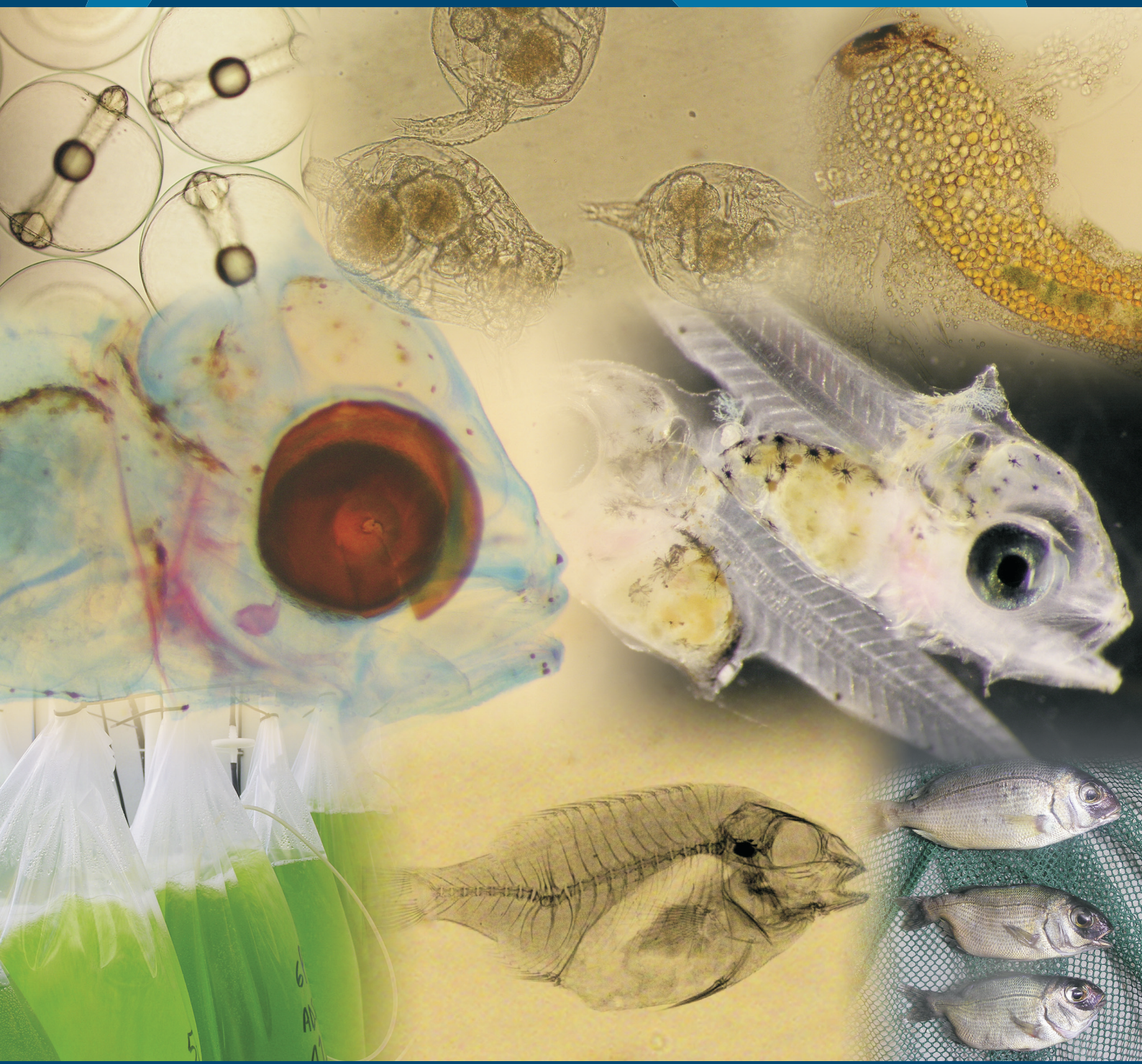
TESIS DOCTORAL

Improvements in the production technology of red porgy (*Pagrus pagrus*) larvae and fry: importance of rearing conditions and diet nutritional value on their quality



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A Raquel, Javier y Carlos.

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Abbreviations

ANOVA: Analysis normal variance

APROMAR: Asociación Empresarial de productores de cultivos marinos (Association of marine aquaculture producers; Spain)

FEAP: Federation of European aquaculture producers

ARA: Arachydonic acid (20: 4n-6)

BHT: Butilated hydroxitoluene

BMP_4: Bone morphogenesis proteins 4

DAH: Days after hatching

DHA: Docosahexaenoic acid (22: 6n-3)

DPA: Docosapentaenoic acid (22: 5n-6)

DW: Dry weight

EFA: Essential fatty acid

EPA: Eicosapentaenoic acid (20: 5n-3)

FAMES: Fatty acid methyl esters

FAO: Food and Agriculture Organization

FFA: Free fatty acid

GC: gas chromatography

HPLC: High polar liquid chromatography

HPTLC: High polar think layer chromatography

ICCM: Instituto Canario de Ciencias Marinas

n-3 HUFA: n-3 series highly unsaturated fatty acid (20 or more carbons)

LA: Linoleic acid (18: 2n-6)

LNA: Linolenic acid (18: 3n-3)

MAPA: Ministerio de Agricultura, Pesca y Alimentación (Ministry of Agriculture, Fisheries and food, Spain).

NL: Neutral lipid

PPAR: Peroxysome proliferators active receptors

RXR: Retinoic acid X receptors

SHH: Hox and sonic hedgehog genes

TFA: Total fatty acid

Units: Along the whole document, the international system of units (SI) was used

Summary

The main objective of this study was ***"to improve the production technology of red porgy (Pagrus pagrus) larvae and fry".*** Specific objectives were divided in two complementary and consecutive phases; the first part was mainly based on a biological approach where specific aspects of the first development stages of this species were described. Followed by a second part where this knowledge was applied to develop a standard larval rearing protocol for commercial production of this species.

In this sense, it was determined that red porgy larvae are visual feeders and hatch with an incomplete and nonfunctional visual and digestive system. Along the third and fourth days post-hatch, major structural changes took place in the visual and digestive systems, with the definition and pigmentation of the primary photoreceptors (cones) and mouth opening. At this moment, the first digestive activity was detected in the midgut, which enables the larva to begin its exogenous feeding. The second most important change in relation to visual system development was detected around 20dah, when second photoreceptors nuclei appeared (rods), these structures prepared larvae with a greater sensitivity and visual acuity, allowing them to prey under restricted light conditions. These changes, agreed with the detection of the first gastric cells and the progressive migration of the larvae, from shallow to depth water, suggesting changes in feeding habits and culture conditions at this stage.

On the other hand, the influence of the culture system over larval development was determined. These differences were showed in a lower growth, from the first week of life, when intensive systems were applied in comparison to semi-intensive ones. This fact is also translated to larval osteological development, since this is better correlated to larval sizes than age. Thus, larvae in more advanced ossification stages were observed in the semi-intensive systems in comparison to the intensive ones, for the same larval age. Furthermore, different patterns of growth throughout the culture period were identified and separated in three main stages, which differs depending on the rearing system. Accordingly, the first stage (5-15 dah) with high growth rates and very sensible to mortalities; a second period (15-30dah) related to completion of metamorphosis with lower growth rates and better control of mortalities and finally, a third post-metamorphic one (30-50dph) considered as a recovery phase in the intensive system with increased growth rates and high survival. Overall, red porgy grows much faster than other sparids such as *Sparus aurata* doubling the size of the juveniles of the later at 95 dah.

Regarding to the meristic variations, intensification of the rearing system does not display any effect on the ribs number, but a higher incidence of individuals with an extra vertebra were

observed in the intensive systems. In addition, present results showed that regardless of the culture conditions, red porgy larvae and fry display a high incidence of morpho-anatomical deformities that might condition their commercial productivity under these culture conditions. In particular, culture intensiveness had a clear influence on the appearance of a lower number of normal shape larvae and this effect was reflected with an increase of larvae with cranial deformities and kyphosis, whereas this factor does not have a significant effect on the apparition of column deformities such as vertebral fusions and lordosis. These anomalies showed the highest impact in this species. Nevertheless, culture system affected vertebral fusions location, thus in the intensive systems reared larvae, most of the fused vertebrae were located in the caudal zone, whereas in the semi-intensive system reared larvae this anomaly were mainly located in the pre-hemal zone. To verify the potential effect of nutrition over this type of deformities, a comparative study were performed, feeding red porgy larvae with rotifers enriched with different enrichment emulsions under intensive culture conditions. The results of this study allowed concluding that an essential fatty acid supplementation in rotifers emulsions was decisive to reduce the incidence of skeletal deformities in this species. Thus, the increase of the DHA content in rotifers, in spite of not modifying the growth of the larvae, clearly affects the biochemical composition of the larva, improving the survival and reducing almost 50% the incidence of skeletal deformities. On the other hand, the presence of elevated content of other fatty acids like DPA (22:5n-6) might have negative consequences on the larval survival in this species. Additionally, in these studies, more abundant fatty acids in red porgy along larval development according to the rearing system were identified: 22:6 n-3, 16:0, 18:1 n-9, 18:0, 18:2 n-6, 20:5 n-3, 16:1 n-7,18: 1n-7, 18:3 n-3 and 20:4 n-6. Hence, the levels of DHA in the red porgy larvae between 12-20dah (15-20%) are higher to the supplemented through the diet when commercial enriching emulsions were used (9-10%), suggesting a selective accumulation of this EFA. The improvements in survival observed in the semi-intensive system could be related to a greater accumulation of DHA in these larvae, consequence of a longer duration of the yolk reserves in the larvae reared under this system, which in turn showed a better resistance to activity test. Indeed, this species under intensive culture conditions seems to be very sensitive to handling and stress. The results of this study allowed the improvement in the rearing protocols for red porgy, increasing the final total length and survival at 50dah from the initial trial to the last ones from 18.9 mm to 25.13 mm and from 4.9 to 12.5% in the intensive system and from 23.52 to 26.4 mm and 4.4 to 28.7% in the semi-intensive system.

Finally, some general culture zoo-technical advices were established for red porgy larval rearing.

In relation to the feeding sequence, present results suggest a prey density over 5 rot.ml^{-1} in semi-intensive system and the extension in the length of rotifer feeding from 20 to 30 dah for both rearing systems facilitating the adaptation to feeding changes. In addition, since this species was found to be very voracious in preying *Artemia*, which was even defecated alive, the application of an early co-feeding and weaning, from 12-15dah, together with a reduction in the *Artemia* amount, markedly decreased mortalities and improved growth rates.

Also, the use of continuous of photoperiod is suggested during the first 20 days, but reduced to 12:12 photoperiod and natural light in later stages, helps to reduce larval stress when the change from green to clear water are conducted.

With all these information, it is possible to conclude that the best larval rearing protocol to sustain a regular and predictable red porgy fingerlings demand with a good biological performance for commercial application is the semi-intensive system technology, but even in this type of systems fry quality is seriously affected by the high incidence of skeletal deformities. However, this problem might be limited with specific formulation of the rotifers enrichment emulsions.

Improvements in the production technology of red porgy (*Pagrus pagrus*) larvae and fry: Importance of rearing conditions and diet nutritional value on their quality

INTRODUCTION

1. INTRODUCTION

1.1.–Aquaculture in the world

Aquaculture has a long history of more than 4,000 years despite its significant contribution to the human feeding is only very recent. Aquaculture has experienced a great development in the last 30 years. Thus, world-wide production of fish, crustaceans and molluscs has increased from 3.9% of total fishing production in 1970 to 43% in 2006, being expected to reach 50% of total fishing production by the year 2025 (FAO, 2004). From 1970, aquaculture production has an average annual grown around 8.7% in comparison to 1.2% of fisheries production or 2.8% in terrestrial animal production. According to FAO previsions, aquaculture seems to be a feasible complement to fisheries in order to satisfy the high demand of marine products. In 2004 worldwide fish consumption rate was 16.6 kg/person/year, Spain being one of the most important fish consumer's with 36.6 kg/person/year (MAP, 2007). Nevertheless, Spanish fish consumption depends on a fishing fleet that for many years has not been able to obtain the necessary captures to supply the national market. The overexploitation of the traditional fishing areas and the restrictions imposed by the changes in the policy and agreements of international fishing areas caused a chronic restriction in marine products supply, which in turns promoted high levels of fish imports. Hence, the increasing demand of food from marine origin ensures a successful future to aquaculture sector making this activity very attractive for investors, as well as an alternative to labor activity for the new generations. Nowadays, labor force working in aquaculture is estimated in more than 12 million people around the world (APROMAR, 2008). In industrialized countries, aquaculture as a complementary sector to fisheries comprises an intensive aquaculture with a high degree of specialization to produce fish, mollusks, crustaceans or plants under controlled conditions. More than 250 fish species and plants were reared around the world in 2006 with almost 50% of them being cultivated species (APROMAR, 2008). European aquaculture is mainly based on production of a reduced number of fish and mollusk species with a high commercial value. In marine fish (excluded salmonids) the last eight year production has experimented an important increase from 125,000 tons in year 2000, to more than 250,000 tons in 2008, this was mainly due to the intensive rearing of 3 species, gilthead seabream (*Sparus aurata*), European seabass (*Dicentrarchus labrax*), and turbot (*Psetta maxima*) (with a small contribution of Atlantic cod (*Gadus morhua*), meagre (*Argyrosomus regius)* and Atlantic bluefin tuna *(Thunnus thynnus)* fattening, included). Smaller productions of new species such as Atlantic cod (*Gadus morhua)*, Senegalese sole *(Solea senegalensis)* and Atlantic halibut (*Hippoglossus hippoglossus),* are being included more recently (Table I), (FEAP, 2008).

Table I: Evolution of European aquaculture marine fish production in metric tons (t), (salmonids not included; source FEAP, 2008).

Common name	**European seabass ***	**Gilthead seabream***	**Turbot**	**Northern bluefin tuna**	**Atlantic cod ***	**Atlantic halibut ***	**Meagre**	**White seabream**	**Senegalese sole**	**Total**
Year	*Dicentrarchus labrax*	*Sparus aurata*	*Psetta maxima*	*Thunnus thynnus*	*Gadus morhua*	*Hippoglossus hippoglossus*	*Argyrosomus regius*	*Diplodus sargus*	*Solea senegalensis*	
2000	57,811	57,272	4,872	6,082	27	135	-	400	-	**126,599**
2001	56,162	74,403	4,640	9,992	111	389	-	400	-	**146,097**
2002	61,093	79,367	5,320	8,816	255	350	-	400	60	**155,661**
2003	62,060	87,940	5,107	6,715	2,940	845	-	400	52	**166,059**
2004	68,679	88,522	6,076	11,792	3,200	884	211	400	75	**179,839**
2005	79,706	93,372	6,085	9,180	6,708	1,319	893	400	60	**197,723**
2006	97,336	119,099	7,101	8,730	8,570	1,839	1,425	400	80	**244,580**
2007	93,425	104,697	7,444	5,380	10,640	1,399	1,138	-	60	**224,183**
2008	102,765	128,943	9,067	5,680	11,680	1,260	2,235	-	90	**261,720**

*The production data includes some countries outside the European Union (Norway, Croatia, Iceland and Turkey).

Table II: Evolution of European aquaculture marine fry production, in Millions (source FEAP, 2008 and authors data).

Common name	**Scientist name**	**Year**								
		2000	**2001**	**2002**	**2003**	**2004**	**2005**	**2006**	**2007**	**2008**
		Fry production (Millions)								
European seabass*	*Dicentrarchus labrax*	214.2	207.9	204.0	220.5	349.8	374.6	389.7	419.7	457.7
Gilthead seabream*	*Sparus aurata*	262.3	293.4	317.0	307.7	318.8	447.3	549.9	507.6	497.7
Turbot*	*Psetta maxima*	4.0	4.0	5.2	5.2	-	6.8	8.5	6.1	13.0
Atlantic cod**	*Gadus morhua*	0.04	0.17	0.40	4.59	5.00	10.48	12.83	-	-
Meagre*	*Argyrosomus regius*	-	-	-	1.3	1.1	3.4	2.4	2.2	3.0
White seabream	*Diplodus sargus*	0.7	0.7	0.7	0.7	0.7	0.7	0.7	-	-
Senegalese sole	*Solea senegalensis*	-	-	-	0.6	0.6	0.6	1.1	0.6	0.6
Atlantic Halibut*	*Hippoglossus hippoglossus*	1.0	-	-	0.10	0.10	0.10	0.10	-	-
Red porgy	*Pagrus pagrus*	1.1	-	-	0.015	0.03	0.10	2.5	2.0	2.0
Sharpsnout seabream	*Puntazzo puntazzo*	6.3	-	-	-	-	-	4.5	4.0	4.0

*The production data includes some countries outside the European Union (Norway, Croatia, Iceland and Turkey).
**Rosenlund pers. com.

With the exception of the Atlantic blue fin tuna *(Thunnus thynnus)* fattening, whose production is based on wild fish capture and fattening in off-shore cages, the production increase in the rest of species has being associated to a better knowledge of reproductive biology and the development of the larval rearing techniques which allowed an important increased in fry availability (Table II).

1.2.-Aquaculture in Spain

1.2.1.-General aspects

Spanish aquaculture is one of the most important of all the European Union with a production of 250-300 thousand tons/year, which accounts 3% of the worldwide production and 25% of the European production. To the traditional modalities of mussel (*Mytilus galloprovincialis*) culture in Galician coast and the extensive aquaculture in Andalucía, a new sector, highly industrialized and in constant expansion has joined from the early 90´s.

Figure 1. Example of extensive traditional aquaculture in Spain: a) group of rafts for mussels (*Mytilus galloprovincialis*) production; b) collection of common cockles (*Cerastoderma edule*) in Ría de Muros y Noya (La Coruña, Spain).

As example, the case of turbot (*Psetta maxima*) production in the north coast of Spain, with 7,512 tons production in 2008 that means 82.8% of the European production, and the Mediterranean and Canary Islands aquaculture mainly based on gilthead seabream (*Sparus aurata*) and European seabass (*Dicentrarchus labrax*) production with 24,790 and 11,760 tons in 2008 respectively (FEAP, 2008). In addition, the introduction of new species to marine aquaculture diversification has to be considered. In the year 2008, 72% of the world production

of meagre (*Argyrosomus regius*) (1,620 tons) was produced in Spain, followed by Italy and France. In addition, Spain is the single producer of blackspot seabream (*Pagellus bogaraveo)* with 134 tons also leads Senegalese sole production (*Solea senegalensis*) in Europe with 90 tons in 2008. Regarding Atlantic blue fin tuna fattening, Spain was the European leader with a 43.5% of the production in 2006 followed by Cyprus and Croatia.

Figure 2. Examples of intensive modern aquaculture in Spain: a) off-shore cages for gilthead seabream (*Sparus aurata*) on growing in Telde, (Las Palmas, Spain); b) off-shore cages for turbot (*Psetta maxima*) on growing in the Ría de Vigo (Pontevedra, Spain).

1.2.2.-Fry production

Actually, 16 commercial marine fish hatcheries produced 113.5 million of fry in 2008 in Spain (APROMAR, 2007), being this country the first European producer of turbot fry with 90.3 % of the production. Additionally, Spain produces 14.8 % of gilthead seabream fry and 5.6 % of sea bass fry (FEAP, 2008). To these data, should be added, the small amounts of fry from other marine species, produced in private companies or research centres: as an example should be considered the Senegalese sole production (600,000 fry in 2008), pollack (*Pollachius pollachius*) and red porgy (*Pagrus pagrus*) (100,000 fry each, in 2006) or black spot seabream production (16,000 fry in 2006). These experimental productions have promoted marine species diversification projects by commercial producers. Moreover, the introduction of new species in the Spanish farms was encouraged by experiences made at pilot scale for several species, i.e. sharp snout seabream (*Puntazzo puntazzo*) in Murcia (Hernández *et al*., 2003), common dentex (*Dentex dentex*) in Catalonia, Valencian Community and Balearic (Abellán, 2000), red banded

seabream (*Pagrus auriga*) in Andalusia and Canary Islands (Cárdenas *et al.*, 2003), or white trevally (*Pseudocaranx dentex*) also in the Canary Islands (Roo *et al*., 2007b). At experimental level with a much lower degree of knowledge an even higher number of species are being tested such as amberjacks (*Seriola dumerilli, Seriola rivoliana or Seriola fasciata*), Atlantic bluefin tuna (*Thunnus thynnus*), pink dentex (*Dentex gibbosus*), striped red mullet (*Mullus surmuletus*), dusky grouper (*Epinephelus marginatus*) or European hake (*Merluccius merluccius*).

1.2.3.-Aquaculture in the Canary Islands

The Canary Islands have very good perspectives of aquaculture expansion due to the exceptional climatic conditions and high quality of their coastal waters. Thus, the production values of the last years showed a continued growth of this activity that was increased from 456 tons in 1998 to more than 8,000 tons in 2006 (APROMAR, 2007). The most important reared species are gilthead seabream (*Sparus aurata)* with 65% of the production and sea bass with 35%, Canary Islands are the second Spanish producer of gilthead seabream with 26% of national production. Besides Canary Islands is the first national producer of European seabass with a production of 31.7% (APROMAR, 2008). Nevertheless, this activity is based in a reduce number of species, which is slowly changing with the introduction of small amounts of fry from other new species such as meagre, Senegalese sole and red porgy with different degree of success.

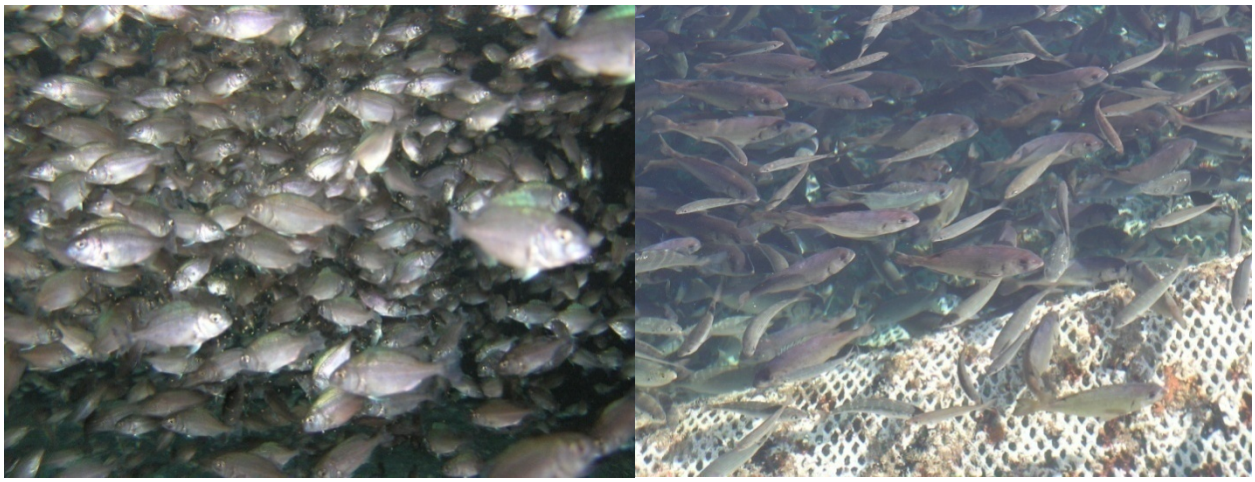


Figure 3. a) Pre-growing of red porgy (*Pagrus pagrus*) on land; b) on-growing of red porgy in offshore cages.

Like counterpoint, fits to mention that marine aquaculture in the Canary Islands continues depending on fry importation, in 2005, around 25 million fry were imported and only a small contribution were locally produced by the ICCM experimental hatchery. These facilities produce

around 250,000 fry of gilthead seabream per year, 50,000 fry of red porgy and has started to produce fry of meagre (*Argyrosomus regius)* (150,000) and Senegalese sole (*Solea senegalensis*) (28,000) in 2008.

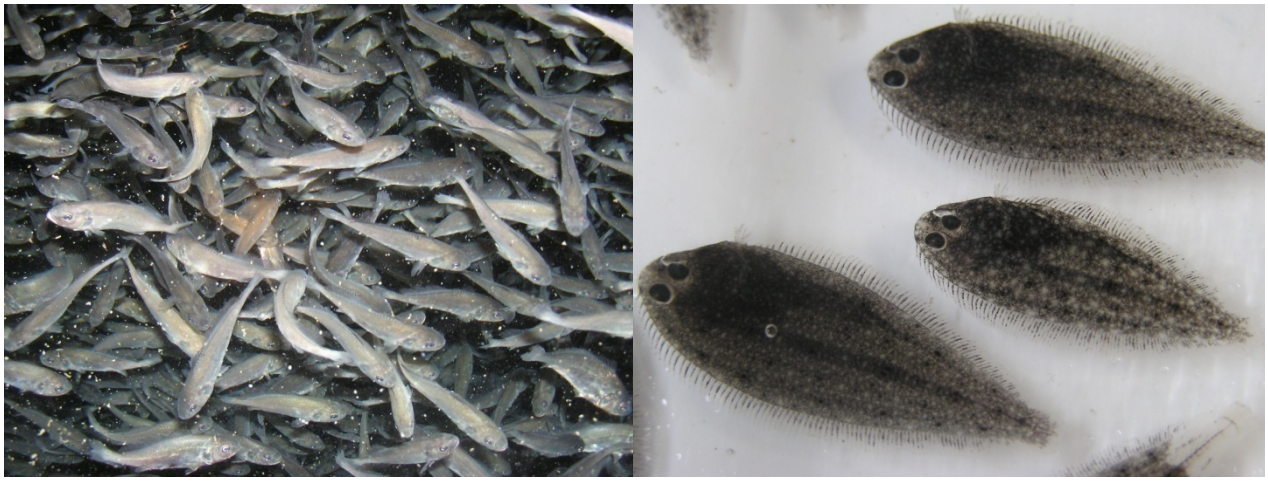


Figure 4. Production of fry for aquaculture diversification in the Canary Islands; a) Meagre (*Argyrosomus regius*) fry; b) Senegalese sole (*Solea senegalensis*) fry.

1.3.-Importance of larval rearing

It is well known that seed capture from the wild stocks is not a sustainable strategy for marine fish intensive aquaculture, being these practice restricted to other marine organisms as certain mollusc and great commercial value fish for extensive production such as tunas, groupers, amberjacks or eels (Ottolengui *et al.*, 2004).

Figure 5. Wild catch fry of major interest species for experimental on growing: a) greater amberjack (*Seriola dumerili*) and (*Seriola fasciata*); b) striped jack (*Pseudocaranx dentex*).

In this sense, one of the main bottlenecks for the whole development of marine aquaculture is the production of a good quality seed in adequate quantities. Larvae from most marine fish species are not fully developed when they hatch and certain structures in visual, nervous, immune or digestive systems or skeleton are incomplete in comparison to the adult. Hence, during the first weeks of life, larvae will suffer important morphologic, functional and physiological changes (Govoni *et al*., 1986). Therefore, at this stage fish larvae are very sensitive and suffer elevated mortality rates. The stress associated to the intensive culture conditions, commonly joined to nutritional unbalances, are the main cause of larval dead.

Despite the important advances in the development of inert diets for larvae (Koven, 2001), commercial mass production of fry, strongly depends on the reproduction of a simplified trophic chain, based in the use of unicellular microalgae, rotifers and *Artemia.* This situation implies the need to maintain parallel facilities to fish larval tanks to produce live prey for larval feeding, increasing the fry production costs.

Optimization of the culture conditions, maintaining controlled biotic and abiotic conditions should tend to be as close as possible to the natural conditions.

1.3.1.-Larval rearing techniques

From the initial stages of the aquaculture, great varieties of larval rearing techniques have been developed. Divanach (1985), established the first classification, this author classified larval rearing techniques based on larval density and tank volume, thus three main categories could be established: intensive, extensive and Mesocosms systems (Figure 5).

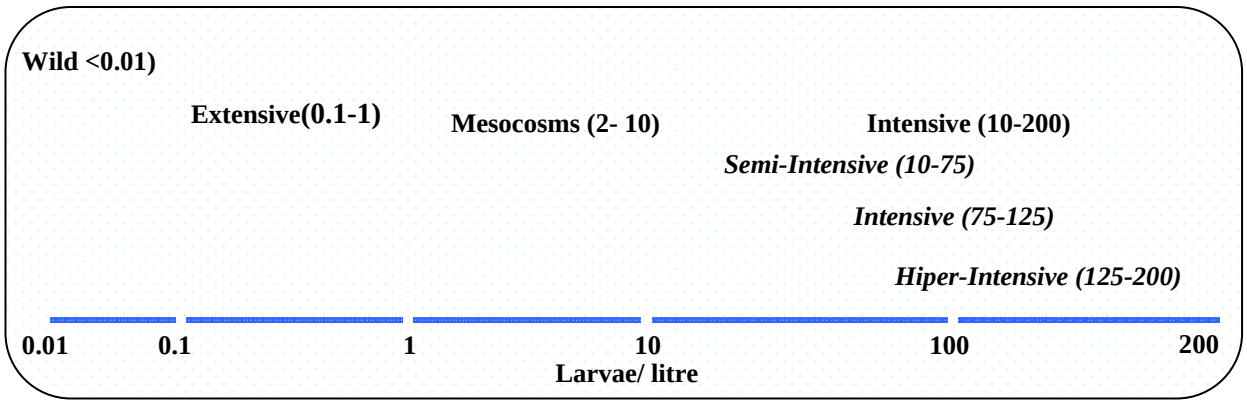


Figure 6. Larval rearing techniques classification according to Divanach, (1985).

Other authors, such as Van der Meeren and Nass (1997), classify the larval rearing techniques according to food supply: intensive systems are described as those exclusively based on exogenous feed, extensive systems on endogenous feed (produce by the own system) and, finally, semi-intensive systems were endogenous feed is complemented by exogenous one. In the present work, the terminology used will be that of Divanach (1985).

1.3.1.1.-Intensive systems

Intensive systems are characterized by high larval density, use of cylinder conical tanks and generally small water volumes (frequently about 0.5-2m^3) despite there is a tendency to use greater water volumes (10-20m^3). Larval density can vary among semi-intensive = 25 - 50 larvae/litre, intensive= 75-125 larvae/litre and hyper-intensive =150-200 larvae/litre with an increase complexity during rearing (Divanach *et al*., 1998).

Figure 7. Larval rearing facilities for intensive production (courtesy of Tinamenor S.A).

The intensive and hyper-intensive techniques are applied with species such as sea bass and gilthead seabream where acceptable survival rates are obtained (Shields, 2001). On the other side, semi-intensive techniques are frequently used in some species of flat fish like turbot (*Psetta maxima*) and Japanese red seabream (*Pagrus major*).

In the intensive systems, temperature, illumination, hydrodynamics, oxygenation and feeding conditions are strictly controlled. Generally, photoperiod, light intensity and quality are artificial, and changed according to the development of larval visual acuity (Roo *et al*., 2001). Tank shape and details as wall colour, position and direction of water inlets and outlets, lamps, feeders,

surface skimmers and air diffusers are previously established, to ensure an homogenous behaviour of the larval population and ergonomic conditions (Divanach *et al*., 2002). The food supply in these systems is completely exogenous and restricted to a diet based on rotifers (*B. plicatilis*) and *Artemia*. The nutritional value of these live preys needs to be improved to satisfy the larval needs of proteins, lipids, vitamins and minerals (Izquierdo and Fernández-Palacios, 1997; Sargent *et al*., 1999). The selection of the correct feeding sequence and the adaptation to the needs of larval batch particularities are essential to obtain high survival and low size dispersion and to reduce the cannibal behaviour. For this reason, specialized personnel and a automation of the processes are crucial to success. With these techniques, most culture parameters are potentially limiting and a good biological knowledge of the species is required to obtain high survival rates. Task specialization and personnel teams' duplication are necessary for a permanent attention 24h of the day. Reduction in the specialized personnel during holidays, weekends and nights, constitute greater risk periods where the specialization and limitations of the intensive system generate great losses (Divanach *et al*., 2002). Adaptation of the larvae during these risk periods may cause changes in shape (deformities), coloration or growth reductions and the production will not be able to fulfil the quality standards demanded by the farmers. For instance the standards applied to gilthead seabream fry produced in the ICCM include less than 20% of size dispersion and less than 5% of total visual deformities, including less than 2% of opercula deformities. Most of these anomalies are produced during the first stages of growth, but they are not visually detected until later stages. Size dispersion, morph-anatomical deformities, coloration and behaviour anomalies, cannibalism, unbalanced sex proportions are frequently observed in the industrial hatcheries where this technique of intensive culture is currently applied (Divanach *et al*., 2002). Thus, the number marine fish species reared with this technology is limited to few of them such as sea bass (*Dicentrarchus labrax*), gilthead seabream (*Sparus aurata*), turbot (*Psetta maxima*) (Shields, 2001) cobia (*Rachycentrum canadum*) (Holt *et al.,* 2007), meagre (*Argyrosomus regius*) (Estévez *et al*., 2007; Roo *et al*., 2007), Senegalese sole *(Solea senegalensis) (*Dinis *et al.,* 1999*)* or cod *(Gadus morhua)* Baskerville-Bridges and Kling, 2000).

In summary, these techniques require sophisticated facilities and elevated investments and the production depends totally on a very specialized personnel and technology, being unsuitable for small producers and the culture of new species (Divanach *et al*., 2002).

1.3.1.2.-Extensive systems

These are based on the stimulated productivity system where the fish larva is the superior hunter of the artificial tropic chain generated. This type of system is based in the use of very low eggs or larval densities (0.1-1 individuals/litre). Larval rearing is performed in big water volumes (hundreds or thousands of cubic meters), coastal lagoons, tanks, great plastic bags or small dumps (Figure 8).

Figure 8. Facilities for extensive Atlantic cod fry production in Øygarden, Norway (courtesy from Professor Terge Van der Meeren).

The procedure starts establishing the new trophic chain with phytoplankton and zooplankton until the systems reach maturity with enough live preys to allow the eggs or larvae addition. Under these natural conditions two variants of this system can be defined: short cycles (1 - 2 months) that produce fry ready to be reared under intensive conditions or long cycles (2 - 6 months) that produce fry usually to be employed in repopulation or extensive aquaculture programs.

Control of environmental conditions is very complicated in such big water volumes and these systems are exposed to environmental fluctuations. Only slight modifications are possible in certain parameters such as photoperiod by artificial light during the night or shadowing nets during the summer, or the use of plastic films to prevent water surface frozen.

This type of systems directly bring untreated seawater from the coastal zone, passed through a gross filtration (250-350µm) to avoid potential fish larvae hunters or competitors. The feeding is exclusively endogenous, generated by the natural zooplankton bloom fed on the primary production in the own pool. Under these conditions, the success of the production is based on the intensity and duration of the generated trophic chain (Gamble *et al*., 1985; Franck and Leggett, 1986). If the producer exceeds the biological capacity of the system, the balance is lost and the system collapse in few days or weeks generating growth problems, size dispersion and cannibalism due to food restrictions (Divanach, 1985). The success in this type of system comes with the expertise of the producer and the knowledge from the ecosystem and the laws that govern the system productivity.

Figure 9. Facilities for extensive Atlantic cod fry production in plastic bags in Lofoten, Norway (courtesy of D. Dag Hansen, Lofilab AS).

Although knowledge is the base of this technology, the human role is less important in contrast to the intensive system. The operations that require a higher human intervention are tank preparation, initiation of the trophic chain and eggs or larvae inoculation. The environmental diversity and nutritional conditions that take place in this type of system allow a multitude of options to the larvae that can choose without forcing them to an anomalous situation (Kentouri and Divanach, 1986). Thus, larvae reared under these systems show natural biological patterns, vertical and horizontal distribution correlated with illumination conditions and prey, currents,

and zooplankton concentration as well as migrations (Pitta *et al.*, 1998). As a result, the success of these systems is usually higher than in the intensive systems.

Frequently, species difficult to be reared under intensive conditions such as in the striped seabream (*Lithoghnathus mormyrus)* or sharp snout seabream *(Puntazzo puntazzo*) with small eggs size or reduced eggs availability are successfully produce in this system. In this type of system, fry quality is excellent and do not show problems such as abnormal swim bladder inflation, skeletal deformities, pigmentation anomalies or deviations in natural behaviour (Divanach *et al.,* 1996).

In the case of the European seabass, a common anomaly when larvae are reared under intensive systems is the apparition of abnormal sex proportion (higher male proportion), which is corrected in the extensive systems. Depending on the water volume, fry harvest can reach several hundreds of thousands or millions of fry. The challenge for its industrial success is the use of megavolumes (megacosmos) with several thousand millions of cubic meters (Gamble *et al.,* 1985),or intermediate volumes (Mesocosm systems) being some commercial experiences conducted in Norway for cod production ((Drenner *et al*., 1990; Van der Meeren and Naas., 1997) (Figure 9).

1.3.1.3.-Semi-extensive (Mesocosms) systems

These systems are commonly applied in semi-outer or inner conditions and are considered as intermediate systems between intensive and extensive (Divanach and Kentouri, 2000; Papandroulakis *et al*., 2004a). Its definition is relatively recent in the form for its present application and was defined by Divanach *et al,* (1998) after the study of the intrinsic qualities and failures of the original models (Grice and Reeves, 1982; Bever *et al.,* 1985; Lalli, 1990).

Mesocosms is a technology quite developed that takes the advantages of both extensive and intensive techniques without its corresponding disadvantages. The larval culture takes place at relatively low densities (2 - 10/litre), in relatively great volumes (30 - 100 m^3) and with certain depth (1.5 – 2.5 m) tanks, located in well organized facilities which assures the quality, ergonomics and security in the culture achieving a high production of fry per tank (50,000 to 300,000 depending on the species) with a high productivity (> 2 million of fry/man/year) (Divanach *et al.,* 2002).

Figure 10. Semi-extensive systems: a) Mesocosm facilities in the Canarian institute of Marine Sciences in Telde (Canary Islands; Spain); b) detail of larval rearing tank (40,000 l) in the Institute of Marine Biology of Crete, (Heraklion, Greece).

The environmental conditions in the tanks are a combination of natural and artificial, avoiding any strong seasonal climatic variation or geographic limitation and allowing the optimization of the operational costs (Divanach and Kentouri, 2000; Papandroulakis *et al*., 2004a). Long photoperiods (> 18 light hours) and warm temperatures (15-21ºC) are used for most of the species. The exogenous and endogenous combined feeding assures fulfill of larval nutritional requirements avoiding risks of nutritional deficiencies. This partial autonomy of the system is very important to obtain a good quality fry and helps to reduce operational cost during nights and weekends (Divanach *et al*., 2002). The results obtained with this technique for already culture marine species, as European seabass (*Dicentrarchus labrax)*, gilthead seabream *(Sparus aurata)* or white seabream (*Diplodus sargus*) are frequently better than the one obtained with intensive or extensive techniques (Papandroulakis *et al*., 2004a). The fry survival after the weaning phase generally ranged between 40-90% from eggs stage. The percentage of total deformities is around 5 to 10%, swim bladder inflation goes up to 95% of the fry and larval growth is between 15 to 20 mg at the first month of life with low size dispersion and with animals that exhibit very homogenous behavior, with a low cannibalism incidence. Weaning is completed at 45-50 days of life (Divanach *et al*., 2002). This technology has been used successfully for the production of fry from more than 25 species of marine fish and 5 hybrids (Divanach *et al*., 2002). Most of them have been produced in mesocosms with extensive approach using pseudo-green water technique, as it is the case of the creek anchovy (*Anchovy mitchilii),* Atlantic cod *(Gadus morhua),* Atlantic herring *(Clupea harengus),* European plaice *(Pleuronectes platessa),* Atlantic halibut

(Hippoglossus hippoglossus), common sole *(Solea solea),* turbot *(Psetta maxima)*. But others like the European seabass (*Dicentrarchus labrax),* gilthead seabream *(Sparus aurata),* sharpsnout seabream *(Puntazzo puntazzo),* white seabream *(Diplodus sargus),* common two-banded seabream *(Diplodus vulgaris),* annular seabream *(Diplodus annularis),* striped seabream *(Lithognathus mormyrus),* red porgy *(Pagrus pagrus),* common dentex *(Dentex dentex),* greater amberjack (*Seriola dumerili)*, thick lipped grey mullet (*Chelon labrosus*), shi drum (*Umbrina cirrosa L.)*, were reared under mesocosm technology with intensive approach (Divanach and Kentouri, 2000; Papandroulakis *et al*., 2004a, 2005; Koumoundouros *et al*., 2005; Zaiss *et al*., 2006; Ben Khemis *et al*., 2006).

Within Mesocosms systems, two variants and four-sub variants according to the origin and quality of the preys were defined:

In the extensive approach, the food chain is mainly endogenous and only complemented with exogenous feeding when symptoms of exhaustion and lack of food occur. In the intensive approach feeding is basically exogenous, although there is a certain capacity for endogenous food production due to the low larval density (low rates of feeding) and the presence of live phytoplankton in the tank.

The four sub variants are characterized by the methods for nutritional chain improvement:

1.3.1.3.1.-Natural-bloom

This sub-variant is based on the generation of a natural trophic chain in the gross filtered seawater that is pumped. The water is fertilized with nutrients to promote phytoplankton growth in the case of very oligotrophic water phytoplankton inoculation with species from *Nannochloropsis* or *Tetraselmis* genus is also found. Water renewal is very low 1-2% day and after 10-15 days, an optimal zooplankton density is obtained. The fish larvae fed themselves on this zooplankton around 20-25 days, a critical time in their development. Afterwards they are ready to continue feeding on exogenous *Artemia* and to begin its weaning.

1.3.1.3.2.-Green water method

It includes the generation of an endogenous trophic chain based in phytoplankton (*Nannochloropsis sp.*) and rotifers (*Brachionus sp.*) which are allowed to grow in filtered and

sterilized seawater. Water is fertilized with nutrients and inoculated with phytoplankton and rotifers which developed until they reach a 500,000 phytoplankton cells.ml^{-1} and >2 rot.ml^{-1}. The water is not renewed until day 4 to 8 when larvae are introduced. When the food chain is correctly developed this last approximately 25 days before *Artemia* and weaning diets are necessary.

1.3.1.3.3.-Pseudo green water

This technique is based on an exogenous food chain, that contains phytoplankton and rotifers, produced in parallel facilities and that are daily introduced according to the larval demand (Divanach *et al*., 2002).

Two variations are found in this technique:

1. Pure: the tank is filled with sterilized seawater and the eggs or larvae are introduced. Later when the larvae initiate their heterotrophic life, phytoplankton and rotifers are added.
2. In the second variant, the first trophic chain is initiated with the introduction of phytoplankton and rotifers before eggs or larvae addition and, water is always renovated, increasing as larvae develops.

Phytoplankton and rotifers, enriched to improve their nutritional value, are added 1-2 times a day. When the larva is able to feed on *Artemia,* addition of phytoplankton is discontinued and rearing continues in clear water.

This technique it is used for species with a well known biological cycle as gilthead seabream (*Sparus aurata*).

1.3.1.3.4- Clear water

Here, phytoplankton is not added to the rearing water and, hence, high water renovation rates of more than 10%tank volume/hour is used to avoid proliferation of endogenous microorganisms. It is used with species which are able to accept *Artemia* from first feeding, like European seabass or associated to automatic feeding to provide food with an elevated nutritious quality (Divanach *et al*., 2002).

1.3.1.3.6- Neo-green water

These technique uses criopreserved, lyophilized or paste phytoplankton, its main limitation being the reduction of the beneficial value of using alive microalgae (Divanach *et al*., 2002).

1.4.–Limiting factors in larval rearing

The selection of an appropriated rearing technology depends on the species specific characteristics and its susceptibility to different parameters affecting the larval rearing success. Some of these parameters, are related to culture conditions such as larval density, tank volume, water renewal, use of phytoplankton and drugs; whereas others refer to the physical-chemical conditions such as temperature, salinity, illumination (photoperiod, intensity and light quality) or waste products; and finally, to larval feeding and nutrition such as amount of food or live preys, prey and feeding sequence or the nutritional value of the food supplied.

1.4.1.-Culture conditions related parameters

1.4.1.1.-Larval density

This factor markedly affects larval growth and survival and determines the type of rearing system selected as as it has been shown in many species like creek anchovy (*Anchovy mitchilli*), lined sole (*Achirus lineatus*) (Houde, 1977), cobia (*Rachycentron canadum*) (Hitzfelder *et al*., 2006; Holt *et al*., 2007) or meagre (*Argyrosomus regius*) (Estévez *et al*. 2007; Roo *et al*., 2007), common seabream (*Pagrus pagrus)* (Hernández-Cruz *et al*., 1999; Roo *et al.,* 2005b), gilthead seabream (*Sparus aurata)* (Parra and Yufera, 1999; Roo *et al*., 2005a), or close species as common dentex, *Dentex dentex* (Giménez and Estévez, 2008a). Larval density and its effect on survival seems to be strongly associated to intracohort cannibalism (Baras and Jobling, 2002). Besides, the increase in larval density also reduces growth rates due to the increase in food competition and the stress caused by an elevated individuals concentration as it has been found in European seabass (*Dicentrarchus labrax*) or Canadian sole *(Paralichthys dentatus)* (Hatziathanasiou *et al*., 2002; Saillant *et al*., 2003; King *et al*., 2000). In other species as Atlantic cod (*Gadus morhua*) the influence of larval density on growth and survival is not so clear because is masked by other associated factors as food availability and water quality deterioration (Baskerville-Bridges and Kling., 2000).

1.4.1.2.-Shape and tank volume

Tank volume strikingly affects larval rearing performance. For instance, early studies on gilthead seabream showed a positive correlation between the increase in tank volume and larval survival (Tandler and Sherman,1981). At present, commercially produced sparids are mainly reared in cylinder-conical shape tanks, usually made of fiberglass with a relatively big volume (1-15m^3,

but frequently greater than $10m^3$). Moreover, the tank depth/surface can also affect larval growth and survival during the first stages of development, as occurs with gilthead seabream (*Sparus aurata*) seven bands grouper (*Epinephelus septemfasciatus*) or the weak "stinger" (*Inimicus japonicus*) (Salhi, 1997; Ruttanapornvareesakul *et al*., 2007).

1.4.1.3.-Water renewal

The water renewal has been suggested to affect the incidence of column deformities (Kihara *et al*., 2002). Increase in water renewal enhances removal of live prey and microalgae affecting the quality of the available food and the water physical-chemical characteristics. On the contrary, poor water renewal boosts the accumulation of waste products (ammonium, nitrites, etc) and the amount of remaining live prey with a poor nutritional value (Reitan *et al*., 1993), negatively affecting larval growth and survival.

However, during the first 10 days of life, water renewal reduces gilthead seabream (*Sparus aurata*) larvae growth and a closed system with no water exchange is recommended (Tandler and Helps, 1985; Tandler and Sherman, 1981; Hernández-Cruz *et al*,, 1990). After this period a water renewal higher than 25% $.day^{-1}$ is recommended. Addition of phytoplankton together with water renewal also enhances gilthead seabream survival (Hernández-Cruz *et al*., 1994). In the last years, an increasing tendency to the use of sophisticated closed systems has being adopted among aquaculturists. In these systems water renewal come from the same systems passed through mechanical and biological filters, ensuring a good water quality and preys with a high nutritional value (Olivar *et al*., 2000; Faulk and Holt, 2005).

1.4.1.4.-Use of phytoplankton in the rearing tank

The beneficial effect of phytoplankton addition in larval rearing tanks, called "green water technique" is supported by hundred of authors (Scott and Baynes, 1979; Hernández-Cruz *et al*., 1990; Koven *et al*., 1990; Salvesen *et al*., 2000). The positive effect of phytoplankton in the tank can be related to its contribution to water oxygenation; reduction of waste substances (ammonium and nitrites) produced by the larvae; control of bacterial flora; nutritional value of preys maintenance; light diffusion and prey color contrast. Algal species used in larval rearing have shown an important antibacterial activity that limits the growth of opportunistic bacteria. In the culture of the phytoplankton species used in the present study, *Nannochloropsis sp*. the main bacterial flora is composed by Alpha and a cluster of *Cytophaga-Flavobacterium* type bacteria, which acts as a biocontrol for other bacterial communities present in the culture tanks (Nakase

and Eguchi, 2007). Bacterial load increase in the larval rearing water results from repeated addition of phytoplankton contributing to the development of larval initial intestinal flora (Skjermo and Vadstein,1993). In addition, the presence of phytoplankton in the rearing water helps to maintain the nutritional value of the live prey. Enriched rotifers, loss 20% of the enrichment in nutritional quality when they are supply to larval tanks using green water technique, whereas in clear water systems the lose is several times higher (Reitan *et al*., 1993; Planas and Cunha, 1999). Thus, larvae reared in green water maintain the yolk sac reserve 2-3 days longer than in clear water and show a 3 times better growth rate and survival (Papandroulakis *et al.*, 2001; Van der Meeren *et al*., 2007). Finally, phytoplankton also acts as a light filter, reducing light reflection in the tank walls. This effect, together with the use dark walls tanks (Hinshaw, 1985), contributes to enhance larval feeding activity of the larva (Naas *et al.,* 1992, 1996; Ramos and Kobayashi, 1985; Muller-Feuga *et al.*, 2003). At present, there is a tendency to use concentrated, pastes, lyophilized and frozen biomass of different phytoplankton strains. There are different comparative studies about the use of this type of products. For instance, Cañavate and Fernández-Diaz (2001) did not found differences in growth and survival when gilthead seabream (*Sparus aurata*) were reared with a microalgae frozen biomass in comparison with alive microalgae. Nevertheless, these authors pointed out water quality deterioration, increasing the ammonium level with the use of inert biomass. On the other hand, the use of this type of products may limit the beneficial effects of the live algae, such as the bacteriostatic effect or the bacterial flora colonization in the tanks and larvae intestine.

1.4.1.5.-Use of antibiotics

The excess of bacterial load in the culture tank due to live prey supply and insufficient water renewal has lead to the use of antibiotics. A great controversy can be found in the ature about the use of these substances in the larval culture. For instance, whereas Hernández-Cruz e*t al*. (1994) showed that the use of oxolinic acid has adverse results in larval survival, enriched rotifers treated with antibiotic showing a lower content of n-3 HUFA. Other studies associated the improvement in Australian trumpeter (*Latris lineata*) larval survival and growth to the use of the oxitetracicline (Bataglene *et al*., 2006).

1.4.2.-Physico-chemical parameters

Several physico-chemical parameters potentially affect larval culture, such as temperature, salinity, illumination, waste products, gases or pH, among others.

1.4.2.1.-Temperature

Within the physico-chemical parameters that affect larval rearing success, temperature is one of the most important for marine fish. Its effect can has been studied along both embryo and larval development.

Incubation temperature during embryo development

The development of fish embryos are strongly influenced by the incubation temperature. Too high temperature, accelerate the embryonic development, whereas low temperatures delayed it (Blaxter, 1988). For gilthead seabream, the embryo is able to adapt to a wide range of incubation temperatures that vary between 14 to 28 ºC, being the optimum around 19º C (Polo *et al.*,1991). The optimal incubation temperature is associated to that corresponding to the latitudes of the spawning grounds. In gilthead seabream, temperatures below 16 ºC and over 22º C reduced survival and increased bone deformities incidence. Similar results have been described for Japanese red seabream (*Pagrus major*) (Mihelakakis and Yoshimatsu, 1998). In the European seabass (*Dicentrarchus labrax*), a slight increase in incubation temperature had a positive effect in muscular growth during later stages (López-Albors *et al*., 2003).

Larval rearing temperature

Tandler and Sherman (1981), determined that gilthead seabream larval survival is not affected by temperature when they range from 17 to 23 ºC, despite highest survival was at 17ºC and larval growth increased with rearing temperature. For the same species, Polo *et al*, (1991) determined that 16 to 22 º C was a suitable range for the same species, the optimum temperature being at 19ºC. A positive correlation was found between temperatures outside this range and larval mortality and skeleton abnormalities incidence. In addition, Tandler *et al*, (1989), showed that elevated temperatures increased size dispersion, reducing larval survival and enhancing cannibalistic behavior from early stages of development.

1.4.2.2.-Salinity

Despite this parameter rarely changes along larval rearing, certain type of facilities occasionally undergo salinity drops because of rain run-offs. Salinity affects eggs hatching and the incidence of skeletal deformities. For instance, in Southern black bream (*Acanthopagrus butcheri*), larval survival is reduced and column deformities increase when salinity drops below 15‰ (Haddy and Pankhurst, 2000). Moreover, skeleton deformities affected 100% of the population when the

salinity dropped to 5 ‰ from 35 ‰. Similar results have been shown gilthead seabream, when salinity drops from 37 to 32 ‰ during the first days of life. Besides, a significant increase in abnormal development of opercula can be obtained (author's unpublished data). On the contrary, in European seabass (*Dicentrarchus labrax*) a salinity drop to 15 ‰ results in an increase of larval survival and improvement in the percentage of larvae with functional swim bladder, similar results were obtained in the case of flathead mullet (*Mugil cephalus)* (Harel *et al*., 1998). Finally, the combined effect of both temperature and salinity had a significant importance to obtain viable larvae. Thus, extreme values of 28ºC and 12 ‰ salinity increase the percentage of larvae with skeleton deformities in red seabream (*Pagrus Major)* (Mihelakakis and Yoshimatsu 1998).

1.4.2.3.-Nitrogen excretion products

Ammonium from protein catabolism is the most important excretion product in fish, being toxic at high levels. The ammonium produced by unconsumed preys and organic detritus. Lethal concentrations for 12 day old gilthead seabream are around 20ppm, being no ionized ammonium ($N\text{-}NH_3$) the most toxic form with 0.24 ppm a lethal concentration for 50% of the population (Lc_{50}) (Parra and Yúfera, 1999). In the case of nitrite (NO_2^-), a 4,500 ppm concentration causes a 100% mortality of the population and 1,997 ppm a 50%. Nevertheless, these levels were directly related to factors like pH, salinity and temperature as well as the reared species. Thus, lethal concentration of $N\text{-}NH_3$ for 7 day old (days after hatching, dah) Senegalese sole (*Solea senegalensis*) larvae was about 80 ppm and 1.32 ppm 24h for Lc_{50}. Besides, the ammonium levels (0.05 – 0.015 ppm of NH_3-H) have been found to be related to damages to cartilaginous structure in red seabream larvae (Mihelakakis and Yoshimatsu 1998).

1.4.2.4.-Light

Most of marine fish larvae are visual feeders (Blaxter and Staines, 1970). Sparids larvae, such as gilthead seabream are visual feeders that need light to effectively attack its preys (Tandler and Mason 1983). Thus, during the first 12 days of life there is a positive correlation between the increase in photoperiod from 12 in 24 hours and larval survival in this species (Tandler and Helps, 1985). Similar results were reported by Barahona-Fernándes (1979) in European seabass larvae. Equally, an increase in light intensity from 205 to 1370 lux has been associated to a better growth and larval survival in gilthead seabream, being optimal light intensity between 1370-

5140 lux when a combination of artificial and natural light, under continuous photoperiod and green water systems, is used (Tandler and Mason, 1984).

From 20dah there are no significant differences on growth and survival caused by the change of photoperiod from 12 to 24 h light. While, from 50-60 dah a reduction of photoperiod length improves growth rates in gilthead seabream (Tandler and Helps, 1985).

The effect of tank wall coloration and light reflection are closely related to light conditions. Black tank walls when provided with an appropriate illumination seem to resemble better natural light conditions (Naas *et al*., 1996; Hinshaw, 1985). Illumination has been also related to larval skeletal development. As an example, the larvae of silver pearl fish (*Carapus homei*) need to pass through a complete dark period to complete metamorphosis. In nature, this species undergo a reduction in the number of vertebrae and a vertebral compression to adopt the definitive fry form (Parmentier *et al*., 2004).

1.4.3.-Larval feeding and nutrition

First feeding is a critical stage in the larval rearing of marine fish. Marine fish larvae hatch with limited endogenous reserves and hence, the ability to catch and digest preys as soon as possible is critical for larval survival. But its small mouth size and the poor development of the digestive system restricts the type of food making first feeding a critical stage for the success of larval rearing.

1.4.3.1.-Prey density

Optimum prey densities in intensive cultures to obtain good larval survival have been determined to be between 10-15 rot.ml^{-1} for gilthead seabream (*Sparus aurata*) reared at 100 larvae.l^{-1} (Tandler and Sherman, 1981) or 5-10 rot.ml^{-1} for common dentex (*Dentex dentex*) reared at 10 to 40 larvae.l^{-1} (Giménez and Estévez, 2008). When larvae are fed copepods densities are higher than 1nauplii.ml^{-1} in the culture of the creek anchovy (*Anchovy mitchilli*) and lined sole (*Achirus lineatus*) (Houde, 1977; O´Connell and Raymond, 1970) or about 4 nauplii.ml^{-1} in Californian anchovy (*Engraulix mordax*).

1.4.3.2.-Feeding sequence and co-feeding

Adequate prey sequence is a critical factor for the success of larval rearing techniques in new species. Mouth size and types of sustainable and mass produced available preys are limiting aspects. Therefore, eurihaline rotifers from the genus *Brachionus* with several morphotypes and

species are used as initial live prey, due to their small lorica size. More common morphotypes include *Brachionus plicatilis* type L (larger and elongated size), *B. ibericus* or S type (small and round form) and *B. rotundiformis* or SS (super small size) (Hagiwara *et al*., 2007). Rotifer feeding will be followed by the crustacean *Artemia* nauplii, to continue with enriched metanauplii and finalizing with inert diets. Species with better developed larvae at hatching and provided with a higher amount of yolk reserves than other species, such as European seabass (*Dicentrarchus labrax*) and Senegalese sole (*Solea senegalensis*), accept the early introduction of preys bigger than rotifers like *Artemia* from the beginning of the exogenous feeding (Cahu *et al*., 1999; Dinis *et al*., 1999). In other species like red drum (*Sciaenops ocellatus*) inert diets can be introduced as early as 10 dah (Buchet *et al*., 2000). Increased labor cost and prey production unpredictability, among other factors, discourage the use of live preys and their substitution by inert diets. But despite the efforts for the last 20 years, complete substitution of live preys from first feeding is only feasible for a reduced number of marine fish species. At present, most species are reared on a co-feeding basis of live prey and inert diets before the completed "weaning" (Rosenlund *et al*., 1997). Indeed, inert diets ingestion is improved in a 120% when *Artemia* is co-fed (Kolkovski *et al*., 1997). In relation to this, live preys provide a visual and chemical stimulant effect in the larvae that decreases with age, when a better development of visual and olfactory capacity is achieved. In addition, prey biochemical composition improves microdiet digestion and assimilation. Thus, the presence in *Artemia* of free amino acids such as alanine, glycine, arginine and betaine acting as attractant factors, stimulate microdiet ingestion, whereas phospholipids, particularly phosphatidilcoline with the trimetil coline chemical group which is also present in betaine, improves microdiet assimilation and digestion.

In certain cases, these traditional live preys are co-fed with others more difficult to obtain such as copepods, which fed during critical stages of larval development improve survival and growth, as it has been seen in turbot (*Psetta maxima*) (Støttrup and Norsker, 1997), west Australian jewfish *Glaucosoma hebraicum* and squired fish (*Pagrus auratus*) (Payne *et al*., 2001) or West Australian seahorse (*Hippocampus subelongatus)* (Payne and Rippingale, 2000). Moreover, copepods fed to Atlantic halibut (*Hippoglossus hippoglossus*) larvae improve the development of digestive systems and its absorption capacity (Luizi *et al.,* 1999). Besides, a smaller incidence of pigmentacion anomalies and a greater success in ocular migration occur in Atlantic halibut larvae fed with copepodos in comparison with *Artemia* fedding (Hamre *et al*., 2002). In Atlantic codfish (*Gadus morhua)* improved growth and lower incidence of skeletal malformations is obtained when larvae are fed copepods in comparison with rotifers (Imsland *et al.*, 2006) . These

advantages have been implemented to commercial hatcheries of both species (Olsen *et al*., 1999; Van der Meeren *et al*., 1997). Another live preys alternative to traditional ones are different larval stages of marine crustaceans. However, the complexity to obtain them limits the use to experimental scale purposes as first feeding preys for species such as common octopus (*Octopus vulgaris),* where a better growth and larval survival is obtained by co-feeding paralarvae with *Artemia* and crustacean zoeas (Villanueva 1995, Iglesias e*t al*., 2000, 2007; Moxica *et al*., 2002; Carrasco e*t al*., 2006). Finally, fish eggs or larvae from commonly reared fish have been also used to produce species with a very high market value such as Pacific blue fin tuna or greater amberjack. These species display a piscivorous phase from early stages of development, usually after notochord flexion, as denoted by their stomach contents studies (Catalan e*t al.,* 2007). At this stage of development, traditional live preys (rotifers or *Artemia)* do not fulfill the nutritional requirements of the larvae, either by an insufficient content of essential nutrients or by their inadequate prey size as it has been seen in Atlantic tuna (*Thunnus thynnus*), Pacific bluefin tuna (*Thunnus orientalis*), Atlantic bonito (*Sarda Sarda*) or greater amberjack (*Seriola dumerili*) (Kaji, 2002; Papandroulakis *et al*., 2006; Seoka e*t al*., 2007; Ortega and De la Gándara, 2007).

1.4.3.3.-Prey nutritional quality

In the wild, marine fish larvae are fed with a wide range of preys of different sizes and nutritional composition according to the different stages of development. In this sense, observed improvements with the use of copepods in marine larval rearing, are associated to their higher nutritional quality in relation to their content in lipids, essential fatty acids, vitamins, carotenes and other nutrients (Evjemo and Olsen, 1997; Evjemo *et al*., 2003; Van der Meeren *et al*., 2008). Nevertheless, the difficulties for massive production of zooplankton species other than rotifers and *Artemia* frequently limit its use to experimental facilities or extensive systems in certain geographical locations (Van der Meeren and Naas, 1997). For that reason, first feeding during early stages of marine fish larvae are based on rotifers and *Artemia sp.,* despite neither of them are natural preys for marine larvae and usually display nutritional characteristics that greatly differ from the natural food of the larvae, even after enrichment processes. This fact limits growth, survival and the normal development of marine fish larvae from early stages (Izquierdo, 1996; Ronnestad *et al*., 1998; McEvoy and Sargent, 1998).One of the most important factors to determine the food quality is its lipid content and particularly n-3 and n-6 series essential fatty acid (Watanabe *et al*., 1983; Izquierdo, 1996).

1.4.3.4.-Importance of lipids in the larval nutrition

In the recent history of aquaculture, a lot of effort has been devoted to the study of the nutritional requirements of marine fish larvae both using live preys and inert diets (Izquierdo, 1996; Sargent *et al*., 1999; Ronnestad *et al*., 1999). Agreat part of this research has been conducted on the essentiality of the highly unsaturated fatty acids (HUFA), especially those from n-3 series. Actually, it is well known, that elevated marine fish larval mortalities are frequently related to essential fatty acid deficiencies (EFA) (Izquierdo, 1996). The essential fatty acids, particularly highly unsaturated fatty acids as docosahexaenoic acid (DHA, 22:6 n-3), eicosapentaenoic acid (EPA, 20:5 n-3) and araquidonic acid (ARA; 20:4 n-6) have been found to be essential components of the cellular membranes, modulating physiological mechanisms as membrane transports process and enzymatic activity, specially at the firsts stages of larval development (Izquierdo, 1996). Freshwater fish have the enzymatic capacity to produce DHA, EPA and ARA from their precursors offered in the diets such as linoleic acid (18:2n-6) and linolenic (18: 3n-3), but most marine fish display a very limited enzymatic capacity (Δ5 and Δ6) to desaturate and elongate its precursors to form ARA, EPA and DHA and consequently these fatty acids need to be incorporated in the diet. Not only the total amount of individual HUFA, but also the relations among them and between n-3/n-6 series are important for correct growth and development of the fish larvae (Izquierdo, 1996; Rainuzzo *et al*., 1997; Sargent e*t al*., 1999). In addition to its structural and functional role in the cellular membranes, the EFA and particularly DHA, are important for functioning and normal development of the nervous system, as it happens with the retina (Bell *et al*., 1995a) whereas ARA and EPA are involved in the regulation and production eicosanoids mechanisms (Bell *et al*., 1995b; Sargent, 1997; Ganga *et al*., 2005). Feeding marine fish larvae with live preys or microdiets deficient in highly unsaturated fatty acids from n-3 series, causes symptoms such as growth and survival reduction, a greater sensitivity to the stress that increase cortisol levels, and immunodeficiency symptoms (Izquierdo, 1996; 2005). Other symptoms of AGE deficiencies are nutritional and swimming activity reduction and presence of hydrops (subcutaneous and subcelomic water accumulation) (Yamashita, 1981), inadequate inflation of the swing bladder (Koven *et al*., 1990) or disintegration of branchial epithelium. Also a diet deficient in HUFA alters retina formation, alters melanocyte stimulating hormone, interrupting melanin formation and giving rise to pigmentation anomalies as it was observed with some flat fish (Kanazawa, 1993). As a consequence, young herring (*Clupea harengus*) fed on DHA deficient diets show a reduction of visual acuity under of low intensity of light, which is translated to a lost in prey capture ability (Bell *et al*., 1995a). In mammals, studies of DHA

deficiencies in the diets show that this FA is strongly retained by the nervous system and at least two generations are needed to induce such deficiencies (Neuringer *et al*., 1986; Neuringer *et al*., 1988). But in fish the development of the brain and the retina takes places in the first days of life, and deficiencies are easily induce if adequate food is not supplied at first feeding (Bell and Dick, 1993). It has been proven that the levels of DHA in brain and eye of the larvae are modified easily with changes of this fatty acid in the diet.

1.5.-Problems in marine fish larval rearing

Nowadays, gilthead seabream (*Sparus aurata*) is one of the best know species in Mediterranean aquaculture what allows the continue increase of on growing production companies. Nevertheless, some aspects of their culture need to be improved, mainly those associated to the first stages of development to optimize the productivity of the commercial hatcheries. Besides, this species is used to develop biological models that will allow the development of rearing techniques for other species.

1.5.1.-Larval survival

The early stages of development of marine fish larvae display different critical points that seriously limit the success of larval rearing. One of the characteristic mortality peaks is observed between the beginning of exogenous feeding and the end of the yolk sac reserves (Tandler *et al*., 1989). The most important causes of early larval mortality are related to nutrition (low quality broodstock diet and n-3 HUFA limitations in live prey) or bacterial diseases related to microbial flora introduce via live food (Skjermo and Vadstein, 1999). Knowledge on larval nutritional requirements, particularly on essential fatty acids, has been improved in the last years and as a consequence a wider variety of live prey enrichments have been developed (Shields, 2001), as well as protocols and products for live prey disinfection (De Wolf *et al*., 1998; Giménez *et al.*, 2006), rotifers being quite susceptible to disinfection methods (Ringo and Birkbeck, 1999). Techniques to reduce bacterial load in the larval rearing tanks suggest the use of mature waters and/or recirculation systems. Besides, the benefits of probiotic or innocuous bacterial stocks to avoid opportunistic or pathogenic bacteria proliferation have been also studied to improve larval survival (Skjermo and Vadstein, 1999; Gatesosupe, 1999). All these advances, towards the control or early mortalities reduction have improved predictability of larval rearing success at a commercial scale. Still, sudden mortalities, probably caused by pathogenic viruses and skeletal deformities have a great impact on commercial hatcheries productivity.

1.5.2.-Incidence of morpho-anatomical disorders

Fry quality in terms of fry resistance to disease and stress and, particularly, of morphological abnormalities in comparison with a wild fish standard remain an important problem in fish larvae production. In intensive commercial hatcheries, around 10 to 30% of the marine fish larvae produced display some type of spinal deviations (Andrades *et al*., 1996). Moreover, a huge variation can be found between and within different production stocks and the type of deformity, its frequency and the economic implications varies not only within reared species but also among production cycles. In species as European seabass (*Dicentrachus labrax*) or sparids as gilthead seabream (*Sparus aurata*), common seabream (*Pagrus pagrus*) and red seabream (*Pagrus major*), the most important skeleton anomalies are those that affect the opercula complex, neurocranium and vertebral column (lordosis and vertebral fusion) (Koumoundouros *et al*., 1997a; Boglione *et al*., 2001; Roo *et al*., 2005b). In the case of flat fish as turbot (*Psetta Maxima*), Atlantic halibut (*Hippoglosuss hipoglosus*) or Senegalese sole (*Solea senegalensis*), additionally to column malformations, important alterations are found in pigmentation as well as incomplete eye migration and mandible deformities (Gavaia *et al*., 2002; Sæle *et al*., 2003; Aritaki and Seikai, 2004; Lewis and Lall, 2006). Thus, within the industrial process for fry production companies are forced to incorporate screening processes to eliminate the non-commercially viable individuals that display abnormalities. Generally, on growing farms do not accept fry batches with over 5% deformed fish after visual evaluation. Screening process adds around 0.01€ per fry to total production cost in gilthead seabream.

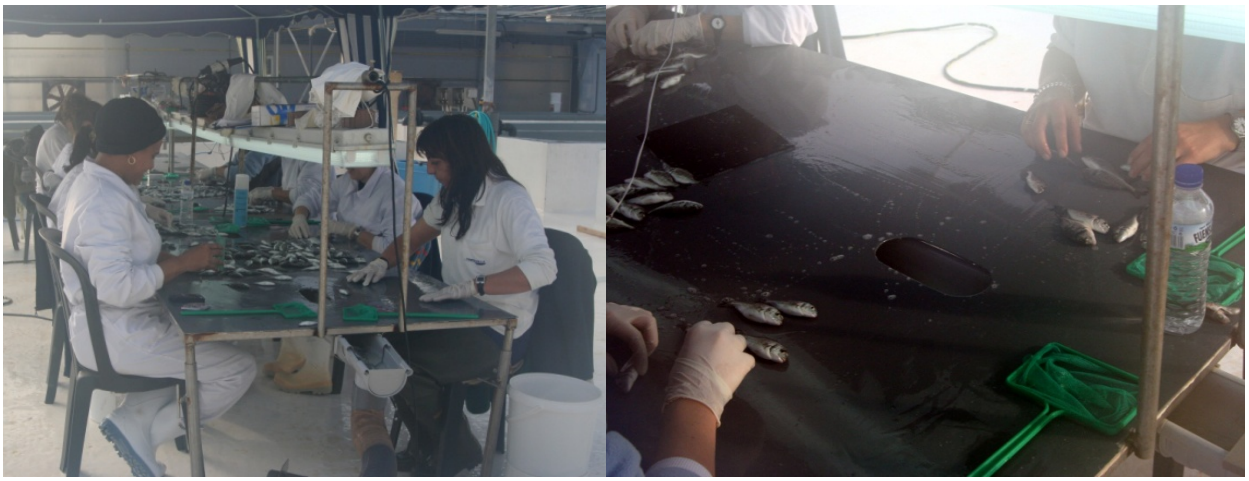


Figure 11. Individual fry screening to eliminate deformed individuals (courtesy of Tinamenor S.A).

The different anomalies affecting fry quality are related either to pigmentation or skeleton anomalies.

1.5.2.1.-Pigmentation anomalies

Pigmentation anomalies include both partial and total alteration of the external body coloration. They have been widely described in flat fish, where they cause problems with important economic repercussion (Gavai *et al*., 2002; Sæle *et al*., 2003; Aritaki and Seikai., 2004; Lewis and Lall, 2006). Hypomelanosis, a lack of pigmentation also known as pseudo albinism, affects upper or ocular side (Figure 12), whereas the hypermelanosis is a hyperpigmentation which particularly affects the down or hidden side of flat fish, also known as ambi-coloration (Venizelos and Benetti, 1999). Pigmentation anomalies are considered a consequence of a differential distribution and or cellular physiological differences in the chromatophores (Kelhs, 2004; Burton, 2005) which have been associated to factors as intensive culture conditions, larval density, illumination, substrate or nutritional alterations (Seikai *et al*., 1987; Estévez *et al*., 1999, 2001; Benetti, 1997). In wild populations both pseudo-albinism (Venizelos and Benetti, 1999) and ambicoloration (Astarloa, 1995) may occur although in a lower frequency than in cultured populations (Bolker and Hill, 2000).

Figure 12. Senegalese sole (*Solea senegalensis*) with normal pigmentation and pseudoalbinism.

1.5.2.2. Skeleton anomalies and factors related to their appearance

Studies about osteological development and skeletal anomalies have been conducted in different sparid species. like gilthead seabream (*Sparus aurata)* (Faustino, 2002; Boglione *et al*., 2001; Koumondouros *et al.*, 1997a), red seabream (*Pagrus major)* (Moteki, 2002; Kihara *et al.,* 2002;

Matsuoka, 2003), common dentex (*Dentex dentex)* (Koumoundouros *et al*., 2001a), common pandora (*Pagellus erythinus)* (Boglione *et al*., 2003; Sfakianakis *et al.,* 2004), sharpsnout seabream (*Diplodus puntazzo)* (Boglione *et al*., 2003; Favaloro and Mazzola, 2003) and white seabream *(Diploduss sargus)* (Koumoundouros *et al*., 2001b; Sfakianakis *et al.,* 2003). In these species, the most important skeleton anomalies with important economic implications are those affecting the opercula complex, neurocranium and vertebral column.

Deformities associated to neurocranium affect to the opercula complex and jaws and they are frequently found in commercial hatcheries. The anomalies of the opercula complex can be present in one or both sides of the fish and consist in a folding or an incomplete formation of the opercula complex (Koumoundourous *et al.,* 1997b) (Figure13). Commonly this anomaly is associated with branchial arcs malformations (Sadler *et al*., 2001) and appear from very early stages of development being able to affect up to 98.3% of the population (Beraldo *et al*., 2003).

Figure 13. Red porgy (*Pagrus pagrus*) fry and meagre (*Argyrosomus regious*) (a,c) with opercula complex anomaly; b) detail of the opercula folding.

Jaw deformities include lower or upper jaw torsions and different degrees of prolongation (Cobcroft *et al*., 2001), being sometimes associated to lethal effects (Barahona-Fernandes, 1982) (Figure 14).

Figure 14. Mandible deformities: a,b) red porgy; c) normal shape gilthead seabream,

In general, ontogeny development of the jaw structures and fins appears at very early stages but is not completed to older stages. Fin deformities are characterized by torsion, partial or total lack and can reach up to 65% of the population in gilthead seabream (*Sparus aurata*) larvae (Koumoundouros *et al*., 1997b) although they have a minor relevance at commercial scale (Figure 15).

Figure 15. Anomalies in the pectoral fins radios: a) gilthead seabream; b) red porgy.

Vertebral column abnormalities are among the most important skeleton aberrations found in aquaculture, not only by their severe effect on fish morphology but also by its clear influence on growth productive characters (Gjerde *et al*., 2005; Kause *et al*., 2005). The main column

deformities are scoliosis, lordosis, kyphosis and vertebral fusions, sometimes several of them combined in the same fish (Afonso *et al*., 2000) (Figure 15).

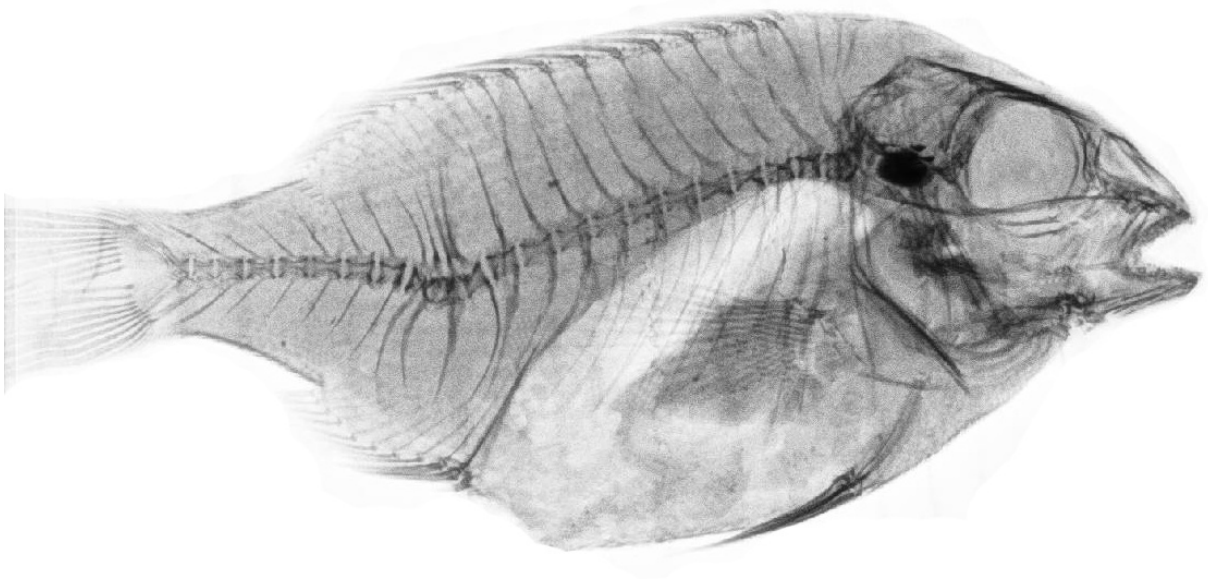


Figure 16. Column anomalies in red porgy (*Pagrus pagrus*).

Column abnormalities have been well documented in freshwater (Akiyama *et al*., 1985; Madsen and Dalsgaard, 1999; Witten *et al*., 2005, 2006) and marine fish species (Paperna 1978; Takashima 1978; Barahona-Fernandes, 1982; Taniguchi *et al*., 1984; Daoulas *et al*., 1991; Andrades *et al*., 1996; Koumoundouros *et al.,* 1997b, 2001a b, 2002; Faustino and Power 1998, 2001; Afonso *et al.*, 2000; Boglione *et al.*, 2001; Faustino 2002 ; Gavaia *et al.*, 2002; Kihara *et al.*, 2002; Moteki 2002; Favaloro and Mazzola 2003; Boglione *et al*., 2003; Matsuoka, 2003; Sfakianakis *et al.,* 2004).

Appearance of skeletal deformities in fish has been related to environmental, nutritional and genetical factors. Environmental factors associated to deformities include abiotic (light intensity, dissolved oxygen, temperature, pH, salinity, tank hydrodynamic, rearing system), biotic (bacteria, virus, fungi and parasites) and xenobiotic (algaecides, fungicides, herbicides, insecticides, industrial effluents and heavy metals). Among nutritional factors, the importance of essential fatty acids, phospholipids, amino acids, proteins and vitamins has being mainly emphasized. In relation to genetic factors both unigenic and polygenic have been described.

1.5.2.2.1-Environmental factors

Light, temperature or salinity has been described as main environmental factors inducing skeleton deformities incidences in marine fish (Battaglene and Talbot, 1990; Polo *et al*., 1991; Mihelakakis and Yoshimatsu, 1998; Cobcroft *et al*., 2001; Sfakianakis *et al*., 2004). Other causes of damage to the larval skeleton integrity include mechanical-shock during embryonic or early larval development. Anomalous development of the swing bladder (Chatain, 1989,1990; Andrades, 1993;) and tank hydrodynamics variations (Chatain, 1994; Divanach *et al*., 1997; Koumoundouros *et al*., 1997a, b; Kihara *et al*., 2002) have been reported as causes of lordosis episodes.

In addition, culture system, and particularly the intensification of the rearing techniques, has been described as a modulator of skeletal deformities occurrence. Thus, extensive and semi-intensive systems have been associated to lower incidence of skeletal deformities than in intensive systems (Divanach and Kentouri, 1983; Divanach *et al*., 1996; Boglione *et al*., 2001; Koumoundouros *et al*., 2001a; Sfakianakis *et al*., 2004; Roo *et al*., 2005b; Giménez and Estévez, 2008b).

Information about the effect of biotic environmental factors on skeletal deformities is scarce, being parasites one of the most studied factors. Parasites from mixosporea family have been described as skeletal deformities promoters in rainbow trout *(Oncorhyncus mykiss),* Korean amberjack *(Seriola quinqueradiata)* and perca *(Perca fluviatilis)* (Lom *et al*., 1991). Among xenobiotic factors related to skeletal deformities incidence, pesticides (Chun *et al*., 1981; Thi Hong Lien *et al*., 1997), herbicides (Koyama 1996), hydrocarbons (Grady *et al*., 1992), organic and organoclorade compounds (Lindesjöö *et al*., 1994) and metals (Slominska and Jezierska, 2000) have been found clearly correlated. Excess of antibiotics to treat several types of diseases has been also related to the induction of skeletal deformities in fish (Toften and Jobling, 1996).

1.5.2.2 2.-Nutritional factors

In addition to environmental factors, nutritional factors such as dietary hormones, vitamins and fatty acids contents might directly act on bone and cartilage metabolism. Other indirect metabolic factors related to these processes are prostaglandins, cytoquines and growth factors (Watkins and Seifert, 2000a). In fish, nutritional factors such as excess or deficiencies of essential fatty acids and vitamins in broodstock diets and/or during the first stages of growth can

markedly alter the osteological development of embryo and larvae (Kanazawa *et al*., 1983; Akiyama *et al*., 1986; Knox *et al*., 1988; Chatain and Ounais-Guschemann, 1990; Afonso *et al*., 2000; Cahu *et al*., 2003; Saele *et al*., 2003; Hamre *et al*., 2005). Although, in fish it has not been shown a clear relation between dietary and cartilage and bone fatty acid composition, studies in other vertebrates as chickens and rats have shown that variations in the relation of n-3/n-6 series in the diet, alters bone and cartilages fatty acids composition (Xu *et al*., 1994; Watkins *et al*., 1991, 2000b; Liu *et al.*, 2004). Nevertheless, skeletal deformities appearance in marine fish larvae have been suggested to be induced by alterations in the dietary essential fatty acids profile (Gapasin and Durai, 2001). However, the mechanisms by which fatty acids control the osteological development are clearly understood. Some authors have suggested that certain fatty acids regulate bone development by acting as gene modulators through specific nuclear active receptors, such as peroxysome proliferators active receptors (PPAR) that are join to DNA molecule as heterodymers with retinoic acid X receptors (RXR). These receptors act like transcription ligand-activated factors (Mangelsdorf *et al*., 1994) regulating genes involved in skeletal development during the larval ontogenesis. In this way, indirectly, essential fatty acids regulate bone development related genes expression and affect skeletogenesis. Still studies must be conducted to clearly elucidate the role of essential fatty acids in bone development and malformations occurrence in order to design effective diets, which are able to prevent these alterations (Cahu *et al*., 2003). More recently it has been suggested that an excess of certain essential fatty acids in the diets accelerates the osteoblast differentiation through a hyper regulation of α-retinoic X receptors and bone morphogenesis proteins (BMP_4), inducing the occurrence of an excess of vertebrae in European seabass *(Dicentrarchus labrax)* (Villeneuve *et al*., 2005, 2006).

In addition, essential amino acids, such as tryptophan, deficiencies have been long ago associated to deformities induction (Walton *et al*., 1984; Akiyama *et al.,* 1985, 1986; Wilson 1989). Moreover, the inclusion of peptides (protein hydrolasates) in the diet along the larval development noticeably reduce skeletal malformations in European seabass larvae, (Cahu *et al*., 1999). Besides, as it is well known in other mammals, unbalanced vitamin C contents induce bone malformations in fish (Halver, 1989; Dedi *et al*., 1995; Takeuchi *et al*., 1995, 1998). Finally, there is also an important terathogenic ability in retinoic acid along human and animal embryogenesis, measured through the expression of Hox and Sonic Hedgehog (SHH) genes, and in fish a dietary excess of retinoic acid or some of its molecular derivatives Japanese flounder

larvae *(Paralichthys olivaceus)* increases the occurrence of vertebrae compression (Takeuchi *et al*., 1998).

1.5.2.3.3.-Genetic factors

Andrades *et al*. (1996) related that most of the lordotic larvae observed *(Sparus aurata)*, probably are lordotic larvae survivors, suggesting that primary causes of the lordosis appearance could be, among others, from genetic origin that can affect eggs during the embryonic development. Afonso *et al*. (2000), described the relation of a severe column deformity with the simultaneous appearance of lordosis, scoliosis and kyphosis to familiar association. Besides Astorga *et al* (2003a, b) demonstrated the effect of consanguinity in the appearance of the skeletal deformities of gilthead seabream, throughout crossing normal individuals with different levels of consanguinity (F=0.125; F=0.25), to the ages of 4, 14 and 35 days post-appearance and to the 194 days of age.

Improvements in the production technology of red porgy (*Pagrus pagrus*) larvae and fry: Importance of rearing conditions and diet nutritional value on their quality

OBJECTIVES

2.-OBJECTIVES

The main objective of this study was ***"to improve the production technology of red porgy (Pagrus pagrus) larvae and fry".*** Specific objectives are grouped in two complementary and consecutive phases: the first part was mainly based in a biological approach where specific aspects of the first developmental stages of this species such as visual and digestive system, osteology and apparition of skeletal deformities according with the rearing system and nutritional value of the live preys were described; whereas the second part, taking advantage of this knowledge aimed to improve the larval rearing protocol for this species.

These objectives are addressed in four different studies included in the thesis:

Study I. **Development of red porgy larvae *Pagrus pagrus* (Linnaeus, 1758) visual system in relation with changes in the digestive tract and feeding habits.**

This study aims to describe in red porgy larvae, the development of the visual system and its correlation with that of the digestive system. This objective was addressed in order to obtain basic information in relation to some general culture conditions as photoperiod and light intensity changes, duration of green water use or feeding sequence. An experiment was done to achieve this objective.

Study II. **Osteological development and occurrence of skeletal deformities in red porgy *Pagrus pagrus* (Linnaeus, 1758) larvae cultured under different rearing techniques.**

This study aims to describe the effect of the intensification of the larval rearing techniques in relation to the osteological development and the incidence of skeletal malformations in red porgy. This objective was addressed in order to obtain basic information about the osteological development pattern and the incidence of the skeletal anomalies in this species that will allow determining the fry quality and suitability of the rearing techniques. Two experiments were done to achieve this objective.

