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Sea bream (*Sparus aurata*) intensive larval rearing. Effects of microalgae and phages addition on the performance of the larvae.

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SEA BREAM (*SPARUS AURATA*) INTENSIVE LARVAL REARING. EFFECTS OF MICROALGAE AND PHAGES ADDITION ON THE PERFORMANCE OF THE LARVAE

JESÚS RUBIO DA COSTA

Trabajo realizado en el Departamento de Acuicultura; Institute of Marine Biology of Crete (Grecia), bajo la dirección del Dr. Nikos Papandroulakis.

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Abstract:

Two different experiments were carried out with *Sparus aurata* larvae. In the first, the effects of microalgae addition (*Chlorella minutissima*) in the rearing system on growth performance (total length and wet weight), quality (% deformities) and survival of the larvae during the first 60 days post hatching were evaluated. Also, the ontogeny of the endogenous antioxidant defenses (superoxide dismutase, glutathione reductase, glutathione peroxidase, glutathione S-transferase and glutathione) and the pancreatic proteases (trypsin and chymotrypsin) was studied. Two groups of *S. aurata* larvae were reared until 60 days post hatch in a system with (pseudo-green water system, PGW) or without microalgae (clear water system, CW) and samples were taken at different developmental stages for the analysis of the enzymes. Additionally, at 120 days post hatching, survival and wet weight was evaluated. Up to our knowledge, this is the first time that the effects of green water on the antioxidant defences are studied. Significantly better growth performance was registered in the PGW group in terms of total length and wet weight. Differences between groups in the various antioxidant and digestive enzymes were discussed.

The second experiment studied the effects of bacteriophages KVP40 addition to the larval rearing system (PH system) on the growth performance, quality and survival of the larvae during the first 120 days post hatch. Phages were added to the PH tanks at 4, 8 and 20 days post hatching. Differences, although not significant, in growth performance were detected between PH and CW groups.

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1. INTRODUCTION

1.1. World Aquaculture

Throughout the history of humanity have been evolving different ways of farming, grazing and ranching to increase food production in order to cover the food requirements necessary for an exponentially growing world population. The aquatic environment has always been a source of exploitable resources in this regard since marine organisms, particularly fish, are a source of high quality nutrients (proteins, essential fatty acids, vitamins, minerals, etc) fundamental in human nutrition, either directly or as by-products and derivatives.

For many years, obtaining a higher number of captures from wild fisheries has been achieved at the expense of an increasing pressure on fish stocks. For this has been developed modern fishing gear, boats with greater capacity and autonomy, satellite detection systems and other new technologies. All this has managed to increase the level of exploitation of marine fisheries resources to the current state, in which an increase in fishing effort generally does not lead to an increase in performance. This has proven that the sea (although it looks like because of its vastness) is NOT an inexhaustible resource.

Aquaculture appears to be the most logical, effective and promising option to increase the production of commercially important aquatic organisms. Although the rate of growth of global aquaculture is decreasing, it is still the sector within the food industry with the fastest growth rate. Currently produces almost half of the total supply of edible fish (FAO, 2010. Figure 1) and FAO estimations for 2030 talk about 65% of the aquatic food coming from aquaculture. Moreover, for some species such as mussels, clams, oysters, sea bream, sea bass, trout, tilapias, and carps, the majority of the production comes almost entirely from aquaculture.

This great development is mainly due to the aforementioned better knowledge in the field of aquatic biology in general and certain technological improvements.

