding base for *S. rivoliana* broodstock, increasing the hatching rates up to 20%, as well as the number of viable eggs and hatched larvae in comparison to the broodstock fed with regimes containing the commercial diets, suggesting a dietary protein level over 70% for *S. rivoliana* broodstock feeds. In this sense, taurine, one of the most abundant free amino acids in fish and implicated in osmoregulation, antioxidation and hormone release, has been previously researched in *Seriola* (Matsunari *et al*., 2003, 2006), and the inclusion of at least 1.0% of taurine to the diet of *S. quinqueradiata* broodstock resulted on improved egg qualities (Matsunari *et al*., 2006).

Carotenoids have an important role in sexual maturation. Since Verakunpiriya and collaborators (1996) observed the presence of the carotenoid zeaxanthin in *S. quinqueradiata* eggs, which is converted from the dietary astaxanthin, its inclusion in feeds has been tested

(Verakunpiriya *et al*., 1997a; Agius *et al*., 2001; Vassallo-Agius *et al*., 2002), obtaining better spawning results, egg quality and final number of normal larvae. Moreover, inclusion of paprika powder as carotenoid source results on even better results than pure astaxanthin inclusion (Vassallo-Agius *et al*., 2002).

Vitamin levels have also been tested in *Seriola*. Spawning quality was improved in *S. quinqueradiata* when dietary vitamin E was supplemented in the diet (Watanabe & Vassallo-Agius, 2003). Contrary to what was expected, the inclusion of krill meal in *S. quinqueradiata* broodstock diet was not beneficial. Fish fed without krill meal supplementation showed a better hatching rate, number of fertilized eggs and final normal larvae (Verakunpiriya *et al*., 1997b).

1.4.1. Lipids in marine fish larvae nutrition.

As a source of metabolic energy, precursors of essential metabolites and components of biological membranes, the lipids in marine fish larvae play an essential biological role (Sargent *et al*., 1989). Being crucial for biomembranes structure and energy supply, dietary lipids provide the phospholipids (PL) into the larvae metabolism. Furthermore, dietary lipids are involved in the absorption of fat-soluble vitamins A, D, E and K, and skeletal development (Cahu *et al*., 2003, 2009; Villeneuve *et al*., 2005; Roo *et al*., 2009a; Izquierdo *et al*., 2010, 2013), as well as being components of hormones or precursors for synthesis of various functional metabolites, such as prostaglandins. However, dietary lipids utilization by body larvae is directly or indirectly affected by several morphological and physiological changes occurring during larval ontogeny and development.

1.4.1.1. Importance of essential fatty acids.

Lipids are constituted by fatty acids (FA), being the LC-PUFA the most abundant in fish. The EFA, particularly DHA (22:6 n-3), eicosapentaenoic acid (EPA, 20:5 n-3) and arachidonic acid (ARA, 20:4 n-6) are PUFA, and are known to be essential components of the cellular membranes, modulating physiological mechanisms as membrane transports and enzymatic activity, especially at the first larval developmental stages (Izquierdo, 1996). In this sense, the importance of dietary lipids for larval rearing performance is extremely associated with their high growth rates, energy demands and structural components needs. Indeed, EFA deficiencies are frequently related to elevate marine fish larval mortalities (Izquierdo, 1996). These EFA need to be incorporated in the diet, due to a very low enzymatic capacity (Δ5 and Δ6) to desaturate and elongate its precursors to from DHA, EPA and ARA. Contrarily, freshwater fish are enzymatically able to produce DHA, EPA and ARA from their precursors such as linoleic (18:2 n-6) and linolenic (18:3 n-3) acids. However, competitive interactions exist between DHA, EPA and ARA due to their marked chemical similarities, especially

between DHA and EPA molecules using the same enzymes to esterify FA into PLs structures (Sargent *et al*., 1999). According to this, amount of individual PUFA and their ratios must be considered, being important for well-balanced feeds and import for the proper growth and fish larval development (Izquierdo, 1996; Sargent *et al*., 1999).

Watanabe (1993) stated that DHA has greater potential as an EFA for marine fish larvae than EPA, and it requirements being more limiting for growth and survival than those for n-3 PUFA (Izquierdo, 1996).

1.4.1.2. Importance of docosahexaenoic acid.

The particular structure of DHA, with a 22-carbon chain and six *cis* double bonds (22:6 n-3), makes it determinant for many important functions in marine fish metabolism (Watanabe, 1993; Izquierdo, 1996, 2005), and its essentiality has been corroborated by its retention in starved or low EFA fed fish (Koven *et al*., 1989; Madsen *et al*., 1999) as well as greater accumulation in ovary, egg and early larval stages (Rønnestad *et al*., 1998, Mourente *et al*., 1999; Laurel *et al*., 2010; Rodríguez-Barreto *et al*., 2012). During early larval developmental stages, DHA content in marine fish larvae rapidly decreases, and its continuous incorporation must be supplied in order to maintain adequate levels for adequate larval performance (Watanabe, 1993). However, larvae DHA supplementation via exogenous live preys feeding becomes difficult, due to long starving periods in rearing tank and its rapid catabolism (Danielsen *et al*., 1995; Evjemo *et al*., 2001; Naz, 2008).

DHA deficiencies may cause deleterious effects in marine fish larvae, such as alterations in neurological system with impaired vision or abnormal behaviour (Bell *et al.,* 1995; Brandsen *et al*., 2005; Benítez-Santana *et al*., 2007), skeletal deformities (Cahu *et al*., 2003; Roo *et al*., 2009a, 2010a; Izquierdo *et al*., 2010, 2013), delay early mineralization (Izquierdo *et al*., 2013; Saleh *et al*., 2015), reduce tolerance to stressful conditions (Izquierdo, 2005; Jalali *et al*., 2008; Mesa-Rodríguez *et al*., 2012; Saleh *et al*., 2013, 2015), reduce larval survival (Copeman *et al*., 2002; Rezek *et al*., 2010; Mesa-Rodríguez *et al*., 2012; Saleh *et al*., 2013, 2015), among others. In the other hand, an excess in dietary DHA without adequate amount of antioxidant nutrients, may suffer oxidative processes and produce skeletal deformities (Mesa-Rodríguez *et al*., 2012; Izquierdo *et al*., 2013), supernumerary vertebrae (Villeneuve *et al*., 2006), appearance of muscular lesions (Betancor *et al*., 2011) among other detrimental effects on larvae (Bradsen *et al*., 2005; Villeneuve *et al*., 2005; Izquierdo *et al*., 2010, 2013).

Marine fish larvae are visual feeders, and the adequate development of their visual capacity directly affects their water column behaviour (Izquierdo, 2005). According to the importance of DHA in larval eye development, Bell & Dick (1993) determined that rods and

cones photoreceptors retain and accumulate DHA in the external segments. Roo and collaborators (1999) stated that the most important changes in the eye structure occur during lecitotrophic stages, underlining the importance of broodstock nutrition and the particular importance of DHA in neural and retinal tissue functions. Moreover, the brains of fish contain large amounts of DHA (Mourente *et al*., 1991; Mourente & Tocher, 1993), and Bell *et al*. (1995) suggested that DHA deficiency impairs retinal development and visual capacity, as has been reported in *S. quinqueradiata* larvae (Masuda *et al.*, 1999). Possible functions of DHA involve the constructions of synapses and formation of neural networks. According to this, DHA deficiencies may result in poor development of central nervous system and the improper development of learning ability, including feeding and schooling behaviour.

For *Seriola*, few studies on DHA requirements have been published (Table IV). As long as dietary DHA is considered to be essential for adequate brain development at early larval stages, studies with *S. quiqueradiata* larvae fed with enriched DHA *Artemia* suggest the essentiality of this EFA for the adequate development of schooling behaviour (Masuda *et al.*, 1998, 1999; Ishizaki *et al*., 2001). The swim bladder inflation is considered to be a critical factor for *Seriola sp*. larval survival, and DHA plays an important role for its proper development. In this sense, Matsunari *et al*. (2012a,b) determined that *S. dumerili* larvae fed rotifers enriched with DHA showed improved swim bladder inflation, as well as growth and final survival. The increase of larval growth and survival due to the elevation of DHA content in live preys enrichment has also been reported for *S. quinqueradiata* (Furuita *et al*., 1996; Ishizaki *et al*., 1996, 1997, 1998; Takeuchi *et al*., 1998), as well as increased the tolerance to stressful conditions in *S. rivoliana* larvae (Mesa-Rodríguez *et al*., 2014a).

Table IV. DHA requirements for *Seriola* larvae.

Specie	Tested Parameter	Objective	Feed	Levels	Optimum Requirement	Reference
S. quinqueradiata	DHA & EPA	Larval requirements	Enrich. *Artemia sp.*	DHA: 0 - 2.63% EPA: 0.5 - 8.9%	DHA: 1.39-2.63% EPA: 3.65%	Furuita *et al*., 1996.
S. quinqueradiata	DHA + *Euglena sp.* & Vit.E	Larval performance	Enrich. Rotifer	-	-	Ishizaki *et al*., 1996.
S. quinqueradiata	≠ levels EPA + DHA	Substitutability between EPA/DHA	Enrich. *Artemia sp.*	DHA: 1.4% EPA: 2.3 - 5.5%	DHA: 1.4 % EPA: 4.0 %	Ishizaki *et al*., 1997.
S. quinqueradiata	≠ levels ARA + DHA	ARA & DHA effect in Larval performance	Enrich. *Artemia sp.*	DHA: 2.1 - 2.5% ARA: 4.0%	DHA: 2.1 - 2.5 % ARA*	Ishizaki *et al*., 1998.
S. quinqueradiata	DHA	Behaviour development	Enrich. *Artemia sp.*	DHA: 1.46 - 2.40%	-	Masuda *et al*., 1998.
S. quinqueradiata	DHA	Larval requirements	Enrich. Rotifer Enrich. *Artemia sp.*	DHA: 0 - 1.6 - 2.6%	DHA: 1.6-2.6%	Takeuchi *et al*., 1998.
S. quinqueradiata	DHA	Schooling Behaviour Ontogeny & Brain	Enrich. *Artemia sp.*	DHA: 0 - 2.13 - 2.53%	DHA: 2.13-2.53%	Ishizaki *et al*., 2001.
S. dumerili	-	Larval requirements; mass production	Enrich. Rotifer Enrich. *Artemia sp.*	DHA Rot: 1.6-4.0% DHA Art: 0.7-1.3%	-	Yamamoto *et al*., 2008.
S. dumerili	-	Rotifers quality in larval rearing Tanks	Enrich. Rotifer	E.R*: 0.8-6.0% T.R*: 3.9-10.3%	-	Yamamoto *et al*., 2009.
S. dumerili	Algae with ≠ DHA content	Larval performance & SB Inflation	Enrich. Rotifer	DHA: 0.04-0.6-1.32%	-	Matsunari *et al*., 2012a.
S. dumerili	Algae with ≠ DHA content	Larval performance & SB Inflation	Enrich. Rotifer	DHA: 0-0.4-1.0-1.9%	DHA: 1.5 %	Matsunari *et al*., 2012b.
S. rivoliana	DHA	Larval performance & Stress resistance	Experimental Microdiet	DHA: 3 - 6.5%	DHA: 3.7-4.5 %	Mesa-Rodríguez *et al*., 2014a.

1.4.2. Microdiets for marine fish larvae.

Supply of an optimal feed which fulfils larval nutritional requirements implies difficulties. As previously mentioned, the utilization of enriched live preys such as *Artemia sp.* becomes difficult due to its rapid catabolism (Danielsen *et al*., 1995; Evjemo *et al*., 2001; Naz, 2008). The early substitution of live prey with a compound diet is a suitable option in order to reduce production costs and avoid unbalanced feeds. Contrarily, early feeding with inert diets resulted in low larval performance, possibly due to its composition, palatability or physical characteristics (Person Le Ruyet *et al*., 1993) or unable to digest it (Kolkovski *et al*., 1993; Zambonino-Infante & Cahu, 1994; Kolkovski, 2001). However, the combination of live prey and inert diet (co-feeding) improve larval performance (Fernández-Díaz & Yúfera, 1997; Kolkovski *et al*., 1997; Hernández-Cruz *et al*., 2007; Roo *et al*., 2010a; Sandel *et al*., 2010).

According to this, the use of experimental microdiets in aquaculture research to identify the marine finfish larvae nutritional requirements has being widely reported (Betancor *et al*., 2011, 2012a, b; Izquierdo *et al*., 2013; Saleh *et al*., 2013, 2015; Hernández-Cruz *et al*., 2015). However, very few studies based on microdiets have been reported for *Seriola*. Mesa-Rodríguez *et al*. (2014a) studied the effect of increasing dietary DHA content in microdiets for *S. rivoliana* larvae, in which the better resistance to acute stress and the incidence of skeletal deformities was correlated with the DHA content. By the way, the incidence of skeletal anomalies has a very important economic implication in aquaculture. The most important anomalies are those affecting the opercula complex, neurocranium and vertebral column, due to its high visual impact. The appearance of skeletal deformities has been widely studied for several marine aquaculture finfish species (Koumoundouros *et al*., 1997a,b, 2001a,b, 2002; Boglione *et al*., 2001, 2003; Sfakianakis *et al*., 2003, 2004; Roo *et al*., 2005a,b, 2009a).

In this sense, the hatchery phase remains the bottleneck of the sector, and the good understanding of species specific larval requirements and bone development becomes indispensable in order to increase fry quality and reduce production costs.

Objectives

3. Objectives

Considered as a relevant specie for the diversification of aquaculture and the limited information available, the main objective of this Thesis was to improve *Seriola rivoliana* larval production. Thus, the following specific objectives were established:

1. To evaluate the effect of larval culture techniques on larvae performance.

First *S. rivoliana* spawn achieved in the Canary Islands were settled under two different culture systems. This study aimed to determine the most appropriate rearing technique (comparison between semi-intensive and intensive systems) at initial culture phases in order to improve larval survival.

2. To describe the skeletal ontogeny of *S. rivoliana* larvae.

Early life stages are strongly affected by undetermined larval rearing needs, feeding protocols and nutritional deficiencies and, consequently low survival and skeletal anomalies occur. For this reason, it is a necessity to obtain bone development information, in order to identify skeletal abnormalities apparition and its causes. Thus, another aim of the present Thesis was to chart the onset of *S. rivoliana* ossification characterizing the skeletal abnormalities in this species.

The onset of bone development was achieved in two sub-objectives, according to the different areas:

1.1. Skeletal development and mineralization pattern of the vertebral column, dorsal, anal and caudal fin complex.

1.2. Bone development of the skull, pectoral and pelvic fins.

3. To evaluate the effect of increasing DHA levels in inert diets.

According to the general importance of DHA as a main dietary lipid for larval marine finfish rearing success, the purpose of the present Thesis was to evaluate the effect of increasing dietary DHA levels on biological performance and larval quality of *S. rivoliana* in terms of growth, final survival, stress resistance as well as the incidence of skeletal deformities.

Material and Methods

3. Material and Methods

3.1. Biological aspects of *Seriola rivoliana*

S. rivoliana belong to the Carangidae family. Carangids are mainly marine teleost's, mostly distributed along tropical and subtropical waters of Atlantic, Indian and Pacific Oceans. Generally present compressed body and small cycloid scales, which often modify into spiny scutes along the lateral line. Carangid juveniles and adults present 2 dorsal fins, with 3-9 spines in the anterior dorsal fin and 1 spine with 18-37 soft rays in the posterior dorsal fin, 3 anal spines with 15-31 soft rays and a widely forked caudal fin. In the Canary Islands, 4 species of *Seriola* genus are described (*S. dumerili, S. rivoliana, S. fasciata* and occasionally *S. carpenteri*).

With the common name of longfin yellowtail or Almaco jack, *S. rivoliana* is a pelagic and benthopelagic species, rarely found at inshore waters. Its circumglobal distribution (Fig.14; Fishbase, 2016), its aquaculture production interest. It can be found in the Eastern Central Atlantic regions from Portugal (Azores and Madeira), Canary Islands to Cape Verde but also sporadically in the Mediterranean Sea (Castriota *et al*., 2002).

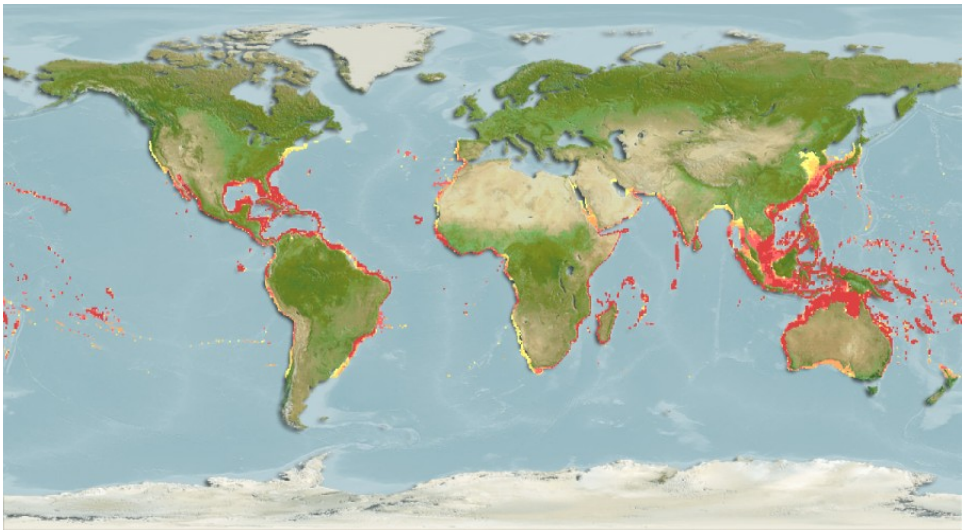


Figure 14. *S. rivoliana* distribution (Fishbase, 2016).

As a fast swimming predator, its diet is mainly based on fish. The body colour is uniformly brownish to olivaceus green with lighter colours at the sides and belly. A dark band is commonly present through the eye to the upper back and sometimes an amber line extends from the eye up to the tail. As a main characteristic, the first rays of the second dorsal fin are about twice longer than the rest dorsal spines (Fig. 15).

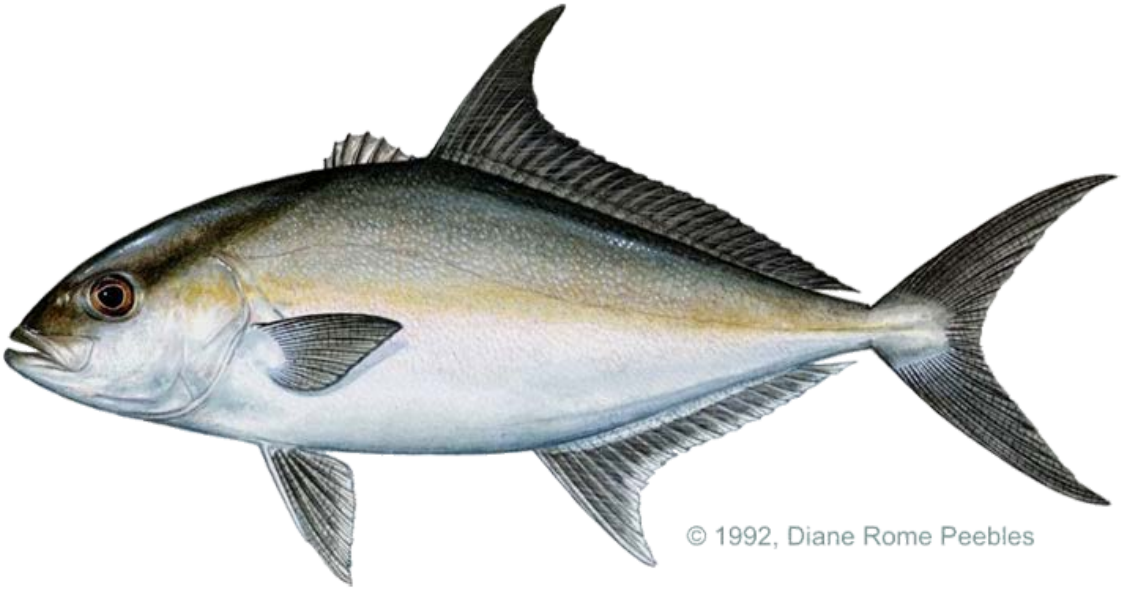


Figure 15. Adult of *S. rivoliana*.

The maximum size registered is 119 cm (TL) and 59.87 kg (IGFA, world record), but common size its around 55 cm fork length (Smith-Vaniz, 2002). Histological studies indicated that the ovarian development of *Seriola sp*. occurs according to a group synchronous pattern (Marino *et al*., 1995), forming aggregations when spawning takes place. *S. rivoliana* presents seasonal maturation, directly influenced by increasing temperature. In the Canary Islands, hormonal injection from late spring (May) to late summer (October) permitted to obtain successful spawns of *S. rivoliana* (Roo *et al*., 2009b, 2012, 2015). In captivity, females tend to grow faster than males, increasing their weigh twice faster every two years, and 66% of the females are considered sufficiently mature (oocytes over 500µm) to be hormonally induced after 4 years (Roo *et al*., 2015) with weights over 6 kg. *S. rivoliana* eggs obtained in captivity are transparent and spherical, with an average egg diameter of 1.1 mm and a single oil droplet of 0.24 ± 0.02 mm (Roo *et al*., 2012).

3.2. Experimental conditions

The experiments and samples analysis described in this document were carried out at the GIAQUA (University of Las Palmas de Gran Canaria, ULPGC) facilities, in Telde, Gran Canaria (Spain).

3.2.1. Broodstock

S. rivoliana broodstock were collected as sub adults from the wild (Fig. 16a) in 2009 with an average weight of 1.7±0.25 kg, and adapted to captivity in $10m^3$ squared glass fibber tanks (3x3x1.5m depth). Fish were kept under natural photoperiod, temperatures ranging from 18 to 24ºC and fed twice a week with commercial pellets (Vitalis Repro™; Skretting, Burgos, Spain) corresponding to 1% of the body weight (BW), supplemented once a week with frozen squid (*Illex argentines*) and mussels (*Mytilus galloprovincialis*) at 2% of BW. The maduration stage was assessed using gonadal biopsy, oocytes from females were taken in vivo and placed in Serra's solution (6:3:1, 70% ethanol, 40% formaldehyde and 99.5% acetic acid) to be measured using a profile projector (Mitutoyo Pj-3000A, Kanagawa, Japan). Mean diameter of the largest oocytes were determined. When the oocyte diameter was over 500µm (Fig. 16b), whole individuals were injected with gonadotropin releasing hormone analogue (GnRHa, desGly 10, [D-Ala6]; Sigma-Aldrich, St. Louis, MO, USA) at a dose of 20 µg/kg of body weight (Mylonas *et al*., 2004). Hormonal treatment was applied every two weeks during the spawning season.

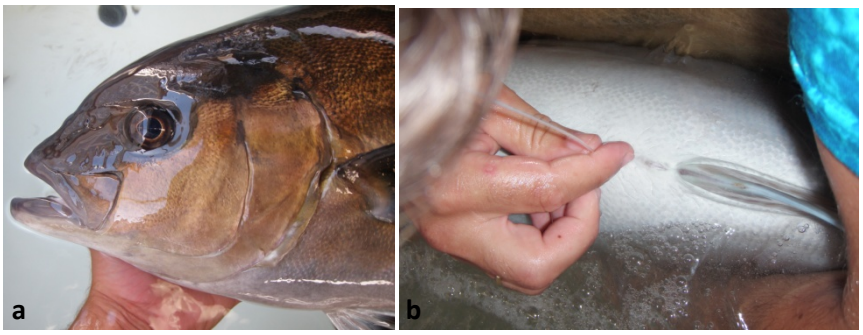


Figure 16 (a, b). a) *S rivoliana* adult; b) *S. rivoliana* gonadal biopsy inserting catheter (Kruuse, Langeskov, Denmark) into the gonadal cavity.

3.2.2. Egg stocking

Floating *S. rivoliana* eggs were collected 32h after hormonal injection. The spawning quality was measured according to the number of fertilized eggs and 3 days old hatched larvae (Fig. 17a,b), following the methodology described by Fernández-Palacios *et al*. (1995). Eggs were directly stocked in the rearing tanks ($40m^3$ - $2m^3$).

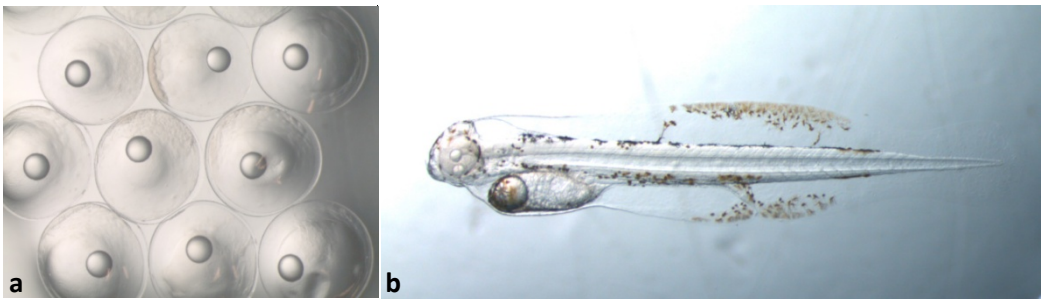


Figure 17(a, b). a) *S. rivoliana* eggs; b) *S. rivoliana* larvae of 1dph.

3.2.3. Larval rearing tanks

The different larval rearing tanks used in the present Thesis were chosen according to the culture systems and volumes.

- Semi-intensive (SIS). SIS tanks presented a cylinder-conical shape with a diameter of 5m, 2.35m depth and a total volume of $40m^3$ (Fig. 18a). The water entrance was located in the lateral bottom part of the tank at fist larval stages and modified to the surface with the age of the larvae. As well as the water entrance, output waters could be located at the bottom and/or the lateral side of the tank, and modified along the larval stage. Airlift is located all around the tank.

- Intensive systems (IS). This cylinder-conical tanks with a diameter of 1.5m, 2.10m depth and a total volume of $2m^3$(Fig. 18b), are frequently used for high larval densities. As well as the SIS tanks, water entrance and output can be modified according to the culture requirements. In both systems, larval rearing was conducted under 12:12 (12 h light:12h dark) photoperiod using mixture of artificial fluorescent lights (Mod. TLD 58W/54-765; Philips, Lyon, France) and natural sun light with intensity between 1.000-3.500 lux. Moreover, water conditions were daily measured (temperature: 24.03 ± 0.26 ºC; oxygen levels: 6.22 ± 0.21 ppm; OxyGuard, Denmark).

Figure 18 (a, b). a) Semi-intensive 40m^3 tank; b) Intensive 2m^3 tank.

- Experimental tanks. For specific feeding trials, larvae were settled in 200 l fibreglass cylinder tanks with conical bottom and painted in a light grey colour (Fig. 19a,b). Water conditions were daily measured (temperature: 22.5 ± 0.6 ºC; oxygen levels: 6.5 ± 0.3 ppm; OxyGuard, Denmark). Photoperiod was kept at 12:12 (12 h light:12 h dark) by fluorescent daylights at 1700lux (digital Lux Tester YF-1065; Powertech Rentals, Osborne, Australia).

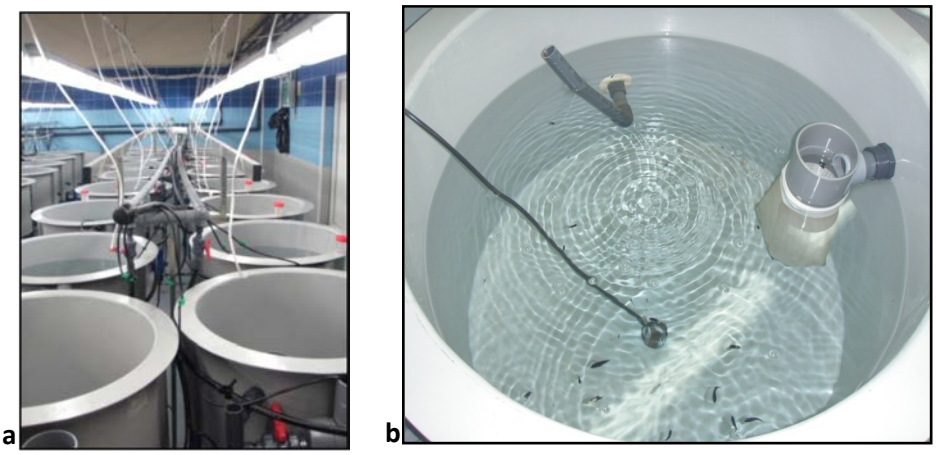


Figure 19 (a, b). Intensive 200l tank.

3.2.4. Phytoplankton and live preys culture

Pseudo-green water technique was used for both SIS and IS, adding live phytoplankton (*Nannochloropsis sp*.) to maintain a concentration of 250.000 cell/ml. In order to preserve this concentration in the rearing tanks, additional phytoplankton culture was maintained. For this massive phytoplankton production, transparent polyethylene bags of 50, 230 and 460 l were conducted in "Bach" system culture, following the phytoplankton culture protocol described by Roo *et al*. (2009b).

Rotifers mass culture was carried out on cylinder conical fibreglass tanks of 1.700 l total volume, with a mixture of fresh and seawater to achieve a salinity of 25 ppt (Roo, 2009b). Rotifers (*Brachionus sp.*; L-strain) were enriched during 6 hours before harvesting with DHA Protein Selco ($INVE_{TM}$), following manufacturer instructions.

Artemia sp. cysts used during the study were ready to hatch without decapsulation phase. This cysts have been treated with SEP-Art technology (Fig. 20a), giving a magnetic coating on the cyst which after pass through separator tube containing passive magnets (Fig. 20b) attract the iron coated shells and obtaining clean nauplii after hatching. Afterwards, *Artemia sp.* nauplii were enriched with Easy DHA Selco (INVE, Dendermonde, Belgium) in a tank at a concentration of 250-300.000 nauplii/l during 18hours.

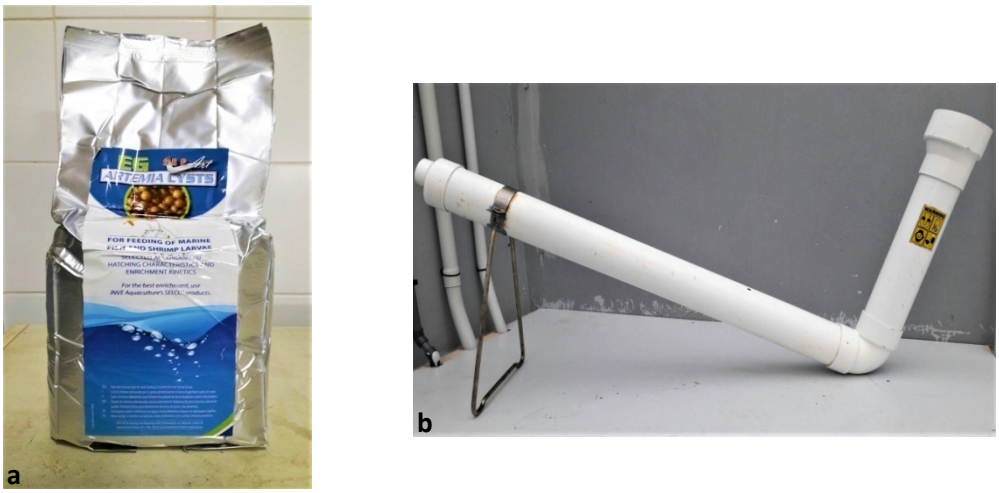


Figure 20 (a, b). a) *Artemia sp.* cyst; b) *Artemia sp.* cysts separator (SEP-Art technology, $INVE_{TM}$).