Study III. **Effect of DHA content in rotifers on the occurrence of skeletal deformities in red porgy *Pagrus pagrus (*Linnaeus*, 1758).***

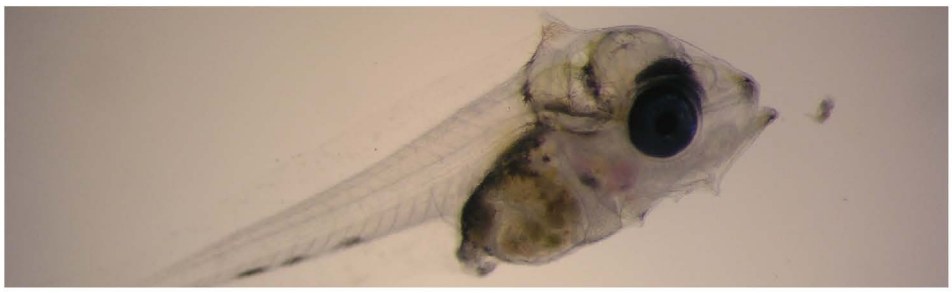
This study aims to describe the effect of certain nutritional factors on growth, survival and the incidence of skeletal malformations in the first stages of development of red porgy. This objective was addressed in order to identify the nutritional factors affecting larval performance and to improve the enrichment products for live preys fed to this species. Two experiments were done to achieve this objective.

Study IV. **Advances in rearing techniques of red porgy *Pagrus pagrus, (*Linnaeus*, 1758):* Comparison between intensive and semi-intensive larval rearing systems**.

This study aims to improve the larval rearing protocol for this species. To achieve this objective three experiments were conducted focusing the effect of light regimes, prey density and co-feeding protocol and comparing larval rearing systems.

Improvements in the production technology of red porgy (*Pagrus pagrus*) larvae and fry: Importance of rearing conditions and diet nutritional value on their quality

MATERIALS AND METHODS



3.-MATERIALS AND METHODS

3.1.-Study location

The experiments described in the present document have been performed at the larval rearing and fry production facilities of the Canary Institute of Marine Sciences (ICCM) (Mesocosms Plant) (Agencia Canaria de Investigación, Innovación y Sociedad de la Información). These facilities were build in the frame of the project Inter Regional Action for Technology Transfer (INTERACTT), co-financed by European Commission (D.G. XVI) and the Government of the Canary Islands, with the objective to transfer a hatchery technology for the production of juveniles marine fish from the Aquaculture Dpt. of the Institute of Marine Biology of Crete (Greece) to Canary and Madeira SME´s in order to sustain the development of local aquaculture industries. At this experimental plant, different activities of *R&D+I* through the use of semi-intensive (Mesocosms) and intensive technologies for marine fish larvae production are conducted. These facilities are located in Melenara, Telde, province of Las Palmas (Canary Islands; Spain), with a geographical coordinates of 27º59´31´´N and 15º22´31´´W, (Figure 17).

Figure 17: Top view of the Canary Islands and location of the facilities of ICCM (Google Earth photo).

3.2.-Facilities description

3.2.1.-General overview

Larval rearing and fry production facilities of the Canary Institute of Marine Sciences (ICCM) constitute a pilot scale plant, which main objective is to develop larval rearing techniques for marine fish diversification, working with new species for aquaculture like common seabream (*Pagrus pagrus*), meagre (*Argyrosomus regius*) and Senegalese sole (*Solea senegalensis*). Other objectives of this experimental lab are the development of research programs for improving the

rearing techniques of consolidated species as gilthead seabream (*Sparus aurata*) and European seabass (*Dicentrarchus labrax*), in order to increase survival, growth and fry quality including the reduction of skeletal deformities incidence. Finally, automation processes are also studied to simplify and better control mass production of juveniles. The dimensions of these facilities allow the production of thousands of fry of the mentioned species, which contributed to the development of commercial research programs and agreements with different companies.

These facilities occupy a 800 m^2 area and comprised two independent units.

Unit A:

- Water treatment area (water filtration and ultraviolet sterilization).
- Live food production area.
- Larval rearing area, (2 x 40,000 l; 4 x 2,000 l; 6 x 500 l tanks).

Unit B:

- Weaning and nursery area, with 8 x 10,000 l tanks.
- Control and storage gases area (oxygen and propane).

General scheme of the facilities are presented in Figures 18 and 19.

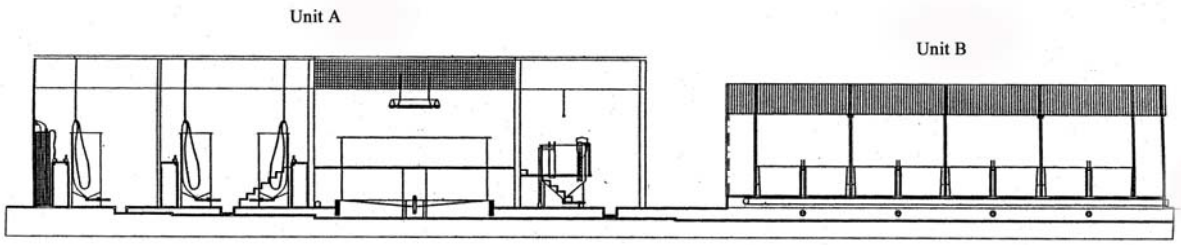


Figure 18. Longitudinal section of the different units.

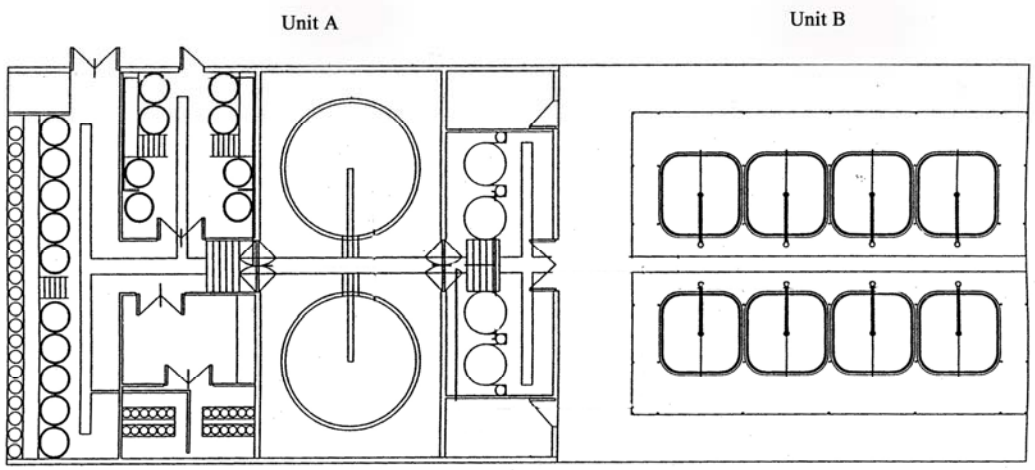


Figure 19.Top view of the different units.

In Figure 20 a detailed view of the different units are presented.

Figure 20. a) General view of units A and B; b) Storage and water treatment area; c) Phytoplankton and rotifers production area; d) Semi-intensive larval tank; e) Intensive larval rearing tanks; f) Weaning and nursery area.

3.2.2 Larval rearing tanks

Three different types of tanks were used for larval rearing experiences, all of them made of polyester reinforced with fibre glass.

3.2.2.1. Tanks of 40.000 litres- Semi-intensive system

Two cylinder-conical tanks with a total volume capacity of 40,000 l are settled in parallel and separated by a central drainage channel (Figure 21). The tanks have a diameter of 5m and a depth of 2.35 m in the central part (Figure 22) water inlet is located in the superior part and two water outlets are located one in the centre at the tank bottom and another in the lateral side. Both inlet and lateral water outlet can be modified with different movable accessories throughout the culture period according to the feeding sequence. Thus, during first stage of feeding with live prey the water inlet is situated near to the tank bottom and the outlet is maintained close to the water surface. Once the inert diets are introduced the system is reversed to favour the tank bottom cleaning.

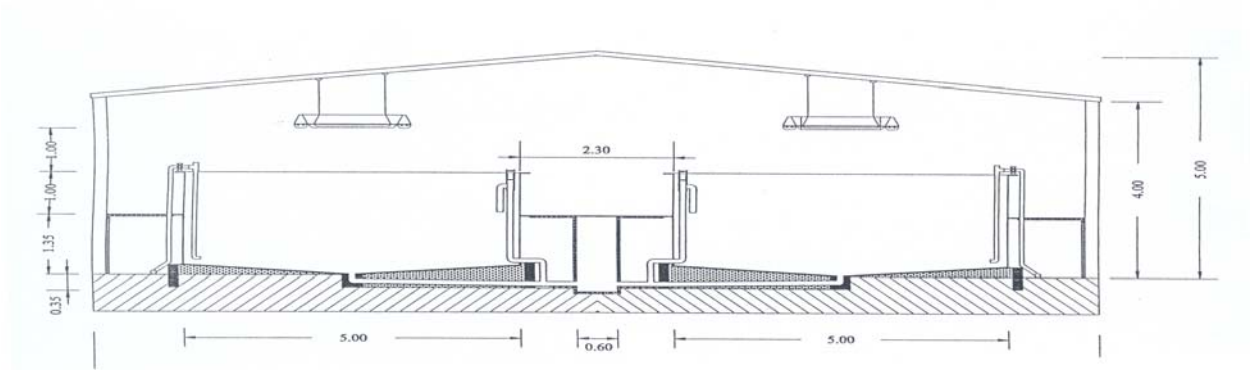


Figure 21. Longitudinal section of the semi-intensive rearing tanks

Figure 22. a) Longitudinal and top view of the tank; b) Semi-intensive rearing tank

3.2.2.2. Tanks of 2.000 litres- Intensive system.

Four cylinder-conical tanks with a total volume capacity of 2,000 l separated by a central drainage channel (Figure 23) constitute this part of the facility. Tanks have a diameter of 1.5 m and a depth of 2.10 m in the central part (Figure 24). Water inlet is located in the superior part and two water outlets are located one in the centre at the tank bottom another one in the lateral side in front of the water inlet. Both inlet and lateral water outlet can be modified with different movable accessories, throughout the culture period according to feeding sequence.

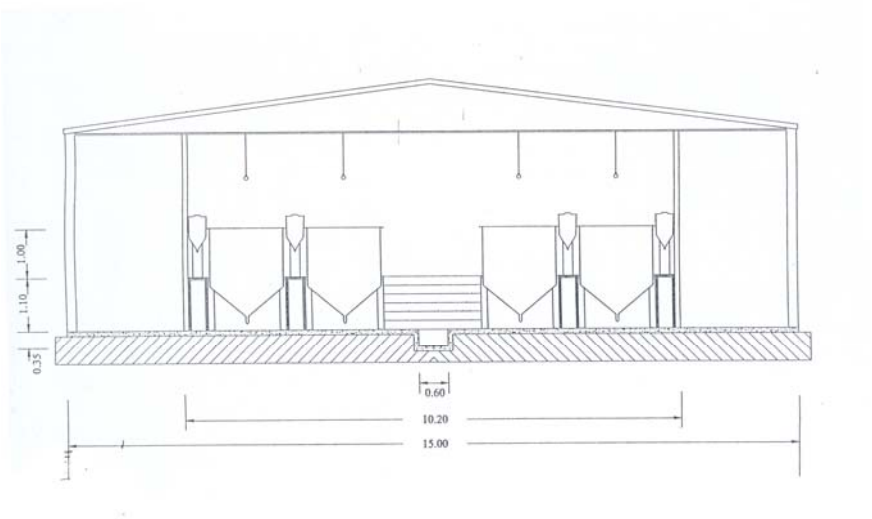


Figure 23. Longitudinal section of the intensive rearing tanks.

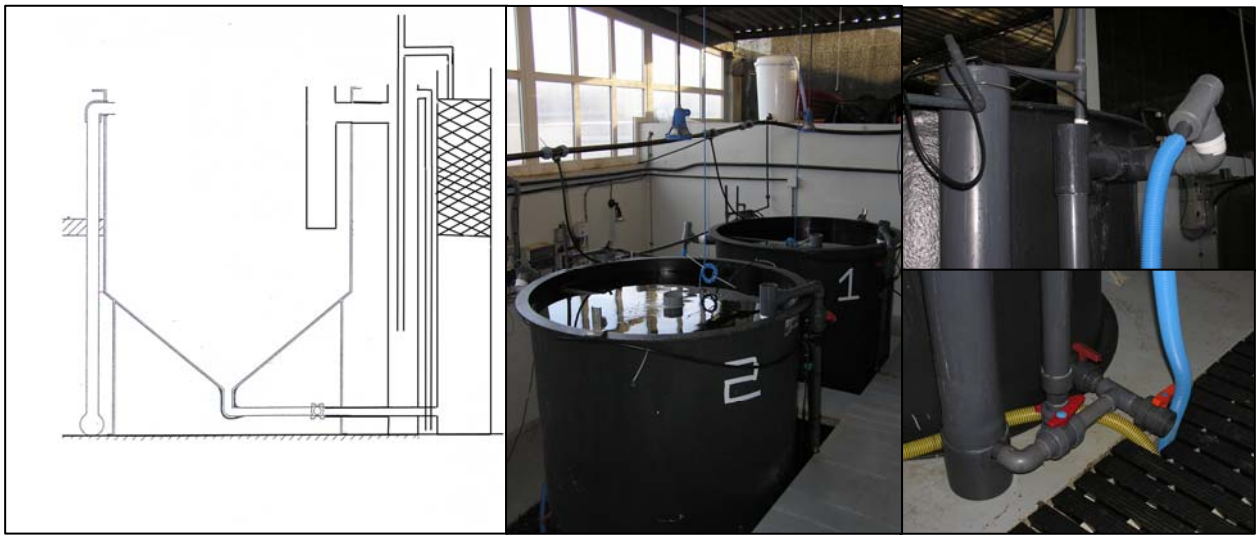


Figure 24. Longitudinal and different details of the water inlet and out lets of the intensive rearing tank.

3.2.2.3. Tanks of 500 litres- Intensive system

Six cylinder conical tanks with a total volume capacity of 500 l are settled in pairs and separated by a central drainage channel. Tanks have a diameter of 1.5 m and a depth of 1.0 m in the central part (Figure 25). Water inlet is located on the tank bottom and water outlet is located near the top of the tank in the lateral side. Both inlet and lateral water outlet can be modified with different movable accessories, throughout the culture period according to the feeding sequence.

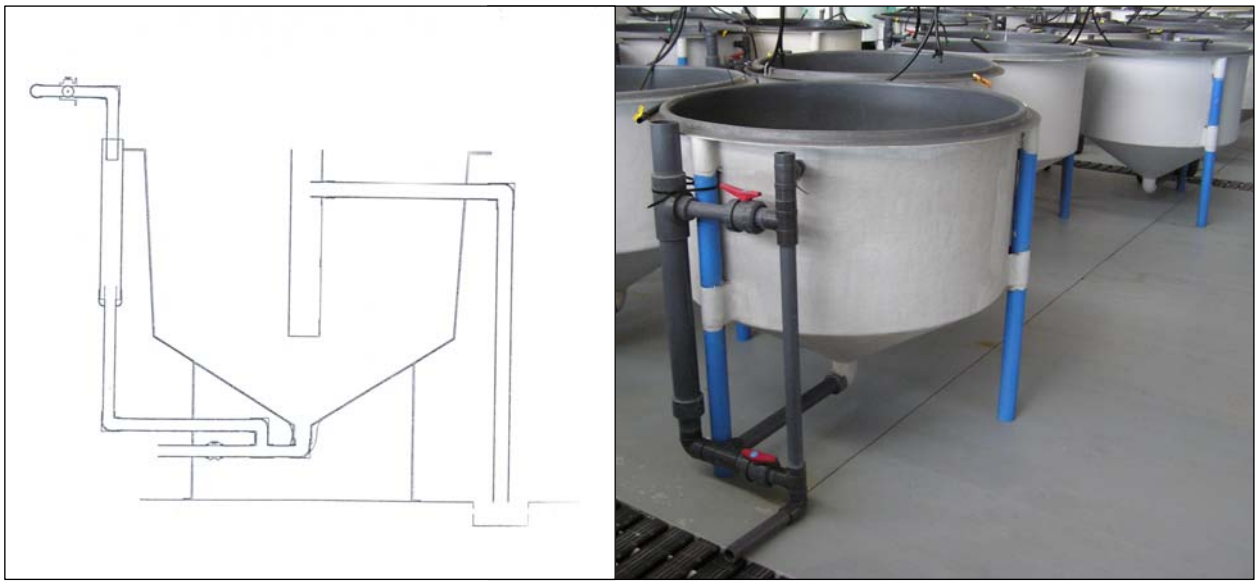


Figure 25. Longitudinal section and different details of the water inlet and out lets of the intensive 500 l rearing tanks.

3.2.2.4. Tanks of 10,000 litres- Weaning and nursery

Eight square tanks with interior rounded corners and light bottom slope towards central water-drainage, with a total volume capacity of 10,000 l were used. These are located in two parallel rows of 4 tanks, separated by the central canal of water-drainage (Figure 26). All the tanks have a lateral length of 3m and a depth of 1.4 m in their central part (Figure 27). Water inlet is located in the superior part and a single water outlet is located in the centre of the tank bottom.

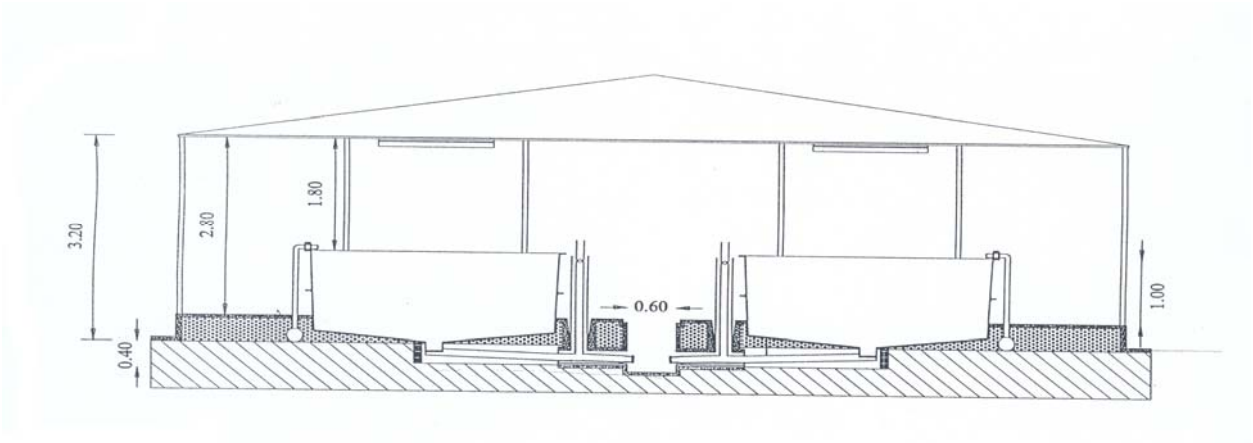


Figure 26. Longitudinal section of the weaning and nursery rearing tanks.

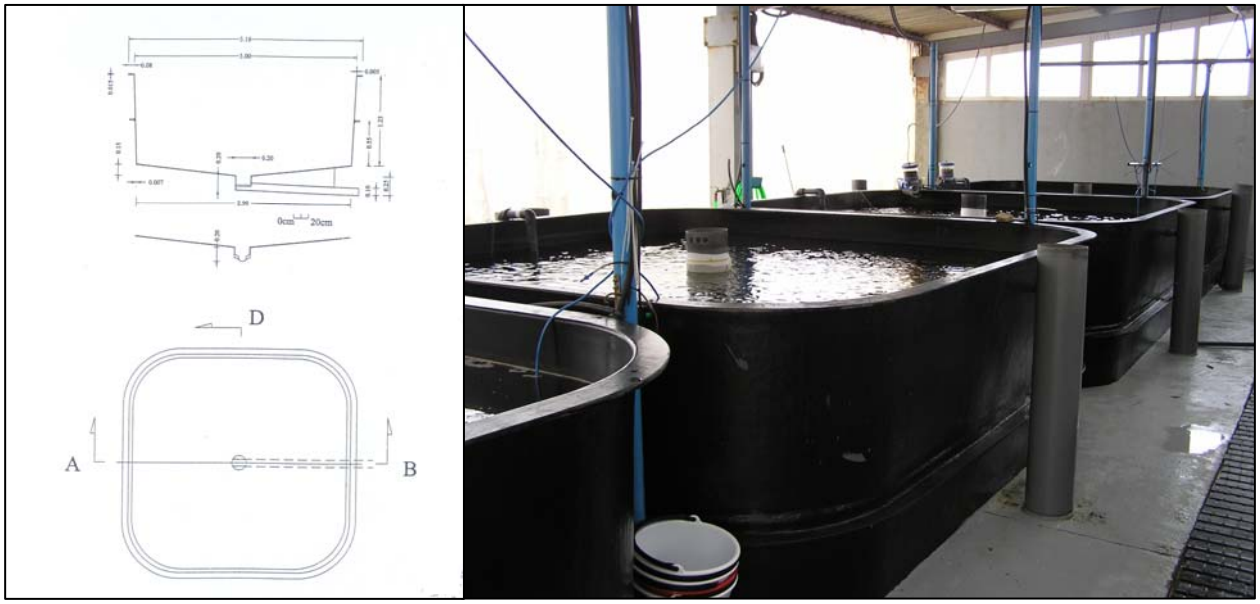


Figure 27. Longitudinal section, top view and different details of the water inlet and out lets of the weaning and nursery rearing tanks.

3.3.-Studied species

Red porgy (*Pagrus pagrus*), is a teleost, belonging to *Sparidae* family, its detailed taxonomic classification is presented bellow.

Phyllum: *Chordata*
Superclass: *Gnathostomata*
Class: *Osteichthyes*
Orden: *Perciformes*
Suborden: *Percoidei*
Family: *Sparidae*
Genus: *Pagrus*
Species: *Pagrus pagrus*

Figure 28. Adult fish, eggs, red porgy larvae and fry; on-land comercial facilities for red porgy on growing test.

3.3.1 Habitat

Red porgy is an euritermal species that adapts to an wide range of temperatures and a great variety of habitats. It is generally found in rocky bottoms in a depth around 50 m, although small size individuals are frequently found in shallow waters (10-30 m). In the wild, red porgy diet is mainly based on mollusc and crustaceans, but in captivity it easily adapts to pellets diets. This is a protogynous species which develops female gonads during the first years of life and whose

spawning season commences in December or January in the western Atlantic (Ciechomski and Weiss, 1973) and can be prolonged until spring in the Canary Islands (Pajuelo and Lorenzo, 1996). In this geographical location and under captivity, spawning season starts at the beginning of March and is extended until the end of May without photo-thermal manipulation (Cejas *et al*., 1997).

3.3.2 Geographical distribution

This species has been reported in different regions of the Mediterranean and Adriatic Sea, in the Atlantic, from British Islands to the south of Angola, including the Canary Islands, Azores and Madeira. It is also possible to locate it in the west Atlantic from New York to the south of Argentina (Manooch and Hassler, 1978) (Figure 29).

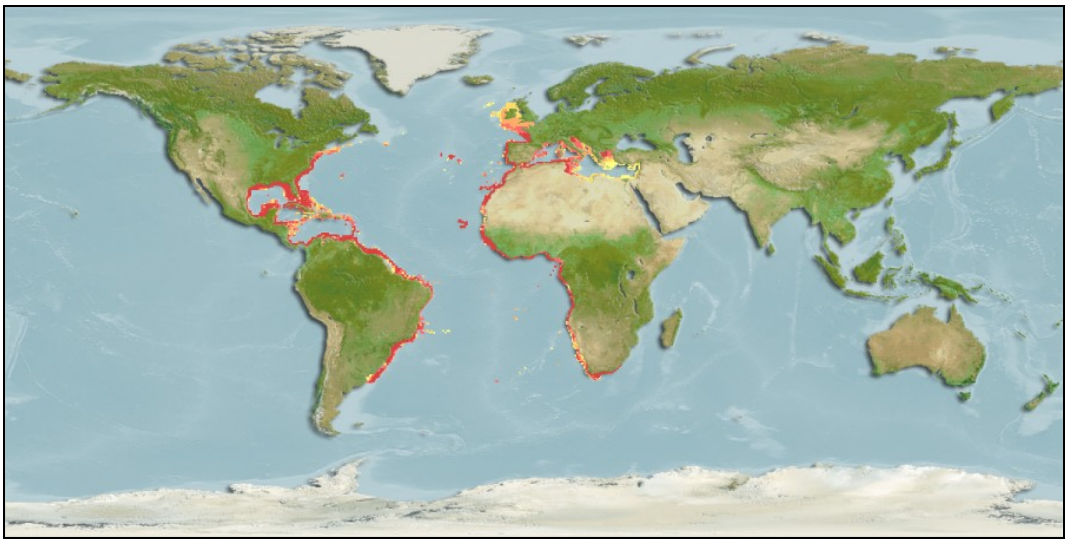


Figure 29. Geographical distribution of *Pagrus pagrus* around the world (Fish Base, 2008).

3.3.3 Aquaculture perspectives

Pagrus pagrus is one of the marine fish species proposed as potential candidate for the marine aquaculture diversification of the Atlantic coast of Spain and Mediterranean sea (Kentouri *et al*., 1995; Hernandez-Cruz *et al*., 1999). It has a high market price and a good acceptance what are indispensable conditions for a new species introduction. Commercial on-growing of common seabream, is still very limited, although some production statistic data were reported in Greece in 1999 with 100 tons (FEAP, 2006). Additionally, during the development of this study different on-growing experiences were carried out both offshore and inland in collaboration with local

commercial farms. Fry transport conditions have been described by Pavlidis *et al*., (2003), and recently data of fry production were reported in Greece with 2 million fry in years 2006, 2007 and 2008 (FEAP, 2008). while no data of on growing production is yet available. On pilot scale, the productions obtained in the Canary Islands have been increased in the last years, with 12.000 fry in 2002 to reach values close to 100.000 regularly produced in 2005 and 2006. Different studies of basic biology have described in detail the ontogeny of different organs (Socorro *et al*., 2001, Roo *et a.*,, 1999, Darias *et al*., 2005, 2007) also the detailed description of the osteological development of common seabream has been published by Socorro (2006). Studies on larval rearing of this species show different degree of success according to the culture conditions applied (Kentouri *et al*., 1995; Hernández-Cruz *et al*., 1999; Mihelakakis *et al*., 2001; Papandroulakis *et al.,* 2004).

3.4.-Experimental conditions

3.4.1.-Live food cultures

"Live food", a term used to define the live organisms that are used as feed for marine fish larvae, were produced under the specific conditions within the different units.

3.4.1.1.-Phytoplankton culture

For all the experimental trials, the *Eustigmatophycea* microalgae, *Nannochloropsis sp.* was used. The most important characteristics of this species were described by Maruyama *et al*., (1989) are shown in table III and Figure 30.

Table III: Biological characteristics of *Nannochlropsis sp.* (Maruyama *et al.,*1989).

Dimensions: 2-4 μm maximun diameter.
Cellular shape: circular or oval shaped.
Chloroplast shape: cup or oval shaped.
Propagation: Binary fission
Endoplasmic reticulum in the chloroplast: present.
Tylacoids disposition: 3-tylacoids.
Pigments: chlorophyll a, carothene, violaxanthyn, vauqueriaxanthyn.

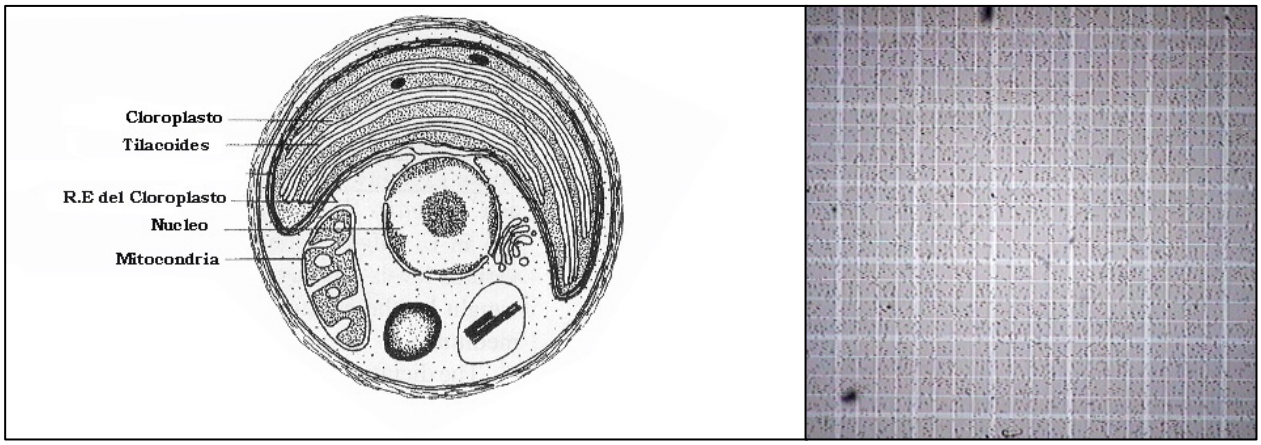


Figure 30: Cell organization of *Nannocloropsis sp*. and cells vision in Neubauer chamber.

The culture system employed for massive phytoplankton production was a "Bach type". This was conducted in the live food production area, under volumes increasing from 50 to 230 and 460 l, using transparent polyethylene bags (Figure 31).

Figure 31. Phytoplankton culture bags of 50, 230 and 460 l volume.

Water was mechanically filtered through a polyester reinforced fibre glass filter (Mod.00689; Astral pool, Barcelona, Spain) filled with a sand bed of diverse sizes. Water was also sterilized by UV radiation at 254nm wavelength with industrial equipment (Mod. M-3PE-300; Wedeco AG, Herford, Germany). Natural salinity (37 ‰) was reduced by addition of fresh water to reach a final salinity of 25 ‰, which was verified by means of a portable refractometer (Mod. SZJ-S, Madrid, Spain). To avoid cells sedimentation, air was supplied throughout ceramics diffusers

settled at 15 cm of the bag bottom. Culture was performed under continuous photoperiod, combining natural with artificial illumination by means of fluorescent lights (Mod. TLD 58W/54-765, Philips, France), maintaining a minimum light intensity during the night of 9,500lux measured with a digital Luxometer (Mod. HT170N; Italy).

Regardless the culture volume, the culture protocol followed a similar pattern: inoculation of microalgae (initial concentration of 1.5 x 10^6 cells.ml^{-1}) in the bag or column previously filled with seawater (25 ‰) and fertilized with a commercial product (Nutri-Phyt; Easy Algae S.L, Puerto de Santa María, Spain) the first day of culture. The growing period lasted 8 to 10 days on average, when culture maximum density was reached (35 to 80 x 10^6cells.ml^{-1}) depending on the culture volume, being generally lower in the larger volumes. At this stage, total volume was harvested and a new cycle started.

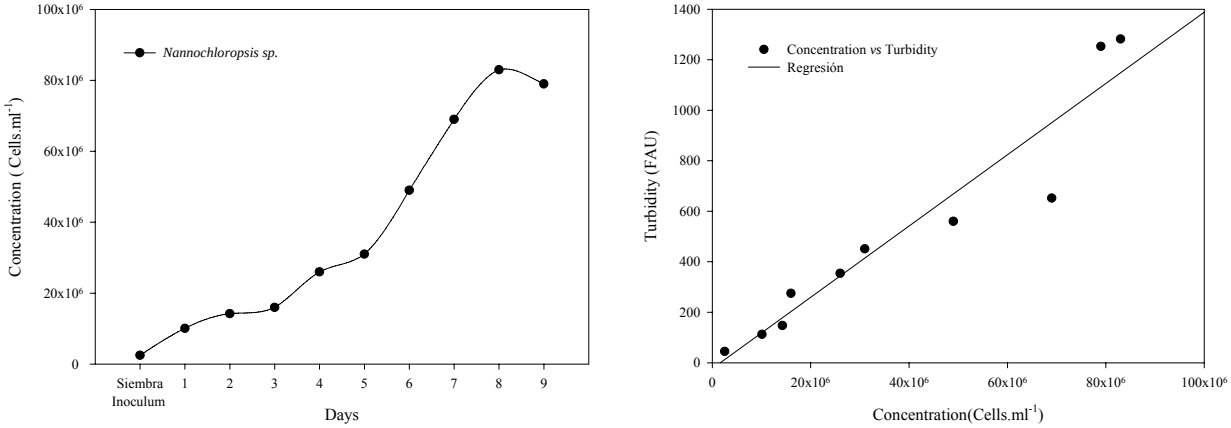


Figure 32. Growth curve and correlation between cell concentration and culture turbidity.

Culture evolution was daily checked by a photometric evaluation testing turbidity with a portable photometer (Mod.PF-11; Macherey-Nagel; Durew, Germany) and individual counts with haemocytometer (Mod. Neubauer, Germany) also, samples of different bags were randomly collected, to determine the cells concentration and to detect the presence of biological contamination agents in the culture media (Figure 32).

Occasionally, contamination with the ciliate *Euplotes sp.* (Figure 33), was observed, being air supply the most common source of contamination. To prevent ciliates contamination, the addition of formaldehyde (38%) (Panreac, Spain) at a dose of 0.05 ml.l^{-1} of culture, gave good results killing 100% of the polluting agents without compromising phytoplankton cell viability.

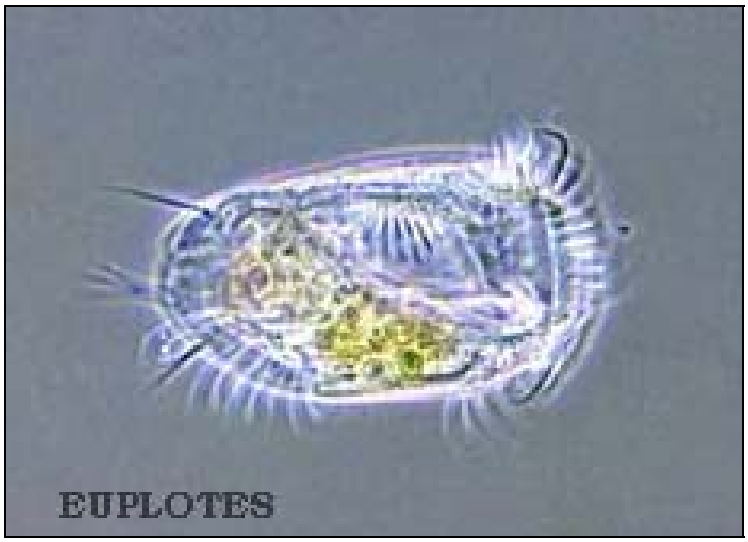


Figure 33. Polluting organism (*Euplotes sp*) found in *Nannochloropsis sp.* culture.

The phytoplankton produced, was used for different purposes within the hatchery process. Thus, 50l bags were commonly used to inoculate and up-scale phytoplankton to 230 l bags. Microalgae coming from 230 l bags were used to inoculate 460 l bags as well as for rotifers strain maintenance. Finally, the phytoplankton produced in 460 l bags was used for green water techniques in larvae culture and occasionally for rotifers feeding.

3.4.1.2.-Rotifers culture

The rotifer *Brachionus plicatilis* was used in larval rearing experiences; adult individuals had an average total length of 240µm, being classified as L morphotype.

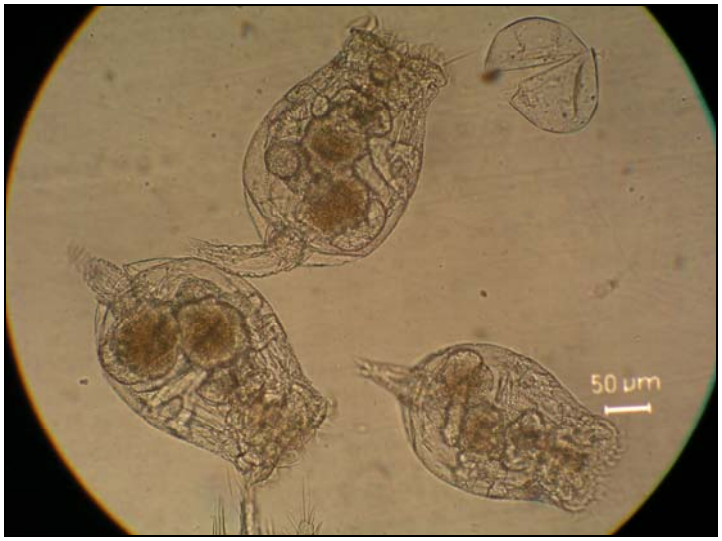


Figure 34. Rotifers (*Brachionus plicatilis*) used in larval cultures.

Culture process comprised several phases (stock maintenance, pre-culture and massive culture) to ensure the quickly re-start of the culture after population failure or accident (Dhert, 1996).

3.4.1.2.1.–Stock maintenance and rotifers pre-culture

Rotifer strain was maintained in 2 to 5l containers filled with *Nannochloropsis sp*. at a density of 20-25 x 10^6 cells.ml^{-1} with an initial concentration of 2-5 rot.ml^{-1}. Culture was supplied with smooth ventilation in a temperate room under continue photoperiod. Once the culture reached 75-100 rot.ml^{-1}, after 5-7 days, total volume was harvested and this was used to inoculate a greater volume, normally, 20 l bottles repeating afterwards the described protocol.

Figure 35: Rotifers pre-culture bottles.

3.4.1.2.2.-Mass culture

Rotifers production was carried out on cylinder conical fibre glass tanks with a total capacity of 1700 l (Figure 36) filled with a mixture of fresh and seawater to attain a salinity of 25 ppt. General culture conditions are presented in Table IV.

Table IV: Culture conditions for rotifers mass production at ICCM experimental hatchery.

Water:	UV sterilized
Temperature:	20-25ºC
Salinity:	25 ppt
Illumination:	Natural light and photoperiod.
Aeration:	Strong, a single air diffuser settled at 80% of tank height.
Oxygen:	Pure O_2 controlled injection when values drop under 3.5ppm.

Production cycles lasted 8 days (Table V). Initial rotifers density was 265 indv.ml^{-1}; from day 4th of culture, harvest of 400 l volume was performed in alternative days, harvested volume being replaced by a mix of seawater and fresh water to reach a 25 ‰ salinity; the 8th day, the total volume was harvested and a new production cycle started. Routinely, after rotifers harvesting, a 1 min freshwater bath was applied to kill potential contaminants such as ciliates. Average total density and percentage of ovigerous females were daily calculated after individual counts (n=3) of 0.5 ml collected with a Micropipette (Mod. Eppendorf Research 100-1000 µl; Hamburg, Germany) from a sample randomly collected from the culture tank. A visual observation of the rotifers mobility was conducted to classify them into three categories: high, medium or low. The content of potential contaminants such as ciliates or copepods was classified in high, medium or low and water quality was checked reflecting the presence of debris and the viscosity increase in a visual estimation (clean, medium or dirty). Similarly, oxygen and temperature were registered twice a day at 9:00 and 15:00 hours by means of a portable probe (Mod. Handy Polaris, OxyGuard; Birkerød, Denmark).

Rotifers feeding consisted in lyophilized baker yeast (*Saccharomyces cerevisiae*) supplied at a dose of 0.4g/10^{6}rotifers. The first day of culture yeast was supplemented with lyophilized microalgae 0.1g/10^{6}rotifers. Food was added manually at 09:00 and 15:00 hours, and automatically at 21:00 and 03:00 hours.

Figure 36: Rotifers culture tanks and food automatic distribution system.

Table V. Average rotifers production sheet, according to described methodology (n=9).

Rotifers production sheet										
Day	**Density (rot/ml)**	**Volume (m^3)**	**Total (mill)**	**Harvest (rot/ml)**	**Egg bearing females (%)**	**Food ration (g/mill)**	**Food (g/tank)**	**Food/fed (g/ration)**	**O_2 (ppm)**	**T^a (ºC)**
1	262.1	1.7	446		19.3%	0.5	225	37.5	7.0	22.9
2	251.5	1.7	428		22.1%	0.4	165	27.4	6.3	22.3
3	281.9	1.7	479		23.3%	0.4	197	32.9	4.3	22.1
4	307.6	1.7	523	72.4	17.9%	0.4	224	37.4	4.3	22.0
5	292.6	1.7	497		19.8%	0.4	187	31.1	5.1	22.0
6	293,0	1,7	498	68.9	21.6%	0.4	198	32.9	4.1	22.2
7	269.9	1.7	459		19.2%	0.4	166	27.6	3.9	22.4
8	265.1	1.7	451	451	17.8%		-	-	4.9	22.3

Rotifers harvesting was performed with a socket bag of 63 µm net size or with a rotifers concentrator made with the same net size.

Figure 37. Rotifers harvesting and detail of harvesting bag.

3.4.1.2.3.-Rotifers enrichment

Rotifer *Brachionus plicatilis* reared under the, previously described protocols are deficient in highly-unsaturated fatty acid (HUFA) and other nutrients, needing to be enriched previously to be fed to the larvae. Enrichment was performed in cylinder-conical fibre glass tanks 500 l

capacity. Rotifers concentration was generally high (over 400 rot.ml^{-1}), and an air diffuser was settled 15cm from the tank bottom centre, to prevent sedimentation and to maintain the oxygen levels, however pure oxygen injection was necessary. Along of the different trials, rotifers enrichment with commercial or experimental products was performed. Generally, different enrichment products were used following manufacturer instructions, including 6 hours enrichment time before harvesting. The enrichment product was spread in two separate doses (0h (initial) and 3 h later). Thus, two tanks were daily used for rotifers enrichment was used daily; the first's one was harvested at 08:30 and fed to the larvae. In this case the enrichment product was automatically added at 02:00 and 05:00 hours. The second enrichment tank was harvested at 14:00, and the enrichment product was manually supplied at 08:00 and 11:00 hours.

After rotifers enrichment, they were harvested and cleaned to eliminate oil emulsions particles, concentrated in a 20 l basket and kept with air supply until larval feeding. A 5 ml sample was taken, diluted in 250 ml, homogenized and counted. Once calculated the needs for each larval tank, rotifer were fed to the larvae and the remaining rotifers discarded. Along the whole experimental period, different samples of the products and rotifers used were stored to study the biochemical composition and fatty acid profile (Table VI).

Table VI. Proximal and main Fatty Acid composition (% Total Fatty Acid, TFA) of rotifers food and enrichment products (n=3); *nd: non detected.

Usage	**Rotifers food**	**Enrichment products**		
Product	**Yeast**	**DHA Protein Selco**	**Red Pepper Paste**	**MorDHA omega-3 I.Q**
% Lipids (dw)	2.41±0.03	30.29±0.16	45.13±0.22	85.13±0.71
% Protein (dw)	48.00±0.40	29.66±0.19	13.26±0.33	6.38±0.21
% Ash (dw)	5.28±0.08	11.21±0.09	8.30±0.04	0.01±0.02
14:0	0.34±0.14	2.43±1.24	5.56±1.44	0.06±0.08
16:0	23.24±8.16	27.85±0.62	21.91±5.31	0.88±0.00
16:1 n-7	27.31±5.51	4.47±0.41	0.75±0.15	0.47±0.04
18:0	19.39±9.25	6.26±0.38	2.99±0.76	5.85±0.72
18:1 n-9	26.33±12.46	12.22±0.49	13.83±0.77	3.68±0.56
18:1 n-7	0.67±0.08	1.29±0.56	1.73±0.82	1.22±0.10
18:2 n-6	0.48±0.12	7.79±0.38	3.93±0.92	0.72±0.08
18:3 n-3	0.08±0.03	0.98±0.18	3.03±0.74	0.22±0.18
20:1 n-9	0.11±0.01	0.81±0.20	0.05±0.07	1.95±0.32
ARA (20:4n-6)	nd	0.97±0.18	1.59±0.33	2.82±0.50
EPA (20:5n-3)	nd	6.40±0.45	3.06±1.40	10.73±2.16
DPA (22:5n-6)	0.30±0.16	1.38±0.27	9.04±1.99	4.79±0.94
DHA (22:6n-3)	nd	19.09±1.23	22.84±1.92	57.32±4.34
∑ saturated [(1)]	43.67±17.64	37.95±0.92	31.79±4.42	7.31±0.07
∑ mono-unsaturated [(2)]	54.59±17.72	19.13±0.93	19.03±6.04	9.62±0.26
∑ n-3 [(3)]	0.43±0.10	30.31±1.17	31.44±3.05	72.44±2.31
∑ n-6[(4)]	0.50±0.13	11.01±0.97	15.87±1.46	9.49±1.89
∑n-9[(5)]	26.47±12.41	13.46±0.80	14.17±0.70	6.23±0.86
∑n-3HUFA[(6)]	0.34±0.10	27.78±1.16	27.66±2.19	71.60±2.85
DHA/22:5 n-6	-	13.84±1.78	2.53±0.55	12.28±3.32
EPA/ARA	-	6.59±0.93	1.93±0.43	3.80±0.09
DHA/EPA	-	2.98±0.25	7.46±2.93	5.49±1.51
DHA/ARA	-	19.67±3.69	14.38±3.45	20.81±5.22
Oleic/DHA	91.19±7.79	0.64±0.02	0.61±0.04	0.06±0.01
Oleic/n-3HUFA	75.73±14.08	0.44±0.00	0.50±0.04	0.05±0.01
n-3/n-6	0.92±0.44	2.75±0.22	1.98±0.17	7.81±1.80

3.4.1.3.-Brine shrimp production

Nauplii of the brine shrimp *Artemia* constitute one of the basic foods items for marine larval rearing. The capacity to produce dormant embryos (cists) that can stay during long periods without a loss in viability, allows its storage to support the demand year around. The use of *Artemia* as live food for fish larvae, implies that eggs or cists should be reared under optimal conditions to obtain nauplii and metanauplii later, which are the main stages used as live food for the marine fish larvae (Van Stappen and Merchie, 1996) (Figure 38).

Figure 38. Different *Artemia* stages, unhatched eggs, nauplii (24h) and metanauplii (48h).

For all the larval rearing experiences *Artemia* nauplii from two different origin were used, *Artemia franciscana* (AF Type; INVE; Dendermode, Belgium) and *Artemia salina* (EG Type; INVE, Dendermode, Belgium). The decapsulation process to eliminate the external corion and to reduce the risk of external pathogens introduction was performed for all the *Artemia* batches. *Artemia* was prepared following the protocol that next is detailed:

3.4.1.3.1.-Hydration

This is the first step for the decapsulation process. Cists were hydrated during 45 min to 1hour and 30 minutes, in a relation of 10-12 l of seawater per kg of dry cists at an average temperature of 20±2°C and 37‰ ambient salinity, for this process, cylinder conical fibre glass tanks ranging from 50-500 l according to the amount of cistes to be hydrated. The system was completed with strong ventilation from the tank bottom that helps to maintain an oxygen concentration over 4

ppm avoiding cists sedimentation. Once hydrated, cists were harvested with a sock net of 125µm mesh light, and residual water was eliminated by hand pressure upon the net (Figures 39).

Figure 39. a) *Artemia cists*; b) hydratation tank; c) hydrated cists.

3.4.1.3.2.-Decapsulation

This process consists in the complete removing of the cist outer layer (corion), by means of the use of a concentrated solution of sodium hypochlorite. Thus, in the same tank used for the hydratation, a relation of 4 l of seawater per kg of dry cist, the necessary amount of commercial bleach to obtain a concentration of 350g of sodium hypochlorite per kg of cists and 66g $NaOH.kg^{-1}$ of dry cists were introduce. Strong aeration was applied and the change of external coloration (initial brown to orange) was controlled, that usually happens in a period of 3 to 8 min. Once external colour change, *Artemia* eggs were harvested in a sock net of 125µm and washed with seawater for 15 to 20 min to eliminate residual chlorine.

This process consists in the complete removing of the outer layer (corion), by means of the use of a concentrated solution of sodium hypochlorite. Thus, in the same tank used for the hydratation, a relation of 4 litres of sea water per kilograms of dry cist, the necessary amount of commercial bleach to obtain a concentration of 350grams of sodium hypochlorite per Kg of cists and 66grams $NaOH.kg^{-1}$ of dry cists were introduce. Strong aeration was applied and the change of external coloration (initial brown to orange) was controlled, that usually happens in a period of 3 to 8 minutes. Once external colour change, *Artemia* eggs were harvested in a sock net of 125µm and washed with sea water for 15 to 20 minutes to eliminate residual chlorine.

3.4.1.3.3.-Storage

Once decapsulated and harvested the extra water was removed by hand pressured over the socked. Wet *Artemia* eggs were introduce in a 60 *l* beaker and salt and seawater were added to obtain a relation 1.17 kg of salt per 4 l of seawater per kg^{-1} of dry cists, to obtain a salt solution with a final volume of 7.17 l (taking into account that cists duplicate its volume when hydratation takes place) and a final concentration of 139g of dry $cists.l^{-1}$ of storage salt solution. Once the storage solution was prepared, it was stored in a cold room (lower 4ºC) under dark conditions were hatching viability could be kept for up 1 month (Figure 40).

Figure 40. a) Beakers with Storage salt solution; b) salt solution with *Artemia* eggs

.

Table VII. Brine shrimp *Artemia* decapsulation and storage protocol sheet

Brine shrimp *Artemia* decapsulation and storage protocol sheet

Hidratation: 45´- 1h 30´. 10-12 l of seawater per kg of dry cists

Decapsulation		***Artemia* (kg)**	**Hypochlorite(g)**	**Bleach(l)**	**NaOH (g)**	**Seawater (l)**	**Total Volume**
Bleach 10 l		0.25	87.5	2.19	16.5	1.31	3.5
Refraction index	1.352	0.50	175	4.38	33	2.6	7.0
Concentration (g/l)	40	1.0	350	8.75	66	5.3	14
		1.5	525	13.13	99	7.9	21
		2.0	700	17.50	132	10.5	28
		2.5	875	21.88	165	13.1	35
		3.0	1.050	26.25	198	15.8	42
		4.0	1.400	35.00	264	21.0	56
		5.0	1.750	43.75	330	26.3	70
		6.0	2.100	52.50	396	31.5	84
		7.0	2.450	61.25	462	36.8	98
		7.5	2.625	65.63	495	39.4	105

Notes

Bleach: 0.35 g hypochlorite per kg of dry cists.
Sodium hydroxide: 66g NaOH per kg of dry cists.
Anti-foam. (Optional) 250-500ppm, 0.25-0.5 g per litter.
Sea water: Quantity needed to obtain a final concentration of 14 l per kg of dry cists.
Time: 5-8 min, according to external color change (brown to orange).
Neutralization: Sea water open bath during 20 min, or 50 g of Tiosulfate per kg of dry cists. Residual Chlorine can be tested with ortotolidina or another chlorine detector.

Salt storage solution	*Artemia* (kg)	Salt (kg)	Seawater (l)	Final volume(l)	Final concentration (g/l)
	0.25	0.29	1.00	1.79	139.4
	0.50	0.59	2.00	3.59	139.4
	1.0	1.17	4.00	7.17	139.4
	1.5	1.76	6.00	10.76	139.4
	2.0	2.34	8.00	14.34	139.4
	2.5	2.93	10.0	17.93	139.4
	3.0	3.52	12.0	21.52	139.4
	4.0	4.69	16.0	28.69	139.4
	5.0	5.86	20.0	35.86	139.4
	6.0	7.03	24.0	43.03	139.4
	7.0	8.20	28.0	50.20	139.4
	7.5	8.79	30.0	53.79	139.4

Notes

Sea water: 4 l of sea water (37%) per kg of dry cists.
Salt: 330 g per l minus quantity of salt added thought sea water.
Final Volume = Seawater + salt + cists (2 x dry weight)
Concentration: Decapsulated cists/Final Volume
Storage: in the dark and at low temperature (4ºC).

Made by: J.Roo		Modification date: 04/10/2008

3.4.1.3.4.-Hatching

Artemia was hatched according to the larval feeding needs. The protocol included tank disinfection and filtered and sterilized seawater filling. Cylinder conical tanks of 1700 l total volume were used and strong air supply and continue photoperiod was applied. Tanks were equipped with a heater to maintain water temperature at 28-29ºC. A density of 1.8 g of cists.l^{-1}, were used, obtaining average hatchability rates of 95% after 24h and *Artemia* nauplii of 450-650µm depending on the *Artemia* strain which were used in the transition feeding from rotifers to *Artemia* metanauplii (Figures 41).

Figure 41. a) *Artemia* introduction in the hatching tanks; b) hatching tanks; c) newly hatched *Artemia* nauplii.

3.4.1.3.5.-Enrichment

To improve nutritional quality of *Artemia*, an enrichment procedure was necessary. For this purpose, cylinder-conical fibre glass tanks 1700 l total volume were employed, strong aeration from the tank bottom and 24 h photoperiod were applied. Besides, an internal heater was used to maintain a culture temperature of 25-26ºC. Just hatched *Artemia* nauplii were introduce in the enrichment tank at a concentration of 250,000-300,000 nauplii.l^{-1}. In all experimental trials the Easy DHA Selco (Inve, Dendermonde, Belgium) enrichment product was used. The enrichment last for 18-24 h with a concentration 0.6 gr.l^{-1}, added in a single dose at the beginning of the enrichment process (h=0).

Enriched nauplii were harvested in a sock net with 125µm mesh size, washed with seawater to eliminate residual lipid particles and concentrated in a 20l beaker to be counted and check the

enrichment success (Figure 42). Enriched nauplii were added to the larval tank both manually or by means of automatic distributors.

Figure 42. a) Enrichment tank; b) enriched *Artemia* cold storage; c) success enrichment checking.

3.4.2.-Larval rearing

3.4.2.1 Eggs

Marine fish eggs are in a biological stage that makes them easy to manipulate and transport. Nevertheless, inadequate manipulation can promote high embryos mortality or later negative effects such as low hatching rates, high larval mortality during the first days of culture or the appearance of different morpho-anatomical anomalies. Consequently, handling of fish eggs must be careful to avoid mortalities and to obtain high quality larvae. In the different trials of larval culture, eggs natural spawned from broodstock belonging to ICCM and the Instituto Español de Oceanografía, Centro Oceanográfico de Canarias (IEO-COC) were used. In both facilities, broodstocks were fed twice per week with commercial diets, complemented once a week with fresh fish and mollusc like cuttlefish, squid and mussels.

Figure 43. Adult red porgy (*Pagrus pagrus*) from ICCM broodstock.

Fertilized eggs display positive buoyancy and they were gathered in a mesh collector of 500 µm, which receives tank water overflow (Figure 44a). Once harvested, eggs were placed in a decantation cone where different fractions were separated, the floating fraction mainly composed by viable eggs and the non floating one mainly composed by nonviable eggs (Figure 44b). When the origin of eggs was IEO-COC, they were transferred by airplane or boat, inside reinforced plastic containers of 20 l capacity inside individual expanded polystyrene boxes, which protect them and maintain stable temperature conditions during transport (Figure 44c). In each container, an average of 250,000 eggs at a concentration of about 25,000 eggs.l^{-1} were introduced. About 50% of the volume was filled with seawater an 50% with saturated oxygen gas (Figure 44d).

Figure 44. a) Eggs collector, b) Decantation cone; c,d) Eggs transport preparation.

After arrival to the facilities, eggs were acclimatized to the local seawater physical-chemical parameters and a new separation to eliminate dead or damaged eggs during the transport was performed. Viable eggs were volumetrically counted and introduced in the rearing tanks (Figure 45). At the same time, six small cylindrical containers provided with water exchange were settled to calculate hatching rates and larval survival by day 3 after hatching before mouth opening (Figure 46).

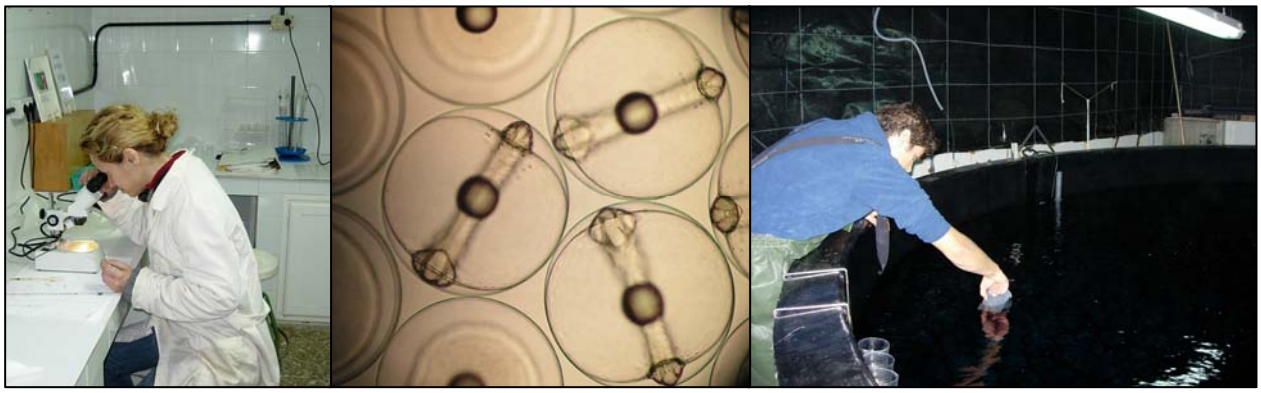


Figure 45. a) Eggs count; b) viable eggs; c) eggs introduction in the rearing tanks.

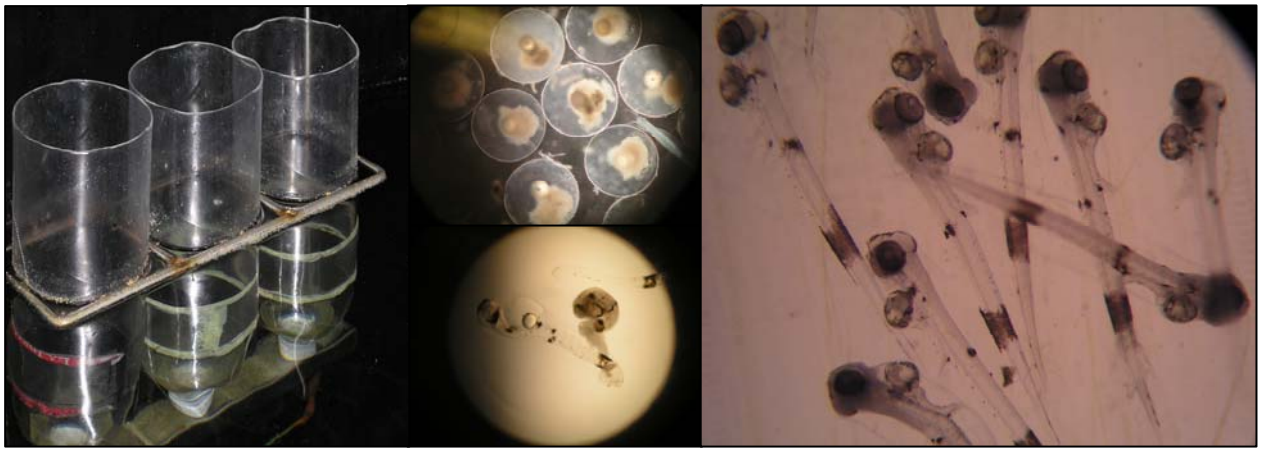


Figure 46. a) Containers for hatching rates and larval survival determinations; b) dead eggs and unviable newly hatched larvae; c) good quality red porgy larvae.

3.4.2.2.-Semi-intensive larval rearing

Semi-intensive system (SMIS) larval rearing was performed in the 40,000 l tanks previously described. In all rearing trials, fertilized eggs were stocked at a density of 5-6 eggs.l^{-1}. Seawater

was previously filtered by a sand filter and pass through a UV sterilizer. Water renewal was increased from 10% of the total volume daily to more than 25% per h^{-1} at 30 dah. Water salinity was kept almost constant (37±0.5 ‰) in all the trials while temperature varied as specified in each trial. Larval culture was conducted under continuous photoperiod and changed to natural photoperiod in later stages (conditions being specify in each trial). Semi-intensive tanks were equipped with 4 fluorescent lights (Mod TLD 36W/54, Philips, France) that in combination with natural light coming from a translucent roof maintained an average light intensity between 1,000-3,500 lux measured in the water surface.

Green water technique was used adding living phytoplankton (*Nannochloropsis sp.*) to maintain a concentration of 250 ± 100 x 10^3 cells.ml^{-1} in the rearing tanks. Feeding protocol included the use of rotifers (*Brachionus plicatilis*) at 4-5 rot ml^{-1} enriched with the different commercial or experimental emulsions from day 3 after hatching (dah) until larvae reached 8.0 mm total length around 25dah. Specific details of feeding periods are given in each trial. Rotifers were added to the larval tank twice a day (09:00; 15:00 hours).

At 13dah, *Artemia* nauplii Instar I (AF type, INVE Aquaculture, Dendermonde, Belgium). were added once a day (11:00), in all the tanks at (0.25 A_0 ml^{-1}). From 15 to 17 dah Artemia Instar II enriched with Easy DHA Selco ® (INVE, Belgium), were added three times a day (09:00;15:00; 20:00 hours) at (0.50 A_1 l^{-1}) being the last day fed automatically distributed (Table VII). Prey concentration in the tanks was determined samplings the tank water collected twice a day (08:00; 14:00 hours) previously to the new fed addition. From 5dah till 25dah a surface skimmer were used to eliminate the lipid film derived from feeding enriched preys.

Generally, larvae were fed commercial diets from 20 dah (Genma Micro;Skretting, France), manually supplied for the first 3 days and by means of automatic feeders (T-Drum feeders, Arvotec, Noruega) afterwards (Table VII).

After 50dah the whole population was transferred to 10,000 l tanks, and kept under the same rearing conditions (1-10 indv l^{-1}) in a flow through water system until 95dah when all the fish were individually counted and skeletal characterization was performed.

3.4.2.3.-Intensive larval rearing

Larval rearing experiments with intensive technology were performed in 2,000 or 500 l tanks previously described. This technology is widely used in commercial hatcheries, with different variants according to general culture conditions such as tank size and form, water turnover, position and number of air diffusers, feeding sequence, prey enrichment products and/or weaning

diets. Generally this technique is based on the use of a high larval density that commonly varies between 50 and 150 larvae l^{-1}.For all the rearing trials fertilized eggs were stocked at a density of 100-125 eggs.l^{-1}. Seawater was previously filtered by a sand filter and pass through a UV sterilizer. Water renewal was decreased from 25% per h^{-1} at hatching to 10% of the total volume daily in the first larval stages, being progressively increased to more than 25% per h^{-1} at 30 dah. Water salinity was kept almost constant (37±0.5‰) for all the experiences, while temperature and oxygen varies along the trials and was daily measured. Larval culture was conducted under continuous photoperiod and changed to natural photoperiod in later stages (these conditions can vary in the different trials as it is specified in each of them). Intensive system rearing tanks were equipped with a single central light (Mod TLD 36W/54, Philips, France) that in combination with natural light coming from a translucent roof maintained an average light intensity between 1500-3500 lux measured in the water surface. With this technology, green water technique was used as well, adding living phytoplankton (*Nannochloropsis sp*.) to maintain a concentration of 250 ± 100 x 10^3 cells ml^{-1} in the rearing tanks. Feeding protocol includes the use of rotifers (*Brachionus plicatilis*) at 5-10 rot ml^{-1} enriched with the different commercial or experimental emulsions from day 3 after hatching (dah) until larvae reached 8.0 mm total length and rotifers were fed twice a day (09:00; 15:00 hours). At 13dah, *Artemia* Nauplii Instar I (AF Type, INVE Aquaculture, Dendermonde, Bélgica) was added once a day (11:00) at (0.25 A_0 ml^{-1}) .From 15-17 dah Artemia Instar II enriched with Easy DHA Selco ® (INVE, Belgium), were added three times a day (09:00;15:00 and 20:00 hours) at (0.50-1.5 A_1l^{-1}) being the last feed of the day automatically distributed (Table VII). Prey concentration in the tanks was measured by sampling the tank water twice a day (08:00; 14:00 hours) before new fed addition. From 5 dah untill 25 dah a surface skimmer was used to eliminate the lipid film introduce with enriched live prey. Generally, larvae were fed commercial diets from 20 dah (Genma Micro, Skretting, Francia), manually supplied for the first 3 days and by means of automatic feeders (T-Drum feeders, Arvotec, Noruega), afterwards (Table VII). After 50 dah the whole population was transferred to 10,000 l tanks, and kept under the same rearing conditions (1-10 ind l^{-1}) in a flow through water system until 95dah when all the fish were individually counted and skeletal characterization was performed.

Table VII. General culture conditions and feeding sequence during red porgy larval rearing

Age (dah)	Degree day	Length (mm)	Light	Filters	Water flow	Tank cleaning	Water	Feeding sequence
Eggs intro Haching			Natural Photoperiod	Superior filter (mesh 315µm)	25%/day		Clear water	Endogenous feeding
2	50	3.00	Photoperiod 24 h Ligth (1500-3000)(Natural+artifitial)		10%-25%/day	Surface cleaning	Green water (250-350.000.cells.ml^{-1})	Rotifers(5-10) Indv.ml^{-1})
5	80-90	3.1-3.5						Nauplii (0,025-0,25)
10	125-135	4.0-5.1						Manual dry Fed Co-feeding Protocol B
15	175-190	5.5-6.4			25%-50%-75%-100%/day	Both		Metanauplii (0,25 -1 indv.ml^{-1})
	180-200							Manual dry Fed Co-feeding protocol A
20	225-250	6.5-8.0		Down filter (mesh 500 µm)				Manual+ automatic dry fed (8-12% biomass day)
	260-275		Photoperiod 12:12 (1500-3000 Lux) (Natural+artifitial light)		5%-15%-25%/hour			
25	280-290	7.5-11.5						
30	350	8.5-12.5				Bottom siphoning	Clear water	
35	375-400	9.5-14.5		Down filter (mesh, 1000µm)				Automatic dry fed (8-12% biomass day)
40	450	15.0-18.5						
45	500	17-22.5						
50	530-550	19.0-30.0						

3.4.2.4.-Nursery

The weaning and nursery phase for all the trials described in the present study were carried out in identical conditions, regardless of the culture technology used during the larval phase but maintaining fish stocks separated according to the culture conditions employed during the larval phase. Tanks of 10,000 capacity previously described were used, (Figure 47). During this stage, unfiltered seawater was used, with a water turnover of 50 % tank volume h^{-1}. Salinity was almost constant during all the experiences (37 ‰) and dissolved oxygen varied around 5.5 to 7.5 ppm being controlled and supply when necessary by means of an automatic control system (Mod. Multi-channel, OxyGuard; Birkerød, Denmark) (Figure 47). The temperature was daily measured with a fluctuation between 20-22ºC in the different experiments. The illumination was a combination of natural and artificial light with a photoperiod of 12 to 18 h. Feeding in this phase was exclusively based on commercial diets (Genma; Skretting; France) combining manual and automatic feeding (Mod. T-Drum feeders, Arvotec; Norway), (Figure 47).

Figure 47. a) Dissolved oxygen control unit; b) automatic feeder.

During this phase when the tank population weight dispersion was higher than 30%, fry grading was performed using commercial bar graders (Mod. Rods, Catvis; Holland) according to the fry size (Figure 48).

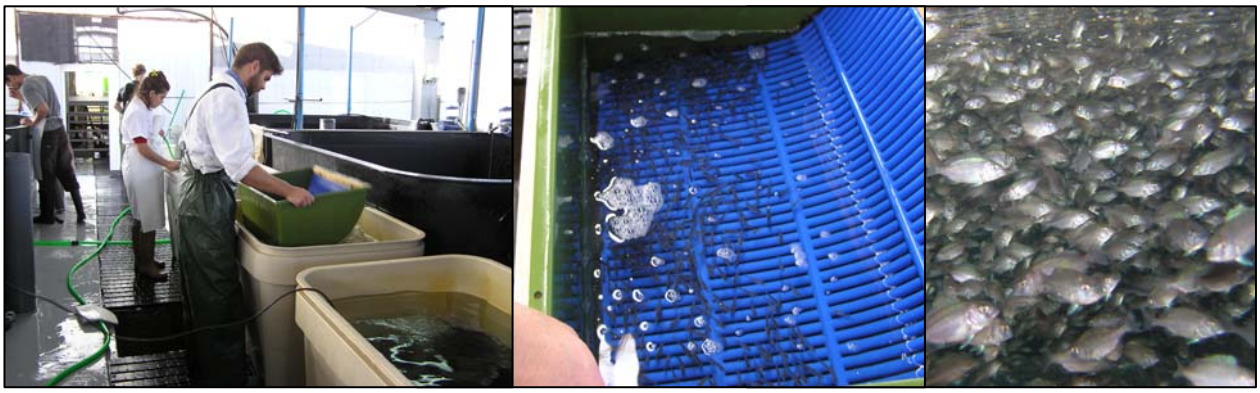


Figure 48. a) Fry grading procedure; b) grids detail; c) red porgy fry.

Tank bottom cleaning was daily performed by siphoning uneaten food and dead fish; that were individually counted for survival adjustment.

3.5.-Measurements

3.5.1.-Growth in length

Larval growth was assessed, measuring the total length of 25 larvae per tank every 5 days, using a profile projector (Mitutoyo PJ-3000A, Kanagawa, Japan) (Figure 49).

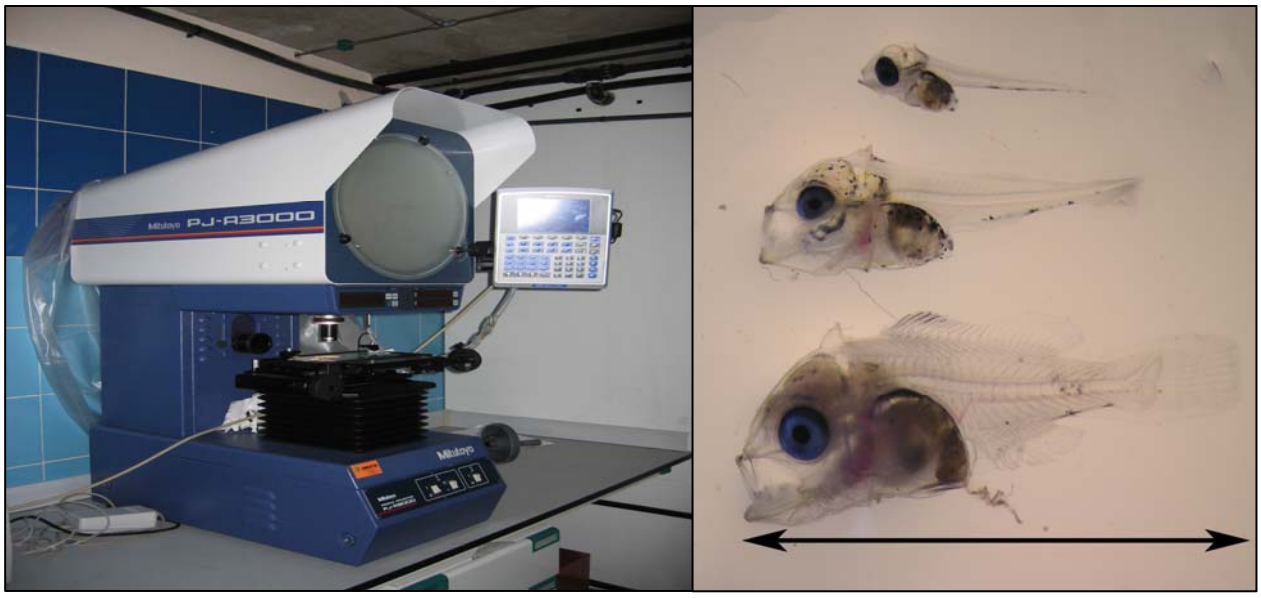


Figure 49. Profile projector and example of total length measured of red porgylarvae from different sizes.

Additionally, length-specific growth rate (SGR) was calculated using the following equation:

$$SGR=([Ln(Tl_t)-Ln(Tl_0)] /t) \times 100;$$

Where Tl_t is the larval total length at the end of time period t, Tl_0 is the total length at the beginning of time period t, and t is the length of the time period in days.

3.5.2.-Growth in weight

Wet weight: To measure wet weight, fish were sacrificed by anesthetic overdose (Clove oil, Guinama S.L, Valencia, Spain). Dead larvae were collected in a 315µm size mesh, washed in tap water first and then in distilled water; larvae were blotted to eliminate residual water. Total larvae number (n=10) from each tank was individually settled in a pre-weight and identified crystal slide (*Pc*), total larval wet weight (L) was obtained in a precision balance (Mod. Mettler,

AG 204, Ohio, USA). To obtain individual larva wet weight (L_{WW}) the following expression was used:

$$L_{WW} = \frac{(Pc + L) - (Pc)}{n}$$

Dry weight: To determine dry weight, the same larvae used for wet weight determination were introduce in an oven, (Jouan EU 28, S. Herblain, France) at 100ºC until constant weight. Before being weighted the dry larvae were introduce in a desecator for 30 min to ambient temperature adaptation. Finally larvae were weighted to obtain final dry weight (L_{DW}) by the following expression.

$$L_{DW} = \frac{(Pc + L) - (Pc)}{n}$$

3.5.3.-Survival

Survival was determined after 95 dah by individually counting of all remaining fish using a fry counter (TPS Fish counter, Type Micro; Impex Agency; Denmark). From 25 dah dead fish were daily recorded and, at the end of the trial, survival was estimated taking into account daily mortality and final alive fish.

Figure 50. a) Automatic fry count; c,d) details of automatic fry counter TPS Fishcounter.

3.5.4.-Histological studies

For histological studies, from hatching to 45dah, 25 individuals from each larval tank were randomly sampled every 5 days and fixed in 10% buffered formalin. Fixed larvae from different ages and treatments were set in individual microcapsules previously identified, to be washed and dehydrated in a series of graded alcohol in a tissues processor (Mod. Histokinette 2000; Leica, Nussloch, Germany) according with the methodology described by Socorro (2006), (Table VIII).

Table VIII. Histological samples manipulation

Step 1: Dehydration		**Step 2: Clarification**		**Step 3: Paraffin infiltration**		**Step 4: Re-hydration**	
	Time		**Time**		**Time**		**Time**
Alcohol 70%	60 min	Xilol I	30 min	Paraffin I	180min	Xilol	15 min
Alcohol 80%	90 min	Xilol II	30 min	Paraffin II	360min	Absolute Alcohol I	5 min
Alcohol 90%	90 min	Xilol III	60 min	Paraffin vacuun	60min	Absolute Alcohol II	5 min
Alcohol 96%	90 min					Alcohol 96%	5 min
Absolute Alcohol I	90min					Alcohol 90%	5min
Absolute Alcohol II	120min					Alcohol 70%	5 min
Absolute Alcohol III	120min					Water	5 min

Once, paraffin infiltration has finalized (Step 3; Table VIII) larvae were remove from the microcapsules and embedded in paraffin using the paraffinic dispenser (Mod. Jung Histoembedder; Leica, Nussloch, Germany). Paraffin blocks were cut in the microtome (Mod. Jung Autocut 2055; Leica, Nussloch, Germany). Initially gross cuts at 20-25 μm (until larvae tissues were reached) and later definitive cuts at 4-5 μm were conducted. The cut series were introduce in a distilled water bath at 45ºC, and the samples were selected, removed from the water bath and dried in an oven (1h at 60ºC). Then, samples were introduced in a xylol bath and re-hydrated in alcohol series of different gradation, finalizing with water (Step 4, Table VIII). For its definitive assembly a drop of synthetic resin was added to the crystal slide with the sample, and a secondary crystal slide were mounted and pressed to eliminate air bubbles. Once dry, the preparations were stored in the dark in a fresh place until staining was done.

Larval tissues were stained with two staining techniques: haematoxylin-eosin (H&E) and peryodic acid-Shiff-Hx reactive (PAS-Hx) according with Martoja and Martoja-Pierson (1970) and García del Moral (1993) methodology, modified by Socorro (2006) (Tables IX; X).

Table IX- Haematoxylin-eosine (H&E) and peryodic acid-Shiff-Hx reactive (PAS-Hx) staining protocol

Step5a:Haematoxylin-eosine staining (H&E)		**Step 5b:Peryodic acid- Shiff reactive-Hx (PAS-Hx)**	
Product	**Time**	**Product**	**Time**
Harris Haematoxylin	15-20 min	HIO_4 Acid (peryodic) 0,5%	5 min
Acid Alcohol	3 short bath	Water	5 min
Water	Baths	Schiff reactive	20 min
Ammonia water	20seg	Water	5 min
Running water	5 min	Haematoxylin	15 min
Puttis Eosin	3-4min	Water	20 min
Water	Wash		

Once staining was end, samples were dehydrated and clarified (Table X).

Table X. Dehydratation and clarification protocol

Paso 6: Dehydration and clarification	
Product	**Time**
Alcohol 96%	5 min
Alcohol 100%	10 min
Alcohol 100%	5 min
Xilol	5 min
Xilol	5 min

When samples were ready, they were observed under a microscopy (Mod. DMBE, Leica, Nussloch, Germany) taking some pictures at the most relevant steps of the larval ontogeny. When digital measures or counts were necessary the image analyze program (Image-pro Plus vers 2.0; Media Cybernetics, Inc., Buckinghamshire, England) were used.

3.5.5.-Osteological study

To study the osteological development, from hatching to 45dah, 25 individuals from each larval tank were randomly sampled every 5 days and fixed in 10% buffered formalin. Fixed larvae were cleared and then stained with alcian blue and alizarin red according with the methodology

described by Taylor and van Dyke (1985) and Dingerkus and Uhler, (1977) modified by Socorro (2006)

Formaldehyde stored larval samples followed the methodology described to be clarified and stained.

Table XI: Cartilage-bone staining methodology

Step 1: Cartilage staining		**Step 2: Hydraingtion**		**Step 3: Clarification**	
	Time		**Time**		**Time**
Alcian-Blue staining	120 min	Alcohol 96%	60 min	Clarification process	60 min
		Alcohol 96%	60 min		
		Alcohol 96%	60 min		
Alcian blue solution preparation 10mg Alcian-blue BGX (c.i. 16230) 80ml de alcohol 96% 20ml glacial acetic acid		Alcohol 75%	60 min	*Tripsine solution preparation* 90 mg tripsin 30 ml de $Na_2B_4O_710H_2O$ (saturated solution) 70 ml distilled water	
		Alcohol 40%	60min		
		Alcohol 15%	60 min		
		Distilled water	60min		

Step 4: Bone staining		**Step 5: Clarification and storage**		
	Time		**Relation**	**Time**
Alizarine Red staining	60min	KOH 0,5% - Peroxide	3:1	60min
		KOH 0,5% - Glycerine	3:1	12-24 hours
		KOH 0,5% - Glycerine	1:1	12-24 hours
		KOH 0,5% - Glycerine	1:3	12-24 hours
		Glycerine + Timol	100%	Storage
Alizarine red solution preparation KOH 0,5% in water. Alizarin red 1 g / l		*Glycerine-Hydrogen Peroxide solution preparation* KOH 0,5% Glycerine (3:1) H_2O_2 1,2 %		

Finally, skeleton structures (in blue cartilaginous structures and in red bone structures) were examined using and stereomicroscopy (Mod. Olympus, Tokyo, Japan) to describe the osteological development, taking digital pictures at most relevant changes.

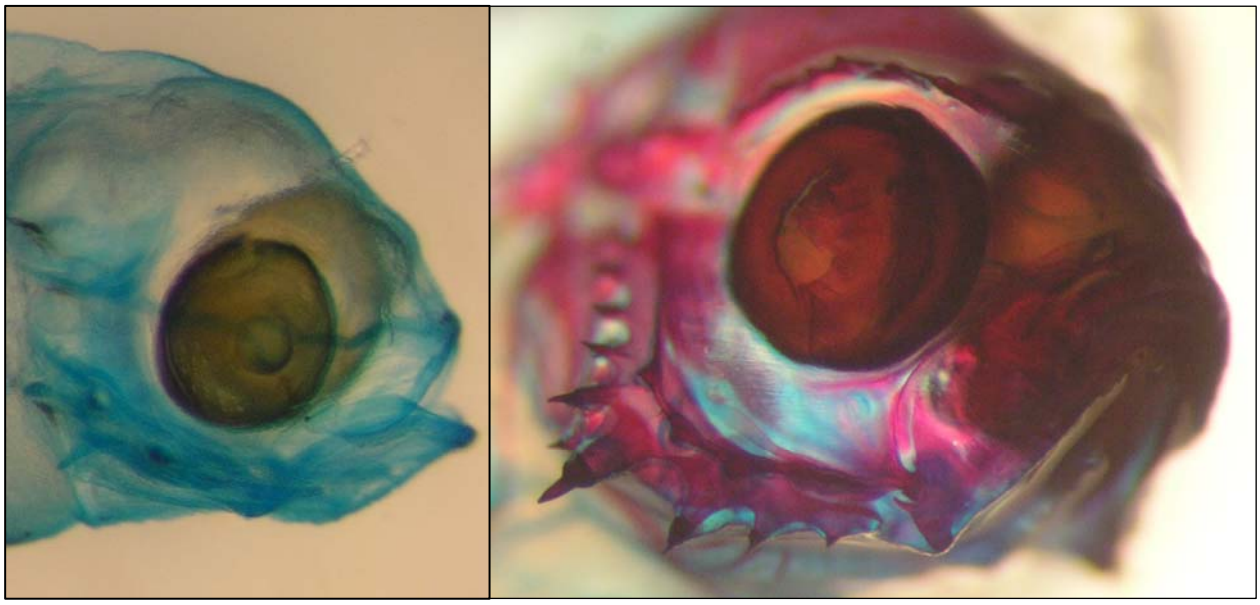


Figure 51. a) Details of red porgy larvae cranial structures at 8dah with cartilaginous structures with alcian blue stained d) details of 27 dah red porgy larvae presenting calcified structures alizarin red stained.

3.5.6.-Characterisation of the different types of deformities

The characterization of the different deformities (lordosis, kyphosis, scoliosis, fused vertebrae, cranial malformations and other defects including asymmetric fins or deformed rays) was performed according with Divanach *et al.* (1996) and Boglione *et al.* (2001) deformities classification, in 300 specimens (mean weight 5.5 g) collected from each treatment at 95 dah. Fish were observed in fresh to detect cranial anomalies and acute deformities that modified external fish shape (Figure 52), and then monitored by soft X-ray (Mod. Senographer-DHR, General electrics, USA) to check internal deformities. The incidence of skeletal abnormalities was performed at 95dah since until this developmental stage is not possible to obtain an accurate identification under soft X-ray plates.

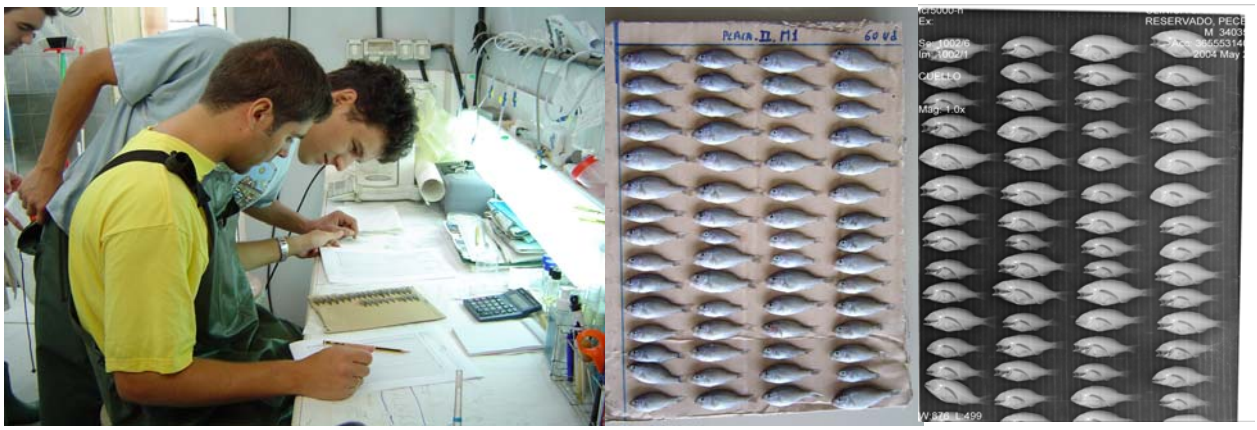


Figure 52. Deformity visual evaluations, X-ray plate's preparation and mammography obtained to conduct detailed studies.

3.5.7.-Meristic determinations

In a specific trial, meristic determinations of total number of vertebrae (including urostyle) and number of pleural ribs were carried out in 95 dah juveniles (n=300) from each treatment. According with Matsuoka (2003), all vertebrae with 2 neural spines and/or 2 hemal spines were considered to be formed by fusion and counted as two vertebrae.

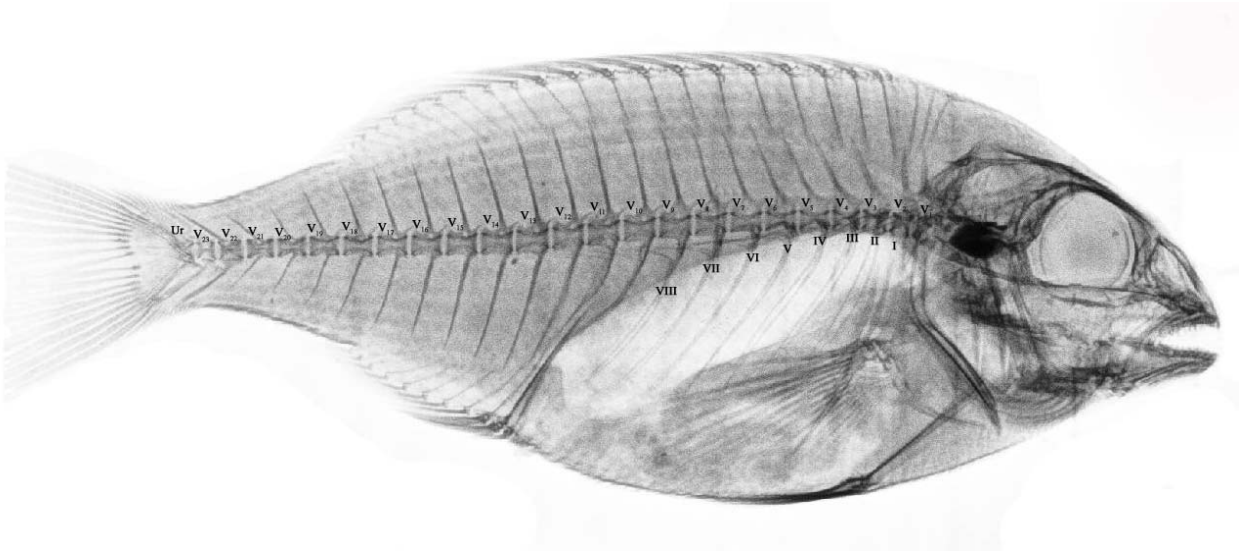


Figure 53. Vertebrae and ribs identification.