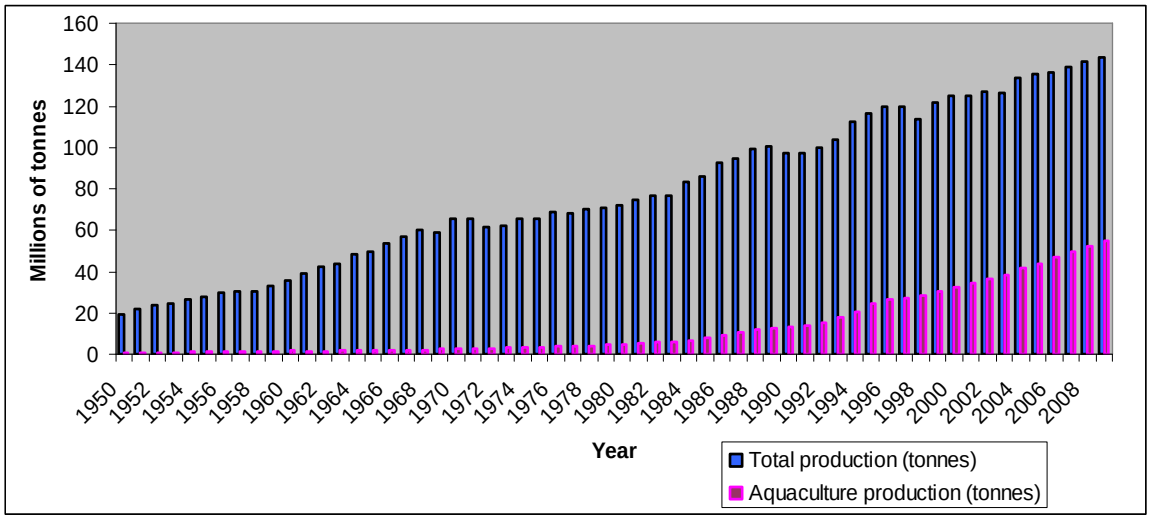


Figure 1: Total global production (FAO, 2010)

Aquaculture is a relatively new multidisciplinary science, coupling expertise not only of biology but also of engineering, systems of production and economics. Its ultimate goal is the controlled production of commercially important aquatic organisms by managing their life cycle and controlling the environmental factors that influence their development. Among the advantages that aquaculture presents, one can emphasize on (i) the better control over the final product quality, (ii) the increased productivity in comparison to agriculture and terrestrial farming, (iii) the limited impact on the environment, (iv) the job creation, (v) the development of rural areas, and (vi) the diversification of the economy.

1.2. European Aquaculture

Aquaculture in the Mediterranean region is an activity which started many centuries ago. There are evidences over aquaculture activities in the Mediterranean since the ancient Egyptian civilization (Basurco and Lovatelli, 2003). The main technique was to capture juveniles and keep them alive until reaching an adult height. Modern marine aquaculture has started effectively 50 years ago and most Mediterranean countries are involved in its development. This new form of aquaculture has developed as a result of significant research mainly on reproduction, larval rearing, feed manufacturing and engineering technology, among other specialties.

As regard to species, recent aquaculture developments focus on the popular carnivorous finfish species with either a low production volume from capture fisheries

or from over-fishing stocks. The technology applied has evolved rapidly as a result of the modifications of existing farming facilities (e.g. water recirculation for land based installations) and the development of new farming concepts (e.g. off-shore submerged netcage technology). As a consequence of such developments, nowadays, in the Mediterranean a wide-range of production activities of marine species co-exist in different environments using a variety of production technologies (Basurco and Lovatelli, 2003).

European aquaculture is considered today as a world leader in the production of some high value species (salmonids, sea bass, sea bream) and contributes to global aquaculture development through knowledge and technology transfer (FAO, 2010). Between 1990 and 2009, the production volume of European aquaculture increased by 55% from 1.6 to 2.5 million tonnes, while the production value doubled (Figures 2, 3). The increase was mainly because of the growth of marine finfish aquaculture (FAO, 2010).