3.2.5. Microdiets (Formulation and Preparation)

Two different types of inert diets were used all along the study. During the first larval experiences (Study I), the weaning protocol included hand feeding a commercial diet (Gemma Micro, Skretting, Vervins, France) at first weaning days, and with automatic feeders afterwards.

For the DHA nutritional requirement experience (Study IV), five isoproteic (54.8%) and isolipidic (24.1%) microdiets, which varied in their DHA content from 0.5-5.0% dry weight (dw), were elaborated. The microdiets were manufactured according to Liu *et al.*, (2002), by mixing squid powder with water soluble compounds, lipids and fat soluble vitamins. Vitamin, mineral and attractant mixes were added according to Betancor *et al.*, (2012a,b). Diluted gelatine was used as a binder. The obtained paste was compressed and dried at 38ºC for 24h (Fig. 21a,b). Next, dried pellets were ground and sieved in two size ranges (250-500µm and 500-710µm).

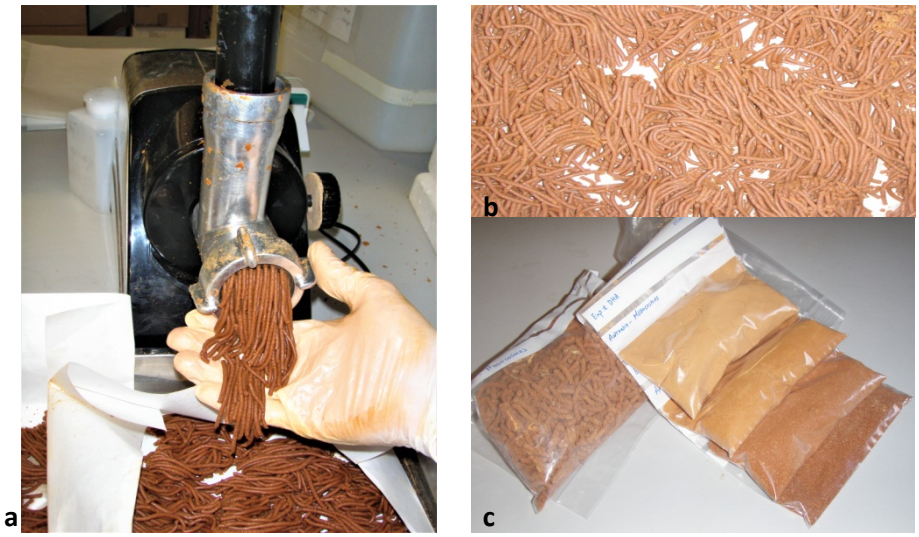


Figure 21(a, b, c). a) Compressing processes of the paste; b) compressed paste; c) grounded pellets in sizes.

In order to evaluate the fatty acid effect and guarantee the desired lipid content, squid powder (Skretting$_{TM}$) was defatted thrice with a chloroform:meal ratio of 3:1 (Fig. 22a,b). The defatted meal was separated from the chloroform fraction after extraction with a vacuum pump, spreaded out in a tray and the remaining solvent was evaporated during 12 h at 38ºC.

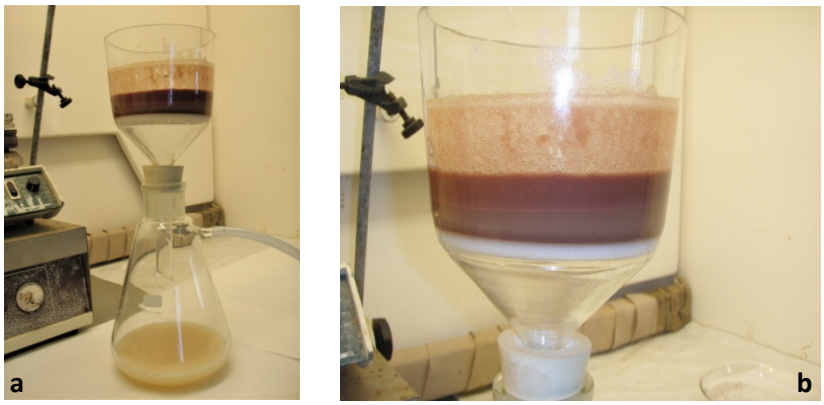


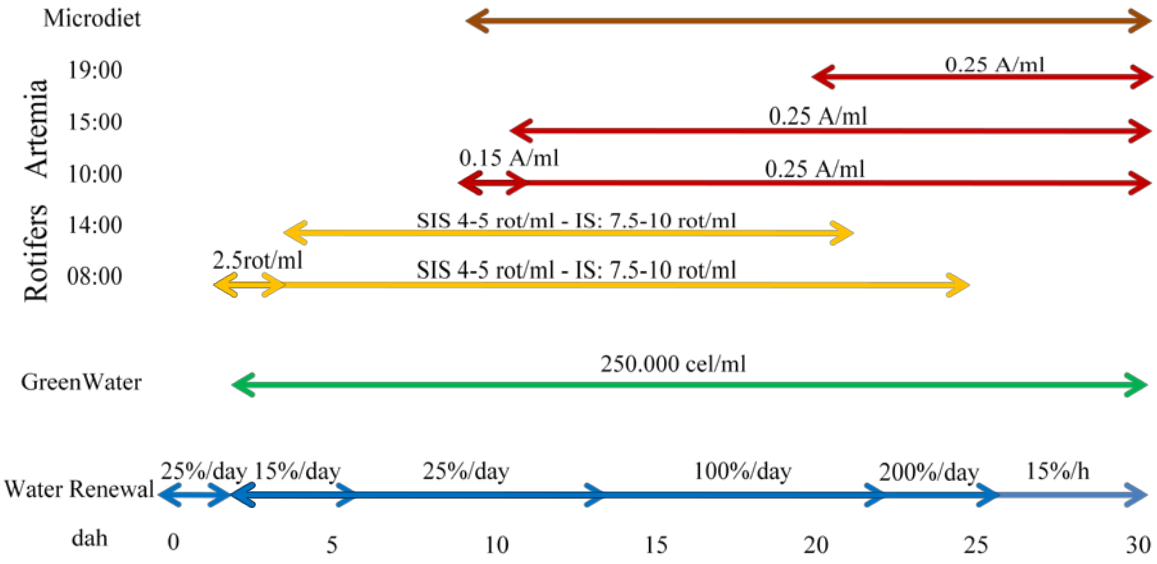
Figure 22(a, b). a) Defatting processes of squid meal; b) extraction of chloroform fraction with a vacuum pump.

3.2.6. Feeding Regimes and Protocol

Both systems (SIS and IS) followed the same rearing protocol (Table V) according to the larval rearing techniques established by Roo *et al.* (2010a). *S. rivoliana* eggs were settled at a density of 4.5 eggs/l in SIS tanks and 125 egg/l in IS tanks. All experimental tanks were supplied with filtered and UV sterilized sea water, being clear water fist up to 2dah and pseudo-green water after until 30dph, at a concentration of 250.000 cells/ml. Water exchange was

progressively increased from 15% to 100% of tank volume per day. Enriched rotifers were added twice a day (08:00; 14:00) to maintain a live prey density of 4-5 rot/ml in SIS and 7.5-10 rot/ml in IS from 2 to 25 dah. Enriched *Artemia sp.* nauplii were added first once a day at 15dah (0.25 Art/ml) and increased up to three times a day after in both systems.

Table V. *S. rivoliana* feeding sequence.



For the dietary DHA experience, 90 larvae per tank (in triplicate) with 30 dah were settled in 200 litre tanks. Diets were manually supplied every hour from 8:30 to 19:30. Initially, *S. rivoliana* larvae were fed twice a day during the first 5 days with un-enriched rotifers and *Artemia* to ensure the adaptation to the new rearing tanks. The daily amount of microdiet was gradually increased from 1.5gr to 2.5gr per tank.

3.3. Measurements

The aim of the different experiences determined the type of measurements carried out as well as the sampling points. At the different samplings larvae were sacrificed by immersion in water and ice according to the current regulations (Spanish Royal Decree 1201/2005) which were accepted by the Spanish Ethic Welfare Committee (Comité Ético del Bienestar) of the University of Las Palmas de Gran Canaria (ULPGC) in 2011.

3.3.1. Larval Length, Weight and Final Survival

In order to evaluate for first time the larval growth of *S. rivoliana* larval growth under two different rearing conditions (SIS and IS), total length (TL) and dry weight (DW) of 25 larvae per tank were measured every 5 days from hatching to 30 dah. TL and other larval body

meristic characters such as standard length (SL), pre-anal length (PAL), eye diameter (ED), cephalic height (CH), yolk sac length (YSL) and lipid globule diameter (LGD) were evaluated (Fig. 23) using a profile projector (Mitutoyo PJ-3000A, Kanagawa, Japan)

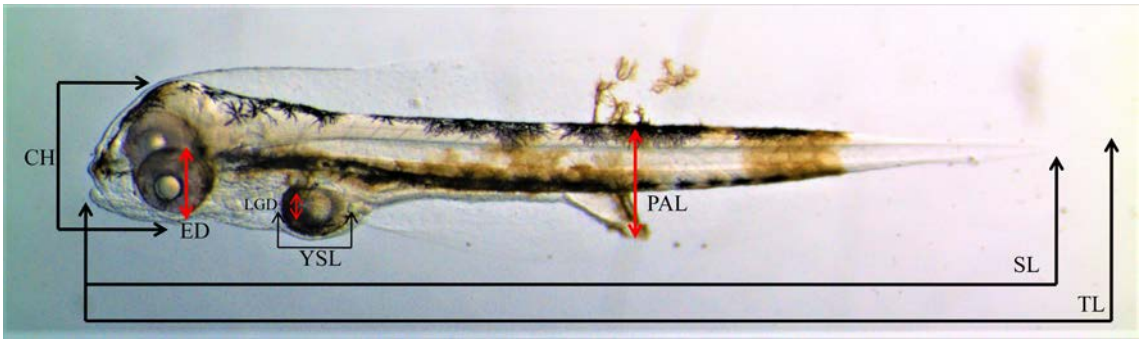


Figure 23. Meristic length measurements of *S rivoliana* larvae.

Larval DW was determined by measuring (in triplicate) the whole body weight of 10 larvae washed with distilled water and dried in a glass slide in an oven (Jouan EU 28, S. Herblain, France) at 100ºC during 24 hours until constant weight in a precision balance (Mod. Mettler, AG 204, Ohio, USA).

At the beginning (30dah), intermediate (40dah) and final (50dah) points of the experimental microdiets trial, growth was assessed by estimating the TL of *S. rivoliana* larvae.

Final survival was determined at 30 dah (SIS and IS) or 50 dah (microdiets trial) counting the remaining alive larvae in the experimental tanks.

3.3.2. Activity Test

An air exposure test was performed on 20 and 30 dah larvae from SIS and IS tanks. On day 20, larvae (n = 45) were individually exposed to air for 15, 30 or 60 seconds in a 500 µm nylon mesh screen (Izquierdo *et al*., 1989). After the air exposure, larvae were transferred to an aerated 2l beaker and survival was recorded 24 h later. At 30 dah, a new set of stress tests were performed. At this point, two clove oil doses (1 and 2 ppt), as anaesthetic, were evaluated in combination with different air exposure times (15, 30, 60, 75 and 90 s). The same procedure (30 seconds of air exposure) was performed at 42 and 50 dah *S. rivoliana* specimens (n = 15) from dietary DHA content experience. Larval survival was recorded 24 h after each stress test.

3.3.3. Osteological Studies

To study the bone ossification, all specimens were fixed in 10% buffered formalin from hatching to 33 dah and individually stained (3.28 ± 0.15 - 16.2 ± 0.73 mm SL). Fixed larvae were cleared and stained with alizarin red (Vandewalle *et al*., 1998) and individually examined using stereomicroscopy (Table VI). The drawings of the different developmental stages were made using Adobe Photoshop CS3-10.0 (1990-2007 Adobe System Incorporated, United States) directly from digital photographs.

Table VI. Staining protocol according to Vandewalle *et al.* (1998).

	Step	**Duration**	**Solutions**
Hydration	Ethanol 95%	1h	E 95%
	Ethanol 95%	1h	
	Ethanol 95%	1h	
	Ethanol 75%	1h	E 75%
	Ethanol 40%	1h	E 40%
	Ethanol 15%	1h	E 15%
	Distilled Water	1h or overnight	
Tissue Digestion	Trypsin Solution	1h	TS
Staining	Alyzarin Red	1h 30'	AR
Clearing	Glycerin : KOH (1:3)	12-24h	Gly (1:3)
	Glycerin : KOH (1:1)	12-24h	Gly (1:1)
	Glycerin : KOH (3:1)	12-24h	Gly (3:1)
Storage	Glycerin		Pure Gly

E 95%	95 ml absolute ethanol + 5 ml destilled water
E 75%	75 ml absolute ethanol + 25 ml destilled water
E 40%	40 ml absolute ethanol + 60 ml destilled water
E 15%	15 ml absolute ethanol + 85 ml destilled water
TS	90mg porcine pancreas trypsin + 70ml destilled water + 30ml saturated solution of $Na_2B_4O_7$
AR	$1gL^{-1}$ Alizarin Red in 0.5% KOH solution
Gly (1:3)	25ml Glycerine + 75ml KOH (0.5%)
Gly (1:3)	50ml Glycerine + 50ml KOH (0.5%)
Gly (1:3)	75ml Glycerine + 25ml KOH (0.5%)
Pure Gly	Pure Gly with some grains of Thymol

Bone description, followed the terminology suggested by different authors (Manod, 1968; Matsuoka, 1985; Collette & Gillis, 1992; Suda, 1996; Cubbage & Mabee, 1996; Faustino & Power, 2001) and their abbreviations are illustrated in the Table VII - IX. The angles of the spine were measured from the beginning of the vertebral body to the tip of the spine. Viscerocranial structures were grouped into different regions (Matsuoka, 1985; Collette & Gillis, 1992; Cubbage & Mabee, 1996; Suda, 1996; Faustino & Power, 2001), depending on their functionality.

Table VII. Pectoral and pelvic fins skeletal abbreviations (Cubbage & Mabee, 1996).

Region	Skeletal elements	Abbreviations
Pectoral Fin	Actinost	Act
	Cleithrum	Cl
	Coracoid	Co
	Distal radial	Dr
	Propterygium	Prop
	Postcleithrum Lower	Pcl
	Postcleithrum Upper	Pcu
	Posttemporal	Pt
	Soft rays	R
	Scapula	Sc
	Scapular foramen	F
	Supracleithrum	Scl
	Supratemporal Lower	Stl
	Supratemporal Upper	Stu
Pelvic Fin	Basipterygium	Bp
	Soft rays	R
	Spine	S

Table VIII. Skeletal elements abbreviations for vertebral column, caudal, dorsal and anal fins (Manod, 1968; Matsuoka, 1985; Suda, 1996).

Region	Skeletal elements	Abbreviations
Vertebral Column	Vertebra centra	Ce
	Notochord	No
	Urostyle	Ur
	Neural Arch	Na
	Neural Spine	Ns
	Haemal Arch	Ha
	Haemal Spine	Hs
	Dorsal Ribs	Eb
	Pleural Ribs	Plr
	Parapophyses	Pp
	Anterior neural zygapophysis	Anz
	Posterior neural zygapophysis	Pnz
	Anterior haemal zygapophysis	Ahz
	Posterior haemal zygapophysis	Phz
Caudal Fin	Hypurals	Hy
	Parhypural	Ph
	Epurals	Ep
	Uroneurals	Un
	Caudal lepidotrichia	PCR
	Caudal Dermatotrichia	SCR
Dorsal Fin	Predorsal	Pd
	Hard Spines	S
	Lepidotrichium	R
	Proximal Pterygiophores	Pr
	Distal Radial	Dr
Anal Fin	Hard Spines	S
	Lepidotrichium	R
	Proximal Pterygiophores	Pr
	Distal Radial	Dr

Table IX. Viscerocranial structures abbreviations grouped into regions according to their functionality (Matsuoka, 1985; Collette & Gillis, 1992; Cubbage & Mabee, 1996; Suda, 1996; Faustino & Power, 2001).

Region	Skeletal elements	Abbreviations
Neurocranium	Basioccipital	Boc
	Basisphenoid	Bas
	Ethmoid	E
	Epioccipital	Eo
	Exoccipital	Eoc
	Frontal	F
	Infraorbitals	Inf
	Intercalar	Ic
	Lateral ethmoid	Le
	Nasal	N
	Parasphenoid	Ps
	Parietal	Pa
	Prootic	Pro
	Pterotic	Pto
	Pterosphenoid	Pts
	Sphenotic	Sph
	Supraoccipital	Soc
	Vomer	V
Jaws	Anguloarticular	Aa
	Dentary	D
	Dentary teeth	Dt
	Maxilla	Mx
	Premaxilla	Pm
	Premaxilla teeth	Pmt
	Retroarticular	Ra

Region	Skeletal elements	Abbreviations
Suspensorium	Ectopterygoid	Ect
	Endopterygoid	En
	Hyomandibula	Hm
	Metapterygoid	Mpt
	Palatine	P
	Palatine teeth	Plt
	Quadrate	Q
	Symplectic	Sy
Hyoid Arch	Branchiostegals	Brs
	Ceratohyal	Ch
	Epihyal	Eh
	Hypohyal	Hh
	Urohyal	Uh
Branquial Arches	Basibranchials	Bb
	Basihyal	Bh
	Ceratobranchials	Cb
	Ceratobranchials teeth	Cbt
	Epibranchials	Eb
	Hypobranchials	Hb
	Pharyngobranchials	Pb
	Pharyngobranchials teeth	Pbt
Opercular Series	Interopercle	Iop
	Opercle	Op
	Preopercle	Pop
	Subopercle	Sop

3.3.4. Meristic counts

S. rivoliana juveniles between 60-120 g (n = 10) were soft X-ray monitored (Mod. Senographer-DHR, General Electric, USA) in order to determine the specific number of the different bone structures. Meristic counts of the different regions can be found in the following tables (Table X - XI).

Table X. Meristic counts for vertebral column, caudal, dorsal and anal fins structures.

Regions	Structure	Nº
Vertebral Column	Vertebra centra	10+13
	Urostyle	1+1
	Neural spine	23
	Haemal spine	13
	Dorsal Ribs	6+6
	Pleural Ribs	8+8
	Parapophyses	6
Caudal Fin	Hypurals	5
	Parhypural	1
	Epurals	3
	Uroneurals	1+1
	Lepidotrichia	10+9
	Dermatotrichia	10+9
Dorsal Fin	Predorsal	3
	Hard Spines	VII+I
	Proximal Pterygiophores	7
	Lepidotrichia	30 / 34
	Proximal Radial	30 / 34
	Distal Radial	30 / 34
Anal Fin	Hard Spines	II+I
	Lepidotrichia	19 / 21
	Proximal Radial	19 / 22
	Distal Radial	19 / 22

Table XI. Meristic counts of the Viscerocranial skeleton. Single (S) or paired (P) structures.

Region	Skeletal element	N. Structures
Neurocranium	Basioccipital	1 - S
	Basisphenoid	1 - S
	Ethmoid	1 - P
	Epioccipital	1 - P
	Exoccipital	1 - P
	Frontal	1 - P
	Infraorbitals	5 - P
	Intercalar	1 - P
	Lateral ethmoid	1 - P
	Nasal	1 - P
	Parasphenoid	1 - S
	Parietal	1 - P
	Prootic	1 - P
	Pterotic	1 - P
	Pterosphenoid	1 - P
	Sphenotic	1 - P
	Supraoccipital	1 - S
	Vomer	1 - S
Jaws	Anguloarticular	1 - P
	Dentary	1 - P
	Maxilla	1 - P
	Premaxilla	1 - P
	Retroarticular	1 - P
Suspensorium	Ectopterygoid	1 - P
	Endopterygoid	1 - P
	Hyomandibular	1 - P
	Metapterygoid	1 - P
	Palatine	1 - P
	Quadrate	1 - P
	Symplectic	1 - P

Region	Skeletal element	N. Structures
Opercular Series	Interopercle	1 - P
	Opercle	1 - P
	Preopercle	1 - P
	Subopercle	1 - P
Hyoid Arch	Branchiostegals	7 - P
	Ceratohyal	1 - P
	Epihyal	1 - P
	Hypohyal	1 - P
	Urohyal	1 - S
Pectoral Fin	Actinost	4 - S
	Cleithrum	1 - S
	Coracoid	1 - S
	Distal radial	19/21
	Propterygium	1 - S
	Postcleithrum Lower	1 - S
	Postcleithrum Upper	1 - S
	Posttemporal	1 - S
	Soft rays	20/22
	Scapula	1 - S
	Supracleithrum	1 - S
	Supratemporal Lower	1 - S
	Supratemporal Upper	1 - S
Pelvic Fin	Basipterygium	1 - P
	Soft rays	5 - P
	Spine	1 - P
Branquial Arches	Basibranchials	3 - S
	Basihyal	1 - S
	Ceratobranchials	5 - P
	Epibranchials	4 - P
	Hypobranchials	3 - P
	Pharyngobranchials	4 - P

3.3.5. Deformities characterization

For the skeletal anomalies characterisation, a total of 15 larvae (50 dah) per tank were fixed in 10% buffered formalin and stained with alizarin red according to the methodology of Vandewalle *et al.* (1998). The different regions were divided (Fig. 24) and evaluated (Table XII) according to Boglione *et al.* (2001).

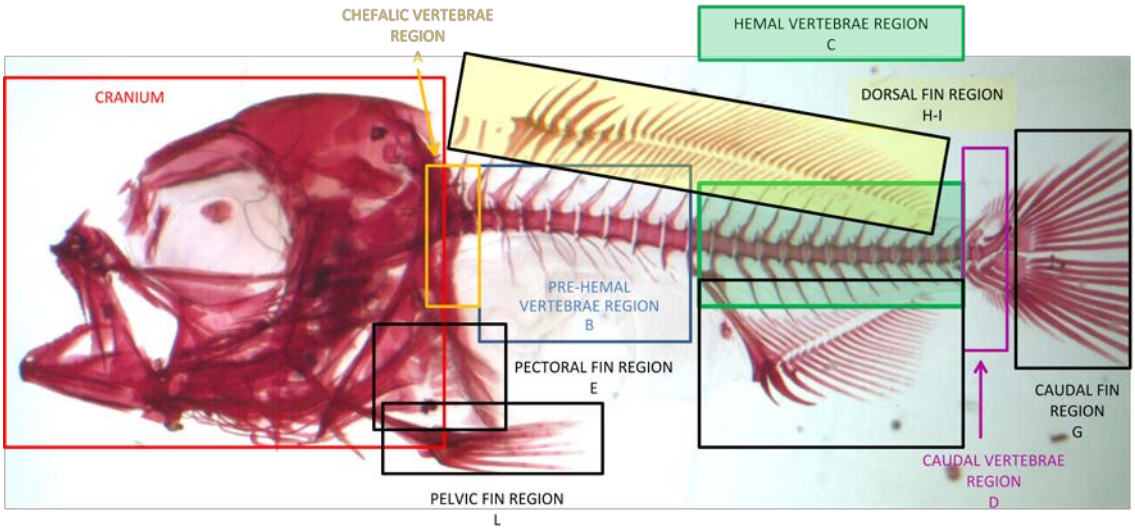


Figure 24. Regions of stained *S. rivoliana* larvae.

Table XII. Skeletal abnormalities classification (Boglione *et al*., 2001).

	Code	Description
Region	A	Cephalic vertebrae (carrying epipleural ribs)
	B	Pre-haemal vertebrae (carrying epipleural and pleural ribs and open haemal arch, without haemal spine)
	C	Haemal vertebrae (with haemal arch closed by haemal spine)
	D	Caudal vertebrae (with haemal and neural arches closed by modified spines)
	E	Pectoral fin
	F	Anal fin
	G	Caudal fin
	H	Dorsal spines
	I	Dorsal soft rays
	L	Pelvic fin
Anomalies	1	Kyphosis
	2	Lordosis
	3	Partial vertebral fusion
	3*	Total vertebral body fusion
	4	Vertebral malformation (deformation, ossification ridges, marked reduction in length or elongation, intervertebral bony plate)
	5	Malformed neural arch and/or spine
	5*	Supernumerary neural elements. Absence of neural elements
	6	Malformed haemal arch and/or spine
	6*	Supernumerary haemal elements Absence of haemal elements
	7	Malformed rib
	7*	Supranumerary pleural rib
	8	Malformed pterygophores (deformed, absent, fused, supernumerary)
	9	Malformed hypural (deformed, absent, fused, supernumerary)
	9*	Deformed or broken parahypural or fused with hypural/hemaspine
	10	Malformed epural (deformed, absent, fused, supernumerary)
	11	Malformed ray (deformed, absent, fused, supernumerary)
	12	Swim-bladder anomaly
	13	Presence of calculi in the urinary ducts
	14	Malformed maxillary and/or pre-maxillary
	15	Malformed dentale
	16	Other cephalic deformities (glossohyal, neurocranium, ..)
	17L/R	Malformed left/right opercle
	17*L/R	Malformed, absent, fused branchiostegal ray
	18	Predorsal bones anomalies
	19	Hypural with decalcifications
	20	Decalcifed pterygophore
	21	Deformed epipleural ribs
	22	Deformed dorsal ribs
	23	Deformed pleural ribs
	24 L/R	Decalcified left/right opercular plate
	25	Epural with decalcifications
	26	Supernumerary bone
	27	Decalcified urostyle
	28	Ossification defects in vertebrae
	29	Deformed postcleithrum
	S	Scoliosis
	CL L/R	Malformed left/right cleithrum
	COR L/R	Malformed left/right coracoid
	SBS	Saddle-back syndrome

3.4. Biochemical Analysis

3.4.1. Proximate Analysis

For biochemical analysis, samples of feeds (rotifer, *Artemia sp.* and microdiets) were collected in all the feeding trials. Larval biochemical composition was analyzed collecting samples at the beginning (30 dah) and the end (50 dah) of the dietary DHA experiment after a starving period of 12 h, washed with distilled water and kept at -80ºC.

3.4.2. Moisture and Ash content

Moisture and ash content was determined according to the Association of Official Analytical Chemists (A.O.A.C., 1995). Samples (about 100mg) were dried at 110ºC during 24 h until constant weight to obtain moisture content. The ash content was determined by drying the samples in an oven at a temperature of 450ºC until a constant ash weight was attained. The moisture and final ash content was obtained applying following the equations:

$$\text{Moisture (\%)} = \frac{100-(B-A)-(C-A)}{B-A} \qquad \text{Ash (\%)} = \frac{100 \text{ x } Ws}{Wa}$$

Where: A = weight of empty container; B = weight of wet sample + container; C = weight of dry sample + container; Ws = weight of sample; Wa = weight of ash.

3.4.3. Total Lipid and fatty acid content

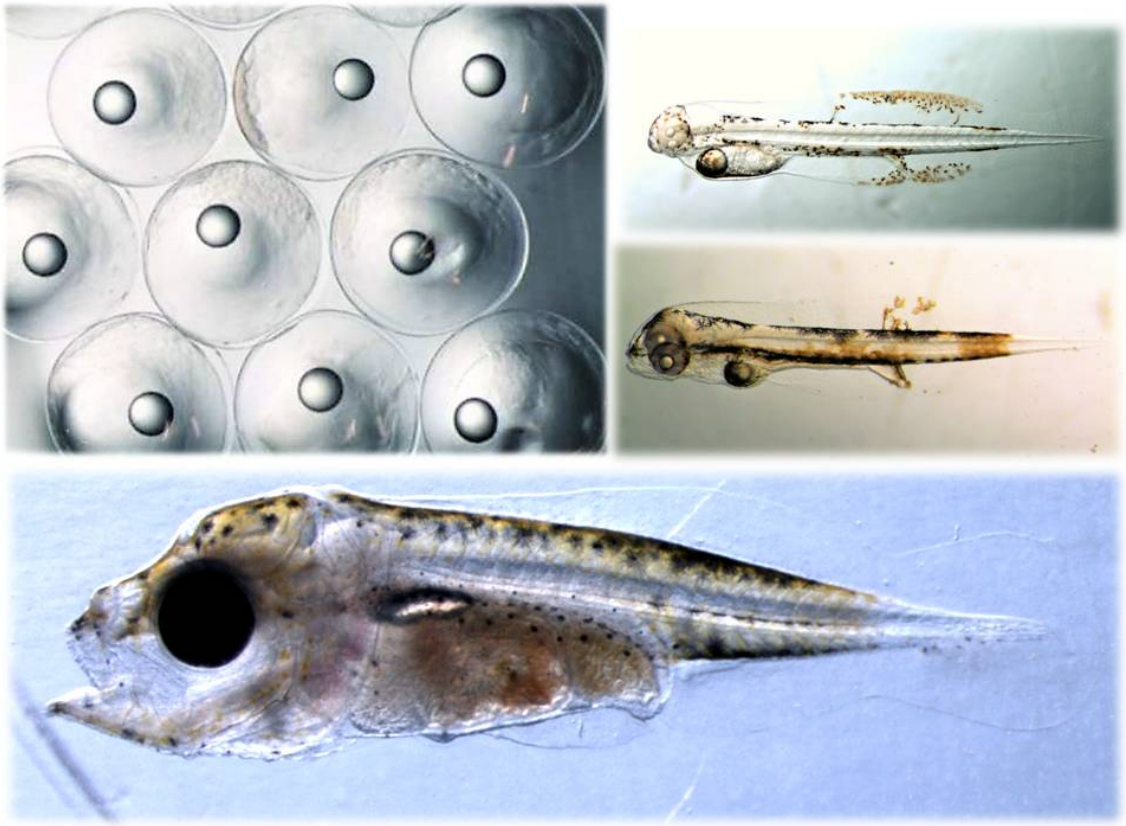
The total lipids were extracted following the method of Folch *et al.* (1957), by homogenising the samples in an Ultra Turrax (IKA-Werke, T25 BASIC, Staufen, Germany) with a solution of 5 ml of Chloroform:Methanol (2:1) and 0.01% of BHT, and filtered after adding KCL to increase water phase polarity. The remnant solvent was dried under nitrogen atmosphere until obtain constant lipid weight.