3.6.-Biochemical analyses

During the course of the different experiences, samples of products used for the production and enrichment of the live prey were taken (rotifers and *Artemia*), dry feed samples and live preys before and after their enrichment were also taken. In addition, larval samples of different ages from each rearing tank and treatments were collected. Once collected, samples of live prey and fish larvae were placed on a 63µm mesh, washed with fresh and distilled water and residual water blotted with dry paper. Samples were frozen (-80ºC) in hermetic bags under nitrogen atmosphere, for its later analysis. The biochemical analyses were made in the laboratory of Instituto Universitario de Sanidad Animal y Seguridad Alimentaria (IUSA; ULPGC). Determinations of dry matter, ash, protein and lipid content as well as fatty acids were performed. All the analyses were performed at least in triplicate.

3.6.1.-Dry matter content

It was determined following the Official methodology of the American Chemical Analysts Association of (AOAC, 1995). Dry matter content was determined after drying the fresh known sample quantity (P_i) in an oven at 105 ºC until constant weight was obtained (P_f). Before being weighted, the samples were introduce in a desecator for 30min to ambient temperature adaptation and finally they were weighted to obtain final data. The dry matter content was obtained by the following expression.

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

3.6.2.-Ash content.

The ash content was determined after incineration of a well-known amount of sample (P_m) in a Muffla oven, at 450ºC during 24 hours, remaining ashes amount was recorded (P_c) and weight until constant weight according to the AOAC (1995). Final ash content was obtained applying the following expression:

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

3.6.3.-Protein content

The protein content, calculated from the total nitrogen content of the samples, was determined by Kjeldahl method. According to AOAC (1995), the technique consists in the sulfuric acid sample digestion at 420ºC in presence of a copper catalyst for one hour. This is, followed by a distillation with NaOH using saturated boric acid 40% as receptor substance in the distilling unit (Mod. Foss Tecator, 1002, Höganäs, Sweden). Finally a valuation with HCl 0.1 M is made. In order to calculate the protein content, the following expression was applied:

$$\%Protein = (V-P) \times N \times Pm \times F/M$$

Where

V = HCl volume for valuation in ml

P = Measured of the valoration of the patterns in ml

N = Normality of the HCl

Pm= Nitrogen molecular weight (14.007)

F = Conversion empirical factor that has a value of 6.25

Ms = Sample weight in mg

3.6.4.-Total lipids content

Total lipids were extracted according to Folch *et al.* (1957). The methodology commenced taking a sample amount between 50-200mg and homogenise it in an Ultra Turrax (IKA-Werke, T25 BASIC, Germany, Staufen) at 11,000 rpm during 5min in a solution of 5ml of Chloroform: Methanol (2: 1) with 0,01% of BHT. The resulting solution was filtered at reduced pressure through glass wool and adding KCl at 0.88%, to increase the water phase polarity. After decantation and centrifugation at 2000rpm during 5min the watery and organic phases were separated. Once watery phase was eliminated, N_2 current was used to evaporate until completed dryness. Finally total lipid content was gravimetrically determined.

3.6.5.-Fatty acids content

The extracted total lipids were trans-esterified according to the method of Christie (1989).

In this procedure a solution of Toluene with BHT and another with Methanol and Sulphuric acid at 1% was added to the sample. The mixture was strongly shaken to improve lipids dissolution. Afterwards, the container was filled with N_2 and sealed. This mixture was left in shaking incubation for 16 hours at 50ºC. After this time, the sample was let cool and pure distilled water and Hexane: Dyetil 1:1 Ether with BHT at 0.01% were added. The purified FAMES were

evaporated to dryness with N_2 and hence weighed. Finally the FAMES concentration was dilute with hexane and stored in vials in a -80ºC fridge.

Fatty acid methyl esters were analyzed using a gas chromatograph (Mod. Shimadzu GC-14A; Analytical instrument division, Kyoto, Japon), with a flame ionization detector and a Supelcowax-10 fused-silica capillary column 30m length x 0.32 mm I.D. (Supelco, Inc., Bellefonte, EE.UU). Helium was used as carrier gas with the following gas pressures: He 1kg.cm^{-2}, H_2 0.5 kg.cm^{-2}, N_2 1 kg.cm^{-2}, air 0.5 kg.cm^{-2}. Conditions were: injector temperature 250 ºC, column temperature 180 ºC during 10 min, increasing afterwards to 215ºC at a rate of 2.5ºC min^{-1} and maintained at 215ºC for 15 min. Fatty acids were identified by reference to a well-characterized fish oil (EPA 28).

3.7.-Statistical analysis

Results are expressed as mean ± standard deviation. The statistical analyses were performed with program SPSS Version 14.0 (SPSS Chicago, Illinois, 1999). The data of each experiment were compared statistically by means of T-Student test (Sokal and Rolf, 1995) when two treatments were established or with variance analysis (ANOVA) if the number of treatments were greater. As general criteria 5% confidence level was applied. If statistically significant differences with the ANOVA were detected, the differences among means were detected the Tukey multiple means comparison test. When the variances were heterogeneous and/or the data were not normally distributed, these were tried to make them homocedastic and the data normally distributed transforming them to logarithms or with arc sine function. If heterogeneity or the normal distribution were not obtained, the non-parametric test of Kolmogorov-Smirnov when two treatments comparison or if a greater nº of treatments the Games-Howell test was used. Finally, to study the fry quality log-linear analysis, with Pearson χi (Sokal and Rolf., 1995) were applied.

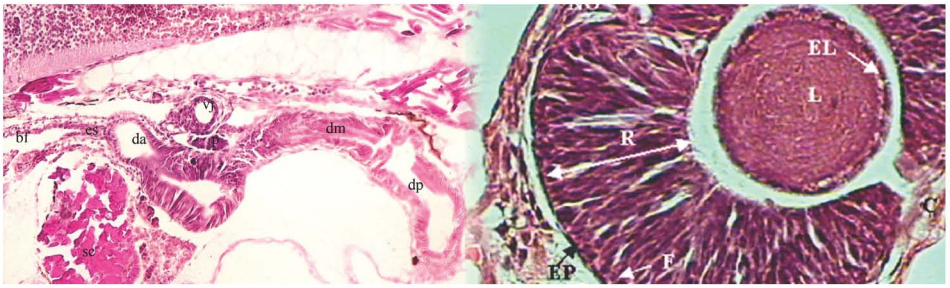
3.8.-Nomenclature of the referenced species

The common names in English of the species referenced in this work were taken from FAO "Fishbase" data base.

Improvements in the production technology of red porgy (*Pagrus pagrus*) larvae and fry: Importance of rearing conditions and diet nutritional value on their quality

Study I.-Development of red porgy larvae *Pagrus pagrus* (Linnaeus, 1758) visual system in relation with changes in the digestive tract and feeding habits

Aquaculture 179 (1999) 499-512



Development of red porgy *Pagrus pagrus* (Linnaeus, 1758) visual system in relation with changes in the digestive tract and larval feeding habits

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Abstract

Red porgy *Pagrus pagrus* larvae, like other sparids such as red sea bream are visual feeders. The normal development of the visual system is essential for successful prey capture and predator avoidance, leading to increased larval growth and survival. The aim of this work is to characterise the development of visual organs in relation to changes in the digestive system and feeding habits.
Twenty five larvae from hatching to day 29 were daily collected from the rearing tank, fixed in formalin, embedded in paraffin, 5 µm sectioned and stained with haematoxylin and eosin (H&E) and Periodic Acid Shift Reactive-Haematoxiline (PAS-Hx). Light microscopy was used to study changes in ocular morphology with respect to digestive system development.
At hatching, eye and digestive system of *Pagrus pagrus* larvae have no function. However, at day 3 post-hatch, when the mouth opens, the larvae must be ready for prey capture and digestion. Despite this, few day 3 larvae had food in the digestive tract. At day 4 photoreceptors were well developed in the eye, pigmentation pattern was complete and thus the visual system was completely ready for prey capture. This development coincided with detection of digestive activity in the midgut and most of larvae starting to take food. The results of this study suggest that the adequate development of the visual system is important to establish the start of exogenous feeding. Besides, the appearance of rod cells increase larval photosensitivity and suggest that changes in lighting regimes could be necessary throughout the larval phase.

Keywords: Pagrus pagrus, Red porgy, New species, Visual system, Digestive tract, Histology

Abbreviations: A: amacrine cells; B: bipolar cells; C: cornea; CR: cartilaginous ring; DHA: docosahexaenoic acid; EFA: essential fatty acids; EPA: eicosapentaenoic acid; G: ganglion cell layer; HG: hindgut; H: Horizontal cells; INL: inner nuclear layer; IPL: inner plexiform layer; ILCV: ileocecal valve; LEC: lens epithelial cells; L: lens; LRM: lens retractor muscle; MG: Midgut; ON: optic nerve; ONL: outer nuclear layer; OPL: outer plexiform layer; P: pigments; PN: photoreceptor nuclei; PRES: photoreceptors external segments; PRIS: photoreceptors inner segments; RN: rod nuclei.

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1. Introduction

Red porgy *Pagrus pagrus* (Linnaeus, 1758) (Osteichthyes, Sparidae) is a species found on Eastern Atlantic coasts: Gibraltar Straits to 15°N (occasionally to 20°N) including Madeira and the Canary Islands; in the Mediterranean and northward to the British Isles, (Bauchot, 1987; Bauchot and Hureau, 1990). It is found over hard (rock and rubble) or sandy bottoms (the young frequently found on seagrass beds) down to about 250 m depth, often above 150 m (Franquet and Brito, 1995). Red porgy is a characteristic pelagic spawner producing pelagic eggs (1.00 $\pm$ 0.02mm diameter). Spawning occurs between January and April, mainly when the water temperature reaches 18 to 22 ºC (Hernández-Cruz *et al*., 1990).

This species is being currently investigated as a potential aquaculture candidate (Kentouri *et al*., 1995; Stephanou *et al*., 1995; Hernández-Cruz *et al*., 1997; Socorro *et al*., 1997).

At hatching the larvae has a non functional visual system, together with a poorly structured mouth and limited locomotion control similar to sea bream *Sparus aurata* (Blaxter, 1986). Larvae hatched with relatively small yolk reserves and, as a result, they must start feeding soon after hatching. This requires the development of organs and systems involved in the capture and digestion of the food. Digestive and visual systems must be developed at the onset of feeding (Govoni *et al*., 1986)

Fish larvae are usually visual feeders, indicating that vision plays an important role in larval orientation at this stage (Blaxter, 1986). In sparids such as *Pagrus major* (Kawamura,1984) and *Pagrus auratus* (Pankhurst,1996) the most important changes in the eye structure occur in the lecitotrophic larvae as a preparation for prey capture. These changes should be coincident with digestive system development to start feeding as in *Sparus aurata* (Sarasquete *et al*., 1995; Hernández-Cruz *et al*., 1990). Other aspects of visual performance in relation to feeding have been investigated, especially the reception distance for food, which increases with body length (Blaxter, 1986).

This study aimed to shows the parallelism between the development of the digestive and visual system and the importance of both for the beginning of prey capture and predator avoidance. It has implications for hatchery management because an appropriate choice of prey size and lighting regimes will contribute to the success of larval rearing.

2. Materials and methods

The experiment was carried out from March 27th to April 26th (1998) at the Canary Institute of Marine Sciences.

Eggs obtained from a wild stock of *Pagrus pagrus* by spontaneous spawning were distributed into 500 l fibreglass tank (100 eggs.l^{-1}) filled with seawater. Larval development occurred between 20.5 to 21.8 ºC. Natural photoperiod of approximately 12h light was used. Larvae were feed with rotifers *Brachionus plicatilis* (L strain) from day 4 after hatching, at a concentration of 10 rot.ml^{-1} in the larval culture tank. Rotifers were mass-cultured using bakers yeast and *Nannochloropsis sp.* (Eustigmatophycea). After day 20, *Artemia* nauplii were offered to larvae. Larval rearing procedure followed protocols described by Hernández-Cruz *et al*, (1990).

Newly hatched larvae of 2.77mm total length were reared up to twenty-nine days and sampled daily. For that purpose, 20 larvae total length (TL), standard length (SL), head height (HH) and eye diameter (ED) were measured using a profile projector (Nikon V-12A).

2.1. Light microscopy

Another 25 red porgy larvae were fixed in 10% buffered formaldehyde for histological studies. All fish were sacrificed during the photo phase so that the eyes were adapted to light at the time of fixation. Larvae were then dehydrated in an ethanol series, embedded in paraffin, serially sectioned at 4-5μm, stained with haematoxylin and eosin (H&E) and Periodic Acid Shift Reactive-Haematoxylin (PAS-Hx) (García del Moral, 1993).

3. Results

At hatching, the eye lens (L) of *P. pagrus* showed undifferentiated cells having a spherical distribution. Retinal cells (R) are arranged radially and optic nerve were observed. At this stage, visual pigments were not present and some precursors of the photoreceptors nuclei cells (PN) were beginning to develop in the external part of the retina (Plate I). In the digestive tract, larvae had a simple undifferentiated straight gut linked to a non-structured mouth and anus.

The lens structure of one day old larva had two differentiated layers: an external layer of epithelial cells (LEC) surrounded by a non nucleated lens fibers layer formed by highly modified epithelial cells. On day 1 the retina also showed well defined layers, outer nuclear layer (ONL) composed by columnar nuclear bodies of photoreceptors (presumptive cones) and an inner nuclear layer (INL). A ganglion cell layer (G) formed by spherical nuclei of ganglion cells. Inner (IPL) and outer plexiform layers (OPL) showed the first signs of formation. At this stage several nuclei of pigment cells (P) were seen up the outer nuclear layer and a cartilaginous ring (CR) was

present (Plate II). At this time in the foregut a short brush border was observed, with a digestive lumen being found in some larvae from the foregut to the hindgut.

In two-day-old larvae, the nuclei photoreceptor in the outer nuclear layer increased in length. Inner and outer plexiform layers were well differentiated and a single layer of horizontal cells were present, down to the external plexiform layer. There was an increase in pigment contents. Lens retractor muscle was sketched and iris structure was better defined than days before. In the lens structure, there was an increase in the content of lens fibers and the lens epithelial cells were slender.

The digestive tract, initially straight, presented at this stage a small curvature in the last third of its length. The hindgut, which was not pressured by the yolk sac, presented a clear digestive lumen. The brush border in the hindgut was less evident than in the foregut.

The third day after hatching the lens was well defined by a single epithelial cell layer with PAS affinity, which covered the lens fibres. Photoreceptor cells were completely differentiated. Internal segment (PRIS) and external segment (PRES) contained visual pigments at this stage. Pigmented epithelial (PE) was well defined. Lens retractor muscle (LRM) was present and reached the lens, and the iris (I) was well defined (Plate III).

At day 3 post-hatch, the digestive system showed an increase in length at the anterior zone by a multiplication of the cell layers. In other parts of the digestive gut there was only a simple epithelium layer.

At this time, the ileocecal valve (ILCV) was formed, which separates midgut (MG) and hindgut (HG) (Plate IV). The brush border was seen from the most anterior part of foregut to the end of the intestinal tract. From day 3 post-hatch, the larval digestive tube could be divided into several parts: an oesophagus composed of a simple plane epithelium layer; a foregut formed by a simple cubical epithelium layer; a midgut where the epithelium layer was columnar, and a hindgut from the ileocecal valve composed of a columnar epithelium also.

By the fourth day, a clear differentiation of the retina layers was found. Pigmentation was completed and the eyes appeared like an adult fish eye (Plate V). The eye diameter of red porgy continued increasing in accordance with body size increment. In this way, during retinal growth, cones and other cell types were added concentrically at the retinal germinal margin. Horizontal and amacrine cells were clearly observed within the inner nuclear layer. There was an increase in length in the outer and inner segment of the photoreceptors.

On day 4, the digestive tract was developed and had several invaginations and an abundant brush border with columnar epithelium. The midgut of four old day larvae had digestive activity showing PAS affinity in the brush border. The ileocecal valve was better developed than on the third day. The larvae had started to ingest food and the anus was opened.

Food in the digestive tract was seen in most of the larvae at this time. During this time there was an increase in eye diameter which was significantly related to larval total length $r^2 = 0.987$ and head height $r^2 = 0.989$ (Figures 1- 3).

At day 10 after hatch, the sclera (S) started to differentiate but there were not significant changes in the retina. Over the following days, an increase in length of the eye occurred but one important change in the visual system occurred at 20 day post-hatch.

At this time spherical nuclei were seen in the basal zone of ONL, this type of cells were rod precursor nuclei (RN) which in following days increased in number (Plate VI).

At the same time there were changes in the digestive tract with gastric glands appearing. On day 25 post-hatch there was a clear increase in rods nuclei cells and a single layer of them were seen under the cones nuclear layer (Plate VII).

Cornea, sclera and iris were totally developed now. From day 25 post-hatch there was an increase in length but no more changes in eye structure were observed.

4. Discussion

Red porgy eye structure was similar to those described by Kawamura, (1984).for *P.major*, lens with undifferentiated cells disposed spherically, retinal cells on radial disposition and nerve optic cup were observed at this stage. The eye was not pigmented and some precursors of the photoreceptors nuclei cells were sketched in the external part of the retina.

One-day-old larvae had all the structural elements necessary for visual function, but most of them were not complete. This was an indication that the eye was about to be functional, Kawamura, (1984) found that the visual system of *P. major* is functional at 36 h after hatch when visual cells and pigments are present and nerve optic fibbers connect with the optic tectum. In red porgy, the visual system could not be functional at 28 h, principally because the pigmentation pattern, responsible for photon absorption was very sparse at this stage. The digestive system had started to differentiate.

The third day after hatching was one of the most important days for the larva, which need to be ready for prey capture and digestion. Although histologically the larvae appears to be prepared and had their mouth opened, only a few of them had food in the digestive tract at this time. This fact could be due to a learning delay between the larvae.

At day 4 in the eye, presumptive cone photoreceptors were well developed, pigmentation pattern was complete and thus the visual system was completely ready for prey capture in coincidence with the digestive activity detected in the midgut. Therefore, most larvae started to feed. From this day, the larvae had good capacity for prey capture, which was increased with body size.

Pure cone retina have been found in the earliest stages of many teleost larvae. At first feeding they are only equipped with simple cones, such as *P. major (*Kawamura, 1984) and *P. auratus* (Pankhurst, 1996) while rods and twin cones appear at metamorphosis (Blaxter and Staines, 1970).

Thus, in the studied species, on day 20 post-hatch spherical nuclei appeared in the basal zone of ONL, this cell type were rod precursor nuclei, which in following days increased in number. From this moment, *P. pagrus* larvae had a duplex retina with cone and rods. These results agree with those found in other sparids. For instance, in *P. auratus* in which presumptive rod precursor cells appear at 18 days post hatch (Pankhurst, 1996)and in red seabream larvae single cones fused to form twin cones and first rods appear at 11 mm TL (Kawamura, 1984).

Rods provide a better vision under low light intensity levels (O'Connell, 1981; Kawamura, 1984; Pankhurst, 1996). Kawamura, (1984) reported many changes in morphology as visual sensitivity increased. Behavior changes like feeding habits and prey selection in wild fish occur in temporal association with its migration from the surface waters to benthic habitat. This is a common behavioral pattern in some sparids fish like *P. major*, *P. auratus* or *S. aurata* which have an early life stage in shallow waters and shift to deep waters later after metamorphosis (Franquet and Brito, 1995). Other species like Pacific salmon hatch at a more advanced stage and some rods are present (O'Connell, 1981).. Northern anchovy have a pure rod-retina at hatch (O'Connell, 1981).

Normally, in culture conditions, daylight-type fluorescent tubes are used. Wild larvae migrate vertically to find the right illumination level according with its visual sensitivity, but in a culture tank, the correct illumination has to be provided (Huse, 1993).

There were high correlations between different morphmetric parameters like total length and head height with eye diameter. This means that the increase in length allows a better swim capacity and the increase in the eye diameter increased the visual capacity and prepares the larvae for the capture of larger prey sizes.

A good parallelism was seen between the development of visual system and digestive tract, until the larvae had no visual system ready, the digestive system was not ready either.

Before exogenous feeding, the larva's eye was completely developed, and it was functional to provide a good vision level that let the larvae start to take food. Different studies attach great importance to docosahexaenoic acid. DHA is present at very high levels in the retina and neural

tissues (Bell and Dick, 1993). In the lecitotrophic larvae, the development of all of its systems is from yolk reserves, this means that, the fish eggs should contain all the essential nutrients, like, essential fatty acids, glycerophospholipids and fat-soluble vitamins required for the development of the embryo and the growth of larvae (Izquierdo, 1996). N-3 highly unsaturated fatty acids (HUFA) are some of the main components of the lipids in fish eggs of several marine species such as red sea bream (Izquierdo *et al*., 1989a) and gilthead seabream (Mourente and Odriozola, 1990). Neuringer *et al,*. (1988) has established a critical role for n-3 polyunsaturated fatty acids and docosahexaenoic acid (DHA) in neural and retinal tissue functions in mammals. Bell and Dick (1993) found that there is a high demand of DHA to form nerve membranes in fish larval stages.

Several authors have shown that the fatty acid composition of total lipids of the eggs reflects those of maternal diet (Watanabe *et al*., 1984, 1985; Mourente & Odriozola, 1990a; Fernández-Palacios *et al*., 1995). Thus, adequate feeding in the broodstock must provide the eggs with all the essential nutrients for correct development during this stage.

Both photoreceptors in the eye, rods and cones accumulate and selectively retain DHA in the external segments. Rods contain twice as much DHA as cones (Bell and Dick, 1993). Rods appear after the start of the exogenous feeding about 18^{th} day after hatch, and important quantities of DHA will be necessary; consequently their requirements for n-3 HUFA should be satisfied by the diet. In nature different varieties of phytoplankton and zooplankton supply n-3 HUFA requirements but in aquaculture these need to be supplemented in the diet.

In addition, the improvement of visual capacity associated with rod apparition occurs with an increase in photosensitivity, this fact could mean that different lighting regimes are required during this phase of larval development for correct development of the visual system. Inadequate lighting regimes may be another stress factor in larvae culture conditions and correct management can contribute to increased larval growth and survival and consequently better success in larval rearing.

5. Acknowledgement

We are very grateful to D. Antonio Valencia for they invaluable contribution in the larval rearing.

6. References

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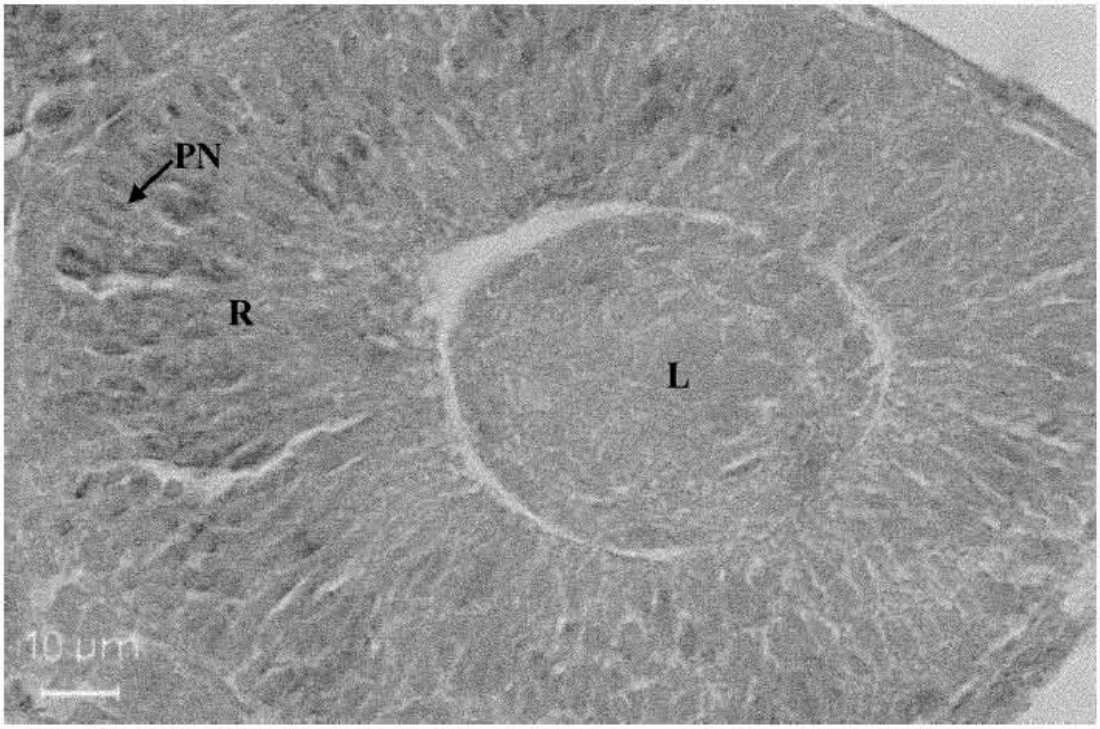

Plate I. Light micrograph of a longitudinal section through the eye of *P. pagrus* larvae at hatching (H&E). Eye lens (L) with undifferentiated cells. Retinal cells (R) on radial disposition. Precursors of the photoreceptors nuclei cells (PN) beginning to develop in the external part of the retina.

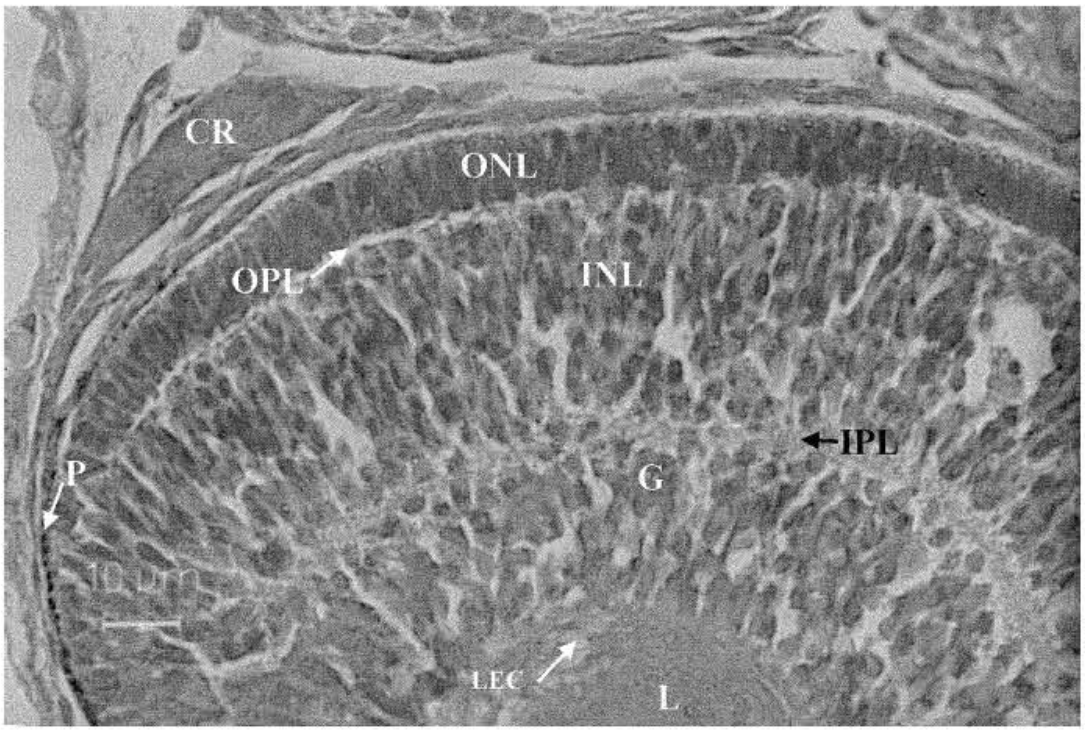

Plate II. Light micrograph of a longitudinal section through the eye of *P .pagrus* larvae at 1 day after hatching (3.65mm TL). Retina differentiate into the outer nuclear layer (ONL), outer plexiform layer (OPL), inner nuclear layer (INL), inner plexiform layer (IPL), ganglion cell layer (G). Nuclei of pigment (P) cells were seen up the outer nuclear layer and cartilaginous ring (CR) was present. Bar equal to 10 µm (H&E).

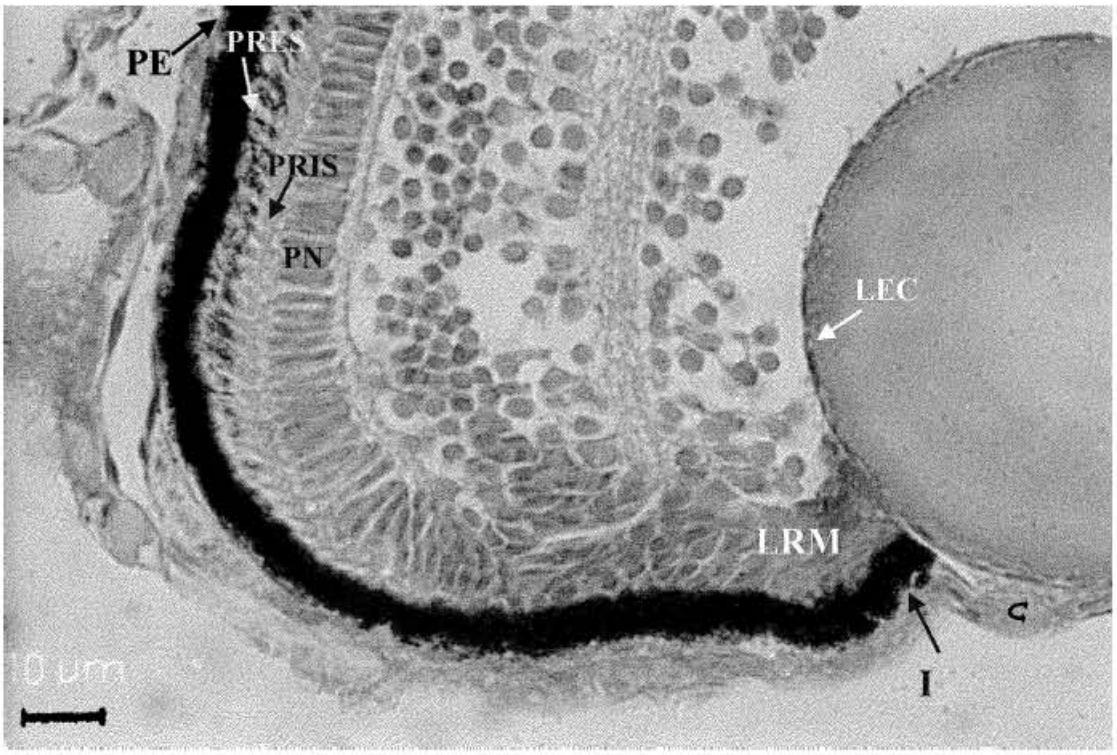

Plate III. Light micrograph of a longitudinal section through the eye of *P. pagrus* larvae at 3rd day after hatching (3.77mm TL) (H&E). Pigmentation pattern was complete (PE), and the iris (I) was present, cornea (C) and lens epithelial cells (LEC) was well differentiated, lens retractor muscle (LRM) was patent.

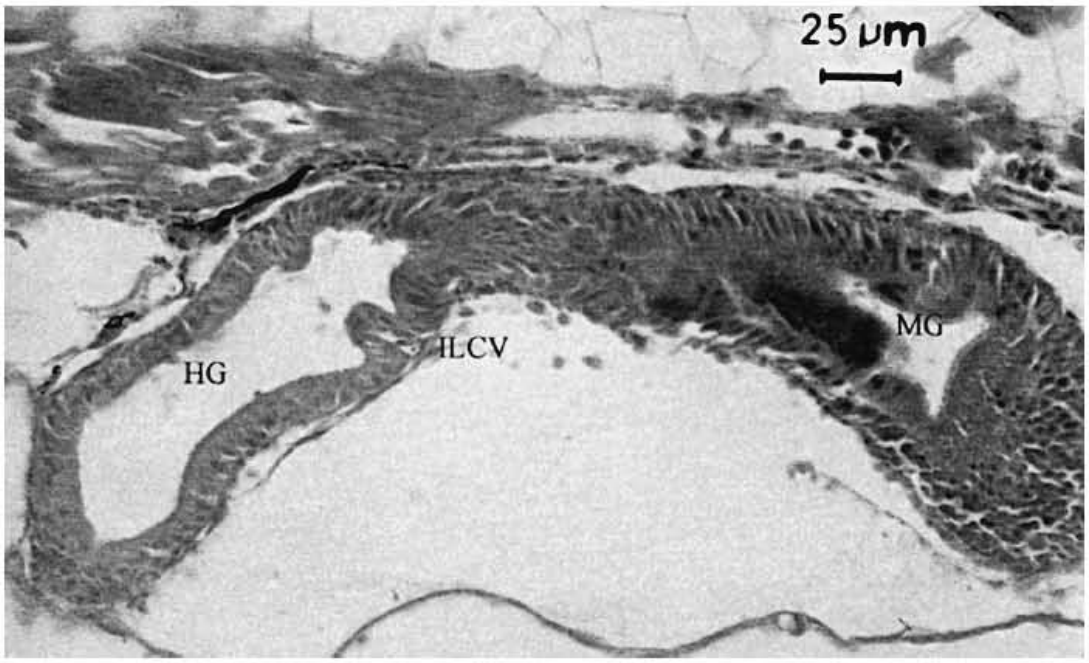

Plate IV. Light micrograph of a longitudinal section through the digestive system of *P. pagrus* larvae at 3rd day after hatching. Midgut(MG),ileocecal valve (ILCV), hindgut (HG), (3.77mm TL)(H&E).

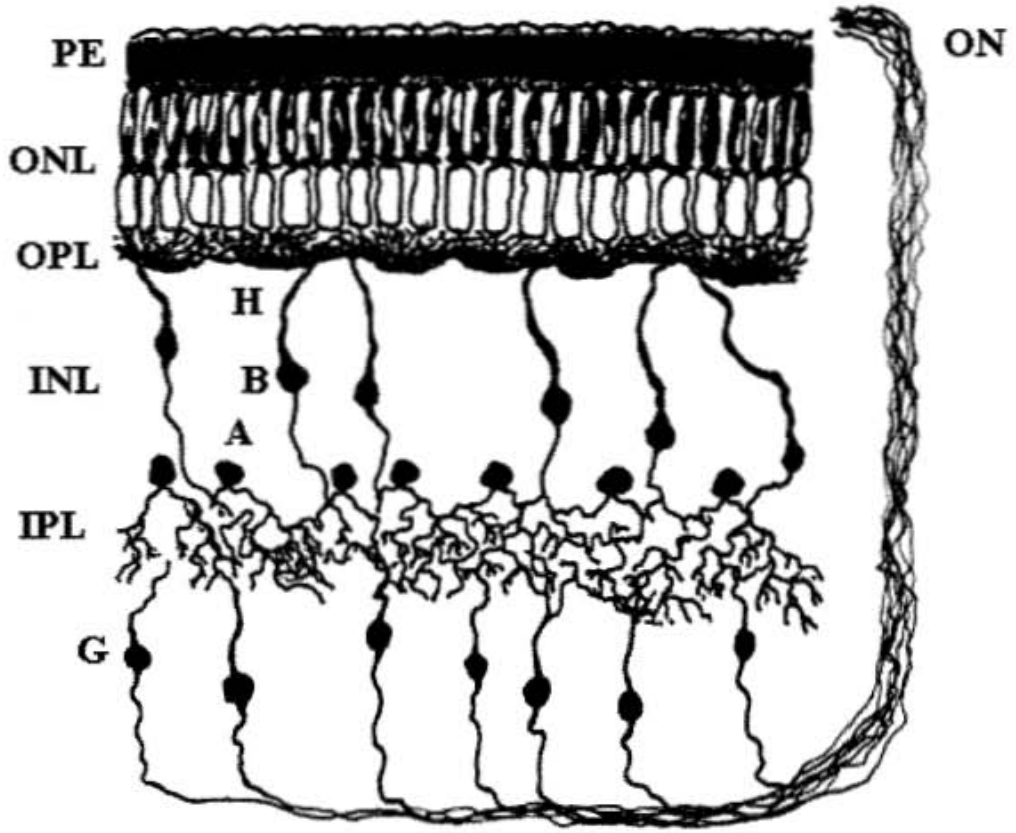

Plate V. Diagram of *P. pagrus* retina layers. Pigment epithelium layer (PE),outer nuclear layer (ONL),outer plexiform layer (OPL),inner nuclear layer (INL),horizontal cells (H),amacrine cells (A),bipolar cells (B), inner plexiform layer (IPL), ganglion cell layer (G), optic nerve (ON).

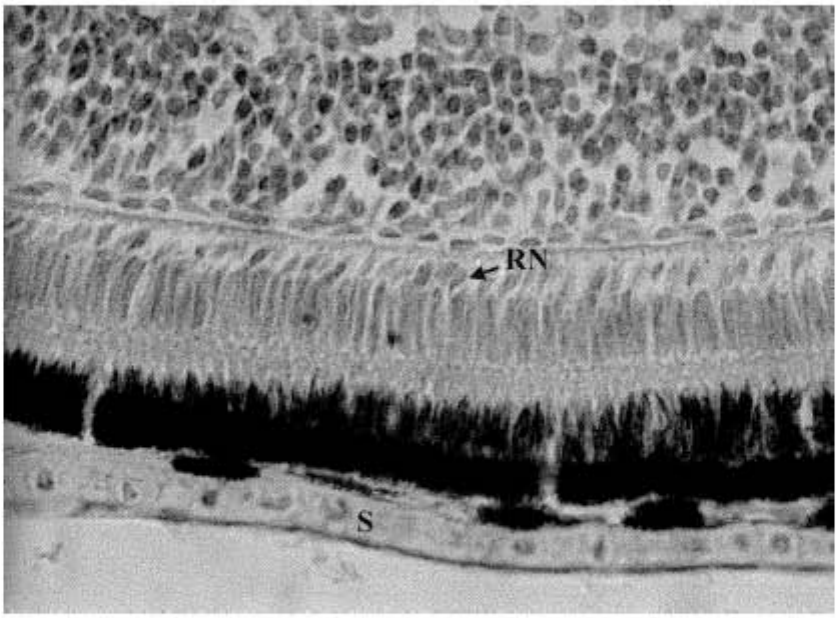

Plate VI. Light micrograph of a longitudinal section through the retina of *P. pagrus* larvae at day 20 after hatching (6.29mm TL). In the retina at day 20 an spherical nuclei were seen added to the basal zone of ONL, this type cells were rod precursor nuclei (RN) and in following days increased in number, sclera was present too (S) (H&E).

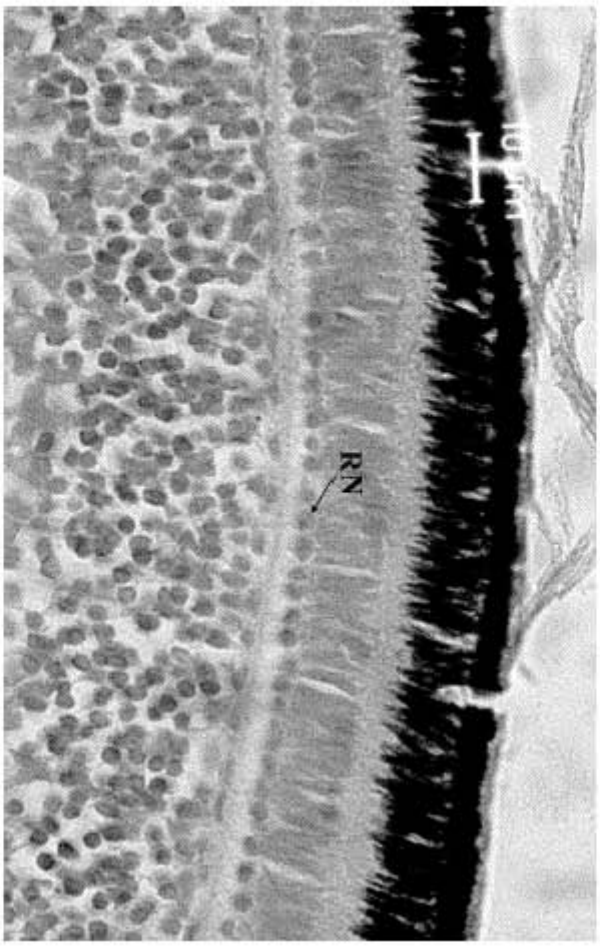

Plate VII. Light micrograph of a longitudinal section through the retina of *P. pagrus* larvae at day 25 after hatching (6.29mm). Rod precursor nuclei layer (RN) (H&E).

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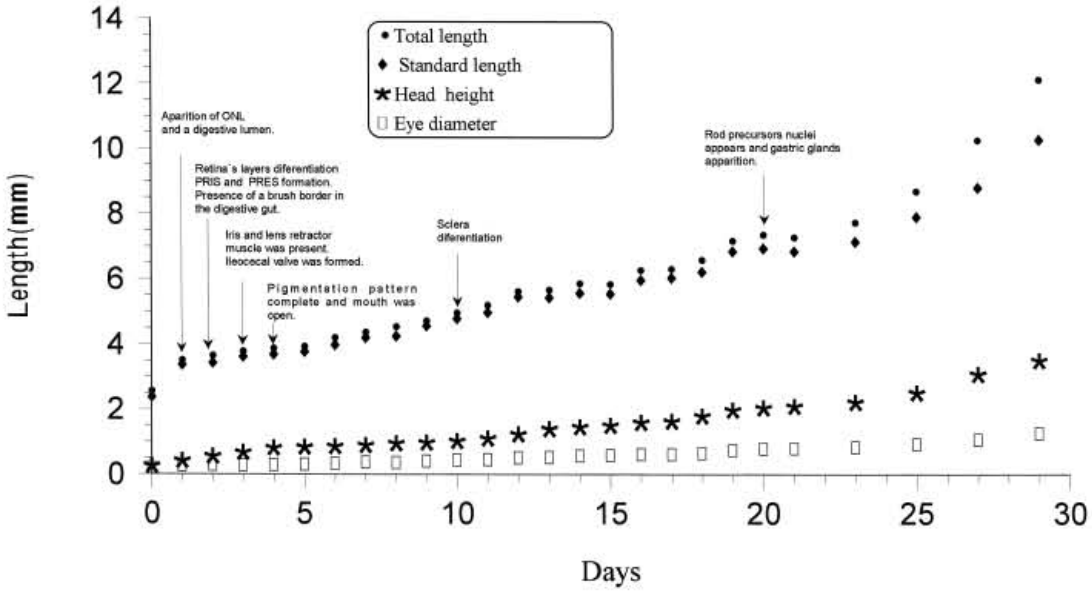

Figure 1. Daily evolution of different morphological parameters, total length, standard length, head height and eye diameter in *Pagrus pagrus* larvae.

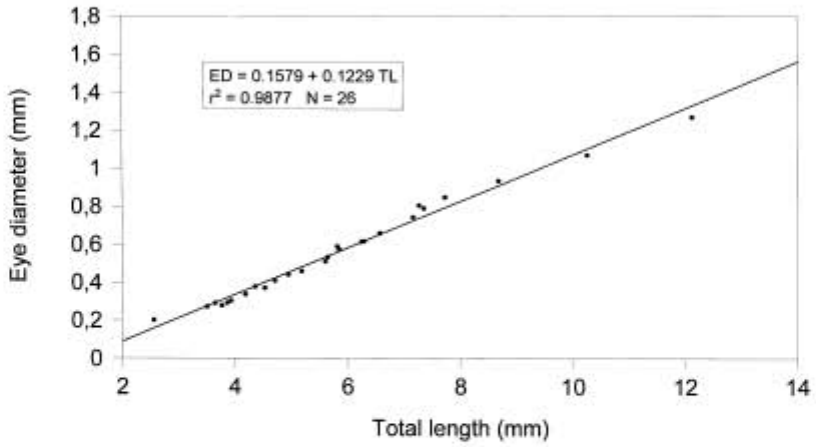

Figure 2. Relationship between the total length (TL) and the eye diameter (DE) for *P. pagrus* larvae.

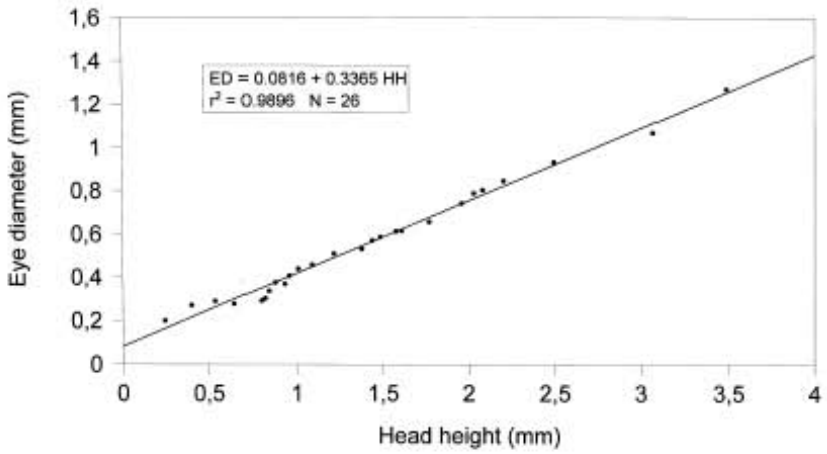

Figure 3. Relationship between the head height (HH) and the eye diameter (DE) for *P. pagrus* larvae.

Improvements in the production technology of red porgy (*Pagrus pagrus*) larvae and fry: Importance of rearing conditions and diet nutritional value on their quality

Study II. Osteological development and occurrence of skeletal deformities in red porgy *Pagrus pagrus* (Linnaeus, 1758) larvae cultured under different rearing techniques

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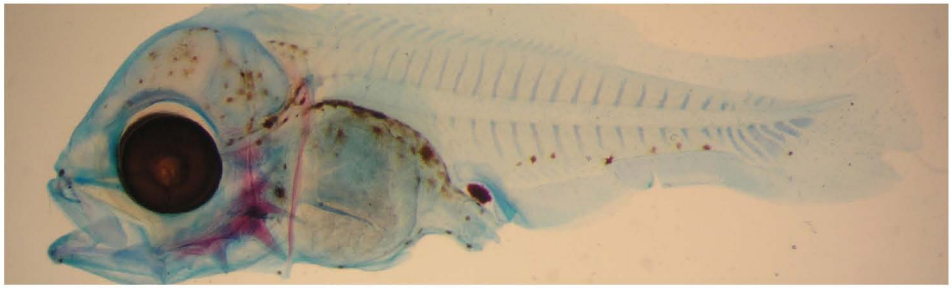

Osteological development and occurrence of skeletal deformities in red porgy *Pagrus pagrus (*Linnaeus, *1758)* larvae cultured under different rearing techniques.

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Abstract

Red porgy is a candidate species for aquaculture diversification, because of its relative fast growth and good adaptability to culture conditions. Standard techniques and feeding products for massive larval rearing have to be improved and their relation to fry quality and skeleton anomalies occurrence have not been studied yet. The objective of the present study was to describe the osteological development and the occurrence of skeletal deformities in *Pagrus pagrus* larvae in relation to the intensification of the rearing system. Eggs obtained from natural spawning, were cultured under two different rearing systems: intensive (100 eggs.l^{-1}) in 2 m^3 and semi-intensive (mesocosm) system, (5 eggs.l^{-1}) in 40 m^3 cylinder-conical tanks. Fish samples were periodically collected along the development from hatching to juveniles (95days after hatching). Osteological development, meristic counts and the presence of skeleton abnormalities were evaluated. Thus, the general pattern of the osteological development for red porgy was similar between fish from both culture systems. However, the ontogeny of the skeleton structures differed between the rearing systems. In addition a significant interaction was found between meristic counts (total number of vertebrae) and the type of rearing system used, fish from the intensive system showing a higher number of fish with an extra vertebrae (10 abdominal+15 caudal). Furthermore, despite the external appearance of the juveniles being similar to wild standards, X-ray studies revealed a high number of fish with skeleton abnormalities regardless of the rearing system (Semi-intensive:38.8%; Intensive:46.5 %). in addition, no significant interaction was found between the highest incidence skeleton anomalies (lordosis and presence of fused vertebrae) and rearing system. However, cranial abnormalities and kyphosis incidence were significantly higher in intensive system cultured red porgy. Moreover, the position of fused vertebrae in this fish was located mainly in the caudal area instead of pre-hemal area for semi-intensive system reared red porgy. Present results, report the osteological description and described for first time the most important skeletal malformation associated with this specie. This information is a useful tool to adapt and improve larval rearing protocols for *Pagrus pagrus*.

Keywords: Pagrus pagrus, Larvae, Development, Skeleton, Abnormalities, Rearing techniques, Mesocosms, Culture system

Abbreviations: ARA: Arachydonic Acid, BMP_4: Bone morphogenetic protein 4; DAH: days after hatching; DHA: Docosahexaenoic Acid; DPA: Docosapentaenoic acid; EFA: Essential fatty acids; EPA: Docosapentaenoic Acid; FA: Fatty acid; HUFA: Highly unsaturated fatty acids; RXR: Retinoid X receptor; SGR: specific growth rate; TFA: Total fatty Acids;

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1. Introduction

Several studies have focused on the osteological development and bone anomalies of different sparids such as *Sparus aurata* (Koumoundouros *et al*., 1997a,b; Faustino, 2002; Boglione *et al*, 2001), *Pagrus major* (Moteki, 2002; Kihara *et al.,* 2002; Matsuoka, 2003), *Dentex dentex* (Koumoundouros *et al*., 2001a), *Pagellus erythinus* (Boglione *et al*., 2003; Sfakianakis *et al.,* 2004), *Diplodus puntazzo* (Boglione *et al*., 2003; Favaloro and Mazzola, 2003) and *Diplodus sargus* (Koumoundouros *et al*., 2001b; Sfakianakis *et al.,* 2003). In teleosts, bone is a regular bony tissue with a chemical composition that does not differ in general from that of other vertebrates (Meunier 1989; Witten, 1997). Two different types of bone have been described in teleosts, acellular bone (lacking osteocytes) in species such as *Oreochromis niloticus*, or cellular bone in species like, *Acanthopagrus australis, Pagrus auratus* and *Rhabdosargus sarba*, *Oncorhynchus mykiss* or *Cyprinus carpio* (Hughes *et al*., 1994; Witten *et al*., 2000). The presence of four types of bone cells, like osteoblasts and osteoclasts has been also described in fish (Witten, 1997 and Witten *et al*., 2000) using histochemical techniques. Bone resorption is described by these authors as a general feature of growing teleost bone, indicating that the process and the characteristics of their resorption cells are quite similar to the mammalian bones. Bone metabolism is directly affected by biological regulators such as hormones, vitamins or autocrine and paracrine factors, including prostaglandins, cytokines and growth factors (Watkins and Seifert, 2000).

Alterations on the osteological development of marine fish larvae have been found to be related to certain environmental factors such as light, temperature, salinity, (Polo *et al*., 1991, Battaglene and Talbot, 1990; Mihelakakis and Yoshimatsu, 1998; Cobcroft *et al.*, 2001; Sfakianakis *et al*., 2004), mechanical shock either during broodstock management or larval development, variations in tank currents (Chatain, 1994; Divanach *et al*., 1997; Koumoundouros *et al*., 1997a,b; Kihara *et al*., 2002), genetic background and nutritional imbalances or deficiencies during early larval development (including live prey feeding) and broodstock feeding (Kanazawa *et al*., 1983; Akiyama *et al*., 1986, Knox *et al*., 1988; Chatain and Ounais–Guschemann, 1990; Afonso *et al*., 2000, Cahu *et al*., 2003, Saele *et al*., 2003; Hamre *et al*., 2005; Roo *et al*., 2008). Furthermore the intensification of the rearing system also seems to increase the incidence of skeletal deformities (Boglione *et al*., 2001; Koumoundouros *et al*., 2001a; Sfakianakis *et al*., 2004; Roo *et al*., 2005; Gimenez and Estévez., 2005), whereas extensive rearing conditions produce fish with a similar incidence of skeleton anomalies to wild juveniles and lower than those obtained in the intensive systems (Divanach and Kentouri,1983 and Divanach *et al*. 1996). The frequency of bone abnormalities has been proposed by Boglione *et al*., (2001) as a fry quality parameter for marine

hatcheries. Impaired osteological development at early stages will be the first sign of a future low quality fry batch with a reduction in the cost-effectively for aquaculture industry.

Red porgy has been proposed as a potential candidate for diversification of aquaculture production (Kentouri *et al*., 1995; Hernández-Cruz *et al.*, 1999) because of its relatively fast growth, good adaptability to culture conditions and high commercial value. Pilot scale on-growing trials have been conducted for this species and the conditions for fry transport have been established by Pavlidis *et al.,* (2003). Besides, the influence of background colour and crowding on the stress response in on-grown fish has been studied by Rotllant *et al*., (2003) and now it is possible to produce red porgy with a similar pigmentation to wild fish (Kalinowski *et al.,* 2005). However, larval rearing techniques described for this species have been adapted from gilthead sea bream culture techniques with different degree of success (Kentouri *et al*., 1995; Hernández-Cruz *et al.,* 1999; Mihelakakis *et al.,* 2001; Papandroulakis *et al.,* 2004), but a reliable standard production technique is not yet available. In addition, information about the ontogeny of different organs and tissues has been previously studied by Roo *et al*. (1999), Socorro *et al*. (2001) and Darias *et al*. (2005). Furthermore, detailed information about the osteological development of red porgy was described by Socorro (2006) although the occurrence of skeleton deformities in this species has not yet been assessed. The objectives of this study were to establish the occurrence of skeletal abnormalities in *Pagrus pagrus* larvae reared in captivity and to determine its relationship to the ossification process and the intensification of the rearing system.

2. Materials and methods

2.1. Experimental conditions

All the experimental labor was performed in the Instituto Canario de Ciencias Marinas facilities (Telde, Las Palmas; Canary Islands, Spain). In order to study the effect of the intensification of the larval rearing system on the osteological development and the occurrence of skeletal abnormalities in red porgy larvae, two different rearing systems were tested, data are a pool of two consecutive production cycles performed in duplicate for each rearing system:

a semi-intensive system (SMIS), similar to the mesocosm system described by Divanach and Kentouri (2000), performed in 2 replicates cylinder-conical 40m^3 tanks, stocked with 5 eggs.l^{-1} and an intensive system (IS) formed by 2 replicates cylinder-conical tanks of 2m^3 capacity, stocked with 100 eggs.l^{-1}. Seawater previously sand filtered and UV sterilized was used in both systems. Water salinity and temperature during the trials were 37 ± 0.5 ‰ and 20 ± 0.5 ºC, respectively, and the oxygen level was kept at 6.6 ± 0.6 ppm. Green water technique was used adding live phytoplankton (*Nannochloropsis sp.*) to maintain a concentration of 250 ± 100 x 10^3

cells.ml^{-1} in the rearing tanks. Larval culture was conducted under continuous photoperiod, by means of natural and artificial light. The light intensity just above the water surface ranged between 1000 to 3500 lux, which is in the optimum range determined by Tandler and Mason (1983) for a closely related species (*Sparus aurata*).

A similar feeding protocol was used in both systems: from day 3 after hatching (dah) until larvae reached 8.0 mm total length, 5-10 rot.ml^{-1} were added twice a day in the intensive system and among 3-5 rot.ml^{-1} in the mesocosm system. Rotifers were enriched with DHA Protein Selco ®, following product guidelines. At 13dah, *Artemia* Instar I (0.25 A_0 mL^{-1}) were added once a day in all the tanks. From 15 dah *Artemia* Instar II enriched with A_1 Selco ® (INVE, Belgium), were added three times a day (500 *Artemia*.ml^{-1}). Larvae were fed commercial diets from 20 dah (Skretting, France), manually supplied for the first 3 days and by means of automatic feeders afterwards. Preys and diet composition is presented in table I. After 50dah the whole population was transferred to 10m^3 tanks, and both populations were kept under the same rearing conditions (10-15 ind L^{-1}) in a flow through water system until 95dah when all the fish were individually counted and skeletal characterisation was performed.

2.2 Growth.

Larval growth was assessed measuring the total length of 25 larvae per tank every 5-7 days, using a profile projector (Nikon V-12A, NIKON, Tokyo, Japan). Length-specific growth rate (SGR) was calculated using the following equation: $SGR=([Ln(L_t)-Ln(L_0)] /t) x 100$; where L_t is the larval length at the end of time period t, L_0 is the length at the beginning of time period t, and t is the length of the time period in days.

2.3. Osteological development, meristic counts and deformities characterisation.

From hatching to 45dah, 25 individuals from each larval tank were randomly sampled every 5 days and fixed in 10% buffered formalin. Fixed larvae were cleared and then stained with alcian blue and alizarin red (Taylor and van Dyke, 1985 and Dingerkus and Uhler, 1977) and individually examined using stereomicroscopy to study the osteological development. Meristic determinations of total number of vertebrae (including urostyle) and number of pleural ribs were carried out in 95 dah juveniles (n=300) from each rearing system. According with Matsuoka (2003), all vertebrae with 2 neural spines and/or 2 haemal spines were considered to be formed by fusion and counted as 2. Deformity characterisation was performed at 95 dah, 300 specimens of 5.5g mean weight collected from each treatment. Fish were observed in fresh to detect cranial and opercula anomalies, and afterwards soft X-ray monitored (Mod. Senographer-DHR, General electrics, USA) to determine the incidence of internal skeletal abnormalities. Bone description and terminology followed Boglione *et al*. (2001).

2.4 Statistical analysis.

All the data was statistically treated using a T-test for simple mean comparison analysis ($P < 0.05$) (Sokal and Rolf, 1995) using a SPSS Statistical Software System ver 12.0 (SPSS Chicago, Illinois, 1999). To evaluate the differences in meristic characters and the frequency of deformities, a log lineal statistical analysis was performed.($P < 0.05$) (Sokal and Rolf., 1995).

3. Results.

3.1 Growth.

Larval growth was significantly affected by the intensification of the rearing system (Figure 1), those larvae reared under SMIS showing a better growth in terms of total length in comparison with IS reared ones.

From 15dah larvae reared in SMIS grew significantly better than IS ones. In addition, the semi-intensive system larvae showed a tendency to have better SGR for the whole rearing period, although without statistically significant differences ($P > 0.05$) with IS larvae (Table 2).

3.2 Osteological development

From hatching to 10dah, the ontogeny of the head region was characterised by the apparition of some skeletal elements like Meckel´s cartilage, maxillary, articular, preopercule, opercula, cleithrum, quadrate, and the characteristic head spine together with other cartilaginous structures related mainly with feeding and respiration (Figure 2a). In both treatments, the first sign of ossification was found in the pre-opercula spines in larvae over 4.8mm TL (Figure 2b) regardless larval age . From hatching to this developmental stage, skeleton anomalies observed were related to head malformation regardless of the rearing system, the most common observations were the presence of larvae with torsion of the trabecula, maxillar and downward curvature of Meckel´s cartilage/ lower jaw in larvae total length (TL 4.1 mm) (Figure 3a), pinched jaw larvae (TL 4.4 mm) (Figure 3b); and upper jaw reduction and longer lower jaw larvae (TL 4.0 mm) (Figure 3c).

Flexion of the notochord tip was observed in larvae over 6.0±0.5mm TL (15 and 20dah for SMIS and IS larvae respectively). At this stage, a clear definition of neural and hemal spines was observed (Figure 4a). Ossification of vertebrae started at 20dah (TL: 6.3 mm ossified vertebrae 1-3) (Figure 4b) and by day 25 after hatching (7.5 - 8.5 mm TL) the differentiation and ossification of the vertebrae became evident (Figure 4c). At 30dah (10.0 mm TL) most of the SMIS larvae had a complete differentiated and ossified column and the scale ossification was obvious (Figure 4d) while this process was not observed until 35dah in the IS reared larvae. At the time of vertebral ossification the first record of vertebral fusion was detected (Figure 4e).

3.2 Meristic counts.

Normal counts of pleural ribs (VIII pairs) were recorded in 96.7% of SMIS fish and 97.4% of IS specimens (Figure 5a), whereas the rest of specimens showed one pair less of pleural ribs. No significant interaction was found between the rearing system and the number of pleural ribs (χ^2 = 0.376; p=0.540).

In general, total number of vertebrae was more variable than the number of ribs and a significant interaction was found between this measure and rearing system (χ^2 = 527.44; P=0.00). Larvae had a normal number of vertebrae (10+14; abdominal + caudal) in 82.8% of the SMIS larvae and 60.8% of the IS larvae, a reduced number of vertebrae (23; (9+14) in 4.4% SMIS and 2.8% IS larvae and, finally, an excess number of vertebrae (25; (10+15) in 12.8 % SMIS and 36.4% in IS (Figure 5b). Thus, intensification of the rearing system induced a higher number of fish with an extra vertebra.

3.3 Skeletal abnormalities.

The most common skeleton abnormalities observed in this species were grouped into one of the following categories: fused vertebrae, lordosis, cranial deformities, vertebrae shortening, kyphosis and others (including lack of neural and haemal spines, saddle back syndrome and vertebrae calcification (Figure 6).

A significant interaction of rearing system on the total number of abnormalities was found (χ^2 = 1211.09; P=0.00). In SMIS group the frequency of normal juveniles (62.2%) was significantly higher than in IS group (54.5%). Rearing system had no effect on the frequency of fused vertebrae (14.2-13.9%) and lordosis (13.3-13.4%) in the SMIS and IS reared larvae, while a significantly higher frequency of cranial deformities (Semi-intensive: 3.1% vs Intensive: 6.8%) and kyphosis (Semi-intensive: 3.9% *vs* Intensive: 8.8%) was observed (Figure 6).

Rearing system also showed a significant interaction on fused vertebrae location (χ^2 = 79.01; P=0.00). Thus, in SMIS reared larvae fused vertebrae were more prevalent (70%) in the pre-hemal region, with the 3rd to 5th vertebrae being most affected, whereas in IS reared fish, the fused vertebrae were mainly located (49%) in the caudal region (Figure 7a). However, the type of rearing system did not affect lordosis location. Lordosis was identified in the pre-haemal and haemal region (Pre-hemal:18-23% *vs* Hemal: 77-82%), the most prevalent location ranging from 8th to 12th vertebrae for this deformity. In addition, the location of kyphosis was identified in the transition between cephalic and pre-haemal region (Figure 8g). Some examples of normal and abnormal skeleton anomalies are shown in Figure 8.

4. Discussion

The incidence of skeleton anomalies and variations on meristic counts are of major interest in commercial and experimental hatcheries as quality descriptors of the reared juveniles (Boglione *et al*., 2001). Reared fishes generally display more meristic variations and higher number of malformations than natural populations (Matsuoka, 1987, Boglione *et al.,* 2001, 2003; Koumoundouros *et al*., 2001a). Most of the juveniles produced in the present study had a normal external appearance, but detailed examinations of the skeleton on stained and radiographed individuals revealed that *Pagrus pagrus* larvae, as other hatchery-reared fish, show considerable variations from their natural standard morpho-anatomical pattern. The general pattern of the osteological development for red porgy was similar between fish from both culture systems, and did not differ significantly from the one described by Socorro (2006) for this species. The first stage of osteological development was related with the apparition of cartilaginous feeding structures in head region (bucal and gill cavity), as it occurs in other species such as Atlantic cod (*Gadus morhua*) or winter flounder (*Pseudopleuronectes americanus*) (Hunt Von Herbing, 2001). Besides the general pattern of osteological development was similar to that detailed for other sparids such as *Pagrus major* (Matsuoka, 1987) and *Sparus aurata* (Faustino and Power, 2001). Different sizes obtained between rearing systems were associated to differences in the degree of osteological development, regardless of larval age, denoting that the ontogeny of the skeleton structures was linked to larval size rather than age, in agreement with the studies of Gavaia *et al*. (2002) in Senegal sole (*Solea senegalensis*) and Faustino (2002) in gilthead sea bream.

Several authors have described lower variability in the meristic counts of wild-caught fish than in reared ones (Koumoundouros *et al*., 2001a ; Boglione *et al*. 2001), for either semi-intensive or intensive systems, and in particular, a higher number of vertebrae in cultured fish (Matsuoka, 1987; Richards, 2006). In red porgy, in contrast with the other species mentioned above, culture intensification increased the number of fish with an extra vertebrae 25 (10 + 15), being *Pagrus pagrus* juveniles semi-intensive reared more similar to the wild standards, with a higher frequency of fish with a total number of 24 vertebrae (Manooch *et al*., 1976). Generally, in teleosts, variations in the number of vertebrae can be attributed to modified physical and chemical parameters during early development and pre-fertilization period (Lindsey and Ali, 1965; Lindsey, 1988), such as temperature which has been pointed out as the most important inductor of this anomaly. For instance, in ayu (*Plecoglossus altivelis*) an increase in the number of vertebrae has been found in relation to low incubation temperature, according to the Jordan's rule which is the tendency for fish from higher latitudes and colder waters to have more vertebrae

than related fish from lower latitudes and warmer waters (Iguchi *et al*., 2006). On the contrary, Georgakopoulou *et al*. (2007) did not find any vertebral number variation when European sea bass larvae were reared under a range of 15-20ºC. Salinity also seems to affect vertebral number as it has been reported by Boumaiza *et al*. (1981) for *A. fasciatus*. Besides, genetically distinct populations as well as siblings may have divergent responses in the number of vertebrae to the same environmental factor (Ali& Lindsey, 1974; Beacham and Murray, 1986). However, in the present study there were no significant differences in water temperature, salinity or genetic background, between the two culture systems assayed and only the culture intensiveness differed. Nevertheless, despite both rearing systems used the same type of food, prey density (Semi-intensive: 3-5 *vs* Intensive: 7.5-10 rot.ml^{-1}) differs and the nutritional quality of the rotifers may become different, once they are in the rearing tank (authors unpublished data). Indeed, supernumerary vertebra in European sea bass larvae has been related to nutritional unbalances such as an excess of HUFA which accelerated the osteoblast differentiation process through the up-regulation of Retinoid X receptor α and BMP_4, (Villeneuve *et al*., 2005, 2006).

Skeletal abnormalities were higher in the intensive system and similar to those reported for other species such as *Sparus aurata*, (Boglione *et al*., 2001), *Diplodus puntazzo* and *Pagellus erythinus* (Boglione *et al*., 2003; Sfakianakis *et al.,* 2004), *Pagrus major* (Hattori *et al*., 2003), and *Solea senegalensis* (Gavaia *et al*., 2002). The effect of the rearing system in relation to system intensification has been pointed out as a cause of skeletal abnormalities in other species, (Divanach *et al*., (1996), Koumoundourous *et al*., (1997a,b, 2001), Boglione *et al*., (2001), (Sfakianakis *et al*., 2004) and Roo *et al*., (2005). Nevertheless, deformities found in semi-intensive reared fish were also considered very high in comparison with the values obtained in other species produced by this system. For instance, historical data from the last six years of studies with *Sparus aurata* in our facilities showed a percent of deformities 3 to 6 fold times higher in larvae reared in the intensive system than semi-intensive ones (Roo *et al*., 2005), whereas in red porgy deformities were only a 8 % higher in the intensive system suggesting the effect of different causative factors, in addition to the rearing system.

The impact of cranial abnormalities and kyphosis increased in fish reared under the intensive system. These anomalies have been described in other species such as larval European sea bass, *Dicentrachus labrax*, (Barahona-Fernandes,1982), striped trumpeter, *Latris lineata*, (Cobcroft *et al*., 2001), bluefin tuna, *Thunnus orientalis*, (Shimizu and Takeuchi, 2002) or yellowtail kingfish, *Seriola lalandi* (Cobcroft *et al*., 2004).

Particularly, the occurrence of cranial abnormalities, such as lack or folding of opercula, upper-lower jaw reduction or twisted jaw are very common in different fish species (Fraser and de Nys,

2005). Beraldo *et al*. (2003) reported the presence of the opercular fold as the first sign of opercular abnormality in 25-40days old gilthead sea bream. Reared red porgy presented a very low incidence of opercula deformities that could be related to the long opercula spines found in this species from early stages, which would restrict the folding of the operculum into the gill chamber. Other cranial deformities mainly related to upper-jaw reduction and cross bite jaw were increased in red porgy reared in the intensive system and could be related to nutritional (highly unsaturated fatty acids, dietary phospholipids and vitamins) and mechanical (nose-walling effect) factors (Kanazawa *et al*., 1983; Cobcroft *et al*., 2001; Roo *et al*., 2008).

Kyphosis in reared larvae has been cited in different culture species, like gilthead seabream or European sea bass. Koumoundouros *et al*., (2002) reported the presence of kyphosis in European sea bass larvae closely associated to branchiostegal ray anomalies. Both skeletal elements are membrane bones, which develop at the same ontogeny phase and probably are sensitive to the same causative factors, being nutrition proposed as an important factor affecting this type of anomaly (Koumoundouros *et al*., 2002). Kyphosis has been associated with swim bladder over-inflation and overfilling of the digestive tract, which induce a dorsal curvature of the notochord in the region above the swim bladder because of excessive pressure on this region (Grotmol *et al*., 2005). In the present study, red porgy larvae showing abnormally large swim bladder were rarely found and most of them, floating in the water surface, were removed by floating skimmers or dead in 1-2 days. Hence, swim bladder over-inflation is unlike to be a main cause of the kyphosis incidence found in red porgy. However, red porgy larvae are very voracious and frequently show an abdomen distended by the digestive tract overfilling with large quantities of *Artemia*, which press the cephalic vertebrae and might contribute to a greater kyphosis incidence (Figure 9g).

In both rearing systems the most common skeleton anomalies were, vertebral column disorders, lordosis and fused vertebrae. Tank hydrodynamic has been considered the most important causative factor for haemal lordosis (Chatain, 1994; Andrades *et al*., 1996; Divanach *et al.,* 1997). In agreement with this hypothesis, lordosis apparition in red porgy was located between the 8^{th} and 12^{th} vertebra, which is the vertebral region, which supports the highest muscle pressure during swimming (Kihara *et al.,* 2002). No effect of the culture system was found in the incidence of lordosis, denoting a similar quality in tank hydrodynamic among systems. Additionally, most of the lordotic specimens showed the presence of another abnormalities such as fused vertebrae, reductions or excessive number of vertebrae, as previously described in *Pagrus major* (Matsuoka, 2003; Hattori *et al*., 2003).

Fused vertebra is one of the most important types of deformity for juvenile quality, since it alters fish shape and length. The high incidence of fused vertebrae found in red porgy was in agreement

with the results found for other species such as *Sparus aurata,* (Boglione *et al*., 2001), *Pagrus major* (Hattori *et al*., 2003) and *Senegalese sole* (Gavaia *et al*., 2002). Despite no effect of the rearing system on the percentage of fish bearing this deformity, a significant relationship was found with the location in the vertebral column. Thus, whereas in the intensive system vertebral fusions were located along the whole vertebral column, particularly in the caudal region, in the semi-intensive system fusions were mainly localized in the pre-hemal vertebrae. Besides, a higher number of vertebral fusions in the caudal region an extra number of vertebrae appeared in this region in the intensive system, suggesting a common factor negatively affecting vertebrae formation during the time of ossification of this vertebral region. Since ossification of vertebrae follows an antero-posterior pattern which lasts from day 20 to day 30 in the semi-intensive system, with ossification of hemal vertebrae around day 25, and until day 35 in the intensive system, these differences in ossification timing and vertebral fusion could be related with the changes in feeding protocol and larval feeding habits which occurred during this period. Feeding with dry diets started on day 20, with a marked increase in dietary intake from day 25 to 30, together with a progressive reduction in *Artemia* ingestion. Since semi-intensive larvae grew, developed and ossified their vertebrae faster, the earlier ability of these larvae to ingest dry feed could be related to the differences in the localization of vertebral anomalies among both systems. Indeed, our previous studies have found a direct relationship between vertebral fusion and nutritional factors in red porgy (Roo *et al.*, 2008). Several nutritional factors have been found to be related with the apparition of fused vertebrae. For instance, inadequate amounts of certain types of vitamin A in *Artemia* have been found to be associated with higher incidence of vertebral fusions (Dedi *et al*., 1995; Takeuchi *et al*., 1998) and skeleton development (Estévez and Kanazawa, 1995; Takeuchi *et al*., 1995; Ronnestad *et al.,* 1998; Suzuki *et al*., 2000; Moren *et al*., 2004) new reference Mazurais . Other nutritional factors involved in osteological development are the essential fatty acids (Cahu *et al*., 2003; Hamre *et al*., 2005; Lall and Lewis-McCrea, 2007). Not only the quantity but also the ratios between DHA and EPA to ARA are considered important for normal growth and development of fish larvae (Izquierdo, 1996; Rainuzzo *et al.,* 1997; Sargent *et al.,* 1999). Although it has been found that dietary lipids affect body fatty acid composition (Izquierdo *et al*., 1996) their effect on cartilage and bone fatty acid composition in fish has not been clearly determined. In chickens and rats dietary n-6/n-3 PUFA alters the fatty acid composition of bone and cartilage and modulates eicosanoid production (Watkins *et al*., 2000b; Xu *et al*., 1994), which in turn modulates the development and resorption of bones. Fish bone has similar cell types (Witten, (1997); Witten *et al*., 2000) and chemical composition to other vertebrates (Meunier 1989; Witten, 1997). In the present study, changes in type of feed

(rotifers, *Artemia* and dry diet) implied changes in dietary fatty acid composition. Also, a significant drop in DHA:EPA ratios of prey FA composition occurred when *Artemia* was introduced and large differences in ARA concentration and DHA/ARA and ARA /EPA were found among rotifers, *Artemia* and dry diet (Table 1). In agreement with these results, low levels of DHA were associated with a significantly higher incidence of vertebral fusions in red porgy (Roo *et al*., 2008). Both n-3 HUFA and particularly DHA have been suggested to be important factors for correct osteological development (Gapasin and Duray 2001, Hamre *et al*., 2005).

5. Conclusions

This osteological description and first report of the most important skeletal malformation associated with this specie is a useful tool to adapt and improve larval rearing protocols for *Pagrus pagrus*. The influence of the rearing system on the apparition of skeletal deformities and their location, suggest a relationship among feeding sequence, osteological development and deformities. Present results showed that despite the fact that a semi-intensive system can be successfully applied for red porgy fry to achieve semi-industrial production with good result in terms of growth, the high incidence of vertebral fusions observed, in this rearing system suggest different causative factors apart from system intensification. Many aspects of red porgy biology remain unknown and further studies are needed in order to reduce the incidence of skeleton anomalies to achieve cost-effective juvenile production of this species at a comercial scale.

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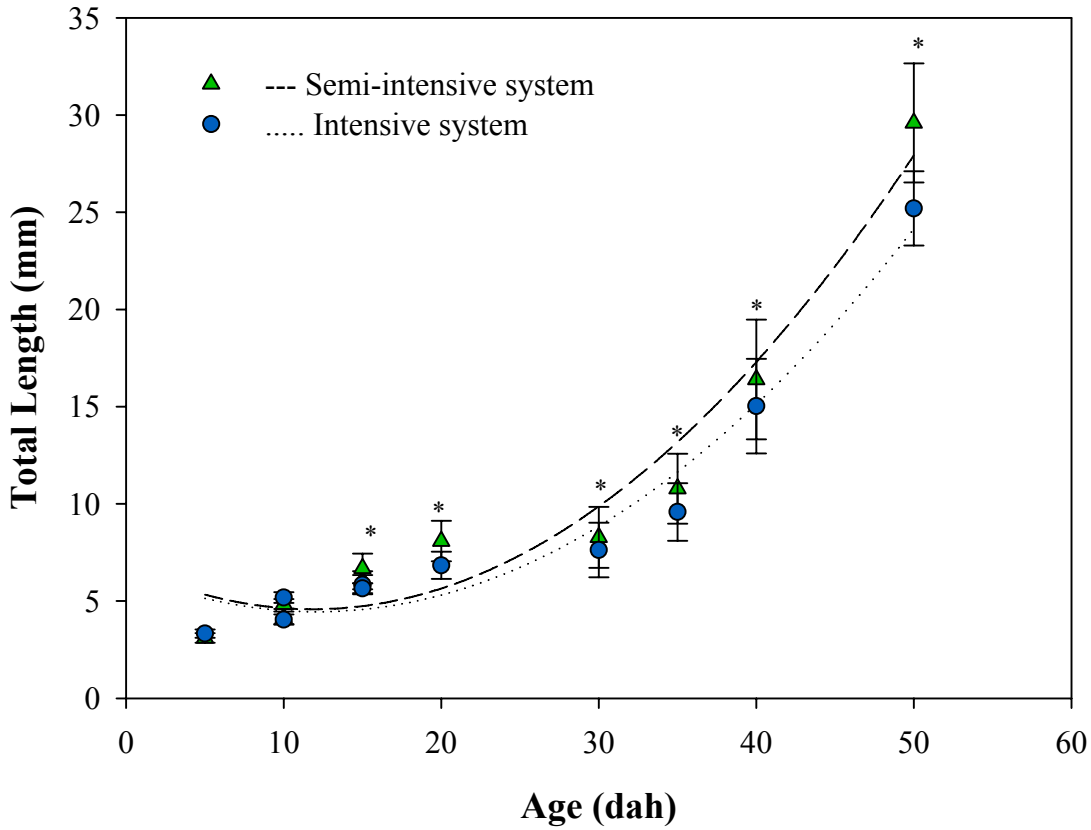

Figure 1. Total length evolution (TL) of red porgy (*Pagrus pagrus*) larvae cultured under different rearing systems. Data are given as mean±SD, (n=25 per tank) from two consecutive production cycles comparing both rearing systems. (*) denotes significant differences (P<0.05).

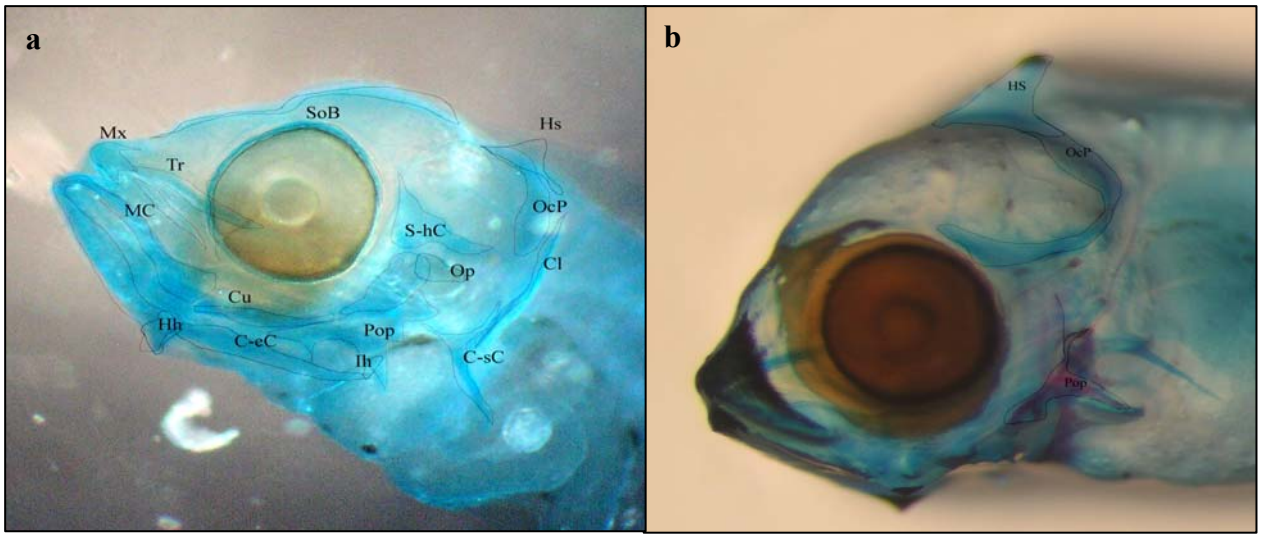

Figure. 2a,b. Initial development of the head region in *Pagrus pagrus*: (a) 10 dah larvae reared under intensive system, total length (TL 4.4 mm); (b) larvae reared under semi-intensive system (TL 4.8 mm); blue area, cartilage; red area, ossification; (a) C-eC, ceratohyalepihyal cartilage; Cl, cleithrum; C-sC, coraco-scapular cartilage; Hh, hypohyal; Hs, head spine; Ih, interhyal; MC, Meckel's cartilage; Mx, Maxillar; OcP, occipital process; Op, opercula; Pop, pre-opercle; Qu, quadrate; S-hC, symplectic-hyomandibular cartilage; SoB, supra-orbital bar; Tr, trabecula.

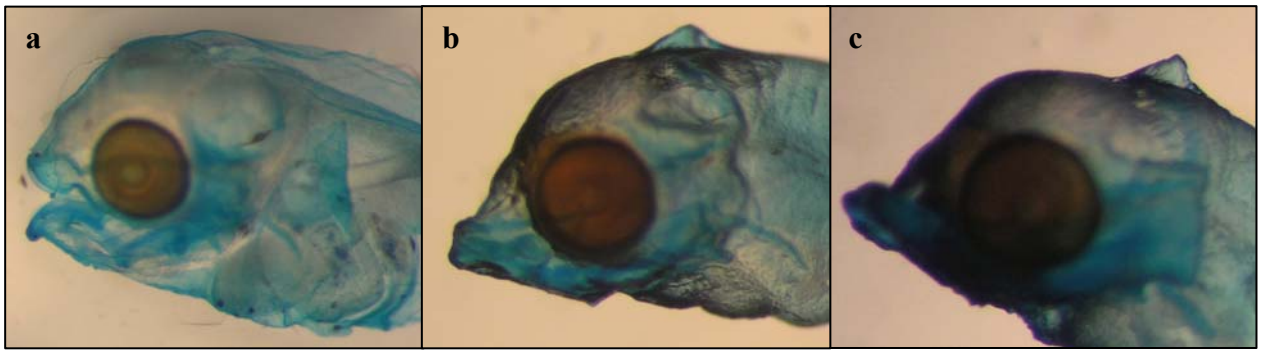

Figure. 3a,b,c. Malformed individuals in 10 dah *Pagrus pagrus* larvae reared under the intensive system: (a) torsion of the trabecula, maxillar and downward curvature of Meckel´s cartilage, lower jaw, total length (TL 4.1 mm); (b) pinched jaw larvae (TL 4.4 mm); (c) upper jaw reduction and longer lower jaw larvae (TL 4.0 mm).

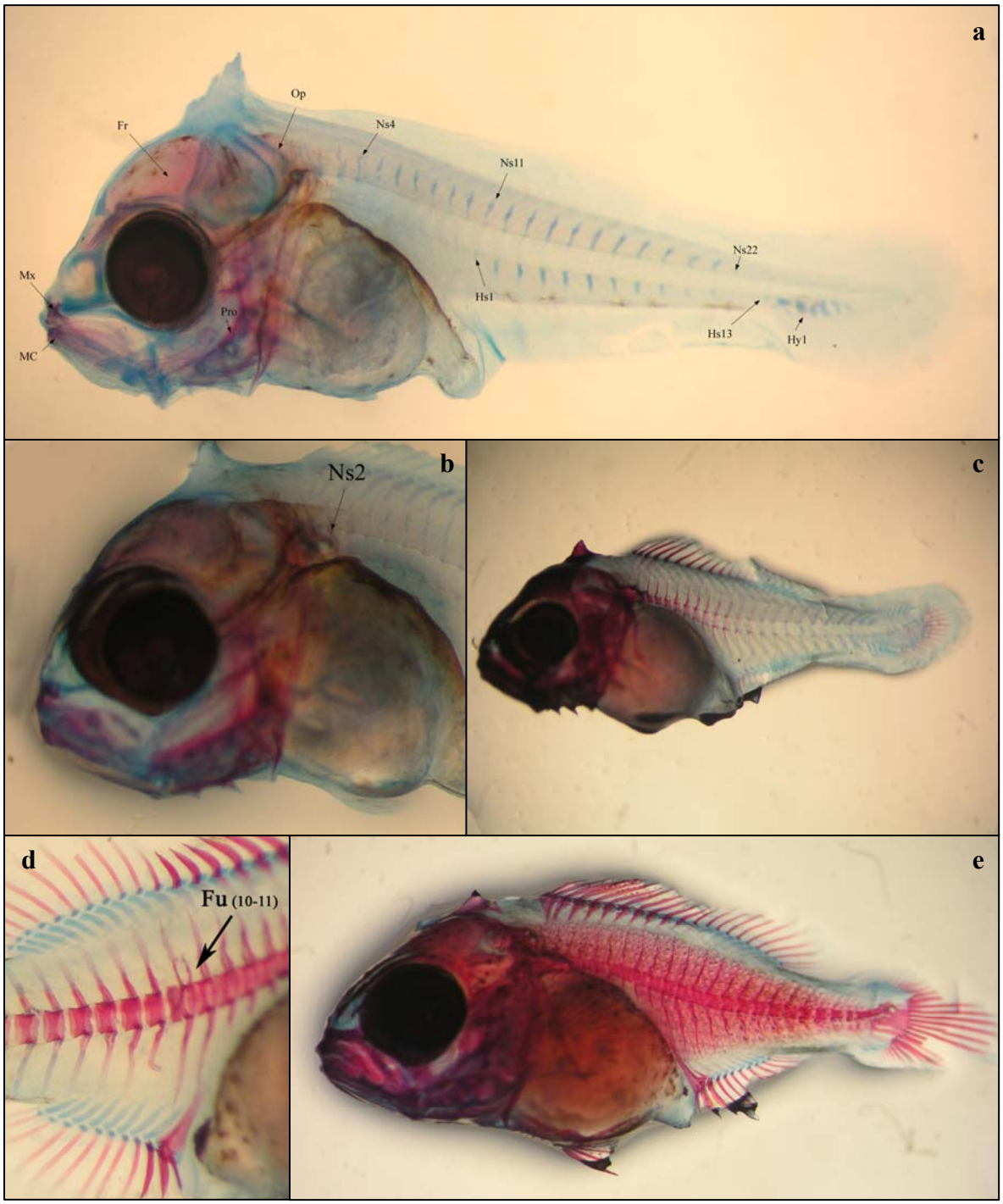

Figure 4. Ossification process in *Pagrus pagrus*. Blue area, cartilage; red area, ossification. (a) 15 dah larvae from intensive system (5.8±0.5 mm total length (TL)), ossified structures: Fr, frontal; Mx, Maxilar; MC, meckel´s cartilage; Pro, Preopercule; Op, occipital; Cartilaginous structures in blue: Hs, Hemal spines (numbered from 1 to 13); Hy, Hypural; Ns, Neural spines (numbered from 1 to 13). (b) 20 dah (6.8±0.7 mm) larvae started to ossify the anterior vertebrae. (c) 25 dah (8.0±0.9 mm TL) vertebral ossification evolution. (d) Vertebral fusion in 30 dah larvae (8.6 mm TL). (e) 30-35 dah, (11.5±1.5 mm TL) larvae with completely ossified column, head and fin rays and initial scale calcification.