Fatty acid methyl esters (FAMEs) were obtained following the method of Christie (1982) by transesterification of total lipid with 1% sulphuric acid in methanol (H2SO4). The reaction was conducted in dark conditions under nitrogen atmosphere for 16 h at 50ºC. Afterwards, fatty acid methyl esters were extracted with hexane and purified by adsorption chromatography on NH2 Sep-pack cartridges (Waters S.A., Massachusetts, USA) as described by Fox (1990). FAMEs were separated by GLC (GC-14A, Shimadzu, Tokyo, Japan) as described Izquierdo *et al.* (1989).

3.5. Statistical Analysis

All the data were statistically treated using SPSS Statistical Software System ver 15.0 (SPSS, Chicago, IL, USA). A t-test for simple mean comparison analysis ($P < 0.05$) (Sokal & Rolf, 1995) was applied to compare differences between rearing systems. When data were not normally distributed, arcsine-transformation was applied, and then Kolmogorov– Smirnov non-parametric test was applied to the non-transformed data. Results are presented as mean values ± SD.

Study I



First results of spawning and larval rearing of longfin yellowtail Seriola rivoliana as a fast-growing candidate for European marine finfish aquaculture diversification.

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First results of spawning and larval rearing of longfin yellowtail *Seriola rivoliana* as a fast-growing candidate for European marine finfish aquaculture diversification.

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Abstract

The present study describes the adaptation of longfin yellowtail Seriola rivoliana as broodstock and first larval rearing trials under intensive and semi-intensive conditions. Fifteen sub-adults were captured in the South coast of Gran Canaria (Canary Islands, Spain) in June 2007. Fish (initial weight 1.76 ± 0.25 kg) reached a weight of 6.0 ± 1.1 kg in July 2010. Once a year, fish were sampled to determine individual growth in weight and size. In addition, the state of sexual maturity was established based on gonadal biopsies. On the basis of repeated hormonal injection (GnRHa, 20 lg/kg), 10 successful spawns were obtained between July and October 2009, with 92.5 ± 5.5% and 72.6 ± 17.2%, fertilization and egg viability respectively. First results of larval rearing under semi-intensive conditions, showed an average survival at 30 DAH of 2.5% as compared with 0.5% under intensive conditions. The low survivals under the two rearing conditions in addition to their failure to pass a stress test could be attributed to deficiencies in essential fatty acids as could be seen in both eggs and feeds. Morphometric parameters showed no significant difference between the two rearing systems in 30 DAH larvae.

Keywords: diversification, seriola, broodstock, spawning, larval rearing, rearing techniques.

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1. Introduction

Longfin yellowtail Seriola rivoliana as other seriola species is considered as one of the most important emerging marine finfish species in Japan, Australia and the United States. In contrast, this species has not been under development for mariculture in Europe. This circumtropical carangid species can be found in Eastern Central Atlantic regions from Portugal (Azores and Madeira), Canary Islands to Cape Verde (Fischer, Bianchi & Scott 1981), whereas some individuals were caught sporadically in the Mediterranean sea (Castriota, Greco, Marino & Andaloro, 2002). As other seriola species, *S. rivoliana* is well known for its fast growth, reaching a maximum standard length (SL) of 160 cm and a maximum weight of 59 kg (IGFA**,** 2001) and its high market value of 7–10 USD per kg (Nakada, 2002). However, the bottleneck to *S. rivoliana* mass production is the unreliable supplies of juveniles resulting from poor spawns and low hatchery survival. Therefore, the industry in Japan, Australia and USA is relying on the collection of fingerlings from the wild, to be ongrown in tank and cages (Nakada, 2002; Yamamoto, Teruya, Hara, Hokazono, Hashimoto, Suzuki, Iwashita, Matsunari, Fuguita & Mushiake, 2008).

A reproduction protocol for this species in terms of culture conditions, maturation and use of hormonal treatment is still not available. Furthermore, larval rearing studies of this species are scarce and limited to some reports in Ecuador (Benetti, 1997; Blacio, Darquea & Rodríguez, 2003) and Hawaii (Laidley, Shields & Ostrowksi, 2004). On the other hand, reproduction and larval rearing protocols have been developed for similar species, such as Japanese yellowtail *Seriola quinqueradiata*, greater amberjack *Seriola dumerili* or yellowtail king fish *Seriola lalandi* (Benetti, 2000; Poortenaar, Hooker & Sharp, 2001; Nakada, 2002; Papandroulakis, Mylonas, Maingot & Divanach, 2005).

To enhance the development of S. rivoliana for aquaculture diversification in Europe, different experimental activities, including broodstock management and larval rearing, are being conducted in the Canary Islands (Spain).

The objective of this study was to test mesocosms or semi-intensive techniques, which have been previously tested and reported successful in the larval rearing of difficult-to-rear fish species, which could be applied later to more intensive, commercial systems to improve biological performance and system productivity (Papandroulakis *et al*., 2005; Jerez, Samper, Santamaría, Villamandos, Cejas & Felipe, 2006; Roo, Hernández-Cruz, Socorro, Fernández-Palacios & Izquierdo, 2010). The comparison between the intensive and semi-intensive techniques, for larval rearing of *S. rivoliana*, will contribute to better understand of the husbandry needs of the species regarding future application in commercial production.

2. Material and Methods

2.1. Broodstock

Fifteen S. rivoliana sub-adults (1.76 ± 0.25 kg) were captured at the South coast of Gran Canaria(Canary Islands, Spain), transported to land facilities and adapted to captivity in 10 m3 squared glass fibber tanks (3 m 9 3 m 9 1.5 m depth). Fish were kept under natural photoperiod and natural sea water with 37 g L^{-1} salinity and temperature ranging from 18 to 24°C year around. After capture, all fish were weighed, sized, individually tagged with PIT tags of 0.1 g and 152 x 12 mm in length (EID Ibérica SA – TROVAN, Madrid, Spain), and sexed by gonadal biopsy inserting a 1.3 mm internal diameter catheter (Kruuse, Langeskov, Denmark) into the gonadal cavity and applying gentle aspiration. Fish were fed twice a week with commercial pellets (13 mm, Vitalis ReproTM; Skretting, Burgos, Spain) corresponding to 1% of the body weight (BW), supplemented once a week with frozen squid (*Illex argentines*) and mussels (*Mytilus galloprovincialis*) at 2% of BW. Once a year (June), the whole population was anaesthetized with clove oil (Guinama S.L, Valencia, Spain; 50 ppm) and standard length (SL), body weight and condition factor (CF) were recorded (Table 1). The maturation stage was assessed using gonadal biopsy, oocytes from females were taken in vivo and placed in Serra's solution (6:3:1, 70% ethanol, 40% formaldehyde and 99.5% acetic acid) to be measured using a profile projector (Mitutoyo PJ-3000A, Kanagawa, Japan). Mean diameter of the largest oocytes

were determined. The per cent of running males was determined (Table 1). When oocyte diameter (OD) was greater than 500 lm, the whole population (males and females) was injected with gonadotropin releasing hormone analogue (GnRHa, des- Gly^{10},[$_{D}$-Ala^{6}]-; Sigma-Aldrich, St. Louis, MO, USA) at a dose of 20 μg kg^{-1} body weight, based on the reported dosage for *S. dumerillli* (Mylonas, Papandroulakis, Smboukis, Papadaki & Divanach, 2004). These hormonal treatments were applied every 2 weeks from 15 June to 15 October 2009. After the hormone injection, fish were left to spawn naturally in the tank. Eggs collectors, located at the perimeter of the tank were monitored daily until spawns were obtained (32 h after hormonal injection, on average). From the total amount of eggs, different spawning quality parameters, such as number of fertilized eggs, hatched larvae, 3-day-old surviving larvae and spawning indexes, such as fertilization rate and hatching rate, were calculated in accordance to the methodology described by Fernández-Palacios, Izquierdo, Robaina, Valencia, Salhi & Vergara (1995).

2.2. Larval rearing

In July 2009, fertilized *S. rivoliana* eggs were obtained for first time and these were used to test semi-intensive (SIS: 4.5 eggs L^{-1} in two 40 m^3 tanks) and intensive (IS: 125 eggs L^{-1} in three 2 m^3 tanks) larval rearing techniques according to Roo *et al.* (2010). Each rearing systems was tested in three consecutive trials, with duration of 1 month each along the spawning season. Experimental tanks were supplied with filtered and UV sterilized seawater. Water exchange progressively increased from 15% of the tank volume per day at 2 DAH for both rearing systems, to 100% from 15 DAH onwards in the IS and from 20 DAH in the SIS one. Water salinity (37 g L^{-1}), dissolved oxygen (6.22 ± 0.21 ppm) and temperature (24.03 ± 0.26°C) were measured daily. In addition, total gas saturation was measured daily using a Weiss saturometer (E-300; Eco Enterprises, Seattle, WA, USA) (DP = 0). Pseudo-green water technique was used adding live phytoplankton (*Nannochloropsis sp.*) to maintain a concentration of 250 000 cells mL^{-1} in both rearing systems. Larval rearing was conducted under 12/12 (dark/light) photoperiod, using mixture of artificial fluorescent lights (Mod. TLD 58W/54-765; Philips, Lyon, France) and natural sun light with a light intensity just above the water surface ranging

between 1000 and 3500 lux. From 2 to 25 days after hatching (DAH) rotifers, *Brachionus plicatilis* L-strain (205 μm mean lorica length, 125 μm mean lorica width) cultured on baker′s yeast, *Saccharomyces cerevisiae* and enriched with DHA Protein Selco (Inve Aquaculture, Dendermonde, Belgium) were added twice a day (08:00; 14:00). Rotifers density was adjusted to 4–5 rot mL^{-1} in the semi-intensive and from 7.5 to 10 rot mL^{-1} in the intensive system. Artemia Instar II nauplii, enriched with A1 Easy Selco (INVE Aquaculture) were given equally from 15 DAH onwards to tanks in the SIS and IS treatments. The weaning protocol included hand feeding of an inert diet from 20 DAH (Genma Micro, Skretting, Vervins, France) four times a day for 5 days and automatic feeding every hour from day 25 until 30 DAH.

2.3. Growth and survival

Larval meristic parameters were recorded along the larval development (Fig. 1): total length (TL), standard length (SL), pre-anal length (PAL), eye diameter (ED), cephalic height (CH), yolk sac length (YSL), lipid globule diameter (LGD). All parameters were monitored from samples of 25 larvae per tank every 5 days. In addition, at the same time intervals, dry weight (DW) was recorded and specific growth rate (SGR) calculated according with Ricker (1958) using the followingequation:

$$SGR=(e^{g} - 1) \times 100\%$$

where $g = ([\ln(DWt) - \ln(DW_0)]/t)$ and DWt is the larval dry weight at the end of time period t, DW_0 is the dry weight at the beginning of time period t and t is the time period in days. On days 2, 5, 8 and 10 after hatching, 25 larvae per tank from each rearing tank and system, were sampled in the morning 9:30 approximately 1:30 hours after rotifer density adjustment. They were looked at in vivo under stereomicroscope to evaluate number of larvae with ingested rotifers. Final survival was determined at 30 DAH counting remaining live larvae in the experimental tanks.

2.4. Stress test

An air exposure test was performed on 20 and 30 DAH. On day 20, larvae (n = 45) were individually exposed to air for 15, 30 or 60 s in a 500 μm nylon mesh screen (Izquierdo, Watanabe, Takeuchi, Arakawa & Kitajima, 1989). After the air exposure, larvae were transferred to an aerated 10-L beaker and survival was recorded 24 h later. At 30 DPH, a new set of stress tests were performed. At this point, two clove oil doses (1 and 2 ppt), as anaesthetic, were evaluated in combination with different air exposure times (15, 30, 60, 75 and 90 s). Larval survival was recorded 24 h after the stress test.

2.5. Biochemical analysis

For biochemical analysis, samples of eggs and feeds (rotifers, Artemia and microdiets) were collected along the feeding trials for biochemical analysis (Tables 2 and 3): Dry matter, ash and protein content were carried out using the methods of analysis of the Association of Official Analytical Chemists (AOAC 1990). Total lipid content was obtained as described by Folch, Lees and Stanley (1957). Fatty acid methyl esters (FAMEs) were obtained by transesterification with H2SO4 (10 mL L^{-1} methanol), (Christie 1982) and purified using adsorption chromatography on NH2 Sep-pack cartridges (Waters, S.A., Milford, MA, USA) as described by Fox (1990), and separated and quantified using gas liquid chromatography (GLC) as described by Izquierdo *et al*. (1989).

2.6. Statistical analysis

All the data were statistically treated using SPSS Statistical Software System ver 15.0 (SPSS, Chicago, IL, USA). A t-test for simple mean comparison analysis ($P < 0.05$) (Sokal & Rolf, 1995) was applied to compare differences between rearing systems. When data were not normally distributed, arcsin-transformation was applied, and then Kolmogorov– Smirnov non-parametric test was applied to the non-transformed data. Results are presented as mean values ± SD.

3. Results

3.1. Broodstock

After 4 years in captivity, survival of the captured S. rivoliana was 73%. Losses in the course of this period were mainly due to repeated parasite outbreaks caused by monogenean ectoparasites (*Neobenedia sp*.). The breeders attained an average weight of 6.0 kg in July 2010 from an initial weight of 1.7 kg in June 2007, reaching a density of ca 10 kg m^3 (Table 1).

The sexual development of the brood stock was followed closely via gonadal biopsies. A year after their capture (2008) the population, independently of the sex, was sexually immature, whereas in 2009, the presence of late developing oocytes (>500 lm diameter) occurred in 33% of the females, in 5.1–6.3 kg BW females and in all males, all of which were running (Table 1). At this point, both males and females were hormonally injected with the GnRHa analogue (20 μg/kg BW) 10 times from July to October 2009, resulting in 100% successful spawns 32 h post injection (average 275 000 eggs per spawn). Eggs were transparent, spherical in shape with an average egg diameter of 1.1 mm (n = 100), and a single oil droplet of 0.24 ± 0.02 mm in diameter (Plate 1a). Along the different spawning events, average fertilization rate was 92.5 ± 5.5% with 72.6 ± 17.2% of viable eggs.

3.2. Larval rearing

Hatching time ranged between 36 and 48 h post fertilization depending on the rearing temperature (22–24°C), with an average hatching rate of 79.0 ± 11.3%. Newly hatched larvae were 3.28 ± 0.13 mm in total length and yolk sac length was 1.27 ± 0.11 mm (Fig. 2; Plate 1b). At 4 DAH, yolk sac reserves were completely absorbed (Fig. 2) and average larval survival was 52.3 ± 19.1%. At this point, survivors showed eye pigmentation and mouth and anus open and ready to start first feeding (Plate 1c). Although yolk sac was quickly depleted, the oil droplet remained in the anterior abdominal cavity until day 8 DAH, and it was not further visible in 10 DAH larvae (Fig. 2; Plate 1d).

Enriched rotifers L-type strain were supplied from 2 DAH onwards, but a low incidence of larvae with rotifers in the gut were recorded from 2 to 5 DAH. Thus, no larvae with ingested rotifers were recorded on day 2, 50% of the larvae were recorded with rotifers inside the gut lumen on day 5, 86% at day 8 and 100% by day 10, regardless of the rearing system utilized.

The transfer to Artemia feeding was successful, and it occurred 15–20 DAH. From 20 to 30 DAH, most of the larvae were observed carrying long faeces packets, identified as live undigested Artemia (Plate 1h,1i). At the end of the rearing trials (30 DAH), average larval survival was 2.3 ± 0.7% and 0.5 ± 0.2% in semi-intensive and intensive rearing conditions respectively. The highest larval mortalities occurred from 8 to 12 DAH. In the following days, a continuous larval mortality was recorded until day 20. At this point, 20 and 30 DAH larvae showed a very high sensitivity to the air exposure stress test; even the lowest air exposure (15 s) was associated with an almost immediate total larval mortality. It is interesting to note that the Seriola larvae resisted better the stress test if exposed to a 1–2 ppm of an anaesthetic.

Growth performance of 5–30 DAH seriola larvae was independent of the rearing system($P > 0.05$; Figs 3 and 4). It is interesting to note that we identified two different growing periods; one, of a slow daily growth rate of 12.1–14.7%, in coincidence with pre-flexion (0–15 DAH) and one with an accelerated daily growth rate of 20.1–22.9%, post-flexion of the notochord (15–30 DAH). Specific growth rate was not significantly different in SIS and IS reared larvae (Fig. 4).

3.3. Proximate composition

Protein content of the live and dry feeds fed to *S. rivoliana* larvae were similar and ranged from 54% to 57% on a dry matter basis. Ash contents were higher in the dry feed than in live preys. On a dry matter basis, total lipid content was 17%, in enriched rotifers and microdiets in comparison with enriched Artemia (29%). Energy content showed a similar trend to lipid content, being higher in enriched Artemia than enriched rotifers and microdiets. This was in contrast with the Protein/ Lipid (P/L) ratio, which was lowest in enriched Artemia (Table 2).

Fatty acid analysis showed that S. rivoliana eggs were high in oleic (18:1n-9), docosahexaenoic (22:6n-3) and palmitic acids (16:0) followed by linoleic (18:2n-6) and eicosapentaenoic (20:5n-3) acids. Grouping the fatty acids by families, n-3 fatty acids were the most abundant, followed by the level of monounsaturated and saturated fatty acids. In comparison, fatty acid profiles of rotifers were extremely high in monounsaturated fatty acids, particularly palmitic and oleic acid, which was 4X higher than in S. rivoliana eggs. Futhermore, n-3 fatty acids were 20% lower in rotifers than in fish eggs, due to their lower DHA content. Artemia fatty acids were particularly high in linolenic acid (18:3n-3), which was 18 times higher than in eggs, and monounsaturated fatty acids and very low in DHA. The dry weaning feed was also very low in DHA, together with ARA, but very high in 18:2n-6 (Table 3).

4. Discussion

4.1. Broodstock

Captured broodstock generally adapted well to culture conditions, but eye anomalies, such as cataracts and exophthalmia, associated with chronic levels of total dissolved gas pressure in water supply (DP = 5–10) were observed. Besides, repeated parasite outbreaks by a monogenean (*Neobenedia sp.*) were the main cause of broodstock mortality. Monogenean infections were identified as a major concern for Seriola industry in Australia and Japan, causing loss of fish growth, decreasing market value and causing fish mortality, as it was reported for different seriola species (Chambers & Ernst 2005; Hirayama, Kawano & Hirazawa, 2009). Regarding the reproductive biology, even after 3 years in captivity (4.08 ± 2.2 kg; 57.19 ± 7.28 cm SL), most of *S. rivoliana* females were sexually immature with ovaries containing only previtellogenic oocytes in 66% of the fish, whereas 100% males were recorded as running, which could be related to the size at first maturity of this species, which was reported to be around 5 kg in Hawaii (Laidley *et al.*, 2004). In the present study, after 4 years in captivity when the fish reached a weight of 6.22 ± 1.78 kg (65.93 ± 5.06 cm SL), 66% of the females carried late developing oocytes (>500 lm in diameter), demonstrating the capacity of this seriola

species to mature in captivity although, no spontaneous spawns were obtained. Lack of oocyte development and maturation in other species in captivity is generally associated with inadequate environmental (photoperiod, temperature) conditions or other stress factors, such as fish density or rearing volume (Micale, Maricchiolo & Genovese, 1999; Benetti, 2000). Indeed, lower fish densities and bigger tanks used in comparison with the present study, for same species in Hawaii and Ecuador, lead to successful spontaneous spawns (Blacio *et al*., 2003; *Laidley* et al., 2004).

In the present study, the use of hormonal induction of oogenesis and egg maturation with the GnRHa analogue was associated with a high rate of egg fertilization without negative effects on brood fish survival. The dosage applied was based on Mylonas *et al*. (2004) for greater amberjack and Duncan, Estevez and Mylonas (2007) for meagre (*Argyrosomus regius*). The fact that in *S. rivoliana*, in the present study, was induced to spawn at a size of about 5.0 kg would suggest that this seriola species has a practical advantage over other seriola species, such as *S. dumerilli*, as the latter needs very large tanks as to reach sexual maturity (Mylonas *et al*., 2004; Jerez *et al*., 2006).

4.2. Larval rearing

Survival rates obtained in this study (0.5–2.5%) were similar to those reported for the same species in Ecuador (Blacio *et al*., 2003), Hawaii (Laidley *et al*., 2004) and other seriola species, such as *S. lalandi* (Tachihara, El-Zibdeh, Ishimatsu & Tagawa, 1997) and *S. dumerili* (Papandroulakis *et al*., 2005; Hamasaki, Tsuruoka, Teruya, Hashimoto, Hamada, Hotta & Mushiake, 2009). In the present study, the highest larval mortalities were observed in the early developmental stages, from 8 to 12 DAH. Different studies attributed the early larval mortalities to an unstable bacterial flora in the rearing water and opportunistic bacteria colonization, the larval digestive tract (Hansen & Olafsen, 1999; Makridis, Fjellheim, Skjermo & Vadstein, 2000; Verner-Jeffreys, Shields, Bricknell & Birkbeck, 2003; Reid, Treasurer, Adam & Birkbeck, 2009) causing massive mortalities mainly when intensive larval rearing systems are utilized

(Skjermo & Vadstein, 1999). The improved larval survival in the SIS in comparison with IS would suggest that seriola larval rearing should be performed following the SIS regime. This could be associated with the use of larger water volumes, with a low renewal rate and low larval density. This regime might help promote the development of a more stable environment, probably associated with a more mature microbial flora, similar to mature waters obtained in recirculation systems (Roo *et al*., 2010; Attramadal, Salvesen, Xue, Øie, Størseth, Vadstein & Olsen, 2012). Indeed, the use of microbial matured water has being reported to improved, larval survival and improved feeding incidence at early stages in different marine species, such as Atlantic halibut *Hippoglossus hippoglossus* (Skjermo, Salvesen, Øie, Olsen & Vadstein, 1997) or Atlantic cod *Gadus morhua*, among others (Attramadal *et al*., 2012). Furthermore, theorical prey availability in relation with initial larval population was tenfold lower in the IS, with around 160 rotifers/larva/day, whereas more than 1600 rotifers/larva/day could be available in the SIS. Similar data where previously reported by Roo *et al*. (2010) in red porgy larvae. Nevertheless, this fact seems not to affect feeding incidence, measured in 5 DAH (50%) and 8 DAH (80%), in the present study in both system regimes. Furthermore, our observations on seriola larval feeding were lower than those reported in similar species, such as *S. dumerilli* (>70% first feeding at 4 DAH; Hamasaki *et al*., 2009), suggest that initial larval mortalities could be also related to the low success for first feeding in *S. rivoliana* under both culture techniques and suggesting a strong dependence on maternal reserves during the early development. Similar results were reported for *S. lalandi* (Chen, Qin, Kumar, Hutchinson & Clarke, 2006) and could be related to nutritional imbalances in broodstock diets, and hence, deficiencies in essential nutrients in the endogenous reserves of the larvae, nutritional imbalance of live prey at first feeding or inadequate culture conditions. In fact, it was suggested that broodstock diets for *Seriola sp*. may need an extra supply of neutral lipids as an important energy source for early larval development (Hilton, Poortenaar & Sewell, 2008). In the present study, *B. plicatilis* L-strain seems to be adequate for first feeding of *S. rivoliana* larvae, which is in agreement with the results reported by Hamasaki *et al*. (2009). These authors reported that larval survival in *S. dumerilli* was independent of rotifer size of different strains, which had a

range of lorica length from 135 to 211 lm. In the present study, copepods (Harpacticoida, Tisbidae) produced endogenously in the semi-intensive systems tanks were observed in the larval digestive tract (Plates 1e, 1f, 1g). This was particularly true for the small naupliar stages of marine copepods, suggesting the preferences for this type of prey by Seriola larvae. These results are similar with those reported by Van der Meeren (1991) when turbot larvae were offered rotifers and copepods. In general, copepods are a suitable prey-size organisms for first-feeding larvae that are rich in a number of biochemical components, such as lipids, highly unsaturated fatty acids, digestible phospholipids, protein, protein-bound amino acids, free amino acids, pigments or vitamins (Støttrup & Norsker 1997; Van der Meeren, Olsen, Hamre & Fyhn, 2008), which are essential nutrients and as such would helped promote seriola larval survival under semi-intensive conditions. In older larval stages (>20 DAH), there was a continuous mortality with weak larvae floating at the water surface. Seriola larvae at this age also succumbed after stress test. The presence of larvae with faeces, including undigested and even live Artemia, demonstrated the low digestibility of this prey (Plate 1i), which leads to larval malnutrition when Artemia is the main source of food (15–30 DAH). Moreover, striking differences were found in the fatty acid profiles of eggs and different feeds utilized in this study, suggesting a potential fatty acid deficiency. This is in agreement with the shock syndrome (sudden mortality) observed after the stress test or even at capture as it was reported by Izquierdo *et al*. (1989). Also, sudden massive mortalities were recorded along the different trials in this rearing period. The relation between larval welfare and essential fatty acids, such as 20:5n-3 (EPA), 22:6n-3 (DHA) and 20:4n-6 (ARA), has been emphasized in early stages of development for different marines species (Izquierdo 1996), with 'shock syndrome' being one of the indication of EFA limitations in the diets (Izquierdo *et al*., 1989). Previous results from Yamamoto *et al*. 2008; suggest that in Artemia feeding stages at least 1.4–2.6% of DHA and 2.3–4.1% of n-3 HUFAs are needed in the diet of the yellowtail *S. quinqueradiata*. Present results showed that *S. rivoliana* eggs contents 16.5% total fatty acids (TFA) (about 4.3% DW) in DHA and n-3 HUFA were as high as 26.9% TFA (7.1% DW) suggesting even higher EFA requirements for this species, than for *S. quinqueradiata*. In agreement with these potential high

EFA requirements, high mortalities could be related with the low DHA and n-3 HUFA content in rotifers (10.3–19.6%), Artemia nauplii (5.3% and 13.3%) and dry feeds (5.7–9.9%). In addition, the ratios of DHA/EPA and oleic/DHA, used to evaluate the essential fatty acids requirements (Izquierdo 1996), greatly differed for feeds (0.93–1.76 DHA/EPA; 1.8-4.1 oleic/DHA) and eggs (2.45 and 1.22 respectively). It is noteworthy to mention, that not only fatty acid composition, but also lipid classes should be taken into consideration in larval feeds. Thus, commercial live prey enrichment, such as the ones utilized in present experiment provides most of EFA as neutral lipids (NL) particularly as triglycerides (TG) and free fatty acids (FFA) forms (data not shown). On the contrary, natural live preys, such as copepods, are rich in phospholipids (PLs) (Van der Meeren *et al*., 2008). It is well known that in marine fish larvae, PL is structural constituents of bio-membranes and therefore highly demanded in the fast-growing larvae. However, there are several indications that fish larvae are unable to efficiently synthesize PL in a rate fast enough to cover their high demand and therefore PL needs to be included in the diet (Izquierdo & Koven, 2011), suggesting that nutritional modification in *S. rivoliana* larval feed could be beneficial for improved larval survival.

Total length of newly hatched larvae averaged 2.54 ± 0.01 mm, which is similar to previous studies in this species (Blacio *et al*., 2003) and *S. dumerilli* (2.88 mm TL) (Papandroulakis *et al*., 2005), but shorter than *S. lalandi* larvae (4.30 mm TL) (Chen *et al*., 2006). Nevertheless, both absolute growth was similar to the reported in these Seriola species (0.45–0.51 mm TL/day) and in the range of values reported for *Thunnus* species (0.44–0.68 mm TL/day) according to Kaji, Tanaka, Oka, Takeuchi, Ohsumi, Teruya and Hirokawa (1999). Notochord flexion started at 15 DAH (5.6–6.1 mm TL), similar to *S. dumerilli* (5.5 ± 0.52 mm TL) (Papandroulakis *et al*., 2005), being completed at 20 DAH (7.7–8.2 mm TL). This change in larval external morphology may be used as an indication of digestive system maturity, as it occurs synchronously with the appearance of digestive glands, as it was reported by Abreu (2010), suggesting changes in larval behaviour and feeding habits, similar to other species, such as red porgy (*Pagrus pagrus*) (Roo, Socorro, Izquierdo, Caballero, Hernández-Cruz, Fernández

& Fernández-Palacios, 1999). Thus, the appearance of gastric glands marked the formation of a functional stomach with a higher capacity to utilize proteins (Govoni, Boehler & Watanabe, 1986) and the capacity to perform a successful early weaning onto dry diets, as it has been found in *S. lalandi* (Chen *et al*., 2006). By day 30 (15.0–16.0 mm TL), juveniles had a wet weight ranging from 0.15 to 0.3 g in wet weight with the characteristic striped pigmentation (Plate 1j). At this point, the elevated mortality recorded in the stress test trials can be reduced by the use of anaesthesia based on the use of low clove oil doses, which was associated with 100% larval survival even after a 90 s air exposure. This protocol could be applied in routine manipulations, such as grading or juveniles transportation. Finally, at 90 DAH juveniles from semi-intensive and intensive systems reached 26.7 ± 4.7 and 14.2 ± 5.2 g in wet weight, respectively, denoting the rapid growth of this species.

In summary, the results of this first experience of *S. rivoliana* culture in Europe showed that this species adapts well to captivity conditions and even with dry commercial feeds, responds with good quality spawns to GnRHa hormonal injection, obtaining similar larval survival and growth behaviour as other Seriola species. Moreover, larval survival results and biochemical analysis of eggs and feeds suggests that this species might have higher n-3 HUFA and particularly DHA requirements during larval stages as compared with other commercial species. Further studies are being conducted to improve the culture performance and the study of EFA requirements of *S. rivoliana* larvae.

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7. List of tables and figures

Table 1. Evolution of body weight, total length and condition factor of *Seriola rivoliana* broodstock.

Year	2007	2008	2009	2010
Females (indv)	8	8	6	6
Body weight (kg)	1.83 ± 0.22	2.90 ± 0.42	4.08 ± 2.02	6.22 ± 1.78
Standard length (SL)	45.05 ± 1.41	54.50 ± 3.54	57.19 ± 7.28	65.93 ± 4.58
Condition factor	2.00 ± 0.04	1.79 ± 0.19	2.01 ± 0.40	2.12 ± 0.24
Oocite diameter (μm)	n.a	<500 (8)	>500 (2)	>500 (4)
Males (indv)	7	7	5	5
Body weight (kg)	1.65 ± 0.39	2.32 ± 0.25	3.36 ± 0.97	5.55 ± 1.10
Standard length (SL)	43.25 ± 3.18	51.38 ± 2.95	54.57 ± 5.16	61.75 ± 5.06
Condition factor	2.02 ± 0.06	1.71 ± 0.26	2.02 ± 0.13	2.37 ± 0.38
Maturation	n.a.	Immature (7)	Running (5)	Running (5)

Mean values ± SD; Condition factor = [body weight (g)/standard length (cm)]3; n.a. no available; (), numbers of individuals.

Table 2. Proximate composition of fertilized S. rivoliana eggs and feeds utilized along the larval rearing (average ± SD, n = 3).

	Moisture	Ash (% DW)	Lipids (% DW)	Protein (% DW)	CHO	Energy (kJ/g)	Ratio P/L
Eggs	90.63 ± 0.18	0.63 ± 0.01	26.92 ± 0.86	68.83 ± 1.42	3.63 ± 2.22	2749.96 ± 27.33	2.56 ± 0.04
Enriched Rotifers	88.69 ± 1.50	2.18 ± 0.91	17.33 ± 3.14	54.95 ± 7.93	25.55 ± 9.32	2420.68 ± 123.97	3.22 ± 0.50
Enriched *Artemia*	91.89 ± 0.88	0.76 ± 0.21	29.29 ± 4.91	54.03 ± 3.75	15.91 ± 5.50	2705.95 ± 105.21	1.90 ± 0.37
Dry feed	5.29 ± 1.44	17.09 ± 0.83	17.70 ± 1.11	57.66 ± 1.09	7.54 ± 1.45	2189.83 ± 19.46	3.27 ± 0.26

Table 3. Fatty acid composition (% total fatty acid) from the total lipid content S. rivoliana fertilized eggs and feeds utilized along the larval rearing (average ± SD, n = 3).

	Eggs	Enriched Rotifers	Enriched *Artemia*	Dry feed
ΣSaturated	23.82 ± 2.18	20.48 ± 1.85	18.31 ± 1.47	27.65 ± 4.21
ΣMonounsaturated	28.55 ± 2.03	41.12 ± 2.67	35.85 ± 0.52	29.09 ± 2.44
Σn-3	29.00 ± 4.27	22.31 ± 2.48	33.78 ± 1.81	14.57 ± 5.20
Σn-6	16.85 ± 2.12	12.23 ± 2.22	9.67 ± 0.06	27.24 ± 1.19
Σn-9	21.18 ± 1.58	23.53 ± 1.47	24.10 ± 0.32	16.19 ± 2.15
Σn-3HUFA	26.91 ± 3.30	19.67 ± 2.17	13.30 ± 0.57	9.97 ± 3.61
14:00	1.03 ± 0.04	1.59 ± 0.11	0.97 ± 0.18	3.53 ± 0.65
16:00	16.19 ± 0.37	14.30 ± 1.58	11.20 ± 0.99	19.36 ± 3.07
16:1 n-7	3.65 ± 0.17	12.40 ± 1.32	3.37 ± 0.10	3.86 ± 0.07
18:00	5.94 ± 1.67	3.38 ± 0.23	5.10 ± 0.25	3.80 ± 0.39
18:1 n-9	20.15 ± 1.34	18.33 ± 0.93	21.82 ± 0.14	10.40 ± 1.64
18:1 n-7	3.57 ± 0.27	3.76 ± 0.42	6.08 ± 0.16	2.15 ± 0.14
18:2 n-6	13.54 ± 1.72	8.67 ± 1.81	6.66 ± 0.06	26.30 ± 1.03
18:3 n-3	0.95 ± 0.02	1.11 ± 1.00	17.71 ± 2.06	3.46 ± 1.19
20:1 n-9	0.49 ± 0.12	2.65 ± 0.22	1.63 ± 0.17	5.48 ± 0.48
ARA	1.48 ± 0.10	1.45 ± 0.05	1.27 ± 0.03	0.38 ± 0.06
EPA	6.72 ± 1.70	5.90 ± 0.24	5.76 ± 0.18	3.54 ± 1.10
DHA	16.50 ± 1.49	10.37 ± 1.97	5.37 ± 0.68	5.71 ± 2.32
DPA (22:5n-6)	0.29 ± 0.02	0.53 ± 0.06	0.29 ± 0.04	0.04 ± 0.02
DHA/22:5 n-6	57.27 ± 73.66	19.42 ± 1.78	18.45 ± 0.29	-
EPA/ARA	4.55 ± 16.24	4.06 ± 0.29	4.54 ± 0.09	9.23 ± 1.37
ARA/EPA	0.22 ± 0.06	0.25 ± 0.02	0.22 ± 0.00	0.11 ± 0.02
DHA/EPA	2.45 ± 0.88	1.76 ± 0.33	0.93 ± 0.14	1.58 ± 0.18
DHA/ARA	11.17 ± 14.22	7.15 ± 1.49	4.24 ± 0.56	14.73 ± 3.72
Oleic/DHA	1.22 ± 0.90	1.83 ± 0.47	4.10 ± 0.51	2.15 ± 1.26
Oleic/n-3HUFA	0.75 ± 0.41	0.94 ± 0.16	1.64 ± 0.06	1.19 ± 0.63
n-3/n-6	1.72 ± 2.02	1.86 ± 0.33	3.49 ± 0.20	0.53 ± 0.17

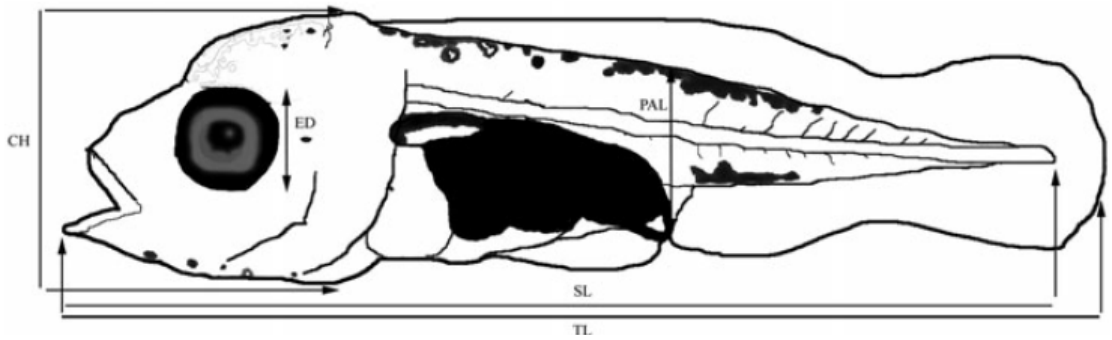


Figure 1. Squematic view of different morphometric determinations.

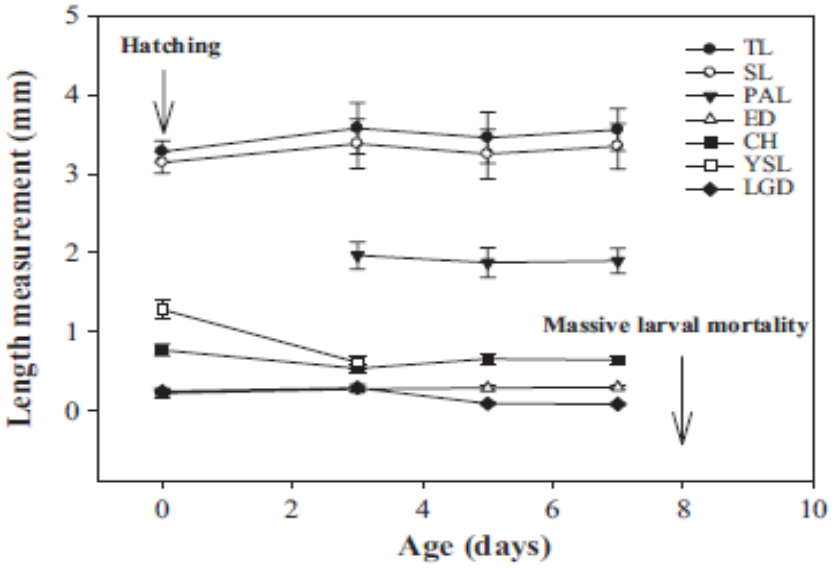


Figure 2. Development of different morphometric indices on starved larvae: total lenght (TL), standard length (SL), pre-anal length (PAL), eye diameter (ED), cephalic height (CH), yolk sac length (YSL), lipid globule diameter (LGD).

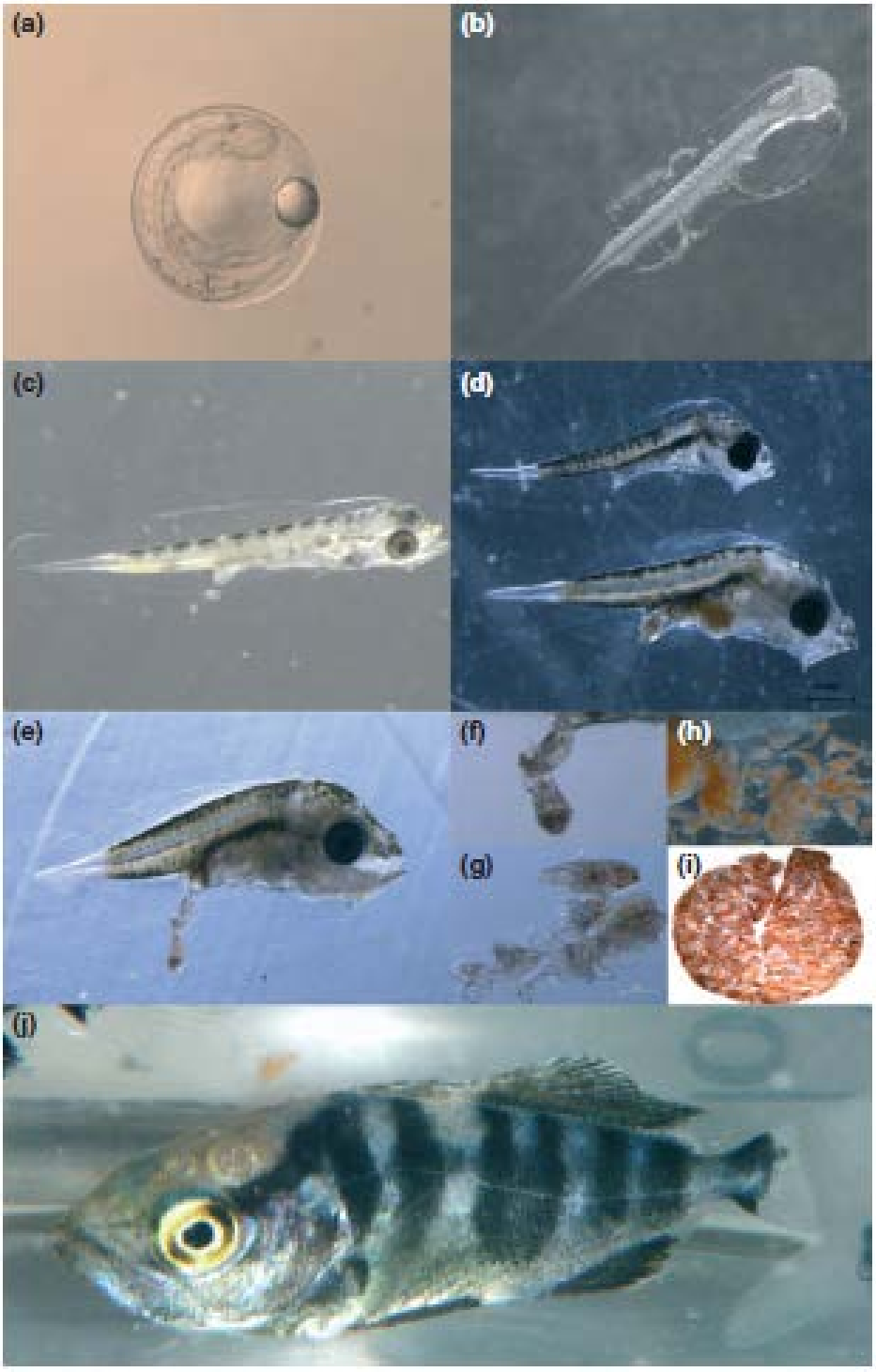


Plate 1 a) *S. rivoliana* egg; b) Newly hatched larvae; c) 3-day old larvae; d) 10-day old fed (down) and starved (up) larvae; e) 20-day old larvae; f) excreted copepods in 20 DAH larvae; i) detail of faeces packets with undigested Artemia in 25 DAH; j) 30 DAH juvenile.

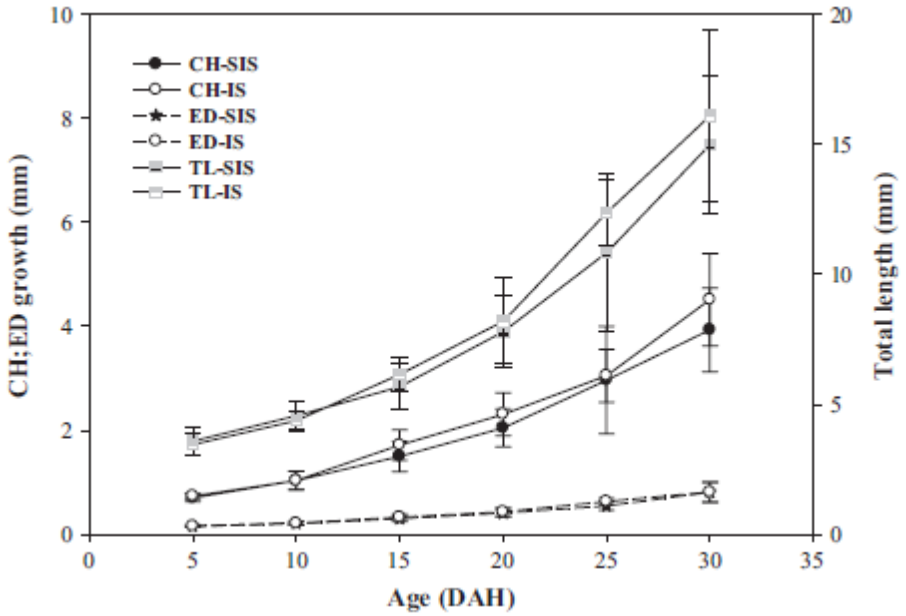


Figure 3. Morphometric development of total length (TL), cephalic height (CH) and eye diameter (ED) of *Seriola rivoliana* larvae cultured under intensive (IS) or semi-intensive (SIS) rearing rechniques.

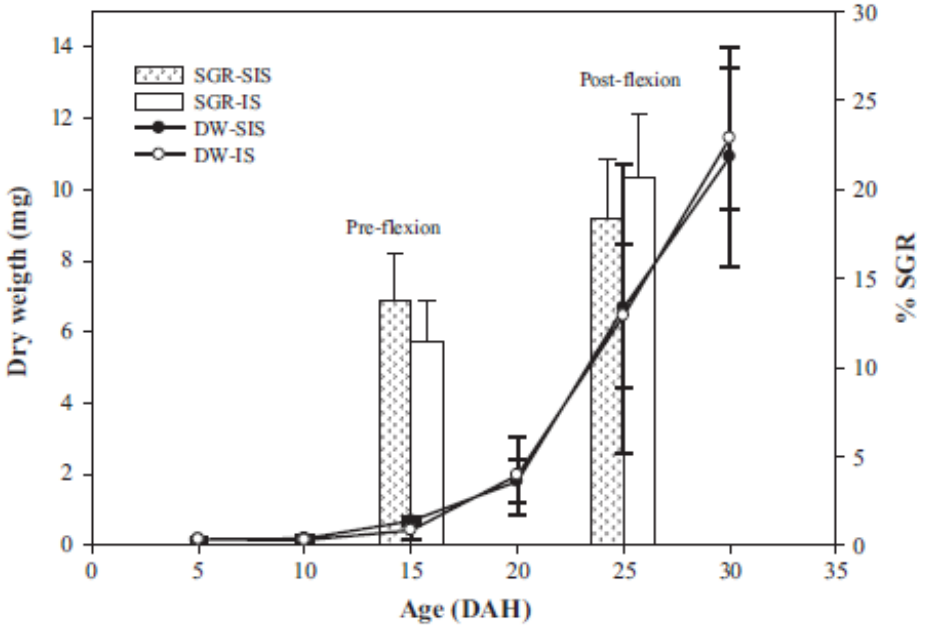
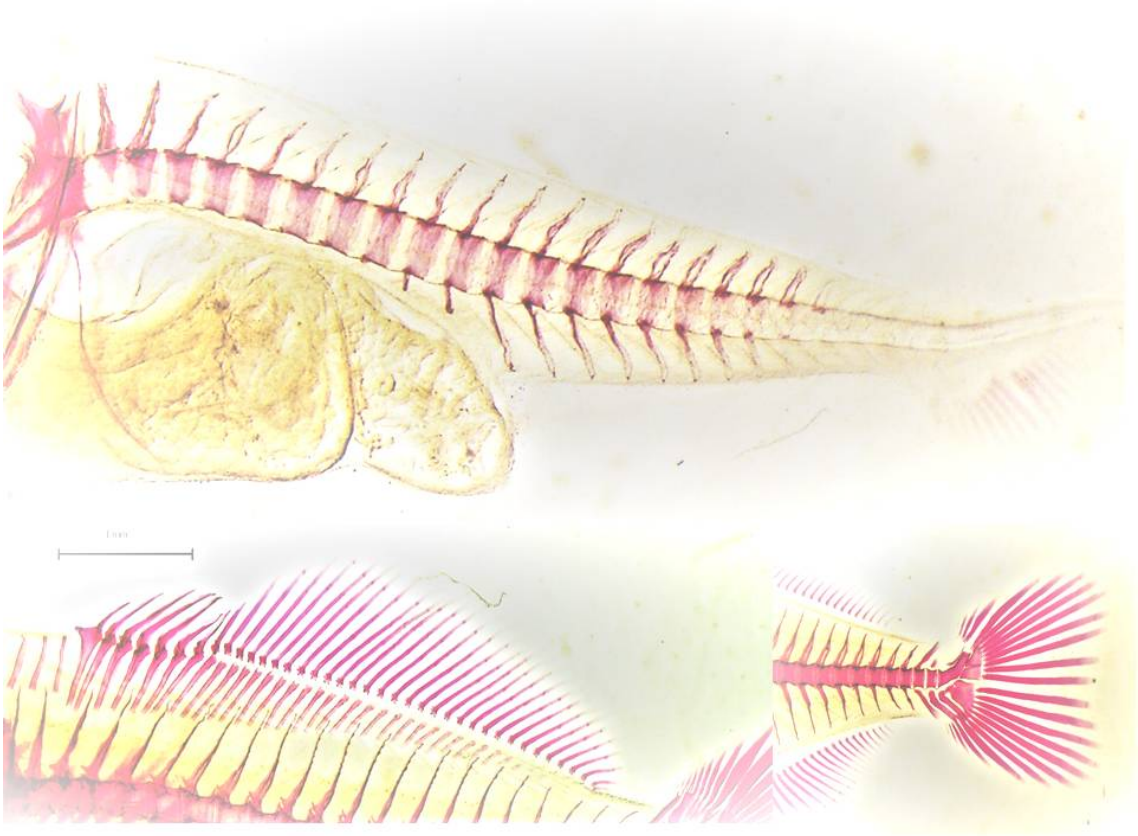


Figure 4. Growth development in dry weight (DW) and standard growth rate (SGR) of *Seriola rivoliana* larvae cultured under intensive (IS) or semi-intensive (SIS) rearing techniques.

Study II



Skeletal development and mineralization pattern of vertebral column, dorsal, anal and caudal fin complex in *Seriola rivoliana* (Valenciennes, 1833) larvae.

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Skeletal development and mineralization pattern of vertebral column, dorsal, anal and caudal fin complex in *Seriola rivoliana* (Valenciennes, 1833) larvae.

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Abstract

Bone and fins development in *Seriola rivoliana* were studied from cleared and stained specimens from 3 to 33 days after hatching. The vertebral column began to mineralize in the neural arches at 4.40 ± 0.14 mm standard length (SL), continued with the haemal arches and centrums following a cranial-caudal direction. Mineralization of the caudal fin structures started with the caudal rays by 5.12 ± 0.11 mm SL, at the same time that the notochord flexion occurs. The first dorsal and anal fin structures were the hard spines (S), and lepidotrichium (R) by 8.01 ± 0.26 mm SL. The metamorphosis was completed by 11.82 ± 0.4 mm SL. Finally, the fin supports (pterygiophores) and the caudal fin were completely mineralized by 16.1 ± 0.89 mm SL. In addition, the meristic data of 23 structures were provided. Results from the present study might be used as a practical guide for future studies on this field with *S. rivoliana* or in related species.

Key words: Amberjack, hatchery, abnormalities, osteology, skeleton.

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1. Introduction

Longfin yellowtail, *Seriola rivoliana* (Valenciennes, 1833) is one of the species proposed for marine aquaculture diversification, mostly due to its fast growth rate [1,2] and worldwide distribution. This specie belongs to Carangidae family, along with other popular species like *Seriola dumerili* (greater amberjack), *Seriola lalandi* (yellowtail king fish) and *Seriola quinqueradiata* (Japanese yellowtail). Even though *S. rivoliana* is commercially produced [3], studies about its biology are scarce and only few reports on larval rearing have been conducted in Ecuador [4-6], Hawaii [7] and more recently in the Canary Islands [8]. In contrast, numerous studies of the genus *Seriola* have been published related to the feeding requirements and nutrition [9-17], reproduction biology [18-21,2] and culture needs [22-26,8].

Regarding osteology studies, previous reports illustrate the bone structure development for other *Seriola* species. The osteological development of the greater amberjack have been described by different authors [27,28] These authors obtained distinct results probably associated to different environmental conditions and/or the number of samples. Also, the caudal skeleton development of the *S. lalandi* has been reported [29]. In addition, numerous studies have described the osteological development of other marine finfish species, such as *Sparus aurata* [30,31], *Pagrus pagrus* [32,33], *Solea senegalensis* [34,35], *Dentex dentex* [36], *Argyrosomus regius* [37], *Epinephelus septemfasciatus* [38] or *Dicentrarchus labrax* [39].

The objective of the present study was to chart the ossification of the vertebral column, dorsal, anal and caudal fin complex in *S. rivoliana* larvae cultured under semi intensive system conditions (mesocosms, [8]). Larvae culture under this type of system usually performed better than those cultures under intensive conditions [32]. The identification of bony structures and mineralization pattern will serve as a tool for future studies, where different factors (zootechnical, nutritional, environmental parameters, etc) may affect the apparition of skeletal abnormalities.

2. Material and methods

S. rivoliana eggs were obtained from induced spawning (hormonally injection, $GnRH_a$; Sigma-Aldrich ™), based on the reported dosage [8]. Larvae were reared under mesocosms rearing system (4.5 eggs.l^{-1} in two 40m^3 tanks [8]), kept under natural photoperiod and filtered natural sea water with 37 g/L salinity and temperature of 23.0 ± 0.9°C. Green water technique was used adding live phytoplankton (*Nannochloropsis sp.*) to maintain a concentration of 250000 cells ml^{-1} in the rearing tanks. From 2 to 25 days after hatching (dah) rotifers, *Brachionus sp.*, L-strain enriched with DHA Protein Selco (INVE ™), were added twice a day (08:00 and 14:00 h). *Artemia* feeding starts at 15 dah, and were enriched with A_1 Easy Selco (INVE ™). Weaning protocol included manual feeding from 20 dah (Genma Micro, Skretting ™) to 25 dah and automatic feeding afterwards.

Larval growth was assessed measuring the standard length (SL) of 25 larvae, every 2 days using a profile projector (Nikon V-12A, NIKON™). A total of 75 specimens were individually stained (3.28 ± 0.15 - 16.1 ± 0.89 mm SL). To study the bone ossification, all specimens were fixed in 10% buffered formalin, from hatching to 33dah. Fixed larvae were cleared and stained with alizarin red [40]. Larvae were individually examined using stereomicroscopy. Drawings of the different developmental stages were made using the Adobe Photoshop CS3-10.0 (1990-2007 Adobe System Incorporated, United States) directly from digital photographs. Bone description, followed the terminology suggested by different authors [41-43], and their abbreviations, are illustrated in the Table I. The angles of the spine were measured from the beginning of the vertebral body to the tip of the spine.

A total of 10 *S. rivoliana* reared juveniles were soft X-ray monitored (Mod. Senographer-DHR, General electric's, USA) for meristic counts.

3. Results

3.1. Vertebral column

In the present study, *S. rivoliana* vertebral column mineralization was initiated with the neural arches (Na_1-Na_3) by 4.40 ± 0.14 mm SL (Fig. 1-A), followed by the haemal arches (Ha_1-Ha_3) and the cephalic vertebrae (Ce_1-$_4$) by 4.74 ± 0.27 mm SL (Fig. 1-B). The ossification of the vertebral column followed a cranial-caudal direction, being totally ossified by 11.82 ± 0.4 mm SL (Fig. 1-G). This size marked the end of metamorphosis. The notochord flexion was initiated at 5.12 ± 0.11 mm SL (Fig. 1-C), at the same time that the caudal complex mineralization was initiated. Initially, urostyle was formed by two independent structures (Ur_1-Ur_2) that fused by 10.23 ± 0.26 mm SL (Fig. 1-F). The neural spine (Ns_{23}), the haemal spine (Hs_{13}) and the Ce_{22}-Ce_{23} were the last structures that mineralized. At 11.82 ± 0.4 mm SL (Fig. 1G), four types of articulation processes were mineralized: anterior neural zygapophyses (Anz), posterior neural zygapophyses (Pnz), anterior haemal zygapophyses (Ahz) and posterior haemal zygapophyses (Phz), (Fig. 1G*).

The vertebral bodies mineralization in the cephalic (without parapophyses) and prehaemal (with parapophyses) region (Ce_1-$_4$ and Ce_{5-10} respectively) proceeded from dorsal to ventral direction and from the surface to internal bone layers (Fig. 2A-B), whereas in the haemal (with Hs) and caudal region (Ns and Hs modified to support caudal fin complex) the mineralization pattern proceeds in both directions dorsal and ventrally (Ce_{11-21} and Ce_{22-23} respectively), joining in the middle of the centra (Fig. 2C). Exceptionally, in Ce_9 and Ce_{10}, the mineralization of the vertebral bodies differed from other vertebral structures of the prehaemal region, proceeding dorsally first and ventrally later on.

The Na and Ha developed from centrum and fused in the middle, forming the neural and haemal canals with rounded shape, later developing into the Ns and Hs (Fig.2A-C). The Ns and Hs angle in relation to the vertebral body varied along the vertebral column, increasing in cranial-caudal direction (Fig. 1G**). The Hs developed according to the angle of the first anal pterygiophore, and decreasing afterward.

The parapophyses (Pp) was first observed with the mineralization of the Pp_6-Pp_5 by 5.12 ± 0.11 mm SL (Fig. 1C). These structures had a caudal-cranial development and were fully ossified at 11.82 ± 0.4 mm SL (Fig.1G). The Pp structures become larger form Ce_4 to Ce_{10}.

The pleural ribs (Plr) were observed for the first time at 10.23 ± 0.26 mm SL (Plr_1-Plr_3) with the caudal development (Fig.1F). Plr_4-Plr_7 developed at 11.82 ± 0.4 mm SL (Fig. 1G). The dorsal ribs (Eb) were first seen at 11.82 ± 0.4 mm SL (Fig.1G), with the mineralization of the Eb_1-Eb_3,following the caudal development.

3.2. Dorsal and anal fins development

The formation and mineralization of the dorsal and anal fins of the longfin yellowtail followed a cranial-caudal direction. The first dorsal fin structures were the hard spines (S) and lepidotrichium (R) by 8.01 ± 0.26 mm SL (Fig. 1D), which initiated its mineralization from the base to the tip of the structure. Predorsal bones (Pd_1-Pd_3) and proximal pterygiophore (Pr) had a dorsal-ventral mineralization pattern (Fig. 1F).

In the anal fin, the two hard spines (S_1-S_2) were first seen at 8.01 ± 0.26 mm SL (Fig. 1D), same as the anal lepidotrichia (R) and Pr. The S_1-S_2 fused into the Pr_1 by 9.92 ± 0.84 mm SL (Fig. 1E). The S_1-S_2 and R mineralized from the base to the tip of the structure, whereas the Pr followed a ventral-dorsal pattern (Fig. 1F).

3.3 Caudal fin development

The first caudal complex structures in mineralized were the upper and lower caudal lepidotrichia (PCR) by 5.12 ± 0.11 mm SL (Fig. 3A). Then, hypurals (Hy) initiated their mineralization as fused structures, first Hy_1+Hy_2, continues with Hy_3+Hy_4 and finally parhypural (Ph) by 5.38 ± 0.11 mm SL (Fig. 3B). At the same time, the first upper caudal dermatotrichia (SCR) started to mineralize, following a base-tip mineralization pattern. The last hypural (Hy_5) delayed its mineralization to 8.01 ± 0.26 SL (Fig.3C). By 9.92 ± 0.84 mm SL uroneurals (Un_1+Un_2) started to mineralize and fused forming a single structure (Uroneural) (Fig. 3D-E). Finally, the last caudal complex structures in mineralized were the epurals (Ep_{1-3}) by 11.82 ± 0.4 mm SL(Fig. 3E).

3.4 Meristic characters

Meristically, *S. rivoliana* had a total number of 23 vertebrae (urostyle not included), 23 neural spines, 13 haemal spines, 16 pleural ribs, 12 dorsal ribs and 6 parapophyses. In the dorsal region, 3 predorsal spines, VII+I hard spines and a variable number (30-34) lepidotrichia were identified. Besides, VII hard spine proximal pterygiophores, 30 to 34 distal radial and proximal pterygiophores were also observed. Within the anal region, II+I hard spines and 19-21 lepidotrichia, same number of distal radial and proximal pterygiophores were identified. Finally, in the caudal complex, 1 parahypural, 5 hypurals, 3 epurals, 2 uroneurals, 10+9 caudal lepidotrichia and 10+9 caudad dermatotrichia were observed (Table II).

4. Discussion

This study reported for first time *S. rivoliana* skeletal development and mineralization. The comparison of present results with other species from the same family and genus, such as *S. dumerili*, suggest some correspondence. Thus, [27] describes first mineralized structure in the vertebra centra for *S. dumerilli* at 6.6 mm (NL) while other result for the same specie [28] identified neural spine (Ns) and centra at 4.8mm (TL). This pattern agrees with present data for *S. rivoliana*, where similar mineralization was obtained (4.74 ± 0.27 mm SL). The differences between vertebra centra mineralization timing for *S. dumerili* could be explained by the different environmental conditions, such as temperature, or rearing protocols applied in those studies [32]. In fact, mineralization pattern is more accurately described when larval growth is used as reference instead of larval age [33].

Also, the present study showed a similar vertebra centra mineralization timing in comparison with other carangid species, such as *Caranx crysos* [44] and *Selene setapinnis* [45], , suggesting that some developmental events during mineralization process may be common for many species. For instance, the dorsal flexion at the posterior end of the notochord could be an external indicator of the initiation of the internal column mineralization for this and other species. In fact, these events also occur in other species such as *S. aurata* larvae (5.7 – 6.0 mm, SL) [30], *Solea senegalensis* larvae (4.7 mm, SL) [35], *Pagrus pagrus* larvae (6.0 ± 0.5 mm, TL) [32] or *Argyrosomus regius* larvae (5.42 – 6.01 mm, TL) [47].