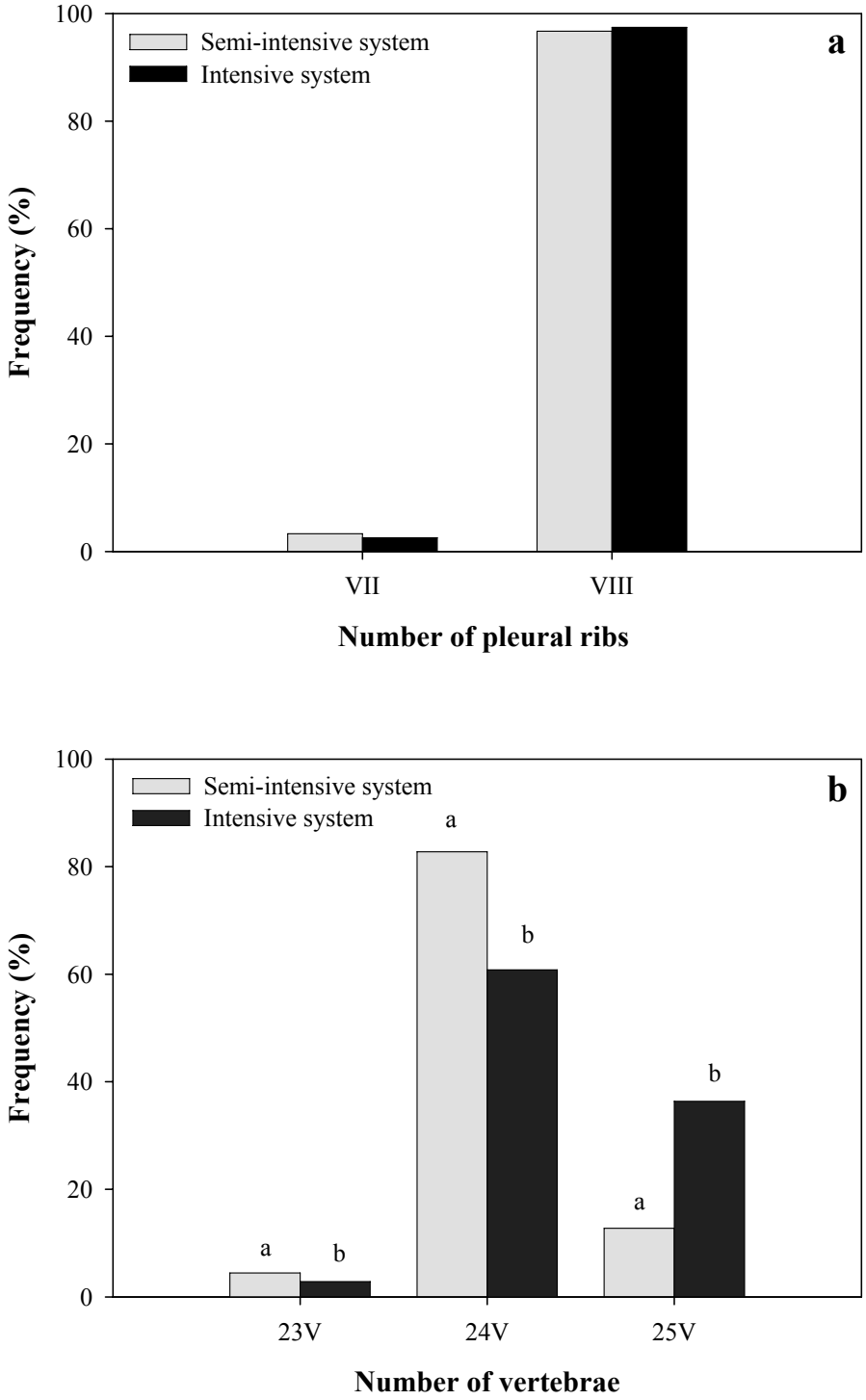

Figure 5a,b. Percent frequency distribution of pleural ribs (a) and number of vertebrae (b) according to the rearing system; Data are given as mean, (n=150 fish per tank), from two consecutive production cycles comparing both rearing systems.VII and VIII denotes the number of pairs of pleural ribs and 23V, 24V, 25V denotes the number of vertebrae). (*) Different letters over each column, denotes significant differences ($P<0.05$).

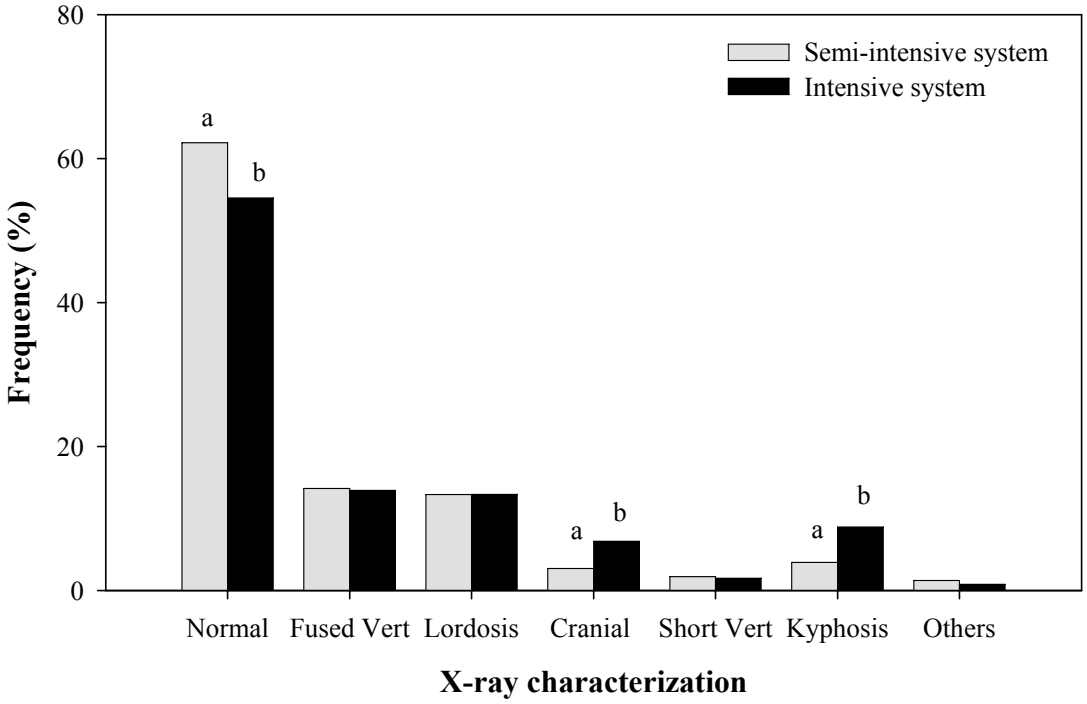

Figure 6. Incidence of skeleton abnormalities in the red porgy *Pagrus pagrus* seedlings reared in different systems Data are given as mean values of two replicates for each production cycles, (n=300 per culture system). (*) Different letters over each column, denotes significant differences ($P<0.05$).

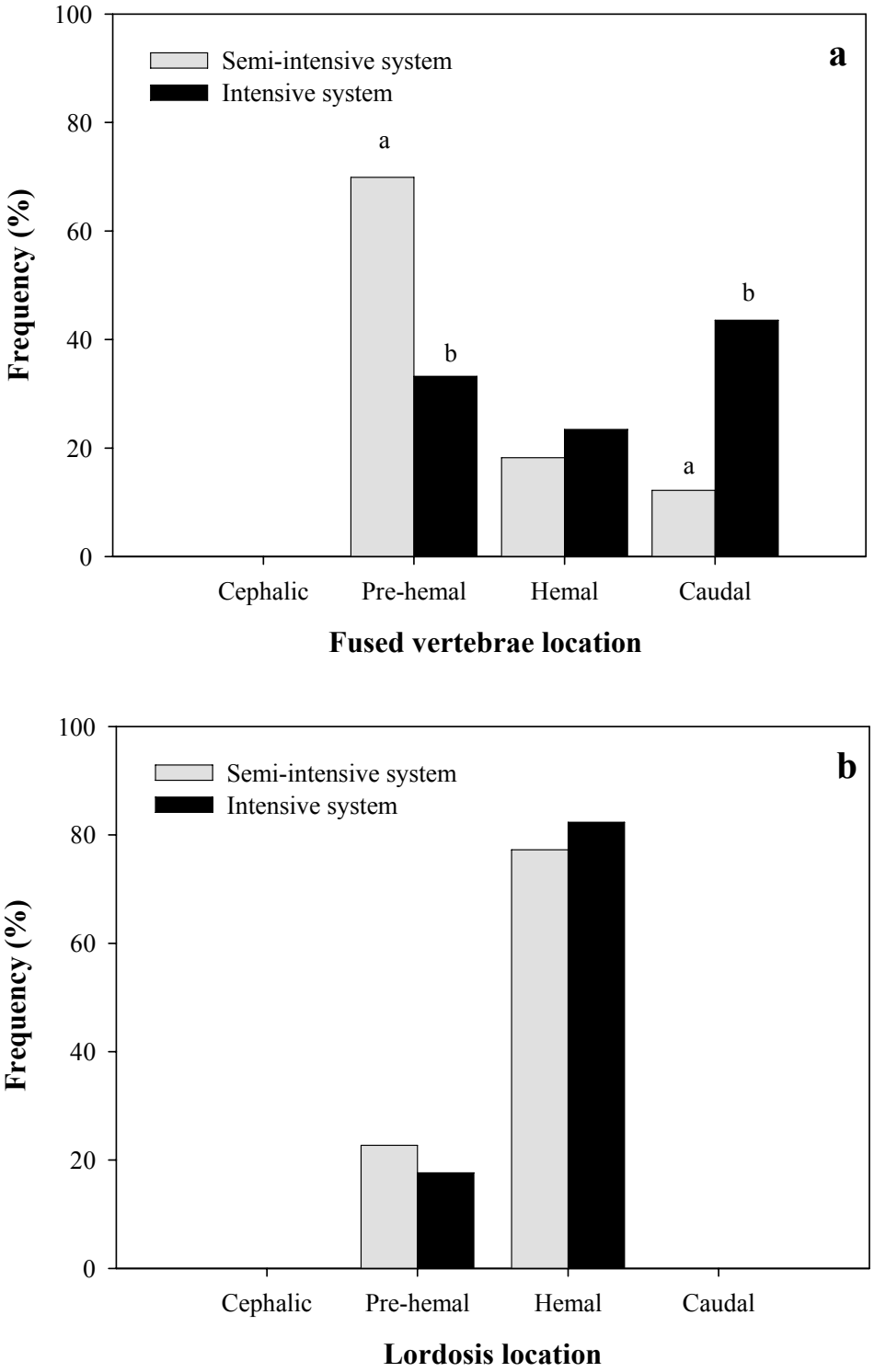

Figure 7a,b. Location in the vertebral column of fused vertebrae (a) and lordosis (b) in red porgy *Pagrus pagrus* seedlings. Data are given as mean values of two replicates for each of the two production cycles presented data (n=300 per culture system). (*) Different letters over each column, denotes significant differences ($P<0.05$).

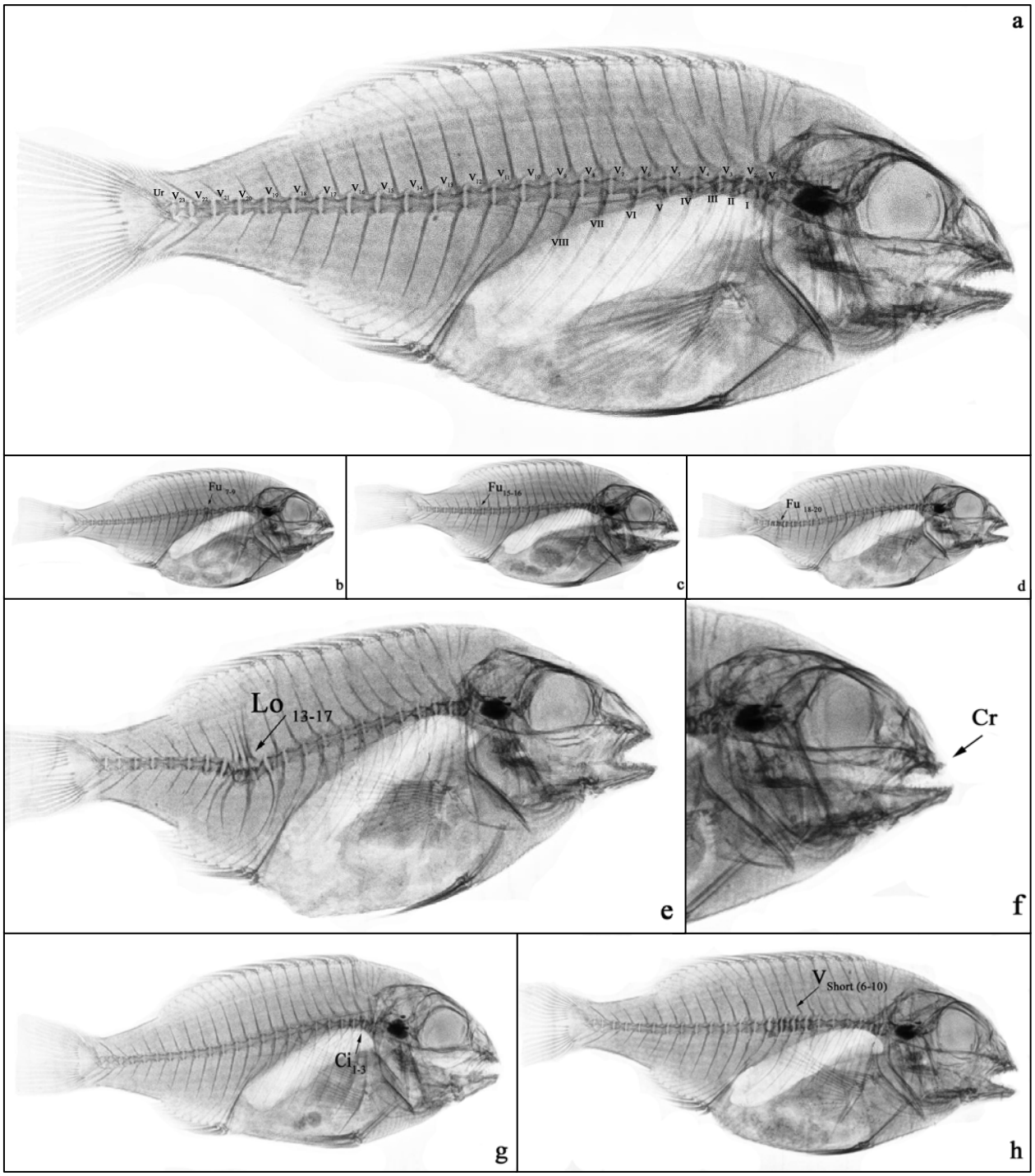

Figure 8. Soft X-ray study in *Pagrus pagrus* fingerlings at 95 dah with a mean weight of 3 g: (a) fish with normal skeleton development and meristic characters identified: V_n: Vertebrae number; I-VIII: Pleural rib number; (b,c,d) fish exhibiting fused vertebrae (Fu_{n-n}) in different regions (n=involved vertebrae number); (e) fish exhibiting acute lordosis between vertebrae 13th to 17th (Lo_{13-17}); (f) detail of cranial (Cr) deformity, fish with upper jaw reduction (Cr); (g) fish showing kyphosis (Ki) (arrow head); (h) example of fish with a vertebral shortening between 6th to 10th vertebrae ($V_{Short\ (6-10)}$).

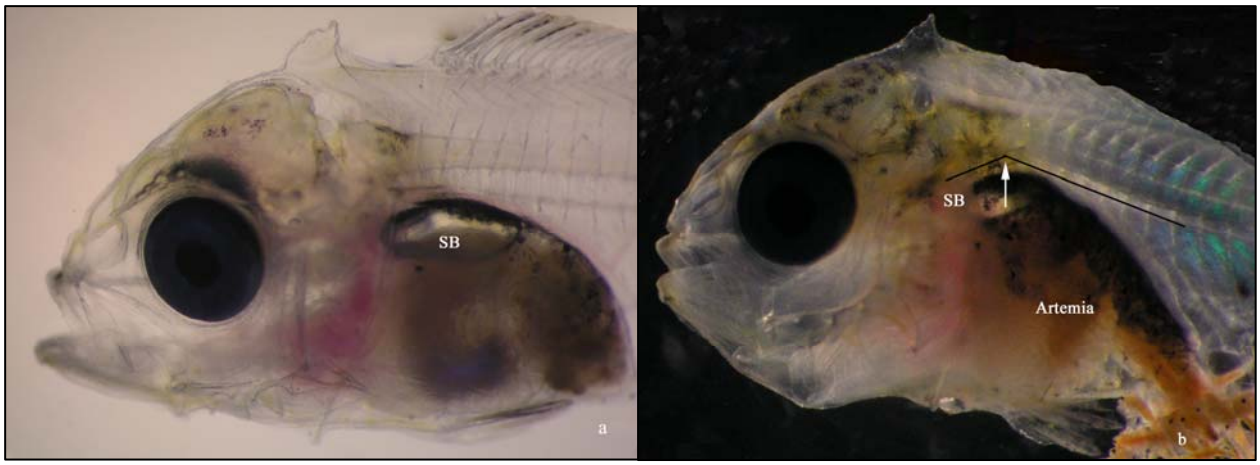

Figure 9. *Pagrus pagrus* larvae at 20dah: (a) fish with normal skeleton development, (SB: Swim bladder); (b) fish showing initial kyphosis (arrowhead) potentially caused by gut overfilling with *Artemia*.

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Table 1. Proximal composition (lipid, protein and ash content; mg/g dry weight) and fatty acid composition (% total fatty acids) of the live preys used, rotifers, *Artemia* (InstarII) and microdiet fed to the larvae. Data are given as mean±SD.

Live preys	Rotifers	*Artemia*	Microdiet
% Lipids (dw)	22.35±2.30 [a]	25.45±2.15 [b]	17.09±0.83 [c]
% Protein (dw)	52.27±4.34 [a]	55.38±2.25 [ab]	57.66±1.09 [b]
% Ash (dw)	1.20±0.27 [a]	0.65±0.06 [b]	17.09±0.83 [c]
∑ saturated [(1)]	23.93±0.63	24.14±3.60	27.19±1.58
∑ mono-unsaturated [(2)]	39.26±1.36 [a]	32.89±1.73 [b]	27.59±1.08 [c]
∑ n-3 [(3)]	21.01±0.52 [a]	31.40±3.14 [b]	20.68±2.89 [a]
∑ n-6[(4)]	10.95±1.23 [a]	9.06±0.48 [a]	22.83±3.57 [b]
∑n-9[(5)]	22.78±0.68 [a]	21.41±0.59 [ab]	20.01±1.06 [b]
∑n-3HUFA[(6)]	18.55±0.56	18.36±2.85	15.78±2.92
16:00	14.60±0.96 [a]	16.19±2.66 [ab]	17.48±1.30 [b]
16:1 n-7	11.51±1.20 [a]	3.47±0.31 [b]	3.72±0.79 [b]
18:00	5.46±0.56	6.47±1.65	4.76±0.71
18:1 n-9	19.12±0.55 [a]	19.06±0.82 [a]	12.23±0.56 [b]
18:1 n-7	3.09±0.17 [a]	5.84±0.25 [b]	2.79±0.85 [a]
18:2 n-6	7.77±1.30 [a]	5.47±0.37 [a]	21.64±3.95 [b]
18:3 n-3	1.35±0.39 [a]	11.31±1.47 [b]	3.51±0.68 [c]
20:1 n-9	2.19±0.10 [a]	1.50±0.09 [a]	3.56±0.67 [b]
ARA (20:4n-6)	1.83±0.23 [a]	1.62±0.16 [a]	0.55±0.15 [b]
EPA (20:5n-3)	6.63±0.34	7.56±0.82	5.98±1.50
DHA (22:6n-3)	10.11±0.32	9.11±1.82	8.61±1.16
EPA/ARA	3.68±0.59 [a]	4.67±0.05 [a]	11.04±1.21 [b]
DHA/EPA	1.53±0.05 [a]	1.20±0.12 [b]	1.48±0.23 [ab]
DHA/ARA	5.61±0.85 [a]	5.59±0.60 [a]	16.29±3.02 [b]
oleic/DHA	1.89±0.07 [ab]	2.15±0.37 [b]	1.44±0.24 [a]
oleic/n-3HUFA	1.03±0.04	1.05±0.13	0.80±0.18
n-3/n-6	1.94±0.21 [a]	3.46±0.26 [b]	0.94±0.27 [c]

dw: dry weight. Values (mean ± S.D) followed by different superscript letters within a row for the same trial were significantly different ($P < 0.05$).

(1) Includes 12:0, 14:0, 15:0, 16:0, 17:0, 18:0, 20:0, 22:0 and 24:0; (2) Includes 14:1n-5, 14:1n-7, 16:1n-9, 16:1n-7, 16:1n-5, 18:1n-9, 18:1n-7, 18:1n-5, 20:1n-9, 20:1n-7, 20:1n-5, 22:1n-11, 22:1n-9 and 22:1n-7; (3) Includes 16:2n-3, 16:3n-3, 16:4n-3, 18:3n-3, 18:4n-3, 20:3n-3, 20:4n-3, 20:5n-3, 22:4n-3, 22:5n-3 and 22:6n-3; (4) Includes 16:2n-6, 18:2n-6, 18:3n-6,18:4n-6, 20:2n-6, 20:3n-6, 20:4n-6, 20:5n-6, 22:3n-6, 22:4n-6 and 22:5n-6; (5) Includes 16:1n-9, 18:1n-9, 18:2n-9, 18:3n-9, 20:1n-9,20:2n-9, 20:3n-9 and 22:1n-9; (6) Includes 20:3n-3, 20:4n-3, 20:5n-3, 22:4n-3, 22:5n-3 and 22:6n-3.

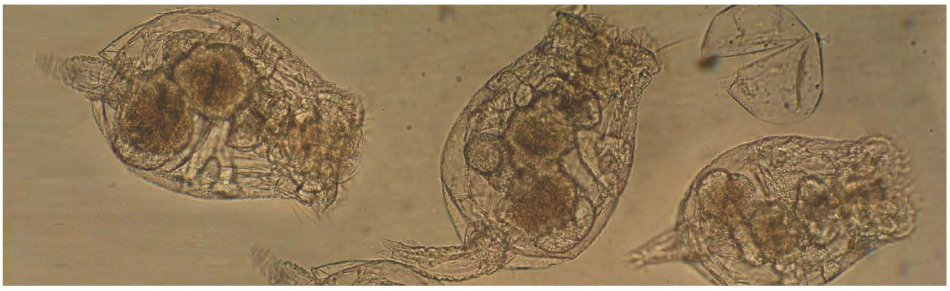
Table 2: Specific growth rate from 5 to 50 days after hatching.

	Specific growth rate in length (%)			
	Age (days)			
	5-15	**15-30**	**30-50**	**Average**
Semi-Intensive	7.10±0.14%	3.03±0.4%	4.60±0.35%	4.74±0.10%
Intensive	5.77±0.31%	2.66±0.28%	4.37±0.12%	4.16±0.17%

Improvements in the production technology of red porgy (*Pagrus pagrus*) larvae and fry: Importance of rearing conditions and diet nutritional value on their quality

Study III.-Effect of DHA content in rotifers on the occurrence of skeletal deformities in red porgy *Pagrus pagrus* (Linnaeus, 1758)

Aquaculture (2008), doi:10.1016/ j.aquaculture.2008.10.010



Effect of DHA content in rotifers on the occurrence of skeletal deformities in red porgy *Pagrus pagrus (*Linnaeus, *1758)*

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Abstract

Despite being proposed as a potential candidate for diversification of marine aquaculture, limited larval survival and the occurrence of elevated levels of skeletal deformities restrict the commercial production of red porgy. The present study was conducted to determine the effect of rotifer enrichment, particularly on DHA, on growth, survival and occurrence of skeleton deformities in this species. The study included two trials, in one the viability of commercial emulsions for rotifer enrichment was evaluated whereas in another the effect of the elevation of DHA in rotifer emulsions on the larvae was studied. No significant differences were found in growth between larvae fed different rotifers in both trials suggesting that 1.9% dw DHA is enough to fulfil the DHA requirements of red porgy larvae for maximum growth. However, a marked positive effect of rotifer DHA content supplementation on larval survival was found. A low larval survival was found when rotifers had a high DPA content, in agreement with the results obtained for other Sparids. This fatty acid was accumulated in red porgy larval tissues in high levels (0.79 % dw DPA) when rotifers rich in DPA were the main source of food. Although the external appearance of the juveniles seemed to be normal, X-ray observations showed elevated levels of bone abnormalities associated, in both trials, to low DHA content in the live prey. Among different anomalies, the presence of fused vertebrae was the most frequent deformity for both rearing trials. Although DHA content in the rotifers used for feeding was the only difference a 50% reduction in the number of deformed fish for each type of deformity studied was obtained when the larvae fed higher DHA levels, denoting the important role of this FA in the prevention of deformities at the rotifer feeding stage. Further studies are needed to elucidate the importance of essential fatty acids on the development of bone deformities in fish, since the functions of HUFA are different, and their absolute levels and ratios among them can lead to very different effects in fish metabolism, including bone formation.

Keywords: Red porgy, *Pagrus pagrus*, Larviculture, Enrichment, Essential fatty acids, Skeletal deformities.

Abbreviations: FA: Fatty acid; TFA: Total fatty Acids; EFA: Essential fatty acids; HUFA: Highly unsaturated fatty acids; DHA: Docosahexaenoic Acid; EPA: Docosapentaenoic Acid; ARA: Araquidonic Acid, DPA: Docosapentaenoic acid; VA: Vitamin A; PGE_2: Prostaglandin E_2 ; PGE_3: Prostaglandin E_3; LT_4: Leukotrienes T_4; LTC_4 : Leukotrienes C_4 ; LTD_4: leukotrienes D_4 ; IGF: Insulin-like Growth Factor; BMP: Bone Morphogenetic Proteins; BMP_4:Bone morphogenetic protein 4 ; BGP: Bone Gla protein; Oc: Osteocalcin; MGP; matrix Gla protein; RXR: Retinoid X receptor; RARγ:Retinoic Acid Receptor; IL-1: Interleukins 1; IL-6: Interleukins 6 and TNF: Tumour necrosis factors; PPAR: Peroxisome proliferators activated receptors; dah: days after hatching; SGR: specific growth rate.

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1.Introduction

Red porgy has been proposed as a potential candidate for diversification of marine aquaculture (Kentouri *et al.,* 1995; Hernández-Cruz *et al.,* 1999) and some on-growing experiences have already been conducted in different Mediterranean and Atlantic areas (Author´s unpublished data). Previous studies focused on the ontogeny of different organs and tissues (Roo *et al.,* 1999; Socorro *et al.,* 2001; Darias *et al.,* 2005) and improvements on larval rearing techniques (Kentouri *et al.,* 1995; Hernández-Cruz *et al.,* 1999; Mihelakakis *et al.,* 2001; Papandroulakis *et al.,* 2004). However, limited larval survival together with high occurrence of skeletal deformities (over 50% of the population) under intensive or semi-intensive systems, constitute major bottlenecks for the production of this species at commercial scale (Author´s unpublished data). There are several studies on the osteological development and skeleton anomalies of different sparids such as *Sparus aurata* (Koumondouros *et al.,* 1997a,b; Boglione, 2001; Faustino, 2002), *Pagrus major* (Moteki, 2001; Kihara *et al.,* 2002; Matsuoka, 2003), *Dentex dentex* (Koumoundouros *et al.,* 1999, 2001a), *Pagellus erythinus* (Boglione *et al.,* 2003; Sfakianakis *et al.,* 2004), *Diplodus puntazzo* (Boglione *et al.,* 2003; Favaloro and Mazzola, 2003) and *Diploduss sargus* (Koumoundouros *et al.,* 2001b; Sfakianakis *et al.,* 2003). Detailed information about osteological development has also been reported for red porgy by Socorro (2006), although the causes for the high incidence of deformities remain unclear. Different authors have studied the relationship between skeletal deformities and environmental factors (light, temperature, salinity), mechanical shock during embryo or larval development, tank currents and the type of rearing system (Battaglene and Talbot, 1990; Polo *et al.,*1991; Chatain, 1994; Divanach *et al.,* 1997; Koumoundouros *et al.,* 1997a,b; 2001a; Mihelakakis and Yoshimatsu, 1998; Cobcroft *et al.,* 2001; Boglione *et al.,* 2001; Kihara *et al.,* 2002; Sfakianakis *et al.,* 2004; Giménez and Estévez, 2005 and Roo *et al.,* 2005). Besides, a genetic component has been also found to contribute to the apparition of some types of acute deformities (Afonso *et al.,* 2000). Several biologically active compounds such as hormones, prostaglandins, cytokines, growth factors and certain nutrients can contribute as biological regulators to control the metabolism of specific bones and cartilages (Watkins and Seifert, 1997, 2000a,b).

Thus, nutritional imbalances on early life stages, such as deficiencies in vitamins, aminoacids or essential fatty acids in feeding regimes of broodstock or larvae may also alter the osteological development of reared larvae (Kanazawa *et al.,* 1983; Akiyama *et al.,* 1986, Knox *et al.,* 1988; Chatain and Ounais–Guschemann, 1990; Cahu *et al.,* 2003, Saele *et al.,* 2003; Hamre *et al.,* 2005).

Specifically, highly unsaturated fatty acids (HUFA) act as essential components of cellular membranes that modulate physiological processes, including membrane transport, nuclear receptors activation and enzymatic activities. Indeed, not only the quantities of the different HUFA, but also the ratios between the n-3/n-6 fatty acids are considered important for normal growth and development of fish larvae (Izquierdo, 1996, Rainuzzo *et al.,* 1997; Sargent *et al.,* 1999; Izquierdo *et al.,* 2000). Docosahexaenoic acid (DHA) plays an important role maintaining structural and functional integrity in fish cell membranes, and it is very important for normal neural development and function (Bell *et al.,*1995b). Arachidonic (ARA) and eicosapentaenoic (EPA) acids are involved in the production and modulation of eicosanoids (Bell *et al.,* 1994,1995a; Sargent *et al.,* 1997). The fatty acid composition of wild marine zooplankton, the natural prey of marine fish larvae, is markedly different from that of rotifers and *Artemia* nauplii used in marine hatcheries, even after enrichment (McEvoy *et al.,* 1998). Little is known about the role of these essential fatty acids on other tissues such as bone and cartilage in fish, but studies in chickens and rats show that the dietary Highly unsaturated fatty acids (HUFA) alter the fatty acid composition of bone and cartilage (Xu *et al.,* 1994; Watkins *et al.,* 1991, 2000b; Liu *et al.,* 2004). The objective of this study was to determine the effect of rotifer enrichment, particularly on DHA, on growth, survival and occurrence of skeleton deformities in red porgy. The study included a first trial testing the viability of commercial emulsions to enrich rotifers with different levels of n-3 HUFA, and a second one testing the specific effect of the elevation of DHA in rotifer enrichment emulsions.

2. Materials and methods

2.1. Rotifer enrichment

Rotifers were stocked at 400rotifers mL^{-1} in 500 L enrichment troncoconical-tanks, containing well-aerated (>5.0 mg $L^{-1}O_2$), filtered and UV-treated seawater (37‰). Emulsions were supplied in two equal rations over the 6h enrichment period. In trial A, two commercial emulsions for rotifer enrichment were used, DHA Protein Selco® (INVE, Belgium, DPS-Rot) and Red Pepper Paste® (Bernaqua bvba, Turnhout, Belgium , RPP-Rot). For trial B, DHA Protein Selco® (INVE, Belgium) was tested in comparison to DHA Protein Selco® mix with purified fish oil capsules MorDHA omega-3 I.Q® (First Vitality Int. Ltd.;United Kingdom)0.125mg L^{-1} of DHA Protein Selco ® supplemented with 3 capsules of MorDHA Omega-3 I.Q) (DHA-Rot). In both trials, rotifer enrichment emulsions were used according to provider instructions (Table 1).

2.2. Larval rearing

Eggs from red porgy broodstock were obtained by natural spawning at the Instituto Canario de Ciencias Marinas (Canary Islands, Spain). In both trials, larval rearing was performed in an intensive system (IS) using triplicate tanks of $2m^3$ capacity per treatment, at a stocking density of 100 eggs L^{-1}. Sea water was previously filtered by a sand filter and pass through a UV sterilizer. Water salinity and temperature along the trials were 37±0.5‰ and 20±0.5ºC and the oxygen level was 6.6±0.6 ppm. Green water technique was used adding live phytoplankton (*Nannochloropsis sp.*) to maintain a concentration of $250±100x10^3$ cells mL^{-1} in the rearing tanks. Larval culture was conducted under continuous photoperiod, by means of natural and artificial light. The light intensity just above the water surface ranged between 1000 and 3500 lx, which is in the optimum range determined by Tandler and Mason (1983) for a close species. Feeding protocol include the use of 5-10 rot mL^{-1} enriched with the different emulsions from day 3 after hatching (dah) until larvae reached 8.0 mm total length . At 13dah, *Artemia* Instar I (0.25 A_0 mL^{-1}) was added once a day in all the tanks. From 17 dah *Artemia* Instar II enriched with A_1 Selco® (INVE, Belgium), were added three times a day (0.50 A_1 L^{-1}) (Table II). Larvae were fed commercial diets from 20 dah (Genma Micra, Skretting, France), manually supplied for the first 3 days and by means of automatic feeders afterwards. After 50dah the whole population was transferred to $10m^3$ tanks, and both populations were kept under the same rearing conditions (10-15 ind L^{-1}) in a flow through water system until 95dah when all the fish were individually counted and skeletal characterisation was performed.

2.3. Growth and survival

Larval growth was assessed measuring the total length of 25 larvae every 5-7 days, using a profile projector (Nikon V-12A, NIKON, Tokyo, Japan). Length-specific growth rate (SGR) was calculated using the following equation: $SGR=([Ln(Lt)-Ln(L0)] /t) x 100$; where L_t is the larval length at the end of time period t, L_0 is the length at the beginning of time period t, and t is the length of the time period in days.

Survival was determined after 95 dah by individual counting using a fry counter (TPS Fish counter, Type Micro; Impex Agency; Denmark). From 25 dah dead fish were daily recorded and, at the end of the trial, survival was estimated taking into account daily mortality and final alive fish.

2.4. Biochemical analysis

For biochemical analysis four sampling point were established. One thousand larvae were collected at 12 and 20 dah and two hundred larvae at 35 and 50 dah from each tank (triplicate per treatment). Besides, samples of enrichment products, rotifers, *Artemia* and microdiets were

collected six times throughout the feeding trials. All the biochemical analysis was conducted in triplicate. Moisture, crude protein and ash were determined according to AOAC (1995). Total lipids were extracted as described by Folch *et al*. (1957). The fatty acid methyl esters were obtained by transesterification with H_2SO_4 (Christie, 1982) and purified by adsorption chromatography on NH_2 Sep-Pack cartridges (Waters, S.A., Milford, MA, USA) as described by Fox (1990), and separated and quantified by Gas-Liquid chromatography as described by Izquierdo *et al.* (1989).

2.5. Characterisation of the different types of deformity.

The characterization of the different deformities (lordosis, kyphosis, scoliosis, fused vertebrae, cranial malformations and other defects including asymmetric fins or deformed rays) was performed according with Divanach *et al.,* 1996 and Boglione *at al* 2001 deformities classification, in 300 specimens (mean weight 5.5 g) collected from each treatment at 95 dah. Fish were observed in fresh to detect cranial anomalies and acute deformities which modified external fish shape (Picture 1), and then monitored by soft X-ray (Mod. Senographer-DHR, General electrics, USA) to check internal deformities. The incidence of skeletal abnormalities was performed at 95dah since until this developmental stage is not possible to obtain an accurate identification under soft X-ray plates.

2.6. Statistical analysis

All the data was statistically treated using a T-test for simple mean comparison analysis ($P<0.05$) (Sokal and Rolf, 1995) using a SPSS Statistical Software System ver 14.0 (SPSS Chicago, Illinois, 1999). To evaluate the differences in the frequency of deformities between groups a log lineal statistical analysis was performed ($P <0.05$)(Sokal and Rolf, 1995).

3. Results

Trial A

3.1Proximate and fatty acid composition of rotifers

No significant differences were found in proximate composition between DPS-Rot and RPP-Rot (Table 3). The fatty acid composition of the rotifers reflected the composition of the enrichment used. Rotifers enriched with RPP emulsion (RPP-Rot) had a significantly ($P<0.05$) lower content in 18:0, 18:1n-9, 18:2n-6, EPA and ∑n-9 whereas DHA, docosapentaenoic acid (DPA, 22:5n-6), ∑n-3 and ∑n-3 HUFA content were higher than in the case of rotifers enriched with DPS emulsion (DPS-Rot) (Table 3).

3.2 Proximate and fatty acid composition of larvae

Total lipid content in red porgy larvae after 12 days of feeding enriched rotifers was significantly lower in RPP larvae (Table 4). This difference was not detected in the larvae after *Artemia* and dry diet feeding (20 and 35 dah). Hence, at the end of the experiment, lipid content of RPP fish was significantly higher, in parallel with a bigger size of the fish. At 12 dah larval FA composition closely reflected that of the rotifers fed (Tables III and IV). Thus, the levels of 16:1n-7, 18:1n-9, 18:2n-6, 18:3n-3 and EPA, ∑ n-9 were significantly ($P<0.05$) lower in RPP-Rot fed larvae, whereas those of ARA, DHA, DPA, ∑ n-3 HUFA, ∑ n-3 and ∑ n-6 were significantly ($P<0.05$) higher than in DPS larvae (Table 4). At 20dah, the effect of rotifer feeding was moderate, with significantly lower levels of 18:1n-9 and 18:2n-6, and significantly higher levels DHA and DPA in RPP larvae. No significant differences were observed for the rest of FA (Table 4). At 35 and 50 dah no significant differences were found in larval fatty acid composition.

3.3 Larval growth and survival.

Larvae fed RPP-Rot showed a marked mortality from 12-20 dah resulting in a significantly lower ($P<0.05$) survival rate from 25 dah. No further peaks in mortality were found in these larvae. Survival rate in the larvae fed DPS rotifers was significantly higher than RPP from25 dah (Figure 1). This difference was kept along the whole experiment, despite the mortality observed in these fish from 25–50 dah, mostly due to cannibalism. This cannibalistic behaviour was not observed in RPP fish due to the lower larval density derived from the high mortality rate indicated above.

During the first month of life larval growth in terms of total length was not affected by feeding enriched rotifers (Figure 2; Table 7). However, at 50 dah, in agreement with the lower survival found in RPP fish, these fish showed a significantly higher total length. Feeding the different enriched rotifers did not significantly affected SGR. In both treatments, the evolution of the SGR showed three periods: high values denoting rapid growth were found between 5-15 dah; a second period of slow growth rate was registered between 15-30 dah and a third period of high growth was again observed from 30-50 dah (Figure 3, Table 7).

3.4 Skeletal abnormalities.

Log-lineal statistical analysis denoted an interaction between rotifer enrichment and the total frequency of skeleton anomalies ($\chi^2 = 325.98$, $P=0.0$). There was a higher number of normal fish in RPP-Rot fed larvae (83.7%; $Z=-2.55$) than in DPS-Rot fed larvae (53.5%). The most prevalent skeleton anomaly was the presence of fused vertebrae (15.8% and 16.3%; Figure 4) in both fish treatments. No other types of deformities were found in RPP-Rot larvae, whereas a wider diversity of deformities was observed in DPS-Rot fed fish. The presence of different osteological

anomalies in the same fish, were commonly observed in DPS fish and were not recorded in RPP fish (Figure 5).

Trial B

3.5Proximate and fatty acid composition of rotifers

In trial B, the inclusion of purified fish oil capsules in the experimental emulsion did not modify the biochemical composition of rotifers (crude lipid, crude protein and ash content). However, the addition of fish oil capsules significantly increased ($P<0.05$) DHA content in the rotifers and consequently different DHA/EPA and DHA/ARA ratios were recorded. Besides, the level of ∑ n-3 and ∑ n-3 HUFA of the DHA-Rot were significantly ($P<0.05$) higher than in DPS-Rot (Table V).

3.6. Proximate and fatty acid composition of larvae

Total lipid content in 12 dah red porgy larvae (around 20% dry weight) was not affected by the type of rotifers fed. A significant reduction in larval lipid content was registered in all the treatments by day 20 and was kept about 15-17% until 50 dah, regardless of the type of rotifer enrichment used (Table 6).

As in trial A, the fatty acid composition of 12 and 20 dah larvae was significantly affected by the rotifer enrichment, whereas this effect was not observed at 35 and 50 dah larvae (Table 6). At 12 dah, the level of 18:2n-6 and 18:3n-3 was significantly lower in DHA-Rot fed larvae while DHA content was significantly ($P<0.05$) higher in this larvae (Table 6). Feeding DHA-Rot significantly increased ($P<0.05$), ∑ n-3 HUFA and ∑ n-3 content and reduced ($P<0.05$) ∑ n-6 content in red porgy larvae. At 20 dah, when larvae were already co-fed rotifers and *Artemia*, DHA-Rot fed larvae showed a significantly ($P<0.05$) higher content of 18:1n-9, 18:1n-7 and 18:2n-6, while no significant ($P<0.05$) differences were detected in the rest of the fatty acids of the larvae (Table 6).

3.7. Larval survival and growth.

In trial B, the increase in DHA content of the rotifers produced a better survival rate the first 25 days of larval rearing (average 18%, Figure 6). However, a drop in mortality starting from day 15 and lasting until day 50 was observed in DHA-Rot fed larvae and as a consequence no significant differences could be found between both treatments at the end of the experiment.

No significant differences were observed in larval growth for the whole rearing period (Figure 7; Table 7).

As in the case of trial A, SGR of the larvae from both treatments showed a three period pattern during larval development: Rapid growth stage between 5 and 15dah, a lower growth stage at 15-30 dah followed by a recovery period from 30-50 dah (Figure 8, Table 7).

3.8 Skeletal abnormalities

In trial B, higher DHA levels in the rotifers significantly improved fry quality (χ^2 = 895,00; P<0,05). In larvae fed DHA-Rot, 79.9% of the population was considered normal (non deformed fish) whereas DPS-Rot fed larvae showed similar results than in trial A (49%; Z=-3.63). Fused vertebrae was the most prevalent deformity among the different types recorded, followed by cranial anomalies, kyphosis, vertebral shortening and lordosis. The comparison between treatments showed a reduction of these anomalies with the use of high doses of DHA in the enrichment (Figure 9). Regarding the apparition of different anomalies in the same fish, fused vertebrae was observed as a sole deformity in 80% of the fish, whereas in 12-13% of the fish this anomaly appeared in combination with cranial deformities and some fish (7-8%) showed it also combined with lordosis. However, no significant differences were observed between treatments (Figure 10).

4. Discussion

The results of larval growth obtained in the present study during rotifer feeding phase until 25dah were similar to those reported by Hernandez Cruz *et al.* (1999) and by Papandroulakis *et al.* (2004). Hernandez Cruz *et al.* (1999) fed rotifers with three levels of EPA and DHA obtaining the best growth using rotifers containing 2.73 % DHA in dry weight (dw). In agreement with these authors, no significant differences in growth could be found in the present study between the larvae fed differently DHA enriched rotifers (1.8 and 4.7% dw in DPS and in RRP, respectively, in trial A and 2.0 and 4.5% dw in DPS and DHA-Rot in trial B), suggesting that even the lowest DHA content (DPS-Rot) can fulfil the requirement of red porgy larvae for maximal growth.

In other species such as red sea bream (*Pagrus major*) (Izquierdo *et al.,* 1989), common dentex (*Dentex dentex)* (Mourente *et al.,* 1999), gilthead seabream (*Sparus aurata*) (Izquierdo *et al.,* 2005), or striped trumpeter (*Latris lineata*) (Bransden *et al.,* 2005) the minimum DHA requirement for optimum growth was found to be 1.2, 2.3, 0.8 and 2.0% dw DHA, respectively. However, a marked positive effect of DHA on survival was found in trial B of the present study during rotifer feeding (until 25 dah). Thus, larvae fed DHA-Rot (4.5% dw DHA) achieved a significantly higher survival rate than larvae fed DPS-Rot (2.0% dw DHA). The relevance of DHA in larval nutrition has been widely described (Izquierdo, 1996; Sargent *et al.,* 1997) and its positive effect on survival in this trial could be related to its important role in stress control (Watanabe *et al.,* 1993; Izquierdo *et al.,* 2005, Ganga *et al.,* 2006) and immune system development (Montero *et al.,* 2003). Indeed, changes in culturing conditions during this period of larval development, such as changes of prey used as food, increase in flow rates, reduction of

green water, etc., impose a high risk of stress to fish larvae, enhancing the importance of adequate feeding. Among other physiological functions, this fatty acid is particularly important for neural and visual capabilities (Neuringer *et al.,* 1988; Sargent *et al.,* 1999) and dietary DHA imbalances elicited changes in behaviour in other species such as larval gilthead sea bream (Izquierdo *et al.,* 2000; Benítez-Santana *et al.,* 2007). Moreover, during this period red porgy larvae undergo important morphological changes such as the development of gastric glands or the addition of rods to the retina (Roo *et al.,* 1999) that demand high quantity of DHA to form new cell membranes. This improvement in survival rate obtained by the higher DHA content in the rotifers in trial B indicates that the lower survival rate of the larvae fed RPP-rot in trial A at the rotifer feeding stage was not related with its DHA content, but to other differences in the enrichment composition, such as vitamins or other fatty acids. For instance, a high level of DPA (22:5n-6) was found in larvae fed RPP-rot, being this fatty acid commonly produced by certain thraustochytrid marine protist such as *Schizochytrium sp*. (Barclay and Zeller, 1996; Parrish *et al.,* 2007). This FA is present at very low levels in animal tissues (around 1% TFA) being synthesized when DHA is not dietary provided (Nettleton, 1994). DPA was accumulated in red porgy larval tissues in high levels (5.73% TFA at 12 dah) when rotifers rich in DPA and also in DHA were the main source of food (Table 4). DPA was also accumulated in similar rates in the larvae of cobia (Faulk and Holt, 2005) and cod (Parrish *et al.,* 2007; García *et al.*, 2008a,b) when rotifers and *Artemia* rich in this FA were administered. DPA has been suggested as an EFA for haddock larvae (Blair *et al.,* 2003) and cod (Parris *et al.,* 2007) and as a precursor of ARA by retro-conversion (Barclay and Seller, 1996, Sargent *et al*, 1997, Koven *et al.,* 2001; Robin *et al.,* 2003).

In trial A, the high larval DPA content appeared in combination with a significantly higher level of ARA in RPP-Rot fed larvae in parallel to the high levels of both FA in RPP-Rot. Although no negative effect of DPA accumulation in cobia or cod larvae has been described, in red porgy the low survival rate of the larvae fed RPP-rot might be related to its high content of DPA, in agreement with the results obtained for other sparids (Roo *et al.,* 2005). An increase of DPA in nervous tissues has been reported to occur in different vertebrates fed low n-3 HUFA levels. These increase was claimed to impair brain function by the structural substitution of DHA by DPA altering the neural membranes properties and modifying the functions of photoreceptor proteins (i.e. rhodopsin) (Hrboticky *et al.,* 1991; Eldho *et al.,* 2003). Additionally, Nettleton (1994) suggested a reduction in the visual function of infant rhesus monkeys that accumulated DPA when fed low n-3 HUFA levels. Thus, being red porgy larvae visual feeders with a not completely developed visual system at this stage (Roo *et al.,* 1999), the reduced survival in

Pagrus pagrus larvae fed rotifers rich in DPA could be related to the high incorporation of DPA in larval neural and visual tissues, affecting (or altering) the physico-chemical properties and functions of bio-membranes as it has been described in other vertebrates.

Regarding to skeleton development, different common anomalies were observed in red porgy such as lordosis, kyphosis, scoliosis, compressed vertebrae and other defects (asymmetric fins, deformed rays, etc). Different causative factors (physiological, environmental, genetic, xenobiotic and nutritional factors) have been associated to the apparition of these anomalies during larval and juvenile stages of cultured freshwater and marine fish (Divanach *et al.*,, 1996; Lall and Lewis., 2007). Among the nutritional factors, lipids have been suggested to play an important role in prevention of bone deformities (Gapasin and Duray, 2001; Cahu and Zambonino-Infante, 2003) and the importance of FA, vitamin A (VA) or phospholipids (Takeuchi *et al.,* 1998; Suzuki *et al.,* 2000, Villeneuve *et al.,* 2005; 2006) provided through rotifer enrichment seem to be determinant in the correct development of specific bone structures such as cranial bones and vertebrae.

In the present study, a reduction in the incidence of skeleton deformities was found in larvae fed RPP-rot in trial A, which could be related to its higher DHA content, despite other differences in the enrichment composition. However, since the only difference between rotifers of trial B was its DHA content, the reduction in the number of deformed fish for each deformity studied (about 50%) when larvae were fed higher DHA levels demonstrates the important role of this FA in the prevention of deformities at the rotifer feeding stage. These findings are in agreement with the results of Gapasin and Duray (2001) who fed rotifers enriched with emulsions or phytoplankton as enrichments and found negative correlations between the DHA content in live prey and the incidence of opercular deformity in *Chanos chanos*. Most skeleton anomalies are originated at the time of chondrogenesis and osteogenesis at early larval stages (Faustino, 2002). In red porgy larvae, the osteological formation takes places mainly at rotifer and *Artemia* feeding stages (Socorro, 2006) and the results of the present study showed that dietary DHA at these stages is determinant in the correct osteological development in this species. In other vertebrates, dietary lipids affect fatty acids composition of bone and cartilage (Watkins *et al.,* 1991; Liu *et al.,* 2004), as well as eicosanoid production in bone cells (Kokkinos *et al*., 1993, Watkings *et al.,* 1997). Furthermore, bone formation and resorption have been found to be regulated by locally produced eicosanoids (Raisz *et al.,* 1993; Meghji *et al.,* 1988). DHA has been suggested to affect eicosanoid production (Nablone *et al.,* 1990) and it is recognized as a precursor of certain biologically active trioxilated derivatives (Hong *et al*., 2005).

Besides, in European seabass dietary HUFA levels have been cited to affect osteoblast differentiation process through regulation of Retinoid X receptor α and BMP4, its excess leading to higher number of vertebral bodies (Villeneuve *et al.,* 2005, 2006). Moreover, expression of osteogenic markers related to the occurrence of skeletal abnormalities, such as OC and MGPC (Karsenty and Wagner, 2002), seem to be affected by the presence of essential fatty acids (Pombinho *et al.,* 2004).

The present study points out the importance of DHA content in the rotifers not only for red porgy survival, but also to reduce bone deformities. Further studies are needed to elucidate the importance of essential fatty acids on the development of bone deformities in fish, since the functions of the various HUFAs are different and their absolute levels and ratios among them can lead to very different effects in fish metabolism, including bone formation.

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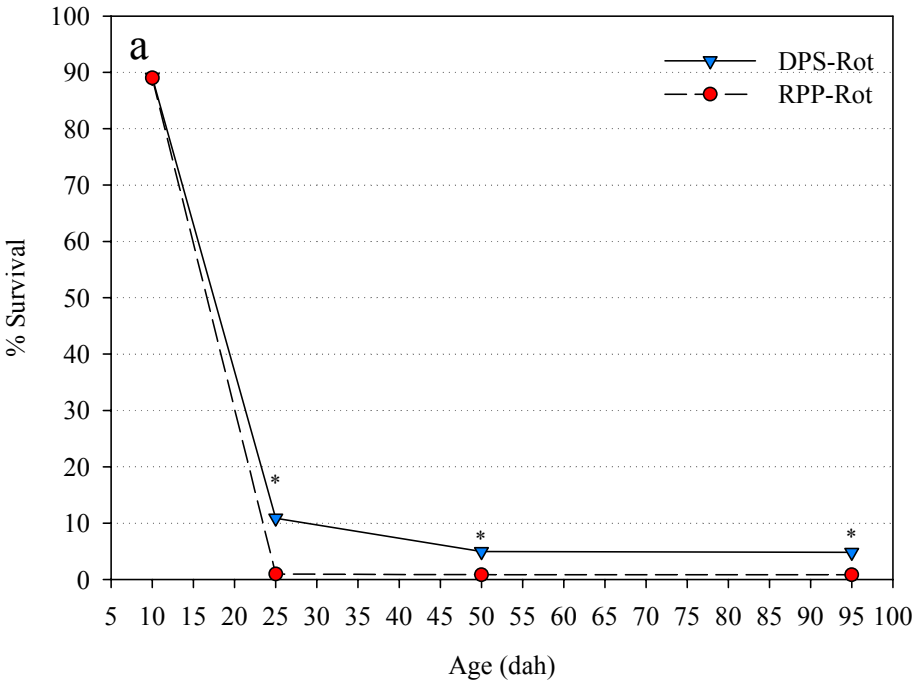

Figure 1. Survival evolution in red porgy (*Pagrus pagrus*) larvae fed different rotifers enrichments in Trial A. (*) shows significant differences.

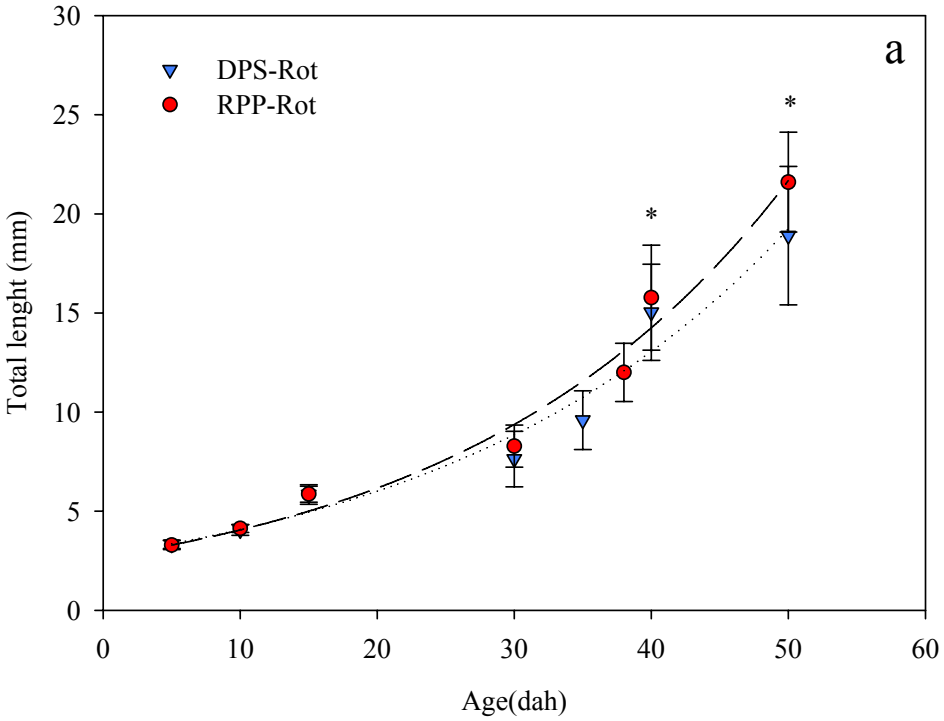

Figure 2. Evolution of growth in terms of total length (TL) in red porgy (*Pagrus pagrus*) larvae fed different enriched rotifers in Trial A. (*) shows significant differences.

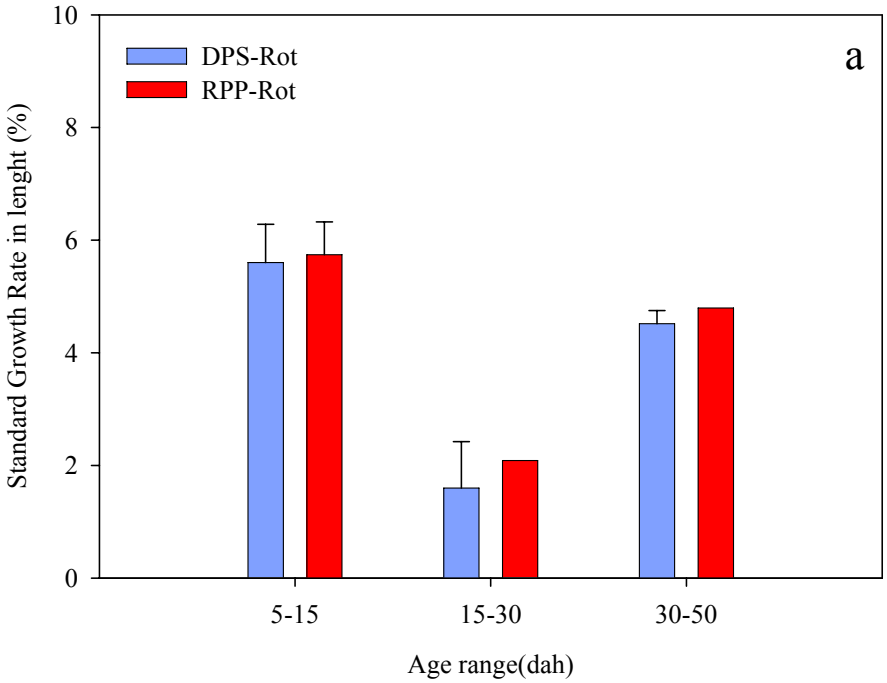

Figure 3. Standard growth rate in red porgy (*Pagrus pagrus*) larvae fed different rotifers enrichments in Trial A.

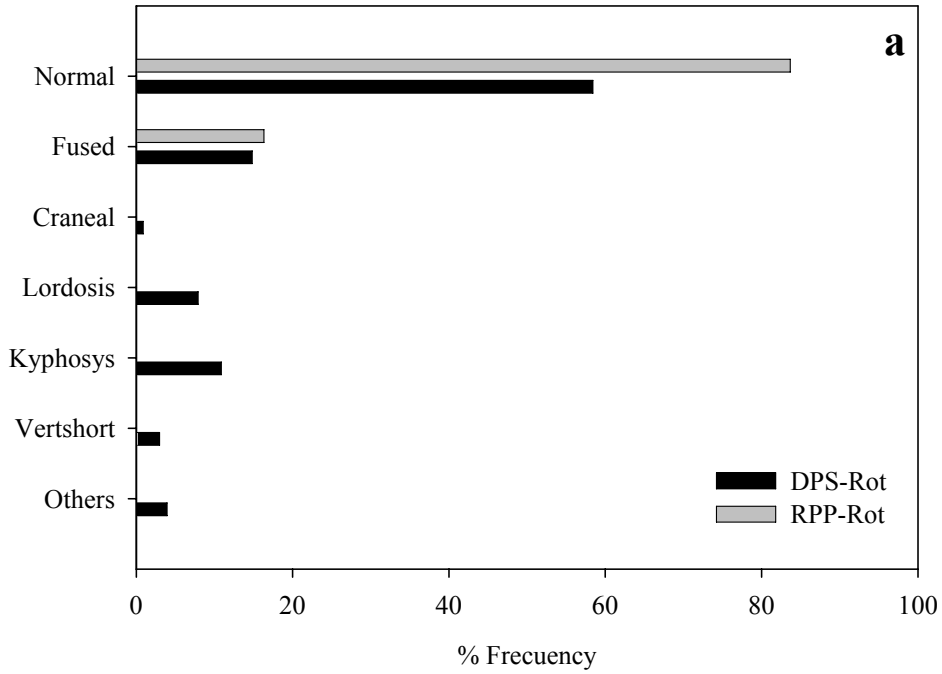

Figure 4. Incidence of skeleton abnormalities in red porgy *Pagrus pagrus* seedlings fed different commercial emulsions in trial A.

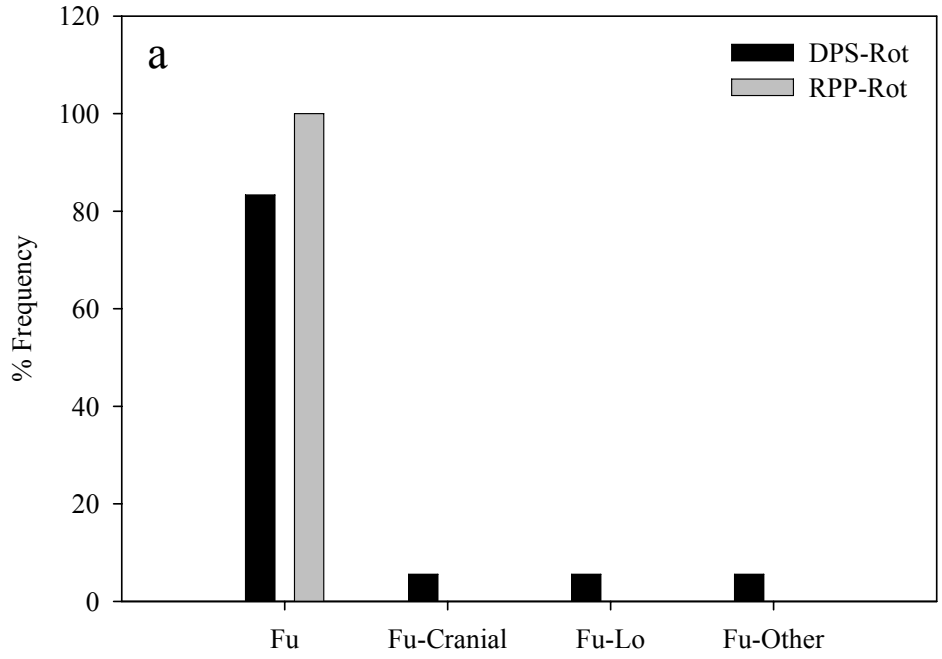

Figure 5. Presence of exclusive fused vertebras and combined with other skeleton anomalies for the same specimen of red porgy fry *Pagrus pagrus* at 95dah in trial A.

Fu: Presence of exclusive fused vertebrae in the same fish*; Fu-Cranial:* Presence of fused vertebrae and cranial deformity in the same fish. *Fu-Lo:* Presence of fused vertebrae and lordosis in the same fish. *Fu-Other:* Presence of fused vertebrae and other type of deformity in the same fish.

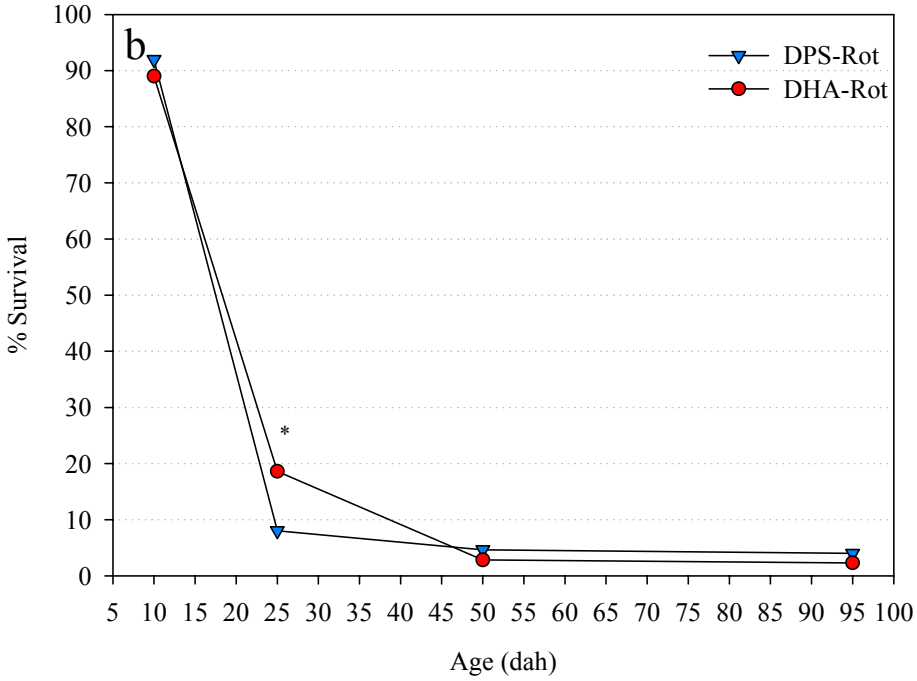

Figure 6. Survival evolution in red porgy (*Pagrus pagrus*) larvae fed different rotifers enrichments in Trial B. (*) shows significant differences.

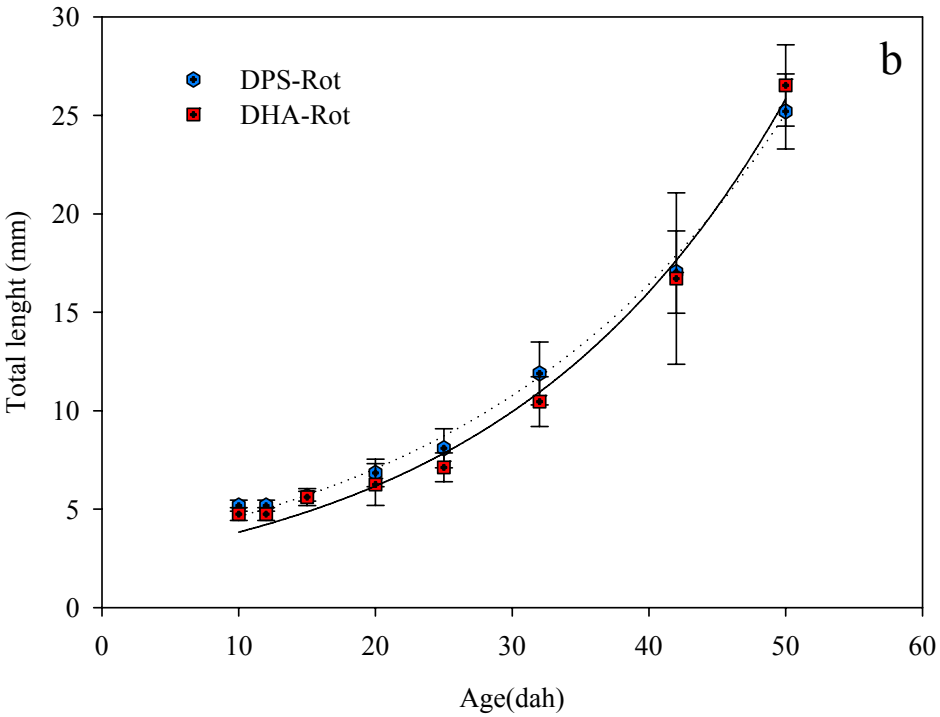

Figure 7. Evolution of growth in terms of total length (TL) in red porgy (*Pagrus pagrus*) larvae fed different enriched rotifers in Trial B.

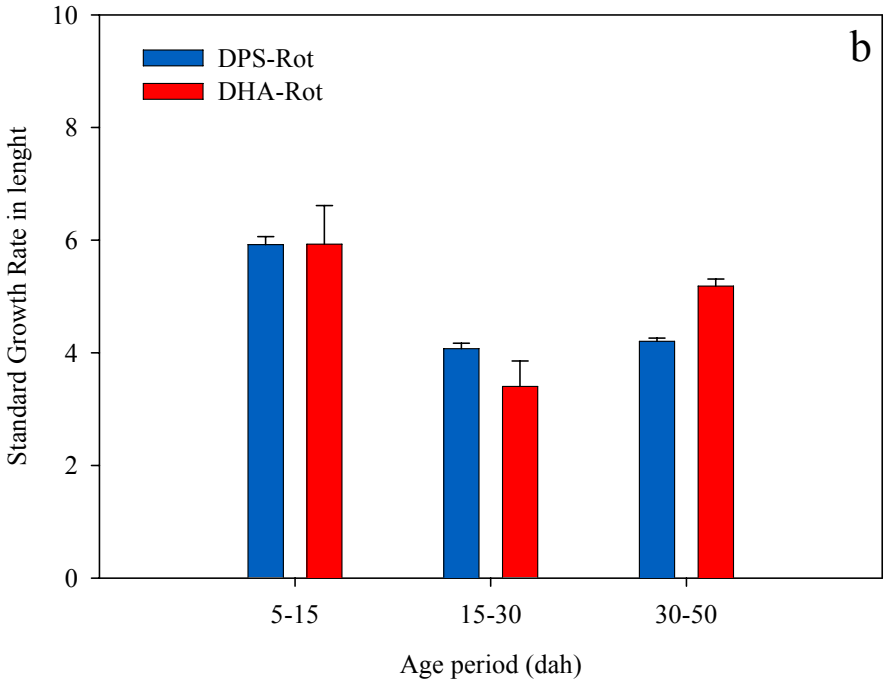

Figure 8. Standard growth rate in red porgy (*Pagrus pagrus*) larvae fed different rotifers enrichments in Trial B

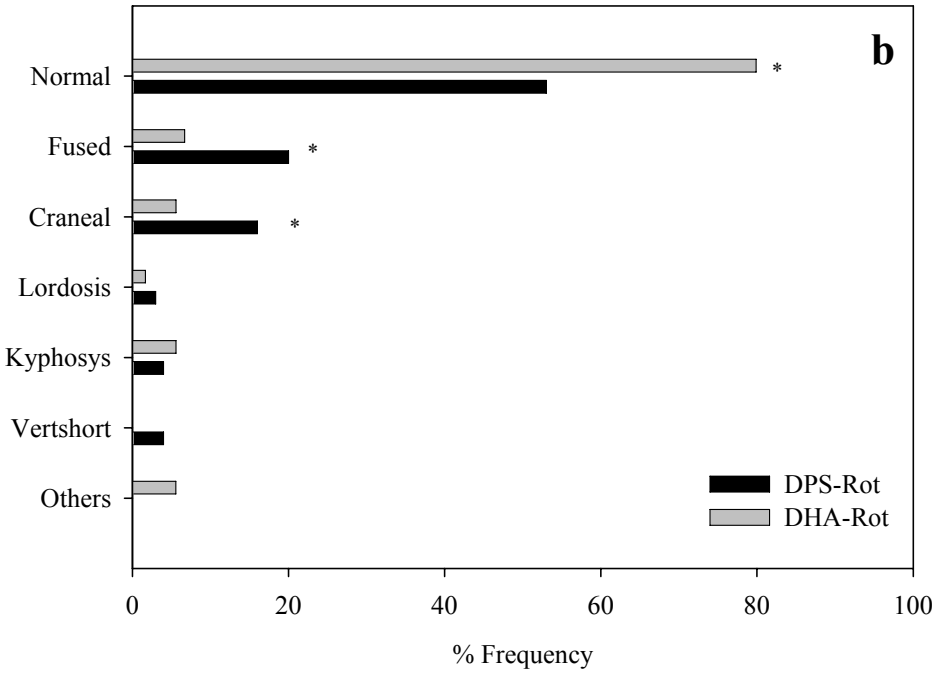

Figure 9. Incidence of skeleton abnormalities in red porgy *Pagrus pagrus* seedlings fed different commercial and experimental emulsions in trial B.

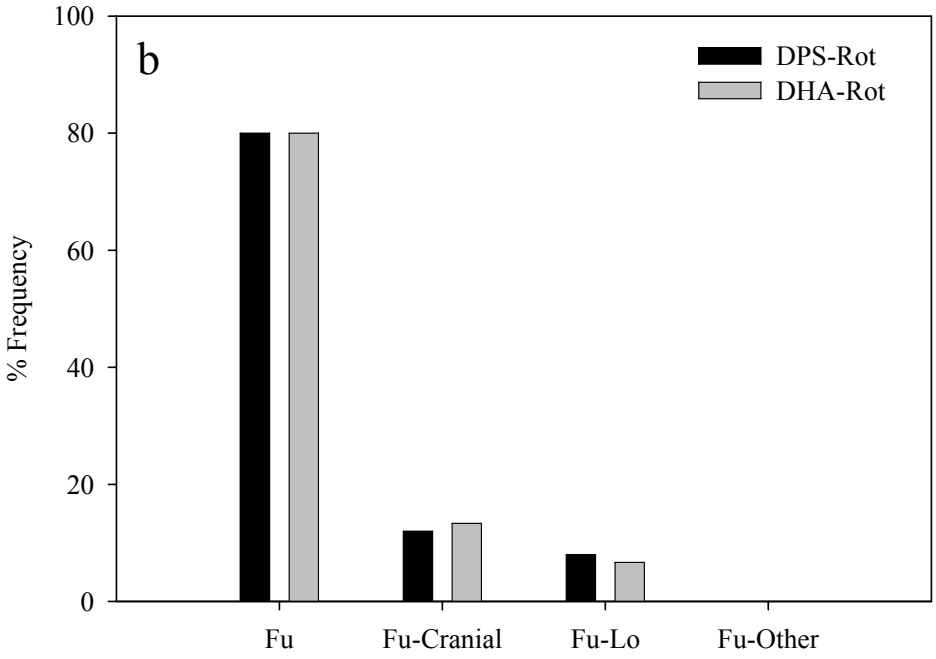

Figure 10. Presence of exclusive fused vertebras and combined with other skeleton anomalies for the same specimen of red porgy fry *Pagrus pagrus* at 95dah in trial B. *Fu:* Presence of exclusive fused vertebrae in the same fish*; Fu-Cranial:* Presence of fused vertebrae and cranial deformity in the same fish. *Fu-Lo:* Presence of fused vertebrae and lordosis in the same fish. *Fu-Other:* Presence of fused vertebrae and other type of deformity in the same fish.

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Table 1. Commercial products proximal composition (manufacturer's data).

Products	DHA Protein Selco	Red Pepper Paste	MorDHA omega-3 I.Q
% Moisture	5%	<70%	-
% Protein	29%	>3%	17%
% Lipids	29%	>10%	80%
% Ash	12%	<10%	20%
Fiber	1%	<2%	-
Phosphorous	1.7%	>1.5%	-
Vit A (IU/kg)	1.500.000	50.000	-
Vit D (IU/kg)	15.0000	10.000	-
Vit E	7.200 mg/kg	1.500 mg/kg	2.500mg/kg
Vit C	20.000 mg/kg	13.500mg/kg	-
Antioxidants	Ethoxyquin	-	Lemon oil (0.9%)
DHA /EPA	>2.5	27.5	6.9
∑ n-3 HUFA	> 7.5%	6%	67.0%

Table 2. Feeding sequences in Trials A, B.

Age(dah)	Degree day	Renewal	Phyto	Rotifers	*Artemia*		Microdiet		
							Perla 6.0*		
					Instar I	Instar II	GM300	GM03	GM05
2DAH	38	10%/day	+						
3DAH	47	10%/day	+	+					
13DAH	145	10%/day	+	+	+				
17DAH	185	25%/day	+	+	+	+			
20DAH	213	15%/hour	+	+		+	+		
30DAH	321	30%/hour				+	+	+	
50DAH	542	50%/hour						+	+

* Trial A

Table 3. Proximal composition (lipid, protein and ash content; mg/g dry weight) and fatty acid composition (% total fatty acids) of different food sources used for larval rearing in Trial A.

Live preys	**Trial A**			
	DPS-Rot	**RPP-Rot**	***Artemia***	***Perla 6.0***
% Lipids (dw)	22.05±3.84	27.31±4.05	26.04±0.41	13.89±0.07
% Protein (dw)	54.28±4.57	57.31±4.00	56.39±4.84	63.98±0.38
% Ash (dw)	1.48±0.50	1.49±0.56	0.75±0.02	1.0±0.02
16:0	13.00±2.48	14.72±1.32	15.22±3.80	19.07
16:1 n-7	13.27±4.21	16.39±3.45	7.93±1.64	3.81
18:0	4.73±1.21[a]	2.02±1.05[b]	4.42±0.37	4.07
18:1 n-9	20.1±1.72[a]	10.08±3.99[b]	20.36±7.38	12.57
18:1 n-7	3.20±0.42	0.66±1.32	5.51±1.17	2.22
18:2 n-6	8.14±1.31[a]	4.08±1.10[b]	3.78±2.61	21.62
18:3 n-3	1.62±0.11	1.92±0.31	10.81±4.23	2.97
20:1 n-9	2.27±0.25	1.85±0.24	0.72±0.41	2.78
ARA (20:4n-6)	1.46±0.73	2.88±0.85	1.49±0.37	0.77
EPA (20:5n-3)	6.51± 0.62[a]	3.99±0.61[b]	11.10±4.27	7.02
DPA (22:5n-6)	0.44±0.07[a]	6.43±0.50[b]	0.14±0.13	0.28
DHA (22:6n-3)	9.68±0.93[a]	20.52±2.80[b]	4.47±1.43	11.72
∑ saturated [(1)]	21.33± 4.41	19.00±2.07	23.95±6.28	26.94
∑ monoenes [(2)]	41.90± 6.53	33.90±2.36	37.16±12.37	23.04
∑ n-3 [(3)]	21.12 ± 0.48[a]	28.59±2.58[b]	31.14±11.43	23.77
∑ n-6[(4)]	10.77± 2.11	14.19±1.70	7.03±3.73	25.13
∑n-9[(5)]	24.84 ±3.16[a]	13.86±3.81[b]	21.26±8.00	15.35
∑n-3HUFA[(6)]	18.23±0.98[a]	25.76±2.69[b]	16.87±6.24	20.30
DHA/22:5 n-6	22.11±1.74[a]	3.23±0.65[b]	30.93±11.14	41.87
EPA/ARA	5.45±2.99	1.48±0.45	7.43±11.53	9.13
DHA/EPA	1.49± 0.01[a]	5.18±0.68[b]	0.40±0.34	1.67
DHA/ARA	8.10± 4.45	7.78±3.01	2.99±3.87	15.26
Oleic/DHA	2.10±0.38[a]	0.50±0.22[b]	4.56±5.15	1.07
Oleic/n-3HUFA	1.11±0.14[a]	0.40±0.17[b]	1.21±1.18	0.62
n-3/n-6	2.02±0.43	2.05±0.39	4.43±3.07	0.95

dw: dry weight. Values (mean ± S.D) followed by different superscript letters within a row for rotifers FA were significantly different (P <0.05).

(1) Includes 12:0, 14:0, 15:0, 16:0, 17:0, 18:0, 20:0, 22:0 and 24:0, (2) Includes 14:1n-5, 14:1n-7, 16:1n-9, 16:1n-7, 16:1n-5, 18:1n-9, 18:1n-7, 18:1n-5, 20:1n-9, 20:1n-7, 20:1n-5, 22:1n-11, 22:1n-9 and 22:1n-7, (3) Includes 16:2n-3, 16:3n-3, 16:4n-3, 18:3n-3, 18:4n-3, 20:3n-3, 20:4n-3, 20:5n-3, 22:4n-3, 22:5n-3 and 22:6n-3, (4) Includes 16:2n-6, 18:2n-6, 18:3n-6,18:4n-6, 20:2n-6, 20:3n-6, 20:4n-6, 20:5n-6, 22:3n-6, 22:4n-6 and 22:5n-6, (5) Includes 16:1n-9, 18:1n-9, 18:2n-9, 18:3n-9, 20:1n-9,20:2n-9, 20:3n-9 and 22:1n-9, (6) Includes 20:3n-3, 20:4n-3, 20:5n-3, 22:4n-3, 22:5n-3 and 22:6n-3.

Table 4. Proximal composition (lipid, protein and ash content; mg/g dry weight) and fatty acid composition (% total fatty acids) of whole body red porgy larvae fed rotifers enriched with different commercial emulsions, at 12, 20 and 35 and 50 dah in trial A (n=3, mean± SD,(P<0.05)).

Days after hatching	**12dah**		**20dah**		**35dah**		**50dah**	
Treatment	**DPS-Rot**	**RPP-Rot**	**DPS-Rot**	**RPP-Rot**	**DPS-Rot**	**RPP-Rot**	**DPS-Rot**	**RPP-Rot**
% Lipids (dw)	18.40±0.52 [a]	16.25±0.69 [b]	17.22±2.10	18.49±1.09	14.50±0.60	14.76±0.16	17.81±0.23 [a]	21.55±0.54 [b]
% Protein (dw)	78.77±3.00	75.13±1.08	76.73±5.06	76.82±2.79	81.23±5.29	81.36±0.25	68.80±2.57	66.95±2.31
% Ash (dw)	1.65±0.34	1.85±0.06	2.21±0.46	2.77±0.03	3.35±0.21	3.38±0.22	3.79±0.47	3.63±0.24
16:0	17.58±0.64	17.16±0.25	17.05±0.33	17.09±0.20	18.61±0.07	16.90±0.45	17.04±0.71	19.40±1.31
16:1 n-7	6.54±0.13 [a]	4.44±0.07 [b]	6.15±0.24	5.44±0.06	3.71±0.26	3.62±0.10	5.39±0.41	4.95±0.43
18:0	10.02±0.86	9.53±0.14	10.17±0.21	9.97±0.12	7.86±0.02	8.59±0.23	7.93±0.87	6.55±0.15
18:1 n-9	12.44±0.31 [a]	9.50±0.29 [b]	12.34±0.40 [a]	11.68±0.14 [b]	10.00±0.14	10.00±0.27	15.57±0.73	14.71±0.20
18:1 n-7	3.95±0.38	3.53±0.10	7.37±0.17	6.87±0.08	4.58±0.37	8.09±0.22	4.22±1.18	3.42±0.29
18:2 n-6	9.08±0.15 [a]	4.42±0.08 [b]	5.48±0.23 [a]	3.38±0.04 [b]	8.22±0.12	7.03±0.19	7.13±0.24	7.55±0.10
18:3 n-3	4.00±0.11 [a]	3.35±0.05 [b]	1.48±0.03	1.49±0.02	8.26±0.43	9.81±0.26	6.67±0.22	6.51±0.28
20:1 n-9	1.53±0.31	1.34±0.02	1.14±0.01	0.93±0.06	1.71±0.01	1.28±0.03	2.58±0.09	2.98±0.04
ARA (20:4n-6)	1.92±0.19 [a]	3.47±0.05 [b]	3.68±0.10	4.22±0.20	2.72±0.11	2.36±0.06	1.38±0.05	1.10±0.01
EPA (20:5n-3)	5.56±0.14 [a]	3.83±0.06 [b]	8.66±0.16	7.57±0.70	8.84±0.24	10.07±0.27	8.32±0.28	8.27±0.11
DPA (22:5n-6)	0.63±0.01 [a]	5.73±0.23 [b]	0.90±0.28 [a]	2.44±0.14 [b]	0.08±0.02	0.04±0.00	0.30±0.01	0.27±0.00
DHA (22:6n-3)	16.13±0.18 [a]	25.41±1.11 [b]	15.91±1.01 [a]	17.34±1.43 [b]	10.55±0.19	10.16±0.27	12.20±0.40	11.37±0.15
∑ saturated [(1)]	30.58±0.35	28.99±0.43	29.87±0.13	29.45±0.30	28.68±0.02	27.36±1.08	28.61±1.47	30.04±1.17
∑ mono-unsaturated [(2)]	26.14±1.01 [a]	19.32±0.29 [b]	27.68±0.84	27.88±0.31	23.05±0.04	25.40±0.37	28.07±0.57 [a]	26.24±0.95 [b]
∑ n-3 [(3)]	28.89±0.96 [a]	35.89±0.95 [b]	29.55±1.05	28.38±0.73	31.97±0.05	33.92±0.79	32.99±1.21	33.06±0.08
∑ n-6[(4)]	12.59±0.14 [a]	14.54±0.22 [b]	11.25±0.09 [a]	10.97±0.13 [b]	12.98±0.25	10.73±0.15	9.34±0.25	9.44±0.13
∑n-9[(5)]	14.95±1.17 [a]	11.15±0.31 [b]	13.74±0.39 [a]	13.07±0.19 [b]	12.64±0.09	12.27±0.48	18.27±0.64	17.87±0.24
∑n-3HUFA[(6)]	24.23±1.12 [a]	32.20±1.01 [b]	27.91±1.01	26.23±0.74	22.47±0.51	22.02±1.08	24.24±1.10	24.09±0.32
DHA/22:5 n-6	25.45±0.79 [a]	4.44±0.37 [b]	18.74±4.77 [a]	7.11±0.19 [b]	136.86±24.54	262.79±0.00	41.26±0.00	42.17±0.00
EPA/ARA	2.92±0.38 [a]	1.10±0.00 [b]	2.36±0.02	1.79±0.08	3.25±0.05	4.27±0.00	6.04±0.00	7.53±0.00
DHA/EPA	2.90±0.10 [a]	6.64±0.39 [b]	1.84±0.14	2.32±0.41	1.19±0.01	1.01±0.00	1.47±0.00	1.38±0.00
DHA/ARA	8.45±0.81 [a]	7.33±0.43 [b]	4.33±0.38	4.12±0.53	3.89±0.09	4.30±0.00	8.85±0.00	10.36±0.00
Oleic/DHA	0.77±0.01	0.37±0.03	0.78±0.07	0.68±0.06	0.95±0.03	0.98±0.00	1.28±0.09	1.29±0.00
Oleic/n-3HUFA	0.51±0.03	0.30±0.02	0.44±0.03	0.45±0.02	0.45±0.02	0.46±0.03	0.64±0.06	0.61±0.00
n-3/n-6	2.30±0.05 [a]	2.47±0.10 [b]	2.63±0.11 [a]	2.59±0.09 [b]	2.46±0.05	3.16±0.04	3.53±0.10	3.50±0.04

(1) Includes 12:0, 14:0, 15:0, 16:0, 17:0, 18:0, 20:0, 22:0 and 24:0, (2) Includes 14:1n-5, 14:1n-7, 16:1n-9, 16:1n-7, 16:1n-5, 18:1n-9, 18:1n-7, 18:1n-5, 20:1n-9, 20:1n-7, 20:1n-5, 22:1n-11, 22:1n-9 and 22:1n-7, (3) Includes 16:2n-3, 16:3n-3, 16:4n-3, 18:3n-3, 18:4n-3, 20:3n-3, 20:4n-3, 20:5n-3, 22:4n-3, 22:5n-3 and 22:6n-3, (4) Includes 16:2n-6, 18:2n-6, 18:3n-6,18:4n-6, 20:2n-6, 20:3n-6, 20:4n-6, 20:5n-6, 22:3n-6, 22:4n-6 and 22:5n-6, (5) Includes 16:1n-9, 18:1n-9, 18:2n-9, 18:3n-9, 20:1n-9, 20:2n-9, 20:3n-9 and 22:1n-9, (6) Includes 20:3n-3, 20:4n-3, 20:5n-3, 22:4n-3, 22:5n-3 and 22:6n-3.