In most Perciforms, the vertebral column follows a bidirectional mineralization pattern (*Pagrus major*, [42]; *S. aurata*, [30]; *Dentex dentex*, [36]; *Diplodus sargus*, [47]*; Pagrus pagrus*, [32]). However, in *S. rivoliana* the vertebral column followed a unidirectional mineralization pattern, in agreement with data reported in *S. dumerili* [28] and in *A. regius* [46].

According to the centrum mineral deposition, three complementary models occur in the vertebral region: in a dorsal-ventral direction (D-V), in a ventral-dorsal direction (V-D) or simultaneously (DVS). In *S. aurata* [30] and *D. sargus* [47], two centra mineral deposition models occur, the first one takes place in a D-V direction from the Ce_1 to Ce_{21} and the second one in a V-D direction in Ce_{22} and Ce_{23}. In *S. rivoliana*, the mineralization expands in a D-V direction from Ce_1 to Ce_{23}, following the same pattern as *S. dumerili* between the Ce_1-Ce_{19} [28]. Additionally, *S. rivoliana* had simultaneously DVS mineralization from Ce_8 to Ce_{23}, whereas *S. dumerili* [28] presents this simultaneous DVS mineralization pattern from Ce_{20} to Ce_{23}. Other marine finfish such as *A. regius* [47] showed a D-V mineral deposition from Ce_1-Ce_4, while the remaining vertebrae had simultaneous DVS mineralization.

About to urostyle (Ur) structure of *S. rivoliana* larvae and other marine finfish such as *P. major*, *C. crysos* and *S. dumerili* [48, 44, 28], this was formed by the fusion of two elements (Ur1+Ur2). In contrast, at least three elements were necessary to form this structure in *S. lalandi* [29]. The results of the present study suggest that the fusion of different structures to form the urostyle is nonspecific of the genus *Seriola sp*.

The development of the parapophyses (Pp) of *S. rivoliana* followed a caudal-cranial development, in concordance with *S. dumerili* [28] and many other perciforms such as S. *aurata* [30], *Lates calcarifer* [49], *Diplodus sargus* [47] or *Pagrus pagrus* [32]. The correlation between the present study and many other marine finfish suggest that the developmental patter for the parapophyses may be common in perciforms.

In many marine finfish species, the mineralization of the anal and posterior dorsal fins starts prior to the anterior dorsal fin [31, 32, 50-52]. Unlike this developmental pattern, but in accordance with *S. dumerili* [28], *S. rivoliana* dorsal and anal structures followed a cranial-caudal development, developing the anterior dorsal fin prior to posterior dorsal fin. However,

despite *S. rivoliana* and *S. dumerili* had the same developmental pattern in dorsal, anal and caudal fins; some differences in structures development have been observed. For instance, in *S. rivoliana*, firsts structures in mineralized were hard spines (S) and lepidotrichium (R) (present study), whereas in *S. dumerili* [28] the dorsal fins development starts with the mineralization of the proximal pterygiophore.

During the process of the caudal complex mineralization of *S. rivoliana*, the fusion of hypurals (Hy_1+Hy_2 and Hy_3+Hy_4) was observed. This developmental pattern is common in other carangid species such as *S. lalandi* [29], *S. setapinnis* [47], *C. crysos* [44] and *S. dumerili* [27, 28]; as well as other perciforms such us *Coryphaena equiselis* [53], *P. major* [42], *S. aurata* [30] and *D. dentex* [36]. The development of three distinct structures (Hy_1+Hy_2, Hy_3+Hy_4, Hy_5) could remain as a characteristic of carangids and *Coryphaena* [44].

In the present study, the development of three epurals and two uroneurals were observed. The number of epurals in the caudal complex of Carangoidei species varies between species[44]: 3 epurals in *S. dumerili* [28], between 3-4 epurals (usually 3 epurals) in *S. lalandi* [29], 2 independent epurals that fused during ontogeny in *C. equiselis* [53] and 3 epurals for *S. rivoliana* (present study). Other authors [54] considered that the presence of uroneurals is a characteristic of the Teleost. The presence of two uroneurals in *S. rivoliana* caudal fin complex is in concordance with other species from the same genus such as *S. lalandi* [29] and *S. dumerili* [28].

Meristically, the vertebral column of longfin yellowtail (*S. rivoliana*) was characterized in this study. Similar results have been reported in *S. dumerili* [28]. Nevertheless, in other carangid species such as *S. setapinnis* [45], *Hemicaranx amblyrhynchus* [55] or *Trachurus japonicas* [43] a total number of 24 vertebral structures were observed, and the first haemal arch was observed at the 10^{th} vertebra [45] instead of at the 11^{th} vertebrae in *S. dumerili* [27, 28] and *S. rivoliana* (present study), indicating that this could be a conserved feature among the genus Seriola (Table II).

Concerning the caudal complex, *S. rivoliana* presented similar results than those observed in *S. lalandi* [29], *S. setapinnis* [45], *C. crysos* [44] and *S. dumerili* [27, 28], although the number of

caudal fin rays is a characteristic for each species. Thus, in this study, *S. rivoliana* had 10+9 caudal lepidotrichia and 10+9 caudad dermatotrichia, while 9+9 caudal lepidotrichia and 11-13+10 caudal dermatotrichia where reported in *S. dumerili* [28] or 9+8 caudal lepidotrichia and 6+5 caudad dermatotrichia were observed in *S. setapinnis* [45].

The importance of the meristic characterization is widely known for the identification not only for marine finfish species, [56, 57] but also in cultured fish species [52, 58, 59].

Results from the present study might be used as practical guide for future studies on this field with *S. rivoliana* or in related species.

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7. List of tables and figures.

Table I. Skeletal elements and their abbreviations.

Region	Skeletal elements	Abbreviations
Vertebral Column	Vertebra centra	Ce
	Notochord	No
	Urostyle	Ur
	Neural Arch	Na
	Neural Spine	Ns
	Haemal Arch	Ha
	Haemal Spine	Hs
	Dorsal Ribs	Eb
	Pleural Ribs	Plr
	Parapophyses	Pp
	Anterior neural zygapophysis	Anz
	Posterior neural zygapophysis	Pnz
	Anterior haemal zygapophysis	Ahz
	Posterior haemal zygapophysis	Phz
Caudal Fin	Hypurals	Hy
	Parhypural	Ph
	Epurals	Ep
	Uroneurals	Un
	Caudal lepidotrichia	PCR
	Caudal Dermatotrichia	SCR
Dorsal Fin	Predorsal	Pd
	Hard Spines	S
	Lepidotrichium	R
	Proximal Pterygiophores	Pr
	Distal Radial	Dr
Anal Fin	Hard Spines	S
	Lepidotrichium	R
	Proximal Pterygiophores	Pr
	Distal Radial	Dr

Table II. Meristic counts in different carangid species. (-) no data; (+) and; (/) between.

Species	Regions																					References
	Vertebral column							Caudal fin						Dorsal Fin					Anal Fin				
	Vertebra centra	Urostyle	Neural spine	Haemal spine	Dorsal Ribs	Pleural Ribs	Parapophyses	Hypurals	Parhypural	Epurals	Uroneurals	Lepidotrichia	Dermatotrichia	Predorsal	Hard Spines	Proximal Pterygiophores	Lepidotrichia	Proximal Radial	Distal Radial	Hard Spines	Lepidotrichia	Proximal Radial	Distal Radial	
S. rivoliana	10+13	1+1	23	13	6+6	8+8	6	5	1	3	1+1	10+9	10+9	3	VII+I	7	30 / 34	30 / 34	30 / 34	II+I	19 / 21	19 / 22	19 / 22	Present Study
S. dumerili	10+13	1+1	23	13	6+6	8+8	6	5	1	3	1+1	9+9	12+10	3	VII-VIII	7-8	31 / 34	35	32	III	19 / 21	20 / 23	20 / 23	Laggis *et al*.,2010
S. fasciata	-	-	-	-	-	-	-	-	-	-	-	-	-	-	VIII+I	-	28 / 31	-	-	II+I	18 / 20	-	-	Bañón & Mucientes, 2009
C. crysos	-	1+1	-	-	-	-	-	5	1	3	1+1	-	-	-	-	-	-	-	-	-	-	-	-	Hilton & Johnson, 2007
S. fasciata	-	-	-	-	-	-	-	-	-	-	-	-	-	-	VIII+I	-	28 / 31	-	-	II+I	18 / 20	-	-	Andaloro *et al*., 2005
S. rivoliana	-	-	-	-	-	-	-	-	-	-	-	-	-	-	VII+I	-	30	-	-	II+I	21	-	-	Castriota *et al*., 2004
S. rivoliana	-	-	-	-	-	-	-	-	-	-	-	-	-	-	VII+I	-	32	-	-	II+I	22	-	-	Castriota *et al*., 2002
S. dumerili	10+13	1	22	13	-	8+8	-	5	1	3	1+1	9+7	12+11	3	VII-VIII		31 / 32	38	-	II-III	20	21	-	Liu, 2001
S. lalandi	-	1+1+1	-	-	-	-	-	5	1	3-4	1+1	-	-	-	-	-	-	-	-	-	-	-	-	Kohno, 1997
S. setapinnis	10+14	-	24	14	-	-	-	5	1	-	-	9+8	5+6	-	-	-	-	-	-	-	-	-	-	Katsuragawa, 1997
H. amblyrhynchus	10+14	-	24	14	-	-	-	-	-	-	-	9+9	8+8	-	VII+I	-	28	-	-	II+I	25	-	-	Flores-Coto *et al*., 1998
T. japonicus	10+14	1	24	14	7+7	10+10	6	5	1	2	1	17	-	3	8	8	27 / 35	27 / 35	27 / 35	II+I	25 / 31	25 / 31	25 / 31	Suda, 1996

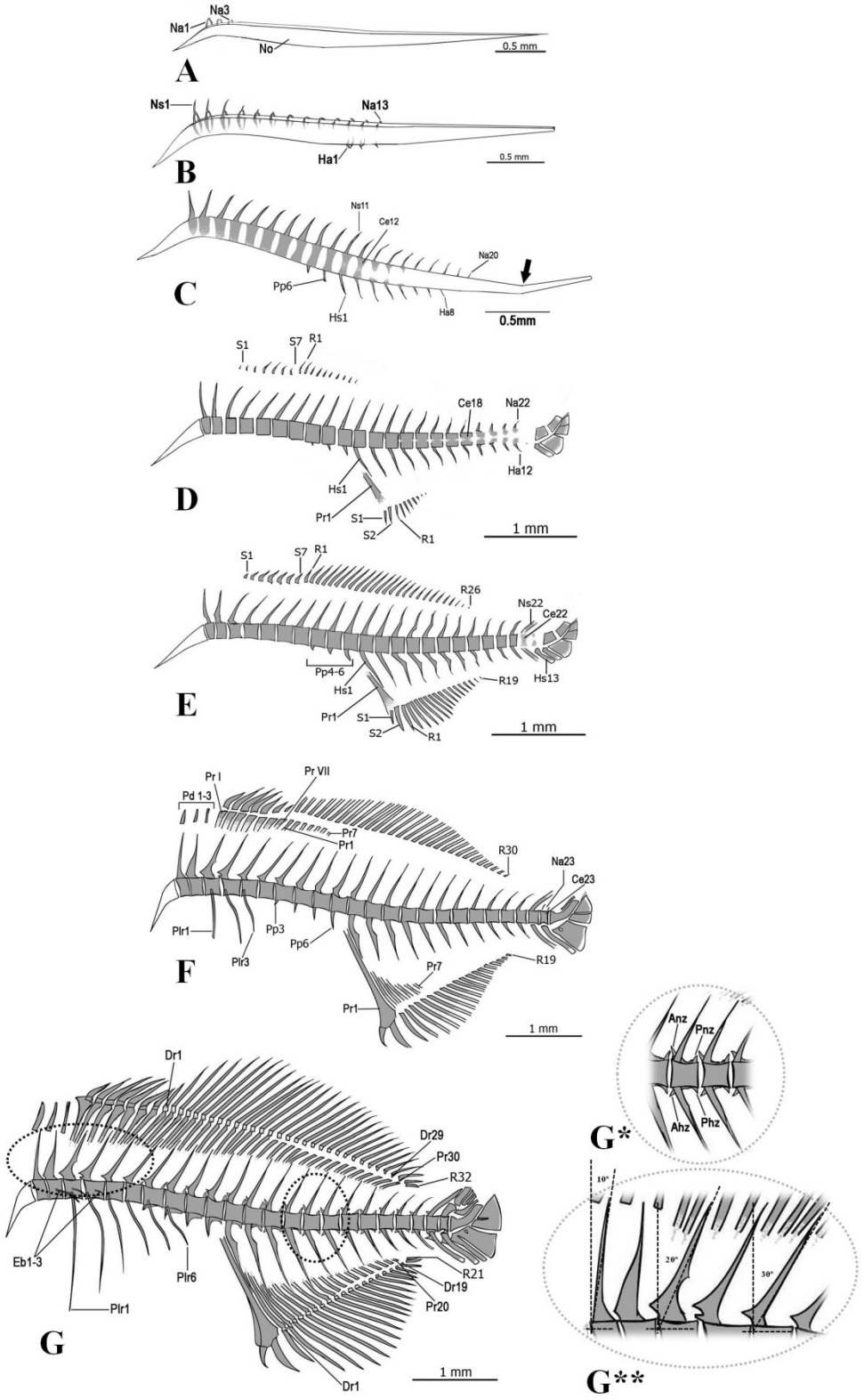


Figure 1. Development of the vertebral column (A - G) in *S. rivoliana* (painted areas, mineralized structures). Structures: Ahz, anterior haemal zygapophysis; Anz, anterior neural zygapophysis; Ha, haemal arch; Hs, haemal spine; No, notochord; Na, neural arch; Ns, neural spine; Pp, parapophyse; Plr, pleural rib; Pd, predorsal; Ph, parhypural; Phz, posterior haemal zygapophysis; Pnz, posterior neural zygapophysis; Pr, proximal pterygiophore; R, lepidotrichium; Dr, distal radial; S, hard spine; Ce, vertebral centra; Ur, urostyle.

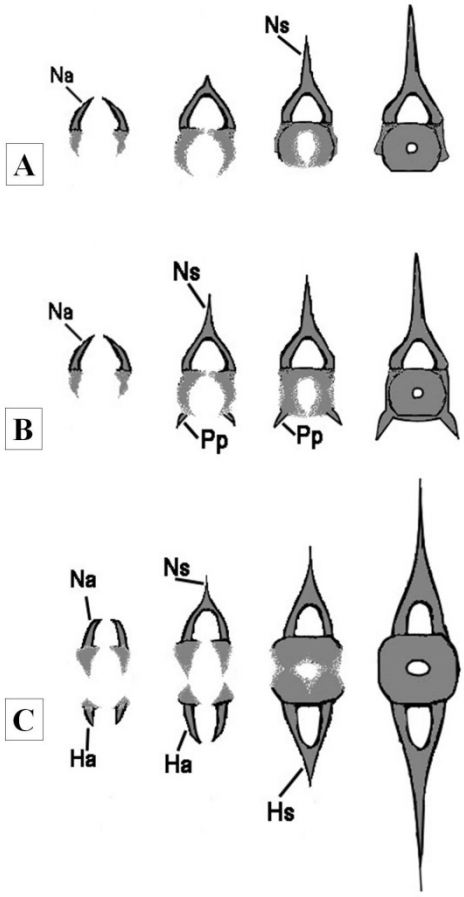


Figure 2. Vertebra mineralization (A - C) (painted areas, mineralized structures). Ha, haemal arch; Hs, haemal spine; Na, neural arch; Ns, neural spine; Pp, parapophyses.

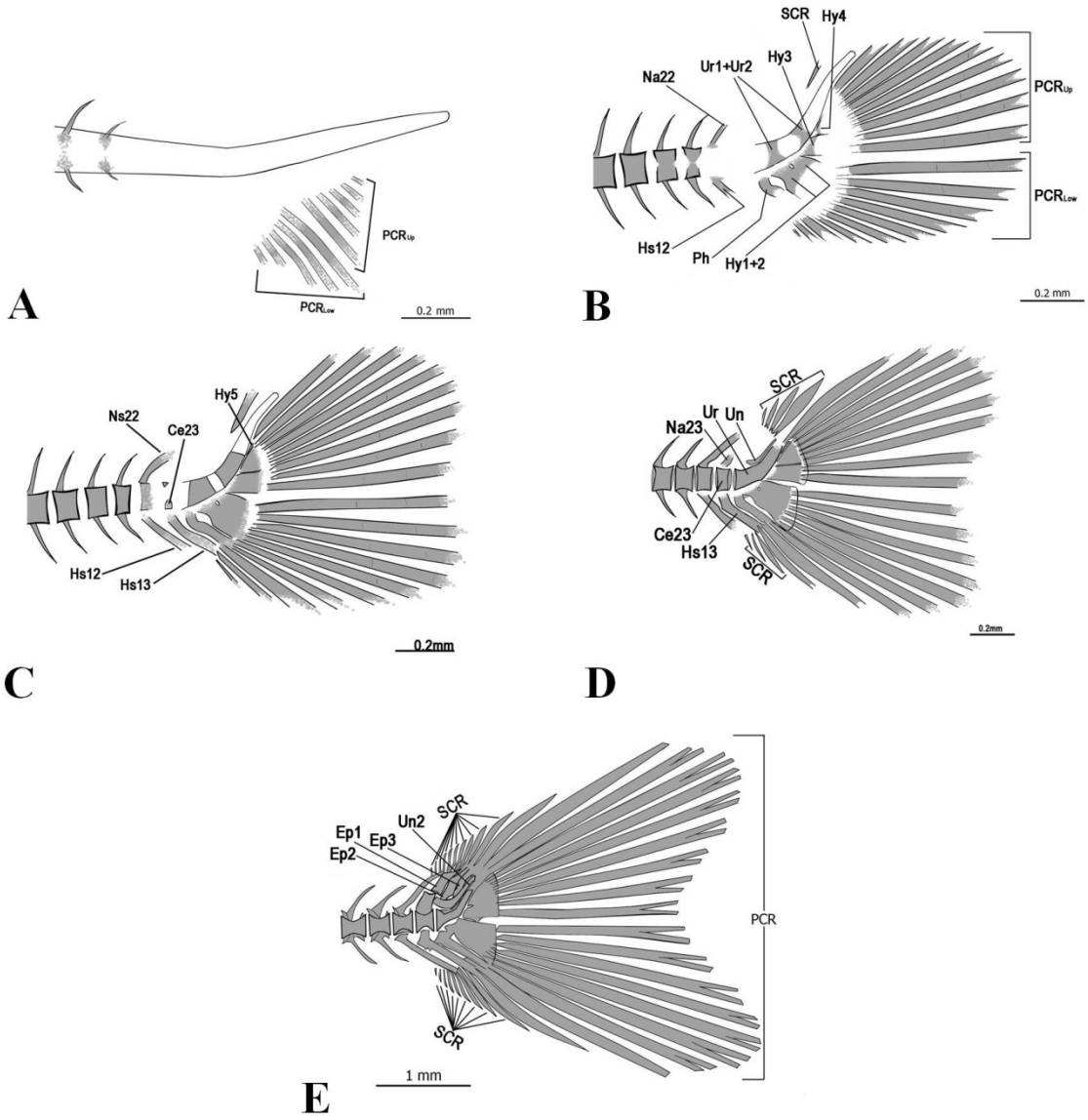
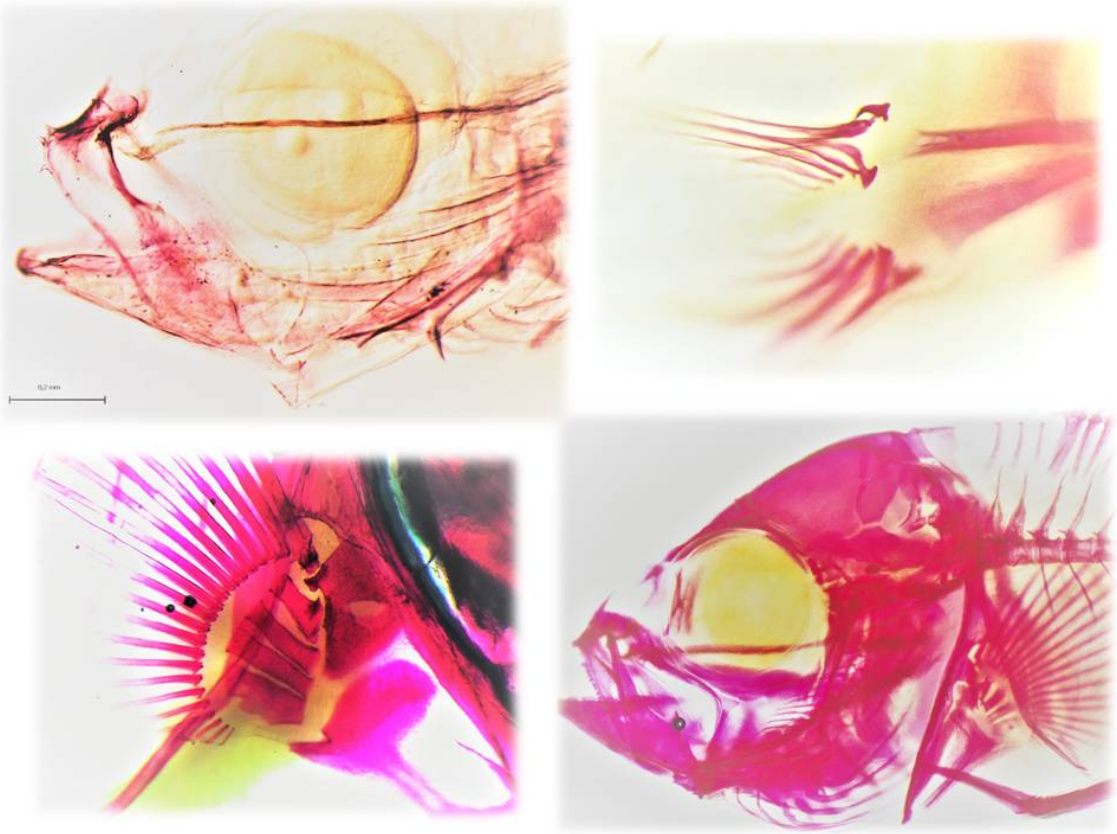


Figure 3. Development of *S. rivoliana* caudal complex (A - E) (painted areas, mineralized structures). Structures: Ep, epurals; Hy, hypurals; PCR, caudal lepidotrichia; Ph, parhypural; SCR, caudal dermatotrichia; Ur, urostyle; Un, uroneural.

Study III



Bone development of the skull, pectoral and pelvic fins in *Seriola rivoliana* (Valenciennes, 1833) larvae.

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Bone development of the skull, pectoral and pelvic fins in *Seriola rivoliana* (Valenciennes, 1833) larvae.

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Abstract

Skull, pectoral and pelvic fins bone structures in longfin yellowtail *Seriola rivoliana* were studied from 3.43 ± 0.15 to 16.20 ± 0.73 mm standard length (SL) specimens. The *S. rivoliana* skull started to mineralize with the appearance of the parasphenoid and maxillary by 3.43 ± 0.15 mm SL at the neurocranium and jaw regions respectively. The first pectoral structure to mineralize was the cleithrum at 3.75 ± 0.14 mm SL, shortly followed by supracleithrum and posttemporal. The pelvic fin started by 6.16 ± 0.32 mm SL with the spine and continued with the soft rays and basipterygium. The present study determined the onset of the skull, pectoral and pelvic fins mineralization. These results might be used as a reference for future studies in *S. rivoliana* or related species.

Key words: Amberjack, hatchery, abnormalities, osteology, skeleton.

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1. Introduction

Longfin yellowtail, *Seriola rivoliana* (Valenciennes 1833) belong to the Carangidae family, the same family as *S. dumerili* (greater amberjack), *S. lalandi* (yellowtail king fish) and *S. quinqueradiata* (Japanese yellowtail). With a high commercial interest due to its fast growth rate (Roo et al. 2012; Mesa-Rodriguez et al. 2014) and worldwide distribution, *S. rivoliana* is one of the species proposed for marine aquaculture diversification. In fact, this species is already commercially produced in Hawaii (Sims & Key 2011) and under pilot scale experimental production in the Canary Islands from 2010.

Nowadays, an increasing amount of literature is available for the *Seriola* genus, particularly in relation to larval biology (Laroche et al. 1984; Sumida et al. 1985), reproduction (Marino et al. 1995a,b; Kozul et al. 2001; Mylonas et al. 2004; Jerez et al. 2006), culture systems (Papandroulakis et al. 2005; Roo et al. 2012; Cobcroft & Battaglene 2013), feeding requirements and nutrition (Garcia-Gomez 2000; Cobcroft et al. 2004; Tomas et al. 2005; Takakuwa et al. 2006; Papadakis et al. 2007; Hamasaki et al. 2009; Fernández-Palacios et al. 2015) as well as skeletal development and deformities (Kohno 1997; Liu 2001; Cobcroft et al. 2004; Laggis et al. 2010; Cobcroft & Battaglene 2013; Mesa-Rodriguez et al. 2014). However, limited information relating to *S. rivoliana* is available (Blacio et al. 2003; Blacio 2004; Laidley et al. 2004; Roo et al. 2012; Mesa-Rodriguez et al. 2014). In this regard, it is well known that for both commercially produced finfish and diversification candidates for aquaculture the regular ontogeny of the skeletal elements can be altered under aquaculture operations, causing skeletal deformities. Similarly to other *Seriola* species, skeletal anomalies constitute one of the most important bottlenecks during the hatchery phase (Cobcroft & Battaglene 2013). Moreover, the high incidence of deformed teleost fish farmed under intensive culture systems has been previously reported (Boglione et al. 2001, 2003; Gavaia et al. 2002; Hattori et al. 2003; Roo et al. 2010b)

Under these perspectives, skeletal studies are important sources of basic knowledge to characterize and understand the osteological development and mineralization process, which in turn would help to understand and prevent the appearance of skeletal abnormalities (Gavia et al.

2002; Cobcroft et al. 2004, 2012; Boglione et al. 2009; Cobcroft & Battaglene 2013; Roo et al. 2005, 2009, 2010b; Izquierdo et al. 2010).

The present study aimed to determine the onset of mineralization in the skull, pectoral and pelvic fins of *S. rivoliana* larvae. These results serve as a reference for the staging of bone development in *S. rivoliana* or related species.

2. Material and methods

S. rivoliana eggs were obtained by induced spawning, using gonadotropin releasing hormone analogue (LHRHa, des-Gly10, [D-Ala6]; Sigma- Aldrich, St. Louis, MO, USA) at a dose of 20 μg kg−1 body weight, based on the reported dosage for longfin yellowtail (Roo et al. 2012). Larvae were reared under mesocosms rearing system following the methodology described by Roo et al. 2012 (4.5 eggs.l^{-1} were stocked in two 40m^3 tanks), kept under natural photoperiod and filtered natural sea water with 37 g l^{-1} salinity and a temperature of 23.0 ± 0.9°C. Green water technique was used by adding live phytoplankton (*Nannochloropsis sp.*) to maintain a concentration of 250000 cells ml^{-1} in the rearing tanks. From 2 to 25 days after hatching (dah) rotifers, *Brachionus sp.*, L-strain enriched with DHA Protein Selco (INVE ™), were added twice daily (08:00 and 14:00 h). *Artemia* feeding started at 15 dah, and was enriched with A_1 Easy Selco (INVE ™). The weaning protocol included manual feeding (Genma Micro, Skretting ™) from 20 dah to 25 dah and automatic feeding afterwards.

Larval growth was assessed by measuring the standard length (SL) of 25 larvae, every 2 days using a profile projector (Nikon V-12A, NIKON™). Bone ossification was studied by fixing in buffered formalin, from hatching to 33dah. A total of 200 fixed larvae (3.43 ± 0.15 - 16.2 ± 0.73 mm SL) were cleared and stained with alizarin red (Vandewalle et al. 1998). Larvae were individually examined using stereomicroscope. Drawings of the different developmental stages were made using the Adobe Photoshop CS3-10.0 (1990-2007 Adobe System Incorporated, United States) directly from digital photographs. Bone description followed terminology previously described (Cubbage & Mabbe, 1996). A list of abbreviations used is detailed in Tables I and II. Structures development sequence is illustrated in Table IV. Thus, viscerocranial

structures were grouped into different regions (Table I), depending on their functionality (Matsuoka 1985; Collette & Gillis 1992; Cubbage & Mabbe, 1996; Suda 1996; Faustino & Power 2001). Also, meristic counts performed on *S. rivoliana* juveniles (n=10; weight: 60-120gr) soft X-ray monitored (Mod. Senographer-DHR, General electric's,USA) were included (Table III).

3. Results

3.1. Neurocranium

The development of the *S. rivoliana* neurocranium started with the mineralization of the parasphenoid (Ps), a large laterally compressed structure across the length of the skull by 3.75 ± 0.14 mm SL (Fig. 1A). This continued with the vomer (V), situated anterior to the Ps, the frontal (F) and exoccipital (Eoc) by 5.38 ± 0.11 mm SL (Fig. 1B). Nasal (N) and lateral ethmoid (Le) started to develop at the same time as the first otic region structures, with the sphenotic (Sph), supraoccipital (Soc), pterotic (Pto) and intercalar (Ic) by 5.76 ± 0.06 mm SL (Fig. 1C). At 8.78 ± 0.64 mm SL infraorbitals (Inf 2-4) started to develop. After 10.35 ± 0.86 mm SL (Fig. 1D), the otic, basicranial and orbital region structures were in the process of developing: parietal (Pa), epioccipital (Eo), intercalar (Ic), prootic (Pro) and infraorbitals (Inf 1-5); basioccipital (Boc); basisphenoid (Bas) and pterosphenoid (Pts) respectively. The ethmoid (E) was the last structure of the ethmoid region that started to develop (12.41 ± 0.43 mm SL, Fig. 1D).

3.2. Jaws

The jaw structures differentiated in two regions, the upper maxilla (Mx) and premaxilla (Pm) and the lower jaw (dentary, D; anguloarticular, Aa; and retroarticular, Ra). The first visible structure in *S. rivoliana* larvae was the Mx by 3.43 ± 0.15 mm SL (Fig. 2A), followed by the D and Pm (3.75 ± 0.14 mm SL; Fig. 2B), with the last structures to develop being the Aa and Ra by 4.60 ± 0.14 mm SL (Fig. 2C). At this time, small premaxillary teeth (Pmt) were first seen, whereas the dentary teeth (Dt) developed by 5.76 ± 0.04 mm SL (Fig. 2D).

3.3. Suspensorium

The ossification of the suspensorium structures started with the symplectic (Sy), hyomandibula (Hm) and quadrate (Q), followed by the palatine (P), endopterygoid (En) and ectopterygoid

(Ect) by 5.76 ± 0.06 mm SL (Fig. 2D). Finally, the metapterygoid (Mpt) and palatine teeth (Plt) began to develop by 10.35 ± 0.86 mm SL (Fig. 2E).

3.4. Opercular Series

The four structures that compound the opercular series are closely distributed. The preopercle (Pop), was the first structure of the opercular series to develop by 3.75 ± 0.14 mm SL (Fig. 2B). The opercle (Op), with a posterior situation developed afterwards by 4.60 ± 0.14 mm SL (Fig. 2C). Ventrally located, the interopercle (Iop) appeared by 5.76 ± 0.06 mm SL (Fig. 2C), at the same time as the subopercle (Sop), located between the Op and Iop.

3.5. Hyoid Arch

At 3.75 ± 0.14 mm SL (Fig. 3A), three simultaneous pairs of branchiostegal rays (Bsr) started to develop in a caudal-rostral direction, up to a total of 7 Bsr pairs. Ceratohyal (Ch) expanded anteriorly and posteriorly from 4.60 ± 0.14 mm SL (Fig. 3B). Between the two Ch and with a ventrally situation the urohyal (Uh) started to ossify by 5.00 ± 0.15 mm SL (Fig. 3C). Finally, Hypohyals (Hh) and epihyal (Eh) ossified by 10.35 ± 0.86 mm SL (Fig. 3D).

3.6. Branchial Arches

This complex is composed by 5 pair of ceratobranchials (Cb1-5), 4 pair of epibranchials (Eb1-4) and pharyngobranchials (Pb1-4), 3 pair of hypobranchials (Hb1-3), 3 basibranchials (Bb1-3) and the basihyal (Bh). The first structure of the branchial arches to develop by 3.75 ± 0.14 mm SL was a tiny pair of ceratobranchial teeth (Cbt) at the Cb5, first seen by 4.60 ± 0.14 mm SL. The Cb´s followed a caudal-rostral development, followed by the Cb4 and Cb3 at 5.00 ± 0.15 mm SL and Cb2 and Cb1 after by 5.38 ± 0.11 mm SL. At the size of 5.76 ± 0.06 mm SL, the upper branchial arches start to develop with the Eb4 and Eb3 first, and Eb2 and Eb1 after by 6.16 ± 0.73 mm SL. Simultaneously to the Eb2 and Eb1, the largest Pb (Pb3), the first Hb (Hb3) and the Bb3 start to develop. Gill rakers become evident on the first Cb (Cb1-2) located at the anterior and posterior border of the structure, and Cb3 and Cb4 after. Cb4 only present gill rakers at the anterior border. Bb2, Hb2, Hb1, Pb2 and Pb4 develop simultaneously by 7.17 ± 0.3 mm SL. The last structures of the branchial complex to develop were the Bb1, Pb1 and Bh at

10.35 ± 0.86 mm SL. At this stage, pharyngobranchial teeth (Pbt) are well seen at Pb´s structure (Fig. 6).

3.7. Pectoral fin-supports

The first elements that start the ossification of the pectoral fin were the cleithrum (Cl) and supracleithrum (Scl) by 3.43 ± 0.15 mm SL and 4.60 ± 0.14 mm SL respectively (Fig. 4A). Above the Cl, the Scl and postemporal (Pt) developed dorsally by 5.76 ± 0.06 mm SL (Fig. 4B). At this time, the ossification of soft rays (R) as well as the upper and lower postcleithrum (Pcu & Pcl) occurred. The coracoid (Co) and the scapula (Sc) ossified by 8.78 ± 0.64 mm SL (Fig. 4C), presenting a dorsal and ventral mineralization until they fused by 16.20 ± 0.73 mm SL (Fig. 4E). The first actinost (Act1) started to ossify by 10.35 ± 0.86 mm SL and expanded dorso-ventrally. The group of four Act presented a gradual ventral development, commencing the mineralization of the third Act by 12.35 ± 0.98 mm SL (Fig. 4D). Finally, supratemporals (Stu & Stl), the fourth Act, the propterygium (Prop) and distal radials (Dr) were ossified by 16.20 ± 0.73 mm SL (Fig. 4E).

3.7. Pelvic fin-supports

At 6.16 ± 0.73 mm SL (Fig. 5A1,2) the spine (S) started to develop, with a caudal mineralization direction. This was followed by the soft rays (R) and the basipterygium (Bp) at 7.17 ± 0.3 mm SL (Fig. 5B1,2). The soft rays developed towards the caudal fin, whereas the Bp mineralized rostrally and caudally (Fig. 5C1,2). The Bp reached the Cl up to 11.77 ± 0.39 mm SL (Fig. 5D1,2), when all the pelvic fin structures were developed.

3.8. Meristic characters

Meristically, major viscerocranial structures of *S. rivoliana* larvae were presented as one pair of each structure (1 - P), with the exception of Boc, Bas, Ps, Soc, V and Uh, which were presented as a single structure (1 - S), while 7 pairs of Bsr (7-P) developed at the hyoid arch. In the pectoral and pelvic fins, a final count of 20/22 and 5 ossified soft rays were identified, respectively (Table III).

4. Discussion

Due to the wide variability between marine teleosts, determination of a common developmental pattern becomes complex. However, a similar skeletal development and mineralization in the neurocranium structures have been found in *S. rivoliana* in comparison with those reported in different species such as *Pagrus major* (Matsuoka 1985), *Spondyliosoma emarginatum* (Beckley 1989), *Dentex dentex* (Koumoundouros et al. 2000), *Sparus aurata* (Faustino & Power 2001), *Anisotremus davidsonii* and *Xenistius californiensis* (Watson & Walker 1992). These findings could be related to the same functional requirements for larval survival at early life stages (Faustino & Power 2001). For example, the structures associated with first feeding, including jaws, neurocranium, suspensorium and gill arches are the first to develop in many different species (Roo et al. 1999) although, the timing of mineralization may differ between species. Thus, some skeletal elements in the neurocranium structure (E, Bas and Pts) and suspensorium (Hm, Sy and Q) share a common developmental pattern with *P. major* (Matsuoka 1985), *A. davidsonii X. californiensis* (Watson & Walker 1992), *D. dentex* (Koumoundouros et al. 2000) or *S. aurata* (Faustino & Power 2001). Furthermore, in addition to the previously mentioned species jaw skeletal structures are also common in species such as *S. emarginatum* (Beckley 1989), *S. setapinnis* (Katsuragawa 1997), *Gadus morhua* and *Pseudopleuronectes americanus* (Hunt von Herbing 2001), *Solea solea* (Wagemans & Vandewalle 2001), *S. dumerili* (Liu 2001) and *Epinephelus septemfasciatus* (Nagano et al. 2007). The tiny variations observed in the ossification timing or sequence of jaw structures ossification may be a consequence of different functional requirements (Faustino & Power 2001), feeding habits or even way of feeding.

Regarding the skeletal elements directly related with breathing action, such as the opercular series and the hyod arches development, these seem to be common in several marine finfish, identifying minor differences in the mineralization timing with species such as *P. major* (Matsuoka 1985), *A. davidsonii* (Watson & Walker 1992) or *S. aurata* (Faustino & Power 2001). On the contrary, other cranial structures such as the Soc crest, which is common in many carangid species (Katsuragawa 1997) such as (*Caranx crysos*, McKenney (1958); *S lalandi*,

Chloroscombrus orqueta, Caranx caballus, Caranx sexfasciatus (Sumida et al. 1985); *Selene setapinnis*, Katsuragawa (1997), *Hemicaranx amblyrhynchus* (Flores-Coto et al. 1998); *Parastromateus niger* (Hilton et al. 2010) was not identified nor in *S. rivoliana* (present study) neither in similar species such as *S. dumerili* (Liu 2001). This type of skeletal element together with opercular spines has been identified as protective structures (Morgan 1989), and might greatly differ between species particularly related to aspects such as type of environment were the larvae growth (estuarine, open ocean) or predators pressure and need of predator avoidance .

Concerning the ossification timing, even within the same species or genus many differences could be addressed. Thus, Liu (2001) described the start of the neurocranium development at 6.6 mm (L_N) with the mineralization of F, Inf, Ps and V simultaneously in *S. dumerili*, whereas *S. rivoliana* (present study) began neurocranium mineralization by 3.75 ± 0.14 mm (SL) starting with Ps, and followed by V, F and Exo at 5.38 ± 0.11 mm (SL). Besides, at 16.8 mm (L_N) the Sph, Pto, Eoc, Pro, Soc, Exo and Bo were the last structures to mineralize in *S. dumerili* (Liu, 2001), whereas in *S. rivoliana* larvae the E was the last structures that started to develop by 12.41 ± 0.43 mm (SL). These developmental patterns and mineralization timing are clearly different between *S. dumerili* (Liu 2001) and *S. rivoliana* (present study). In this regard, not only the species but also the environmental conditions (Boglione et al. 2009; Cobcroft et al. 2012), and zootechniques applied (Roo et al. 2010a,b) might delay or advanced the larval ontogeny, promoting differences in the larval stages of stained specimens that would explain these differences.

The ontogeny and mineralization of feeding and breathing structures should be combined with those skeletal elements involved in swimming activity, related to prey capture and escape abilities. Thus *S. rivoliana* larvae initiated the ossification of the dorsal and anal fin at 8.01 ± 0.26 mm SL (Mesa-Rodriguez, et al. 2014), and were shortly followed by the pelvic fins at 8.60 ± 0.66 mm SL during the notocord post-flexion stage. This developmental pattern occur after the mineralization of the main jaw and breathing structures between 3.75 ± 0.14 mm SL and 6.16 ± 0.73 mm SL, supporting the importance of feeding and breathing. Moreover, bones

involved in main functions such as feeding and breathing have been conserved among many different Teleost (*Danio rerio*, Cubbage & Mabee, 1996; *Betta splendens*, Mabee & Trendler, 1996*; Gadus morhua* and *Pseudopleuronectes americanus*, Hunt von Herbing, 2001; *Solea solea*, Wagemans & Vandewalle, 2001; *Epinephelus septemfasciatus*, Nagano et al. 2007). The onset of similar ossification pattern in swimming structures such as the dorsal, anal and pelvic fins between *S. dumerili* (Laggis et al. 2010), *S. lalandi* (Sumida et al. 1985) and *S. rivoliana* (Mesa-Rodriguez, et al. 2014) at post-flexion stage suggest that this characteristic is common for the *Seriola* genus. Therefore, identification of pre-flexion and post-flexion stages are useful external indicators of the initiation of the internal column mineralization (Mesa-Rodriguez, et al. 2014) and fins. This fact improves larval swimming ability to capture prey and predator avoidance. In addition, notochord flexion is paired with the appearance of the first gastric glands and new photoreceptors in the eye (rods) providing a higher digestive capacity and improving visual acuity respectively (Roo, personal com.), which is probably related with changes in feeding habits and environmental needs, as happens with other species (Roo et al. 1999). Furthermore, notochord flexion was also an indicator to distinguish different genus from the carangid family during the formation of pelvic fin elements (Laroche et al. l984; Ahlstrom & Sumida 1985). For example, the presence of single Scl and Pt spines in the pectoral fin support of *S. rivoliana* is in concordance with that found for *S. setapinnis*, *S. vommer* (Katsuragawa 1997) and *C. sexfasciatus* (Sumida et al. 1985), but differs from *Trachurus lathami*, *Decapterus punctatus*, *Chloroscombrus chrysurus* (Katsuragawa 1990), *C. caballus*, *C. orqueta* and *S. lalandi* (Sumida et al. 1985), in which the number of spines of each structure varies. According to the presence and number of spines, there is no tendency between carangid species. In this regard, obtention of meristic data of *S. rivoliana* are also relevant to identify deviation from the regular pattern under culture conditions. In fact, meristic counts are considered quality descriptors of reared juveniles (Boglione et al. 2001). Generally, cultured juveniles display a higher variability of meristic characters than natural populations. In this study, *S. rivoliana* were cultured under semi-intensive system conditions, a condition where juvenile finfish were previously reported to be more prone to present similar characteristics to wild juveniles (Roo et

al. 2009). The meristic data obtained for *S. rivoliana* identified a number of 7 paired Bsr, similar to other carangid species such as *S. dumerili* (Liu 2010) and *P. niger* (Hilton et al. 2010), and in common with *A. davidsonii* and *X. californiensis* (Watson & Walker 1992). Also, the number of spines in the pelvic fin (I+5) and soft rays in the pectoral fin (20-23), are common in several carangids such as *S. setapinnis* (Katsuragawa 1997), *S. dumerili* (Liu 2001; Laggis et al. 2010), *P. niger* (Hilton et al. 2010), *S. lalandi* (Sanchez-Ramirez & Flores-Coto 1993), *S. rivoliana* (Castriota et al. 2004; present study), *Seriola fasciata* (Bañón & Mucientes 2009), *C. caballus*, *C. orqueta* and *C. sexfasciatus* (Sumida et al. 1985). Whereas for many other marine finfish the soft rays from the number of pectoral fin varies from 14-22 (*S. emarginatum*, Beckley 1989; *A. davidsonii* and *X. californiensis*, Watson & Walker 1992; *Dicentrarchus labrax*, Marino et al. 1993; *D. dentex*, Koumoundouros et al. 2000; *S. aurata*, Faustino & Power 2001; *D. sargus*, Koumoundouros et al. 2001; *P. erythrinus*, Sfakianakis et al. 2004). These results may indicate that the total number of 20-23 pectoral fin soft rays is characteristic from the carangid fish.

Results from the present study determined that the onset of structures ossification is directly related with the main functionalities of the different structures, suggesting that some developmental events during mineralization process may be common for many different species. In addition, the first description of meristic data and mineralization timing for this species might be a useful practical guide for future studies on this field with *Seriola* genus or related species.

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7. List of tables and figures

Table 1. Viscerocranial structures and their abbreviations.

Region	Skeletal elements	Abbreviations
Neurocranium	Basioccipital	Boc
	Basisphenoid	Bas
	Ethmoid	E
	Epioccipital	Eo
	Exoccipital	Eoc
	Frontal	F
	Infraorbitals	Inf
	Intercalar	Ic
	Lateral ethmoid	Le
	Nasal	N
	Parasphenoid	Ps
	Parietal	Pa
	Prootic	Pro
	Pterotic	Pto
	Pterosphenoid	Pts
	Sphenotic	Sph
	Supraoccipital	Soc
	Vomer	V
Jaws	Anguloarticular	Aa
	Dentary	D
	Dentary teeth	Dt
	Maxilla	Mx
	Premaxilla	Pm
	Premaxilla teeth	Pmt
	Retroarticular	Ra
Suspensorium	Ectopterygoid	Ect
	Endopterygoid	En
	Hyomandibula	Hm
	Metapterygoid	Mpt
	Palatine	P
	Palatine teeth	Plt
	Quadrate	Q
	Symplectic	Sy
Hyoid Arch	Branchiostegals	Brs
	Ceratohyal	Ch
	Epihyal	Eh
	Hypohyal	Hh
	Urohyal	Uh
Branquial Arches	Basibranchials	Bb
	Basihyal	Bh
	Ceratobranchials	Cb
	Ceratobranchials teeth	Cbt
	Epibranchials	Eb
	Hypobranchials	Hb
	Pharyngobranchials	Pb
	Pharyngobranchials teeth	Pbt
Opercular Series	Interopercle	Iop
	Opercle	Op
	Preopercle	Pop
	Subopercle	Sop

Table II. Fins structures and their abbreviations.

Region	Skeletal elements	Abbreviations
Pectoral Fin	Actinost	Act
	Cleithrum	Cl
	Coracoid	Co
	Distal radial	Dr
	Propterygium	Prop
	Postcleithrum Lower	Pcl
	Postcleithrum Upper	Pcu
	Posttemporal	Pt
	Soft rays	R
	Scapula	Sc
	Scapular foramen	F
	Supracleithrum	Scl
	Supratemporal Lower	Stl
	Supratemporal Upper	Stu
Pelvic Fin	Basipterygium	Bp
	Soft rays	R
	Spine	S

Table III. Meristic counts of *S. rivoliana*. (S) single, (P) paired.

Region	Skeletal element	N. Structures
Neurocranium	Basioccipital	1 - S
	Basisphenoid	1 - S
	Ethmoid	1 - P
	Epioccipital	1 - P
	Exoccipital	1 - P
	Frontal	1 - P
	Infraorbitals	5 - P
	Intercalar	1 - P
	Lateral ethmoid	1 - P
	Nasal	1 - P
	Parasphenoid	1 - S
	Parietal	1 - P
	Prootic	1 - P
	Pterotic	1 - P
	Pterosphenoid	1 - P
	Sphenotic	1 - P
	Supraoccipital	1 - S
	Vomer	1 - S
Jaws	Anguloarticular	1 - P
	Dentary	1 - P
	Maxilla	1 - P
	Premaxilla	1 - P
	Retroarticular	1 - P
Suspensorium	Ectopterygoid	1 - P
	Endopterygoid	1 - P
	Hyomandibular	1 - P
	Metapterygoid	1 - P
	Palatine	1 - P
	Quadrate	1 - P
	Symplectic	1 - P

Region	Skeletal element	N. Structures
Opercular Series	Interopercle	1 - P
	Opercle	1 - P
	Preopercle	1 - P
	Subopercle	1 - P
Hyoid Arch	Branchiostegals	7 - P
	Ceratohyal	1 - P
	Epihyal	1 - P
	Hypohyal	1 - P
	Urohyal	1 - S
Pectoral Fin	Actinost	4 - S
	Cleithrum	1 - S
	Coracoid	1 - S
	Distal radial	19/21
	Propterygium	1 - S
	Postcleithrum Lower	1 - S
	Postcleithrum Upper	1 - S
	Posttemporal	1 - S
	Soft rays	20/22
	Scapula	1 - S
	Supracleithrum	1 - S
	Supratemporal Lower	1 - S
	Supratemporal Upper	1 - S
Pelvic Fin	Basipterygium	1 - P
	Soft rays	5 - P
	Spine	1 - P
Branquial Arches	Basibranchials	3 - S
	Basihyal	1 - S
	Ceratobranchials	5 - P
	Epibranchials	4 - P
	Hypobranchials	3 - P
	Pharyngobranchials	4 - P

Table IV. Structure sequence of *S. rivoliana*.

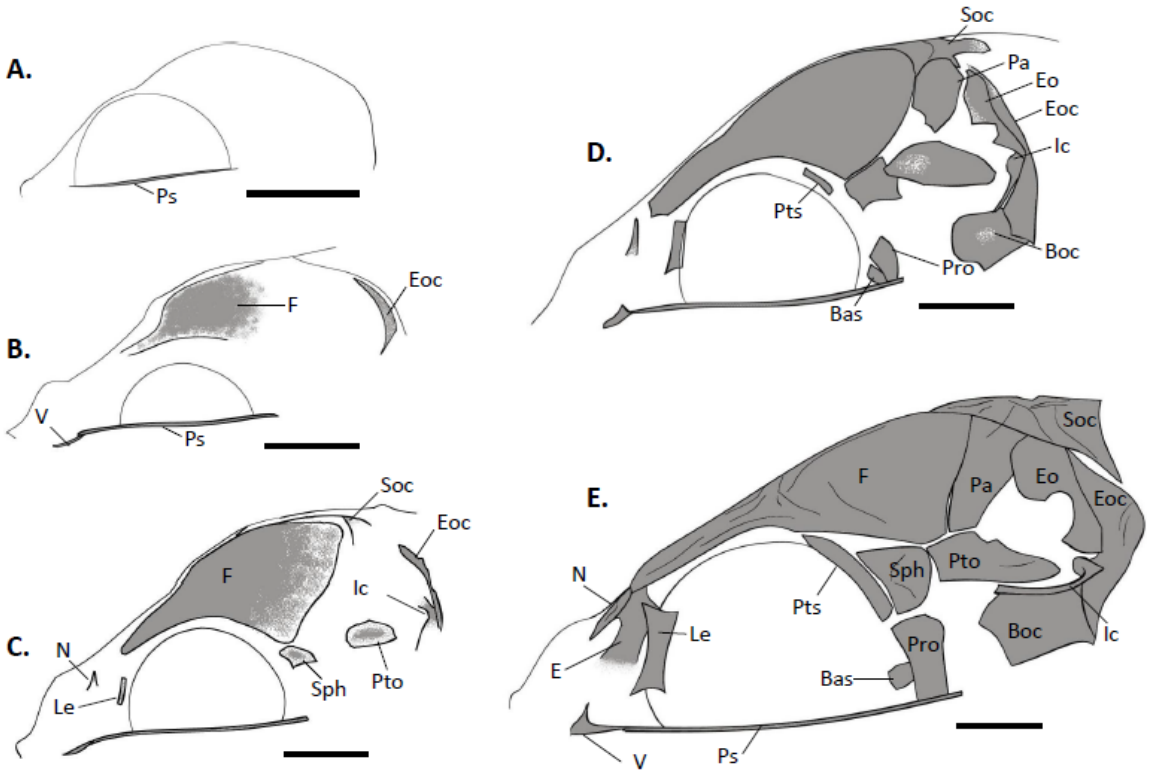


Figure 1. Neurocranium development (A-E) of S. rivoliana (painted areas, mineralized structures). Structures: Boc, basioccipital; Bas, basisphenoid; Eo, epioccipital; E, ethmoid; Eoc, exoccipital; F, frontal; Ic, intercalar; Le, lateral ethmoid; N, nasal; Ps, parasphenoid; Pa, parietal; Pro, prootic; Pto, pterotic; Pts, pterosphenoid; Sph, sphenotic; Soc, supraoccipital; V, vomer.

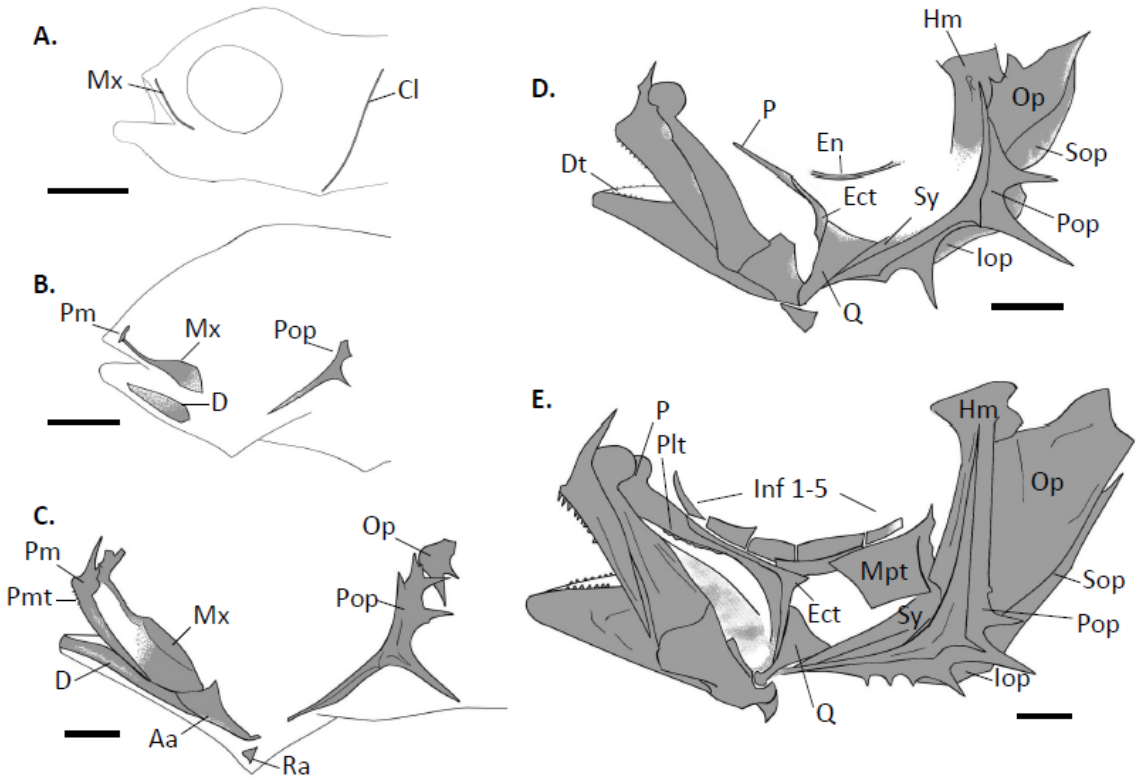


Figure 2. Jaws and opercular series development (A-E) of S. rivoliana (painted areas, mineralized structures). Structures: Aa, anguloarticular; D, dentary; Dt, dentary teeth; Ect, ectopterygoid; En, endopterygoid; Hm, hyomandibular; Inf, infraorbitals; Iop, interopercle; Mpt, metapterygoid; Mx, maxillary; Op, opercle; P, palatine; Pm, premaxillary; Pmt, premaxillary teeth; Pop, preopercle; Plt, palatine teeth; Q, quadrate; Ra, retroarticular; Sop, subopercle; Sy, sympletic.

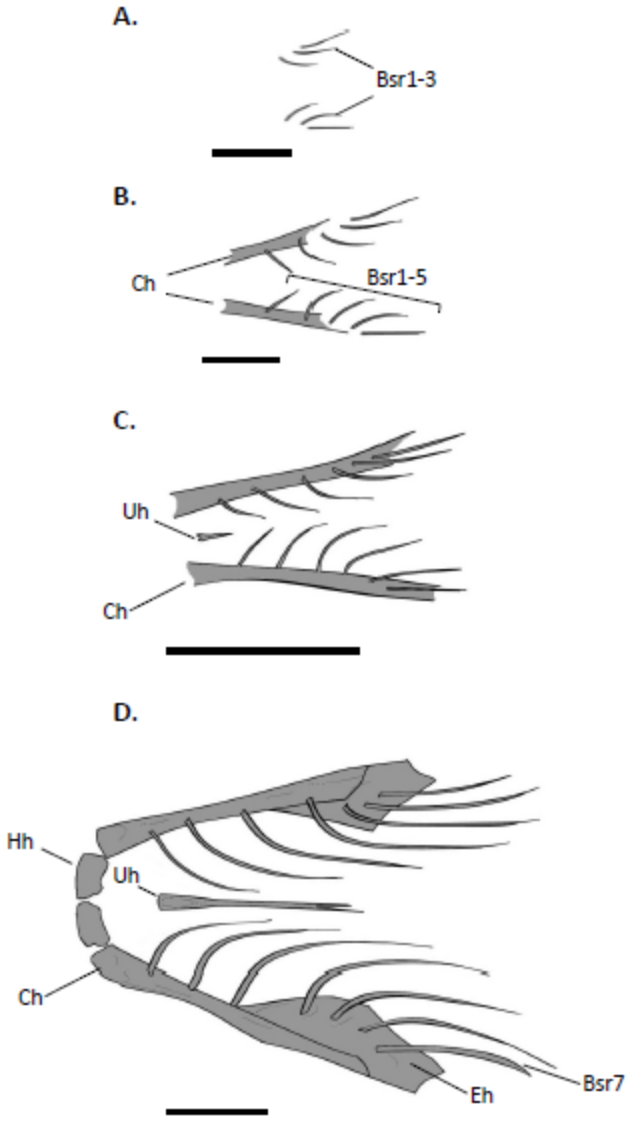


Figure 3. Hyoid arch development (A-D) of *S. rivoliana* (painted areas, mineralized structures). Structures: Bsr, branchiostegals; Ch, ceratohyal; Eh, epihyal; Hh, hypohyal; Uh, urohyal.

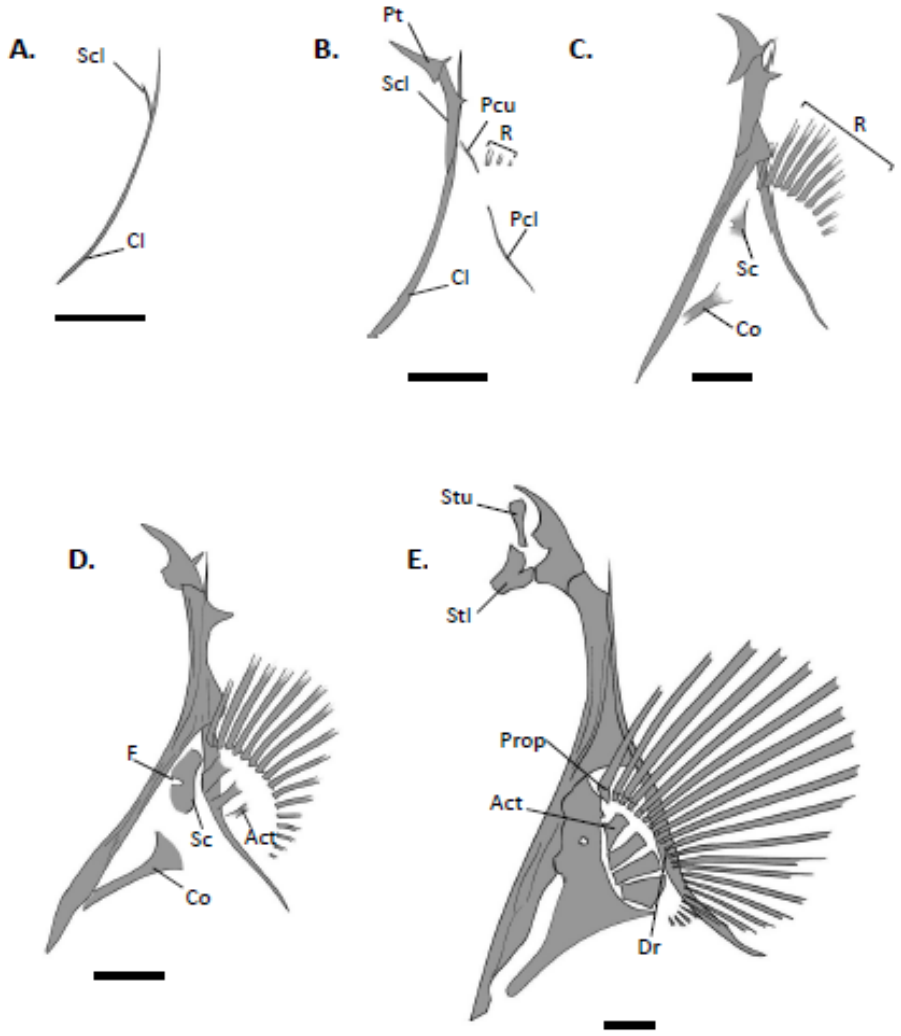


Figure 4. Pectoral fin development (A-E) of *S. rivoliana* (painted areas, mineralized structures). Structures: Act, actinost; Cl, cleithrum; Co, coracoid; Dr, distal radial; F, scapular foramen; Pcl, postcleithrum low; Pcu, postcleithrum up; Prop, propterygium; R, soft rays; Sc, scapula; Scl, supracleithrum; Stl, supratemporal lo; Stu, supratemporal up.