Table 5. Proximal composition (lipid, protein and ash content; mg/g dry weight) and fatty acid composition (% total fatty acids) of different food sources used for larval rearing in Trial B.

Live preys	**Trial B**			
	DPS-Rot	**DHA-Rot**	***Artemia***	**GM 300™**
% Lipids (dw)	23.32±2.25	23.80±1.66	25.45±2.15	17.09±0.83
% Protein (dw)	51.21±3.80	50.36±3.00	55.38±2.25	57.66±1.09
% Ash (dw)	1.56±0.30	1.68±0.08	0.65±0.06 [b]	17.66±0.11
16:0	14.60±0.96	15.49±4.76	16.19±2.66	17.48±1.30
16:1 n-7	11.51±1.20	9.52±1.42	3.47±0.31	3.72±0.79
18:0	5.46±0.56 [a]	3.83±0.22 [b]	6.47±1.65	4.76±0.71
18:1 n-9	19.12±0.55 [a]	14.46±1.83 [b]	19.06±0.82	12.23±0.56
18:1 n-7	3.09±0.17 [a]	2.17±0.13 [b]	5.84±0.25	2.79±0.85
18:2 n-6	7.77±1.30 [a]	5.14±0.35 [b]	5.47±0.37	21.64±3.95
18:3 n-3	1.35±0.39	1.55±0.92	11.31±1.47	3.51±0.68
20:1 n-9	2.19±0.10 [a]	1.72±0.14 [b]	1.50±0.09	3.56±0.67
ARA (20:4n-6)	1.83±0.23 [a]	1.33±0.10[b]	1.62±0.16	0.55±0.15
EPA (20:5n-3)	6.63±0.34	6.83±0.57	7.56±0.82	5.98±1.50
DPA (22:5n-6)	0.45±0.04 [a]	0.92±0.57 [b]	0.59±0.11	n.d
DHA (22:6n-3)	10.11±0.32 [a]	22.51±2.06 [b]	9.11±1.82	8.61±1.16
∑ saturated [(1)]	23.93±0.63	21.86±5.21 [a]	24.14±3.60	27.19±1.58
∑ monoenes [(2)]	39.26±1.36 [a]	30.66±3.86 [b]	32.89±1.73	27.59±1.08
∑ n-3 [(3)]	21.01±0.52[a]	34.51±3.74 [b]	31.40±3.14	20.68±2.89
∑ n-6[(4)]	10.95±1.23 [a]	8.47±0.14 [b]	9.06±0.48	22.83±3.57
∑n-9[(5)]	22.78±0.68 [a]	17.97±2.12 [b]	21.41±0.59	20.01±1.06
∑n-3HUFA[(6)]	18.55±0.56 [a]	31.97±2.67 [b]	18.36±2.85	15.78±2.92
DHA/22:5 n-6	22.40±1.49 [a]	18.89±1.21[b]	15.56±0.58	n.d
EPA/ARA	3.68±0.59 [a]	5.16±0.39 [b]	4.67±0.05	11.04±1.21
DHA/EPA	1.53±0.05 [a]	3.30±0.25 [b]	1.20±0.12	1.48±0.23
DHA/ARA	5.61±0.85 [a]	16.98±1.33 [b]	5.59±0.60	16.29±3.02
Oleic/DHA	1.89±0.07 [a]	0.65±0.11 [b]	2.15±0.37	1.44±0.24
Oleic/n-3HUFA	1.03±0.04 [a]	0.46±0.08 [b]	1.05±0.13	0.80±0.18
n-3/n-6	1.94±0.21 [a]	4.08±0.44 [b]	3.46±0.26	0.94±0.27

dw: dry weight. Values (mean ± S.D) followed by different superscript letters within a row for rotifers FA were significantly different ($P < 0.05$).

(1) Includes 12:0, 14:0, 15:0, 16:0, 17:0, 18:0, 20:0, 22:0 and 24:0, (2) Includes 14:1n-5, 14:1n-7, 16:1n-9, 16:1n-7, 16:1n-5, 18:1n-9, 18:1n-7, 18:1n-5, 20:1n-9, 20:1n-7, 20:1n-5, 22:1n-11, 22:1n-9 and 22:1n-7, (3) Includes 16:2n-3, 16:3n-3, 16:4n-3, 18:3n-3, 18:4n-3, 20:3n-3, 20:4n-3, 20:5n-3, 22:4n-3, 22:5n-3 and 22:6n-3, (4) Includes 16:2n-6, 18:2n-6, 18:3n-6,18:4n-6, 20:2n-6, 20:3n-6, 20:4n-6, 20:5n-6, 22:3n-6, 22:4n-6 and 22:5n-6, (5) Includes 16:1n-9, 18:1n-9, 18:2n-9, 18:3n-9, 20:1n-9,20:2n-9, 20:3n-9 and 22:1n-9, (6) Includes 20:3n-3, 20:4n-3, 20:5n-3, 22:4n-3, 22:5n-3 and 22:6n-l.

Table 6. Proximal composition (lipid, protein and ash content; mg/g dry weight) and fatty acid composition (% total fatty acids) of whole body red porgy larvae fed rotifers enriched with different emulsions, at 12, 20 and 35 and 50 dah in trial B (n=3, mean± S.D, (P<0.05)).

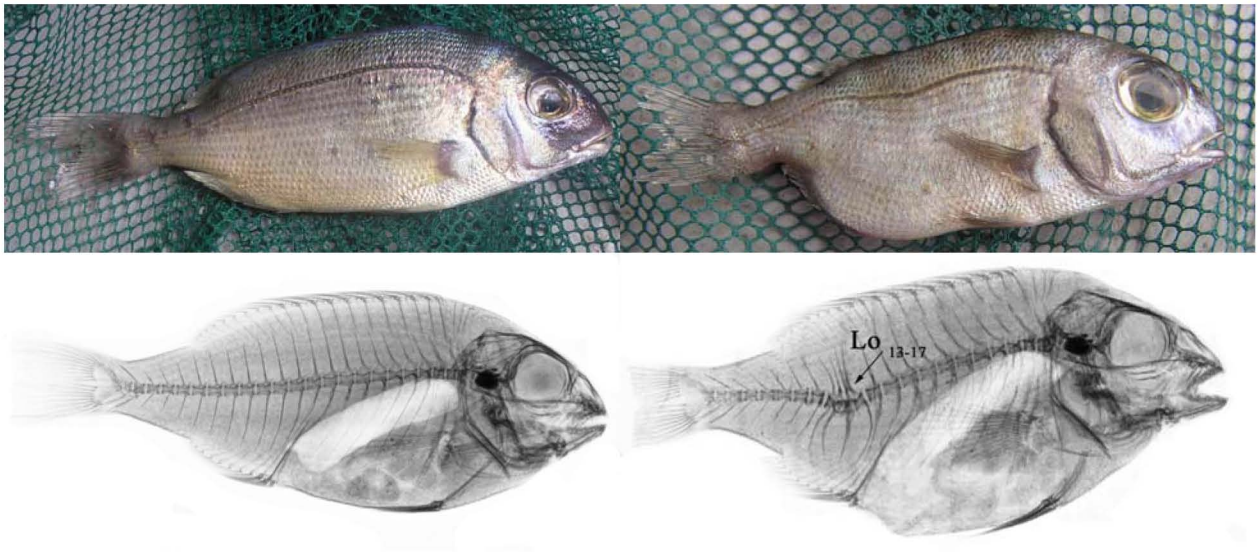
Days after hatching	**12dah**		**20dah**		**35dah**		**50dah**	
Treatment	**DPS-Rot**	**DHA-Rot**	**DPS-Rot**	**DHA-Rot**	**DPS-Rot**	**DHA-Rot**	**DPS-Rot**	**DHA-Rot**
% Lipids (dw)	20.45±1.03	19.03±1.81	14.68±0.63	16.07±2.15	17.13±1.32	15.93±0.15	16.07±0.57	15.87±0.21
% Protein (dw)	71.10±3.46[a]	78.70±7.31[a]	79.27±2.62	78.40±4.77	67.25±4.50	67.04±2.51	63.89±2.05	65.69±2.27
% Ash (dw)	1.64±0.18	1.37±0.32	2.12±0.10[a]	1.66±0.44[a]	3.48±0.22	3.49±0.22	3.43±0.14	3.64±0.01
16:0	18.12±1.26	19.41±3.26	20.87±0.88	22.16±0.08	20.96±0.43	21.25±2.61	19.88±2.12	20.00±3.61
16:1 n-7	4.98±0.47	3.94±0.34	4.03±0.42	3.53±0.14	2.93±0.30	3.58±0.84	3.14±0.65	3.85±0.91
18:0	9.87±0.36	8.43±0.65	12.21±0.25	9.68±0.19	9.81±1.26	9.17±0.71	8.42±1.17	8.26±1.81
18:1 n-9	11.25±0.09	11.98±2.22	11.70±0.69[a]	13.23±0.02[b]	14.76±0.30	14.25±1.61	14.59±0.46	14.04±1.61
18:1 n-7	4.24±0.22	3.55±0.37	4.77±0.13[a]	5.30±0.07[b]	4.62±0.39	4.37±0.94	4.37±0.63	4.02±1.06
18:2 n-6	8.57±0.16[a]	5.14±0.35[b]	6.14±0.35[a]	4.72±0.29[b]	10.62±3.06	10.73±0.17	12.57±1.64	12.51±3.08
18:3 n-3	3.47±0.34[a]	2.01±0.07[b]	3.25±0.21	3.43±0.47	4.43±0.88	3.94±2.10	4.42±0.90	3.40±2.20
20:1 n-9	1.18±0.07	0.91±0.19	0.97±0.04	1.05±0.06	1.65±0.26	1.95±0.26	2.11±0.64	2.20±0.48
ARA (20:4n-6)	1.82±0.03	2.07±0.20	2.05±0.02	2.24±0.03	1.91±0.43	1.52±0.40	1.53±0.32	1.37±0.46
EPA (20:5n-3)	4.59±0.13	3.77±0.29	4.37±0.05	4.71±0.88	4.94±0.31	5.64±0.40	5.37±0.81	5.79±0.45
DPA (22:5n-6)	0.54±0.02	1.03±0.10	0.55±0.11	1.11±0.21	0.52±0.10	0.43±0.14	0.44±0.09	0.38±0.15
DHA (22:6n-3)	16.30±0.25[a]	25.76±2.66[b]	15.98±0.15	17.45±0.99	12.74±2.26	11.63±0.06	12.04±1.06	12.31±1.16
∑ saturated [(1)]	29.40±1.65	30.20±2.97	35.30±0.60	34.20±0.04	33.05±1.04	33.51±4.22	30.62±2.81	31.06±5.80
∑ mono-unsaturated [(2)]	23.16±0.95	21.80±1.35	23.07±0.13	24.69±0.17	25.96±0.89	26.73±1.13	27.07±0.61	28.65±0.69
∑ n-3 [(3)]	27.38±0.28[a]	34.31±3.10[b]	26.41±0.12	28.00±0.16	24.48±1.97	23.54±2.08	24.40±1.86	24.06±2.34
∑ n-6[(4)]	12.73±0.20[a]	9.50±0.60[b]	10.38±0.40[a]	9.52±0.35[b]	13.92±2.43	13.59±0.89	15.74±1.18	15.37±2.77
∑n-9[(5)]	14.42±0.29	14.23±1.96	13.84±0.55[a]	15.17±0.18[b]	16.97±0.54	16.84±1.46	17.23±0.28	16.81±1.46
∑n-3HUFA[(6)]	23.37±0.13[a]	31.68±3.14[b]	22.51±0.16	23.82±0.29	19.30±2.59	18.84±0.32	18.94±1.99	19.71±1.47
DHA/22:5 n-6	30.00±1.57	25.10±0.22	28.99±0.78	16.14±3.65	24.52±2.47	29.47±10.39	28.62±8.01	35.44±12.47
EPA/ARA	2.53±0.11[a]	1.82±0.04[b]	2.13±0.00	2.11±0.43	2.66±0.51	3.94±1.41	3.66±1.23	4.58±1.56
DHA/EPA	3.56±0.15[a]	6.83±0.18[b]	3.66±0.07	3.81±0.86	2.58±0.45	2.07±0.16	2.26±0.15	2.13±0.20
DHA/ARA	8.97±0.02[a]	12.43±0.08[b]	7.80±0.15	7.79±0.33	6.74±0.62	8.02±2.25	8.16±2.31	9.67±3.16
Oleic/DHA	0.69±0.01	0.47±0.13	0.73±0.05	0.76±0.05	1.18±0.19	1.22±0.13	1.22±0.13	1.15±0.18
Oleic/n-3HUFA	0.48±0.00	0.39±0.10	0.52±0.03	0.56±0.01	0.77±0.10	0.76±0.10	0.78±0.09	0.72±0.11
n-3/n-6	2.15±0.01	3.61±0.10	2.55±0.10	2.94±0.09	1.81±0.46	1.73±0.04	1.56±0.20	1.58±0.18

(1) Includes 12:0, 14:0, 15:0, 16:0, 17:0, 18:0, 20:0, 22:0 and 24:0, (2) Includes 14:1n-5, 14:1n-7, 16:1n-9, 16:1n-7, 16:1n-5, 18:1n-9, 18:1n-7, 18:1n-5, 20:1n-9, 20:1n-7, 20:1n-5, 22:1n-11, 22:1n-9 and 22:1n-7, (3) Includes 16:2n-3, 16:3n-3, 16:4n-3, 18:3n-3, 18:4n-3, 20:3n-3, 20:4n-3, 20:5n-3, 22:4n-3, 22:5n-3 and 22:6n-3, (4) Includes 16:2n-6, 18:2n-6, 18:3n-6,18:4n-6, 20:2n-6, 20:3n-6, 20:4n-6, 20:5n-6, 22:3n-6, 22:4n-6 and 22:5n-6, (5) Includes 16:1n-9, 18:1n-9, 18:2n-9, 18:3n-9, 20:1n-9, 20:2n-9, 20:3n-9 and 22:1n-9, (6) Includes 20:3n-3, 20:4n-3, 20:5n-3, 22:4n-3, 22:5n-3 and 22:6n-3.

Table 7. Specific growth rate from 5 to 50 days after hatching. Data are given as average growth rate for *Pagrus pagrus* seedlings reared in different trials.

		Specific growth rate in length (%)			
		Age (dah)			
		5-15	**15-30**	**30-50**	**Average**
Trial A	**DPS-Rot**	5.6%	1.7%	4.5%	3.8±2.0%
	RPP-Rot	5.8%	2.3%	4.8%	4.2±1.8%
Trial B	**DPS-Rot**	5.9%	4.0 %	4.2%	4.0±0.1%
	DHA-Rot	5.9%	3.4 %	5.1%	4.4±0.0%

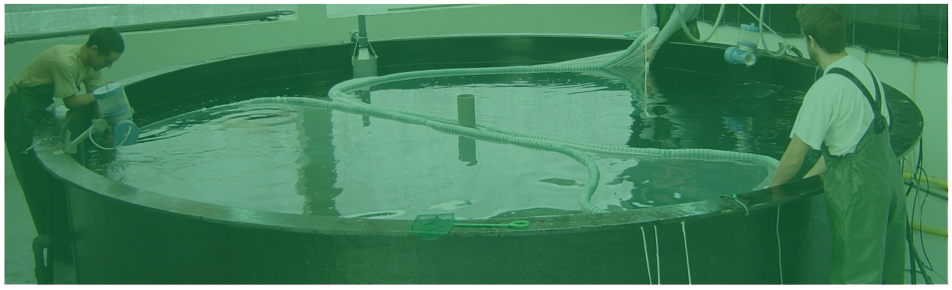
Picture 1. Red porgy fry in fresh to detect cranial anomalies and after X-ray study to detect internal deformities.



Improvements in the production technology of red porgy (*Pagrus pagrus*) larvae and fry: Importance of rearing conditions and diet nutritional value on their quality

Study IV.-Advances in rearing techniques of red porgy *Pagrus pagrus*, (Linnaeus, 1758): Comparison between intensive and semi-intensive larval rearing systems

Aquaculture Research (2008), (submitted)



Advances in rearing techniques of red porgy *Pagrus pagrus,* (Linnaeus, 1758): Comparison between intensive and semi-intensive larval rearing systems

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Abstract

Red porgy *(Pagrus pagrus*) constitutes a potential candidate for marine finfish diversification on commercial Mediterranean and Atlantic coastal aquaculture. However one of the major bottlenecks for the development of this species is the availability of fingerling for on growing mainly associated to limited information regarding larval rearing protocols for this specie. This paper described the development of a suitable larval rearing protocol for commercial aquaculture implementation. Three larval rearing trials were conducted (Trial A,B,C) where each new trial applied the results and benefits obtained in the previous one.

Red Porgy, eggs obtained from natural spawning, were incubated in the rearing tanks and reared until 50 days after hatching (dah). Two different rearing systems were used: Intensive (125 indv.l^{-1} in 2m^3 tanks) and Semi-intensive (7 indv.l^{-1} in 40m^3 tanks). In addition, on trial C two different weaning protocols were tested for each rearing technology. The effect of rearing system and weaning protocol applied on the growth, survival and whole body biochemical and fatty acid composition of red porgy larvae were evaluated. Significant differences on growth but not in survival at 50 days after hatching were detected in trial A for semi-intensive and intensive rearing system (23.5-18.9mm; 4.4-4.9%), however modifications on initial prey density and illumination conditions, implemented in trials B (29.5-25.2mm; 21.8-5.3%), and C (26.2-24.6mm; 22.7-3.8%), significantly improved survival and growth rates in semi-intensive rearing system. Furthermore, trial C results, confirmed the feasibility of a partial reduction in *Artemia* use and the significant improvement in survival rates with the new weaning protocol applied (26.4-24.1mm; 28.7-12.5%) in the intensive systems. Significant differences in fatty acid composition of the larvae were found among rearing systems. In trial A at early stages (12dah) larvae from semi-intensive system contained higher levels of highly unsaturated fatty acids than those reared in the intensive system, however no significant differences were found in trial B. Furthermore, a negative correlation was found between larval whole body tissues content on docosahexaenoic acid (DHA) with age. The results of this study concluded that with actual rearing technology and available commercial products, the best larval rearing protocol for commercial production of red porgy fingerlings, should included the use of semi-intensive system technology in order to avoid early mortalities (5-15dah). Furthermore, the use of intensive system technology could be also suitable when is made in combination with an early co-fed and weaning from 15dah in order to reduce *Artemia* use and improves survival rates. However, further studies are needed to elucidate the importance of initial diet on the poor activity, low SGR observed in the window from 12-20dah.

Keywords: Pagrus pagrus, Red porgy, New species, Larval rearing, Technology, Mesocosm

*Abbreviations:*FA: Fatty acid; TFA: Total fatty Acids; EFA: Essential fatty acids; PUFA: Polyunsaturated fatty acids; HUFA: Highly unsaturated fatty acids; DHA: Docosahexaenoic Acid; EPA: Eicosapentaenoic Acid; ARA: Araquidonic Acid, dah: days after hatching; SGR: specific growth rate.

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1. Introduction

Red porgy *(Pagrus pagrus*) constitutes a potential candidate for marine finfish diversification on commercial Mediterranean and Atlantic coastal aquaculture (Kentouri *et al*., 1995). The adaptation of wild caught fish as broodstock has been successfully held since 1995 and natural spawning occurs within January till May in Canary Islands with elevated hatching and survival rates at first feeding (Cejas *et al*., 1997). Larval rearing of this species is based in an adaptation of intensive seabream culture techniques with different degree of success (Hernández-cruz *et al.,* 1990; Papandroulakis *et al*., 2004b; Kentouri *et al*., 1995; Hernández-cruz *et al.,* 1999; Mihelakakis *et al.,* 2001).

Larval rearing technologies have been classified by Divanach *et al*., (1995, 1998) and Van der Meeren and Naas, (1997) take into account factors such us microalgae addition, prey sources, tank volume, larval stoking density and water supply. However, practically larval rearing protocols must be adapted to the ontogeny of each species and the morphological and physiological changes which take place along larval development. Moreover, larval ontogeny as well as growth and survival will be affected not only by environmental conditions, but also by prey nutritional quality and availability. Live preys, mainly rotifers (*Brachionus sp.*) and brine shrimp (*Artemia*) are widely used in massive culture of most marine species (Dhert *et al*., 2001; Sorgeloos *et al*., 2001) including red porgy. This reduced range of food organisms fed to marine fish larvae, may lead to nutritional imbalances or nutritional deficiencies (Izquierdo, 1996). Actually, there is very limited information about nutritional requirements of red porgy, but the importance of long chain fatty acids such as docosahexaenoic acid (22:6n-3), eicosapentaenoic acid (20:5n-3) and arachidonic acid (20:4n-6) has been widely studied in marine species (Izquierdo, 1996, Sargent *et al*.,1997) where they play an important role in cell membrane structure and function, affecting the development of different tissues and organs at early stages such as neural and visual systems. In red porgy larvae, previous results obtained in our laboratory suggest that this specie have high specific requirements for DHA during early stages significantly affecting skeletal development (Roo *et al*., 2008).

Generally, the improvement in larviculture techniques for a given fish species was accompanied by the intensification in the rearing methods (Shields, 2001). However alternative rearing techniques such as Mesocosms techniques, an intermediate semi-intensive production technology has been successfully applied to rear more than 20 species (Divanach and Kentouri, 2000) such as sharpsnout sea bream (*Diplodus puntazzo)*, white sea bream (*Diplodus sargus)*, greater amberjack (*Seriola dumerili)* (Papandroulakis *et al*., 2004a, 2005), thick lipped grey mullet (*Chelon labrosus*) (Khemis *et al*., 2006), shi drum (*Umbrina cirrosa L.) (*Zaiss *et al*., 2006), or European

sea bass (*Dicentrarchus labrax*) (Zouiten, D., *et al*., 2004). A more extensive version of mesocosm has been also used in Norway for larval rearing of saithe (*Pollachius virens,* L.), goldsinny (*Ctenolabrus rupestris,* L.), corkwing (*Crenilabrus melops*, L.) and cod larvae (*Gadus morhua.* L.) by Van der Meeren and Lønøy, (1998) and Van der Meeren and Jørstad, (2001).

In the present study, in order to improve larval rearing techniques of red porgy and better understand its biological performance, Mesocosms technique was used as a model system for fry production of this species in comparison with intensive culture. Moreover, in view of the high DHA requirements of these species and since the residual time of enriched live preys in the Mesocosms system is higher than in the intensive system due to the lower water turnover, life preys biochemical composition, particularly in terms of essential fatty acid content, was also study to determine if they differ between both systems.

2. Materials and methods

Rearing techniques.

Two rearing techniques, mainly differing on tank capacity and larval density were applied: A semi-intensive system (mesocosm) similar to the one described by Divanach and Kentouri (2000) using cylinder-conical 40m^3 tanks, stocked with 7 eggs.l^{-1} and an intensive system, using cylinder-conical tanks of 2m^3 capacities stocked with 125 eggs.l^{-1}. In order to compare the larval rearing success in both types of systems, three different trials (A, B, C) were conducted.

In trial A, both systems were compared, following a similar protocol than that described by Hernández Cruz *et al*. (1999) and Papandroulakis *et al*. (2004b) and differing only in their respective initial egg density, prey density and tank volume. In trial B, in order to determine if rotifer density and feeding period was related to the different results obtained between systems, rotifers density was increased in the semi-intensive system as well as rotifers feeding period in both systems in comparison to trial A. Finally, in trial C, the effect of early co-feeding with microdiets and weaning was compared between both systems.

2.1 Experimental conditions

Red porgy eggs obtained from natural spawning (Figure 1), were distributed into rearing tanks provided with filtered and UV sterilized seawater. Mean dissolved oxygen and temperature were registered (Table I). Different photoperiod regimes, combining natural and artificial light was used according to the trials (Table I). Light intensity just above the water surface was measured (Light meter, Mod. HT170N; Italy) and ranged along the day (9:00, 12:00; 21:00) between 1000-3500 lux. Live phytoplankton (*Nannochloropsis sp*) was added once a day adjusted to a concentration of 250±100x10^3 cells.ml^{-1}. From day 2 after hatching (dah) rotifers (*Brachionus*

plicatilis) were added twice a day adjusted to 5-10 rot.ml^{-1} in the intensive system and in the semi-intensive system adjusted to 1-2 rot.ml^{-1} in trial A and to 4-5 rot.ml^{-1} in trials B and C. Rotifers feeding period lasted from 2-20dah in trial A and it was extended from 2-30dah in trials B and C. Rotifers were growing out on baker´s yeast (*Saccaromices cerevisiae*) and enriched with DHA Protein Selco (Inve Aquaculture, Dendermonde, Belgium) following the manufacturer instructions.

In trials A and B, the weaning protocol included manual feeding from 20dah (Genma Micro, Skretting, France) four times a day for 5 days and automatic feeding every hour from day 25. In trial C two co-feeding and early weaning protocols using *Artemia* and microdiets for different periods were compared as showed in Table I. Therefore, the quantity and onset of *Artemia* Instar I (25-250 A_0 . l^{-1}) (AF type, INVE Aquaculture, Dendermonde, Bélgica), *Artemia* Instar II (EG tipe, INVE Aquaculture, Dendermonde, Bélgica) enriched with A_1 Easy Selco (INVE Aquaculture, Dendermonde, Bélgica) and commercial diet (Genma Micro, Skretting, France) varied according the co-feeding and early weaning protocols (Table I).

2.2 Sample collection

Larval growth was assessed measuring the total length of 25 larvae every 5-7 days, using a profile projector (Nikon V-12A, NIKON, Tokyo, Japan). Length-specific growth rate (SGR) was calculated using the following equation: $SGR=([Ln(Lt)-Ln(L0)] /t) x 100$; where L_t is the larval length at the end of time period t, L_0 is the length at the beginning of time period t, and t is the length of the time period in days.

Survival was determined after 95dah by individual counting all the remaining fish using a fry counter (TPS Fish counter, Type Micro; Impex Agency; Denmark). Form 15 dah, dead fish were daily recorded and from that day, survival was accordingly estimated taking into account daily mortality and final alive fish.

2.3 Activity test.

In trial C, activity test was performed on days 20 (10 seconds air exposure) and 30 dah (60 seconds air exposure). Larvae (n=25) in triplicates from each replicate tank were air exposed in a 500µm nylon mesh screen. Later, larvae were transferred to a net container (1liter) in their original rearing tank and survival was registered 24h after the test.

2.4 Biochemical analysis

For biochemical analysis, one thousand larvae per tank were collected at 12 and 20dah and two hundred fish at 35 and 50dah from each tank and treatment. In order to determine prey nutritional quality in the larval rearing tanks, in trial B, samples of the remaining preys (rotifers) in the larval tank were collected previous morning first feeding addition from both rearing systems at 8, 10

and 12 days after hatching. Besides, samples of enrichment products, rotifers, *Artemia* and microdiets were collected, at least three times throughout each feeding trial. All the biochemical analyses were conducted in triplicate. Protein and ash were determined according to AOAC., (1995). Total lipids were extracted as described by Folch *et al*. (1957). The fatty acid methyl esters were obtained by transesterification with H_2SO_4 (Christie, 1989) and purified by adsorption chromatography on NH_2 Sep-Pack cartridges (Waters, S.A., Milford, MA, USA) as described by Fox (1990), and separated and quantified by Gas-Liquid chromatography as described by Izquierdo *et al.* (1990).

2.5 Statistical analysis.

All the data were statistically treated using SPSS Statistical Software System ver 15.0 (SPSS Inc., 2000, Chicago, IL, USA). A T-test for simple mean comparison analysis ($P < 0.05$) (Sokal and Rolf, 1995) was applied between systems. Variances were tested for normality and homogeneity. Results are presented as mean ± standard deviation.

3. Results

3.1 Growth and survival.

Trial A

In trial A, despite the average total length of red porgy larvae at 5,10, 15, 35 and 40 dah was not significantly different between rearing systems, at 50 dah total length of the larvae from semi-intensive system was significantly higher than that of intensive system (Figure 2).

Along the whole rearing period, semi-intensive system larvae had a higher SGR, although without statistical significant differences ($P>0.05$) between rearing systems. Three different growth periods in terms of SGR were observed: an early rapid growth period (5-15dah) (5.8-6.7% SGR in the intensive and semi-intensive systems, respectively) when preys source were restricted to rotifers, a second period with a strong reduction of SGR (15 to 30dah) when new prey types were incorporated (*Artemia* and microdiets), and a third growth period with increased of SGR values (4.5%-5.3%) from 30 to 50dah when larvae were actively fed on microdiet (Figure 3).

Survival up to 50dah was not significantly different between rearing systems (4.4 - 4.9% in the semi-intensive and intensive systems, respectively; Figure 4) being the highest larval mortality period from 3-15 dah (Figure 4).

Trial B

From 15 to 50 dah, larvae from semi-intensive system (Figure 5) were significantly higher in TL than intensive system larvae. Moreover, these larvae were bigger than those obtained in trial A

for the same age and rearing system (23.5 *vs* 29.59mm TL) in semi-intensive system 50 old larvae in trial A and B respectively.

Again three different growth periods were found in terms of SGR (Figure 6), however an important improvement in larval growth was obtained in the second period (15-30dah), in comparison to previous trial for both rearing systems.

Overall, survival in the semi-intensive system in trial B was significantly higher than in trial A (4.4% *vs* 21.8 in trial A and B respectively). Besides, the highest larval mortality period from 3-15 dah was noticeably reduced in the semi-intensive system, to reach a 38% higher survival than in trial A for the same age (Figure 7). However, no noticeable improvements were obtained in final survival for larvae reared in the intensive system (4.9% *vs* 5.3% in trial A and B respectively).

In this trial, the estimation of available prey in relation to estimated larval density, at different ages was reflected in important differences among rearing system, thus for the period from 3 to 35 dah mesocosms reared larvae showed an average prey availability of 6 times higher (Figure 8).

Trial C

In trial C, mean total lengths for 50-day-old larvae were not significantly different neither between rearing systems nor between weaning protocols applied for each system (Table III). The average total length (25.04 mm) obtained for this trial at 50dah was quite similar to trial B and bigger than in trial A, regardless the rearing system (Figure 9a,b).

In trial C, the evolution of SGR followed a similar tendency than in previous trial, showing a hyperbolic curve evolution along the whole study while a decreasing trend was observed in the semi-intensive one regardless of the weaning protocol. Finally, average SGR were no significantly different between rearing systems or weaning protocols (Figure 10a,b). Survival was higher in semi-intensive system larvae regardless the weaning protocol (Figure 11a,b). However, in weaning protocol 2 (W2) survival significantly improved, particularly in the intensive system (12.5% in W2; Table III).

3.2 Activity test for trial C.

At 20dah, regardless of the weaning protocol applied, larvae reared under Semi-intensive system conditions showed higher survival after the activity test than larvae reared in the intensive system (Figures 12, 13). Regarding the effect of weaning protocol, larvae fed according to the weaning protocol 1 showed a higher survival after activity test than those fed protocol 2 for both rearing systems.

At 30 dah, in larvae fed according to weaning protocol 1, no significant differences were observed in survival among rearing systems (Figure 14). However, in larvae fed following weaning protocol 2, survival of the larvae from semi-intensive system was significantly higher than larvae from intensive system (Figure 15).

3.3 Fatty acid composition of Pagrus pagrus larvae.

Trial A

In Trial A, the lipid content of intensive system reared larvae were significantly higher that semi-intensive ones at 12 and 20dah. The levels of n-3, n-3 HUFA and DHA content of red porgy larvae was significantly higher in Semi-intensive than in the intensive system at this days (12-20dah), whereas no significant variation was observed at older stages (35 and 50dah) (Table 4). Consequently, DHA/ARA, Oleic/DHA, Oleic/n-3 HUFA and n-3/n-6 ratios were significantly affected at 12 dah. At 50dah, the level 18:2n-6 was significantly lower and the level of 18:3n-3 significantly higher in Semi-intensive reared larvae in comparison to intensive reared ones.

Trial B

In Trial B, at 12dah, the lipid content of intensive system reared larvae was significantly higher than in semi-intensive ones. At this day, larval lipid level was higher than the level observed previous trial A for both rearing systems. As in previous trial, the levels of n-3, n-3 HUFA and DHA of red porgy larvae were higher in semi-intensive than in the intensive system at 12 and 20dah, but not statistically significant. The FA profile of the larvae was not significantly affected by the rearing systems at older stages (Table 5). As occurred in trial A, at 50dah, the level of 18:2n-6 was significantly higher in the intensive system reared larvae in respect to semi-intensive system ones.

Trial C

In Trial C, at 20dah the lipid content of intensive system reared larvae was higher than in semi-intensive ones, although only significantly in the protocol 2 fed larvae. As in previous trial, the levels of n-3 were higher in semi-intensive than in the intensive system at 20dah regardless of the weaning protocol. No effects were observed in n-3 HUFA and other individual FA at 20dah when weaning protocol 1 was used, however some differences in proportions of total saturates and monounsaturated FA among systems were found when weaning protocol 2 was applied. Differences were mainly due to 16:0 and 18:0 which were 16.8% and 10.3% in the semi-intensive larvae and 19.4% and 11.3% in the intensive reared larvae. Besides, a significantly higher level of 18:3n-3 was observed in Semi-intensive reared larvae, moreover a generally lower level of 18:2n-6 were observed in this system in comparison to intensive reared larvae when weaning protocol 2 were applied. In addition, a lower content of 18:3n-3 and higher 18:2-6 level were associated

with the early microdiet introduction for both rearing systems. In relation to EFA, the level of docosahexaenoic acid (19.8%) was significantly increased in larvae from intensive system at 20dah when protocol 2 was used, moreover this increased was evident in comparison to larvae fed on weaning protocol 1 and cultured on the same rearing system.

4. Discussion

Major fatty acids in whole body of red porgy larvae were 22:6n-3, 16:0, 18:1n-9, 18:0, 18:2n-6, 20:5n-3, 16:1n-7,18:1n-7, 18:3n-3 and 20:4n-6. Similar results have been reported for other marine species such as gilthead seabream (*Sparus aurata)* (Roo *et al.,* 2005), barramundi *Latris lineata* (Brandsen *et al*., 2005) or Atlantic cod (*Gadus morhua)* (Plante *et al*., 2007). The importance of long chain fatty acids such as docosahesaenoic acid (22:6n-3), eicosapentanoic acid (20:5n-3) and arachidonic acid (20:4n-6) had been widely studied in marine fish species (Izquierdo 1996, Sargent *et al*., 1997), playing an important role in cell membrane structure, function and development of different tissues and organs as neural and visual systems at early stages. The proportion of DHA in red porgy larvae at 12 dah, were almost 2 times higher than those observed in the live preys. Thus, the level of DHA in larval tissues (15-20%) in early stages (12-20dah) largely exceeded the values found in the diet (9%-10%). These results agreed with the observations of Izquierdo,(1996) suggesting a selective incorporation of this fatty acid into both lipid reserves and membranes. In contrast, ARA remains almost constant and EPA clearly reflects dietary changes during ontogeny. The proportion of EPA in red porgy feeding on *Artemia* and microdiet increased significantly in comparison to values recorded for early stages due to a higher EPA content in this type of food. Consequently, the DHA/EPA decreased with age and EPA/ARA ratio tends to increased in older larvae.

Regardless the rearing system and trial, three different periods could be found along larval development: a first one (5-15 dah) with high growth rates (6%) despite being a very sensitive stage for larval survival; a second period (15-30dah), related to completion of metamorphosis, with lower growth rates (2, 3% for trial A and around 4%, for B and C) and also quite sensitive to mortalities; and, finally, a third post-metamorphic one (30-50dph) with increased growth rates (5%) and low mortality.

During the first period (5-15 dah), larval performance seemed to be closely related to the success after the onset of exogenous feeding and the yolk reserves depletion. Thus, the low growth and survival ratios obtained in semi-intensive larvae in trial A could be due to an insufficient prey intake, which was also reflected in the larval fatty acid composition. Indeed, retention of DHA together with reduction of 18:0 and 18:1n-9 has been found to be associated to starvation in

marine fish larvae (Koven *et al*., 1989; Izquierdo, 1988; Rodríguez *et al*., 1994). Accordingly, the increase in prey density during this period in semi-intensive system in trial B (4-5 rot.ml^{-1}) in comparison with trial A (1-2 rot.ml^{-1}) markedly decreased mortalities and improved growth rates. Moreover, in one hand, wild zooplankton aggregations are usually over 2 indv.ml-1 (Browman, 2005) and in the other hand, the larval visual perception field (VPF; water volume where the larvae is able to visually detect a prey) (Gallager *et al*., 2004) for a marine fish larvae of this size (5 mm) is not higher than 1 ml (Browman and Skiftesvik 1996; Galbraith *et al*.2004). Therefore, prey densities under 2 rot.ml-1 in semi-intensive system in trial A would be too low to promote predator–prey encounters. Accordingly, in Trial B rotifer density was increased over 2 rot.ml-1 to satiate feed intake of red porgy which besides seems to be more voracious than other sparids (Hernández-Cruz *et al*., 1999). However, the low growth and high mortalities recorded during this period in the intensive system could be more related to other causes, such an unsuccessfully use of prey nutrients under a more stressful rearing conditions or related to changes in the bacterial community structures in the rearing water as it was recently reported by Nakase *et al.*, 2008. These authors, found that poor growth and early mortality (4-14dah) in a close species to red porgy (*Pagrus pagrus)* such as Japanese red seabream (*Pagrus major)* reared under intensive system was associated to the increase in γ-proteobacteria in seawater despite the daily addition of *Nannochloropsis sp.*, and suggested a marked effect of the microalgae physiological condition on the bacterial community structures. In the present study *Nannochloropsis sp.* was daily added in boh systems, but the concentration and physiological condition of this microalga was markedly affected by the specific characteristics of each system (tank volume and depth, water turnover and turbulence, light distribution) could affect the bacterial community structures to a less favorable one in the intensive system.

In the second period (15-30dah), morphological and physiological changes lead to an improvement in many biological functions such as digestion and vision. Thus, during this period extracellular digestion improves, gastric glands start to be functional (Govoni *et al*., 1986; Segner *et al*., 1994; Roo *et al*., 1999, Socorro *et al*., 1999; Darias *et al*., 2005) and a new type of photoreceptors (rods) develops increasing retina sensitivity necessary for wild fish to shift from shallow to deep waters (Roo *et al*., 1999). At the same time, important changes in the rearing techniques occur in feeding (switch in live prey, from rotifer to *Artemia*, and weaning to dry feed) and water quality (green to clear water). Very low growth rates (2%) were found during this period in both rearing systems in trial A, which were increased in trial B (4%) when rotifer feeding was prolonged up to 30 dah (20 dah in trial A) and 24 photoperiod was reduced to 12 h from 20 dah (40 dah in trial A). Inadequate illumination levels with continuous photoperiods and

the progressive change to clear water in such a sensitive phase could act as a stressor over larval population (Boeuf y Le Bail, 1999). But the better growth rate found in trial B, could be also related to a better nutritional value of rotifers in comparison to *Artemia.* Indeed, red porgy ability to digest *Artemia* was very deficient in both trials A and B, as it was observed under the binocular microscope (Figure 1). The poor digestion of *Artemia* could be related to the poor development of red porgy gut at this stage, in where, despite having a very rudimentary stomach and gastric glands as early as 19 dah (Roo *et al*., 1999; Socorro *et al*., 1999, 2006), acid digestion is not completely functional until 35 dah (Darias *et al*., 2005, 2007). In addition, this reduced *Artemia* digestion could be also related to an excessive intake of this prey by the voracious red porgy larvae which would reduce intestinal transit of *Artemia* in larval gut.

In agreement with the observed reduction in prey digestion when larvae were fed *Artemia*, the red porgy DHA content remained constant during rotifer feeding (12-20dah) and was markedly reduced with *Artemia* feeding (35-50dah), despite a similar content of this FA was recorded in both preys. Previous studies have shown that red porgy has a high DHA requirement during larval development, its reduction in live preys causing severe skeletal anomalies (Roo *et al*., 2008). Moreover, in weaning protocol 2, co-feeding with dry diet was onset as early as 15 dah and this could be also related to the improvement in survival in this second period, denoting the superior nutritional quality of the commercial diet over *Artemia* at this larval stage. In fact the use of an early co-feeding protocol, significantly affected larval fatty acid composition, increasing the level of DHA in early co-fed larvae reared under intensive system respect to control larvae. The benefits of co-feeding protocols were previously reported in other species such as gilthead seabream (Rosenlund *et al* 1997; Kolkowski *et al* 1997; Koven *et al* 2001), or barramundi (*Lates calcacifer*) (Curnow *et al*., 2006). In this study, the early microdiet introduction was found to be more effective in the intensive system than in the semi-intensive one, and was also reflected in larval fatty acid composition which was higher in 18:2n-6 in early weaned larvae.

After complete metamorphosis and weaning to dry diet, third period (30-50dph), growth rates were good in all trials and similar to the second stage. These results seemed to be related to the higher maturation of the fish, particularly in digestive, neural, sensorial and endocrine systems (Roo *et al*., 1999; Socorro *et al*., 1999, 2006; Darias *et al*., 2005, 2007). Overall, growth rates until older stages allowed to get a body weight at 95 dah (6.2±1.5g) values significantly higher than in *Sparus aurata* juveniles reared under similar conditions (Author´s unpublished data), and in agreement with results found by Papandroulakis *et al.* (2004b). During this third period the main factor threatening survival seemed to be the apparition of cannibalistic behavior, which in this study was observed at 8-8.5mm total length although did not caused significant losses.

Cannibalistic behavior appears also in other sparids such as common dentex (*Dentex dentex)* (Koumoundouros *et al*., 1999) or gilthead seabream. Despite in the later cannibalism is reduced by grading and separation of different fish sizes, manipulation at this stage causes sudden mortalities in several species such as common dentex (*Dentex dentex*) (Mourente *et al*., 1999). In this sense, sudden mortalities by shock syndrome were also found in red porgy when an acute stress (activity test) was applied, but being lower in fish reared under the semi-intensive system. The apparition of shock syndrome after acute stress has been suggested to be a consequence of nutritional unbalances during previous larval stages (Izquierdo *et al*., 1989), in relation to essential fatty acids, particularly DHA, as it seems to be the case of red porgy (Roo *et al*., 2008) which had a higher DHA content when reared in semi-intensive systems in agreement with the higher survival after acute stress test.

In a wide range of marine species, the use of semi-intensive technology for larval rearing, results in higher survival and growth performance than in intensive larval rearing techniques (Papandroulakis *et al*., 2004a, Roo *et al*., 2005). Several factors have been claim to be responsible for this marked difference in larval performance between both systems such as the more stable rearing conditions in the semi-intensive one which allows to develop more mature bacterial flora buffering the effect of daily bacterial charges included with live preys addition. Nutrition during second period could be also an important factor related to the better performance of fish in semi-intensive system. Thus, survival of intensive system red porgy was much closer to that of semi-intensive ones only when weaning was moved ahead and *Artemia* Instar II density was reduced. The main difference in the larval success between both systems for this species still remains in the rotifers feeding period, when the density of larvae is reduced from 125 to about 17 larvae.l^{-1} at 15 dah, and in the semi-intensive system from 7 larvae.l^{-1} to 3 larvae. l^{-1}. At this stage, one of the most important differences observed was related to prey availability in the intensive system (around 425 rotifers/larva/day in the intensive system and 1700 rotifers/larva/day in the semi-intensive), together with those related to water turnover, green water condition and tank management. Hence, higher survival during rotifer feeding period in semi-intensive system could be related to a better utilization of prey nutrients under more favorable environmental conditions and hence, lower energy demands. This hypothesis is supported by the longer yolk and lipid reserves duration (Papandroulakis *et al*., 2004a) and the higher DHA content in semi-intensive system reared larvae in the present study. Moreover, the higher levels of 18:3n-3 semi-intensive larvae in comparison with intensive ones denoted a higher intake of rotifers which were particularly rich in this fatty acid. On the contrary, less favorable culture conditions such as increasing stocking density may induce lower food consumption and utilization as reported for

juvenile gilthead seabream (*Sparus aurata*) and white sea bream (*Diplodus sargus*) (Montero *et al*., 1999, 2001; Papoutsoglou *et al*.,2006) and higher energy demands, which could led to higher nutritional requirements.

5. Conclusions

The results of this study allowed the improvement in the rearing protocols for red porgy, increasing the final total length and survival at 50dah from the initial trial to the last ones from 18.9 mm to 25.13 mm and from 4.9 to 12.5% in the intensive system and from 23.52 to 26.4 mm and 4.4 to 28.7% in the semi-intensive system. At present, the best larval rearing protocol to sustain a regular and predictable red porgy fingerlings demand is the semi-intensive system technology. Besides, early co-feeding and weaning together with a reduction in stressors during rotifer feeding is recommended in this species to improve larval performance under intensive system.

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List of tables.

Table 1. Summary of the rearing parameter modifications applied in the different trials.

	Trial A	Trial B	Trial C	
			W1	W2
Oxygen (ppm)	6.5±0.5	6.6±0.6	6.0±0.8	6.0±0.8
Temperature (ºC)	20.0±0.5	20.0±0.5	20.4±0.7	20.4±0.5
Photoperiod				
24 h: (Natural+ artificial)	2-40dah	2-20dah	2-20dah	2-20dah
12 :12 h (Natural+ artificial)	40-50dah	20-50dah	20-50dah	20-50dah
Phytoplankton (age)	2-25dah	2-25dah	2-25dah	2-25dah
SMIS & IS (Cells.ml^{-1})	250. $10^{3.}$	250. $10^{3.}$	250. $10^{3.}$	250. $10^{3.}$
Rotifers (age)	3-20dah	3-30dah	3-30dah	3-30dah
SMIS (indv.ml^{-1})	1-2	4-5	4-5	4-5
IS (indv.ml^{-1})	5-10	5-10	5-10	5-10
Instar I (age)	13-20dah	13-16dah	13-16dah	13-16dah
SMIS (indv.l^{-1})	25-250	25-250	25-250	25-250
IS (indv.l^{-1})	25-250	25-250	25-250	25-250
Instar II (age)	18-50dah	15-50dah	15-50dah	15-40dah
SMIS (indv.l^{-1})	500	500	500	250
IS (indv.l^{-1})	1000	1000	1000	500
Weaning (onset)	20dah	20dah	20dah	15dah

*Values express range of larval age in days after hatching; W: weaning protocol.

Table 2. Proximal composition (lipid, protein and ash content; mg/g dry weight) and fatty acid composition (% total fatty acids) of enriched rotifers, *Artemia Instar II* and microdiets fed to red porgy larvae.

Live preys	**Enrich Rotifers**	**Enrich *Artemia***	**Microdiet**
% Lipids (dw)	22.35±2.30 [a]	25.45±2.15 [b]	17.09±0.83 [c]
% Protein (dw)	52.27±4.34 [a]	55.38±2.25 [ab]	57.66±1.09 [b]
% Ash (dw)	1.20±0.27 [a]	0.72±0.26 [b]	17.09±0.83 [c]
16:0	14.60±0.96 [a]	16.19±2.66 [ab]	17.48±1.30 [b]
16:1 n-7	11.51±1.20 [a]	3.47±0.31 [b]	3.72±0.79 [b]
18:0	5.46±0.56	6.47±1.65	4.76±0.71
18:1 n-9	19.12±0.55 [a]	19.06±0.82 [a]	12.23±0.56 [b]
18:1 n-7	3.09±0.17 [a]	5.84±0.25 [b]	2.79±0.85 [a]
18:2 n-6	7.77±1.30 [a]	5.47±0.37 [a]	21.64±3.95 [b]
18:3 n-3	1.35±0.39 [a]	11.31±1.47 [b]	3.51±0.68 [c]
20:1 n-9	2.19±0.10 [a]	1.50±0.09 [a]	3.56±0.67 [b]
ARA (20:4n-6)	1.83±0.23 [a]	1.62±0.16 [a]	0.55±0.15 [b]
EPA (20:5n-3)	6.63±0.34	7.56±0.82	5.98±1.50
DHA (22:6n-3)	10.11±0.32	9.11±1.82	8.61±1.16
∑ saturated [(1)]	23.93±0.63	24.14±3.60	27.19±1.58
∑mono-unsaturated	39.26±1.36 [a]	32.89±1.73 [b]	27.59±1.08 [c]
∑ n-3 [(3)]	21.01±0.52 [a]	31.40±3.14 [b]	20.68±2.89 [a]
∑ n-6[(4)]	10.95±1.23 [a]	9.06±0.48 [a]	22.83±3.57 [b]
∑n-9[(5)]	22.78±0.68 [a]	21.41±0.59 [ab]	20.01±1.06 [b]
∑n-3HUFA[(6)]	18.55±0.56	18.36±2.85	15.78±2.92
EPA/ARA	3.68±0.59 [a]	4.67±0.05 [a]	11.04±1.21 [b]
DHA/EPA	1.53±0.05 [a]	1.20±0.12 [b]	1.48±0.23 [ab]
DHA/ARA	5.61±0.85 [a]	5.59±0.60 [a]	16.29±3.02 [b]
oleico/DHA	1.89±0.07 [a]	2.15±0.37 [b]	1.44±0.24 [ab]
oleico/n-3HUFA	1.03±0.04	1.05±0.13	0.80±0.18
n-3/n-6	1.94±0.21 [a]	3.46±0.26 [b]	0.94±0.27 [c]

Values (mean ± S.D) followed by different superscript letters within a row for the same trial were significantly different (P <0.05).

Table 3. Proximal composition (lipid, protein and ash content; mg/g dry weight) and fatty acid composition (% total fatty acids) of unconsumed rotifers in the rearing tank in trial B. (values express average of pool samples from each tank replicate at days 8-10-12 dah).

Unconsumed preys	Semi-intensive	Intensive
% Lipids (dw)	12.37±1.22 [a]	16.97±1.81 [b]
% Protein (dw)	64.41±13.70	71.22±4.13
% Ash (dw)	0.91±0.28 [a]	0.35±0.12 [b]
16:0	18.66±0.33	17.60±1.12
16:1 n-7	9.58±0.32	8.80±0.32
18:0	4.02±0.11	4.26±0.41
18:1 n-9	12.07±0.47	11.91±0.50
18:1 n-7	2.53±0.00	2.82±0.03
18:2 n-6	10.26±0.57	10.76±0.78
18:3 n-3	11.16±0.94	9.99±0.83
20:1 n-9	1.99±0.01	1.91±0.01
ARA (20:4n-6)	0.83±0.00	0.91±0.04
EPA (20:5n-3)	3.81±0.10	4.10±0.25
DHA (22:6n-3)	4.40±0.19 [a]	6.70±0.61 [b]
∑ saturated [(1)]	26.68±0.41	25.04±1.65
∑mono-unsaturated	29.53±1.55	29.63±0.79
∑ n-3 [(3)]	24.86±0.78	25.77±1.87
∑ n-6[(4)]	12.35±0.43	13.70±1.24
∑n-9[(5)]	16.83±0.69	17.17±0.88
∑n-3HUFA[(6)]	11.14±0.15 [a]	12.95±0.98 [b]
EPA/ARA	4.58±0.15	4.51±0.07
DHA/EPA	1.15±0.02 [a]	1.63±0.05 [b]
DHA/ARA	5.28±0.25 [a]	7.37±0.33 [b]
oleico/DHA	2.74±0.01 [a]	1.79±0.24 [b]
oleico/n-3HUFA	1.08±0.03	0.93±0.11
n-3/n-6	2.01±0.01 [a]	1.88±0.03 [b]

Values (mean ± S.D) followed by different superscript letters within a row for the same trial were significantly different ($P < 0.05$).

Table 4. Total length (5, 15, 30, 40 and 50 days) and survival (50day) of red porgy larvae reared in different systems and weaned with two different protocols in trials C.

Trial C	**Total lenght (mm)**					**Survival (%)**
	Age (dah)					
	5	***15***	***30***	***40***	***50***	***50***
SMISW1-TC	3.4 ± 0.2	6.4 ± 0.7	12.5 ± 0.7	17.6 ± 3.0	26.2 ± 2.6	$22.7 \pm 1.9_{a}^{A}$
ISW1-TC	3.1 ± 0.4	6.3 ± 0.6	10.2 ± 1.5	16.3 ± 2.9	24.6 ± 3.8	$3.8 \pm 0.7_{a}^{B}$
SMISW2-TC	3.4 ± 0.2	6.4 ± 0.6	11.5 ± 0.7	16.6 ± 2.4	26.4 ± 2.3	$28.7 \pm 5.4_{a}^{A}$
ISW2-TC	3.5 ± 0.1	6.3 ± 0.3	10.3 ± 1.5	18.8 ± 3.7	24.1 ± 3.9	$12.5 \pm 1.5_{b}^{B}$

Values (mean ± S.D) followed by different subscript or superscript capital letters within a column are significantly different ($P < 0.05$). Subscript letters compare the use of a different weaning protocol for the same rearing system, while superscript capital letters compare the effect of the rearing system for the same weaning protocol.

Table 5. Growth constants.

Treatment	**Constants**		$\mathbf{r^2}$
	a	***b***	
ISW1-TC	3.0394	0.0425	0.9977
SMISW1-TC	3.6137	0.0399	0.9967
ISW2-TC	3.2482	0.0404	0.9942
SMISW2-TC	3.3708	0.0413	0.9966

Table 6. Proximal composition (lipid, protein and ash content; mg/g dry weight) and selected fatty acid composition (% total fatty acids) of whole body red porgy larvae at 12. 20. 35 and 50DAH. in trial A.

Days after hatching	**12dah**		**20dah**		**35dah**		**50dah**	
Treatment	**SMI-TA**	**SI-TA**	**SMI-TA**	**SI-TA**	**SMI-TA**	**SI-TA**	**SMI-TA**	**SI-TA**
% Lipids (dw)	14.67±0.27[a]	18.40±0.52[b]	16.77±0.12	17.22±2.10	15.07±0.85	14.50±0.60	19.21±0.57[a]	17.81±0.23[b]
% Protein (dw)	83.27±4.07	78.77±3.00	81.07±4.94	76.73±5.06	74.03±7.82	81.23±5.29	64.59±4.77	68.80±2.57
% Ash (dw)	1.64±0.32	1.65±0.34	2.07±0.23	2.21±0.46	2.75±0.29[a]	3.35±0.21[a]	3.43±0.48	3.79±0.47
16:00	15.19±1.63	17.58±0.64	17.51±0.50	17.05±0.33	21.16±0.99	18.61±0.07	16.87±0.49	17.04±0.71
16:1 n-7	5.79±0.32	6.54±0.13	5.01±0.09	6.15±0.24	2.27±0.09	3.71±0.26	5.07±0.43	5.39±0.41
18:00	9.88±0.58[a]	10.02±0.86[b]	9.78±0.21[a]	10.17±0.21[b]	8.66±0.09	7.86±0.02	7.56±0.20	7.93±0.87
18:1 n-9	11.81±0.12	12.44±0.31	12.08±0.24	12.34±0.40	10.97±0.25	10.00±0.14	15.05±0.07	15.57±0.73
18:1 n-7	5.06±0.85[a]	3.95±0.38[b]	7.94±0.47	7.37±0.17	5.45±0.27	4.58±0.37	6.69±0.63[a]	4.22±1.18[b]
18:2 n-6	7.68±0.91	9.08±0.15	5.90±0.09	5.48±0.23	10.04±0.23	8.22±0.12	6.51±0.16	7.13±0.24
18:3 n-3	3.81±0.24	4.00±0.11	2.19±0.04	1.48±0.03	5.18±0.06	8.26±0.43	8.49±0.76[a]	6.67±0.22[b]
20:1 n-9	1.28±0.08	1.53±0.31	1.19±0.41	1.14±0.01	1.46±0.10	1.71±0.01	2.15±0.24	2.58±0.09
ARA (20:4n-6)	2.21±0.04	1.92±0.19	2.74±0.04	3.68±0.10	2.05±0.07	2.72±0.11	1.41±0.21	1.38±0.05
EPA (20:5n-3)	6.83±0.93	5.56±0.14	5.90±0.09	8.66±0.16	6.36±0.55	8.84±0.24	7.88±0.14	8.32±0.28
DHA (22:6n-3)	17.87±0.62[a]	16.13±0.18[b]	16.04±0.21[a]	15.91±1.01[b]	13.41±0.41	10.55±0.19	11.79±0.14	12.20±0.40
∑ saturated [(1)]	28.28±1.43	30.58±0.35	30.49±0.85	29.87±0.13	33.97±1.22	28.68±0.02	27.83±0.13	28.61±1.47
∑ mono-unsaturated [(2)]	25.37±1.83	26.14±1.01	29.18±0.97	27.68±0.84	21.28±0.20	23.05±0.04	30.57±1.37	28.07±0.57
∑ n-3 [(3)]	32.20±0.31	28.89±0.96	26.93±0.25	29.55±1.05	28.82±0.12	31.97±0.05	31.92±1.79	32.99±1.21
∑ n-6[(4)]	11.85±0.68	12.59±0.14	10.39±0.53	11.25±0.09	14.06±0.29	12.98±0.25	8.75±0.33	9.34±0.25
∑n-9[(5)]	13.53±0.31	14.95±1.17	13.92±0.55	13.74±0.39	12.62±0.09	12.64±0.09	17.31±0.22	18.27±0.64
∑n-3HUFA[(6)]	27.70±0.84	24.23±1.12	24.18±0.34	27.91±1.01	21.62±0.28	22.47±0.51	21.74±0.91	24.24±1.10
EPA/ARA	3.08±0.38	2.92±0.38	2.15±0.00	2.36±0.02	3.10±0.17	3.25±0.05	5.70±0.95	6.04±0.00
DHA/EPA	2.66±0.49	2.90±0.10	2.72±0.04	1.84±0.14	2.12±0.25	1.19±0.01	1.50±0.01	1.47±0.00
DHA/ARA	8.08±0.40[a]	8.45±0.81[b]	5.85±0.07	4.33±0.38	6.54±0.41	3.89±0.09	8.52±1.37	8.85±0.00
oleico/DHA	0.66±0.03[a]	0.77±0.01[b]	0.75±0.01	0.78±0.07	0.82±0.04	0.95±0.03	1.28±0.01	1.28±0.09
oleico/n-3HUFA	0.43±0.01[a]	0.51±0.03[b]	0.50±0.00	0.44±0.03	0.51±0.01	0.45±0.02	0.69±0.03	0.64±0.06
n-3/n-6	2.72±0.16[a]	2.30±0.05[b]	2.60±0.11	2.63±0.11	2.05±0.04	2.46±0.05	3.66±0.34	3.53±0.10

Dw: dry weight. Values (mean ± S.D) followed by different superscript letters within a row for the same trial were significantly different (P <0.05).

Table 7. Proximal composition (lipid, protein and ash content; mg/g dry weight) and selected fatty acid composition (% total fatty acids) of whole body red porgy larvae at 12. 20. 35 and 50DAH. in trial B.

Days after hatching	**12dah**		**20dah**		**35dah**		**50dah**	
Treatment	**SMI-TB**	**IS-TB**	**SMI-TB**	**IS-TB**	**SMI-TB**	**IS-TB**	**SMI-TB**	**IS-TB**
% Lipids (dw)	17.37±0.91 [a]	20.45±1.03 [a]	16.20±1.35	14.68±0.63	15.71±0.30	17.13±1.32	17.64±1.10	16.07±0.57
% Protein (dw)	85.95±5.94 [a]	71.10±3.46 [a]	87.43±9.69	79.27±2.62	69.29±2.79	67.25±4.50	63.12±0.85	63.89±2.05
% Ash (dw)	1.69±0.25	1.64±0.18	2.27±0.13	2.12±0.10	3.23±0.11 [a]	3.48±0.22 [b]	3.70±0.22	3.43±0.14
16:0	19.47±1.10	18.12±1.26	20.14±1.94	20.87±0.88	20.54±0.46	20.96±0.43	20.60±0.09	19.88±2.12
16:1 n-7	4.55±0.53	4.98±0.47	3.98±0.01	4.03±0.42	2.69±0.01	2.93±0.30	5.23±0.04	3.14±0.65
18:0	8.59±0.30	9.87±0.36	10.86±0.12	12.21±0.25	9.31±0.37	9.81±1.26	7.59±0.25	8.42±1.17
18:1 n-9	12.81±0.18 [a]	11.25±0.09 [b]	13.27±2.16 [a]	11.70±0.69 [b]	14.37±0.23	14.76±0.30	14.65±0.71	14.59±0.46
18:1 n-7	3.61±0.20	4.24±0.22	4.28±0.16	4.77±0.13	4.78±0.31	4.62±0.39	3.17±0.48	4.37±0.63
18:2 n-6	7.79±0.10 [a]	8.57±0.16 [b]	6.16±0.74	6.14±0.35	12.34±1.06	10.62±3.06	11.24±1.05 [a]	12.57±1.64 [b]
18:3 n-3	3.16±0.10	3.47±0.34	2.27±0.69	3.25±0.21	4.80±0.25	4.43±0.88	2.36±0.03	4.42±0.90
20:1 n-9	1.12±0.05	1.18±0.07	0.95±0.11	0.97±0.04	1.54±0.01	1.65±0.26	2.27±0.22 [a]	2.11±0.64 [b]
ARA (20:4n-6)	1.79±0.01	1.82±0.03	2.30±0.18	2.05±0.02	1.77±0.16	1.91±0.43	1.32±0.27	1.53±0.32
EPA (20:5n-3)	4.58±0.17	4.59±0.13	4.82±0.63	4.37±0.05	5.23±0.34	4.94±0.31	6.86±0.65	5.37±0.81
DHA (22:6n-3)	16.92±0.47	16.30±0.25	17.27±0.68	15.98±0.15	11.52±0.41	12.74±2.26	10.95±0.19	12.04±1.06
∑ saturated [(1)]	29.56±1.38	29.40±1.65	33.10±1.89	35.30±0.60	32.18±0.13	33.05±1.04	32.00±0.70	30.62±2.81
∑ mono-unsaturated [(2)]	23.35±0.84	23.16±0.95	23.45±1.85	23.07±0.13	25.92±0.38	25.96±0.89	28.56±0.28 [a]	27.07±0.61 [b]
∑ n-3 [(3)]	28.14±0.63	27.38±0.28	27.48±2.49	26.41±0.12	24.02±1.14	24.48±1.97	22.80±1.39	24.40±1.86
∑ n-6[(4)]	11.68±0.11	12.73±0.20	10.55±0.99	10.38±0.40	15.27±0.78	13.92±2.43	13.84±0.81	15.74±1.18
∑n-9[(5)]	15.63±0.02	14.42±0.29	15.41±2.01	13.84±0.55	16.74±0.45	16.97±0.54	17.50±0.37	17.23±0.28
∑n-3HUFA[(6)]	24.36±0.62	23.37±0.13	24.63±1.72	22.51±0.16	18.33±0.87	19.30±2.59	20.05±1.34	18.94±1.99
EPA/ARA	2.56±0.09 [a]	2.53±0.11 [b]	2.09±0.12	2.13±0.00	2.95±0.07	2.66±0.51	5.29±0.60	3.66±1.23
DHA/EPA	3.70±0.04	3.56±0.15	3.61±0.34 [a]	3.66±0.07 [b]	2.21±0.07	2.58±0.45	1.60±0.12	2.26±0.15
DHA/ARA	9.48±0.24 [a]	8.97±0.02 [b]	7.53±0.28 [a]	7.80±0.15 [b]	6.52±0.36	6.74±0.62	8.53±1.62	8.16±2.31
oleico/DHA	0.76±0.03 [a]	0.69±0.01 [b]	0.77±0.16 [a]	0.73±0.05 [b]	1.25±0.06	1.18±0.19	1.34±0.04	1.22±0.13
oleico/n-3HUFA	0.53±0.02	0.48±0.00	0.54±0.13	0.52±0.03	0.79±0.05	0.77±0.10	0.73±0.01	0.78±0.09
n-3/n-6	2.41±0.07	2.15±0.01	2.60±0.02 [a]	2.55±0.10 [b]	1.58±0.15	1.81±0.46	1.66±0.20	1.56±0.20

n.d: Not detected. dw: dry weight. Values (mean ± S.D) followed by different superscript letters within a row for the same trial were significantly different ($P < 0.05$).

Table 8,9: Proximal composition (lipid, mg/g dry weight) and selected fatty acid composition (% total fatty acids) of whole body red porgy larvae at 20. 35 and 50DAH. in trial C.

Days after hatching		**20dah**		**35dah**	**50dah**	
Treatment	**SMIW1-TC**	**ISW1-TC**	**SMIW1-TC**	**ISW1-TC**	**SMIW1-TC**	**ISW1-TC**
% Lipids (dw)	15.21±1.25	16.58±3.30	17.42±1.35	18.73±1.46	19.63±5.99	17.03±0.44
16:0	17.06±0.52	17.22±0.35	15.90±1.55	18.95±5.15	19.30±0.81 [a]	20.55±0.16 [a]
16:1 n-7	4.14±0.25	3.89±0.02	3.37±0.14 [a]	2.35±0.44 [b]	5.34±0.17	5.43±0.32
18:0	9.88±0.27	10.21±0.13	8.05±0.72 [a]	10.82±1.48 [b]	6.88±0.23	7.99±0.72
18:1 n-9	13.33±0.27	12.79±0.09	15.72±0.52	15.86±0.98	11.98±0.40 [a]	14.10±1.24 [a]
18:1 n-7	5.04±0.22	4.35±0.00	4.74±0.25	4.51±0.71	3.61±0.13	3.34±0.56
18:2 n-6	6.16±0.21	7.99±0.00	7.42±0.52	6.52±1.06	10.49±0.30	11.53±1.16
18:3 n-3	4.19±0.20	2.06±0.03	6.89±0.64	8.64±1.34	2.84±0.15	1.98±0.66
20:1 n-9	1.19±0.09	1.35±0.01	1.55±0.09 [a]	1.09±0.18 [b]	1.83±0.01	2.13±0.34
ARA (20:4n-6)	2.64±0.10	2.20±0.00	2.08±0.07	1.62±0.30	1.22±0.03	1.34±0.27
EPA (20:5n-3)	6.24±0.16	4.87±0.02	6.41±0.10	4.97±0.95	8.33±0.05	7.21±0.87
DHA (22:6n-3)	15.25±0.80	17.31±0.22	14.28±0.21	10.68±1.79	13.60±0.28	12.38±2.44
∑ saturated [(1)]	29.27±0.70	30.86±0.38	26.75±2.28	32.74±7.14	30.50±1.11	32.08±0.72
∑ mono-unsaturated [(2)]	26.49±0.34	25.50±0.05	27.70±0.95	26.30±1.46	25.40±0.79	27.46±1.88
∑ n-3 [(3)]	29.98±0.14	27.83±0.22	31.47±1.06	28.44±4.53	29.10±0.36 [a]	23.99±2.43 [a]
∑ n-6[(4)]	11.05±0.48	11.94±0.05	11.13±0.48	9.74±1.43	13.12±0.04	14.22±1.04
∑n-9[(5)]	15.46±0.25	15.98±0.16	18.18±0.61	18.04±0.94	14.02±0.44	16.54±1.60
∑n-3HUFA[(6)]	24.08±0.06	24.35±0.24	22.75±0.34	17.55±3.08	23.99±0.44	21.54±2.83
EPA/ARA	2.36±0.05	2.22±0.01	3.09±0.10	3.07±0.06	6.82±0.10	5.46±0.64
DHA/EPA	2.45±0.07	3.55±0.06	2.23±0.03	2.15±0.07	1.63±0.02	1.71±0.22
DHA/ARA	5.78±0.21	7.87±0.09	6.87±0.14	6.61±0.15	11.13±0.00	9.44±2.15
oleico/DHA	0.87±0.02	0.74±0.00	1.10±0.03	1.52±0.35	0.88±0.01	1.18±0.28
oleico/n-3HUFA	0.55±0.01	0.53±0.00	0.69±0.02	0.93±0.22	0.50±0.01	0.66±0.12
n-3/n-6	2.72±0.10	2.33±0.01	2.83±0.12	2.92±0.17	2.22±0.03 [a]	1.69±0.20 [b]

n.d: Not detected. dw: dry weight. Values (mean ± S.D) followed by different superscript letters within a row for the same trial were significantly different ($P < 0.05$).

Days after hatching	20dah		35dah		50dah	
Treatment	**SMIW2-TC**	**ISW2-TC**	**SMIW2-TC**	**ISW2-TC**	**SMIW2-TC**	**ISW2-TC**
% Lipids (dw)	15.87±2.01[a]	18.00±1.47[b]	15.33±5.29	17.23±2.42	14.84±2.02	16.00±2.11
16:0	16.81± 0.14[a]	19.49±0.83[b]	17.59±0.85[a]	19.09±0.40[b]	18.82±0.33	20.33±1.28
16:1 n-7	3.99±0.16	3.54±0.08	3.17±0.09	2.56±0.13	5.43±0.28	5.26±0.03
18:0	10.13± 0.35[a]	11.34±0.37[b]	8.56±0.33	8.91±1.29	6.89±0.24	7.56±0.62
18:1 n-9	12.39± 0.42	12.05±0.05	13.67±0.28	11.84±0.22	11.81±0.41	12.15±0.73
18:1 n-7	4.60±0.09[a]	3.84±0.12[b]	3.96±0.18	3.38±0.16	3.43±0.14	3.20±0.08
18:2 n-6	7.74±0.40	7.42±0.78	8.84±0.53	14.48±2.98	11.07±0.30	11.30±0.18
18:3 n-3	2.69±0.15[a]	1.21±0.16[b]	4.11±0.48[a]	2.38±0.54[b]	2.00±0.13[a]	1.28±0.02[b]
20:1 n-9	1.19±0.08	1.15±0.19	1.47±0.02	1.64±0.35	2.02±0.04	2.14±0.57
ARA (20:4n-6)	2.41±0.04	2.24±0.06	2.21±0.10	1.78±0.33	1.17±0.03	1.20±0.02
EPA (20:5n-3)	5.59±0.06	4.40±0.10	5.95±0.13	5.39±0.51	8.61±0.35[a]	7.84±0.13[b]
DHA (22:6n-3)	16.81±0.41[a]	19.08±0.67[b]	17.38±0.25	17.71±1.32	13.97±0.42[a]	14.94±0.22[b]
∑ saturated [(1)]	29.99± 0.43[a]	34.40±1.35[b]	29.18±1.47	30.57±1.87	30.31±0.70	32.02±2.10
∑ mono-unsaturated [(2)]	25.34± 0.62[a]	23.43±0.52[b]	24.50±0.48	21.29±0.16	25.39±0.81	25.34±1.98
∑ n-3 [(3)]	28.98± 0.43	28.07±0.87	31.00±0.88[a]	28.50±0.47[b]	28.86±0.13[a]	27.61±0.29[b]
∑ n-6[(4)]	12.19± 0.32	11.45±0.96	12.58±0.51[a]	17.35±2.50[b]	13.32±0.22	13.37±0.18
∑n-9[(5)]	14.70± 0.36	14.14±0.24	16.08±0.31[a]	14.19±0.30[b]	13.93±0.35	14.29±1.30
∑n-3HUFA[(6)]	24.85± 0.52	25.45±0.93	25.30±0.36	24.84±0.93	24.68±0.10	24.69±0.32
EPA/ARA	2.32±0.03[a]	1.96±0.01[b]	2.69±0.08	3.13±0.78	7.39±0.46[a]	6.51±0.12[b]
DHA/EPA	3.00±0.06[a]	4.34±0.10[b]	2.92±0.06	3.32±0.58	1.63±0.12[a]	1.90±0.03[b]
DHA/ARA	6.98±0.07[a]	8.53±0.19[b]	7.87±0.29[a]	10.10±1.02[b]	11.97±0.15[a]	12.40±0.02[b]
oleico/DHA	0.74±0.04[a]	0.63±0.02[b]	0.79±0.01	0.67±0.06	0.85±0.05	0.81±0.05
oleico/n-3HUFA	0.50±0.03	0.47±0.02	0.54±0.00	0.48±0.03	0.48±0.02	0.49±0.03
n-3/n-6	2.38±0.10	2.47±0.27	2.47±0.12[a]	1.67±0.29[b]	2.17±0.04[a]	2.06±0.04[b]

n.d: Not detected. dw: dry weight. Values (mean ± S.D) followed by different superscript letters within a row for the same trial were significantly different ($P < 0.05$).

List of figures.

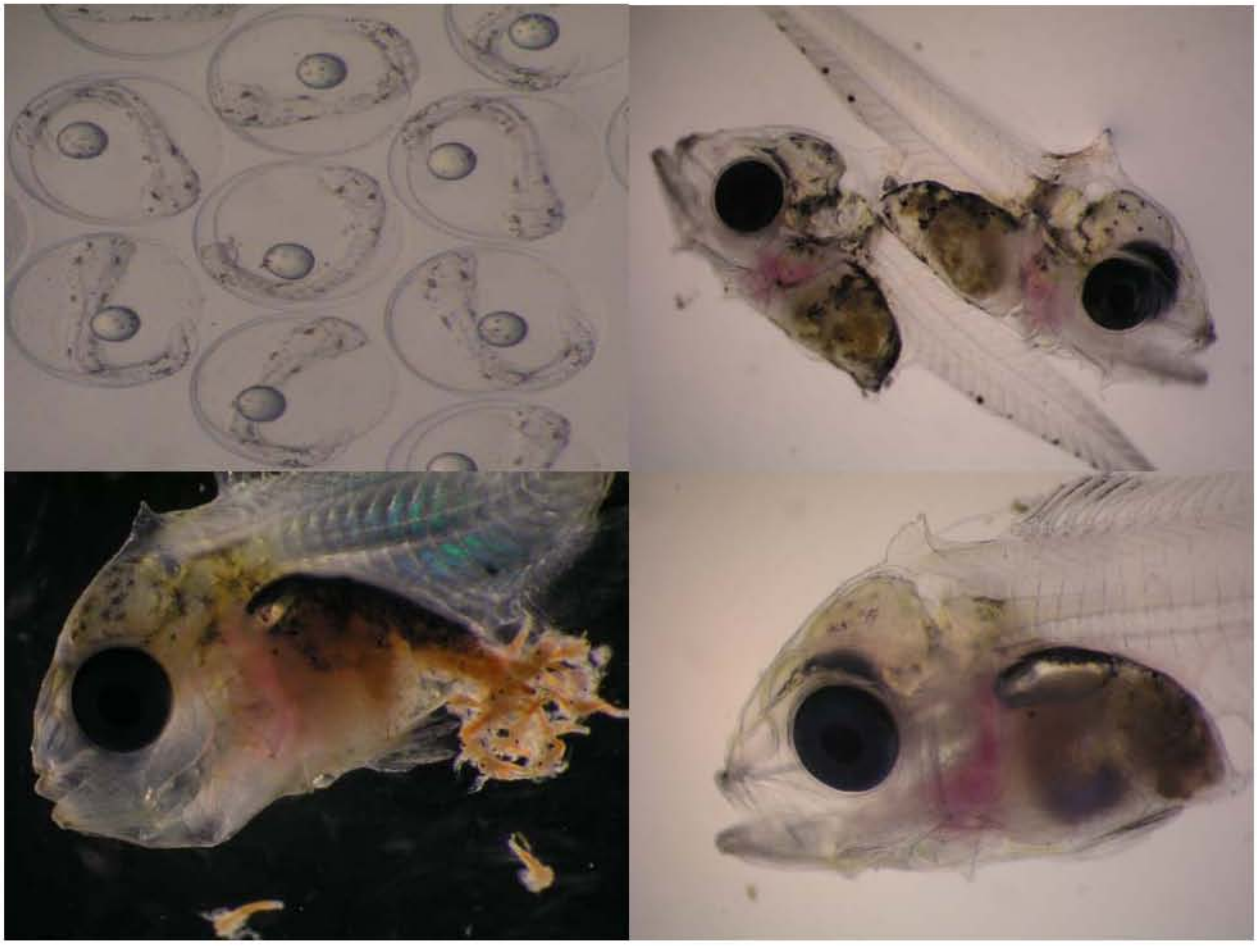


Figure 1. Different larval stages of red porgy; eggs, rotifers fed larvae; *Artemia* fed larvae and early weaned larvae in trial C.

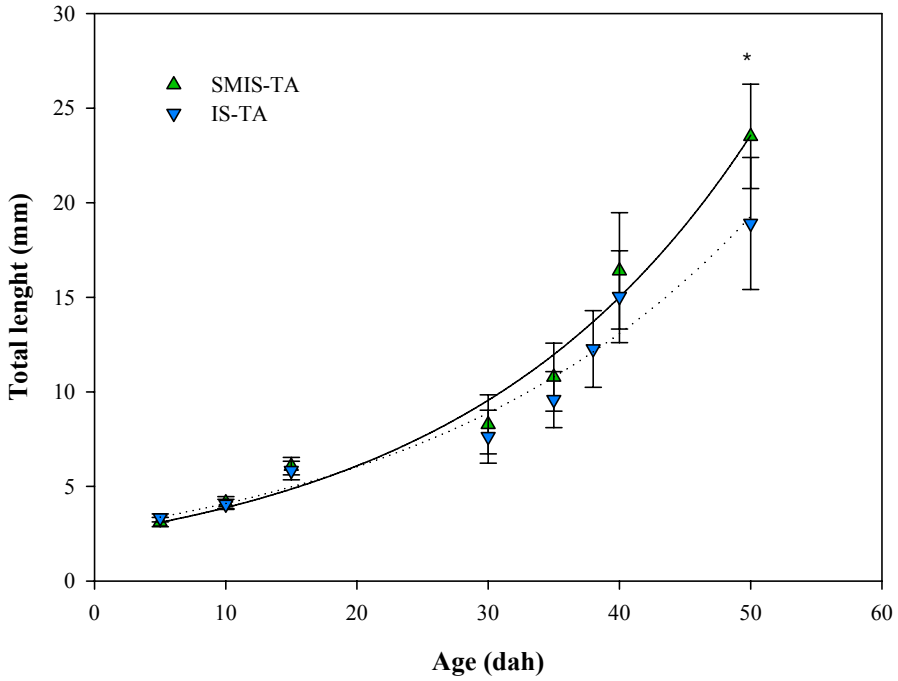


Figure 2. Total length evolution (TL) of red porgy (*Pagrus pagrus*) larvae culture under different rearing systems in Trial A. (*) shows significant differences. Larval growth followed an exponential equation. ($TL = 2.4677\ e^{0.0451d}$; $r^2 = 0.9939$ in semi-intensive system and $TL = 2.7639\ e^{0.0388d}$; $r^2 = 0.9903$ in the intensive system. where TL= total length (mm) and d= age (days after hatching)).

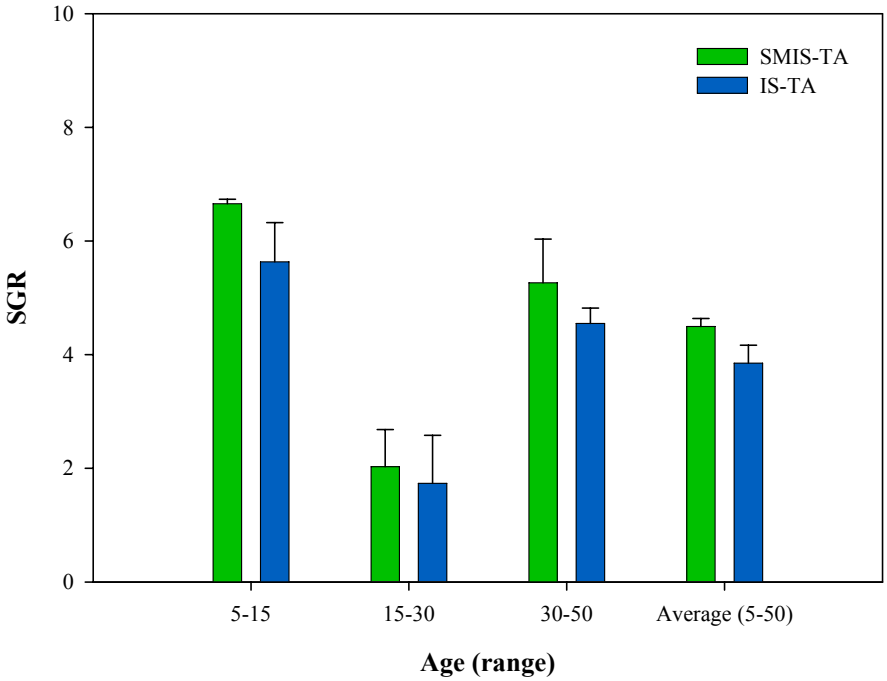


Figure 3. Specific growth rate evolution according to the rearing system in trial A.

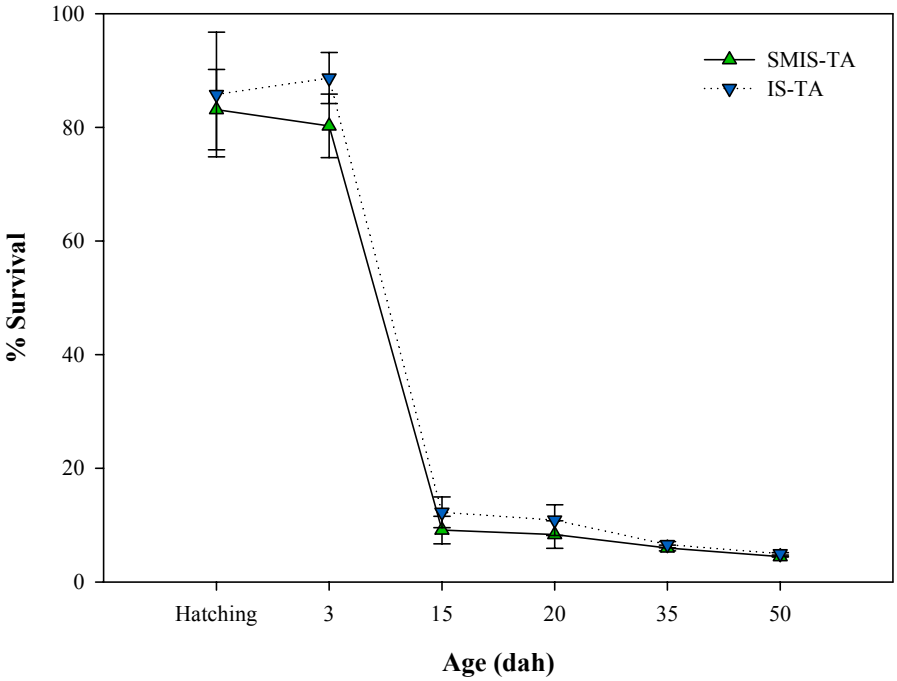


Figure 4. Survival evolution according to the rearing system in trial A.

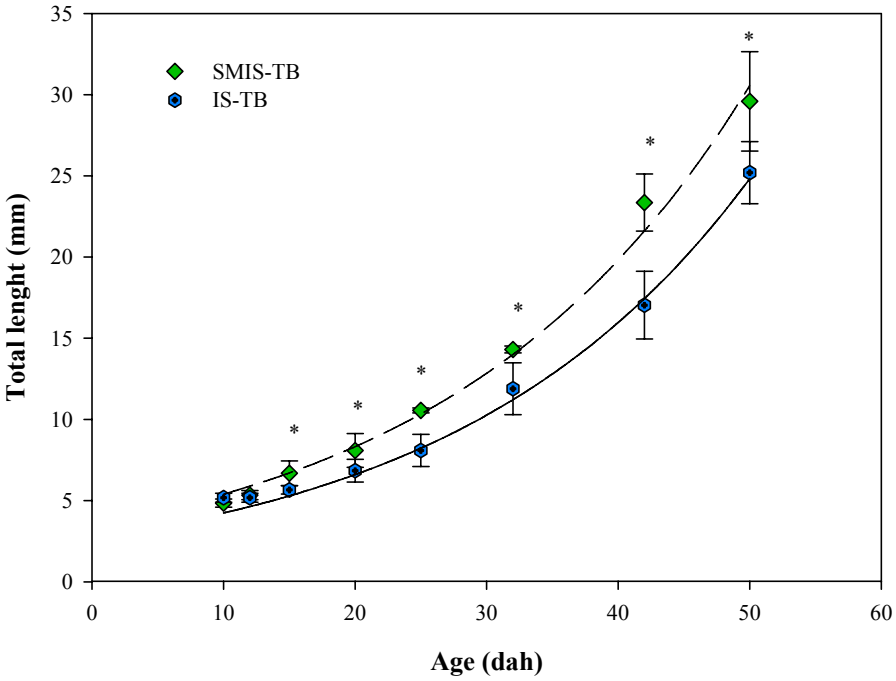


Figure 5. Total length evolution (TL) of red porgy (*Pagrus pagrus*) larvae culture under different rearing systems in trial B. (*) shows significant differences. Larval growth follows and exponential equation. TL = 3.5464 e $^{0.0392d}$; r^2 = 0.959 in Semi-intensive system and TL = 3.0267 $e^{0.0886d}$; r^2 = 0.999 in the intensive system. where TL= total length (mm) and d= age (days after hatching).

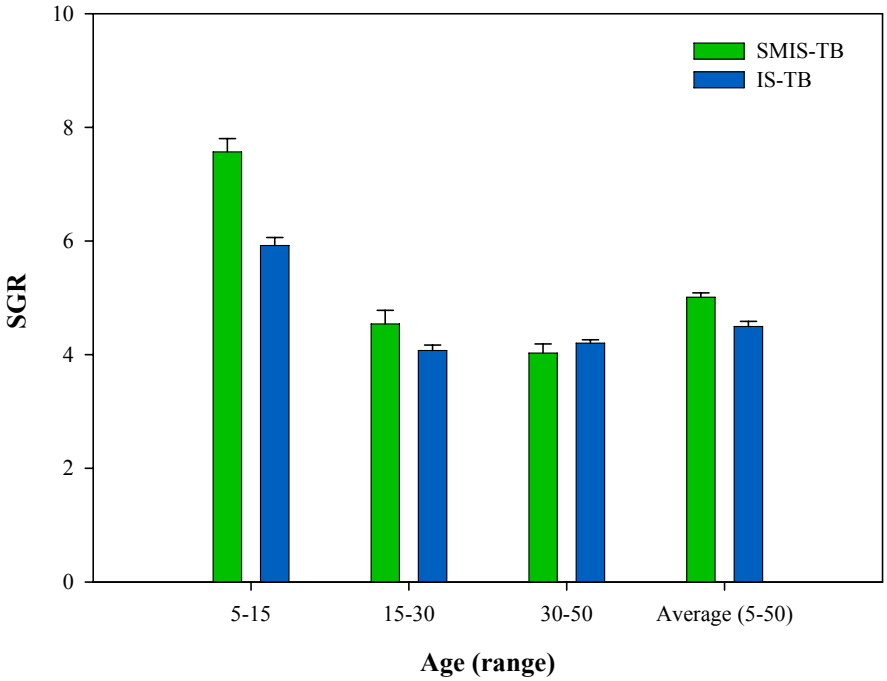


Figure 6. Specific growth rate evolution according to the rearing system in trial B.

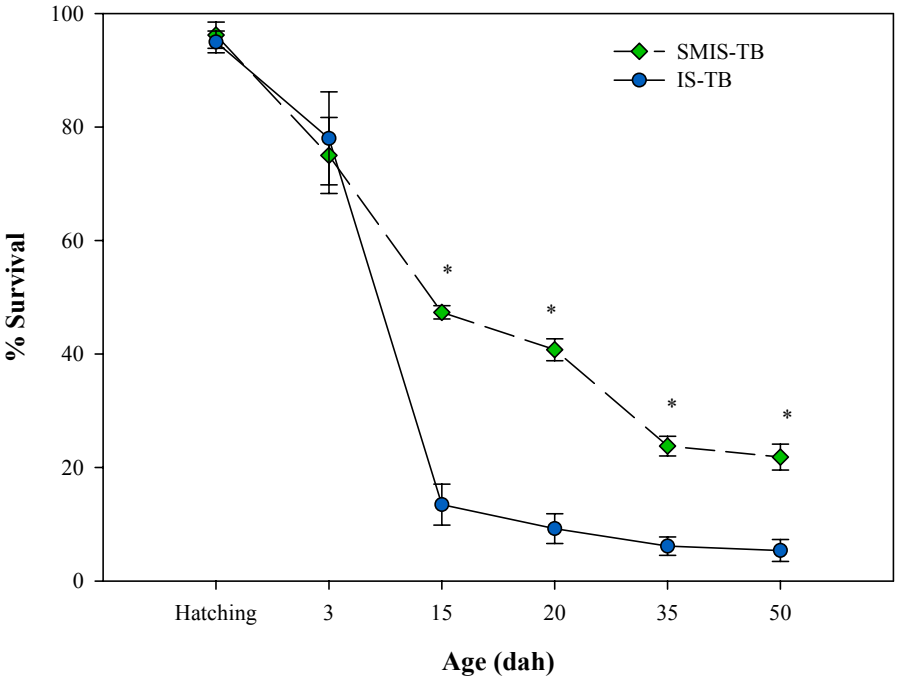


Figure 7. Survival evolution according to the rearing system in trial B.

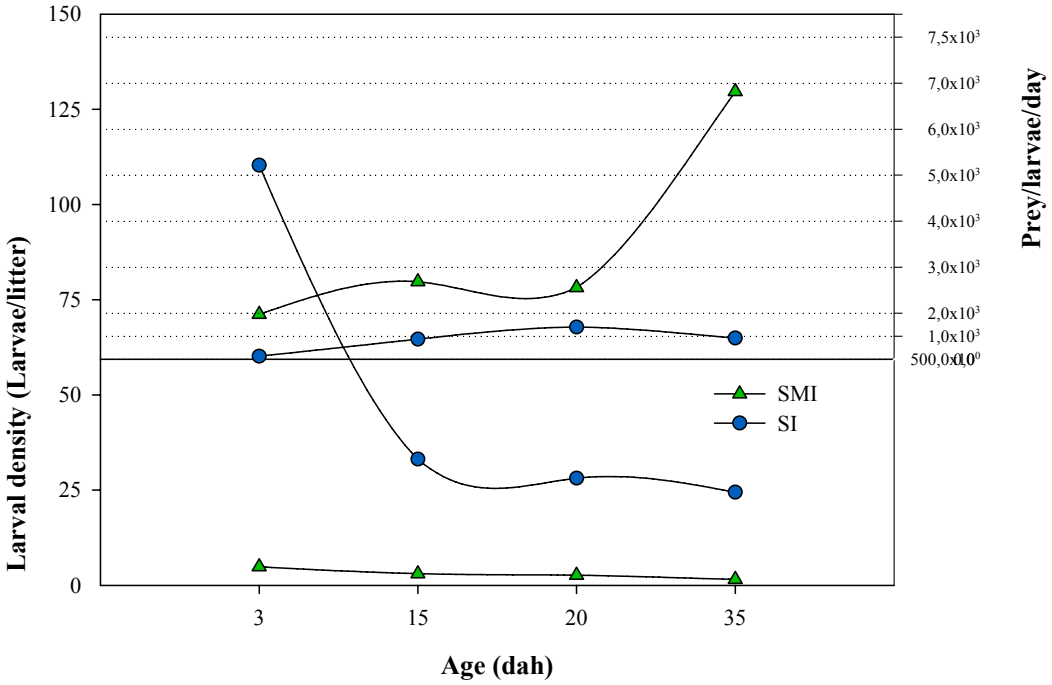
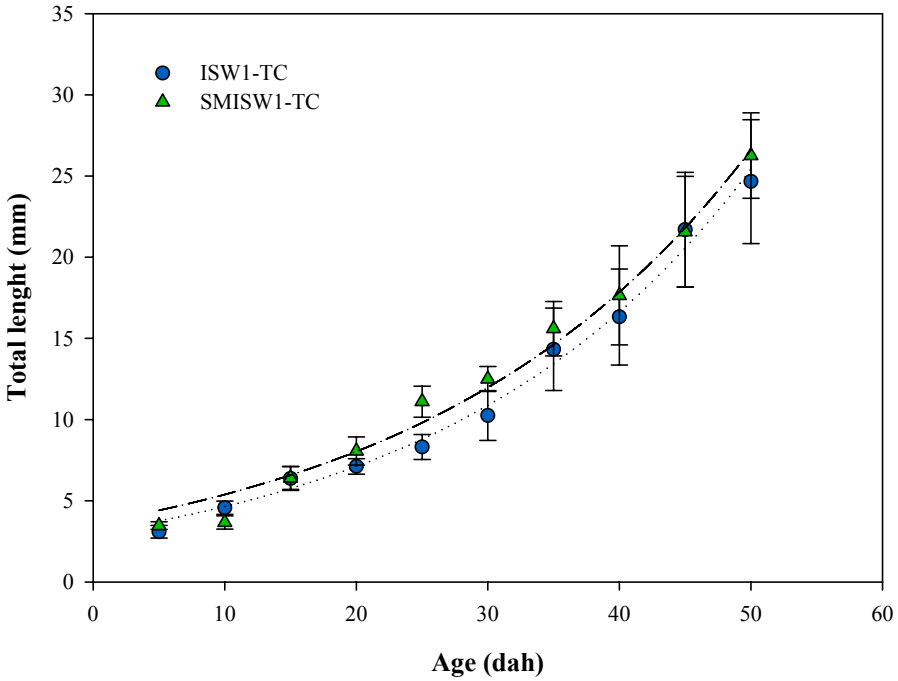


Figure 8. Estimated larval density evolution and available live prey (rotifers+ *Artemia*) per day according to the rearing system.



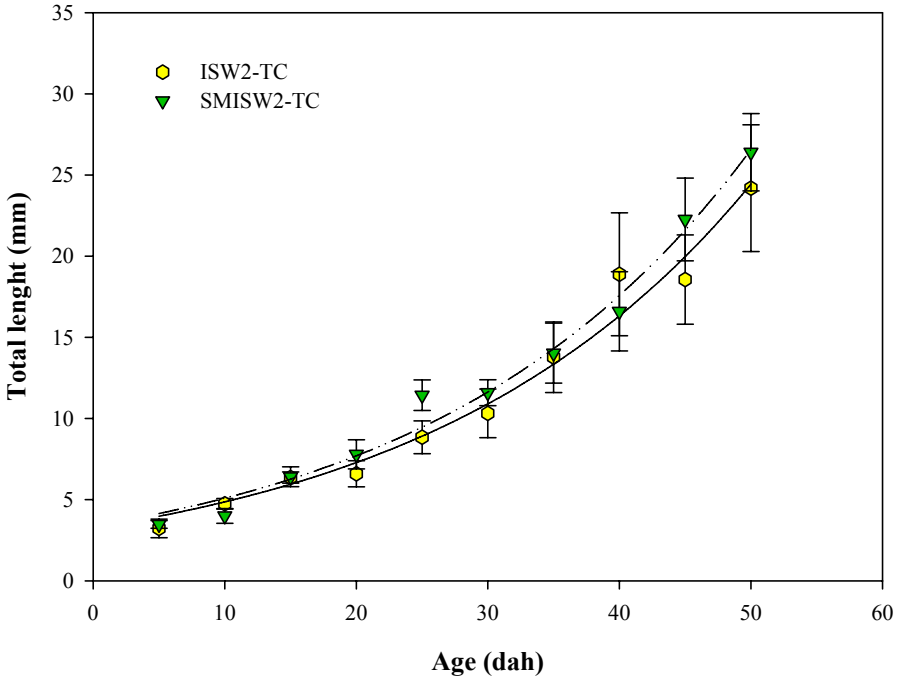
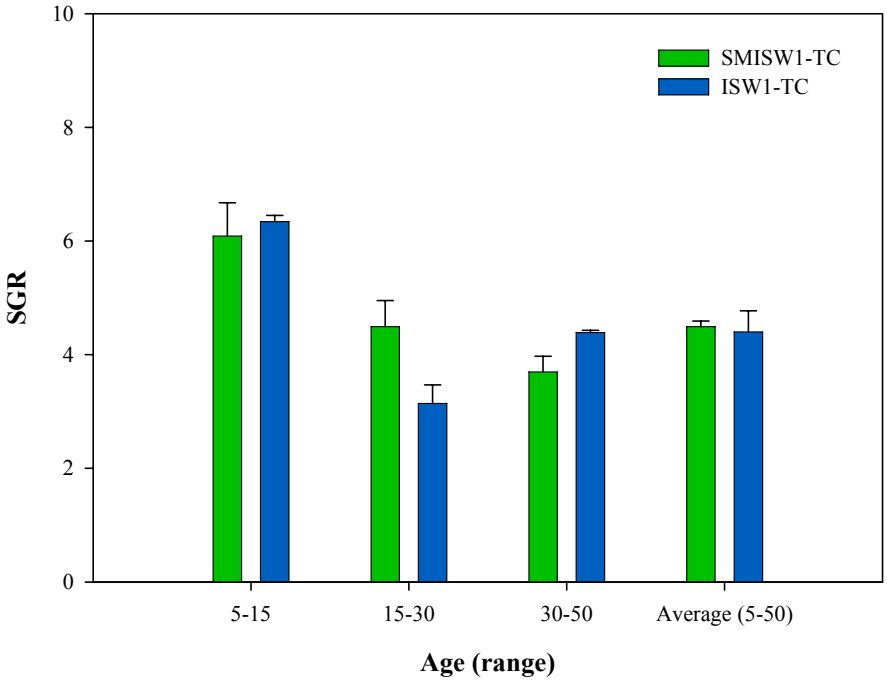


Figure 9a,b. Total length evolution (TL) of red porgy (*Pagrus pagrus*) larvae culture under different rearing systems and weaning protocols in trial C. (*) shows significant differences. Larval growth. fits to an exponential equation. TL= a*exp ^{bd} where TL= total length (mm). d= age (days after hatching) and a.b were constants (Table IV).



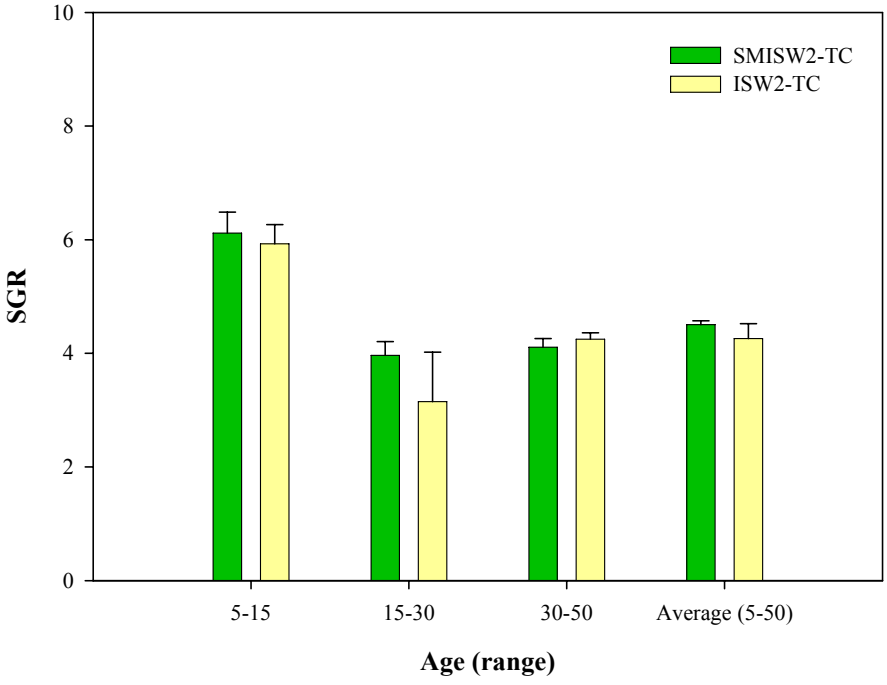
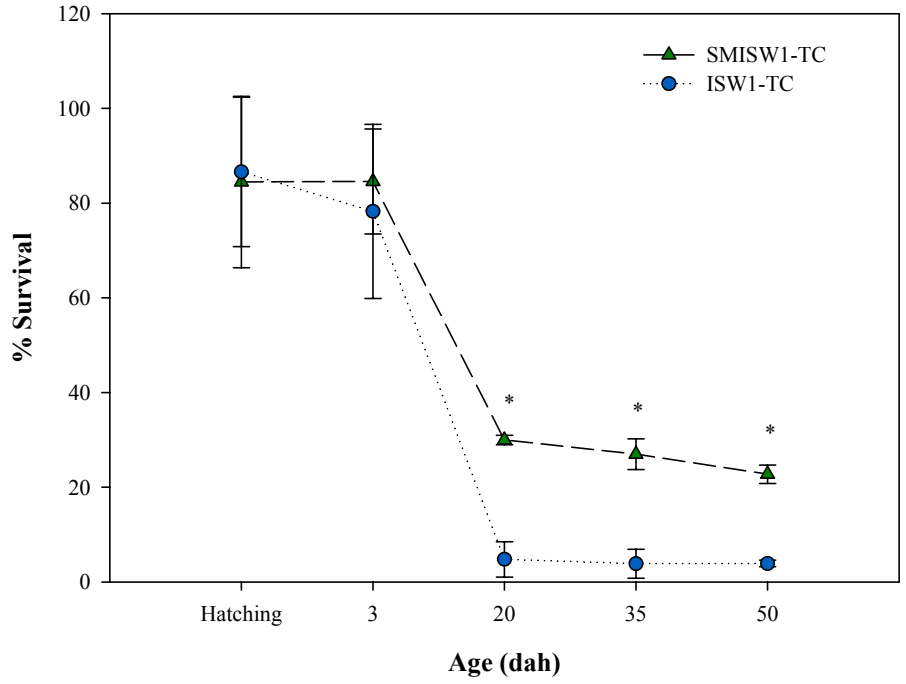


Figure 10a,b. Specific growth rate evolution according to the rearing system and weaning protocol in trial C.



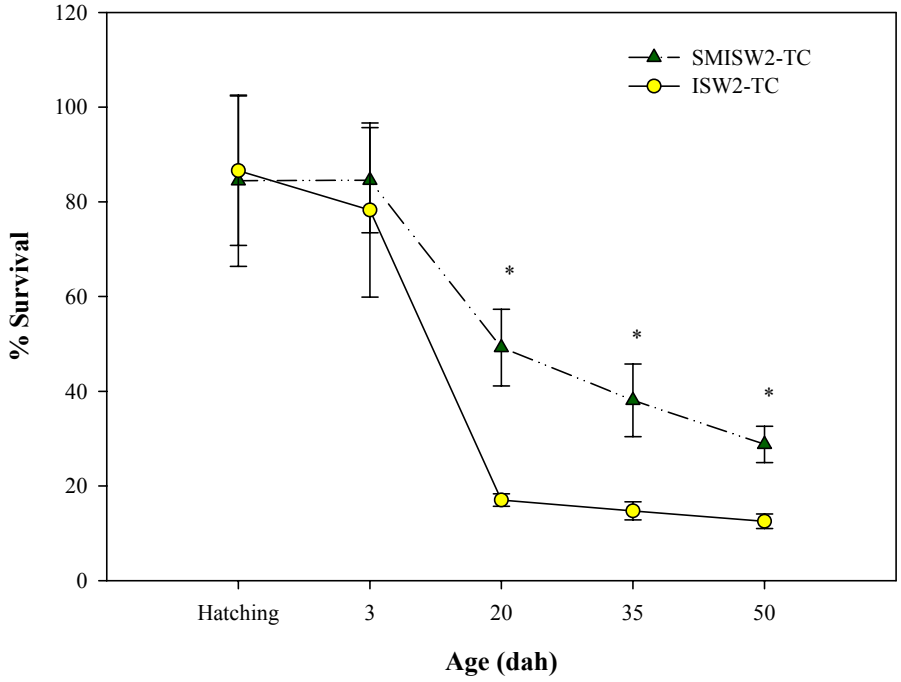


Figure 11a,b. Survival evolution according to the rearing system and weaning protocol in trial C.

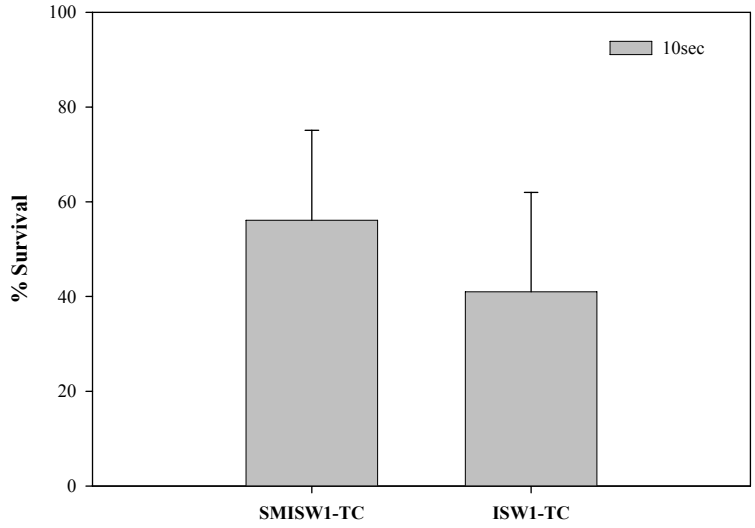


Figure 12. Survival 24h after activity test (10seconds air exposure) of 20 old day red porgy larvae according to the rearing system in weaning protocol 1.

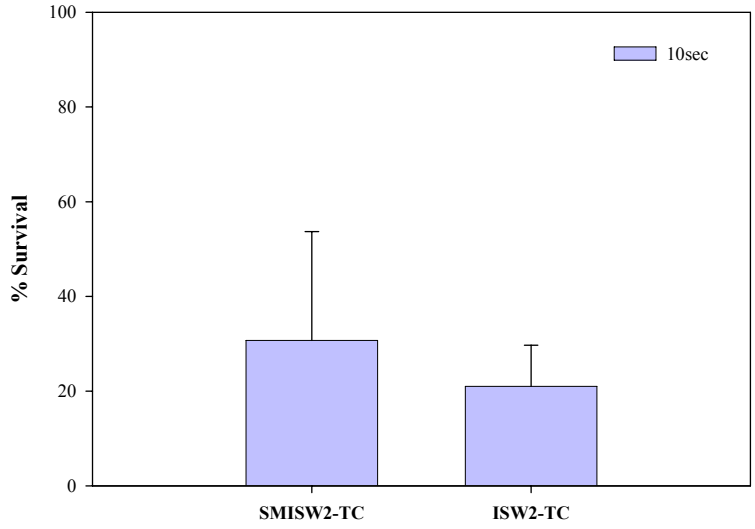


Figure 13. Survival 24h after activity test (10seconds air exposure) of 20 old–day red porgy larvae according to the rearing system in weaning protocol 1.

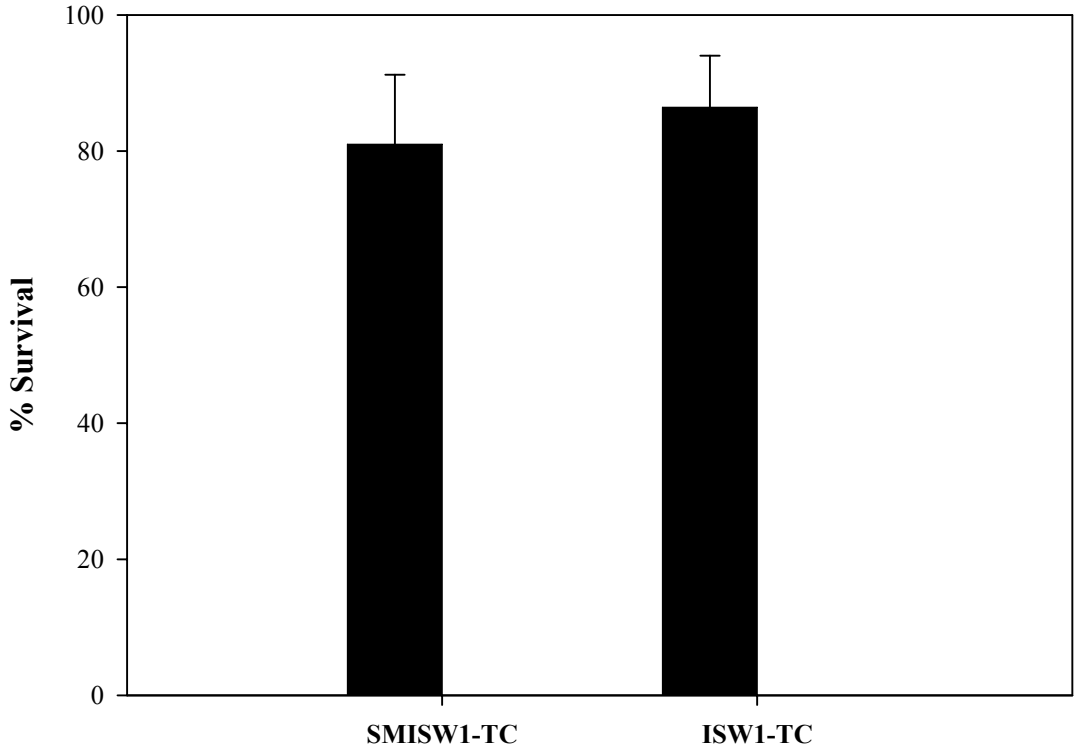


Figure 14. Survival 24h after activity test (1 minute air exposure) of 30 old–day red porgy larvae, according to the rearing system in weaning protocol 1.

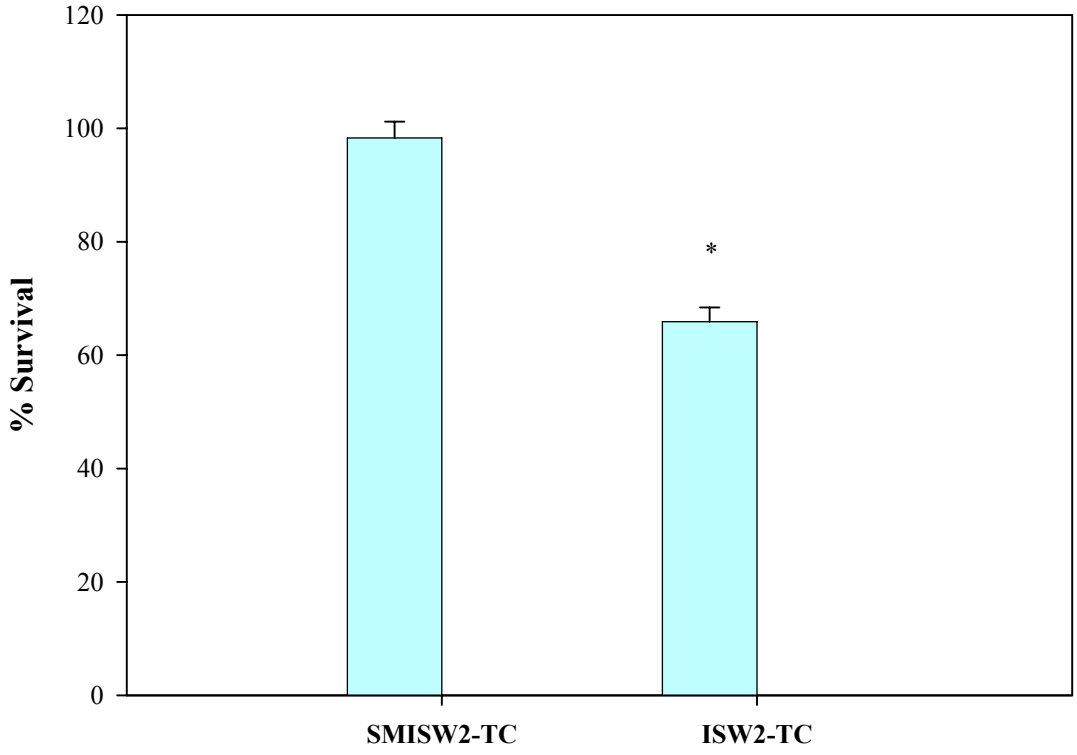
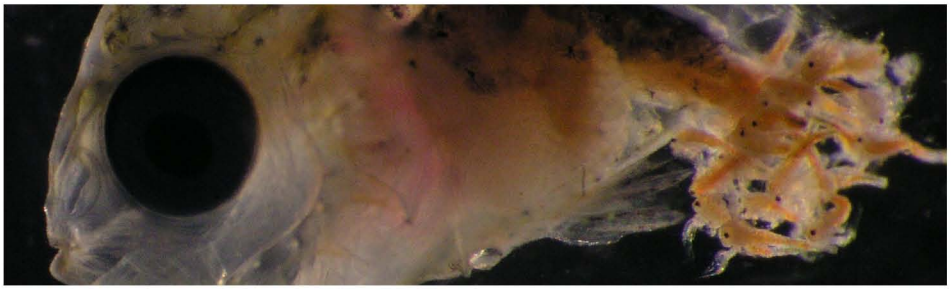


Figure 15. Survival 24h after activity test (1 minute air exposure) of 30 old–day red porgy larvae according to the rearing system in weaning protocol 2.

Improvements in the production technology of red porgy (*Pagrus pagrus*) larvae and fry: Importance of rearing conditions and diet nutritional value on their quality

CONCLUSIONS



8.-CONCLUSIONS

Study I. **Development of red porgy *Pagrus pagrus* (Linnaeus, 1758) visual system in relation with changes in the digestive tract and larval feeding habits.**

1. Red porgy larvae are visual feeders that hatch with an incomplete and non functional visual and digestive system.

2. Along the third and fourth days post-hatch, major structural changes took place in the visual and digestive system such as definition and pigmentation of the primary photoreceptors (cones) that are in coincidence with mouth opening and first digestive activity detection in the midgut, meaning that the lavae are ready to start exogenous feeding.

3. The second most important change in relation to visual system development was detected around 20 dah (7.0-7.5mm TL) when a new type of photoreceptors nuclei appeared (rods). From this moment *Pagrus pagrus* larvae had a duplex retina with cones and rods.

4. Rods pigmentation is in coincidence with the detection of the first gastric cells and the progressive migration of the larvae from shallow to deep water, suggesting changes in feeding habits and culture conditions at this stage.

Study II. **Osteological development and occurrence of skeletal deformities in red porgy *Pagrus pagrus* (Linnaeus, 1758) larvae cultured under different rearing techniques.**

5. The general pattern of the osteological development for red porgy was not affected by the culture system. However, timing of skeletogenesis processes was linked to larval size rather than age and differed between the rearing systems. Hence, ossification was earlier completed in semi-intensive system, which showed higher growth rates.

6. A high incidence of morpho-anatomical deformities was registered on histological and x-ray studies in this species, regardless the system employed. Lordosis and fused vertebrae were the most frequent skeleton anomalies, whereas operculum deformities were low,

probably due to the presence of long opercular spines from early stages preventing operculum folding into the gill chamber. Lordosis appeared in the vertebral region supporting the highest muscle pressure during swimming (8th and 12th vertebra).

7. Culture intensification affected vertebral fusions localization, which in the intensive system appeared along the whole vertebral column and, particularly, in the caudal region, while in the semi-intensive system appeared mainly in the pre-hemal vertebrae.

8. Culture intensification caused a higher incidence of individuals with an extra vertebra, kyphosis and cranial abnormalities mainly registered as upper-jaw reduction and cross bite jaw.

Study III. Effect of DHA content in rotifers on the occurrence of skeletal deformities in red porgy *Pagrus pagrus* (Linnaeus, 1758).

9. The DHA requirement of red porgy larvae for maximal growth was lower than 1.9%. DPA (22:5n-6) was easily accumulated in red porgy larval tissues when this fatty acid was present in the rotifers and a lower survival was found in these larvae, what could be related to the high incorporation of DPA in larval neural and visual tissues membranes affecting their functioning as described in other vertebrates.

10. Dietary DHA contents were associated to high survival and a reduction of deformed fish, regardless the type of deformity indicating the importance of this FA in osteological development.

***Study IV*. Advances in rearing techniques of red porgy *Pagrus pagrus*, (Linnaeus, 1758): Comparison between intensive and semi-intensive larval rearing systems.**

11. Major fatty acids in whole body of red porgy larvae were 22:6n-3, 16:0, 18:1n-9, 18:0, 18:2n-6, 20:5n-3, 16:1n-7,18:1n-7, 18:3n-3 and 20:4n-6.

12. DHA is selectively accumulated in red porgy larvae even when it is present in low amounts in the live preys, denoting the importance of this fatty acid for this species.

However, larval EPA contents reflect dietary levels and ARA remains constant along ontogeny.

13. In the development of red porgy three different periods can be identified: a first one (5-15 dah) with high growth rates and very sensible to mortalities; a second period (15-30dah) related to completion of metamorphosis with lower growth rates and better control of mortalities; and a third post-metamorphic one (30-50dph) with increased growth rates and high survival.

14. During the first period, increase in prey density in semi-intensive system from 1-2 rot.ml^{-1} to 4-5 rot.ml^{-1} markedly decreased mortalities and improved growth rates.

15. During the second period, increasing the length of rotifer feeding from 20 to 30 dah improved growth rates, facilitating adaptation to feeding changes.

16. During the third period, mortalities seemed to be related only to the cannibalistic behaviour of this species.

17. This species was found to be very voracious in preying *Artemia*, filling the gut, reducing intestinal transit and digestion of *Artemia*, which was even defecated alive.

18. Overall, red porgy grows much faster than other sparids such as *Sparus aurata* doubling the size of the juveniles of the later at 95 dah.

19. In semi-intensive systems, a longer duration of the yolk reserves was observed, as denoted by the greater accumulation of DHA in this larvae, which in turn showed a better resistance to activity test. Indeed, this species under intensive culture conditions seems to be very sensitive to handling and stress.

20. In intensive system, early co-feeding protocol markedly increased survival, larvae being able to adapt to the inert diet more rapidly than in the semi-intensive ones, as showed by their fatty acid composition.

21. The main difference in the larval culture success between both systems for this species still remains in the rotifers feeding period, when the density of larvae is reduced in a 86% in intensive system and in a 57% in the semi-intensive system at 15 dah, leading to a higher survival rate in the semi-intensive system in relation to a better utilization of prey nutrients under more favorable environmental conditions and hence, lower energy demands. The most important technical differences between the intensive and semi-intensive systems were related to prey availability, water turnover, green water condition and tank management.

22. The results of this study allowed the improvement in the rearing protocols for red porgy, increasing the final total length and survival at 50dah from the initial trial to the last ones from 18.9 mm to 25.13 mm and from 4.9 to 12.5% in the intensive system and from 23.52 to 26.4 mm and 4.4 to 28.7% in the semi-intensive system. At present, the best larval rearing protocol to sustain a regular and predictable red porgy fingerlings demand is the semi-intensive system technology.

Improvements in the production technology of red porgy (*Pagrus pagrus*) larvae and fry: Importance of rearing conditions and diet nutritional value on their quality

SPANISH SUMMARY

Mejora en la tecnología de producción de larvas y alevines de bocinegro (Pagrus pagrus): Importancia de las condiciones de cultivo y el valor nutritivo de la dieta en su calidad

9.1.-Resumen

El objetivo principal de este trabajo fue **"la mejora de la tecnología de producción de larvas y alevines de bocinegro".** Los objetivos específicos se dividen en dos etapas consecutivas que se complementan, una primera parte del trabajo en la que se describen aspectos específicos sobre la biología y desarrollo de las primeras etapas de vida de esta especie, seguida de una segunda parte donde el conocimiento adquirido se aplicó al desarrollo de un protocolo estándar para la producción comercial de esta especie.

En este sentido, se ha podido determinar que las larvas de bocinegro son predadores visuales, que eclosionan con un sistema visual y digestivo incompleto y no funcional. Entre el tercer y cuarto día post eclosión se producen los mayores cambios en el sistema visual, con la aparición de pigmentación en los fotoreceptores primarios (conos) y apertura de la boca, lo que capacita a la larva para comenzar su alimentación exógena. Igualmente en este momento se detecta la primera actividad digestiva a la altura del intestino medio. El segundo hito mas importante en el desarrollo del sistema visual se detecta en torno a los 20dpe, cuando aparecen los núcleos de los segundo fotoreceptores (bastones) que dotan a las larvas de una mayor agudeza y sensibilidad visual, permitiéndole predar en condiciones de una menor intensidad lumínica, estos cambios coinciden con la detección de las primeras células gástricas y la progresiva migración de las larvas desde las capas superficiales a mayor profundidad en la columna de agua, lo que permite determinar la necesidad de un cambio de alimentación y condiciones de cultivo en esta etapa.

Por otro lado, se ha determinado la influencia del sistema de cultivo en el desarrollo de las larvas, que se refleja en un menor crecimiento de las mismas a partir de la segunda semana de vida, cuando se cultivan en sistemas intensivos frente a los sistemas semi-intensivos. Este hecho se traduce a su vez, en un efecto sobre el desarrollo osteológico de las larvas, ya que este, se encuentra más ligado a la talla de la larva que a la edad de la misma. En este sentido, se observaron larvas en un estadio de osificación más avanzado en los sistemas semi-intensivos que en los intensivos para una misma edad larvaria.

Igualmente, se han identificado diferentes patrones de crecimiento a lo largo del periodo de cultivo divido en tres etapas principales, las cuales difieren dependiendo del sistema de cultivo empleado. Así una primera etapa (5-15dpe) se caracteriza por ser una fase de rápido crecimiento y elevada mortalidad, una segunda etapa (15-30dpe) relacionada con el final de la metamorfosis donde se produce un fase de ralentización del crecimiento y un mejor control de la mortalidad,

finalmente un tercer periodo post-metamórfico (30-50dpe) considerado como un periodo de recuperación en el sistema intensivo, con un aumento de las tasas de crecimiento y elevada supervivencia. En general el crecimiento del bocinegro es mucho más rápido que otros espáridos como la dorada, llegando a doblar el peso de los juveniles de esta especie a los 95dpe.

En cuanto a las anomalías merísticas, la intensificación del sistema no presenta un efecto sobre el número de costillas pero si sobre el número de vértebras de las larvas, con una mayor incidencia de individuos que presentan una vértebra extra cuando se producen en sistemas intensivos. En cuanto a la incidencia de deformidades esqueléticas, de manera general se pudo determinar, la existencia de una clara influencia de la intensificación sobre la aparición de un menor número de larvas normales. De manera particular el efecto intensificación se ve más claramente manifiesto en un aumento de las deformidades craneales y aparición de cifosis. Mientras que este factor no tiene un efecto significativo sobre las deformidades de columna, tipo fusiones vertebrales o la aparición de lordosis, con diferencia las anomalías que más afectan a esta especie con valores medios en torno al 15%. Sin embargo, se pudo observar un efecto del sistema de cultivo sobre la localización de las fusiones vertebrales. Así en las larvas cultivadas en sistemas intensivos hay una clara predisposición a localizarlas en la zona caudal, mientras que en el sistema semi-intensivo se localizan en la zona pre-hemal. En este sentido, los resultados alcanzados han verificado que en las condiciones de cultivo aplicadas las larvas de bocinegro presentan una elevada incidencia de deformidades morfo-anatómicas, que condiciona de manera determinante la rentabilidad de su producción. Con el fin de verificar la existencia de factores nutricionales que pudieran influenciar el desarrollo de las deformidades con mayor impacto en esta especie, en las etapas tempranas de cultivo, se realizo una experiencia de cría donde se planteó un estudio comparativo de producción larvaria de bocinegro alimentado con rotíferos enriquecidos con diferentes emulsiones enriquecedoras en condiciones de cultivo intensivo. Los resultados de dicho estudio permiten concluir que la alimentación desde la fase de rotíferos y en concreto un suplemento de ácidos grasos esenciales, son determinantes para reducir la incidencia de deformidades esqueléticas en esta especie. Así el aumento de los niveles de DHA, en el rotífero a pesar de no modificar el crecimiento de las larvas si afecta, a la composición bioquímica de la larva, mejorando la supervivencia y reduciendo casi en un 50% la incidencia de deformidades esqueléticas. Por otro lado, la presencia de concentraciones elevadas de otros ácidos grasos como el DPA (22:5n-6) podría tener consecuencias negativas sobre la supervivencia larvaria en esta especie.

Así mismo, a lo largo de los diferentes estudios se ha podido identificar los ácidos grasos más abundantes en las larvas del bocinegro: 22:6n-3, 16:0, 18:1n-9, 18:0, 18:2n-6, 20:5n-3, 16:1n-7,18:1n-7, 18:3n-3 and 20:4n-6 y su evolución de acuerdo al crecimiento y sistema de cultivo empleado. Los niveles de DHA en el tejido de las larvas, entre los días 12-20 (15-20%), son netamente superiores a los aportados en la dieta cuando se utilizan emulsiones enriquecedoras comerciales (9-10%), lo que sugiere una acumulación selectiva de este AGE. Las mejoras en supervivencia observadas en el sistema semi-intensivo podrían estar relacionadas con una mayor acumulación de DHA en estas larvas como consecuencia de un mejor aprovechamiento y duración de las reservas vitelinas de las larvas cultivadas en este sistema, las cuales además mostraron una mayor resistencia al test de actividad. De hecho, las larvas cultivadas en el sistema intensivo presentaron una elevada sensibilidad al manejo y el stress.

Los resultados de este trabajo han permitido mejorar los protocolos de cultivos larvario de bocinegro, incrementando la talla total final y la supervivencia a los 50dpe desde las primeras experiencias hasta la últimas de 18.9 mm a 25.13 mm y desde 4.9 hasta 12.5% en el sistema intensivo y de 23.52 a 26.4 mm y desde 4.4 hasta 28.7% en el sistema semi-intensivo.

En cuanto a la zootecnia de cría del bocinegro, se pueden extraer diferentes recomendaciones para su cultivo: en cuanto a la secuencia alimentaria, los resultados obtenidos sugieren una densidad de rotíferos de 5 $rot.ml^{-1}$ para el sistema de de cultivo semi-intensivo y una extensión de la alimentación con rotífero desde los 20 a los 30dpe que mejora la adaptación a los cambios de alimentación. Además, la elevada voracidad que presenta sobre la *Artemia*, la cual incluso llega a defecar viva, sugiere la aplicación de un protocolo de co-alimentación y destete temprano, iniciado desde los 12-15dpe acompañado de una reducción en la cantidad de *Artemia* y que conduce a la mejora de la supervivencia y crecimiento de las larvas. Igualmente se sugiere el uso de fotoperiodo continuo durante los primeros 20 días pasando a un sistema con fotoperiodo reducido 12:12 e iluminación natural en las etapas posteriores para contrarrestar los cambios en las técnicas de agua verde y reducir el estrés de las larvas en esta etapa.

Con la información generada, se puede concluir que el mejor protocolo de cultivo para sostener una demanda regular y predecible de alevines de bocinegro con un buen rendimiento biológico y aplicable a nivel comercial, es el sistema semi-intensivo. Sin embargo, la calidad de los alevines está seriamente comprometida aun con el uso este tipo de sistema, debido a la elevada incidencia de deformidades esqueléticas, si bien este problema puede ser mitigado con el uso de enriquecedores de rotíferos formulados acorde con los requerimientos de esta especie.

9.2.-Introducción

2.1. –La acuicultura en el mundo.

La acuicultura tiene una antigüedad de más de 4.000 años, sin embargo, su contribución significativa en la alimentación humana, es mucho más reciente. La acuicultura ha experimentado un enorme crecimiento en los últimos 30 años. Así, la producción mundial de pescado, crustáceos y moluscos de acuicultura ha pasado de constituir el 3,9% de la producción pesquera total en 1970 a ser de 43% en 2004, con una previsión de que alcance el 50% en el 2025 (FAO, 2008). Así desde 1970, la producción acuícola es el sector productivo que más ha crecido con un 8,7% anual frente al 1,2% en la producción pesquera o el 2,8% de la producción cárnica de animales terrestres. Ante esta situación, según la FAO, la acuicultura se presenta como una alternativa factible para complementar el grado de abastecimiento de la pesca extractiva y satisfacer la enorme demanda existente de productos del mar, permitiendo mantener las actuales proporciones de pescado en la dieta. Según la FAO, en el año 2004 el consumo mundial de pescado fue de 16,6 kg/persona/año, siendo en la actualidad España uno de los primeros consumidores de pescado a nivel mundial con 36,6kg/persona/ año (MAPA, 2007). Sin embargo, nuestro suministro depende de una flota pesquera, que desde hace años no puede conseguir las capturas necesarias para abastecer el mercado. La clara sobreexplotación a la que han sido, sometidos los diferentes caladeros de pesca tradicionales, y las restricciones impuestas por los cambios en la política de acuerdos para el uso de caladeros internacionales hace que exista un desabastecimiento crónico de estos productos, lo que se traduce en unos elevados niveles de importaciones. Es precisamente este gran aumento en la demanda actual de alimentos de origen marino el que hace de la acuicultura un sector empresarial en expansión con un gran atractivo para los inversores, siendo a su vez una alternativa para las nuevas generaciones como fuente de actividad laboral, al dar empleo actualmente a más de 12 millones de personas en el mundo (Apromar, 2008).La definición de acuicultura como sector complementario a la pesca extractiva en Europa se refiere a un tipo de acuicultura de producción intensiva con un alto grado de especialización donde se producen peces, moluscos, crustáceos o plantas de forma controlada. A nivel mundial, en el año 2006, se contabilizaron más de 250 especies de animales y plantas cultivadas, siendo los peces el grupo mayoritario en diversidad, con casi un 50% de las especies cultivadas (Apromar, 2008). En el caso concreto de la acuicultura Europea, las principales especies que se producen, son peces y moluscos de alto valor comercial. Sin embargo, se trata de un sector que sigue basado en la explotación de un reducido número de especies, cuyas técnicas

de cultivo han podido establecerse gracias al esfuerzo dedicado tanto al trabajo empírico, como a la investigación aplicada al desarrollo tecnológico.

En el caso particular de los peces marinos (excluyendo Salmónidos) la producción en los ocho últimos años ha experimentado un importante aumento pasando de poco mas de 125.000 toneladas en el año 2000, concentradas en el cultivo intensivo de 3 especies fundamentalmente, dorada (*Sparus aurata*), lubina (*Dicentrarchus labrax*) y rodaballo (*Psetta máxima*) y se incluyen también las producciones de bacalao (*Gadus morhua)*, corvina (*Argyrosomus regious)* y el engorde de atún Atlántico *(Thunnus thynnus)***,** a algo más de 250.000 toneladas estimadas en el año 2008. A las especies anteriormente mencionadas se han incorporado el despegue en las producciones de lenguado senegalés *(Solea senegalensis)*, fletán (*Hippoglossus hippoglossus)* y sargo (*Diplodus sargus(FEAP, 2008)* (Tabla I).

Tabla I.- Evolución de la producción de peces marinos más representativos de la acuicultura europea (sin salmónidos, fuente FEAP, 2008).

Nombre común	**Lubina***	**Dorada***	**Rodaballo***	**Atún Atlántico**	**Bacalao ***	**Fletán***	**Corvina**	**Sargo**	**Lenguado Senegalés**	**Total**
Año	*Dicentrarchus labrax*	*Sparus aurata*	*Psetta maxima*	*Thunnus thynnus*	*Gadus morhua*	*Hippoglossus hippoglossus*	*Argyrosomus regius*	*Diplodus sargus*	*Solea senegalensis*	
2000	57.811	57.272	4.872	6.082	27	135	-	400	-	**126.599**
2001	56.162	74.403	4.640	9.992	111	389	-	400	-	**146.097**
2002	61.093	79.367	5.320	8.816	255	350	-	400	60	**155.661**
2003	62.060	87.940	5.107	6.715	2.940	845	-	400	52	**166.059**
2004	68.679	88.522	6.076	11.792	3.200	884	211	400	75	**179.839**
2005	79.706	93.372	6.085	9.180	6.708	1.319	893	400	60	**197.723**
2006	97.336	119.099	7.101	8.730	8.570	1.839	1.425	400	80	**244.580**
2007	93.425	104.697	7.444	5.380	10.640	1.399	1.138	-	60	**224.183**
2008	102.765	128.943	9.067	5.680	11.680	1.260	2.235	-	90	**261.720**

*Los datos de producción incluyen algunos países fuera de la Unión Europea (Noruega, Croacia, Islandia y Turquía).

Tabla II: Evolución de la producción de alevines de peces marinos en la acuicultura europea (FEAP, 2008 y datos propios).

Nombre común	**Nombre científico**	**Año**								
		2000	**2001**	**2002**	**2003**	**2004**	**2005**	**2006**	**2007**	**2008**
		Producción de alevines (Millones)								
Lubina*	*Dicentrarchus labrax*	214.2	207.9	204.0	220.5	349.8	374.6	389.7	419.7	457.7
Dorada*	*Sparus aurata*	262.3	293.4	317.0	307.7	318.8	447.3	549.9	507.6	497.7
Rodaballo*	*Psetta maxima*	4.0	4.0	5.2	5.2	5.2	6.8	8.5	6.1	13.0
Bacalao**	*Gadus morhua*	0.04	0.17	0.40	4.59	5.00	10.48	12.83	-	-
Corvina	*Argyrosomus regius*	-	-	-	1.3	1.1	3.4	2.4	2.2	3.0
Sargo	*Diplodus sargus*	0.7	0.7	0.7	0.7	0.7	0.7	0.7	-	-
Lenguado senegalés	*Solea senegalensis*	-	-	-	0.6	0.6	0.6	1.1	0.6	0.6
Fletán*	*Hippoglossus hippoglossus*	1.0	-	-	0.10	0.10	0.10	0.10	-	-
Bocinegro	*Pagrus pagrus*	1.1	-	-	0.015	0.03	0.10	2.6	2.0	2.0
Sargo picudo	*Puntazzo puntazzo*	6.3	-	-	-	-	-	4.5	4.0	4.0

*Los datos de producción incluyen algunos países fuera de la Unión Europea (Noruega, Croacia, Islandia y Turquía).
**Rosenlund pers. com.

2.2.-Acuicultura marina en España

2.2.1.-Generalidades

La acuicultura española está considerada como una de las más importantes de toda la Unión Europea, con una producción de 200-300 mil tm/año, lo que supone el 3% de la producción mundial y el 25% de la europea. A las modalidades tradicionales de cultivo de mejillón (*Mytilus galloprovincialis*) y acuicultura extensiva en las rías gallegas y en los esteros gaditanos, se ha incorporado un nuevo sector, altamente industrializado y en expansión.

Figura 1. Representación de acuicultura extensiva tradicional; a) Imagen de un grupo de bateas para el cultivo de mejillón (*Mytilus galloprovincialis*); b) Cosecha de berberecho (*Cerastoderma edule*) en la Ria de Muros y Noya (La Coruña, España).

Sirva como ejemplo el cultivo de rodaballo en el norte con una producción de 7.512 toneladas en el año 2008 lo que supone el 82,8 % de la producción Europea, o la expansión en el Mediterráneo y Canarias del cultivo de la dorada y lubina, con 24.790 y 11.760 toneladas en el año 2008 respectivamente (FEAP, 2008). En lo que se refiere a especies marinas de mas reciente introducción, España lidera con 1.620 toneladas la producción de corvina con un 72% de la producción por delante de Italia y Francia en el año 2008, siendo además el único productor de besugo (*Pagellus bogaraveo*) y lenguado senegalés (*Solea senegalensis*) de Europa con 134 y 90 toneladas en el año 2008, respectivamente. Además, España encabezaba el engorde de atún Atlántico (*Thunnus thynnus*) con un 43,5% de la producción en el año 2006 por delante de Chipre y Croacia.

Figura 2. Representación de acuicultura intensiva moderna; a) Imagen de un grupo de jaulas oceánicas de cultivo de dorada (*Sparus aurata*) en Telde, (Las Palmas, España); b) Imagen de un grupo de jaulas de cultivo de rodaballo (*Psetta maxima*) en la Ría de Vigo (Pontevedra, España).

2.2.2.-Producción de alevines

España cuenta con 16 criaderos comerciales de peces marinos que produjeron un total de 113,5 millones de alevines en el año 2008 (Apromar, 2007), siendo el primer productor europeo de alevines de rodaballo con un 90,3% de la producción total. Además, produce un 14,8 % de los alevines de dorada y un 5,6 % de los alevines de lubina (FEAP, 2008). A estos ha de añadirse la producción de pequeñas cantidades de alevines de otras especies, en empresas o centros de investigación, como es el caso del lenguado senegalés (*Solea senegalensis*) con algo más de 600.000 alevines, el abadejo (*Pollachius pollachius*) y el bocinegro o pargo (*Pagrus pagrus*) con valores cercanos a los 100.000 alevines, o el besugo (*Pagellus bogaraveo*) con poco mas de 16.000 alevines en el año 2005, y que han incentivado a los productores en su apuesta por la diversificación de especies marinas.

De manera generalizada existe una clara tendencia a la introducción de nuevas especies. En el caso de España, a las especies anteriormente mencionadas, hay que sumar, aquellas en las que ya se han realizado diferentes experiencias de engorde a escala piloto como son el sargo picudo *(Puntazzo puntazzo)* en la comunidad de Murcia (Hernández *et al*., 2003), dentón (*Dentex dentex*) en Cataluña, Comunidad Valenciana y Baleares (Abellán, 1995), sama roquera o hurta (*Pagrus auriga*) en Andalucía y Canarias (Cárdenas *et al*., 2003), o jurel dentón (*Pseudocaranx dentex*) también en las islas Canarias (Roo *et al*., 2007b). A nivel experimental se trabaja con un amplio número de especies, cuyo nivel de conocimiento se encuentra en fases más tempranas,

como es el caso de las distintas especies de medregal o seriola (*Seriola dumerilli, Seriola rivoliana o Seriola fasciata),* atún Atlántico *(Thunnus thynnus),* sama de pluma (*Dentex gibbosus*), salmonete de roca (*Mullus surmuletus*) o merluza (*Merluccius merlucius*).

2.2.3.-Acuicultura en las Islas Canarias.

Las Islas Canarias, presentan unas enormes perspectivas de expansión dadas las excepcionales condiciones climatológicas y la calidad de sus aguas. Los valores de producción de los últimos años muestran un crecimiento continuado de esta actividad que ha pasado de una producción de 456 toneladas en 1998 a más de 8.000 tm en el 2006 (Apromar, 2008). Las especies cultivadas son dorada (*Sparus aurata*), con un 65% de la producción y lubina con un 35%. Las Islas Canarias son la segunda región productora nacional de dorada con un 26% de la producción total de dorada por detrás de la Comunidad Valenciana con el 34% del total, y la primera en la producción de lubina con un 31,7% (Apromar, 2008). Sin embargo, siguiendo la tendencia nacional, este escaso número de especies, está comenzando a cambiar lentamente con la introducción de pequeñas cantidades de alevines de otras especies como es el caso de la corvina, el lenguado senegalés y el bocinegro con resultados alentadores.

Figura 3. a) Pre-engorde de alevines de bocinegro (*Pagrus pagrus*) en tanques; b) Engorde de bocinegro en jaulas.

Como contrapunto, cabe mencionar, que la acuicultura marina en Canarias sigue dependiendo de la importación de los alevines del exterior, en el año 2005 se importaron alrededor de 25 millones de alevines de las especies cultivadas, y únicamente existe una pequeña contribución local procedente de la planta piloto de producción de alevines del Instituto Canario de Ciencias Marinas, que viene produciendo en los últimos años una media de 400.000 alevines anuales de dorada, 50.000 de bocinegro y donde se ha comenzado la producción a escala piloto de alevines de corvina (*Argyrosomus regius)*, con 150.000 alevines en el 2008 y algo más 28.000 de lenguado senegalés (*Solea senegalensis*), para las empresas canarias.

Figura 4. Producción de alevines de nuevas especies para la diversificación de la acuicultura en Canarias; a) Alevines de corvina (*Argyrosomus regius*); b) Alevines de lenguado senegalés (*Solea senegalensis*).

2.3.-Importancia del cultivo larvario

Cualquier proyecto de cultivo de una especie marina parte de un principio común, que es la obtención de la semilla o alevines de la especie que se pretende cultivar. Se ha comprobado que la obtención de ésta, del medio natural, no es una estrategia viable en los cultivos intensivos de peces, siendo adecuada en otros sistemas de cultivo y otros organismos, como moluscos y algunos peces de gran valor comercial como pueden ser ciertas especies de atún, mero, seriola y anguila (Ottolengui *et al*., 2004).

Figura 5. Ejemplares de alevines capturados para su engorde; a) Seriola (*Seriola dumerili*) y (*Seriola fasciata*); b) Jurel dentón (*Pseudocaranx dentex*).

En este sentido uno de los principales problemas que sigue frenando el desarrollo de la acuicultura marina comercial para la diversificación de especies, como las anteriormente mencionadas, es la obtención de alevines de buena calidad y en cantidades suficientes. Las larvas de peces marinos no están completamente desarrolladas cuando eclosionan, por lo que ciertos tejidos y órganos, como los sistemas nervioso, digestivo y estructuras óseas, no han completado su desarrollo y deben experimentar importantes cambios morfológicos, funcionales y fisiológicos durante las primeras semanas de vida (Govoni *et al*., 1986).

Las primeras etapas de desarrollo de estos organismos serán con diferencia las que presentan un manejo más delicado, siendo habitual elevadas tasas de mortalidad. La situación de estrés inherente a las condiciones de cultivo intensivo, unido a los requerimientos nutricionales no siempre debidamente cubiertos, hace que la obtención de alevines de buena calidad en cantidades industriales no sea siempre posible. Aunque se han hecho grandes avances en el

desarrollo de dietas inertes (Cahu y Zambonino Infante, 2001), por el momento la producción industrial de alevines de peces marinos depende todavía de la reproducción simplificada de una cadena trófica natural, basada en el uso de algas unicelulares, rotíferos y *Artemia.* Esta situación implica la necesidad de mantener cultivos paralelos a los peces para obtener las presas vivas, necesarias para la alimentación de las larvas, aumentando los costes de producción de los alevines. La optimización de las condiciones de cultivo, pasa por mantener unas condiciones bióticas y abióticas adecuadas que deben tender a aproximarse en lo posible, a las condiciones del medio natural. En el éxito de las distintas técnicas de cultivo, intervienen un gran número de variables que son condicionantes de las mismas.

2.3.1.-Técnicas de cultivo larvario

Desde los inicios de la acuicultura se han desarrollado una gran variedad de técnicas de cultivo larvario de peces marinos, mediante las cuales se han establecido los parámetros que pueden ayudar al éxito del cultivo. La clasificación establecida por Divanach, (1985) define las técnicas de cultivo larvario teniendo en cuenta la densidad larvaria y el volumen del tanque utilizado, diferenciando así tres categorías principales: sistemas o técnicas intensivas, técnicas extensivas y sistemas o técnicas mesocosmos (Figura 6).

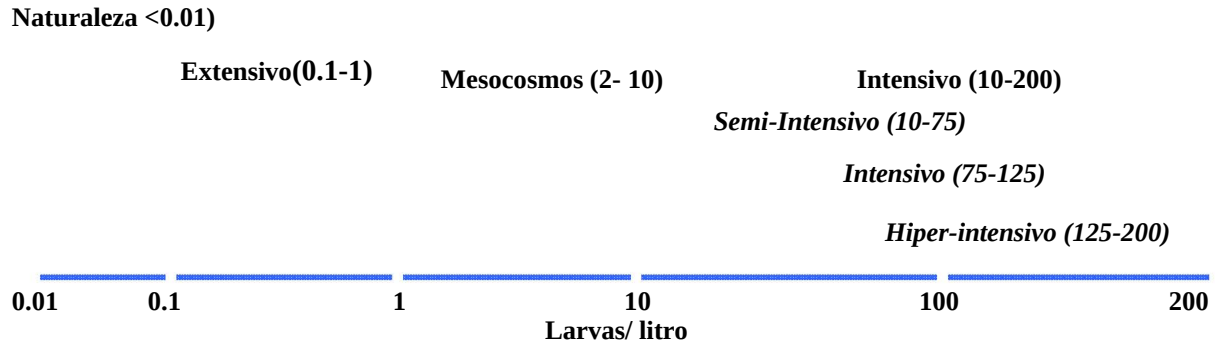


Figura 6. Clasificación de las técnicas de cultivo larvario modificado de Divanach, (1985).

Otros autores como Van der Meeren y Nass, (1997) clasifican las técnicas de cría larvaria de acuerdo al origen de la alimentación, definiendo los sistemas o técnicas intensivas como los que son exclusivamente dependientes de la aportación de presas exógenas, mientras que los sistemas extensivos se basan en la alimentación con presas que se producen de manera endógena dentro del propio sistema. Finalmente los sistemas semi-intensivos serán aquellos que basan su alimentación en ambos tipos de presas. La última revisión de definición de técnicas de cría larvaria realizada por Divanach *et al.* (1998) incorpora a su clasificación inicial diferentes características estructurales y operacionales que permiten definir la productividad y eficiencia del sistema. Tras esta revisión se pudo observar que las clasificaciones de los diferentes autores coinciden plenamente en la definición de sistemas extensivos e intensivos. Sin embargo la definición de semi-intensivo de Van der Meeren y Naas (1997) es lo que Divanach *et al*. (1998) definen como sistema semi-extensivo. Así para evitar posibles confusiones, la terminología que se utiliza en este estudio, hace referencia a la establecida por Divanach *et al.,* (1998).

2.3.1.1.-Sistemas intensivos

Se caracterizan por la alta densidad larvaria usada en los tanques que por lo general tienen una forma determinada (cilindro-cónicos) y, a pesar de que todavía es frecuente el uso de pequeños volúmenes (0,5-1 m^3), la tendencia actual es la utilización de volúmenes mayores (10-20m^3). Esta técnica se puede subdividir en tres variantes (Semi-intensivo = 25-50 larvas/litro; Intensivo= 75-125 larvas/litro; Hiper-intensivo =150-200 larvas/litro) en las que la complejidad del cultivo se incrementa con el aumento de densidad larvaria (Divanach *et al*.,1998).

Figura 7. Instalaciones de cultivo larvario de peces marinos en técnicas intensivas (fotografía cortesía de Tinamenor S.A).

Las técnicas intensivas e híper-intensivas se aplican con especies como la lubina y la dorada, con las cuales aun a estas densidades de cultivo se pueden alcanzar elevadas supervivencias (Shields, 2001). Por el contario, las técnicas semi-intensivas suelen estar más indicadas en algunas especies de peces planos como el rodaballo (*Psetta máxima*) y otros sistemas de producción, como el comúnmente aplicado para la producción de dorada japonesa (*Pagrus major).* Los parámetros de cultivo en las técnicas intensivas son condiciones controladas de temperatura, iluminación, hidrodinámica, oxigenación y alimentación. Generalmente el fotoperiodo, intensidad y espectro lumínico son artificiales, y se cambian de acuerdo al desarrollo del las larvas y a la agudeza de su sistema visual (Roo *et al*., 2001). La forma y organización del tanque (color, posición, orientación de las entradas y salidas de agua, lámparas, alimentadores, limpiadores de superficie y aireadores son establecidos previamente con el fin de favorecer un comportamiento homogéneo de la población de larvas, y unas condiciones ergonómicas que faciliten el manejo diario (Divanach *et al*., 2002). La alimentación en estos sistemas es

totalmente exógena y restringida, a una dieta que se basa en el uso del rotífero *B. plicatilis* y *Artemia*, cuyo valor nutritivo es necesario mejorar a través de enriquecedores comerciales que aportan diversos nutrientes (proteínas, ácidos grasos altamente insaturados, vitaminas y minerales) con el fin de cubrir los requerimientos nutritivos de las larvas (Izquierdo y Fernández-Palacios, 1997; Sargent *et al.,* 1999). La selección de la correcta secuencia alimentaria y la flexibilidad en su aplicación, de acuerdo a las necesidades del lote de larvas cultivado, es una herramienta indispensable para obtener elevadas tasas de supervivencia, baja dispersión de tallas y reducir el comportamiento caníbal. Para ello, es indispensable contar con un personal especializado y una adecuada automatización de los procesos.

Con estas técnicas, la mayoría de los parámetros de cultivo son potencialmente limitantes y es condición indispensable un profundo conocimiento de la biología de las especies a cultivar para obtener elevadas tasas de supervivencia. La especialización de tareas y duplicación de equipos de personal, son necesarias para una atención permanente las 24h del día. Noches, fines de semana y periodos vacacionales, donde frecuentemente hay una reducción en el personal especializado, son por lo general periodos de mayor riesgo. En caso de fallo, la especialización y limitaciones del sistema generan grandes pérdidas (Divanach *et al*., 2002). Por otro lado, cuando la demanda biológica no es correctamente satisfecha con los medios tecnológicos disponibles las larvas deben adaptarse o morir. Esta adaptación en muchas ocasiones conlleva cambios en su forma, coloración o reducción en el crecimiento, que hacen que estos animales no cumplan los estándares de calidad demandados por los productores (sirva como ejemplo las aplicadas a los diferentes lotes de alevines que se producen habitualmente en el ICCM, donde se exigen menos de 20% de dispersión de talla, menor de 5% de deformidades totales de las cuales no se admiten más del 2% de deformidades operculares, (Roo; datos no publicados). Gran parte de estas anomalías tienen lugar durante los primeros estadios de crecimiento del animal y no son observables hasta etapas posteriores. La dispersión de tallas, deformidades morfo-anatómicas, anomalías de coloración y comportamiento, canibalismo, selección de animales de crecimiento lento, proporciones de sexos anormales, son problemas que se observan con frecuencia en lo criaderos industriales que utilizan esta técnica de cultivo intensivo (Divanach *et al*., 2002). Así, el número especies de peces marinos que se cultivan con esta tecnología, es reducido y se limita a especies como lubina (*Dicentrarchus labrax),* dorada *(Sparus aurata),* rodaballo *(Psetta máxima),* (Shields, 2001); cobia *(Rachycentrum canadum),(*Holt *et al*., 2007), corvina (*Argyrosomus regius*) (Estevez *et al*, 2007; Roo *et al*., 2007), lenguado senegalés *(Solea*

*senegalensis) (*Dinis *et al.,* 1999*),* y bacalao *(Gadus morhua)* Baskerville-Bridges B. y Kling, J. L., 2000.

Se puede concluir que son técnicas que requieren unas instalaciones sofisticadas e inversiones elevadas, donde la producción depende totalmente del personal muy especializado y la tecnología disponible, no siendo recomendadas para pequeños productores ni para la producción de nuevas especies (Divanach *et al*., 2002).

2.3.1.2.-Sistemas extensivos

Se basan en la productividad de un sistema artificial que simula la naturaleza, donde la larva es el eslabón superior de la pirámide alimenticia que se genera. Los volúmenes de cultivo se inoculan con fitoplancton y zooplancton y posteriormente se añaden los huevos o larvas de las especies objetivo (van der Meeren and Naas, 1997; Divanach *et al*., 2002). Bajo estas condiciones naturales se establece una cadena trófica en la cual de forma natural se establecen los flujos de materia necesarios hasta que el crecimiento del alevín termine por colapsar el sistema por agotamiento de las presas. Se han establecido dos variantes de este sistema: ciclos cortos (1 – 2 meses) que producen larvas listas para ser criadas en sus fases posteriores en sistemas intensivos; o ciclos largos (2 - 6 meses) que producen alevines o juveniles para repoblación o acuicultura extensiva (Divanach *et al*., 2002).

Figura 8. Estanque de producción extensiva de bacalao en Øygarden, Noruega (fotografía cortesía del profesor Terge Van der Meeren).

Este tipo de larvicultura se realiza con bajas densidades (0.1 – 1 indv/ litro) en grandes estanques exteriores (cientos o miles de metros cúbicos) y con profundidades que van de 2 a 5 metros, otra variante es el uso de grandes bolsas plásticas y ocasionalmente jaulas o tanques construidos con mallas muy finas las cuales se preparan para soportar una adecuada alimentación de las larvas.

Figura 9. Sistema de producción extensiva en bolsas utilizado para la producción de larvas de bacalao en Lofoten, Noruega (fotografía cortesía de D. Dag Hansen, Lofilab AS).

Estos volúmenes de cultivo tan grandes impiden o dificultan en gran medida las modificaciones medioambientales. Generalmente estos sistemas están expuestos a las fluctuaciones climáticas y adaptadas al medioambiente natural que será específico de cada región, estación y geografía del lugar elegido para su desarrollo. Solo serán viables pequeñas modificaciones como variaciones del fotoperiodo (luz artificial durante la noche, sombreados parciales durante el verano) uso de películas plásticas para evitar que se hiele la superficie del agua en zonas y épocas frías), pero que pueden producir un incremento de la productividad del sistema. Cuando las condiciones climáticas son demasiados rudas (inviernos muy fríos en las regiones del norte y veranos excesivamente calurosos en las regiones del sur), la producción es imposible en los tanques y son usados para otras tareas.

El medio de cultivo es agua de mar tomada directamente de la zona costera, lagunas o pozos litorales, que tras una filtración gruesa (250-350µm) para eliminar los predadores potenciales ó competidores se introduce en el estanque. Eventualmente, se procede a la adición de fertilizantes para promover un desarrollo más rápido de la producción primaria en el estanque. En ocasiones

previo al inicio de la actividad de cría, se procede a la cloración del agua del estanque cuando esta contiene organismos indeseables para el cultivo y se re-inocula nuevamente para que se desarrolle la deseada. Una vez que el estanque se ha llenado la renovación de agua es nula o muy baja.

La alimentación es exclusivamente endógena, es decir la que se genera por el bloom natural de zooplancton consecuencia de la producción primaria en el propio estanque. En estas condiciones el éxito de la producción se basa en la intensidad y duración de la cadena trófica que se genera (Gamble *et al*., 1985; Franck y Leggett 1986). Cuando el productor comete el error de sobrepasar la capacidad biológica del sistema este equilibrio se rompe y el colapso del sistema se produce en pocos días o semanas generando problemas de crecimiento, dispersión de tallas y canibalismo como consecuencia de la falta de alimento en el tanque, lo que da lugar a una producción muy limitada (Divanach 1985). El éxito en este tipo de sistema viene dado por el conocimiento del ecosistema y las leyes que rigen la productividad del mismo. En este sentido la organización oligo-específica de la cadena trófica que favorece el desarrollo de la población pelágica frente a la bentónica, la protección frente a posibles competidores y predadores, adecuación a la geografía regional y las variaciones climáticas estaciónales, así como respetar la densidad inicial y edad de las larvas que se introducen, son los principales factores que condicionan la productividad del sistema.

El know-how es la base de esta tecnología aunque el papel del hombre es menos importante en este sistema frente al sistema intensivo. Los problemas que se generan a consecuencia de los días festivos, fines de semana o durante la noche en el sistema intensivo, no se dan en el sistema extensivo. Las principales operaciones que requieren de la intervención de las personas se refieran a la preparación del tanque, inicio de la cadena trófica, inóculo de huevos o larvas y la pesca final de los alevines producidos.

Aun cuando el manejo del tanque no es el más adecuado, la diversidad medioambiental y condiciones alimenticias que se producen en este tipo de sistema permiten una multitud de opciones que la larva puede encontrar sin forzarla a una situación anómala (Kentouri y Divanach, 1986). Las larvas en estos sistemas muestran ritmos biológicos naturales, agrupamientos, repartición diferencial correlacionada con las condiciones de iluminación, corrientes, concentraciones de zooplancton así como migraciones verticales y horizontales (Pitta *et al*, 1998). Como consecuencia de esto, las probabilidades de éxito son más elevadas que en los sistemas intensivos. Aproximadamente son 20 especies las que se producen habitualmente con estas técnicas y muchas de ellas presentan huevos muy pequeños como la herrera (*Lithoghnathus*

*mormyrus),*el sargo picudo *(Puntazzo puntazzo*) y son consideradas especies de cultivo complicado. Además, la calidad de los alevines producidos es excelente, ya que no aparecen problemas como peces sin vejiga, deformidades esqueléticas, anomalías de coloración o comportamiento (Divanach *et al.* 1996). En el caso de la lubina, las anomalías de proporción de sexos no son observadas y el crecimiento en etapas posteriores de los alevines producidos en estos sistemas es mejor que aquellos que se producen en los sistemas intensivos. Aunque bajas, las densidades de cultivo con estas técnicas son cientos incluso miles de veces superiores a las naturales. La cosecha de los alevines, cuando se produce con éxito puede ser de varios cientos de miles o incluso de millones. El reto para su aplicación industrial es la aplicación en volúmenes medios (Técnicas Mesocosmos) (Drenner *et al*., 1990) o la aplicación a mega volúmenes (mega-cosmos) de varios miles o millones de metros cúbicos (Gamble *et al.,* 1985) (Divanach *et al*., 2002). En términos económicos se trata de técnicas baratas y más ecológicas.

2.3.1.3.-Sistemas semi-extensivos (Mesocosmos)

Son técnicas de cría que se aplican bien en condiciones interiores (a cubierto) o bien semi-exteriores. Se consideran intermedias entre el sistema intensivo y el extensivo siendo consideradas técnicas semi-extensivas de producción masiva (Divanach y Kentouri, 2000; Papandroulakis *et al*., 2004a). Su definición es relativamente reciente en la forma de su aplicación actual y fue revisada por (Divanach *et al.* 1998) después del estudio de las cualidades intrínsecas y fallos de los modelos originales de Grice y Reeves (1982), Bever y colaboradors (1985) y Lalli, (1990).

El cultivo larvario tiene lugar a densidades relativamente bajas (2–10 larvas/litro) en volúmenes relativamente grandes (30-100 m^3) y con cierta profundidad (1,5-2,5m), tanques bien diseñados, situados en instalaciones bien organizadas. Esto asegura la calidad, ergonomía y seguridad en el cultivo, una elevada producción de alevines por tanque (50.000 a 300.000 dependiendo de la especie) y una productividad alta (> 2 millones de alevines/persona/año) (Divanach *et al*., 2002).

Figura 10. Sistemas semi-intensivos; a) Planta piloto de producción de alevines en el instituto Canario de Ciencias Marinas en Telde (Islas Canarias, España). b) Detalle de tanque de cultivo larvario de 40m^3 en el Instituto de Biología Marina de Creta, (Heraclion, Grecia).

Las condiciones medioambientales de los tanques son una combinación de naturales y artificiales, lo que aporta una gran flexibilidad, evitando variaciones climáticas estacionales o geográficas y permitiendo la optimización de los costes operacionales (Divanach y Kentouri, 2000; Papandroulakis *et al*., 2004a). Fotoperiodos largos (> 18 horas) y temperaturas medias (15-21ºC) son utilizadas para la mayoría de las especies. La alimentación combinada exógena y endógena complementa la calidad nutritiva del alimento para las larvas, reduciendo los riesgos de deficiencias nutricionales a consecuencia de un exceso de alimentación o falta de presas. Esta autonomía parcial es muy importante para obtener una buena calidad y mejor control de los costes de producción durante las noches y los fines de semana (Divanach *et al*., 2002).

El rendimiento obtenido con esta técnica para el cultivo de especies conocidas como lubina (*Dicentrarchus labrax),* dorada *(Sparus aurata)* o sargo (*Diplodus sargus*) son frecuentemente mejores que los obtenidos con técnicas intensivas o extensivas. La supervivencia tras el destete de los alevines generalmente está entre un 40-90% de los huevos con una media de 60 %, el porcentaje de deformidades totales se sitúa en torno a 5-10 % (de las que las operculares están en torno al 2-3% en el caso de la dorada), el porcentaje de éxito en la inflación de vejiga natatoria ronda el 100%; el crecimiento es entre un 15 a 20 mg al mes de vida, con unos índices de dispersión bajos; los animales presentan un crecimiento muy homogéneo, con una menor incidencia de canibalismo (no necesitan ser cribados o estas operaciones se reducen considerablemente respecto a los sistemas intensivos) durante los 40-50 primeros días; el destete

se completa a los 45-55 días de vida (Divanach *et al*., 2002). En el caso de los peces planos como el rodaballo, casi el 100% de la población no presenta anomalías de pigmentación.
La validez del mesocosmos para nuevas especies es similar a los sistemas completamente extensivos. Esta tecnología ha sido empleada con éxito para la producción de alevines de más de 25 especies de peces marinos y 5 híbridas (Divanach *et al*., 2002). La mayoría han sido producidos solo en mesocosmos con filosofía extensiva y cadenas naturales, como la anchoa de caleta (*Anchoa mitchilii),* bacalao *(Gadus morhua),* arenque *(Clupea harengus),* solla *(Pleuronectes platessa),* fletán *(Hippoglossus hippoglossus),* lenguado *(Solea solea),*o rodaballo *(Psetta máxima)*. Pero otros como la lubina (*Dicentrarchus labrax),* dorada *(Sparus aurata),* sargo picudo *(Puntazzo puntazo),* sargo *(Diplodus sargus),* mojarra *(Diplodus vulgaris),* raspallón *(Diplodus annularis),* herrera *(Lithognathus mormyrus),* bocinegro *(Pagrus pagrus),* dentón *(Dentex dentex),* seriola *(Seriola dumerilli),* verrugato (*Umbrina cirrosa L);* Lisa *(Chelon labrosus)* han sido cultivados con técnicas de agua verde o mesocosmos con filosofía intensiva con técnica de agua pseudo verde (Divanach y Kentouri, 2000); Papandroulakis *et al*., (2004a); (2005); Koumoundouros *et al*., 2005; Zaiss *et al*., (2006); Khemis *et al*., 2006)..
Dentro de la técnica Mesocosmos se diferencian dos variantes y cuatro subvariantes de acuerdo al origen y calidad de la cadena alimenticia (Divanach *et al*., 2002).

En la primera, la **filosofía extensiva**, la cadena alimenticia es básicamente endógena y esta es complementada con alimentación exógena cuando se presentan síntomas de agotamiento y falta de alimento. En la segunda, la filosofía intensiva, la alimentación es básicamente exógena pero presenta una cierta capacidad de producción endógena debido a la baja densidad de las larvas (baja tasas de alimentación) y presencia de fitoplancton en el tanque. Las 4 subvariantes se caracterizan por los métodos para la mejora del medio y /o la cadena alimenticia y se describen a continuación.

2.3.1.3.1.-Bloom natural- Filosofía extensiva.

Se basa en la generación de una cadena trófica natural, cuyo inóculo se obtiene directamente a partir del agua de mar que se bombea (Divanach *et al*., 2002). Para ello, se utiliza una filtración del agua gruesa, 250-350μm que permite eliminar posible competidores y posibilita el paso de pequeño plancton salvaje que será usado como inóculo. El agua es fertilizada con nutrientes para que se desarrolle el fitoplancton presente de forma natural, o bien en el caso de regiones muy

oligotróficas incluso se procede a introducir un inóculo de fitoplancton con especies de los géneros *Nannochloropsis* o *Tetraselmis.*

La renovación en este sistemas es muy baja 1-2% día, y después del llenado del tanque, esta se mantiene en torno a 10-15 días para permitir un óptimo desarrollo del zooplancton. Las larvas se alimentaran de este zooplancton alrededor de 20-25 días, tiempo crítico en el desarrollo de la larva y tras el cual ya están preparadas para continuar alimentándose de *Artemia* y comenzar su destete. Sin embargo, el uso de este sistema puede encontrarse a veces con dificultades como bloom inesperado de otros organismos como medusas, anélidos, o incluso ciertos ciliados parásitos que pueden dar al traste con la producción.

2.3.1.3.2.-Método de agua verde- Filosofía extensiva

Al igual, que el descrito anteriormente, consiste en la generación de una cadena trófica, sin embargo, esta será de origen endógeno (Divanach *et al*., 2002). En este caso, el tanque se llena con agua de mar previamente filtrada y esterilizada. Se fertiliza con nutrientes y se introduce un inóculo de fitoplancton y rotíferos permitiendo que se desarrollen ambos hasta una media de 500.000 cells/ml y >2 rot/ml. No se renueva el agua del tanque, manteniendo el cultivo de 4 a 8 días hasta que se introducen los huevos o larvas.

Cuando la cadena alimenticia se desarrolla correctamente esta dura aproximadamente 25 días pasando a usarse *Artemia* y dietas de destete y circuito abierto de agua. Los inconvenientes de este sistema estriban en los desequilibrios que se pueden establecer a consecuencia de una siembra demasiado baja, los rotíferos alcanzan un bloom rápidamente consumiendo el fitoplancton y pudiéndose dar condiciones de bajada de pH y oxígeno durante la noche. Por otro lado un exceso en el número de larvas puede conllevar un agotamiento prematuro de las presas y el bloom de fitoplancton remanente puede dar lugar a una hiper-oxigenación superficial en el tanque y condiciones anóxicas en el fondo (baja penetración de luz) lo que puede originar enfermedades en las larvas, como la sobresaturación de gases o la mortalidad por hipoxia.

En la segunda, la **filosofía intensiva**, la alimentación es básicamente exógena pero presenta una cierta capacidad de producción endógena debido a la baja densidad de las larvas (baja tasas de alimentación), presencia de fitoplancton en el tanque o a la introducción de inóculos de otros tipos de presas como pueden ser copépodos marinos.

2.3.1.3.4.- Agua pseudo verde- Filosofía intensiva

En ella, el cultivo larvario se desarrolla en un medio con una composición más estable que los anteriores, que contiene fitoplancton y rotíferos de origen exógeno, producidos en instalaciones paralelas y que se aportan diariamente según la demanda de las larvas (Divanach *et al*., 2002).

De esta técnica existen dos variantes:

Puro: el tanque se llena con agua de mar esterilizada y se introducen los huevos o larvas recién eclosionadas. Posteriormente cuando las larvas inician su vida heterotrófica, se adicionan el fitoplancton y los rotíferos.

En la segunda variante se inicia primero la cadena trófica añadiendo fitoplancton y rotíferos antes de sembrar los huevos o larvas. En este caso, el periodo de vida autotrófico de la larva puede verse perjudicado por la presencia de un medio muy poblado.

En estos casos, el circuito de agua es siempre abierto y su renovación se incrementa a medida que la larva se desarrolla. La adición de fitoplancton y rotíferos se realiza 1-2 veces al día y se utilizan rotíferos previamente enriquecidos con preparados comerciales para mejorar su valor nutritivo. Cuando la larva alcanza el tamaño necesario para poder alimentarse de *Artemia* cesa el uso del fitoplancton y el cultivo se continúa en técnica de agua clara.

Suele utilizarse con especies de las que se conoce bien su ciclo biológico como es el caso de la dorada (*Sparus aurata*), y presentan unos rendimientos muy elevados.

2.3.1.3.5.-Agua clara- Filosofía intensiva.

Es un método en el cual no se utiliza fitoplancton y esta siempre asociado a altas tasas de renovación de agua >10%/h lo que imposibilita el desarrollo de microorganismos endógenos. Se utiliza generalmente con especies, como la lubina, que aceptan rápidamente los nauplios de *Artemia* en su primera alimentación. O bien asociado a sistemas de alimentación automática capaces se suministrar alimento con una calidad nutritiva elevada (Divanach *et al*., 2002).

2.3.1.3.6.-Técnicas neo-verde.

Son técnicas que todavía están siendo testadas en muchas instalaciones a nivel experimental, utilizando generalmente fitoplancton crioconservado o producido en sistemas de cultivos heterotróficos, como pastas y liofilizados de microalgas (Divanach *et al*., 2002)..

La principal limitación de este tipo de técnica se basa en la perdida de los valores beneficiosos que se apuntan con el uso de microalgas vivas .

2.4.-Factores condicionantes en el cultivo larvario

Se han realizado infinidad de estudios conducentes a determinar cuáles son los parámetros óptimos para el cultivo larvario de las especies marinas. Hay que destacar que existe una clara influencia de la especie a cultivar sobre la tecnología más apropiada para la misma. Entre los parámetros que se han considerado como más importantes están aquellos que se pueden englobar dentro de las condiciones generales de cultivo: densidad de siembra, volumen del tanque, renovación del medio, presencia de fitoplancton o uso de antibióticos. Los parámetros físico-químicos que han sido más estudiados como temperatura, salinidad, iluminación (fotoperiodo, intensidad y calidad de luz) o los productos de excreción. Y finalmente, los que afectan a la nutrición larvaria: cantidad de presas aportadas, secuencia alimentaria y el considerado como uno de los factores más importantes como es el valor nutricional del alimento suministrado.

2.4.1.-Parámetros generales

2.4.1.1.-Densidad larvaria

La influencia de la densidad de cultivo, además de ser un factor que condiciona el tipo de sistema, ha sido señalado como parámetro condicionante del crecimiento y la supervivencia durante la etapa larvaria de peces marinos, como se ha señalado en el caso de la anchoa de caleta (*Anchoa mitchilli*), lenguado americano (*Achirus lineatus*) (Houde,1977), cobia (*Rachycentron canadum*) (Hitzfelder *et al*., 2006, Holt *et al*., 2007) o corvina (*Argyrosomus regius*) (Estevez *et al*, 2007; Roo *et al*., 2007). En este mismo sentido, se han pronunciado otros autores que han trabajado con la misma especie objeto de este estudio como el bocinegro (*Pagrus pagrus*) (Hernández-Cruz *et al*., 1999; Roo *et al*., 2005b), dorada, (*Sparus aurata)* (Parra y Yúfera, 1999; Roo *et al*., 2005a), o especies cercanas como dentón, (*Dentex dentex)* (Giménez y Estevez, 2008a). Uno de los factores asociados al aumento de densidad, que puede condicionar la supervivencia es el canibalismo intracohorte como señalan Baras y Jobling, (2002). Sin embargo, en lubina (*Dicentrarchus labrax*) y lenguado canadiense (*Paralichthys dentatus*) se asocia el incremento de la densidad larvaria únicamente a la disminución del crecimiento como consecuencia de la gran competencia por el alimento y el estrés inherente a la gran acumulación de individuos (Hatziathanasiou *et al*., 2002; Saillant *et al*., 2003; King *et al*., 2000). Trabajos más recientes con una especie como bacalao (*Gadus morhua*) muestran que la densidad de cultivo no parece ser un factor que limite el crecimiento y la supervivencia, porque su influencia se ve enmascarada por otros factores asociados a la alta densidad de cultivo, como son la

disponibilidad del alimento y el riesgo en el deterioro de la calidad de agua a altas densidades (Baskerville-Bridges B. y Kling, J. L., 2000).