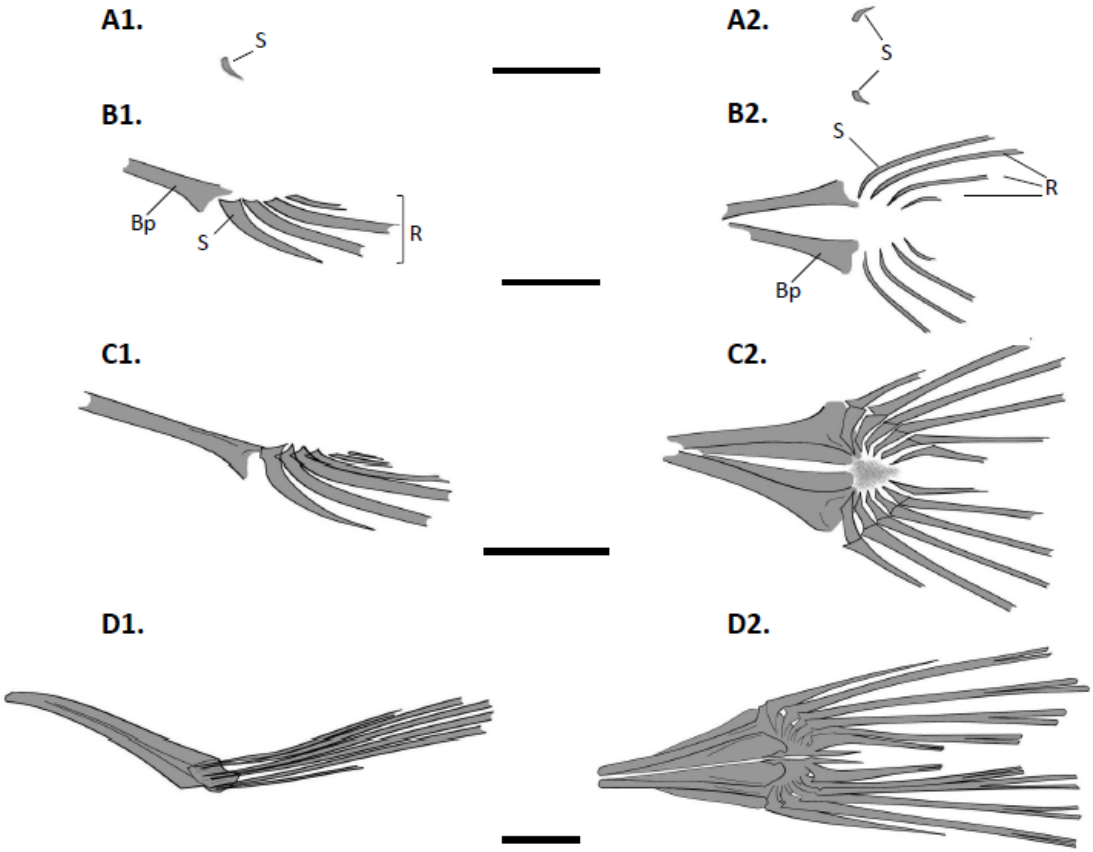


Figure 5. Pelvic fin development (A-D) of S. rivoliana (painted areas, mineralized structures). Ventral and lateral view. Structures: Bp, basipterygium; R, soft rays; S, spine.

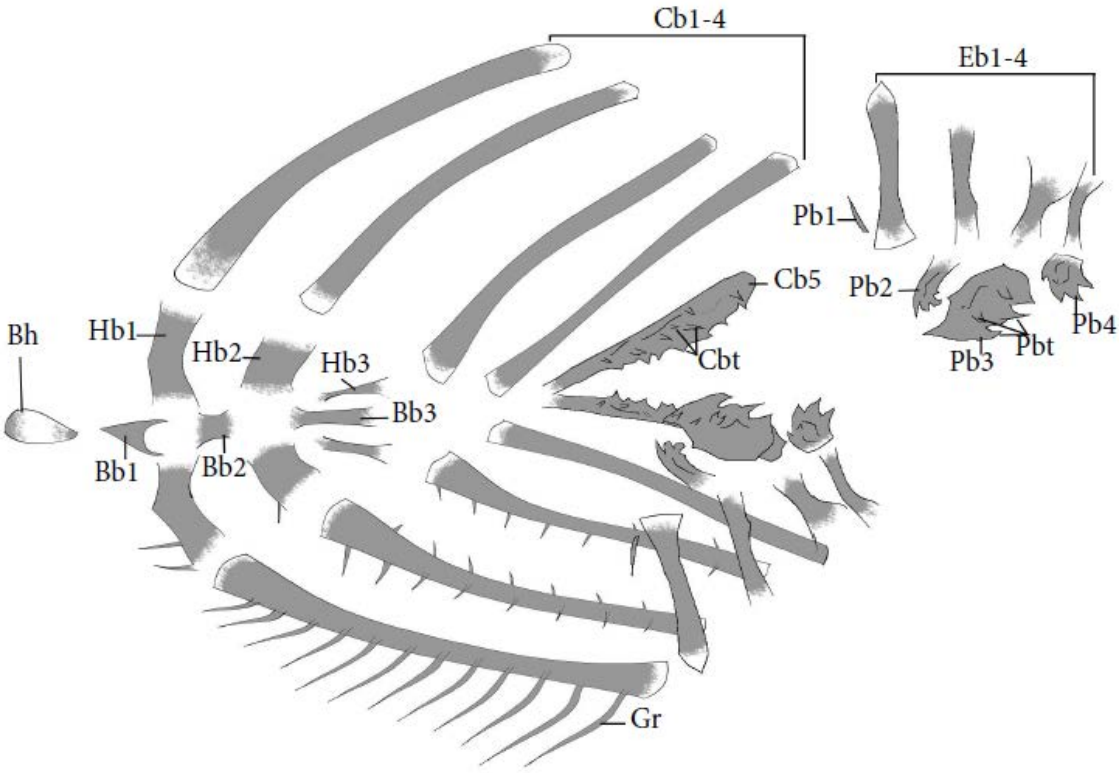
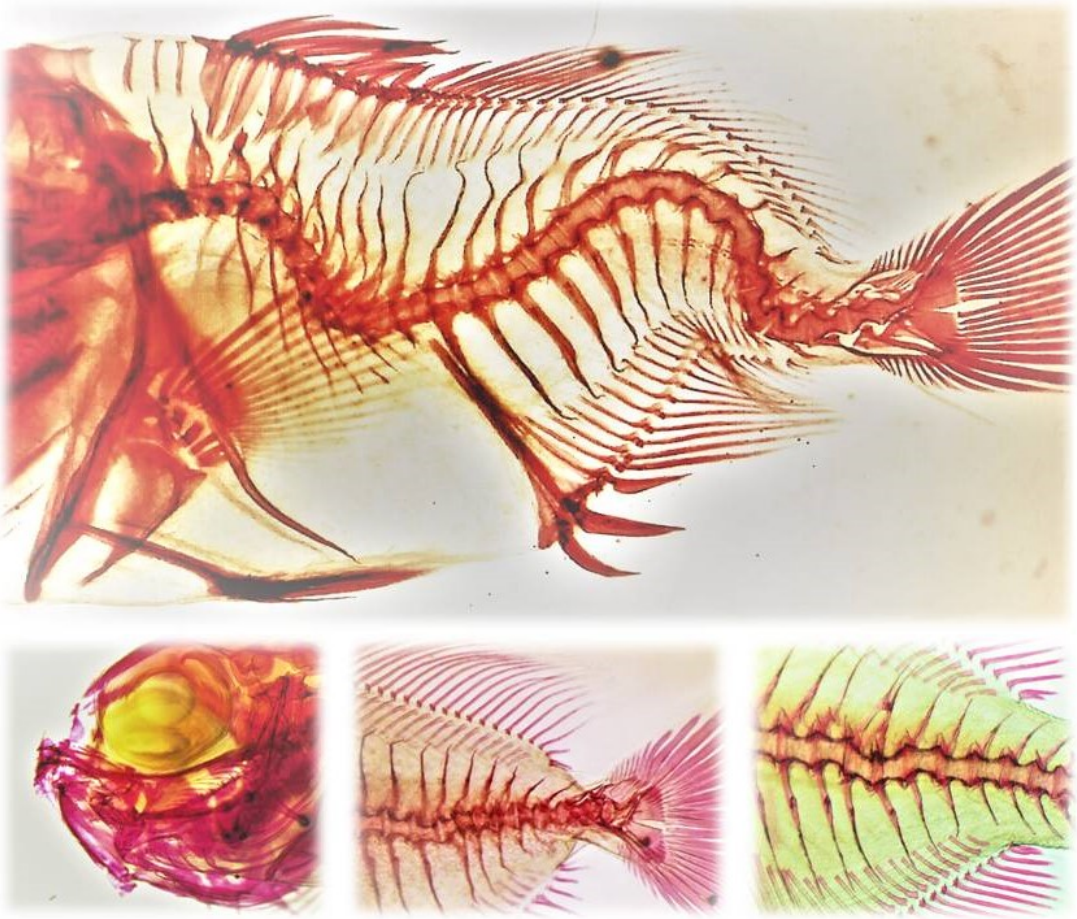


Figure 6. Branchial Arches development of *S. rivoliana* (painted areas, mineralized structures). Structures: Bb, basibranchial; Bh, basihyal; Cb, ceratobranchial; Eb, epibranchial; Hb, hypobranchial; Pb, pharyngobranchial.

Study IV



Effect of increasing DHA content in weaning feeds for longfin yellowtail (*Seriola rivoliana*).

Submitted

Effect of increasing DHA content in weaning feeds for longfin yellowtail (*Seriola rivoliana*).

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Abstract

Five isoproteic (54.8%) and isolipidic (24.1%) microdiets, which varied in their docosahexaenoic acid (DHA) content from 0.25-3.17% (dw), were manufactured to determine its effects on longfin yellowtail *Seriola rivoliana* larvae in terms of fish biological performance, whole body fatty acid profile and incidence of skeletal anomalies. The inclusion of dietary DHA up to a 3.17% (dw) improved larval resistance to air exposure. Although DHA did not significantly affect fish final growth or final survival. Indeed, high levels of dietary DHA (2% and 3%, dw) tended to increase the incidence of skeletal anomalies in *S. rivoliana* larvae, albeit no significant differences were observed. Furthermore, the occurrence of severe anomalies such as kyphosis and lordosis, was mainly associated to the larvae fed with the highest levels of dietary DHA. In terms of survival, increasing dietary DHA levels did not significantly affect longfin yellowtail survival rate, although tended to enhance with increasing dietary DHA.

Keywords: longfin yellowtail, fish larvae, docosahexaenoic acid, microdiets, skeletal anomalies.

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1. Introduction

The recent interest on marine fast-growing teleost species for aquaculture diversification has lead to research in fish such as bluefin tuna (*Thunnus thynnus*), greater amberjack (*Seriola dumerili*), yellowtail kingfish (*Seriola lalandi*), Japanese yellowtail (*Seriola quinqueradiata*) or meagre (*Argyrosomus regius*). Longfin yellowtail, (*Seriola rivoliana*, Valenciennes 1833) is a carangid with a high commercial interest due to its fast growth rate (Roo *et al*. 2012; Mesa-Rodriguez *et al*. 2014, 2016) and worldwide distribution. Moreover, *S. rivoliana* is already commercially produced in Hawaii (Sims & Key 2011) and under pilot scale experimental production in Gran Canaria (Canary Islands; Spain) from 2010 (GIA, 2011).

Nonetheless, very few studies have been performed in order to determine *S. rivoliana* nutritional requirements (Roo *et al*., 2012; Fernández-Palacios *et al*., 2015). In this sense, several studies have been reported for other species from the same genus, such as *Seriola dumerili* (Garcia-Gomez, 2000; Tomas *et al*., 2005; Takakuwa *et al*., 2006; Papadakis *et al*., 2007; Hamasaki *et al*., 2009; Matsunari *et al*., 2012, 2013), *Seriola lalandi* (Cobcroft *et al.*, 2004) and *Seriola quinqueradiata* (Masuda *et al*., 1998; Ishizaki *et al*., 2001; Yamamoto *et al*., 2008; Takeuchi, 2014).

Long chain polyunsaturated fatty acids (LC-PUFAs) are determinant for the success of larvae rearing (Izquierdo, 2005). Moreover, the adequate performance of marine fish larvae is related to the inclusion of the n-3 LC-PUFA docosahexaenoic acid (DHA; 22:6n-3) in the diet, due to its direct relationship with tissues and cell functioning (Izquierdo & Koven, 2011). Not only DHA is an essential fatty acid (EFA) for larval rearing success, but also the importance of the omega 3 (n-3) and n-6 LC-PUFA eicosapentaenoic acid (EPA; 20:5n-3) and arachidonic acid (ARA; 20:4n-6) has been emphasized (Izquierdo, 1996). Besides, several studies indicated that DHA had a greater potential than EPA as an EFA for marine fish larvae (Watanabe *et al.*, 1989; Takeuchi, 2001; Izquierdo & Koven, 2011), being the DHA requirements more limiting for growth, survival (Izquierdo, 1996) and development of schooling behaviour (Masuda *et al*.,

1998; Ishizaki *et al*., 2001) than EPA. Contrarily, some studies observed that high levels of dietary DHA may cause muscular dystrophy (Betancor *et al*., 2011) or lead to the appearance of supernumerary vertebrae (Villeneuve *et al.,* 2006) in *Dicentrarchus labrax* larvae due to the peroxidation of DHA and the formation of toxic oxidized compounds.

On the other hand, the effects of dietary DHA deficiency have been reported in a variety of marine fish species, being characterized by an increase in the incidence of skeletal deformities in larvae of *Sparus aurata* (Roo *et al*., 2010; Izquierdo *et al*., 2013) and *Pagrus pagrus* (Roo *et al*., 2009; Izquierdo *et al*., 2010), as well as jaw anomalies in *Latris lineata* (Cobcroft *et al.*, 2001). The alteration in gut and liver tissues has also been described in *Latris lineata* (Bransden *et al*., 2005) due to DHA deficiency, as well as malpigmentation and irregular eye migration in flatfish (Bell *et al*., 2003) or reduction in stress resistance in *Huso huso* (Jalali *et al*., 2008).

Despite of the varied negative effects of inadequate dietary DHA levels in larval feeds, the low culture performance and survival in different larval species (Watanabe *et al*., 1989; Furuita *et al*., 1996a,b; Copeman *et al.*, 2002; Rezek *et al*., 2010) are still considered the main negative effects.

Due to the generally importance of DHA as a main dietary lipid for larval marine finfish rearing success, the purpose of this study was to evaluate the effect of increasing dietary DHA levels on biological performance and larval quality of *S. rivoliana*. In order to do so, five feeds containing increasing levels of DHA were fed to longfin yellowtail larvae from 30 to 50 dah and larvae growth, final survival, survival after a stress challenge, larvae fatty acid profile and incidence of skeletal anomalies were evaluated.

2. Material and methods

2.1. Broodstock and Larval rearing

S. rivoliana eggs were obtained from induced spawning of wild adults adapted to captivity at GIA (Grupo de Investigación en Acuicultura) facilities, using gonadotropin releasing hormone

analogue (LHRHa, des-Gly10, [D-Ala6]; Sigma- Aldrich, St. Louis, MO, USA) at a dose of 20 μg kg^{-1} body weight, based on the reported dosage for longfin yellowtail (Roo *et al.*, 2012). Larvae were reared under mesocosms rearing system following the methodology described by Roo *et al.*, (2012). In this way, 4.5 eggs l^{-1} were stocked in two 40m^3 tanks up to 29 days after hatching (dah). At 30 dah (11.31 ± 1.79 Total Length, TL), larvae were settled in 200 litre fibreglass cylinder tanks with conical bottom and painted a light grey colour (90 larvae per tank, in triplicates). Filtered seawater was supplied (37 gl^{-1} salinity) and water conditions were daily measured (temperature: 22.5 ± 0.6 ºC; oxygen levels: 6.5 ± 0.3 g l^{-1}; OxyGuard, Denmark). Photoperiod was kept at 12:12 (12 h light:12h dark) by fluorescent daylights at 1700 lux (digital Lux Tester YF-1065; Powertech Rentals, Osborne, Australia).

2.2. Experimental diets

Five isoproteic and isolipidic formulated diets, which varied in their DHA content were produced (Table 1). To the common ingredients of the formulated diets, DHA, EPA (DHA-50 and EPA-50, Croda Chemicals Ltd. Goole, U.K.) and ARA (Vevodar DSM Food Specialities, Netherlands) oils were added in graded amounts in substitution of oleic acid to maintain a constant lipid content (Table 1). Microdiets were manufactured according to Betancor *et al.* (2012) by mixing squid meal and water-soluble components, then the lipid and fat soluble vitamins and, finally, warm water dissolved gelatin. The paste was pelleted and dried in oven at 38ºC for 24h. Pellets were ground and sieved to obtain two particle sizes, from 250 to 500μm and from 500-710μm. Formulated diets were analyzed for proximate and fatty acid composition.

2.3. Growth, survival and activity test

Larval growth was assessed by estimating the TL of the larvae using a profile projector (Nikon V-12A, NIKON™, Tokyo, Japan) at 30, 42 and 50 dah. Final larvae survival was calculated by individually counting the larvae at the beginning and at the end of the trial. Additionally, an activity test was performed by subjecting fifteen larvae per tank to 30 seconds of air exposure at 42 and 50 dah and counting all the remaining surviving larvae after 24h (Izquierdo *et al.*, 1989).

A sample of larvae from each tank were washed with distilled water and kept at -80ºC for proximate analysis and fatty acid composition.

2.4. Osteological studies

For the skeletal anomalies characterisation, a total of 15 larvae (50 dah) per tank were fixed in 10% buffered formalin and stained with alizarin red according to the methodology of Vandewalle *et al.* (1998). Terminology described by Mesa-Rodriguez *et al.* (2014, 2016) was used for *S. rivoliana* bone structures identification. The different regions of the axial column were divided and evaluated according to Boglione *et al.* (2001).

2.5. Statistical analysis

All data were statistically treated using a SPSS Statistical Software System 15.0 (SPSS, www.spss.com). The significant level for all the analysis was set at 5% and results are given as mean values and standard deviation. All values presented as percentage were arcsine transformed. Also, all variables were checked for normality and homogeneity of variance, using the Kolmogorov-Smirnoff and the Levene tests, respectively. To compare means, the group data were statistically tested using one-way ANOVA. When variances were not homogenate, a non-parametric Kruskal-Wallis test was accomplished. To evaluate the differences in skeletal frequency of deformities log lineal statistical analysis were performed (Sokal & Rolf, 1995).

3. Results

S. rivoliana larval survival was positively correlated with increasing dietary DHA levels ($y=1.137x^2 - 4.121x + 73.48$; $R^2 = 0.890$); with values ranging from 69.63% at 0.25% (dw) dietary DHA to 81.48% with 3.17% (dw) dietary DHA (Fig. 1). In addition, the increase of dietary DHA significantly ($p < 0.05$) enhanced resistance to stress test (Fig. 2). On the other hand, no significant differences among treatments were observed in growth (Table 2) at mid (15.08±0.48 mm TL) and final sampling points (19.80±0.58 mm TL).

Increasing dietary levels of DHA lead to several changes in larval fatty acid profile after 20 days of feeding the experimental microdiets (Table 3). Total sum of saturated fatty acids showed to be the highest in larvae fed the highest DHA levels (3.17%, dw; Diet 5), showing intermediate values in the larvae fed with a 2% (dw; Diet 4) of DHA. Differences were also found in total monounsaturated fatty acid contents, finding the highest levels in larvae fed the lowest DHA levels (Diet 1), mainly due to increased contents of oleic acid (18:1n-9). The main saturated fatty acids (SFA) present in total body *S. rivoliana* larvae was palmitic acid (16:0) and stearic acid (18:0). Larval tissue showed a positive correlation on its DHA content with dietary DHA content, finding the lowest levels in fish fed with Diet1 and the highest in Diet5 (Table 3). ARA levels showed minor variations among dietary treatments, while a significant progressive decrease of EPA content was observed along with the increasing dietary DHA ($p< 0.05$). Total n-3 and total n-3 PUFA levels were positively correlated with the DHA increase in dietary treatments. All the FA ratios were significantly ($p< 0.05$) affected by dietary treatment, thus ARA/EPA, DHA/EPA, DHA/ARA and n-3/n-6 ratios were increased according to the DHA contents in microdiets while the opposite trend were observed in oleic/DHA and oleic/n-3 PUFA ratios (Table 3).

Regarding the characterization of skeletal anomalies, scores showed no significant differences among dietary treatments ($p>0.1$). The occurrence of cranial (jaw) abnormalities (6.7 - 4.4%) was only observed in larvae fed with the lowest dietary DHA treatments (Diet1 and Diet2, respectively). However, a reduced incidence of skeletal deformities was obtained in larvae fed the lowest dietary DHA treatment (Diet1), whereas increasing dietary DHA content seemed to promote an increase in the number of total skeletal anomalies (kyphosis, lordosis, abnormal vertebra and cranial), being the larvae fed with diet Diet4 the ones that showed the highest number of total anomalies. Furthermore, severe anomalies such us kyphosis and lordosis were absent in larvae fed with the Diet1 treatment. The occurrence of kyphosis and lordosis increased along with the dietary DHA contents (Fig. 3). Moreover, the occurrence of kyphosis was only observed in larvae fed with the highest dietary DHA treatments (Diet4 and Diet5). Additionally,

the incidence of abnormal vertebra centra was also in concordance with the increasing dietary DHA content.

4. Discussion

The inclusion of dietary DHA in inert diets up to a 3.71 % (dw) increased the final survival in *S. rivoliana* larvae (81.5%), being higher than previous studies with other marine fin fish species such as *S. aurata* (48%, Saleh *et al*., 2013; 45%, Hernández-Cruz *et al*., 2015) or *Dicentrarchus labrax* (73%, Cahu et al., 2003). Species from the same genus fed during early larval stages with live preys enriched with DHA displayed enhanced final larval survival (Furuita *et al*., 1996b; Ishizaki *et al*., 1998; Takeuchi *et al*., 1998; Yamamoto *et al*., 2008; Matsunari *et al*., 2012). For instance, *S. quinqueradiata larvae* fed with DHA enriched *Artemia sp*. (2.5%, dw), showed enhanced final survival (88.5%) at 13 dah (Ishizaki *et al*., 1998). Another study in *S. dumerili* found the highest larval survival during first 7 days (22%), with DHA content up to 2.0% (dw; Matsunari *et al*., 2012). In the other hand, Yamamoto and collaborators (2008) stated that DHA contents between 0.7-1.3% (dw) in rotifers and 1.2-2.1% (dw) in *Artemia sp*. did not satisfy DHA larval requirements for *S. dumerili*.

The increase of both dietary DHA and EPA in the feed has been proved to improve, not only larval performance, but also stress resistance (Liu *et al*., 2002; Izquierdo, 2005). In this sense, EFA play an important role as eicosanoids precursors (Ganga *et al*., 2005) which play a pivotal role in stress response and immune system (Sargent *et al*., 1995). In the present study, *S. rivoliana* larvae fed increasing DHA levels from 0.25% to 3.17% (dw) showed improved resistance to air exposure along with the dietary increase of DHA. Similar results have been observed for *S. aurata* (Saleh *et al*., 2013, 2015) in which larvae fed with high levels of dietary marine phospholipids with higher levels of DHA showed better survival rate after handling. Additionally, the deficiency of DHA may reduce the tolerance to stressful conditions as observed in *Huso huso* larvae (Jalali *et al*., 2008). It is known that deficiencies in structural components due to nutritional privation may produce a range of effects in the membrane of

immune cells. These structural changes caused by component deficiencies in the membrane can alter eicosanoids production and membrane permeability. Moreover, cell membrane changes can also modulate the alternative complement pathway (ACP) activity as well as the immune response in fish (Montero *et al*., 1998).

On the other hand, inclusion of dietary DHA did not significantly affect *S. rivoliana* larval growth. Similar results have been reported in other marine teleost species, such as *Sparus aurata* (Izquierdo *et al*., 2013; Hernández-Cruz *et al*., 2015), *Pagrus pagrus* (Roo *et al*., 2009), *Coryphaena hippurus* (Kraul, 1993) and *Centropomus parallelus* (Seiffert *et al*., 2001), where fish performance was not influenced by increasing dietary levels of DHA. Contrarily to what could be expected taking into account other studies from the *Seriola* genus (Furuita *et al*., 1996b; Takeuchi *et al*., 1998; Matsunari *et al*., 2012), larval growth was slightly higher in the lowest DHA dietary content (Diet1), albeit no significant differences were observed. This could be explained due to the decreased population density which in turn translates into higher amount of feed available per larvae, as larvae fed Diet1 displayed the lowest final survival although no significant differences were detected. Moreover, an unbalanced DHA/EPA ratio seems to affect the growth in certain fish species (Izquierdo, 1996,2005; Takeuchi *et al*., 1997; Shiozawa *et al*., 2003;), indicating that not only the increasing levels of dietary DHA could promote the larvae final survival and growth, but also an adequate ratio DHA to EPA. In this sense, Matsunari and collaborators (2012) observed the maximum total length in *S. dumerili* larvae fed a DHA/EPA ratio between 1.4 and 2.9, being this ratio much lower than the ones used in the present trial (up to 7.2).

The DHA/EPA ratio has been correlated with the dietary DHA supplementation. Due to the importance of handling stress resistance of the larvae, in the present study, the best result were obtained when the DHA/EPA ratio was above 3.1. This result is in agreement with the ratio obtained in the tissues of wild specimens of the same genus such as *S. lalandi* and *S. dumerili* with DHA/EPA ratios of 3.5 and 5.6 respectively (O'neill *et al*., 2015; Haouas *et al*., 2010). Whitmore, *S. rivoliana* larvae fed with Diet 1 and Diet2 with a DHA/EPA ratio lower than 1.4

showed significantly poor survival after activity test (Fig. 2), being in concordance with the minimum ration suggested by Matsunary *et al*. (2012) of at least 1.4 for *S. dumerili* larvae. However, in other marine fish species, the optimum dietary DHA/EPA ratio during larval development seemed to be about 1.4 as it is the case for *Pagrus pagrus* (Hernández-Cruz *et al*., 1999), 0.32 for *Dentex dentex* (Mourente *et al*., 1999), 1.2 for *S. aurata* (Rodríguez *et al*., 1997) and 1.5 for *Lateolabrax japonicus* (Xu *et al*., 2014). In these sense, it seems that *S. rivoliana* larvae needs higher DHA/EPA ratios than other commercially produced marine species.

As expected, the fatty acid compositions of larvae whole body mirrored the increasing dietary DHA levels. Therefore, larvae fed with high DHA contents consequently accumulated higher DHA and total n-3 LC-PUFA levels. Whitmore, the increase of monounsaturated fatty acid (MUFA) levels, mainly oleic acid (18:1n-9) in larvae, was correlated with the low dietary DHA inclusion, given that olive oil, naturally rich in 18:1n-9, was used to equalize the lipid levels in the feeds. Contrarily, total body larvae fatty acid profile displayed increasing levels of total SFA when dietary DHA levels were increased, instead of decreasing its content with the minor amount of oleic. This is in agreement with other studies from species of the same genus, in which the comparison between wild and reared specimens showed that main MUFA presented in muscle samples of both wild and reared fish was the 18:1n-9, being the total amount of MUFA higher in the wild rather than reared fish (*S. lalandi*, O'Neill *et al*., 2015; *S. dumerili*, Rodriguez-Barreto *et al*., 2012, 2014). In this sense, a comparison between reared and wild specimens of *S. quinqueradiata* determined that the triglycerides content observed in reared fish was higher than in wild fish, as well as the amount of n-3PUFA, particularly DHA (Arakawa *et al*., 2002). Curiously, in other marine teleost species, increased DHA levels did not result in alterations in the total SFA content in larvae tissue (Izquierdo *et al*., 2013; Hernández-Cruz *et al*., 2015).

Regarding skeletal abnormalities, the occurrence of cranial abnormalities in *Seriola sp*. has been previously reported (Cobcroft *et al*., 2004). This author suggested that the inclusion of high DHA/EPA ratios, particularly around notochord flexion stages, and certain environmental

factors such as light conditions may contribute to "Wall-nosing" behaviour and the apparition of jaw malformations in yellowtail kingfish (*Seriola lalandi)* culture. In the present study, the reduction of cranial abnormalities was concomitant with the increased dietary DHA content. In previous studies, the appearance of skeletal muscle lesions (Betancor *et al*., 2011) and the occurrence of skeleton anomalies (Villeneuve *et al*., 2005; Izquierdo *et al*., 2010, 2013) were associated with increased dietary DHA levels. In this way, the incidence of skeletal anomalies in *S. rivoliana* larvae in the present study was could be related with the high dietary DHA levels supplementation, albeit no significant differences were observed. Furthermore, the occurrence of severe anomalies such as kyphosis and lordosis, was mainly correlated to the larvae fed with the highest levels of dietary DHA (Spearman correlation, ρ=0.9). In this sense, severe deformities of the vertebral column always involve abnormalities over a relative wide range of vertebrae, which can appear fused and deformed mostly in the region of maximal axis curvature (Boglione *et al*., 2011), being in agreement the number of severe abnormalities with abnormal vertebral bodies observed in the present study.

In general, other studies demonstrated that an excessive dietary DHA content increases peroxidation risks (Betancor *et al*., 2011) and consequently, the proliferation of reactive oxygen species (ROS) and the formation of toxic compounds that negatively affect bone development (Izquierdo *et al*., 2010). This peroxidation risk seems to be directly related to the pellet sizes, being more exposed the microdiets during larval feeding due to major surface area in contact with sea water than volume ratio of the pellet (Betancor, 2012).

The relationship between n-3 LC-PUFA and the bone formation mechanism is still unknown. Previous studies in sea bream larvae indicated that DHA inclusion increased the n-3/n-6 ratio and could promote ossification (Izquierdo *et al*., 2013), reduce vertebral fusion and cranial deformities in *P. pagrus* (Roo *et al*., 2009) and decrease the incidence of opercular deformities in *Chanos chanos* (Gapasin & Duray, 2001). Moreover, low dietary DHA levels can delay early mineralization and increase the risk of cranial and axial skeletal deformities in sea bream larvae

(Izquierdo *et al*., 2013). Thus, high dietary DHA levels and adequate balance between pro and antioxidant nutrients seem to promote good skeletal health.

In summary, the results of the present study proved that the inclusion of dietary DHA in inert diets up to a 3.17% (dw) and a DHA/EPA ratio above 3.1 increased the final survival and stress resistance in *S. rivoliana* larvae. Further studies on EFA requirements are required in order to enhance *S. rivoliana* larval production.

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7. List of tables and figures

Table 1. Ingredients and proximate composition of the experimental microdiets containing increasing levels of DHA.