2.4.1.2.-Forma y volumen del tanque.

Se ha encontrado que el volumen del tanque de cultivo tiene diferentes efectos en los sistemas de cría de larvas de espáridos como la dorada, así Tandler y Sherman (1981) encuentran una correlación positiva, entre el incremento del volumen del tanque de cultivo y la supervivencia. En la actualidad, la mayoría de criaderos que se dedican a la producción intensiva de espáridos utilizan un modelo de tanque cilindro cónico de fibra de vidrio y con volúmenes relativamente grandes 1-15m^3 al menos en las primeras etapas larvarias, siendo la tendencia generalizada el uso de volúmenes mayores de 10m^3. Del mismo modo, al igual que con el volumen, se ha encontrado que la relación profundidad/superficie del tanque puede afectar a la supervivencia larvaria en las primeras etapas de desarrollo, como se ha visto en el mero de siete bandas (*Epinephelus septemfasciatus*) y el escorpénido "devil stinger" (*Inimicus japonicus*) (Ruttanapornvareesakul *et al*., 2007).

2.4.1.3.-Renovacion de agua.

Un exceso de renovación de agua, del tanque, afectará la aparición de deformidades de columna (Kihara *et al*., 2002) ó la rápida eliminación de presas o microalgas que estén presentes en el tanque, limitando la calidad del alimento suministrado y las condiciones físico-químicas del medio. Por el contrario, un defecto en la renovación, puede contribuir a la acumulación de metabolitos (amonio, nitritos, etc.) en el sistema de cultivo, o la acumulación de presas vivas con un valor nutritivo escaso (Reitan *et al*., 1993) que puede tener efectos negativos en las tasas alimenticias de la larva y consecuentemente en el crecimiento y supervivencia de las mismas.

Autores como Tandler y Helps, (1985), Tandler y Sherman, (1981) o Hernández-Cruz *et al*, (1990), correlacionan el uso de sistema de circuito cerrado con un mejor crecimiento de la larva de dorada (*Sparus aurata*), durante los 10 primeros días de vida. Sin embargo, sugieren una renovación del 25%.$día^{-1}$ a partir del día 20 de vida. Otros trabajos muestran que la combinación de circuito abierto y agua verde mejora significativamente la supervivencia en los cultivos de dorada (Hernández-Cruz *et al*., 1994). En los últimos años, se ha establecido una tendencia creciente al uso de sofisticados sistemas de circuito cerrado, donde se realiza una renovación del medio con agua del propio sistema pasada por filtros mecánicos y biológicos que aseguran el

mantenimiento de una buena calidad de agua y el valor nutritivo de las presas vivas (Olivar *et al*., 2000, Faulk y Holt, 2005,).

2.4.1.4.-Presencia de fitoplancton en el tanque de cultivo larvario

Numerosos trabajos defienden el uso de fitoplancton o la denominada técnica de agua verde para el cultivo larvario de especies de peces marinos (Scott y Baynes,1979; Hernández-Cruz *et al*., 1990; Koven *et al*., 1990, Salvesen *et al*., 2000). Los efectos beneficiosos de la presencia de fitoplancton en el tanque se pueden resumir en que contribuye a una mayor oxigenación del agua del tanque; ayuda a la eliminación de sustancias de desecho (amonio y nitritos) producidos por las larvas y presas presentes en el tanque; controla la flora bacteriana, ya que la mayoría de microalgas presentan actividad antibacteriana a través de la producción de exudados orgánicos y metabolitos tóxicos para las bacterias oportunistas. En el caso particular de *Nannochloropsis sp.* la especie utilizada en este estudio, la principal flora bateriana esta compuesta por Alfa-proteobacterias y el grupo de bacterias tipo *Cytophaga-Flavobacterium,* las cuales actúan como herramienta de biocontrol de otras comunidades bacterianas que se desarrollan en los tanques de cultivo larvario (Nakase y Eguchi, 2007). A su vez, el incremento de la carga bacteriana en el agua de cultivo, como consecuencia de la repetida adición de fitoplancton al tanque, puede contribuir al desarrollo de flora intestinal inicial de las larvas (Skjermo y Vadstein 1993). Además, la presencia de fitoplancton en el tanque de cultivo, ayuda al mantenimiento del valor nutritivo de las presas vivas. En rotíferos enriquecidos con emulsiones la pérdida de lípidos se estima en un 20% diario en sistemas de cultivo en agua verde, mientras que en los sistemas de cultivo en agua clara hay un descenso más acentuado de la calidad nutritiva de las presas (Reitan *et al*., 1993; Planas y Cunha., 1999). Se ha observado también, que las larvas cultivadas en agua verde mantienen las reservas vitelinas 2-3 días, tienen una tasa de crecimiento mayor y sufren de 2 a 3 veces menor mortalidad larvaria que cuando se cultivan en agua clara (Papandroulakis *et al*, 2001; Van der Meeren *et al*, 2007). Finalmente, se puede decir que el fitoplancton actúa como un filtro lumínico, ya que las microalgas reducen el efecto de la reflexión de la luz en las paredes de tanque, que junto con el uso de tanques con paredes oscuras (Hinshaw,1985), contribuye a una mejora de la actividad alimenticia de la larva, al favorecer un mayor contraste de las presas, (Naas *et al* 1992,1996, Ramos y Kobayashi, 1985; Muller-Feuga *et al*, 2003). Actualmente existe una tendencia al uso de concentrados, pastas, liofilizados y biomasa congelada de diferentes microalgas. Hay diferentes estudios comparativos entre el uso de este tipo de productos y las microalgas vivas, así Cañavate y Fernández-Díaz, (2001) no encuentran diferencias en el

crecimiento y supervivencia en la larva de dorada (*Sparus aurata*) con el uso de biomasa congelada frente al alga viva. Sin embargo, estos autores mencionan un deterioro en la calidad de agua del tanque, por el incremento de los valores de amonio, con el uso de biomasa inerte en los sistemas que trabajan en circuito cerrado. Por otro lado, el uso de este tipo de productos limita los efectos beneficiosos exclusivos de la biomasa viva, como son el efecto bacteriostático o la colonización de la flora intestinal de la larva y tanque larvario.

2.4.1.5.-Uso de antibióticos

El exceso de carga bacteriana en los tanques de cultivo, como consecuencia de la introducción de presas vivas y el uso de sistemas con limitada o nula renovación, hace que haya sido frecuente el uso de antibióticos para su control. El uso de estas sustancias en el cultivo larvario ha sido un amplio tema de discusión entre diferentes autores y trabajos con diferentes especies. Así, Hernández-Cruz *et al*.,1994 muestran que el uso de ácido oxolínico tiene resultados adversos en la supervivencia larvaria, es más los rotíferos enriquecidos sometidos a tratamientos con antibiótico muestran un menor contenido en n-3 HUFA que los no tratados. Sin embargo, otros trabajos, correlacionan el uso de la oxitetraciclina, con un incremento de la supervivencia y crecimiento de las larvas de trompetero australiano *(Latris lineata)* (Bataglene *et al*.,2006).

2.4.2.-Parámetros físico-químicos

Entre el elevado número de parámetros físico químicos que, potencialmente, pueden afectar o condicionar el cultivo larvario (temperatura, salinidad, iluminación, productos de excreción, gases disueltos, pH etc.) se han seleccionado aquellos considerados como más determinantes del crecimiento, la supervivencia y la calidad de las larvas.

2.4.2.1.-Temperatura

Dentro de los parámetros físico-químicos uno de los que tienen mayor importancia en el cultivo larvario es la temperatura. Su efecto se puede separar en dos etapas: durante el desarrollo del embrión y durante el desarrollo de la larva.

Temperatura de incubación

El desarrollo embrionario y larvario de los peces está altamente influenciado por la temperatura de incubación del embrión, así las temperaturas elevadas aceleran el desarrollo embrionario, mientras que las bajas temperaturas lo disminuyen (Blaxter, 1988). Los trabajos de autores como

Polo *et al*. (1991) señalan, que la dorada europea admite un amplio rango de temperatura de incubación que va, desde 14 a 28 ºC, siendo el óptimo en torno a 19º C. Otros autores han relacionado la temperatura óptima de incubación con las condiciones naturales asociadas a las latitudes de los stocks de reproductores considerados. Se indica sin embargo que, por debajo de 16 ºC y por encima de 22º C las tasas de eclosión se reducen y los porcentajes de larvas con deformidades se incrementan notablemente. Resultados similares han sido descritos para otras especies de espáridos como el pargo japonés (*Pagrus major)*, (Mihelakakis y Yoshimatsu, 1998). En el caso de la lubina (*Dicentrarchus labrax*) se ha señalado que un ligero incremento de la temperatura durante la incubación tiene un efecto positivo en el crecimiento muscular en etapas posteriores (López-Albors *et al*., 2003).

Temperatura de cultivo larvario

Tandler y Sherman, (1981), determinaron que la supervivencia larvaria de la dorada (*Sparus aurata*) no se ve afectada por el efecto de la temperatura de cultivo larvario empleada que puede ir desde 17º a 23 ºC, aunque la mayor supervivencia se obtuvo a 17ºC, asociando un incremento de la temperatura con un mayor crecimiento de las larvas. De la misma manera, Polo *et al*., (1991) determinan el rango de 16-22 º C como idóneo para el cultivo de esta especie, situando el óptimo en 19ºC y correlacionando las temperaturas fuera del mismo con un aumento en la mortalidad y un incremento de la incidencia de anormalidades en las larvas. Otros estudios demuestran que la temperatura puede tener efectos negativos sobre la supervivencia larvaria así, Tandler *et al*, (1989), correlacionan el efecto de la temperatura de cultivo con la estructura de tallas de la población, señalando que a temperaturas más elevadas el efecto de dispersión de la talla de la población puede ser mayor lo que contribuye a un aumento de la mortalidad por el efecto del canibalismo a edades tempranas.

2.4.2.2.-Salinidad

A pesar de que suele ser un parámetro que se mantiene relativamente estable, algunos criaderos presentan ocasionalmente problemas de bajadas de salinidad a consecuencia de las escorrentías en épocas de lluvias. Se ha visto que produce efectos sobre el cultivo larvario condicionando las tasas de eclosión del huevo e incluso incidiendo en el desarrollo de deformidades esqueléticas. Tal es el caso del "Southern black bream" (*Acanthopagrus butcheri*) donde la reducción de la salinidad por debajo de 15‰ se refleja con una reducción en la eclosión y un incremento del porcentaje de larvas con deformidades de columna, que puede afectar hasta al 100% de la

población a una salinidad de 5‰ y hasta un 20%, a salinidades de 20‰ (Haddy y Pankhurst, 2000). Resultados similares se han evidenciado en el caso de la dorada, donde una bajada brusca de salinidad de 37 a 32‰ durante los primeros días de vida de la larva se refleja en un incremento significativo del porcentaje de anomalías operculares respecto al control mantenido a salinidad constante en la misma etapa (datos propios, no publicados). Por el contario en especies como la lubina (*Dicentrarchus labrax*) la bajada de salinidad a 15‰ resulta en un aumento de la supervivencia y una mejora en el porcentaje de larvas con vejiga natatoria, resultados similares fueron obtenidos en el caso de la lisa pardete (*Mugil cephalus)* (Harel *et al*., 1998).

Por otro lado, la combinación de los dos parámetros anteriormente mencionados temperatura y salinidad tiene una importancia significativa en la obtención de larvas viables. Así valores extremos de 28ºC y 12‰ de salinidad aumentan el porcentaje de larvas que presentan deformidades en el pargo japonés *Pagrus major* (Mihelakakis y Yoshimatsu, 1998).

2.4.2.3.-Productos de excreción nitrogenados

El amonio es el principal producto de excreción de los peces, como resultado del catabolismo proteico, siendo especialmente tóxico, en el caso de las larvas. La producción de amonio y detritos orgánicos por el alimento no consumido como pueden ser las microalgas y presas muertas, contribuyen al incremento de la cantidad de amonio en los tanques larvarios. Parra y Yúfera, (1999), sitúan en 20ppm las concentraciones letales de amonio en cultivos de larvas de dorada (*Sparus aurata*) de 12 días, siendo la forma más tóxica el amonio no ionizado ($N\text{-}NH_3$) con una concentración letal para el 50% de la población de 0,24 ppm. En el caso del nitrito (NO_2^-) la mortalidad del 100% de la población se observa a 4.500 ppm, y el 24h LC50 en 1.997 ppm. Sin embargo, estas concentraciones son dependientes de factores como el pH, salinidad ó temperatura, así como de la especie, ya que en el caso de las larvas de lenguado senegalés (*Solea senegalensis*) de 7 días estos mismo autores citan una concentración letal para el $N\text{-}NH_3$ de 80 ppm y la 24h-Lc 50 de 1,32 ppm. En el caso del NO_2^- la mortalidad del 20% de la población se observa a concentraciones de 5.000 ppm. Cabe mencionar además la relación que se ha encontrado entre los niveles de amonio de (0,05-0,015 ppm de NH_3-H) y la aparición de daños en las estructuras cartilaginosas de las larvas de pargo japonés (Mihelakakis y Yoshimatsu, 1998).

2.4.2.4.-Iluminación

La mayoría de larvas de peces marinos son predadores visuales (Blaxter y Staines., 1970). Por ejemplo, se ha demostrado que la larva de dorada es un predador visual que necesita luz para ser efectiva en sus ataques (Tandler y Mason 1983). Así, Tandler y Helps (1985) demostraron que durante los primeros 12 días de vida de la larva hay una correlación positiva entre el aumento del foto período de 12 a 24 h de luz con la supervivencia en larvas de dorada europea, lo mismo encuentra Barahona-Fernándes, (1979) en larvas de lubina. También se correlaciona un aumento de la intensidad luminosa de 205 a 1370 lux con un mayor crecimiento y supervivencia larvaria, con óptimos que se sitúan entre 1370-5140 lux cuando se combina luz artificial con luz natural en fotoperiodo continuo y sistemas de cultivo en agua verde (Tandler y Mason, 1984).

A partir del día 20 de vida no se encuentran diferencias significativas con el cambio de foto período de 12 a 24 horas lo que está relacionado con el desarrollo del sistema visual de la larva (Roo *et al*., 1999, 2001). Las larvas de espáridos como dorada (*Sparus aurata*) y bocinegro (*Pagrus pagrus*) nacen con un sistema visual simple únicamente conformado por fotoreceptores denominados conos, los cuales les capacitan para ver solo en condiciones de alta intensidad de luz. Sin embargo, el sistema visual continúa desarrollándose y aumentando su complejidad, y así al día 18-20 de vida aparecen los precursores de los fotoreceptores de tipo bastones, que mejoran la agudeza visual de la larva permitiéndole predar en condiciones de baja intensidad de luz. Este sistema dual compuesto por conos y bastones no se completa hasta el día 25-30 de vida según la temperatura de cultivo de la larva. Para el periodo comprendido entre los 50-60 días Tandler y Helps 1985 obtienen los mejores crecimientos con una reducción del foto período en el caso de la dorada europea.

Otro de los factores que hay que tener en cuenta y que se relacionan con la iluminación en los tanques larvarios es la coloración y reflexión de las paredes del tanque, Naas *et al*., (1996) y Hinshaw, (1985) describen que los tanques de paredes negras son los que mejor asemejan las condiciones de iluminación natural cuando están provistos de una iluminación adecuada. Finalmente, cabe mencionar la relación encontrada entre la iluminación y el desarrollo esquelético de las larvas, en casos particulares, como el "silver pearfish" (*Carapus homei*), las larvas de esta especie necesitan pasar por un periodo de oscuridad para completar su metamorfosis, este cambio lo realizan en el interior del pepino de mar ocelado (*Bohadschia argus)* en el interior del cual tiene lugar una reducción natural del número de vertebras así como una compresión de las mismas para adoptar su forma definitiva de alevín (Parmentier *et al*., 2004).

2.4.3.-Nutrición larvaria

Un elemento crítico en el cultivo larvario de peces marinos es la primera alimentación. Las larvas de peces marinos generalmente eclosionan con un cantidad muy limitada de reservas endógenas, por lo que su supervivencia va a estar enormemente condicionada a la alimentación suministrada. El reducido tamaño de la boca de la mayor parte de las larvas de peces marinos, unido a un limitado desarrollo del sistema digestivo, hacen del comienzo de la alimentación exógena una etapa crítica para el éxito del cultivo larvario.

2.4.3.1.-Cantidad de presas

Tandler y Sherman, (1981), determinaron que la cantidad de presas óptimas para obtener buenas supervivencias larvarias se encuentran entre 10-15 rot/ml para cultivos intensivos de dorada (*Sparus aurata*) a una densidad larvaria de 100indv.l^{-1}. Resultados similares han sido citados por Giménez y Estévez (2008) quienes apuntan una densidad de 5-10rot.ml^{-1}, para el cultivo larvario de dentón (*Dentex dentex*) a una densidad de entre 10 a 40indv.l^{-1} . Autores como Houde, (1977) y O´Connell y Raymond (1970) indican las concentraciones para el uso de otras presas como copépodos. Así densidades superiores a 1nauplio.ml^{-1} son necesarias para el cultivo de la anchoa de caleta (*Anchoa mitchilli*) y el lenguado americano (*Achirus lineatus*) mientras que concentraciónes de 4 nauplios.ml^{-1} se asocia con las mejores tasas de supervivencia (68%), en larvas de anchoa del pacífico (*Engraulix mordax*), lo que da idea a su vez de las diferencias interespecificas para este parámetro.

2.4.3.2.-Secuencia alimentaria-tipos de presas-co-alimentación

La secuencia de presas vivas a utilizar es uno de los elementos críticos para obtener el éxito en el cultivo larvario de nuevas especies de peces marinos. Generalmente, la elección de la secuencia alimentaria adecuada, vendrá determinada fundamentalmente por el nivel de desarrollo del organismo a cultivar, que puede variar según las características intrínsecas de las diferentes especies. Generalmente, será el tamaño de la boca y la disponibilidad de distintos tipos de presas que puedan ser producidas de una manera masiva y sostenible, uno de los principales limitantes. Así, en el caso de la mayoría de los espáridos es frecuente el uso de una presa inicial de menor tamaño, como es el rotífero eurihalino del genero *Brachionus,* que puede ser de diferentes especies o morfo tipos basados en el tamaño de la lorica; *Brachionus plicatilis*, tipo L (grande), *B. ibericus* o tipo S (Pequeño) y *B.rotundiformis* ó SS(Super pequeño) (Hagiwara *et al*., 2007). Al rotífero le sigue un periodo corto de transición a nauplios de *Artemia*, para continuar con

metanauplios enriquecidos y pasando finalmente a la incorporación de dietas inertes. En el caso de la lubina (*Dicentrarchus labrax*) y el lenguado senegalés (*Solea senegalensis*), sus larvas nacen con un nivel de desarrollo y cantidad de reservas vitelinas superior a otras especies, que hace posible la introducción directa de presas de mayor tamaño como la *Artemia* desde el inicio de la alimentación exógena (Cahu et al., 1999 ; Dinis *et al*., 1999). En el caso de otras especies como el corvinón ocelado (*Sciaenops ocellatus*) se puede comenzar la alimentación con dietas inertes a partir del día 10 de vida (Buchet *et al*., 2000). A pesar de que uno de los principales objetivos buscados desde hace años por los acuicultores es la eliminación completa de las presas vivas, se ha visto que esta, resulta factible en un reducido número de especies marinas, y en muchos casos supone una reducción de la supervivencia y calidad de las larvas. Es por ello que, a excepción de determinadas pruebas experimentales, las mayoría de las técnicas de cría de peces marinos se basan en la co-alimentación de presas vivas y dietas inertes, en el tramo final de la secuencia alimentaria en el momento del "destete" cuando se sustituye el alimento vivo por el alimento inerte en vez de la sustitución total (Rosenlund *et al*., 1997). Kolkovski *et al*, (1997) demuestran que la ingestión de las microdietas por las larvas de dorada mejora en un 120% en presencia de *Artemia*. Las presas vivas ejercen un estimulo visual y químico, que decrece con la edad de la larva, y sugieren que un mejor desarrollo de la capacidad visual y olfatoria las capacita para una mayor agudeza en la predación. Por otro lado, se ha atribuido también una cierta influencia de la composición bioquímica de las presas sobre la digestión y asimilación de las microdietas. Así, la presencia de aminoácidos libres tales como la alanina, glicina, arginina y la betaína que son metabolitos presentes en los medios de cultivo de la *Artemia*, se consideran estímulos químicos que favorecen la ingesta de la microdieta. Otros factores relacionados con la dieta como los fosfolípidos, afectan la asimilación y la digestión. Así, la fosfatidilcolina, y más concretamente el grupo químico trimetil colina que también esta presente en la betaina, mejora la asimilación y digestión de las microdietas. Este mismo metabolito ha sido aislado en la *Artemia,* interviniendo en la síntesis de lipoproteínas y en el transporte de los lípidos.

Otra de las técnicas que se está utilizando mas habitualmente, es la combinación de las tradicionales presas vivas, con otras de mas difícil obtención como son los copépodos, ofreciéndolos en determinadas etapas críticas de la larva y con sensibles mejoras en la supervivencia y el crecimiento, como en el caso del cultivo de rodaballo (*Psetta maxima*) (Støttrup, J. G., Norsker, N. H. 1997), el "westralian jewfish" *Glaucosoma hebraicum* y el pargo australiano *Pagrus auratus*. En el caso de *G. hebraicum*, la co-alimentación con copépodos mejora la supervivencia de un 5% en la dieta control a un 37% (Payne *et al*., 2001). Resultados

similares se han obtenido en el caso del "West Australian seahorse" (*Hippocampus subelongatus)* (Payne y Rippingale, 2000). Ademas, se ha observado que la alimentación con copépodos, en el caso de las larvas de fletán (*Hippoglossus hippoglossus),* en comparación con la *Artemia* mejora el desarrollo del sistema digestivo, así como la capacidad de absorción (Luizi *et al.,* 1999). De la misma manera, Hamre *et al*, (2002) reportan una menor incidencia de anomalías de pigmentación y un mayor éxito en el proceso de migración ocular para esta especie, cuando las larvas son alimentadas con copépodos en comparación con el uso de *Artemia*. Igualmente, Imsland *et al*, (2006) registran un mejor crecimiento y una menor incidencia de malformaciones esqueléticas en el caso de las larvas de bacalao (*Gadus morhua)* cuando se alimenta con copépodos en comparación con el uso de rotíferos. Estas ventajas han dado lugar a que en los criaderos comerciales de ambas especies sea frecuente el uso de este tipo de estrategias de alimentación para la producción larvaria (Olsen, *et al*., 1999, Van der Meeren *et al*., (1997). Otro tipo de presas menos comunes por la complejidad que presenta su captura o producción son los diferentes estadios larvarios de crustáceos marinos que han sido utilizados para el cultivo de una de las especies marinas que más problemas presenta en sus primeras etapas de cultivo como es el pulpo de roca (*Octopus vulgaris)* los escasos trabajos que han obtenido éxito en su cultivo relacionan los mejores crecimientos y supervivencia de las paralarvas con la co-alimentación con *Artemia* y zoeas de crustáceos (Villanueva 1995, Iglesias *et al*., 2000, 2007; Moxica *et al.*, 2002; Carrasco *et al.*, 2006). Finalmente, citar el uso de huevos o larvas de peces cultivados para la cría de especies marinas tipo grandes pelágicos como Atún o Medregal. Estas especies presentan una alimentación netamente piscívora, desde etapas tempranas del desarrollo como han puesto de manifiesto Catalán *et al,* (2007), tras la evaluación de contenidos estomacales de larvas de albacora (*Thunnus alalunga*) y donde se detecta el comportamiento piscívoro tras el proceso de flexión de la notocorda. A partir de este momento, las presas vivas habituales como rotíferos o *Artemia* no llegan a satisfacer de una forma adecuada las necesidades alimenticias del animal, bien por la cantidad de nutrientes esenciales necesarios o bien por la limitación en los tamaños de las mismas, más aún, cuando la adaptación a la ingesta de microdietas es limitada, como ocurre con el atún Atlántico *Thunnus thynnus*, el atún Pacífico (*Thunnus orientalis*), el bonito (*Sarda sarda*) o la seriola (*Seriola dumerili*) (Kaji, (2002); Papandroulakis *et al*., (2006); Seoka *et al*., (2007); Ortega y De la Gándara., (2007).

2.4.3.3.-Calidad nutritiva de las presas

Se ha demostrado que los parámetros anteriormente mencionados tienen una influencia clara en el éxito del cultivo larvario, tanto en cantidad como en la calidad de los alevines producidos. Si bien, este éxito está siempre condicionado mucho más a la calidad nutritiva de las presas utilizadas. En el medio natural, las larvas se alimentan de un gran abanico de presas con un rango de tamaños y composición nutritiva adecuada a los distintos estadios de desarrollo de las larvas. En este sentido, las mejoras observadas con el uso de copépodos en la alimentación larvaria, se asocian a una mayor calidad nutritiva general, asociada al contenido de lípidos, ácidos grasos, vitaminas, carotenos y otros nutrientes esenciales presente en estas presas naturales (Evjemo y Olsen., 1997; Evjemo *et al*., 2003; Van der Meeren *et al*., 2008).

Sin embargo, la dificultad que presentan las técnicas de producción masiva de este tipo de zooplancton marino, hacen que su uso en los criaderos se vea muy limitado a instalaciones experimentales o sistemas de cría extensivos en determinadas localizaciones (Van der Meeren y Naas, 1997). Es por ello, que los procesos de cría de peces marinos en sus primeras etapas se basan en la alimentación con dos tipos de presas vivas: el rotífero (*Brachionus sp*.) y el crustáceo *Artemia sp.,* que generalmente no forman parte de la dieta natural de las larvas en el mar y presentan unas características nutricionales que difieren enormemente del alimento natural de las larvas, incluso después de ser sometidas a los procesos de enriquecimiento, lo cual limita el crecimiento, supervivencia y correcto desarrollo de las larvas durante las primeras etapas (Ronnestad *et al*., 1998, McEvoy y Sargent., 1998).

Uno de los principales parámetros que se han considerado clave para determinar la calidad del alimento es el contenido lipídico de los mismos y mas concretamente, el contenido de ácidos grasos esenciales de las series n-3 y n-6 (Izquierdo, 1996).

2.4.3.4.-Importancia de los lípidos en la nutrición larvaria

En la historia reciente de la acuicultura, se ha dedicado un gran esfuerzo a los estudio de los requerimientos nutricionales de las larvas de peces marinos, usando presas vivas y dietas inertes (Izquierdo, 1996; Sargent *et al*., 1999; Ronnestad *et al*., 1999). Muchos de estos trabajos, se han centrado en los estudios de la esencialidad de los ácidos grasos altamente insaturados, especialmente de la serie n-3, ya que, es bien sabido, que las elevadas mortalidades en las primeras etapas de vida de las larvas de peces marinos, son frecuentemente debidas a deficiencias de ácidos grasos esenciales (AGE) (Izquierdo, 1996). Los ácidos grasos esenciales y más concretamente los altamente insaturados (HUFA) como el ácido docosahexaenoico (DHA,

22:6n-3), ácido eicosapentaenoico (EPA, 20:5n-3) y ácido araquidónico (ARA, 20:4n-6) se ha demostrado que son componentes esenciales de las membranas celulares, modulando mecanismos fisiológicos, como los proceso de trasporte de membrana, funciones de receptores o la actividad enzimática, especialmente en las etapas de desarrollo larvario (Izquierdo, 1996). En el caso de los peces de agua dulce, estos tienen la capacidad enzimática suficiente para producir DHA, EPA y ARA a partir del suministro en la dieta de precursores tales como el ácido linoleico (18:2n-6) y linolénico (18:3n-3). Sin embargo, los peces marinos presentan una capacidad muy restringida de las enzimas responsables de desaturar y elongar los precursores (Δ5 y Δ6) para formar ARA, EPA y DHA y consecuentemente estos ácidos grasos han de ser incorporados a través de la dieta.

No solo la cantidad total de cada uno de los diferentes HUFA, sino también de las distintas relaciones entres las series n-3/n-6 y en particular de los distintos ácidos grasos como el DHA, EPA y ARA se consideran importantes para el crecimiento y correcto desarrollo de las larvas de peces (Izquierdo, 1996, Rainuzzo *et al*., 1997; Sargent *et al*., 1999). Además de su papel estructural y funcional en las membranas celulares de los peces, los AGE como el DHA son particularmente importantes para el funcionamiento y desarrollo normal del sistema nervioso, como ocurre con la retina (Bell *et al*., 1995a) mientras que el ARA y EPA están más involucrados en los mecanismos de regulación y producción de eicosanoides (Bell *et al*., 1995b; Sargent, 1997; Ganga *et al*., 2005).

De manera general, la alimentación de larvas de peces marinos con presas vivas, o microdietas deficientes en ácidos grasos altamente insaturados de la serie n-3 HUFA, provoca síntomas tales como una reducción en el crecimiento y en la supervivencia, además de una mayor sensibilidad al estrés que se manifiesta con un aumento del cortisol y síntomas de inmunodeficiencia para algunas especies marinas (Izquierdo, 1996; 2004). Otros síntomas de las deficiencias de AGE en las larvas son, la disminución de la actividad natatoria y alimenticia, hidropesía (depósitos de agua subcutaneos y subcelómicos) (Yamashita, 1981), shock y mortalidad con el test de actividad de exposición al aire, inadecuada inflación de la vejiga natatoria (Koven *et al*., 1990) o la disgregación del epitelio branquial. Es más, con una dieta deficiente en HUFA la formación de la retina es defectuosa, la información visual que llega al sistema nervioso central no es correcta, por lo que la hormona estimuladora de los melanocitos no es secretada por la glándula interna, y la formación de melanina se interrumpe, dando lugar a problemas de falta pigmentación como ha ocurrido con algunos peces planos, lo que les hace poco atractivos cara al consumidor (Kanazawa, 1993). Otros problemas relacionados con la deficiencia de AGE se centran en los

daños irreversibles que se pueden causar al propio sistema visual en desarrollo de las larvas deficitarias en DHA en la retina, lo que se traduce en las altas mortandades. Por otro lado, juveniles de arenque (*Clupea harengus*) sometidos a dietas deficitarias en DHA muestran una reducción de la visión en condiciones de baja intensidad de luz, lo que se traduce en una perdida en la habilidad para la captura de presas (Bell *et al*., 1995a). En mamíferos, estudios de deficiencias de DHA en las dietas muestran que este es fuertemente retenido por el tejido nervioso y se necesitan al menos dos generaciones para inducir deficiencias (Neuringer *et al*., 1986; Neuringer *et al*., 1988). Pero en peces, el desarrollo del cerebro y la retina ocurre en los primeros días tras la eclosión, por lo que, resulta más fácil inducir deficiencias (Bell y Dick, 1993). Se ha probado que los niveles de DHA en cerebro y ojo de las larvas se modifican fácilmente con cambios de este ácido graso en la dieta.

2.5.-Problemática asociada al cultivo larvario de peces marinos

Incluso en la dorada (*Sparus aurata*) que es una especie bien estudiada existen todavía aspectos de su cultivo, fundamentalmente asociados a las primeras etapas de desarrollo, que han de seguir siendo mejorados para optimizar la productividad de los criaderos comerciales y asi servir como modelo, para aplicación en otras especies cuyo conocimiento general no está tan avanzado.

En este sentido, merecen especial atención los estudios referentes al desarrollo de técnicas de cultivo larvario que permitan un mayor control y estabilidad de las tasas de supervivencia larvaria, así como profundizar en el conocimiento de los factores y mecanismos que determinan la aparición de deformidades esqueléticas que condicionan la calidad de las larvas.

2.5.1.-Supervivencia larvaria

Las primeras etapas de cultivo de las larvas de peces marinos, como la dorada, presentan diferentes fases críticas en su desarrollo que pueden comprometer seriamente el éxito del cultivo al verse afectadas por mortalidades masivas. Uno de los picos de mortalidad característico del cultivo larvario de dorada se observa entre el final de la absorción del saco vitelino y comienzo de alimentación exógena (Tandler *et al*., 1989). Los principales factores a los que se atribuye la mortalidad larvaria son: la alimentación deficiente de los reproductores, las deficiencias de n-3 HUFA en las presas vivas, o la excesiva entrada de carga microbiana vía alimento vivo cuando se introduce en el tanque de cultivo, siendo esta una de las mayores causas de mortalidad en las etapas tempranas (Skjermo y Vadstein, 1999). En los últimos años se ha hecho grandes avances en el conocimiento de los requerimientos nutricionales de las larvas, así como en el desarrollo de productos enriquecedores y microdietas (Shields, 2001). Igualmente se han desarrollado

protocolos y productos para la desinfección de las presas vivas como *Artemia* y el rotífero (De Wolf *et al*., 1998; Gimenez *et al*., 2006), si bien este último se ha mostrado mas susceptible a los métodos de desinfección (Ringo y Birkbeck, 1999). Igualmente, se han desarrollado técnicas tendentes a reducir la carga bacteriana del tanque de cultivo mediante el empleo de aguas maduras y a través de sistemas de recirculación. Finalmente, la introducción del uso de probióticos o cepas bacterianas beneficiosas, que eviten la proliferación de bacterias oportunistas o patógenos obligados es otro de los avances desarrollados para la mejora de la supervivencia (Skjermo y Vadstein, 1999; Gatesosupe, 1999). Todos estos avances, encaminados a la reducción de las mortalidades tempranas del cultivo han permitido el desarrollo de técnicas de cría a escala comercial con una buena predictibilidad, si bien estas siguen en gran parte basadas en la repetitibilidad de un proceso con un gran esfuerzo más que en el control del mismo.

Otro de los grandes retos a los que se enfrenta la producción de larvas de peces marinos es el creciente aumento de otras patologías, como son la incidencia de malformaciones esqueléticas, que a pesar de no provocar mortalidades masivas si tienen una gran repercusión sobre la productividad del criadero.

2.5.2.-Incidencia de deformidades

Hoy en día uno de los factores que más preocupa a los productores comerciales de peces marinos es la calidad de los alevines producidos. El término calidad hace referencia a la no incidencia de anormalidades morfológicas, que son alteraciones de la morfología del pez, normalmente irreversibles, en comparación con un estándar de calidad.

De una manera general, en los criaderos comerciales intensivos, entre un 10 y un 30 % de la población de larvas de peces marinos presenta desviaciones espinales (Andrades *et al*., 1996), si bien estos porcentajes son orientativos, debido a que existe mucha variación entre y dentro de los criaderos en cuanto al tipo de deformidad, la incidencia, la repercusión económica y la especie. En especies como la lubina (*Dicentrachus labrax*) o espáridos como dorada (*Sparus aurata*), bocinegro (*Pagrus pagrus*) y pargo japonés (*Pagrus major*), las malformaciones más relevantes son las que afectan al complejo opércular, neurocráneo y columna vertebral (lordosis y fusión de vértebras) (Koumoundouros *et al*. 1997a; Boglione *et al*. 2001; Roo *et al*. 2005b). En el caso de peces planos como rodaballo (*Psetta máxima*), fletán (*Hippoglosuss hipoglosus*) o lenguado senegalés (*Solea senegalensis*), además de las malformaciones de columna, tienen especial relevancia las anomalías de pigmentación, la migración incompleta del ojo y las deformidades mandibulares (Gavai *et al*., 2002, Sæle *et al*., 2003; Aritaki y Seikai 2004; Lewis y Lall,. 2006).

Así, dentro del proceso industrial de cría de alevines, las empresas se ven obligadas a implantar procesos de criba para eliminar los individuos que presentan algún tipo de anormalidad no comercial, ya que de manera generalizada las granjas de engorde no aceptan lotes con más de un 5% de peces deformes en valoración visual. Este proceso añade unos costes asociados que se estima, repercuten en 0,01€ en el coste de producción de un alevín. Hay criaderos que niegan la existencia de deformidades en sus lotes, que por una estrategia de marketing necesariamente va acompañada de un elevado grado de selección de los animales antes de su venta.

Figura 11: Criba manual de individuos deformes (fotografía cortesía de Tinamenor S.A).

Las diferentes anomalías que afectan a la calidad de los peces, en mayor o menor medida, pueden ser agrupadas principalmente en dos grupos: de pigmentación y de esqueleto.

2.5.2.1.-Anomalías de pigmentación

Las anomalías de pigmentación consisten en una alteración parcial o total de la coloración del cuerpo. Es en peces planos, donde han sido ampliamente descritas por su importancia económica (Gavai *et al*., 2002; Sæle *et al*., 2003; Aritaki y Seikai., 2004; Lewis y Lall., 2006). La pigmentación anormal consiste en hipomelanosis o pseudoalbinismo cuando afecta a la cara cara dorsal e hipermelanosis o ambicoloración si afecta a la ventral (Venizelos y Benetti 1999) (Figura 12). Las anomalías de pigmentación son consecuencia de una distribución diferencial y o diferencias fisiológicas celulares de los cromatóforos (Kelhs 2004; Burton 2005) que han sido asociadas a factores como las condiciones intensivas de cultivo, densidad, iluminación, el

sustrato o la dieta (Seikai *et al*. 1987; Estévez *et al*. 1999, 2001; Benetti 1997). En poblaciones naturales suceden tanto el pseudoalbinismo (Venizelos y Benetti 1999) como la ambicolaración (Astarloa 1995), con inferior incidencia que en poblaciones de cultivo (Bolker y Hill 2000).

Figura 12. Ejemplares de lenguado senegales (*Solea senegalensis*) con pigmentación normal y con pseudoalbinismo.

2.5.2.2.-Anomalías de esqueleto

Existen diferentes estudios acerca del desarrollo osteológico y la aparición de anomalias esqueléticas en espáridos, como dorada (*Sparus aurata)* (Faustino, 2002; Boglione *et al*., 2001; Koumondouros *et al*., 1997a), el pargo japonés (*Pagrus major)* (Moteki, 2002; Kihara *et al.,* 2002; Matsuoka, 2003), dentón (*Dentex dentex)* (Koumoundouros *et al*., 2001a), breca (*Pagellus erythinus)* (Boglione *et al*., 2003; Sfakianakis *et al.,* 2004), sargo picudo (*Diplodus puntazo)* (Boglione *et al*., 2003; Favaloro and Mazzola, 2003) y sargo *Diploduss sargus* (Koumoundouros *et al*., 2001b; Sfakianakis *et al.,* 2003). De manera general, las anomalías de esqueleto más importantes en peces marinos son las que afectan al neurocráneo, espina dorsal y esqueleto apendicular ya que alteran de manera severa la morfología de estos y tienen una importante repercusión económica en las empresas.

Las deformidades asociadas al neurocráneo afectan fundamentalmente al complejo opercular y mandíbulas y son encontradas frecuentemente en los criaderos comerciales. Las anomalías del complejo opercular pueden estar presentes a uno o ambos lados del pez y consistir en un plegamiento o una formación incompleta del complejo opercular (Koumoundourous, *et al.,*

1997b) a menudo esta anomalía está asociada con malformaciones de los arcos branquiales (Sadler *et al*., 2001) (Figura 13). Estas deformidades suceden desde edades muy tempranas en el desarrollo, sin asociación con un lado corporal concreto, y pudiendo alcanzar incidencias de hasta el 98,3% de la población (Beraldo *et al*., 2003).

Figura 13. Ejemplares juveniles de pargo o bocinegro (*Pagrus pagrus*) y corvina (*Argyrosomus regious*) con anomalía del complejo opercular (a,c); b) detalle del plegamiento hacia el interior.

Las deformidades de las mandíbulas, consisten en torsiones de la mandíbula inferior y superior o prolongación de éstas en diferente magnitud (Cobcroft *et al*., 2001), que en ocasiones han sido asociadas a efectos letales (Barahona-Fernandes, 1982) (Figura 8).

Figura 14. Deformidades mandibulares en:a,b) pargo o bocinegro, c) dorada.

En general, el desarrollo ontogénico del neurocráneo y de las aletas es previo a la eclosión, siendo completado a etapas más avanzadas del desarrollo. Las deformidades de las aletas se caracterizan por torsión, falta parcial o total de las mismas y puede alcanzar incidencias de hasta un 65% en larvas de dorada (*Sparus aurata*) (Koumoundouros *et al*.,1997b) aunque tienen una relevancia menor en su repercusión comercial (Figura 14).

Figura 15. Anomalías en los radios de las aletas pectorales en; a) dorada, b) pargo ó bocinegro.

Las anormalidades de la columna vertebral son una de las más relevantes en piscicultura no sólo por su severo efecto sobre la morfología del pez sino también por la influencia que ejercen sobre caracteres productivos como el crecimiento (Gjerde *et al*. 2005; Kause *et al*. 2005). Las principales deformidades de columna son la escoliosis, lordosis, cifosis y fusiones vertebrales, que en ocasiones es posible encontrar en un mismo pez de forma combinada (Afonso *et al*., 2000) (Figura 16).

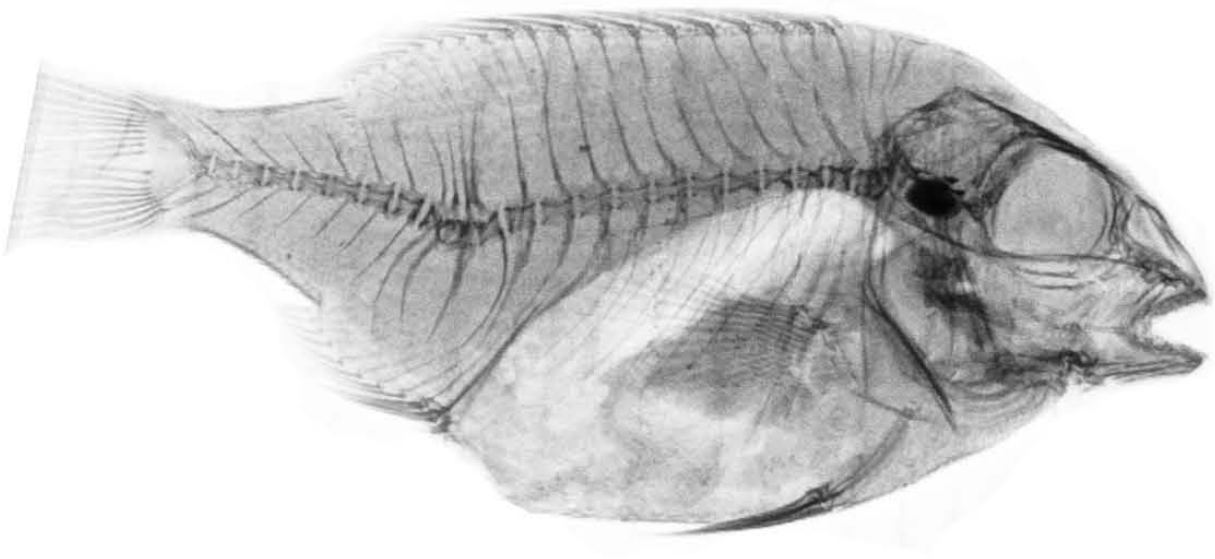


Figura 16. Anomalías de columna en bocinegro (*Pagrus pagrus*).

Las anormalidades de la columna han sido documentadas en especies de aguas dulce (Akiyama *et al*., 1985; Madsen y Dalsgaard 1999) y en especies marinas (Paperna, 1978; Takashima, 1978; Barahona-Fernandes, 1982; Taniguchi *et al*., 1984; Daoulas *et al*.,1991; Andrades *et al*., 1996; Koumoundouros *et al*.,1997b, 2001a,b, 2002; Faustino y Power, 1998, 2001; Afonso *et al*., 2000; Boglione *et al*., 2001; Faustino, 2002; Gavaia *et al*., 2002; Kihara *et al*., 2002; Moteki, 2002; Favaloro y Mazzola, 2003; Boglione *et al*., 2003; Matsuoka, 2003; Sfakianakis *et al*., 2004).

2.5.2.3.-Factores condicionantes de la aparición de anomalías de esqueleto

La aparición de deformidades esqueléticas en peces está relacionada con factores medioambientales, nutricionales y genéticos. Entre los factores ambientales están los abióticos (intensidad de la luz, oxígeno disuelto, temperatura, pH, salinidad, flujo de agua, asociados al cultivo), bióticos (bacterias, virus, hongos y parásitos) y xenobióticos (alguicidas, fungicidas, herbicidas, insecticidas, efluentes industriales y metales pesados). Entre los nutricionales están los relacionados con los ácidos grasos esenciales, fosfolípidos, aminoácidos, proteínas, vitaminas y minerales, mientras que entre los factores genéticos están los unigénicos y los poligénicos.

2.5.2.3.1.-Factores medioambientales

Dentro de los factores ambientales abióticos se ha descrito la luz, la temperatura o la salinidad como causantes de la aparición de deformidades, en peces marinos (Battaglene and Talbot, 1990; Polo *et al*.,1991; Mihelakakis y Yoshimatsu, 1998; Cobcroft *et al*., 2001; Sfakianakis *et al*., 2004). Otros factores que puede provocar repercusiones negativas en la integridad del esqueleto-vértebras en larvas son los traumas mecánicos durante el desarrollo embrionario, o durante las primeras etapas de cultivo larvario, consecuencia de la manipulación de huevos y larvas. El desarrollo anómalo de la vejiga natatoria, (Chatain,1989,1990; Andrades, 1993;) y las variaciones en la hidrodinámica del tanque (Chatain, 1994; Divanach *et al*., 1997; Koumoundouros *et al*., 1997a,b; Kihara *et al*., 2002) se ha visto que favorecen la aparición de lordosis.

De manera general, el sistema de cultivo ha sido descrito como factor modulador de la aparición de deformidades esqueléticas, fundamentalmente relacionado con la intensificación de las técnicas de cría, asociando el uso de sistemas extensivos y semi-intensivos a la obtención de peces con muy baja incidencia de deformidades esqueléticas más similares a las de los peces salvajes, cuando se comparan con los obtenidos en ejemplares cultivados mediante técnicas intensivas (Divanach y Kentouri, 1983; Divanach *et al*.,1996; Boglione *et al*., 2001; Koumoundouros *et al*., 2001a; Sfakianakis *et al*., 2004; Roo *et al*., 2005b; Giménez y Estévez, 2008b) (Figura 7).

Los antecedentes de factores medioambientales bióticos como generadores de deformidades esqueléticas son escasos, siendo los parásitos los que tienen una mayor influencia. Parásitos pertenecientes a la familia mixosporea han sido descritos como causantes de deformidades esqueléticas en trucha arcoiris *(Oncorhyncus mykiss),* seriola coreana *(Seriola quinqueradiata)* y perca de río *(Perca fluviatilis)* (Lom *et al*., 1991). La relación de factores xenobióticos con la aparición de deformidades esqueléticas ha sido señalada para pesticidas (Chun *et al*. 1981; Thi Hong Lien *et al*., 1997), herbicidas (Koyama, 1996), hidrocarburos (Grady *et al*. 1992), compuestos orgánicos y organoclorados (Lindesjöö *et al*. 1994) y metales (Slominska y Jezierska, 2000). También el exceso de antibióticos en los tratamientos de enfermedades ha sido relacionado con la inducción de deformidades esqueléticas (Toften y Jobling, 1996).

2.5.2.3.2.-Factores nutricionales

Además de los factores ambientales, son muy numerosos los factores nutricionales que actúan directamente sobre el metabolismo del hueso y el cartílago, como las vitaminas y ácidos grasos, además de otros factores que de manera indirecta condicionan estos procesos como son las hormonas, prostaglandinas, citoquinas y factores de crecimiento (Watkins y Seifert, 2000a). En el caso particular de los peces, se ha visto que los factores nutricionales tales como las deficiencias y excesos de vitaminas y ácidos grasos esenciales durante las primeras etapas de de crecimiento o las dietas de los reproductores pueden alterar el desarrollo osteológico de los embriones y larvas (Kanazawa *et al*., 1983; Akiyama *et al*., 1986, Knox *et al*., 1988; Chatain and Ounais–Guschemann, 1990; Afonso *et al*., 2000, Cahu *et al*., 2003, Saele *et al*., 2003; Hamre *et al*., 2005). Aunque, en los peces no se ha establecido una relación entre la composición de ácidos grasos de la dieta y la del cartílago y hueso, diferentes estudios con otro vertebrados como pollos y ratas han demostrado que las variaciones en la relación de las series n-3/n-6 PUFA en la dieta alteran la composición de los ácidos grasos del hueso y cartílagos (Xu *et al*., 1994; Watkins *et al*., 1991,2000b; Liu *et al*., 2004). En este sentido, Gapasin y Durai, (2001), han encontrado una relación entre la aparición de deformidades esqueléticas y los ácidos grasos esenciales de la dietas en los peces marinos larvas, juveniles y reproductores. Aunque, los mecanismos por los cuales los ácidos grasos controlan el desarrollo osteológico no son bien conocidos. Sin embargo, su implicación a nivel molecular ha sido demostrada, actuando como moduladores del genoma a través de receptores nucleares específicos, como los receptores activados de proliferación de Peroxisomas (PPAR) que se unen a la molécula de ADN como heterodímero con el receptor X del ácido retinoico (RXR). Este receptor actúa como factor de trascripción ligando-activado (Mangelsdorf *et al*., 1994) y los genes que lo regulan están involucrados en el desarrollo esquelético durante la ontogénesis, lo que pone de manifiesto que los HUFA pueden regular la expresión de genes del desarrollo y de este modo afectar el desarrollo del esqueleto, esto tiene muchas implicaciones en la salud humana y ha sido muy poco estudiado en el desarrollo de los peces (Cahu *et al*., 2003). Sin, embargo recientemente Villeneuve *et al*., 2005, 2006 han reflejado que el exceso de HUFA en las dietas acelera la diferenciación de los osteoblastos a través de una sobre-regulación del Receptor X retinoico α y las proteínas de morfogénesis del hueso (BMP4), lo que se refleja con la aparición de un exceso de vértebras en las larvas de lubina *(Dicentrarchus labrax).*

En cuanto a los aminoácidos esenciales, las deficiencias en triptófano han sido asociadas con la presencia de deformidades (Walton *et al*., 1984; Akiyama *et al*., 1985, 1986; Wilson, 1989). La deficiencia de péptidos en la dieta también influye en la incidencia de malformaciones esqueléticas en el desarrollo larvario (Cahu *et al*., 1999). En cuanto a las vitaminas, ha sido demostrada la influencia de los excesos o defectos las vitaminas A y C en las incidencias de las deformidades esqueléticas en peces (Halver, 1989; Dedi *et al*., 1995; Takeuchi *et al*., 1995, 998). En este sentido, es importante resaltar que el ácido retinoico tiene capacidades teratogénicas con influencia en los sucesos que acontecen durante la embriogénesis de animales y humanos, viéndose los efectos de éste, medidos a través de la expresión de los genes Hox y Sonic Hedgehog (shh). Así, un exceso de ácido retinoico en la dieta de las larvas de lenguado del Pacífico *(Paralichthys olivaceus)* provoca la aparición de compresión de las vértebras (Takeuchi *et al*., 1998).

2.5.2.3.3.-Factores genéticos

Andrades *et al*. (1996) explican que la mayoría de los juveniles lordóticos observados en una granja de engorde de dorada *(Sparus aurata)*, provenían probablemente de larvas lordóticas supervivientes, sugiriendo que las causas primarias de la aparición de lordosis podrían ser, entre otras, de origen genético que pueden afectar a los huevos durante el desarrollo embrionario. La primera asociación familiar de una deformidad severa en dorada se debe a Afonso *et al*. (2000), quienes describieron la aparición simultánea de una triple deformidad de columna (*LEC;* lordosis, escoliosis y cifosis) en todas las réplicas de descendientes de una misma familia. Astorga *et al*. (2003a,b) estudiaron el efecto de la consanguinidad en la aparición de las deformidades esqueléticas de dorada, mediante el cruce de reproductores normales con distintos niveles de consanguinidad (F=0,125; F=0,25), a las edades de 4, 14 y 35 días post-eclosión y a los 194 días de edad.

9.3.-Objetivos

El objetivo principal de este trabajo fue **"la mejora de la tecnología de producción de larvas y alevines de bocinegro".** Los objetivos específicos se dividen en dos etapas consecutivas que se complementan, una primera parte del trabajo en la que se describen aspectos específicos sobre la biología y desarrollo de las primeras etapas de vida de esta especie como son el desarrollo del sistemas visual y digestivo, el desarrollo osteológico o la aparición de deformidades esqueléticas en relación al sistema de cultivo empleado y al valor nutricional de las presas vivas. A continuación en la segunda parte, se ha aplicado el conocimiento adquirido para la mejora del protocolo de cría de esta especie.

Los objetivos planteados se han logrado mediante la realización de 4 estudios que se incluyen en esta trabajo de tesis doctoral.

Estudio I. **Desarrollo del sistema visual de las larvas de bocinegro *Pagrus pagrus* (Linnaeus, 1758) en relación a los cambios del sistema digestivo y hábitos alimenticio.**

En este estudio se pretende describir la formación del sistema visual de las larvas de bocinegro, y su correlación con el desarrollo del sistema digestivo. Este objetivo se plantea con el fin de obtener información básica, como herramienta para la búsqueda de las condiciones de cultivo generales para esta especie: como son la necesidad de cambios de iluminación, uso de agua verde y determinación de la secuencia alimentaria idónea. Se realizó una experiencia de cultivo para lograr este objetivo.

Estudio II. **Desarrollo osteológico y aparición de deformidades esqueléticas en las larva de de bocinegro *Pagrus pagrus* (Linnaeus, 1758) bajo diferentes técnicas de cultivo.**

En este estudio se pretende describir el efecto de la intensificación de las técnicas de cría larvaria en relación al desarrollo osteológico de esta especie y la incidencia de malformaciones esqueléticas. Este objetivo se plantea con el fin de obtener información básica a cerca del patrón de desarrollo osteológico y la incidencia de las anomalías esqueléticas de esta especie, que permitirá determinar la calidad de los alevines producidos e idoneidad de las técnicas de cría aplicadas. Se realizaron dos experiencias de cultivo para lograr este objetivo.

Estudio III*. Efecto del contenido de DHA en los rotíferos sobre la incidencia de deformidades esqueléticas del bocinegro *Pagrus pagrus (*Linnaeus*, 1758).

En este estudio se pretende describir el efecto ciertos factores nutricionales sobre el crecimiento, supervivencia e incidencia de las malformaciones esqueléticas en los primeros estadios de desarrollo de esta especie. Este objetivo se plantea con el fin de identificar factores nutricionales que afectan al desarrollo larvario y mejorar los productos enriquecedores de presas vivas para esta especie. Se realizaron dos experiencias de cultivo para lograr este objetivo.

***Estudio IV*. Avances en las técnicas de cultivo de bocinegro *Pagrus pagrus (*Linnaeus*, 1758)*: Comparación de sistemas de cultivo larvario intensivos y semi-intensivos.**

En este estudio se pretende mejorar los protocolos de producción larvaria de esta especie: Para alcanzar este objetivo se realizaron tres experiencias centradas en el estudio de los cambios en los regimenes de luz, la densidad de presas y el protocolo de co-alimentación, comparando a su vez diferentes sistemas de cultivo.

9.4.-Material y métodos

4.1.-Localización del estudio

Las experiencias que se describen en este estudio han sido realizadas en la planta experimental de cría larvaria y producción de alevines del Instituto Canario de Ciencias Marinas (ICCM) (Planta Mesocosmos) perteneciente a la Agencia Canaria de Investigación, Innovación y Sociedad de la Información de la Presidencia del Gobierno de Canarias, donde se desarrollan diferentes actividades de I+D+I mediante la aplicación de tecnologías de cultivo semi-intensivas (Mesocosmos) e intensivas, para la producción de larvas y alevines de peces marinos a escala pre-industrial. Dicha instalación se encuentra ubicada en la localidad de Melenara, Municipio de Telde, provincia de las Palmas (Gran Canaria), con una situación geográfica de latitud 27º59´31´´ y longitud 15º22´31´´(Figura 17).

Figura 17: Fotografía satélite de las Islas Canarias y ubicación de las instalaciones de ICCM (Google Earth foto).

4.2.-Instalaciones

4.2.1.-Descripción general

La planta experimental de cría larvaria y producción de alevines, es un criadero a escala piloto, cuya principal finalidad es el desarrollo de técnicas de cultivo larvario de nuevas especies para la diversificación de la acuicultura marina, tales como es el caso de bocinegro (*Pagrus pagrus*), corvina (*Argyrosomus regius*) y lenguado senegalés (*Solea senegalensis*). Otra de las funciones de esta instalación es el desarrollo de proyectos encaminados a la mejora de los procesos de cría de las especies ya consolidadas como son dorada (*Sparus aurata*) y lubina (*Dicentrarchus labrax*), los principales objetivos con estas especies se han dirigido a la reducción del porcentaje

de deformidades esqueléticas, mejora de la supervivencia y crecimiento o la automatización de los procesos de cría. Las dimensiones de estas instalaciones, permiten la producción de alevines a escala semi-industrial de las citadas especies, lo que ha contribuido al desarrollo de proyectos mediante convenios de colaboración con diferentes empresas del sector.

La planta experimental ocupa una superficie de 800 m^2 y se componen de dos naves independientes que incluyen las siguientes unidades.

Nave A:

- Unidad de tratamiento de agua (filtración y esterilización de agua por ultravioleta).
- Unidad de producción de alimento vivo.
- Unidad de cultivo larvario (2 tanques de 40.000 litros, 4 de 2.000 litros y 6 de 500 litros).

Nave B:

- Unidad de alevinaje y pre-engorde 8 tanques de 10.000 litros.
- Unidad de control y suministro de gases (oxígeno y propano).

En las siguientes figuras 18 y 19 se muestra un esquema general de las instalaciones.

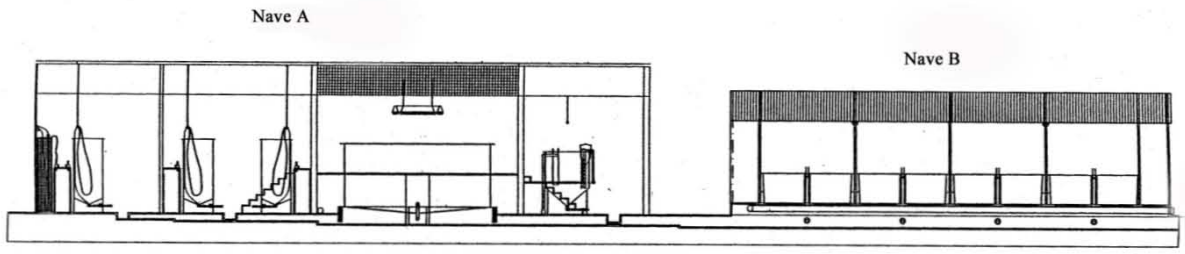


Figura 18: Sección longitudinal de las instalaciones.

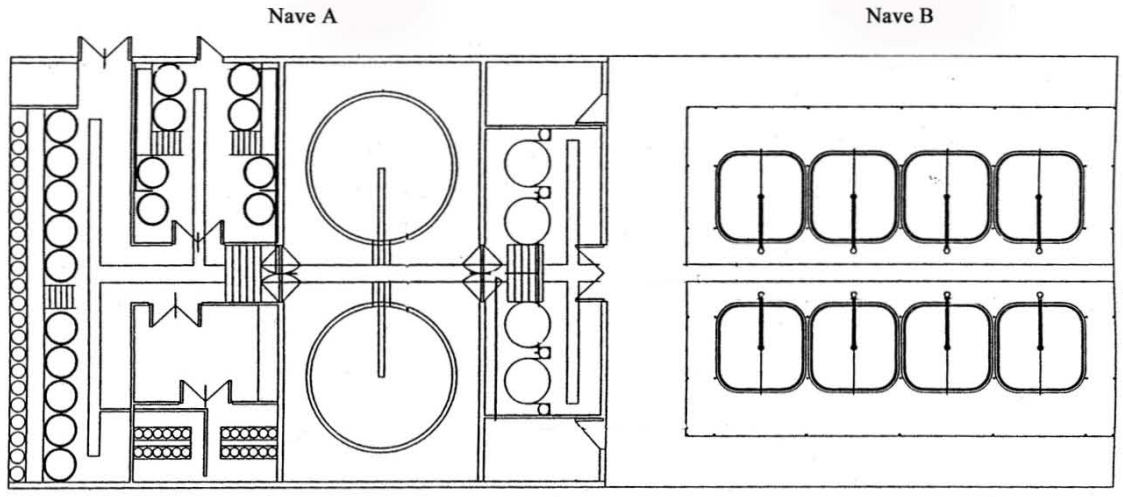


Figura 19: Vista en planta de las instalaciones.

En la figura 20 se muestran detalles de las diferentes unidades de las instalaciones de cría.

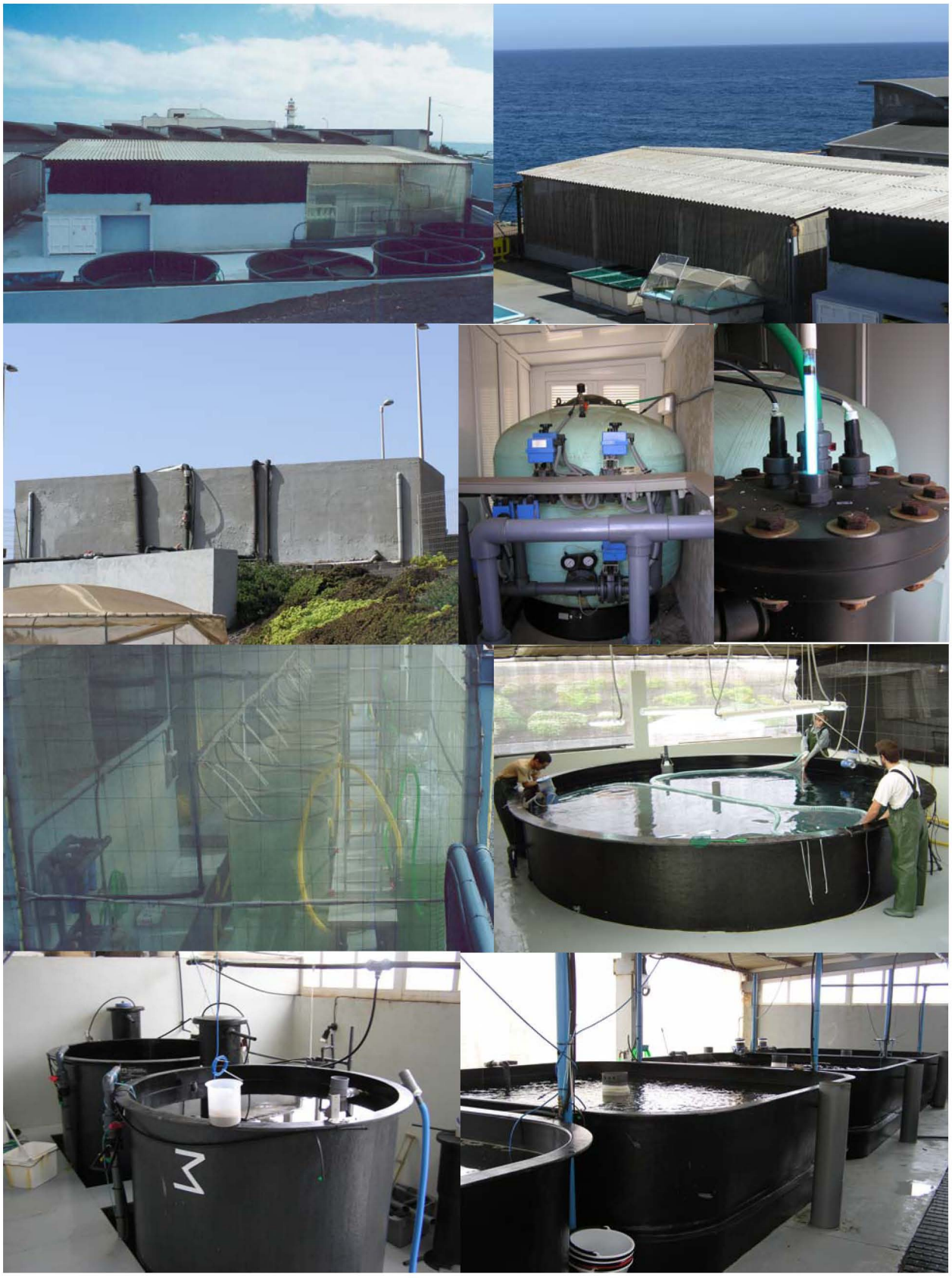


Figura 20. a) Vista general de las naves A,B; b) Unidad de almacenamiento y tratamiento de agua; c)Unidad de producción de fitoplancton y rotíferos; d) tanque de cultivo larvario semi-intensivo; e) Tanques de cultivo larvario intensivo) Tanques de alevinaje.

4.2.2 Descripción de los tanques de cultivo

Para las experiencias de cultivos larvario se emplearon tres tipos de tanques fabricados de polyester reforzado con fibra de vidrio.

4.2.2.1. Tanques de 40.000 litros-Cultivo semi-intensivo

Se utilizaron 2 tanques cilíndricos con el fondo en ligera pendiente hacia el desagüe central, con una capacidad de 40.000 litros situados en paralelo y separados por una canaleta central de drenaje (Figura. 21). Los tanques tienen un diámetro de 5 m y una profundidad de 2,35m en la parte central (Figura 22) y cuentan originalmente con una entrada de agua en su parte superior y dos desagües situados en el fondo, uno de ellos en posición central y el otro en posición lateral enfrentado a la entrada de agua. La entrada y salida de agua, se modifican con diferentes accesorios móviles, a lo largo del periodo de cultivo, de acuerdo con la secuencia alimentaria. Así, durante las primeras fases de alimentación con presas vivas, la entrada de agua se realiza por el fondo y la salida por la superficie. Una vez se introducen las dietas inertes el sistemas se invierte para favorecer la limpieza del tanque.

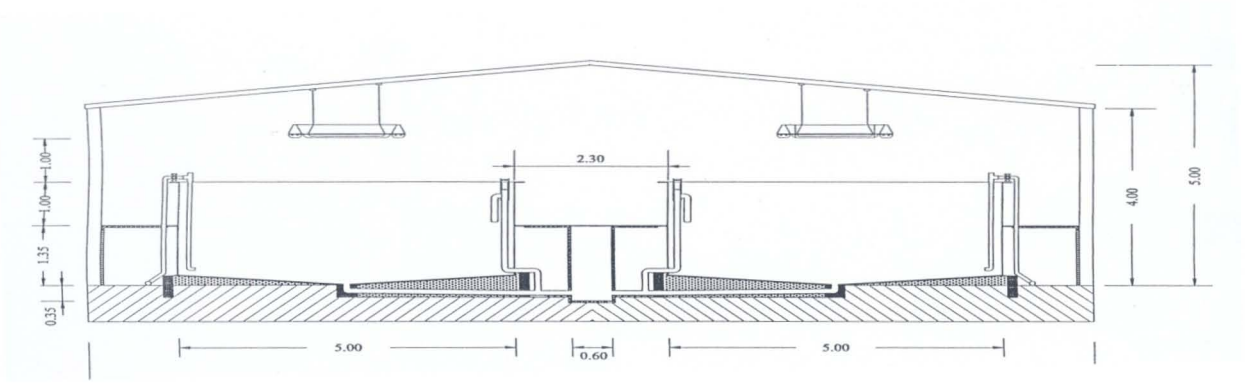


Figure 21. Sección longitudinal de los tanques de cultivo semi-intensivo.

Figura 22. a) Vista longitudinal, planta del tanque; b) Tanque de cultivo semi-intensivo.

4.2.2.2. Tanques de 2.000 litros-Cultivo intensivo.

Se utilizaron 6 tanques cilindro-cónicos con una capacidad de 2.000 litros situados en paralelo y separados por la canaleta central de desagüe (Figura. 23). Los tanques tienen un diámetro de 1,5m y una profundidad de 2 m, en su parte más profunda (Figura 24). Los tanques cuentan con una entrada de agua en su parte superior y dos salidas de agua una situadas en la parte inferior, una de ellas en posición central y la otra en posición lateral enfrentada a la entrada de agua. Al igual que en los tanques de 40.000 litros, la posición de entrada y salida de agua se modifica con diferentes accesorios móviles a lo largo del periodo de cultivo, de acuerdo a la secuencia alimentaria.

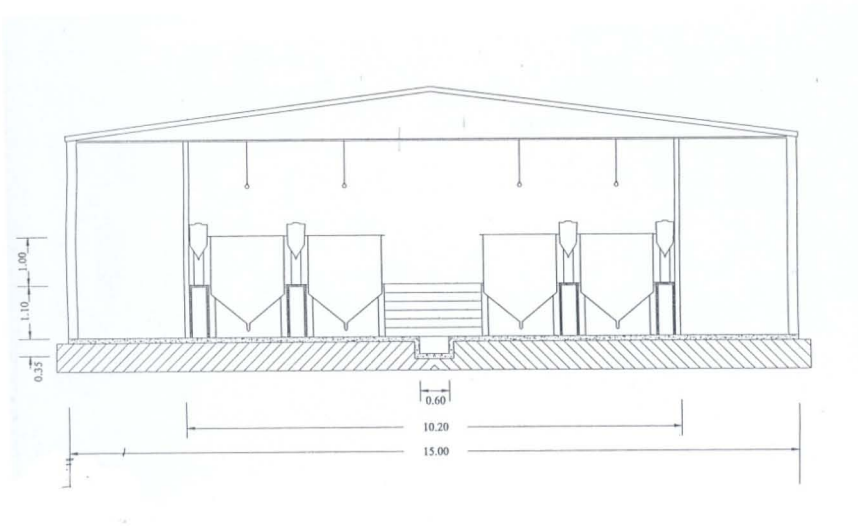


Figura 23. Sección longitudinal de los tanques de cultivo intensivo.

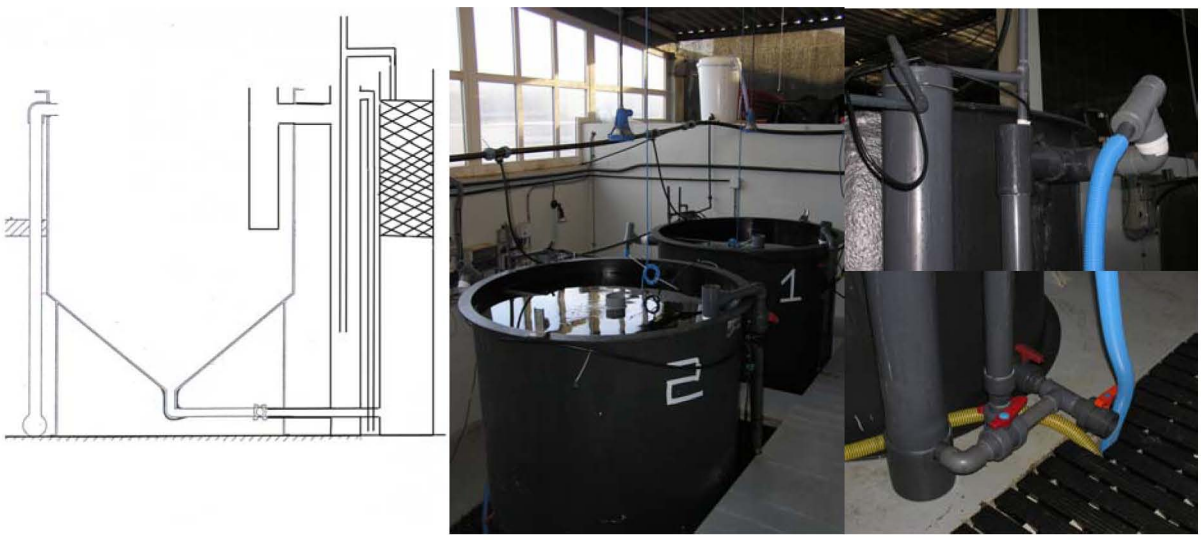


Figura 24. Vista longitudinal y fotografías de tanques de cultivo intensivo y detalles de entradas y salidas de agua.

4.2.2.3. Tanques de 500 litros- Cultivo intensivo

Se utilizaron 6 tanques cilindro-cónicos con una capacidad de 500 litros. Los tanques tienen un diámetro de 1,5 m y una profundidad de 1m, en su parte más profunda (Figura 25). Los tanques cuentan con una entrada de agua en su parte inferior y la salida de agua está situada en la parte superior. Al igual que en los tanques de 2.000 litros, la posición de entrada y salida de agua se modifica con diferentes accesorios móviles a lo largo del periodo de cultivo, de acuerdo a la secuencia alimentaria.

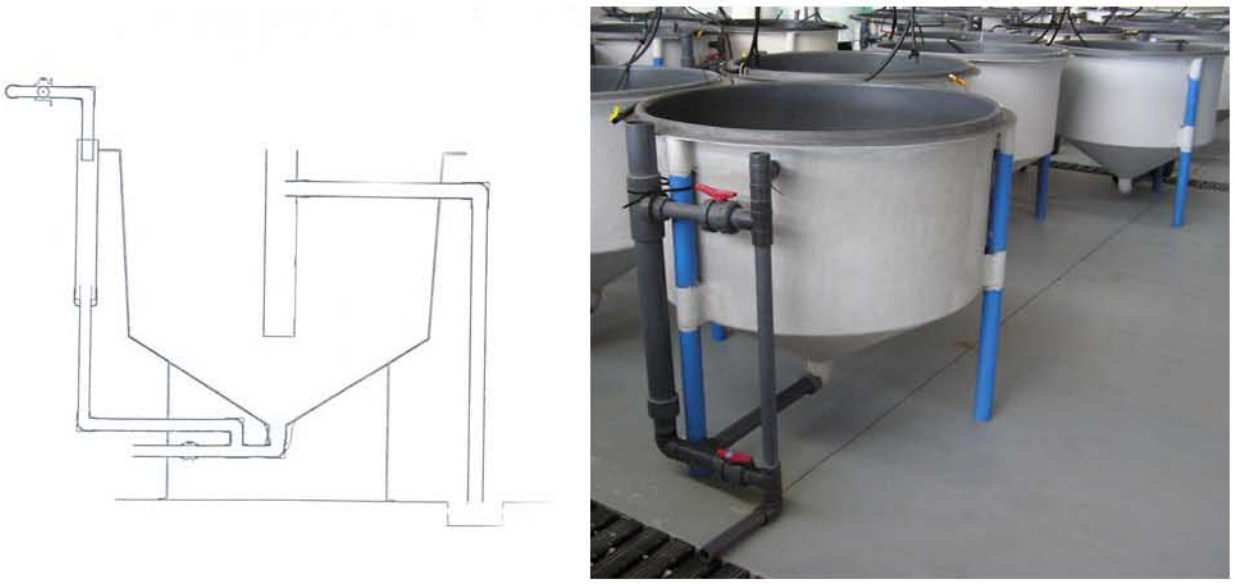


Figure 25. Vista longitudinal y fotografía de tanques de 500 litros para cultivo intensivo.

4.2.2.4. Tanques de 10.000 litros- Alevinaje

Se utilizaron 8 tanques cuadrados con esquinas redondeadas y fondo en pendiente hacia el desagüe central, tienen una capacidad de 10.000 litros que se encuentran dispuestos en dos filas paralelas de 4 tanques, separados por la canaleta central de desagüe (Figura 26). Todos los tanques tienen una longitud lateral de 3 m y una profundidad de 1,4 m en su parte central (Figura 27). Los tanques cuentan con una entrada de agua en su parte superior y una única salida en el fondo posición central

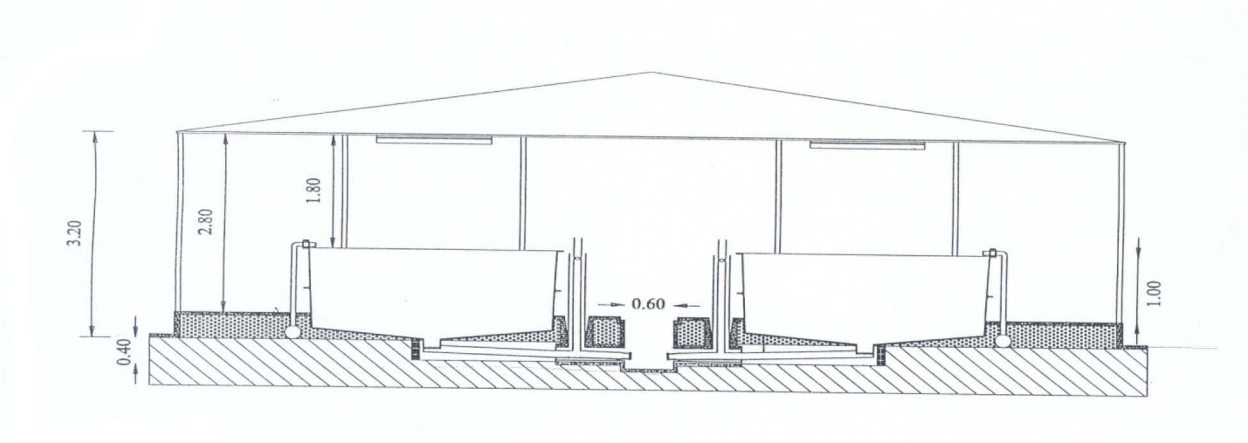


Figure 26: Sección longitudinal de los tanques de alevinaje.

Figura 27. a) Vista longitudinal, planta y fotografía de tanques de alevinaje.

4.3.-Especie objeto de estudio

El bocinegro o pargo (*Pagrus pagrus*), es un teleósteo, que pertenece a la familia *Sparidae*, su clasificación taxonómica detallada se especifica a continuación:

Phyllum: *Chordata*
Superclase: *Gnathostomata*
Clase: *Osteichthyes*
Orden: *Perciformes*
Suborden: *Percoidei*
Familia: *Sparidae*
Género: *Pagrus*
Especie: *Pagrus pagrus*

Figura 28. Ejemplar reproductor, huevos, larvas y alevines de bocinegro e instalaciones comerciales de engorde en tanques donde se realizaron experiencias piloto de engorde de esta especie.

4.3.1 Habitat

Se trata de una especie euriterma que se adapta a un amplio rango de temperaturas y a una gran variedad de hábitats, generalmente se encuentra en fondos rocosos y pedregales en una batimetría media 50 m de profundidad, los ejemplares jóvenes (denominados palletes en Canarias) es frecuente encontrarlos a menor profundidad en un rango de 10-30m. En el medio natural, es un

pez que se alimenta de moluscos y crustáceos principalmente, gracias a su potente mandíbula, mientras que en condiciones de cultivo se adapta con facilidad al alimento seco. Se trata de una especie hermafrodita proterogínia (hembra en su primea etapa de vida y macho posteriormente) y la época de puesta, en el medio natural, oscila entre los meses de diciembre y enero en el Atlántico occidental (Ciechomski y Weiss, 1973), y en el caso de Canarias se puede prolongar hasta comienzo de primavera (Pajuelo y Lorenzo, 1996). En esta misma localización y en condiciones de cultivo la época de puesta da comienzo a principios de marzo pudiéndose prolongar hasta finales mayo sin manipulación foto-térmica (Cejas *et al*., 1997).

4.3.2 Distribución geográfica

Esta especie ha sido citada en diferentes regiones, pudiéndose encontrar en el Mar Mediterráneo y Adriático; en el Atlántico este desde las Islas Británicas hasta el sur de Angola, incluyendo las Islas Canarias, Azores y Madeira; y en el Atlántico oeste se puede localizar desde Nueva York, hasta el sur de Argentina (Manooch y Hassler, 1978) (Figura 29).

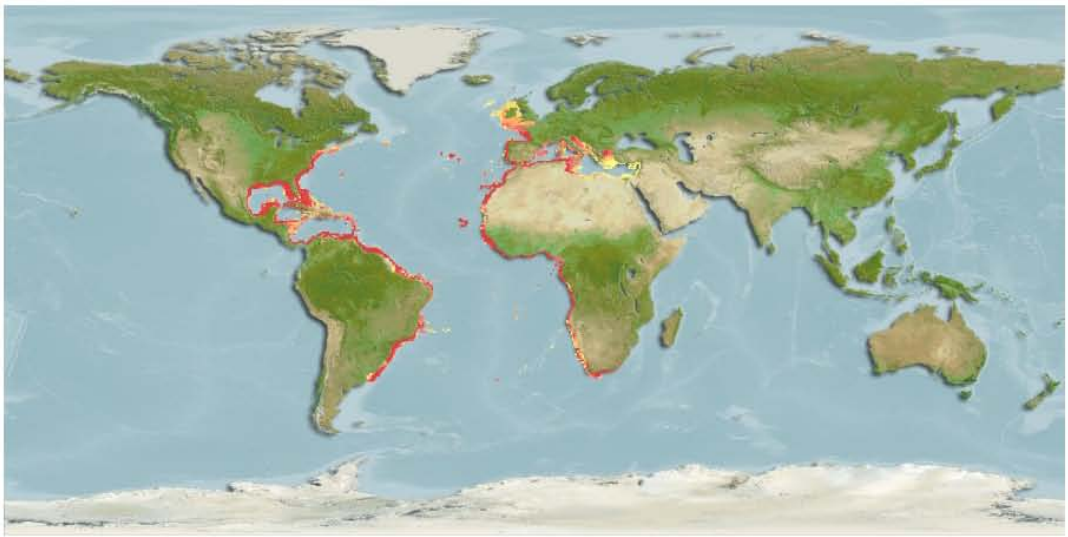


Figura 29. Distribución geográfica de la especie *Pagrus pagrus (*Fish Base, 2008).

4.3.3 Perspectivas de cultivo

El bocinegro (*Pagrus pagrus*) desde hace algunos años, es uno de los peces marinos que han sido propuestos como candidato potencial para la diversificación de la acuicultura marina (Kentouri *et al*., 1995; Hernández-cruz *et al.*, 1999). Tiene un alto precio y buena aceptación en el mercado, lo que es una condición indispensable para la introducción de una nueva especie ya que la rentabilidad del cultivo comercial es la última finalidad de la investigación en acuicultura.

El engorde comercial de bocinegro, todavía no es una realidad, si bien esta estadísticamente señalada una primera producción en Grecia en el año 1999 con 100 toneladas (FEAP, 2006), además durante el desarrollo de este trabajo se han realizado diferentes experiencias de engorde a escala piloto, tanto en jaulas como en tanques, con empresas locales con producciones muy reducidas. Aunque, las condiciones para el transporte de alevines han sido descritas por Pavlidis *et al,* (2003), los datos de producción de alevines son escasos y se puede resaltar la producción de 1,1 millones de alevines en Grecia en el año 2000 y la reciente publicación de datos correspondientes a los años 2006, 2007 y 2008 señalan una producción de alrededor de 2 millones de alevines anuales en este país (FEAP, 2008), sin embargo no se han encontrado datos a cerca de las producciones obtenidas con estos alevines. A escala experimental se han de destacar las producciones obtenidas en Canarias que se han incrementado en los últimos años, pasando de 12.000 unidades en el año 2002 hasta los cerca de 100.000 alevines que se han producido de manera regular en los años 2005 y 2006.

Los estudios de cultivo larvario de esta especie muestran resultados dispares según las condiciones de cultivo empleadas (Papandroulakis *et al*., 2004; Kentouri *et al*., 1995; Hernández-Cruz *et al.,* 1999; Michelakakis *et al.,* 2001; Papandroulakis *et al.,* 2004). Y diferentes estudios de investigación básica han descrito en detalle la ontogenia de diferentes tejidos y órganos (Socorro *et al*., 2001, Roo *et al*., 1999, Darias *et al*., 2005, 2007) así mismo la descripción detallada del desarrollo osteológico del bocinegro ha sido recientemente publicada por Socorro (2006).

4.4.-Condiciones experimentales

4.4.1.-Cultivos auxiliares

Para el desarrollo de las diferentes experiencias de cultivo larvario fue necesario la producción de "*alimento vivo*", término que define al conjunto de organismos que se utilizan como dieta de las larvas de peces marinos en sus etapas más tempranas, cuando todavía no es viable la alimentación con dietas inertes, y que por sus características biológicas necesitan ser producidos en condiciones específicas dentro de las diferentes unidades de la instalación.

4.4.1.1 Cultivo de fitoplancton.

En todas las experiencias de cultivo larvario se utilizó la microalga Eustigmatoficea, *Nannochloropsis sp.* Las principales características de esta especie han sido descritas por Maruyama *et al*., (1989) y se presenta en la siguiente tabla III.

Tabla III. Características biológicas de *Nannochlropsis sp* (Maruyama *et al*., 1989).

Dimensiones: 2-4 μm.
Forma celular: circular a ovalada.
Forma del cloroplasto: copa u ovalada.
Propagación: fisión binaria.
Retículo endoplásmico en el cloroplasto: presente.
Disposición de los tilacoides: 3-tilacoides.
Pigmentos predominantes: clorofila a, caroteno, violaxantina, éster de vauqueriaxantina.

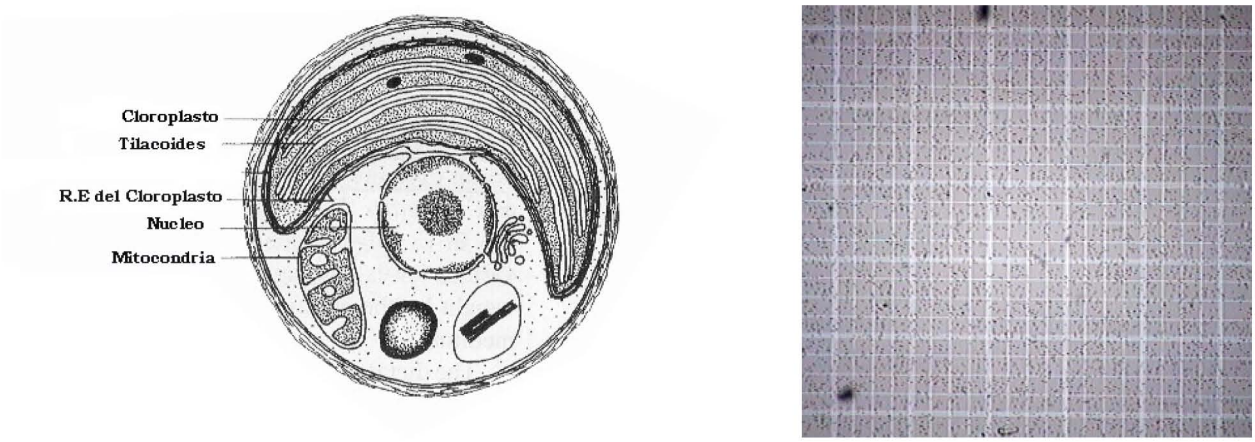


Figura 30: Esquema de una célula de *Nannocloropsis sp*. y visión microscópica en cámara Neubauer.

El sistema de cultivo empleado para la producción masiva de fitoplancton, fue de tipo "Bach" o sistema cerrado. Este se realizó en la unidad de producción de alimento vivo, en volúmenes crecientes de 50, 230 y 460 litros, utilizando bolsas de polietileno transparentes (Figura 31). En el cultivo se utilizó, agua de mar filtrada mecánicamente a través de un filtro de polyester reforzado y fibra de vidrio (Mod.00689; Astral pool, Barcelona, España) relleno con un lecho de arena de diversa granulometría. Seguidamente, el agua fue esterilizada por radiación UV a una longitud de onda de 254 nm con un equipo de desinfección industrial (Mod. M-3PE-300; Wedeco AG,

Herford, Alemania). La salinidad natural (37‰) se redujo, para el proceso de cultivo mediante la adición de la cantidad necesaria de agua dulce para alcanzar una salinidad final de 25‰, que se comprobó mediante un refractómetro portátil (Mod. SZJ-S, Madrid, España). A cada bolsa de cultivo se le introdujo uno o dos difusores de cerámica según el tamaño de la misma, ubicados a una distancia de 15cm del fondo, a través de los cuales se suministró aireación para favorecer la mezcla del medio y evitar la sedimentación de las células. Los cultivos, se mantuvieron con fotoperiodo continuo, combinando la iluminación natural con luz artificial, mediante luminarias fluorescentes (Mod. TLD 58W/54-765, Philips, Francia), que mantenían un intensidad mínima durante la noche de 9.500 lux medida con un luxómetro digital (Mod. HT170N; Italia).

Figura 31. Bolsas de cultivo de fitoplancton de 50, 230 y 460 litros de volumen.

Independientemente del volumen de cultivo, el protocolo consistió en la introducción de una cantidad de inóculo (concentración inicial de $1.5*10^6$ cells.ml^{-1}) en las bolsas previamente llenas con el agua de mar preparada (25‰) y la adición de fertilizante comercial (Nutri-Phyt; Fitoplancton marino S.L, Puerto Santa María, España) únicamente el primer día de cultivo. Los ciclos de cultivo tuvieron una duración media de 8 a 10 días tras los cuales se cosechó el volumen total de la bolsa, que alcanzaba unas concentraciones de 35-80*10^6cells.ml^{-1} según el volumen de cultivo, siendo generalmente menor (35*10^6cells.ml^{-1}) en los volúmenes mayores. Diariamente se comprobó el estado del cultivo mediante una valoración fotométrica de la turbidez con un fotómetro portátil (Mod. PF-11; Macherey-Nagel; Durew, Alemania) y contaje con hemocitómetro (Mod. Neubauer, Alemania) de muestras de diferentes bolsas al azar, que permitió determinar la concentración y la presencia de contaminantes biológicos en el medio (Figura 32).

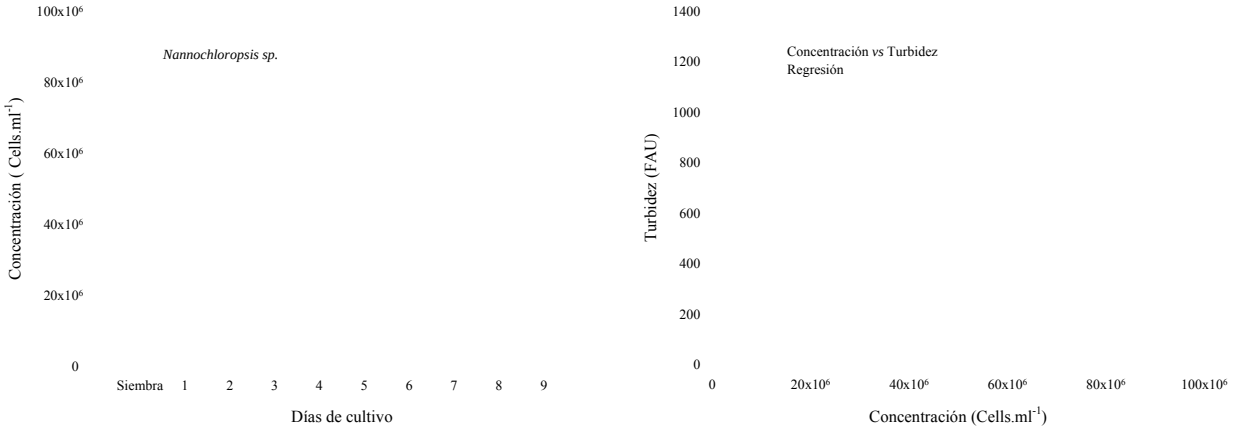


Figura 32. Curva de crecimiento tipo y correlación entre medidas de concentración y turbidez del cultivo.

Ocasionalmente se encontraron contaminantes como cianobacterias y ciliados tipo *Euplotes sp.* (Figura 33), en el caso de este último, la adición de formaldehido (38%) (Panreac, España) a una dosis de 0.05 $ml.l^{-1}$ de cultivo dio excelentes resultados eliminando el 100% de los contaminantes sin comprometer la viabilidad del cultivo de fitoplancton.

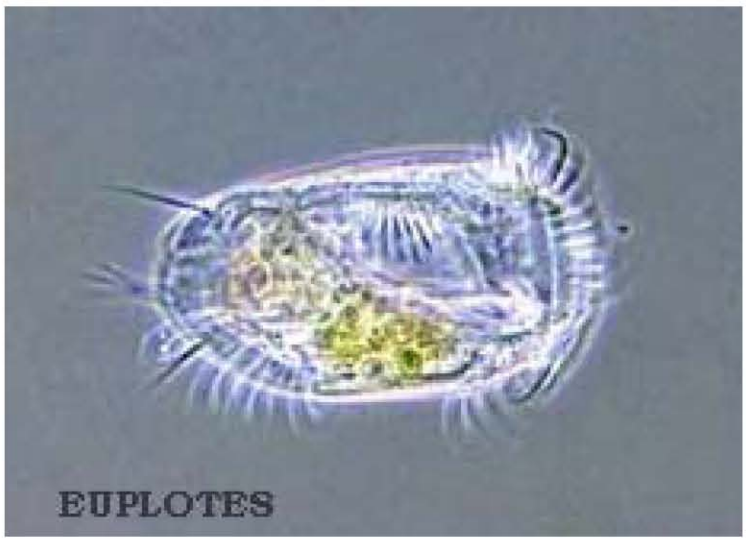


Figura 33. Organismo contaminante (*Euplotes sp).*encontrado en los cultivos de *Nannochloropsis sp.*

El fitoplancton producido se utiliza para diferentes acciones dentro del proceso de cría de larvas

de peces. Así, las bolsas de 50 l se utilizaron como inóculo para el escalado del cultivo a 230 l. El procedente de las bolsas de 230 se utilizó a su vez, como inóculo de las bolsas de 460 l y como medio de cultivo para el mantenimiento de las cepas de rotíferos. Finalmente, el fitoplancton producido en las bolsas de 460 l se utiliza en el proceso de cultivo de larvas para la aplicación de las técnicas de agua verde y ocasionalmente para la alimentación de rotíferos.

4.4.1.2 Cultivo de rotíferos.

El rotífero utilizado en las experiencias de cultivo larvario fue *Brachionus plicatilis*. cepa tipo L con una longitud total media de los individuos adultos de 240 µm.

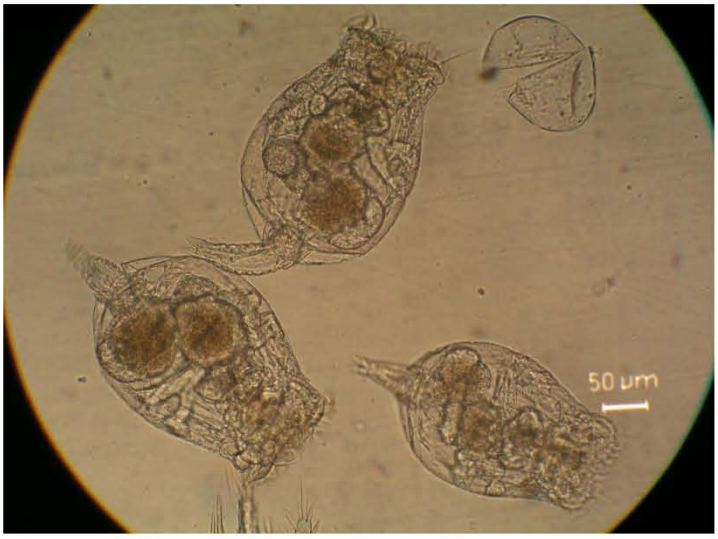


Figura 34. Rotíferos (*Brachionus plicatilis*) utilizados en los cultivos de larvas.

El proceso del cultivo consta de varias fases (mantenimiento de la cepa, pre-cultivo y cultivo masivo) lo que permite, por un lado, autonomía en la producción y por otro, la posibilidad de re-comenzar el cultivo de forma rápida en caso de fallo o accidente.

4.4.1.2.1.-Mantenimiento de la cepa y pre-cultivo de rotíferos.

Generalmente la cepa se mantuvo en recipientes de 2 a 5 l de capacidad que se llenaron con *Nannochloropsis sp*. a una concentración de 20-25*10^6 cells.ml^{-1}, al que se añadió la cantidad de rotíferos necesario, para obtener una concentración inicial de 2-5 rot.ml^{-1}. El cultivo se mantuvo con aireación suave a temperatura ambiente y fotoperiodo continuo de 24h.

Una vez que el cultivo alcanzó una concentración de 75-100 rot.ml^{-1}, alrededor del 5º-7º día, se cosechó el volumen total y se pasó a un volumen mayor, normalmente, botellas de 20 l repitiendo

el mismo protocolo descrito anteriormente.

Figura 35. Recipientes para el pre-cultivo de rotíferos.

4.4.1.2.2.-Cultivo masivo.

En el proceso de producción masiva de rotíferos se utilizaron tanques cilindro-cónicos de fibra de vidrio, con una capacidad de 1700 l (Figura 36). Las condiciones generales de cultivo se muestran en la tabla IV.

Tabla IV. Condiciones generales de la producción de rotíferos en las instalaciones experimentales del ICCM.

Agua:	Esterilizada con UV
Temperatura:	20-25ºC
Salinidad:	25 ppt
Iluminación:	Fotoperiodo natural, sin iluminación directa.
Aireación:	>2 l/min con un único difusor central, de manguera porosa, situado al 80% de altura del tanque.
Oxígeno:	Inyección de O_2 puro, a través de difusor de manguera porosa, cuando el nivel era inferior a 3,5ppm.

Los ciclos de producción (Tabla V) fueron de 8 días de cultivo, iniciándose el proceso con una densidad aproximada de 265 rot.ml^{-1}, a partir del 4º día de cultivo se cosechó en días alternos 400 l de volumen del tanque que fueron repuestos con agua previamente mezclada para alcanzar la salinidad establecida (25ppt). Al 8ºdía se procedió a la cosecha del volumen total y reinicio de un

nuevo ciclo. Como medida rutinaria, tras la cosecha, los rotíferos eran sumergidos durante 1 minuto en agua dulce para eliminar posibles contaminantes. Diariamente se determinó la densidad media y el porcentaje de hembras ovígeras en tres contajes individuales de 0,5ml con una Micropieta (Mod. Eppendorf Research 100-1000µl; Hamburgo, Alemania), tomados de una muestra recogida en la zona central del tanque de cultivo, para asegurar una buena homogeneización de la muestra en cada medida. Se anotó la actividad de los individuos, con una estimación visual de la movilidad de los mismos identificándola como (alta-media-baja). Se anotó también la presencia de posibles contaminantes, como ciliados y copépodos, con una estimación visual de concentración (alta-media-baja), y se determinó la calidad del medio de cultivo por la presencia de flóculos o partículas en suspensión y el aumento de viscosidad del mismo, con una estimación visual (Limpio-Medio-Sucio). De la misma manera, a diario se tomaron medidas de temperatura y oxígeno disuelto a las 9:00 y a las 15:00 con una sonda portatil (Mod. Handy Polaris, OxyGuard; Birkerød, Dinamarca).

En cuanto a la alimentación, de manera general se utilizó levadura de panificación (*Saccharomyces cerevisiae*) añadiendo 0,4g/10^6rotíferos. Únicamente el día de inicio del ciclo se suplementó la levadura con fitoplancton liofilizado, en una concentración de 0,1g/ 10^6rotíferos. La alimentación se distribuyó manualmente a las 09:00 y a las 15:00 horas y mediante un distribuidor automático a las 21:00 y 03:00 horas.

Figura 36. Tanques de producción masiva de rotíferos y sistema de distribución automática del alimento.

Tabla V. Parámetros medios de la producción de rotíferos con la metodología descrita (n=9).

Producción de rotíferos

Día	Densidad (Rot/ml)	Volumen (m^3)	Total (mill)	Cosecha (Rot/ml)	Ovígeras (%)	Alimento (g/mill)	Alimento (g/tanque)	Alimento (g/ración)	O_2 (ppm)	Tª (ºC)
1	262.1	1.7	446		19.3%	0.5	225	37.5	7.0	22.9
2	251.5	1.7	428		22.1%	0.4	165	27.4	6.3	22.3
3	281.9	1.7	479		23.3%	0.4	197	32.9	4.3	22.1
4	307.6	1.7	523	72.4	17.9%	0.4	224	37.4	4.3	22.0
5	292.6	1.7	497		19.8%	0.4	187	31.1	5.1	22.0
6	293,0	1,7	498	68.9	21.6%	0.4	198	32.9	4.1	22.2
7	269.9	1.7	459		19.2%	0.4	166	27.6	3.9	22.4
8	265.1	1.7	451	451	17.8%		-	-	4.9	22.3

En el proceso de cosecha se utilizó una bolsa de malla de 63 micras de luz o bien un concentrador de rotíferos con malla de la misma medida.

Figura 37. Proceso de cosecha de los tanques de cultivo masivo de rotíferos y detalle de malla del concentrador.

4.4.1.2.3.-Enriquecimiento de rotíferos

Los rotíferos cultivados, en las condiciones anteriormente descritas, son deficiente en ácidos grasos poli-insaturados y aminoácidos, por lo que es necesario enriquecerlos antes de suministrarlos a las larvas. El enriquecimiento se realiza en tanques de fibra de vidrio opacos de 500 l de capacidad. Estos tanques tienen un sistema de aireación central mediante un difusor de

manguera porosa, la concentración de rotíferos que se mantiene es generalmente elevada (>400 rot.ml^{-1}) por lo que en determinadas circunstancias puede ser necesario el uso de oxígeno puro. En este proceso se utilizaron productos comerciales o experimentales, de acuerdo a las necesidades de cada uno de las experiencias planteadas. De manera general, los diferentes productos se utilizaron siguiendo las instrucciones del fabricante, que incluían un tiempo de enriquecimiento 6h, y un reparto en dos dosis (h=inicio y a las 3h) de la cantidad necesaria. Diariamente se utilizaron dos tanques de enriquecimiento, uno de ellos para la primera comida de la mañana, que era cosechado a las 08:30. En este caso el producto enriquecedor se suministró mediante un dosificador automático programado para dar el producto a las 02:00 y a las 05:00. Al segundo de los tanques, que era cosechado a las 14:00, se le suministró el enriquecedor de forma manual a 08:00 y a las 11:00.

Tras el enriquecimiento, los rotíferos eran filtrados con una bolsa de malla de 63 μm de luz, enjuagados para eliminar los restos de la emulsión enriquecedora y concentrados en un cubo de 20 l donde se mantenían con aireación para proceder a su contaje. Se tomaba una muestra de 5 ml de la zona central del cubo y se diluía en un vaso con agua de mar en un volumen de 250 ml, una vez homogeneizada la mezcla se realizaban tres contajes individuales de 0.5 ml con una Micropieta (Mod. Eppendorf Research 100-1000 μl; Hamburgo, Alemania). A lo largo de todo el desarrollo experimental se tomaron muestras del producto utilizado para la alimentación de los rotíferos y para su enriquecimiento en las diferentes experiencias (Tabla VI).

Tabla VI. Analítica proximal y composición de AG (% ácidos grasos totales) del alimento de los rotíferos y productos enriquecedores (n=3).

Uso	**Alimento de rotíferos**	**Productos enriquecedores**		
Producto	**Levadura**	**DHA Protein Selco**	**Red Pepper Paste**	**MorDHA omega-3 I.Q**
% Lípidos (dw)	2.41±0.03	30.29±0.16	45.13±0.22	85.13±0.71
% Proteínas (dw)	48.00±0.40	29.66±0.19	13.26±0.33	6.38±0.21
% Cenizas (dw)	5.28±0.08	11.21±0.09	8.30±0.04	0.01±0.02
14:0	0.34±0.14	2.43±1.24	5.56±1.44	0.06±0.08
16:0	23.24±8.16	27.85±0.62	21.91±5.31	0.88±0.00
16:1 n-7	27.31±5.51	4.47±0.41	0.75±0.15	0.47±0.04
18:0	19.39±9.25	6.26±0.38	2.99±0.76	5.85±0.72
18:1 n-9	26.33±12.46	12.22±0.49	13.83±0.77	3.68±0.56
18:1 n-7	0.67±0.08	1.29±0.56	1.73±0.82	1.22±0.10
18:2 n-6	0.48±0.12	7.79±0.38	3.93±0.92	0.72±0.08
18:3 n-3	0.08±0.03	0.98±0.18	3.03±0.74	0.22±0.18
20:1 n-9	0.11±0.01	0.81±0.20	0.05±0.07	1.95±0.32
ARA (20:4n-6)	nd	0.97±0.18	1.59±0.33	2.82±0.50
EPA (20:5n-3)	nd	6.40±0.45	3.06±1.40	10.73±2.16
DPA (22:5n-6)	0.30±0.16	1.38±0.27	9.04±1.99	4.79±0.94
DHA (22:6n-3)	nd	19.09±1.23	22.84±1.92	57.32±4.34
∑ saturados	43.67±17.64	37.95±0.92	31.79±4.42	7.31±0.07
∑ mono-insaturados	54.59±17.72	19.13±0.93	19.03±6.04	9.62±0.26
∑ n-3 (3)	0.43±0.10	30.31±1.17	31.44±3.05	72.44±2.31
∑ n-6(4)	0.50±0.13	11.01±0.97	15.87±1.46	9.49±1.89
∑n-9(5)	26.47±12.41	13.46±0.80	14.17±0.70	6.23±0.86
∑n-3HUFA(6)	0.34±0.10	27.78±1.16	27.66±2.19	71.60±2.85
DHA/22:5 n-6	-	13.84±1.78	2.53±0.55	12.28±3.32
EPA/ARA	-	6.59±0.93	1.93±0.43	3.80±0.09
DHA/EPA	-	2.98±0.25	7.46±2.93	5.49±1.51
DHA/ARA	-	19.67±3.69	14.38±3.45	20.81±5.22
Oleico/DHA	91.19±7.79	0.64±0.02	0.61±0.04	0.06±0.01
Oleico/n-3HUFA	75.73±14.08	0.44±0.00	0.50±0.04	0.05±0.01
n-3/n-6	0.92±0.44	2.75±0.22	1.98±0.17	7.81±1.80

4.4.1.3.-Producción de Artemia

La *Artemia* sigue siendo uno de los alimentos básicos sobre los que se sustenta la cría larvaria de peces marinos actualmente. Debido principalmente a su capacidad de producir cistes que pueden mantenerse en estado de vida latente durante largos periodos de tiempo sin disminuir su viabilidad de manera importante, lo que permite que sean procesados, envasado y almacenado evitando así la realización de un cultivo continuo, como en el caso del rotífero. Esto implica que para su uso como alimento de las larvas de peces, los huevos o cistes se pongan en las condiciones adecuadas para la obtención de nauplios o metanauplios que son los principales estadios que se utilizan como alimento de las larvas de peces (Figura 38).

Figura 38. *Artemia* en diferentes estadios: huevo no eclosionado, nauplio (24h) y metanauplio (48h).

En todas las experiencias de cultivo se utilizaron cistes de *Artemia* de dos calidades, *Artemia franciscana* (Tipo AF; INVE; Dendermode, Bélgica) y *Artemia salina* (Tipo EG; INVE, Dendermode, Bélgica). Es recomendable, realizar la descapsulación, un proceso que consiste en la eliminación del corion o cubierta protectora del huevo, con lo que se evita la introducción de posibles patógenos asociados a estas cubiertas de resistencia. Del mismo modo, el ciste sin descapsular puede ser ingerido por la larva y dada su baja digestibilidad puede causar una obstrucción del tubo digestivo de la misma. Por último, el exceso de materia orgánica que suponen estos cistes si son introducidos en el tanque de larvas, contribuye a la disminución de la calidad de agua.

La *Artemia* se prepara siguiendo el protocolo que a continuación se detalla:

4.4.1.3.1.-Hidratación

En el primer paso del proceso de descapsulación, los cistes son hidratados durante 45min-1:30 minutos, en una relación de 10-12 litros de agua del mar por kg de cistes seco a temperatura y salinidad ambiente 20±2°C y 37‰, en este proceso se utilizan tanques cilindró-cónicos de 50-500 litros, según la cantidad de cistes a decapsular. El sistema se completa con una aireación fuerte desde la base del tanque, que ayuda a mantener una concentración de oxígeno superior a 4 ppm y evita la sedimentación de los cistes. Una vez hidratados, los cistes son cosechados con una bolsa de 125µm de luz de malla, lavados con agua de mar, eliminando la mayor cantidad de agua mediante presión de la malla (Figura 39).

Figuras 39. a) Cistes de *Artemia*; b) Tanque de hidratación; c) Cistes hidratados.

4.4.1.3.2.-Descapsulación

Una vez hidratados se procede a la descapsulación propiamente dicha de los cistes, este proceso consiste en la eliminación completa de la capa exterior (corión), mediante el uso de una solución concentrada de hipoclorito sódico. Así, en el mismo tanque utilizado para la hidratación, se añaden 4 litros de agua del mar.kg^{-1}de cistes seco, la cantidad de lejía comercial necesaria para obtener una concentración de 350gramos de hipoclorito sódico.kg^{-1} de cistes secos y 66 gramos de NaOH.Kg^{-1} de cistes secos. Se aplica una aeración fuerte y se controla el cambio de coloración del pardo inicial a naranja que suele suceder en un periodo de 3 a 8 minutos. Una vez observado el cambio de color se procede a la cosecha, en una bolsa de 125µm de luz de malla y al lavado con agua del mar durante 15 a 20 minutos para eliminar posibles restos de cloro.

4.4.1.3.3.-Conservación

Una vez descapsulados, se elimina la mayor parte de agua de los cistes. Se añade agua de mar y sal marina en una relación de 1.17kg de sal/4l de agua de mar.kg^{-1} de cistes secos, de manera que obtenemos una solución de salmuera con un volumen final de 7,17 litros (teniendo en cuenta la duplicación del volumen del ciste una vez ha sido hidratado) y una concentración final de 139g de cistes.l^{-1} de salmuera. Una vez preparada la salmuera, se mantiene en la cámara de frío en ausencia de luz y a baja temperatura (<4˚C) donde los cistes descapsulados se mantienen viables durante al menos 30 días (Figura 40).

Figura 40. a) Recipientes de almacenamiento de salmuera de *Artemia*; b) Solución de huevos en salmuera.

Tabla VII. Protocolo de descapsulación de *Artemia* y conservación en salmuera de la planta de producción de alevines

Protocolo Descapsulación-Conservación en salmuera de *Artemia*

Hidratación: 45´- 1h 30´. 10-12 litros de agua por kg de ciste seco.

Descapsulación		***Artemia* (Kg)**	**Hipoclorito(g)**	**Lejía(l)**	**NaOH (g)**	**Agua de mar(l)**	**Vol total**
Lejías varias 10 l		0,25	87,5	2,19	16,5	1,31	3,5
Indice de Refracción:	1,352	0,50	175	4,38	33	2,6	7
Conc. Gram/l	40	1,0	350	8,75	66	5,3	14
		1,5	525	13,13	99	7,9	21
		2,0	700	17,50	132	10,5	28
		2,5	875	21,88	165	13,1	35
		3,0	1.050	26,25	198	15,8	42
		4,0	1.400	35,00	264	21,0	56
		5,0	1.750	43,75	330	26,3	70
		6,0	2.100	52,50	396	31,5	84
		7,0	2.450	61,25	462	36,8	98
		7,5	2.625	65,63	495	39,4	105

Notas

Lejía: 0.35 gramos de hipoclorito por gramo de cistes secos(INVE).
Sosa: 66g de NaOH por kilogramo de cistes secos.
Antiespumante. Opc: 250-500ppm, 0.25-0.5 g/l.
Agua de mar: La necesaria para alcanzar una concentración final de 14 l/kg de ciste secos.
Tiempo:5-8min, según cambio de color (pardo-naranja).
Neutralización : Lavar con agua de mar abundante durante 20 min, tb puede utilizarse 50g Tiosulfato/kg ciste. Se puede comprobar la presencia de restos de Cl con ortotolidina u otro detector de cloro.

Salmuera	*Artemia* (Kg)	Sal (kg)	Agua (l)	Vol final(l)	Conc. Final
	0,25	0,29	1,00	1,79	139,4
	0,50	0,59	2,00	3,59	139,4
	1,0	1,17	4,00	7,17	139,4
	1,5	1,76	6,00	10,76	139,4
	2,0	2,34	8,00	14,34	139,4
	2,5	2,93	10,0	17,93	139,4
	3,0	3,52	12,0	21,52	139,4
	4,0	4,69	16,0	28,69	139,4
	5,0	5,86	20,0	35,86	139,4
	6,0	7,03	24,0	43,03	139,4
	7,0	8,20	28,0	50,20	139,4
	7,5	8,79	30,0	53,79	139,4

Notas

Agua: 4l agua de mar(37%) por kg de cistes secos.
Sal:330 g sal/litro - g de sal en agua de mar.
Cistes:
VT= Agua + sal + Cistes(2*peso en seco)
Concentración: Kg descapsulado/VT
Producción teórica: HE* gramos sembrados
Conservar en ausencia de luz en lugar fresco(<10º).

Elaborado por: J.Roo		Fecha Modif. 02/02/2003

4.4.1.3.4.-Eclosión

Según las necesidades de alimentación de las larvas se determinó el volumen de cistes en salmuera a eclosionar. El procedimiento se iniciaba con la desinfección del tanque de eclosión, para posteriormente proceder a su llenado con agua del mar filtrada y esterilizada. Se utilizaron tanques cilíndro-cónicos de fibra de vidrio, de 1700 l de capacidad, con aireación central fuerte, iluminación durante 24 horas con una intensidad de 2000 lux. Estos tanques estaban equipados con un intercambiador de calor que permitía mantener la temperatura del cultivo en torno a 28-29ºC. El día anterior a la siembra de los cistes en salmuera, se llenaba el tanque y se introducía un sensor de temperatura (Mod. OxyGuard; Birkerød, Dinamarca) que unido a un sistema automático de regulación de temperatura (Mod. AKO Electrómecanica, Barcelona, España) permitía que el nuevo tanque alcanzase y mantuviese la temperatura requerida para la eclosión. La densidad de siembra se fijó en 1.8g.l^{-1}, el 95% de los huevos eclosionaban en la siguientes 24h, obteniéndose nauplius de *Artemia* con un tamaño de 450-650µm, dependiendo del tipo de *Artemia*, y que era el adecuado para la primera fase de transición de alimento vivo de las larvas, paso de rotífero a *Artemia* (Figura 41).

Figura 41. a) Siembra de salmuera; b) Tanques de eclosión; c) Nauplio de *Artemia salina* recién eclosionado.

4.4.1.3.5.-Enriquecimiento

Para mejorar el valor nutritivo de la *Artemia*, se procedió a su enriquecimiento, al igual que en proceso de eclosión se utilizaron tanques cilindro-cónicos de fibra de vidrio, de 1700l de capacidad, con aireación central fuerte, iluminación durante 24 horas, con una intensidad de 2000 lux, equipado con un intercambiador de calor que permitía mantener la temperatura de cultivo en

torno a 25-26ºC. Los nauplios de *Artemia* recién cosechados, se introducían en el tanque de enriquecimiento, lleno con la cantidad necesaria de agua del mar filtrada y esterilizada para mantener una concentración de 250.000-300.000 nauplios.l^{-1} . De modo general el enriquecedor utilizado fue Easy DHA Selco (Inve, Dendermonde, Bélgica), producto que se presenta en forma de emulsión lipídica. El tiempo de enriquecimiento de los nauplios fue de 18-24 horas a una concentración de 0.6gr.l^{-1}, y añadido en una única dosis al inicio del enriquecimiento (h=0).

Una vez enriquecidos los metanauplius de 18-24horas, se filtraron en una bolsa de 125µm de luz de malla, se lavaron con agua de mar abundante para eliminar los posibles restos de emulsión y se concentraron en un cubo de 20l procediendo a su contaje y comprobando visualmente su correcto enriquecimiento por la presencia de gotas lipídicas en el tracto de la *Artemia* (Figura 42). Los metanauplius enriquecidos se añadían manualmente y mediante distribuidores automáticos de acuerdo a las necesidades de los cultivos larvarios.

Figura 42. a) Tanque de enriquecimiento; b) Mantenimiento de *Artemia* en frío; c) Comprobación del correcto enriquecimiento de los metanauplios.

4.4.2.-Cultivo larvario

4.4.2.1.-Huevos

Por lo general, los huevos de peces marinos son un estadío biológico fácil de manipular y transportar. Sin embargo, unas condiciones inadecuadas de manipulación pueden dar lugar a una elevada mortalidad de los mismos, y a efectos negativos posteriores tales como bajas tasas de eclosión, elevada mortalidad larvaria durante los primeros días de cultivo o aparición de diferentes anomalías morfo-anatómicas. En consecuencia, el manejo de los huevos de peces debe ser cuidadoso para evitar mortalidades y obtener larvas de calidad.

En las distintas experiencias de cultivo larvario, que se presentan en este documento se utilizaron huevos procedentes de puestas naturales de stocks de reproductores pertenecientes al ICCM y al Instituto Español de Oceanografía-Centro Costero de Canarias (IEO-COC). En ambas instalaciones, los ejemplares reproductores fueron alimentados dos veces por semana con dietas comerciales, que se complementaron una vez a la semana con pescado fresco y moluscos como sepia, calamar y mejillón (Figura 43).

Figura 43. Ejemplares reproductores de bocinegro (*Pagrus pagrus*).

Independientemente del origen, la manipulación de las puestas siguió una secuencia similar. Los huevos se recogieron en un colector de malla de 500µm dispuesto en la salida superior de agua de los tanques de cultivo, los huevos fertilizados presentan flotabilidad positiva y caen por rebose en dicho colector (Figura 44a). Una vez recogida la puesta se realizó la separación de huevos en un cono de decantación, diferenciándose, la fracción flotante como huevos mayoritariamente viables y la no flotante como mayoritariamente no viables (Figura 44b). Cuando el origen de los huevos

no fue del stock del ICCM, estos se trasladaron por avión o barco, en el interior de cubitainers de plástico reforzado, de 20 litros de capacidad, que se mantuvieron en el interior de cajas individuales de poliestireno expandido, que protegen de los golpes y mantienen unas condiciones de temperatura estables durante el transporte (Figura 44c). En cada cubitainer, se introducen una media de 250.000 huevos a una concentración a 25.000 huevos.l^{-1}, llenando únicamente el 50% del volumen del recipiente con agua de mar. Tanto el agua como el 50% restante del volumen del cubitainer se saturaron con oxígeno gas (Figura 44d).

Figura 44. a) Colector de huevos, b) Embudo de decantación para separación de fracción de puesta no viable; c,d) Llenado de cubitainer y cajas para transporte de huevos.

Una vez en las instalaciones, se procedió a la aclimatación de los huevos a los parámetros físico-químicos existentes y se hizo una nueva decantación para eliminar los huevos muertos o dañados durante el transporte. Se realizó un recuento de los huevos viables y se procedió a su siembra, por volumetría, en los tanques de cultivo (Figura 45).

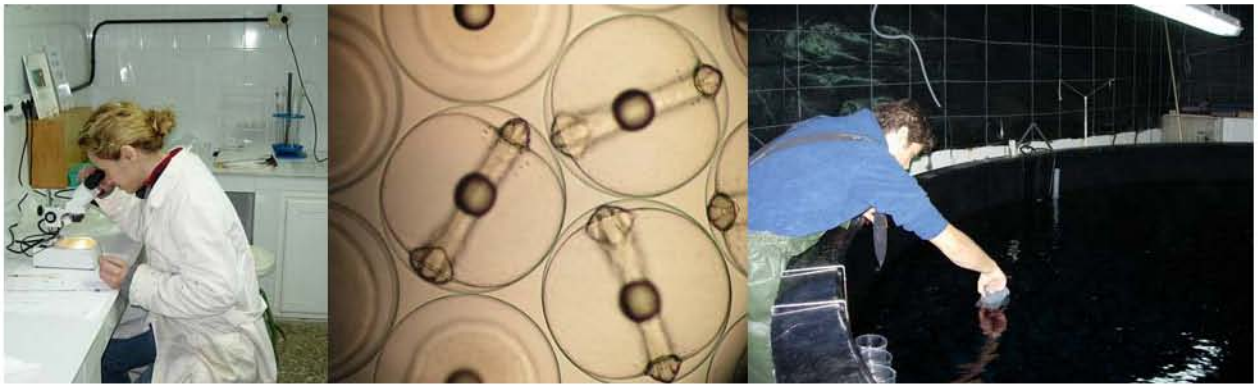


Figura 45. a) Conteo de huevos; b) Huevos viables para siembra; c) Siembra de huevos en tanques de cultivo.

Paralelamente se sembraron seis pequeños recipientes cilíndricos provistos de intercambio de agua y aire para calcular los índices de eclosión y de supervivencia larvaria, al tercer día de vida, justo antes de la apertura de la boca (Figura 46).

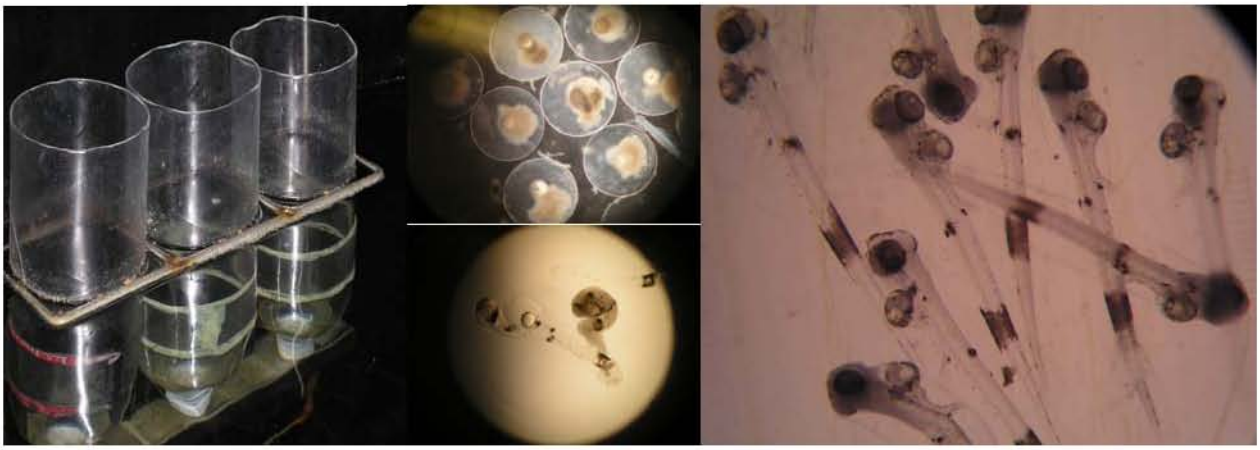


Figura 46. a) Recipientes usados para determinar porcentajes de eclosión y supervivencia larvaria; b) Huevos muertos y larvas recién eclosionadas no viables; c) Larvas viables.

4.4.2.2.-Cultivo larvario semi-intensivo

Las experiencias de cultivo larvario, con tecnología semi-intensiva (Mesocosmos), se realizaron en los tanques de 40.000 litros de capacidad, anteriormente descritos. En todas las experiencias se sembraron huevos fertilizados de 12 a 24h de vida, a una densidad que osciló entre 5-6,5 huevos.l^{-1}. En todos los casos, durante la fase de cultivo larvario se utilizó agua de mar previamente filtrada y esterilizada como se describe en el apartado de cultivos auxiliares. La renovación de agua se incrementó desde un 10% del volumen diario del tanque en las primeras etapas hasta un 25%.h^{-1} a partir de los 30 días post eclosión (dpe). La salinidad del agua de mar se mantuvo constante durante todas las experiencias (37‰). El oxígeno disuelto y la temperatura se determinaron diariamente, fluctuando entre los diferentes experimentos. De manera general, la iluminación, consistió en una combinación de luz natural y luz artificial con un fotoperiodo 24h horas (el fotoperiodo se varió en experiencias concretas de cultivo larvario como se detalla en las diferentes experiencias), pasando posteriormente a fotoperiodo natural. Los tanques están equipados con 4 luminarias fluorescentes (Mod TLD 36W/54, Philips, Francia) que en combinación con luz natural que penetra a través del techo traslucido mantenían una intensidad de entre 1.000-3.500 Lux en la superficie del agua.

En cuanto a la secuencia alimentaria, la tecnología de mesocosmos, incluye el uso de agua verde,

por lo que desde el día 2 dpe, se añadió diariamente fitoplancton vivo (*Nannochloropsis sp.*) para mantener una concentración media de 250-500.000 células.ml^{-1}. Desde el día 2 hasta el 25dpe, la alimentación consistió en rotíferos (*Brachionus plicatilis*) alimentados con levadura de panificación (*Saccharomyces cerevisiae*) y posteriormente enriquecidos con diferentes productos comerciales o experimentales según el experimento, manteniendo una concentración 4-5 rotíferos.ml^{-1} en el tanque larvario y ajustado dos veces al día (08:00;15:00). Desde el día 13 hasta el 20 una vez al día (11:00), se añadieron 175 nauplios.l^{-1} de *Artemia* (Tipo AF, INVE Aquaculture, Dendermonde, Bélgica). Desde el día 15 hasta el día 50 se añadieron metanauplios de *Artemia* (Tipo EG, INVE Aquaculture, Dendermonde, Bélgica), enriquecidos con Easy DHA Selco (INVE Aquaculture, Dendermonde, Bélgica) a una concentración que se incrementó de 250 a 1000 metanauplios.l^{-1}) tres veces al día (09:00;15:00 y 20:00) siendo la última toma de *Artemia* distribuida mediante un distribuidor automático. La concentración de presas en el tanque (rotíferos y *Artemia*) se evaluó mediante la toma de muestras, dos veces al día (08:00;14:00), de 3-5 puntos del tanque de cultivo, antes de la adición de presas nuevas para restablecer la concentración predeterminada. A partir del 5dpe y hasta los 25dpe se limpio superficie de los tanques mediante el uso de un limpiador de superficie, para la eliminación de la película lipídica que se genera con la adición de presas vivas enriquecidas. Generalmente, el destete dio comienzo el día 20 utilizándose micro dietas de la gama Genma Micro (Skretting, Francia) diseñadas para peces marinos y se dio por finalizado a los 45-50dpe. La alimentación con microdietas se realizó inicialmente de forma manual y posteriormente, cuando se observó una buena aceptación de las mismas, se distribuyeron de forma automática mediante alimentadores automáticos (T-Drum feeders, Arvotec, Noruega), inicialmente cada hora y finalmente cada 15min, durante 24h al día. (Tabla VII). A los 50 días de edad los alevines fueron transferidos a tanques de 10.000 litros en el área de alevinaje, donde se mantuvieron en las mismas condiciones de cultivo (1-10 ind.l^{-1}) en circuito abierto hasta 95 días, cuando los peces fueron contados y se llevó a cabo la caracterización esquelética.

4.4.2.3.-Cultivo larvario intensivo

Las experiencias de cultivo larvario, con tecnología intensiva, se realizaron en los tanques de 2.000 litros de capacidad, descritos previamente. Este tipo de tecnología es ampliamente utilizada en los criaderos comerciales, con diferentes variantes en cuanto a las condiciones generales del cultivo como pueden ser el tamaño y forma de los tanques, flujos de agua, posición y número de aireadores o la secuencia alimentaria y productos comerciales y dietas empleadas para el enriquecimiento de presas y destete de las larvas. De manera general, esta técnica se basa en el

uso de una densidad larvaria elevada que generalmente oscila entre 50 y 150 larvas.l^{-1}.

En el presente trabajo, en todas las experiencias se utilizó una densidad de siembra que osciló entre 100-125 huevos.l^{-1}. Se utilizó agua de mar previamente filtrada y esterilizada, al igual que en el técnica semi-intensiva. La renovación de agua, se redujo de un 25%.h^{-1} durante la eclosión, a un 10% del volumen del tanque diario en las primeras etapas, incrementándose gradualmente hasta un 25%.h^{-1}. A partir de los 30 días post eclosión. La salinidad del agua de mar se mantuvo constante durante todas las experiencias (37‰) y el oxígeno disuelto y la temperatura se determinaron diariamente, fluctuando entre los diferentes experimentos. De manera general, la iluminación, consistió en una combinación de luz natural y luz artificial, con un fotoperiodo 24h horas (el fotoperiodo se varió en experiencias concretas de cultivo larvario como se detalla en las diferentes experiencias), pasando posteriormente a foto periodo natural. Los tanques están equipados con una única luminaria incandescentes (Mod TLD 36W/54, Philips, Francia) que aportaban una intensidad de entre 1500-3500 Lux en la superficie del agua.

Esta tecnología incluyó también el uso de agua verde, por lo que desde el día 2 dpe, se añadió diariamente fitoplancton vivo (*Nannochloropsis sp.*) para mantener una concentración media de 250-500.000 cells.ml^{-1}. Desde el día 2 hasta el 25dpe, la alimentación consistió en rotíferos (*Brachionus plicatilis*) alimentados con levadura de panificación (*Saccharomyces cerevisiae*) y posteriormente enriquecidos, con diferentes productos comerciales o experimentales según las experiencias, manteniendo una concentración 5-10 rotíferos.ml^{-1} en el tanque larvario y ajustada dos veces al día (08:00;15:00). Desde el día 13 hasta el 20, una vez al día (11:00), se añadieron 250 A_0.l^{-1} Nauplios de *Artemia* (Tipo AF, INVE Aquaculture, Dendermonde, Bélgica), posteriormente desde el día 15 hasta el día 50, se añadieron metanauplios de *Artemia* (Tipo EG, INVE Aquaculture, Dendermonde, Bélgica), enriquecidos con el producto A_1 DHA Selco (INVE Aquaculture, Dendermonde, Bélgica) a una concentración que se incrementó de 250 a 2500 metanauplios.l^{-1}) tres veces al día (09:00;15:00 y 20:00) siendo la última toma de *Artemia* distribuida mediante un distribuidor automático. La concentración de presas en el tanque, se evaluó mediante la toma de muestras, dos veces al día (08:00;14:00), de 3 puntos del volumen del tanque de cultivo, antes de la adición de presas nuevas al tanque para restablecer la concentración de presas indicada. A partir del 5dpe y hasta los 20dpe se procedió a la limpieza de la superficie de los tanques mediante el uso de un limpiador de superficie, para la eliminación de la película lipídica que se genera con la adición de presas vivas enriquecidas. Generalmente, el destete dio comienzo el día 20 utilizándose microdietas de la gama Genma Micro (Skretting, Francia) diseñadas para peces marinos y se dio por finalizado a los 45-50dpe. La alimentación con microdietas se realizó inicialmente de forma manual y posteriormente cuando se observó una

buena aceptación de las mismas, se distribuyó de forma automática mediante alimentadores automáticos (Mod. T-Drum feeders; Arvotec, Noruega), inicialmente cada hora y finalmente cada 15min, durante 24h al día. (Tabla VII). A los 50 días de edad los alevines fueron transferidos a tanques de 10.000 litros en el área de alevinaje, donde se mantuvieron en las mismas condiciones de cultivo (1-10 ind.l^{-1}), en circuito abierto hasta 95 días, cuando los peces fueron contados y se llevo a cabo la caracterización esquelética.

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Tabla VII. Condiciones de cultivo generales y secuencia alimenticia durante el cultivo larvario de bocinegro sistema intensivo semi-intensivo

Edad (dpe)	Grados día	Talla (mm)	Luz	Filtros	Flujo	Sifonado	Cultivo	Alimentación		
Siembra Eclosión			Fotoperiodo Natural	Filtro superior (luz de malla 315µm)	25%/día		Agua clara	Alimentación endógena		
2	50	3.00	Fotoperiodo 24 h (1500-3000 Lux) (Natural+artificial)		10%-25%/día	Limpieza de superficie	Agua verde (250-350.000.cells.ml^{-1})	Rotifers(5-10) Indv.ml^{-1})		Piens. Man. Coaliment. Protocolo B
5	80-90	3.1-3.5							Nauplio (0,025-0,25)	
10	125-135	4.0-5.1			25%-50%-75%-100%/día	Ambos			Metanauplio (0,25 -1 indv.ml^{-1})	Piens.Man Coaliment Protocol A
15	175-190	5.5-6.4								Pienso manual+ automático (8-12% biomasa día)
	180-200									
20	225-250	6.5-8.0		Filtro inferior (luz de malla 500 µm)						
	260-275		Fotoperiodo 12:12 (1500-3000 Lux) (Natural+artificial)							
25	280-290	7.5-11.5			5%-15%-25%/hora	Sifonado de fondo	Agua clarar		Pienso automático (8-12% biomasa día)	
30	350	8.5-12.5								
35	375-400	9.5-14.5		Filtro inferior (luz malla 1000 µm)						
40	450	15.0-18.5								
45	500	17-22.5								
50	530-550	19.0-30.0								

4.4.2.4.-Alevinaje

La fase de alevinaje, de todas las experiencias, que se describen en el presente trabajo se llevó a cabo en condiciones idénticas, independientemente de la tecnología de cultivo empleada durante la fase larvaria y manteniendo separados los ejemplares de acuerdo a su origen en el cultivo larvario. Se emplearon los tanques de $10m^3$ de capacidad, previamente descritos. Durante esta etapa, se utilizó agua de mar sin filtrar, la renovación fue de $50\%.h^{-1}$ y la salinidad se mantuvo constante durante todas las experiencias (37‰) y el oxígeno disuelto se mantuvo en un rango de 5,5-7,5 ppm mediante un sistema automático de control y suministro de O_2 (Mod. Multicanal, OxyGuard; Birkerød, Dinamarca) (Figura 47). La temperatura se determinó diariamente, fluctuando entre 20-22ºC en los diferentes experimentos. La iluminación, fue una combinación de luz natural y luz artificial, con un fotoperiodo 12-18h horas. La alimentación se basó exclusivamente en dietas comerciales tipo Genma (Skretting; Francia), en toda su gradación de tamaños (0,2-1,6mm), combinando alimentación manual y automática mediante alimentadores (Mod. T-Drum feeders, Arvotec; Noruega) (Figura 47).

Figura 47. a) Sistema de control de oxígeno disuelto; b) Alimentador automático.

Durante el proceso de alevinaje, cuando la dispersión de pesos de la población dentro del tanque superaba el 30%, se procedió a la clasificación de los alevines con cribas comerciales (Mod. Varillas, Catvis; Holanda), de diferente tamaño acorde con el peso del alevín (Figura 48).

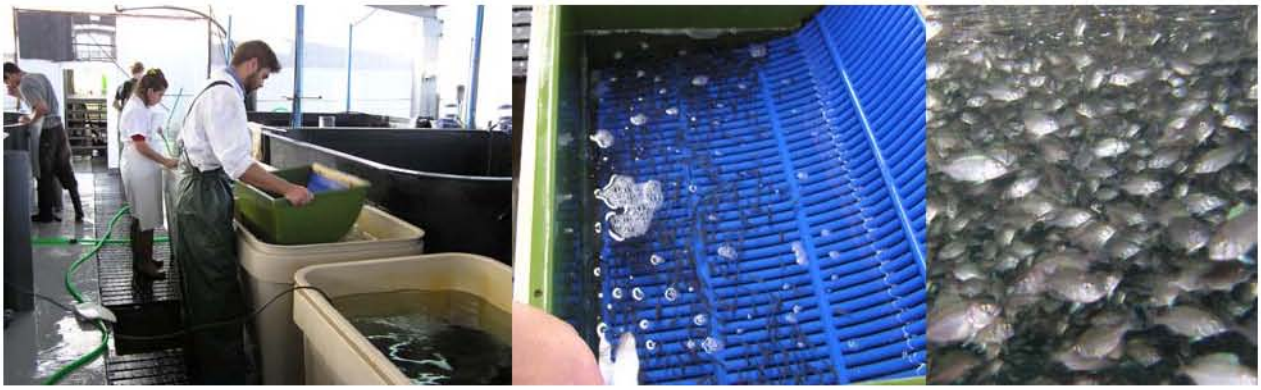

Figura 48. a) Proceso de criba de alevines; b) Detalle de criba; c) Alevines de bocinegro.

Diariamente se procedió a la limpieza del fondo del tanque mediante sifonado del mismo, realizándose un contaje de los individuos muertos para el posterior ajuste de supervivencia.

4.5.-Medidas

4.5.1.-Crecimiento en talla

Durante la primera etapa de cultivo (0-50dpe), cada 5días, se determinó la talla total de 25 individuos por tanque, utilizando un proyector de perfiles (Mitutoyo PJ-3000A, Kanagawa, Japón) (Figura 49).

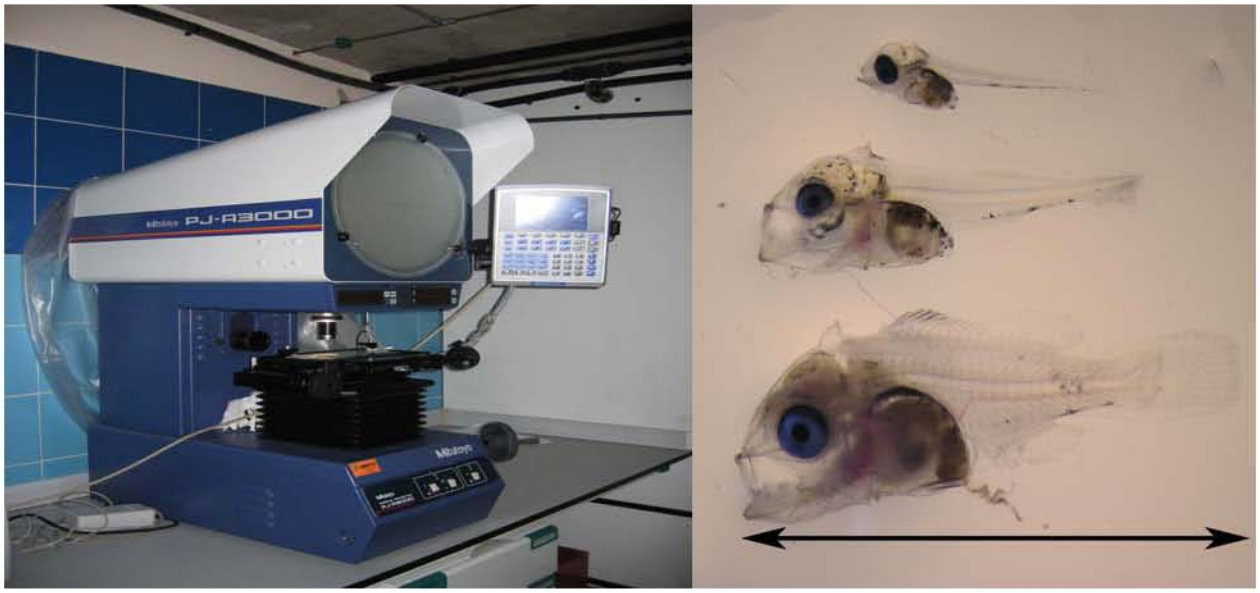

Figura 49. Proyector de perfiles y medida de longitud total en ejemplares de bocinegro de diferente talla.

A su vez, se calculó la Tasa de crecimiento específico en talla (SGR) con la siguiente ecuación:

$$SGR=([Ln(Lt)-Ln(L0)] /t) \times 100$$

donde L_t es la talla total al final del tiempo t, L_0 es la talla total al inicio, t es el periodo de tiempo en días.

4.5.2.-Crecimiento en peso

Peso húmedo: Para determinar el peso húmedo, de las larvas, se sacrificaron mediante una sobredosis de anestésico (Aceite de clavo, Guinama S.L, Valencia, España) y se recogieron sobre una red de malla de 315µm de luz. Se lavaron primero con agua dulce y después con agua destilada, eliminando de la muestra de larvas la mayor cantidad de agua posible con papel

secante. Las larvas fueron colocadas una a una (n=10) en un portaobjetos de cristal previamente marcado y seco, del cual se conocía su peso (*Pc*), se determinó el peso húmedo del conjunto *(Pc)* y larvas (L) en una balanza de precisión (Mettler Toledo AG 204, Ohio, USA). Para determinar el peso individual de las larvas se aplico la siguiente expresión:

$$Pl = \frac{(Pc + L) - (Pc)}{n}$$

Peso seco: Para la determinación del peso seco, el conjunto porta+ larvas se secó a 100ºC, en una estufa (Jouan EU 28, S. Herblain, Francia) hasta alcanzar peso constante: Posteriormente el porta con las larvas secas se sacaron de la estufa dejándolas en un desecador durante 30min para adaptarlas a la temperatura ambiente. Finalmente se pesó, el conjunto, porta más larvas secas (*L*) y el peso individual de cada larva (*Pl*) se obtuvo mediante la expresión siguiente.

$$Pl = \frac{(Pc + L) - (Pc)}{n}$$

4.5.3.-Supervivencia

La supervivencia final se determinó a los 95dpe, mediante el contaje individual con un contador industrial de alevines (TPS Fishcounter, Type Micro; Impex Agency; Dinamarca). La mortalidad diaria se determina por contaje de los alevines muertos y recogidos mediante el sifonado de fondo de los tanques.

Figura 50. a) Operación de contaje de alevines. c,d) Detalle de contador industrial de alevines TPS Fishcounter.

4.5.4.-Estudios histológicos

Para la realización de los diferentes estudios histológicos se tomaron, periódicamente, muestras que fueron fijadas en una solución de formol tamponado al 10% hasta el procesado de las mismas. Se siguió la metodología descrita por Socorro (2006) iniciándose con la identificación y colocación de las muestras fijadas en microcápsulas, que permitirán el lavado y deshidratación en alcohol de diferente gradación en un procesador de tejidos (Mod. Histokinette 2000; Leica, Nussloch, Alemania) (Tabla VIII).

Tabla VIII. Procesamiento de las muestras histológicas

Paso 1: Deshidratación		**Paso 2: Clarificación**		**Paso 3: Infiltración en parafina**		**Paso 4: Re-hidratación**	
	Tiempo		**Tiempo**		**Tiempo**		**Tiempo**
Alcohol 70%	60 min	Xilol I	30 min	Parafina I	180min	Xilol	15 min
Alcohol 80%	90 min	Xilol II	30 min	Parafina II	360min	Alcohol absoluto I	5 min
Alcohol 90%	90 min	Xilol III	60 min	Parafina en vacio	60min	Alcohol absoluto II	5 min
Alcohol 96%	90 min					Alcohol 96%	5 min
Alcohol absoluto I	90min					Alcohol 90%	5min
Alcohol absoluto II	120min					Alcohol 70%	5 min
Alcohol absoluto III	120min					Agua	5 min

Una vez infiltradas (Paso 3; Tabla VIII) las larvas se retiran de las microcápsulas y se procede a la elaboración de bloques de parafina en un dispensador (Mod. Jung Histoembedder;Leica, Nussloch, Alemania). Los bloques se liberan de los respectivos moldes y son tallados para eliminar los restos de parafina de los bordes y permitir así el correcto anclaje de los mismos en el micrótomo (Mod. Jung Autocut 2055; Leica, Nussloch, Alemania). Inicialmente se procedió a un tallado grueso a 20-25 μm hasta alcanzar el tejido y posteriormente se realizaron los cortes definitivos a 4-5 μm. Los cortes fueron recogidos por flotación con un portaobjetos en un baño con agua destilada a 45ºC, secados en estufa (1h a 60ºC). Las muestras se introdujeron en un baño de xilol y se re-hidrataron en alcohol de diferente gradación hasta finalizar con agua (Paso 4, Tabla VIII).

Para el montaje definitivo de las muestras, se añadió una gota de resina sintética sobre un cubreobjetos, montando el portaobjetos con la muestra sobre el cubreobjetos y presionando hasta eliminar las posibles burbujas de aire. Una vez secas, las preparaciones se conservaron en un lugar fresco y en oscuridad hasta la tinción de las mismas.

Las muestras de tejidos obtenidas se tiñeron con dos tipos de tinción; hematoxilina - eosina (H&E) y ácido peryódico-reactivo de Shiff-Hx (PAS-Hx) según las técnicas de Martoja y

Martoja-Pierson (1970) y de García del Moral (1993) modificadas por Socorro (2006) (Tablas IX, X).

Tabla IX. Protocolo de tinción hematoxilina–eosina y con ácido peryódico-reactivo de Shiff-Hx (PAS-Hx)

Paso 5a: Tinción con Hematoxilina-eosina (H&E)		**Paso 5b:Tinción con ácido peryódico-reactivo de Shiff-Hx (PAS-Hx)**	
Producto	**Tiempo**	**Producto**	**Tiempo**
Hematoxilina de Harris	15-20 min	Acido HIO_4 (peryódico) 0,5%	5 min
Alcohol ácido	3 baños cortos	Agua	5 min
Agua	Lavado	Reactivo de SCHIFF	20 min
Agua amoniacal	20seg	Agua	5 min
Agua en continuo	5 min	Hematoxilina	15 min
Eosina de Puttis	3-4min	Agua	20 min
Agua	Lavado		

Una vez finalizada la tinción, se procedió a la deshidratación y clarificado de las muestras (Tabla X).

Tabla X. Protocolo de deshidratación y clarificado

Paso 6: Deshidratación y clarificado	
Producto	**Tiempo**
Alcohol 96%	5 min
Alcohol 100%	10 min
Alcohol 100%	5 min
Xilol	5 min
Xilol	5 min

Finalizado el proceso de tinción, las preparaciones fueron observadas y fotografiadas en un fotomicroscopio (Mod. DMBE, Leica, Nussloch, Alemania) para la realización de los estudios descriptivos de las diferentes estructuras. A su vez, cuando fue necesaria la realización de mediciones y contaje de estructuras particulares, se utilizó un programa informático de análisis de imagen (Image-pro Plus versión 2.0; Media Cybernetics, Inc., Buckinghamshire, Inglaterra).

4.5.5.-Estudio osteológico

Para estudiar el desarrollo osteológico de las larvas desde el día 0 al 50dpe se cogieron 25 individuos, por tanque de cada tratamiento cada 5 días. Estos se fijaron en una solución de formol tamponado al 10% y posteriormente se sometieron al proceso de tinción de estructuras de cartílago-hueso siguiendo la metodología de Taylor y Dyke (1985) y (Dingerkus y Uhler (1977) modificadas por Socorro (2006).

Las larvas fijadas en formol se extraen del recipiente de conservación y se introducen en un recipiente de fijación de acuerdo con siguiente procedimiento:

Tabla XI. Protocolo de tinción cartílago-hueso

Paso 1: Tinción de cartílago		**Paso 2: Hidratación**		**Paso 3: Clarificación**	
	Tiempo		**Tiempo**		**Tiempo**
Tinción con Azul Alcian	120 min	Alcohol 96%	60 min	Proceso de clarificación	60 min
		Alcohol 96%	60 min		
		Alcohol 96%	60 min		
Preparacion de Solución de azul Alcian 10mg azul alcián BGX (c.i. 16230) 80ml de alcohol 96% 20ml ácido acético glacial		Alcohol 75%	60 min	*Preparación de la solución de tripsina* 90 mg tripsina 30 ml de $Na_2B_4O_710H_2O$ (solución saturada) 70 ml agua destilada	
		Alcohol 40%	60min		
		Alcohol 15%	60 min		
		Agua destilada	60min		

Paso 4: Tinción de hueso		**Paso 5: Clarificacion y conservación**		
	Tiempo		**Relación**	**Tiempo**
Tinción con Rojo Alizarina	60min	KOH 0,5% - Peróxido	3:1	60min
		KOH 0,5% - Glicerina	3:1	12-24 horas
		KOH 0,5% - Glicerina	1:1	12-24 horas
		KOH 0,5% - Glicerina	1:3	12-24 horas
		Glicerina + Timol	100%	Conservación
Preparacion de Solución de rojo alizarina KOH 0,5% en agua. Rojo de alizarina 1 g / litro		*Preparación de la solución de Glicerina-Peróxido de hidrógeno* KOH 0,5% Glicerina (3:1) H_2O_2 1,2 %		

Una vez terminado el proceso, las larvas presentan las estructuras esqueléticas teñidas (azul estructuras cartilaginosas y rojo las estructuras óseas) y fueron estudiadas individualmente bajo la

lupa (Mod. Olympus, casa pais) para describir su desarrollo osteológico, tomando fotografías digitales,en los momentos más importantes del desarrollo.

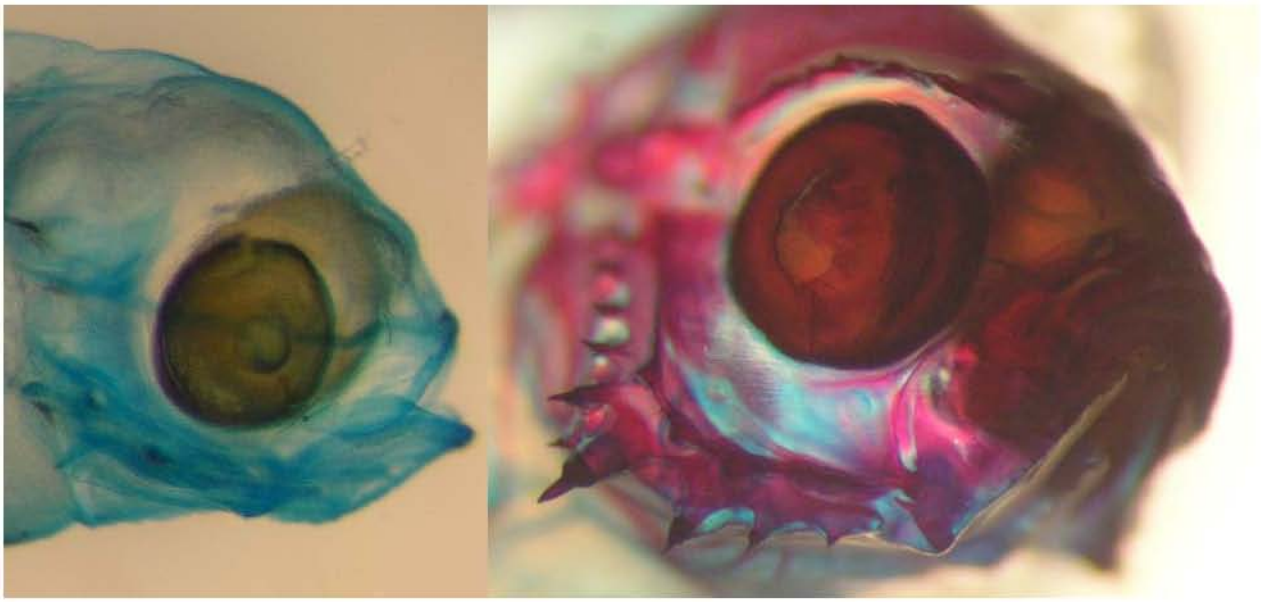

Figura 51. a) Detalle de estructura craneal de una larva de bocinegro de 8dpe presentado únicamente estructuras cartilaginosas teñidas con azul alcian; d) Larva de 27 días con estructuras calcificadas teñidas con rojo alizarina.

4.5.6.-Caracterización de deformidades

Para la caracterización de las deformidades esqueléticas, se tomaron muestras de manera rutinaria a diferentes edades, en todas las experiencias de cultivo, así al finalizar el destete 50dpe, se llevó a cabo una valoración visual de 500 larvas por tanque y por tratamiento. En etapas posteriores, además de la valoración visual, se realizaron valoraciones de placas radiológicas de baja intensidad realizadas con un mamógrafo digital (Mod. Senographer-DHR, General electrics, USA) de las deformidades morfo-anatómicas que aparecían en 100 individuos de cada tratamiento. Los anomalías detectadas se clasificaron de acuerdo con Divanach *et al*., (1996).

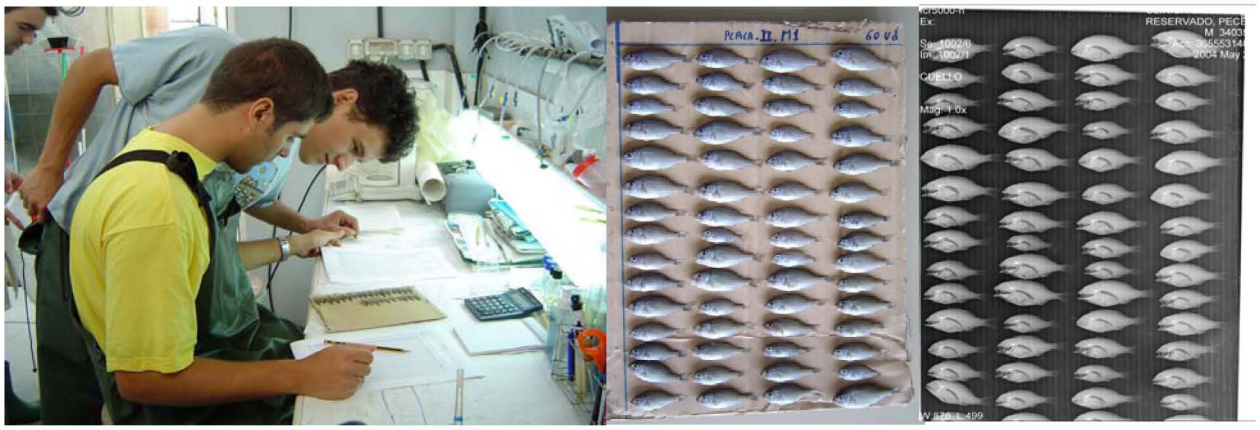

Figura 52. Evaluación visual de deformidades, preparación de placas y mamografías resultantes para caracterización en detalle.

4.5.7.-Determinaciones merísticas

En determinadas experiencias se realizaron valoraciones merísticas del nº total de vertebras incluyendo el urostilo y nº de costillas pleurales en 250 ejemplares de 95dpe, siguiendo la metodología descrita por Matsuoka (2003). Así, las vertebras con dos espinas neurales y /o con dos espinas hemales se consideraron compuestas por la fusión de dos vertebras.

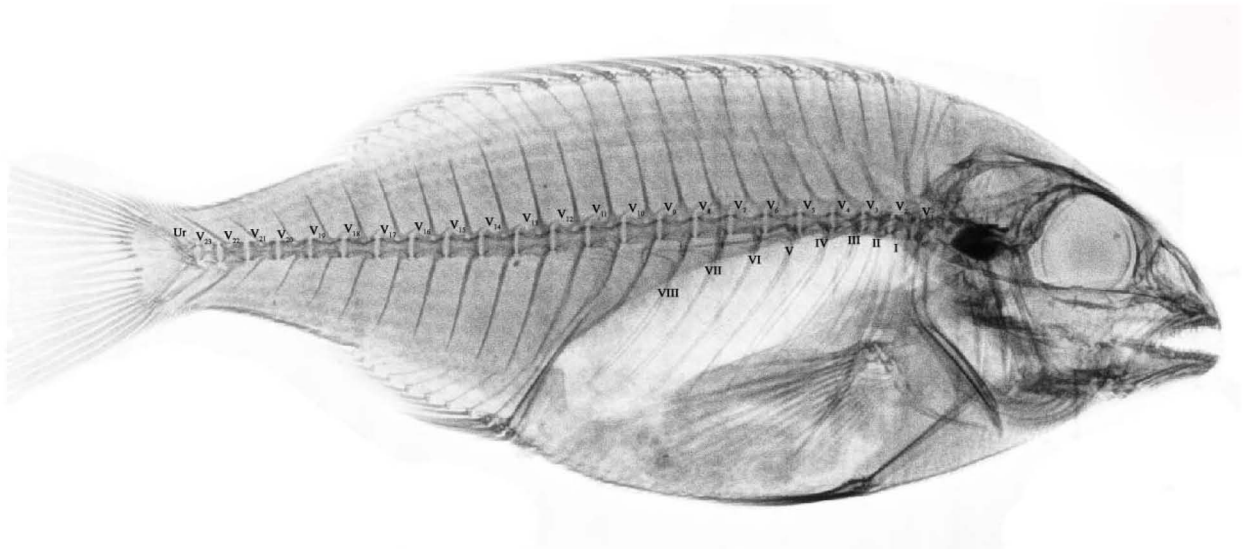

Figura 53. Identificación de vertebras y costillas.

4.6.-Analisis bioquímicos

Durante el transcurso de las diferentes experiencias, se tomaron muestras de los productos utilizados para la producción y enriquecimiento de las presas vivas (rotíferos y *Artemia*), piensos y de las propias presas vivas utilizadas, antes y después de su enriquecimiento. Así mismo, se recogieron muestras de larvas de diferentes edades, de cada uno de los tratamientos ensayados. Una vez recogidas, las muestras de presas vivas y larvas, se colocaron sobre una malla de 63µm, se lavaron con agua dulce y posteriormente con agua destilada. A continuación se eliminaba la mayor cantidad de agua posible con papel secante y se procedia a su congelación a -80ºC en bolsas herméticas bajo atmósfera de nitrógeno, para su posterior análisis. Los análisis bioquímicos se realizaron en el laboratorio del Instituto Universitario de Sanidad Animal y Seguridad Alimentaria (IUSA). Se hicieron determinaciones del contenido en humedad, cenizas, proteínas, lípidos totales y ácidos grasos. Todas las determinaciones, se realizaron al menos por triplicado.

4.6.1.-Determinación de la humedad

Se determinó siguiendo el método de la American Oficial de Analis Chemisty (AOAC,1995). El procedimiento consiste en secar en una estufa a 110ºC una cantidad de muestra conocida (P_i) hasta peso constante (P_f). Posteriormente se saca la muestra, se introduce en un desecador 30 min y se realiza la pesada. El porcentaje de humedad de la muestra se obtiene con la expresión:

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

4.6.2.-Determinación de las cenizas

El contenido de cenizas se determinó, por medio de la incineración de una cantidad conocida de muestra (P_m) en un horno mufla, a una temperatura de 450ºC durante 24 horas, pesando posteriormente la cantidad de cenizas remanente (P_c) hasta peso constante según la AOAC (1995) y aplicando la siguiente expresión.

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

4.6.3.-Determinación de las proteínas

El contenido proteico se calculó a partir del contenido de nitrógeno total de las muestras, determinado por la técnica de Kjeldhal. Según el método de la AOAC (1995), la técnica consiste en la digestión de las muestras con acido sulfúrico a 420ºC con presencia de un catalizador de cobre durante una hora, seguido de una destilación con Na(OH) al 40% utilizando acido bórico saturado como sustancia receptora en una unidad destiladora (Mod. Foss Tecator, 1002, Höganäs,

Suecia). Finalmente se realiza una valoración con HCl 0,1 M. Para calcular el porcentaje de proteína se aplicó la siguiente expresión.

$$\%Proteina = (V-P) \times N \times Pm \times F/M$$

Siendo:

V = Volumen de HCl usado en la valoración en ml
P = Media de la valoración de los patrones en ml
N = Normalidad del HCl
Pm= Peso molecular del nitrógeno que es 14,007
F = Factor de conversión empírico que tiene un valor de 6,25
M = Peso de la muestra en mg

4.6.4.-Determinación de los lípidos totales

La extracción de los lípidos se realizó según el método de Folck *et al.* (1957), adaptado por Izquierdo y Gil (1998). El método consistió en tomar una cantidad de muestra entre 50-200 mg, que se homogeneizó en un Ultra Turrax (IKA-Werke, T25 Basic, Germany, Staufen) a 11.000 rpm durante 5 min en una solución de 5 ml de Cloroformo: Metanol (2:1) con 0.01% de BHT. A continuación, la solución resultante, se filtró a presión reducida a través de lana de vidrio y añadiendo KCl al 0.88%, para aumentar la polaridad de la fase acuosa. Por decantación y tras un centrifugado a 2000 rpm durante 5 min se separaron las fases acuosa y orgánica. Una vez retirada la fase acuosa se evaporó a sequedad con una corriente de N_2 y se determinó el contenido de lípidos totales de la muestra por gravimetría.

4.6.5.-Determinación de ácidos grasos

Los lípidos totales extraídos, se transesterificaron según el método de Christie,(1982). En este procedimiento se añadió Tolueno con BHT y una solución de Metanol:Sulfúrico al 1%. La mezcla se agitó fuertemente para favorecer la disolución de los lípidos, llenando el recipiente con N_2 y sellándolo posteriormente. Se dejó incubando 16 horas a 50ºC en agitación. Transcurrido este tiempo, se dejó enfriar la muestra y se le añadió agua destilada ultra pura y Hexano:Dietil eter 1:1 con BHT al 0.01%. Los FAMES purificados se evaporaron a sequedad con N_2 y se pesaron. Finalmente, se diluyeron a una concentración de 20mg de FAMES por ml de Hexano, pasándolos a microviales, que se congelaron a -80ºC hasta el momento de su identificación y cuantificación en el cromatógrafo de gases, (Mod. Shimadzu GC-14A; Analytical instrument division, Kyoto, Japon), equipado con un detector de ionización de llama y un integrador Shimazu (CR-5A).

Las características de la columna son las siguientes: columna capilar de sílice fundida, de 30m x

0.32mm D.I. con supelco-10 como fase estacionaria, (Supelco, Inc., Bellefonte, EE.UU). Actuando como Gas portador:helio. La presión de los gases: He 1 $Kg.cm^{-2}$, H_2 0.5 $Kg.cm^{-2}$, N_2 1 $Kg.cm^{-2}$, aire 0.5 $Kg.cm^{-2}$. Y la temperatura: en el inyector 250 ºC, columna según rampa con las siguientes características: temperatura inicial 180ºC durante 10min, tasa de incremento de temperatura 2.5ºC.min^{-1}, temperatura final 215ºC durante 10min.

La identificación de los ácidos grasos se llevó a cabo mediante la utilización de EPA 28 como aceite estándar. Comparando los tiempos de retención y las distancias de los picos del análisis de las muestras con el estándar.

4.7.-Análisis estadístico

Los resultados obtenidos se han expresado siempre como media ± desviación estándar de la media. Los análisis estadísticos se realizaron con el programa SPSS Versión 14.0 (SPSS Chicago, Illinois, 1999). Los datos de cada experimento, se compararon estadísticamente mediante test de la T-Student (Sokal and Rolf, 1995), cuando había solo dos tratamientos o con un análisis de varianza (ANOVA) si era mayor el número de tratamientos ensayado. Como criterio general se tomó el 5 % como nivel de significación. Una vez habían sido detectadas diferencias estadísticamente significativas con el ANOVA, las diferencias entre medias fueron puestas de manifiesto mediante el test de comparación múltiple de Tukey. Cuando las varianzas eran heterogéneas y/o los datos no se distribuían normalmente se intenta hacerlas homocedásticas y/o que los datos se distribuyeran normalmente transformándolas variables en sus logaritmos o bien con la función arco seno. Si la heterogeneidad o la no distribución normal de los datos persistían, se empleaba el test no paramétrico de Kolmogorov-Smirnov, cuando solo había dos replicados, o si era mayor el nº de tratamientos el test Games-Howell. Finalmente, para el estudio de calidad de los alevines se aplicó un análisis log lineal, con la χi cuadrado de Pearson (Sokal and Rolf., 1995).

4.8.-Nomenclatura de especies citadas

Los nombres vulgares de las especies, utilizados en este trabajo fueron tomados del "Diccionario multilingüe de especies marinas para el mundo hispano" de Vera, (1992). En caso de no figurar, la especie, en dicho diccionario se utilizó la denominación FAO en español de la base de datos "Fishbase" y en el caso de no existir el nombre en español, se utilizó la denominación FAO en inglés de esa base de datos.

9.5.-Conclusiones

Estudio I. **Desarrollo del sistema visual de las larvas de bocinegro *Pagrus pagrus (Linnaeus, 1758)* en relación a los cambios del sitema digestivo y hábitos alimenticios.**

1. Las larvas de bocinegro son predadores visuales, que eclosionan con un sistema visual y digestivo incompleto y no funcional.

2. Entre el tercer y cuarto día post eclosión se producen los mayores cambios en el sistema visual y digestivo, como la pigmentación de los fotoreceptores primarios (conos) que coincide con la apertura de la boca y la detección de acividad digestiva en en el intestino medio, lo que es un indicativo de que las larvas están preparadas para comenzar la alimentación exógena.

3. El segundo hito mas importante en el desarrollo del sistema visual se detecta en torno a los 20 dpe, (7,0-7,5mm LT) cuando aparecen los núcleos de los segundo fotoreceptores (bastones). Desde este momento, las larvas de bocinegro presentan una retina con doble sistema de fororreceptores (conos y bastones).

4. La pigmentación de los bastones coincide con la aparición de las primeras células gástricas y la progresiva migración de las larvas desde las capas superficiales a una mayor profundidad en el tanque de cultivo, lo que sugiere cambios en los hábitos alimenticios de las condiciones de cultivo a partir de esta etapa.

Estudio II. **Desarrollo osteológico y aparición de deformidades esqueléticas en las larva de de bocinegro *Pagrus pagrus* (Linnaeus, 1758) bajo diferentes técnicas de cultivo.**

5. El patrón general de desarrollo osteológico del bocinegro, no se ve afectado por el sistema de cultivo. Sin embargo, el momento de aparición de las diferentes estructuras esqueléticas, que se encuentra mas ligado al crecimiento en talla que a la edad de las larvas, si se vio afectado por el sistema de cultivo empleado. Así, la osificación se completa antes en los sistemas semi-intensivos, donde se obtiene una mayor tasa de crecimiento.

6. Independientemente del sistema de cultivo empleado se observó una elevada incidencia de deformidades morfoanatomicas en esta especie. Siendo las aparición de lorsosis y fusiones vertebrales las anomalías más frecuentes. Se registró una baja incidencia de anomalías operculares, lo cual se asoció a la presencia de unas largas espinas operculares desde etapas tempranas de su desarrollo que previenen la curvatura del opérculo hacia el interior de la cámara branquial. La lordosis, fue localizada mayoritariamente entre la 8ª y 12ª vertebra, región de la columna que soporta una mayor presión muscular durante la natación.

7. El sistema de cultivo, tiene efecto sobre la localización de las fusiones vertebrales. Así, en las larvas cultivadas en sistemas intensivos, las fusiones se localizan a lo largo de toda la columna y particularmente en la zona caudal, mientras que en el sistema semi-intensivo se localizan mayoritariament en la zona pre-hemal.

8. La intensificación del sistema de cultivo se reflejo en la aparición de un mayor número de individuos con una vertebra extra, una mayor incidencia de cifosis y anomalías craneales principalmente registradas como acortamiento de la mandibula superior y aparición de mandibulas cruzadas.

***Estudio III*. Efecto del contenido de DHA en los rotíferos sobre la incidencia de deformidades esqueléticas del bocinegro *Pagrus pagrus (*Linnaeus, *1758)*.**

9. Los requerimientos de DHA para obtener un buen crecimiento en las larvas de bocinegro fueron inferiore as 1.9%. El DPA (22:5n-6) se acumula fácilmente en los tejidos de las larvas de bocinegro cuando estas se alimentan con rotíferos con un elevado contenido en este ácido graso, registrandose una baja supervivencias de las larvas, lo que puede estar relacionado con la elevada incorporación de DPA en el tejido nervioso y visual, afectando a funciones de las biomembranas, como se ha descrito en otros vertebrados.

10. La suplementacion de DHA en la dieta, fue asociada a una mejora en la supervivencia y una reducción en la incidencia de deformidades esqueléticas en general, lo que indica la importancia de este ácido graso en el desarrollo osteológico.

***Estudio IV*. Avances en las técnicas de cultivo de bocinegro *Pagrus pagrus (*Linnaeus, *1758)*: Comparación de sistemas de cultivo larvario intensivos y semi-intensivos.**

11. Los ácidos grasos más abundantes en las larvas del bocinegro son el 22:6n-3, 16:0, 18:1n-9, 18:0, 18:2n-6, 20:5n-3, 16:1n-7,18:1n-7, 18:3n-3 y 20:4n-6.

12. El DHA es acumulado selectivamente en los tejidos de las larvas de bocinegro, en niveles superiores a los aportados en las presas vivas, lo que sugiere la importancia de este ácido graso para esta especie. Los niveles de ácido eicosapentaenoico refleja los cambios en la dieta durante la ontogenia de las larvas de bocinegro mientras que el ácido araquidónico permanece casi constante a lo largo del desarrollo larvario.

13. Durante el desarrollo larvario del bocinegro, se han identificado tres estadios de crecimiento: una primera etapa (5-15 dpe) de elevado crecimiento y periodo muy sensible en la supervivencia larvaria; una segunda etapa (15-30dpe), que se correlaciona con la finalización de la metamorfosis, con un bajo crecimiento y un mejor control de la mortalidad; y finalmente, un tercer perido post-metamorfosis (30-50dpe) con un ligero incremento del crecimiento y baja mortalidad.

14. Durante la primera etapa (5-15 dpe), el incremento en la densidad de presas en el sistema de cultivo semi-intensivo de 1-2 rot.ml^{-1} a 4-5 rot.ml^{-1} disminuye la mortalidad larvaria e incrementa las tasas de crecimiento.

15. Durante la segunda etapa, el incremento en el periodo de alimentación con rotíferos desde 20 a 30 dpe mejora las tasas de crecimiento facilitando los cambios de alimentación.

16. Durante la tercera etapa, la mortalidad registrada esta relacionada con el comportamiento canival de esta especie.

17. Las larvas de bocinegro presentan una elevada voracidad sobre la *Artemia*, la excesiva ingestión de la misma, reduce el tiempo de tránsito de la *Artemia* a través del digestivo llegando incluso a defecarla viva.

18. Las tasa de crecimiento en el bocinegro es mas elevada que la de otros Espáridos comerciales como la dorada *Sparus aurat*, doblando incluso el peso de los juveniles a los 95 dpe.

19. En el sistema semi-intensivo, se observó una mayor duración de las reservas vitelinas de las larvas, lo que se refleja en una mayor acumulación de DHA en estas larvas, las cuales muestran una mayor resistencia al test de actividad de exposición al aire. De hecho las larvas cultivadas en sistema intensivo presentaron una elevada sensibilidad al manejo.

20. En el sistema intensivo, el protocolo de co-alimentación con microdietas temprano, incrementa la supervivencia, siendo aceptada las microdietas más fácilmente que en el sistema semi-intensivo, como se reflejo en la composición de ácidos grasos.

21. Las principales diferencias entre los sistemas se reflejan en el periodo de alimentación con rotíferos, cuando la densidad de larvas se reducen en un 86 % en el sistema intensivo y en un 57 % en el semi-intensivo a los 15 dpe. Lo que sugiere una mejor utilización de los nutrientes de las presas en unas condiciones de cultivo más favorables y una menor demanda energética. Las principales diferencias de ambos sistemas, se relacionaron con la disponibilidad de presas, tasas de renovación, cambio de agua verde y manejo de los tanques.

22. Los resultados de este estudio, permitieron mejorar los protocolos de producción de larvas de bocinegro, incrementando la talla total y la supervivencia a los 50 dpe desde la primera prueba hasta la última de 18.9 mm a 25.13 mm y desde 4.9 a 12.5% en el sistema intensivo y de 23.52 a 26.4 mm y de 4.4 a 28.7% en el sistema semi-intensivo. Asi mismo en estos momentos, el sistema semi-intensivo se muestra como el mejor sistema de cultivo de larvas para mantener una producción regular y sostenible de alevines de bocinegro.

Improvements in the production technology of red porgy (*Pagrus pagrus*) larvae and fry: Importance of rearing conditions and diet nutritional value on their quality

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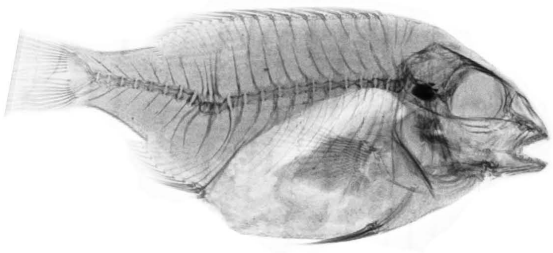

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