Diet	***Diet 1***	***Diet 2***	***Diet 3***	***Diet 4***	***Diet 5***
			Ingredients (g kg^{-1}diet)		
Defatted Squid meal [a]	626.9	626.9	626.9	626.9	626.9
DHA-50 [b]	0	20	500	70	90
EPA 50 [b]	20.5	17.5	12.5	10.0	6.5
ARA [c]	12.5	12.5	10.0	10.0	8.0
Oleic acid	114.5	97.5	75.0	57.5	43.0
Soy Lecithin	30.0	30.0	30.0	30.0	30.0
Vitamin mixture [d]	64	64	64	64	64
Mineral mixture [d]	45.7	45.7	45.7	45.7	45.7
Attractant [d]	55.9	55.9	55.9	55.9	55.9
Gelatin	30	30	30	30	30
			Proximate and FA analysis (g kg^{-1}diet)		
Proteins (N×6.25)	517.7	590.3	592.2	596.4	603.9
Lipids	205.4	194.6	204.9	191.1	185.2
Moisture	33.6	32.6	27.8	27.2	27.9
Ash	64.1	64.1	65.0	63.7	65.7
Energy (MJ/kg)[f]	1,638.92	1,719.44	1,761.45	1,716.44	1,706.72
DHA (%TFA/DW)	2.76/ 0.25	8.90/ 0.75	18.35/ 1.64	25.83/ 1.99	35.26/ 3.17
EPA	6.42/ 0.58	6.58/ 0.56	5.91/ 0.53	5.64/ 0.44	4.88/ 0.44
ARA	3.36/ 0.3	3.73/ 0.32	3.76/ 0.94	4.14/ 0.32	4.11 / 0.37
Saturated	15.83/1.43	15.04/1.27	14.20/1.27	12.97/1.00	11.59/1.04
Monosaturated	56.74/5.12	50.87/4.3	42.07/3.75	36.00/2.78	28.40/2.55

[a] Squid meal (Agramar, Lorient, France),
[b] DHA-50, EPA-50 Croda Chemicals Ltd. Goole, U.K.
[c] VEVODAR Oil.
[d] Betancor et al., 2012
Energy calculated as: fat×37.7 MJ/kg; protein×16.7 MJ/kg;

Table 2. *S. rivoliana* total length from 30 to 50 dah fed formulated diets with increasing levels of dietary DHA (0.25, 0.75, 1.65, 2.0 and 3.17 $g.kg^{-1}$dw DHA). No significant differences were observed ($p<0.05$).

	30 dah	**42 dah**	**50 dah**
Diet 1	11.31 ± 1.79	15.91 ± 2.18	20.78 ± 3.54
Diet 2	11.31 ± 1.79	14.87 ± 2.01	19.82 ± 3.49
Diet 3	11.31 ± 1.79	15.02 ± 2.00	19.47 ± 2.86
Diet 4	11.31 ± 1.79	14.66 ± 2.09	19.60 ± 3.35
Diet 5	11.31 ± 1.79	14.94 ± 2.07	19.32 ± 2.59

Table 2. Total fatty acid composition (%TFA) of 50dph larvae fed microdiets with increased levels of DHA (0.25, 0.75, 1.65, 2.0 and 3.17 g.kg^{-1}dw DHA).

	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5
			Fatty acid content (%TFA)		
14:0	0.26	0.27	0.29	0.30	0.39
14:1n-5	0.03	0.02	0.05	0.04	0.04
14:1n-7	0.01	0.01	0.01	0.01	0.01
15:0	0.12	0.13	0.16	0.16	0.21
15:1n-5	0.01	0.01	0.01	0.01	0.01
16:0iso	0.02	0.03	0.03	0.03	0.04
16:0	13.44	12.8	13.83	14.43	16.78
16:1 n-7	0.84	0.65	0.61	0.53	0.60
16:1n-5	0.07	0.07	0.10	0.13	0.16
16:2n-6	0.02	0.03	0.02	0.03	0.03
16:2n-4	0.17	0.21	0.24	0.31	0.38
17:0	0.03	0.03	0.03	0.04	0.04
16:3n-4	0.18	0.15	0.15	0.15	0.16
16:3n-3	0.04	0.04	0.05	0.06	0.07
16:3n-1	0.47	0.71	0.71	0.89	0.97
16:4n-3	0.45	0.64	0.58	0.68	0.65
16:4 n-1	0.05	0.10	0.11	0.13	0.14
18:0	5.8	6.48	6.90	8.01	9.19
18:1 n-9	41.11^{d}	31.02^{c}	23.79^{b}	20.58^{ab}	17.67^{a}
18:1 n-7	1.19	1.99	2.06	2.02	2.19
18:1 n-5	0.04	0.04	0.04	0.05	0.06
18:2n-9	0.09	0.09	0.08	0.09	0.11
18:2 n-6	12.18^{b}	10.73^{b}	10.72^{a}	8.66^{a}	8.40^{a}
18:2n-4	0.09	0.09	0.07	0.07	0.06
18: 3n-6	0.30	0.31	0.29	0.18	0.22
18:3n-4	0.06	0.063	0.05	0.03	0.04
18:3 n-3	1.30	1.16	1.18	0.94	0.94
18:3n-1	0.006	0.007	0.004	0.004	0.002
18:4 n-3	0.30	0.33	0.31	0.25	0.22
18:4 n-1	0.037	0.033	0.024	0.024	0.028
20:0	0.35	0.32	0.34	0.40	0.47
20:1 n-9	0.041	0.044	0.06	0.06	0.07
20: 1n-7	0.95	0.88	0.89	0.98	1.11
20: 1n-5	0.065	0.076	0.08	0.09	0.12
20: 2n-9	0.04	0.041	0.04	0.037	0.046
20:2 n-6	0.27	0.25	0.26	0.31	0.35
20:3n-9+n-	0.02	0.02	0.015	0.017	0.015
20:3 n-6	0.35	0.31	0.24	0.24	0.20
20:4 n-6 (ARA)	4.68^{ab}	5.25^{b}	4.74^{ab}	4.92^{ab}	4.51^{a}
20: 3n-3	0.17	0.18	0.20	0.22	0.24
20:4 n-3	0.31	0.26	0.21	0.18	0.17

Table 2. Total fatty acid composition (%TFA) of 50dph larvae fed microdiets with increased levels of DHA (0.25, 0.75, 1.65, 2.0 and 3.17 g.kg^{-1}dw DHA); (Continued).

	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5
	Fatty acid content (%TFA)				
20:5 n-3 (EPA)	5.34^{c}	5.23^{c}	4.19^{ab}	3.28^{bc}	2.55^{a}
22:1 n-11	0.05	0.07	0.10	0.08	0.12
22:1 n-9	0.23	0.25	0.24	0.25	0.30
22:4 n-6	0.28	0.32	0.34	0.40	0.42
22:5 n-6	0.20	0.69	1.13	1.37	1.49
22:5 n-3	1.28	1.30	1.12	1.06	0.98
22:6 n-3 (DHA)	6.68^{a}	16.26^{b}	23.26^{c}	27.23^{c}	26.97^{c}
Satured	19.97^{a}±2.66	20.04^{a}±1.19	21.55^{a}±1.49	23.35^{ab}±0.97	27.09^{b}±3.47
Monoenoics	44.63^{d}±4.44	35.12^{c}±0.98	28.05^{b}±1.89	24.82^{ab}±0.83	22.47^{a}±2.49
Total n-3	15.87±3.36	25.40±2.28	31.10±2.35	33.91±1.93	32.80±5.22
Total n-6	18.27±0.92	17.90±1.10	17.76±0.86	16.11±0.78	15.62±1.37
Total n-9	41.51±3.26	31.44±0.71	24.20±1.55	21.01±0.62	18.19±1.78
Total n-3PUFA	13.78±3.01	23.22±2.18	28.98±2.16	31.98±1.80	30.91±5.09
ARA	4.67±0.44	5.25±0.19	4.74±0.08	4.92±0.04	4.51±0.25
EPA	5.34±0.93	5.23±0.26	4.18±0.39	3.28±0.20	2.55±0.37
DHA	6.68±1.68	16.26±1.78	23.26±1.70	27.23±1.57	26.97±4.53
ARA/EPA	0.88^{a}±0.10	1.01^{a}±0.72	1.13^{a}±0.21	1.50^{b}±0.19	1.76^{b}±0.69
DHA/EPA	1.25^{a}±0.11	3.11^{b}±0.21	5.55^{c}±0.62	8.30^{d}±0.25	10.55^{e}±0.63
DHA/ARA	1.43^{a}±0.27	3.09^{b}±0.22	4.90^{c}±0.27	5.53^{c}±0.28	5.97^{c}±0.74
oleic/DHA	6.16±1.91	1.91±0.38	1.02±0.87	0.76±0.37	0.65±0.37
oleic/n-3PUFA	2.98±1.07	1.34±0.31	0.82±0.69	0.64±0.3	0.57±0.33
n-3/n-6	0.87^{a}±0.16	1.42^{b}±0.15	1.75^{bc}±0.15	2.11^{c}±0.17	2,10^{c}±0.23

PUFA, polyunsaturated fatty acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; ARA, arachidonic acid. Data expressed as means ± SD (n = 3). Different superscript letters within a row denote significant differences among diets ($p < 0.05$).

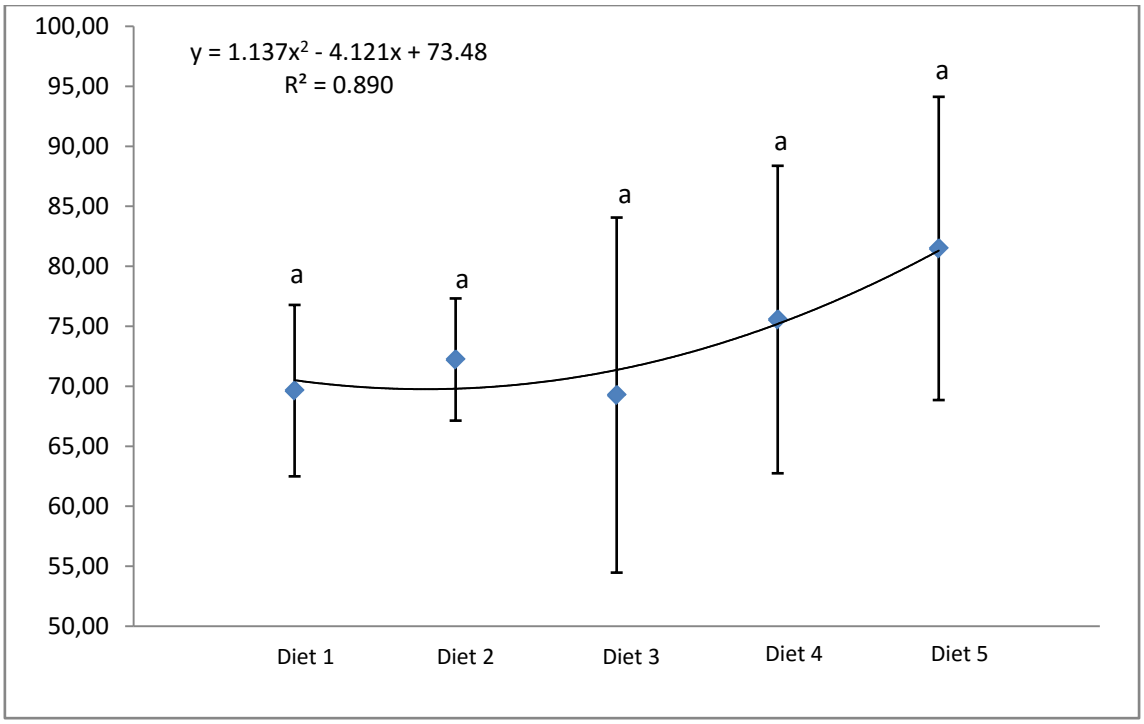

Figure 1. Survival rates (% of initial population) of *S. rivoliana* larvae fed formulated diets with increasing levels of dietary DHA (0.25, 0.75, 1.65, 2.0 and 3.17 g.kg^{-1}dw DHA) from 30 to 50 dah. Points show mean ± standard deviation of three replicate tanks per diet, same letters denote that data are not significantly different (P >0.05).The regression model represented by a line: survival = 1.137*(DHA)^2 - 4.121*DHA + 73.48, where DHA is g kg^{-1} of dietary DHA (polynomial regression, order 2).

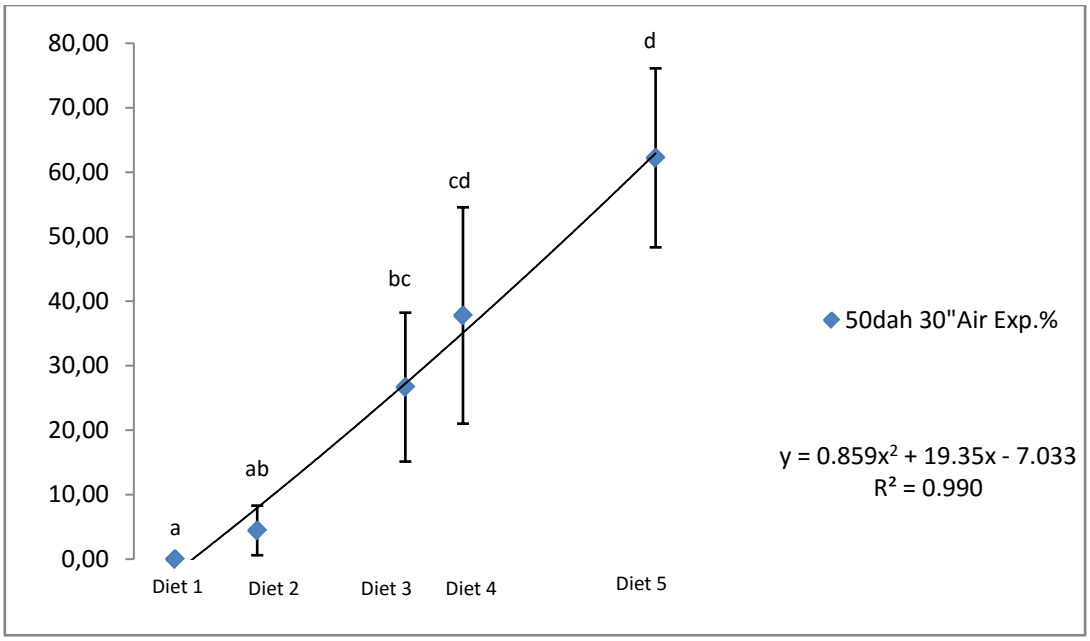

Figure 2. Survival rates 24 h after activity test of *S. rivoliana* larvae fed formulated diets with increasing levels of dietary DHA (0.25, 0.75, 1.65, 2.0 and 3.17 g.kg^{-1}dw DHA) from 30 to 50 days after hatch. Activity test at 50 dah consisted of 30 s air exposure. Points show mean ± standard deviation of different treatments, different letters denote that data were significantly different ($P < 0.05$). (Pearson correlation is 0.99 with a significance of p=0.001). The regression model represented by a line: survival = 0.859*(DHA)^2 - 19.35*DHA + 7.033, where DHA is g. kg^{-1} dw of dietary DHA (polynomial regression, order 2).

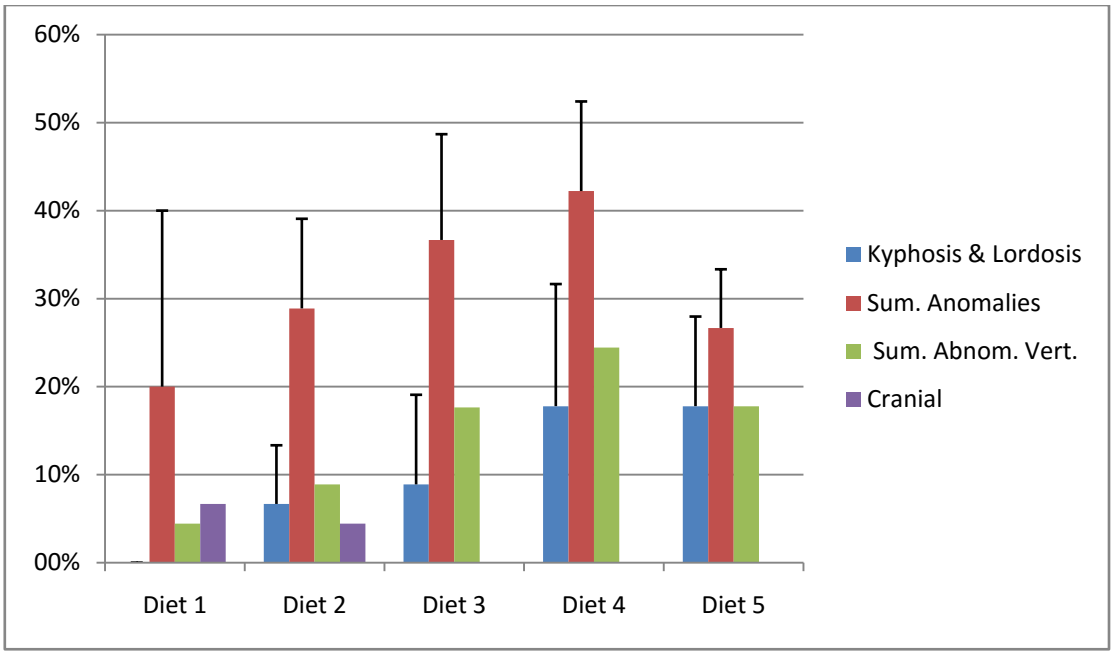

Figure 3. Incidence of skeletal deformities in *S. rivoliana* larvae fed formulated diets with increasing levels of dietary DHA (0.25, 0.75, 1.65, 2.0 and 3.17 $g.kg^{-1}$dw DHA) at 50 dah. Sum. Anomalies (cranial + abnormal vertebra + fusion of vertebra + Kyphosis + Lordosis); Sum. Abnormal vertebra (fusion of vertebra + abnormal vertebra); Cranial (abnormal jaw).

Conclusions

1. Fist report of *S. rivoliana* larval rearing in Europe.

2. Yolk sac reserves of S. rivoliana larvae are completely absorbed at 4dah. At this age, the larvae shows eye pigmentation and mouth and anus are opened. The oil droplet remained present until 8dah.

3. *Brachionus sp.* (L-strain) seem to be adequate for first feeding of *S. rivoliana* larvae. The presence of larvae with faeces, including undigested and even live Artemia, demonstrated the low digestibility of this prey, which leads to larval malnutrition when Artemia is the main source of food.

4. Theoretical prey availability in relation with initial larval density between the intensive and semi-intensive systems does not seem to affect feeding incidence.

5. Two different growing periods could be identified during the development of *S. rivoliana*: one slow daily growth in coincidence with the pre-flexion (0-15 dah) stages, and one with accelerated daily growth post notochord flexion (15-30 dah).

6. Two main mortalities periods could be identified: from 8 to 12 dah, that could be associated to low success at first feeding and nutritional imbalances in broodstock; and a second period after 20 dah, with the apparition of aggressive behaviour and cannibalism.

7. The improved larval survival in the semi-intensive system in comparison with intensive system would suggest that *S. rivoliana* larval rearing should be performed following the semi-intensive system regime. At 30 dah, weaned larvae presented a TL between 15.0-16.0 mm, wet weight ranging from 0.15 to 0.3 g.

8. This Thesis reported for first time the skeletal development and mineralization of *S. rivoliana*. The first structures in mineralize are those associated with the feeding and breathing (3. 43 ± 0.15 and 3.75 ± 0.14 mm SL, respectively), supporting the importance of feeding and breathing at early stages.

9. Identification of pre and post-flexion stages is an useful external indicator of the mineralization of vertebral column, caudal complex and paired fins (5.12 ± 0.11 mm SL).

10. The first fin structure in mineralize was the cleithrum at 3.75 ± 0.14 mm SL, and shortly followed by the supracleithrum and posttemporal, from the pectoral fin. The pelvic fin started to mineralized by 6.16 ± 0.32 mm SL with the spine and continued with the soft rays (lepidotrichium) and basipterygium. The first dorsal and anal fin structures were the hard spines and soft spines (lepidotrichium) by 8.01 ± 0.26 mm SL, which initiated its mineralization from the base to the tip of the structure.

11. The fusion of different structures to form the urostyle suggest that is nonspecific of the genus *Seriola*, nontheless, the development of three distinct hypural (Hy) structures during caudal complex mineralization (Hy1+Hy2, Hy3+Hy4, Hy5) could remain as a characteristic of carangids.

12. In *S. rivoliana*, a total of 24 vertebral structures were observed, following a unidirectional mineralization pattern, differing from the bidirectional pattern common in most Perciforms.

13. The inclusion of dietary DHA in feed up to a 3.17% (dw) improved larval resistance to air exposure. Also, DHA did not significantly affect fish final growth or final survival. The results of the present Thesis suggest that an adequate DHA/EPA ratio should be above 3.1.

14. The incidence of skeletal anomalies in *S. rivoliana* larvae was correlated with the high dietary DHA levels supplementation, albeit no significant differences were observed. The occurrence of severe anomalies such as kyphosis and lordosis, was mainly associated to the larvae fed with the highest levels of dietary DHA.

15. DHA/EPA ratio and the role of n-3 LC-PUFA role in bone formation in *S. rivoliana* bone should also be studied to understand the requirements of this emerging fast growth species.

Spanish Summary

Resumen

Seriola rivoliana está considerada como una de las especies más relevante para la diversificación en acuicultura, sin embargo son pocos los estudios realizados con esta especie. Es por ello que el principal objetivo del presente trabajo fue mejorar y puesta a punto las técnicas de producción larvaria de medregal negro (*S. rivoliana*). En este sentido, se establecieron tres objetivos específicos, en los que se evaluaron las técnicas de cultivo más apropiadas, un estudio específico del desarrollo de los huesos y el incremento en los niveles de ácido docosahexaenoico (22:6n-3; DHA) en la microdieta.

Durante el presente estudio se realizó por primera vez en Europa un cultivo larvario de medregal negro. Se identificaron dos períodos de crecimiento larvario, un primer período de crecimiento lento durante los primeros 15 días post eclosión (dpe) y previos a la iniciación de la flexión de la notocorda, y un segundo período con crecimiento mucho más rápido comprendido entre la post flexión y los 30 dah. Por otro lado, se pudo identificar dos picos de mortalidad bien diferenciados, siendo un primer pico entre los 8 y 12 días, pudiendo ser asociado a la baja eficiencia predatoria y deficiencias nutricionales en el stock de reproductores; y un segundo pico de mortalidad después de los 20 días, asociado a la aparición de comportamientos agresivos y la presencia de larvas débiles flotando en la superficie. La utilización de *Brachionus sp.* (L-strain) a lo largo de todo el cultivo indica la idoneidad de esta presa viva como alimento en los primeros estados larvarios. Por el contrario, la continua aparición de *Artemia sp*. sin digerir evidencia su poca digestibilidad y las consecuencias nutricionales en las larvas de *S. rivoliana* durante su alimentación (15-30 dpe). Los resultados obtenidos entre sistema intensivo y semi-intensivo con larvas de *S. rivoliana* sugieren que el cultivo deba realizarse en condiciones semi-intensivas.

En la presente Tesis se describe por primera vez el desarrollo y patrón de mineralización de los huesos de *S. rivoliana*. Las primeras estructuras en mineralizar fueron las estructuras relacionadas con la respiración y las mandíbulas (3.75 ± 0.14 mm y 3. 43 ± 0.15 mm longitud estándar, SL), corroborando la importancia de la alimentación y respiración en estadios larvarios inicial. La identificación de los estadios pre y post flexión se presenta como un indicador externo de la mineralización de la columna vertebral, la aleta caudal y aletas pares (5.12 ± 0.15 mm SL). Merísticamente, *S. rivoliana* presenta un total de 24 estructuras vertebrales cuya mineralización sigue un patrón unidireccional, a diferencia del patrón bidireccional mayoritario descrito en Perciformes.

En relación al incremento en los niveles de DHA en las microdietas, su aumento hasta un 3.17% (peso seco, dw) mejoró la tolerancia de exposición al aire de las larvas. Además, el crecimiento y la supervivencia finales no se vieron significativamente afectadas por el incremento en DHA. Por el contrario, altos niveles de DHA tienden a incrementar el índice de anomalías esqueléticas en larvas de *S. rivoliana*, pero sin diferencias estadísticamente significativas. Sin embargo, la aparición de anomalías severas como cifosis o lordosis están directamente relacionadas con el incremento de DHA en la microdieta.

Adicionalmente, un ratio adecuado de ácidos grasos poliinsaturados de cadena larga (LC-PUFA) omega 3 (n3) como el DHA y el ácido eicosapentaenoico (20:5n-3; EPA) deben de ser superior a 3.1. Este ratio y el rol que desempeñan los n-3 LC-PUFA en el desarrollo osteológico de *S. rivoliana* deberían de ser estudiados para una mejor comprensión de los requerimientos en especies de rápido crecimiento. Lo que es más, sería conveniente desarrollar estudios basados en los requerimientos de ácidos grasos esenciales (EFA) para mejorar la producción larvaria de *S. rivoliana.*

Objetivos

Seriola rivoliana está considerada como una de las especies más relevante para la diversificación en acuicultura, sin embargo son pocos los estudios realizados con esta especie. Es por ello que el principal objetivo de la presente Tesis fue la mejora y puesta a punto las técnicas de producción larvaria de medregal negro (*S. rivoliana*). Para ello, se establecieron los siguientes objetivos:

1. Evaluación de las distintas técnicas de cultivo en el desarrollo larvario.

Las primeras puestas de *S. rivoliana* obtenidas en las Islas Canarias fueron sembradas bajo dos técnicas de cultivo diferentes. En este estudio se pretende determinar cuál de las técnicas de cultivo larvario (comparación entre los sistemas intensivo y semi-intensivo) es la más apropiada para la obtención de una mayor supervivencia en la fase inicial del cultivo.

2. Descripción del desarrollo osteológico de las larvas de S. rivoliana.

La obtención de un correcto desarrollo larvario está directamente relacionado con los requerimientos nutricionales, protocolos de cultivo y alimentación. Una deficiencia en cualquiera de estos aspectos conlleva a la obtención de bajas tasas de supervivencia y la aparición de anomalías esqueléticas. Teniendo en cuenta la importancia del desarrollo osteológico para la correcta identificación de anomalías esqueléticas a edades tempranas, se plantea el estudio de su desarrollo en larvas de *S. rivoliana*.

El desarrollo osteológico se dividió en dos sub-objetivos, atendiendo a las diferentes regiones estudiadas:

2.1. Desarrollo esquelético y patrón de mineralización en la columna vertebral y aleta dorsal, anal y caudal.

2.2. Desarrollo esquelético de la zona craneal, aletas pectorales y pélvicas.

3. Evaluación del incremento en los niveles de DHA en microdietas.

Teniendo en cuenta la importancia del DHA en los estadios larvarios de peces marinos, en este estudio se pretende evaluar el efecto del incremento en los niveles de DHA en el desarrollo de las larvas, así como de la supervivencia final, tolerancia al manejo y la incidencia en la formación de anomalías esqueléticas.

1. Se describe por primera vez el cultivo larvario de *S. rivoliana* en Europa.

2. Las reservas del saco vitelino de S. rivoliana queda totalmente absorbida a los 4 días post eclosión (dpe). A esta edad, el ojo está pigmentado y tanto el ano como la boca están abiertos.

3. *Brachionus sp.* (L-strain) parece ser una adecuada presa viva inicial para larvas de *S. rivoliana*. La presencia de larvas con heces, incluyendo *Artemia sp.* sin digerir e incluso viva, demuestra la baja digestibilidad de esta presa, lo que conlleva a una mala nutrición de las larvas durante su alimentación.

4. La disponibilidad teórica de presas vivas en relación con la densidad larvaria inicial correlacionadas con los sistemas intensivos y semi-intensivos parece no afectar el índice de ingesta.

5. Se identificaron dos periodos de crecimiento durante el desarrollo de *S. rivoliana*: un primer período de crecimiento lento durante los primeros 15 dpe y previos a la iniciación de la flexión de la notocorda, y un segundo período con crecimiento mucho más rápido comprendido entre la post flexión y los 30 dpe.

6. Se identificaron dos picos de mortalidad bien diferenciados, un primer pico entre los 8 y 12 días, pudiendo estar asociado a la baja eficiencia predatoria y deficiencias nutricionales relacionadas con en el stock de reproductores; y un segundo pico de mortalidad después de los 20 días, asociado a la aparición de comportamientos agresivos y la presencia de larvas débiles flotando en la superficie.

7. La mejora de la supervivencia larvaria un en sistema de cultivo semi-intensivo en comparación con el sistema de cultivo intensivo sugiere que, el cultivo de las larvas de *S. rivoliana* debería realizarse bajo condiciones de cultivo semi-intensivo. A los 30 dpe, las larvas destetadas presentaban una longitud total entre 15.0-16.0 mm, y un peso húmedo entre 0.15 y 0.3 g.

8. En la presente Tesis se describe por primera vez la mineralización y el desarrollo esquelético de *S. rivoliana*. Las primeras estructuras en mineralizar son las relacionadas con la mandíbula y respiración (3. 43 ± 0.15 y 3.75 ± 0.14 mm longitud estándar, LS; respectivamente), corroborando la importancia de la alimentación y respiración en estadios larvarios inicial.

9. La identificación de los estadios pre y post flexión se presenta como un indicador externo de la mineralización de la columna vertebral, la aleta caudal y aletas pares (5.12 ± 0.15 mm LS).

10. La primera estructura del conjunto de aletas en mineralizar fue el cleithrum a los 3.75 ± 0.14 mm LS, seguido del supracleithrum y posttemporal, pertenecientes a la aleta pectoral. Las aletas pélvicas comienzan a mineralizarse con las espinas a los 6.16 ± 0.32 mm LS, seguido de los radios blandos(lepidotrichium) y el basipterygium, al igual que ocurre con las aletas dorsales y anales, empezando con 8.01 ± 0.26 mm LS y con una mineralización característica de la base hasta el extremo de las mismas.

11. La fusión de diferentes estructuras para formar el urostilo no sugiere una especificidad dentro del género *Seriola*, mientras que el desarrollo de tres estructuras independientes de hipurales (Hy) para formar el complejo (Hy1+Hy2, Hy3+Hy4, Hy5) podría mantenerse como un patrón característico de los carángidos.

12. *S. rivoliana* presenta un total de 24 estructuras vertebrales cuya mineralización sigue un patrón unidireccional, a diferencia del patrón bidireccional mayoritario descrito en Perciformes.

13. El incremento de DHA en las microdietas para *S. rivoliana* hasta un 3.17% (peso seco) mejoró la tolerancia de exposición al aire. Además, el crecimiento y la supervivencia finales no se vieron significativamente afectadas por el incremento en DHA. Los resultados de la presente Tesis sugieren que un ratio adecuado de DHA/EPA debe ser superior de 3.1.

14. El índice de anomalías esqueléticas en larvas de *S. rivoliana* se correlacionan con los altos niveles de DHA presentes en la dieta, pero no se observaron diferencias estadísticamente significativas. La aparición de anomalías severas como cifosis o lordosis están directamente relacionadas con el incremento de DHA en la microdieta.

15. El ratio de DHA/EPA y el rol que desempeñan los n-3 LC-PUFA en el desarrollo osteológico de *S. rivoliana* debería de ser estudiado para una mejor comprensión de los requerimientos nutricionales en especies de rápido crecimiento

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