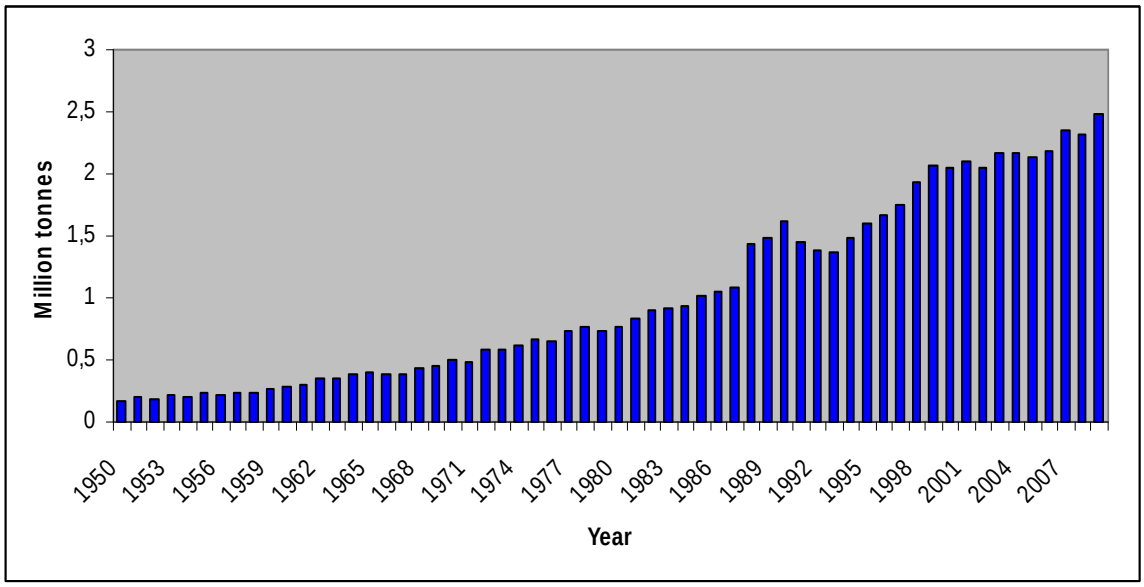


Figure 2: European aquaculture production (tonnes). (FAO, 2010)

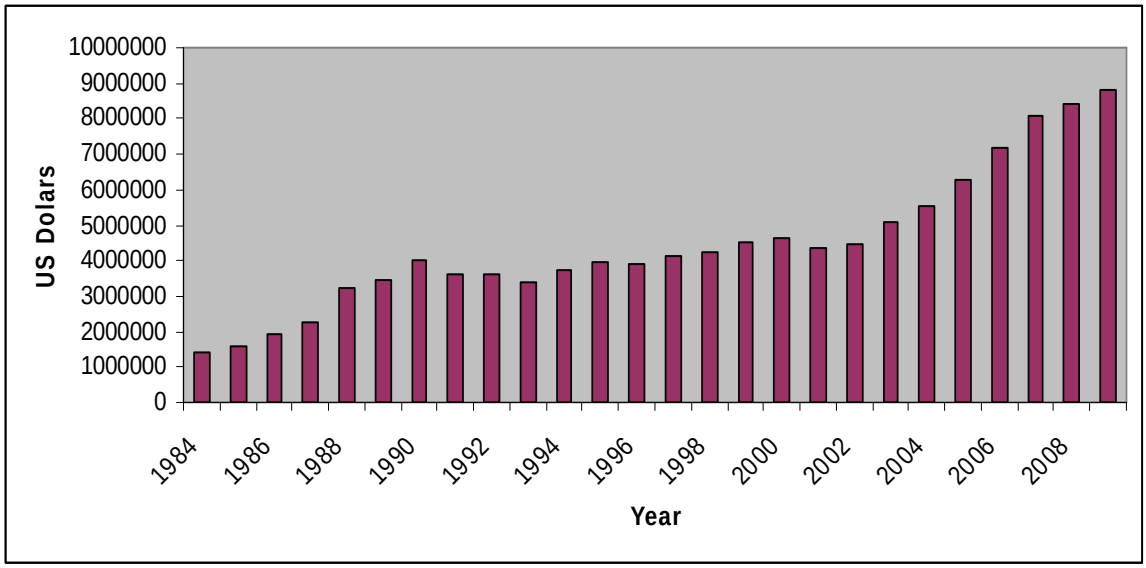


Figure 3: European aquaculture production (value). (FAO, 2010)

1.3. Aquaculture in Spain

Spain has almost 8000 km of coast, with varied topography and climate, which give the country the characteristics necessary for the development of marine aquaculture. It has also numerous fluvial resources, lakes and reservoirs, which provide ideal conditions for developing inland aquaculture also (FAO, 2010).

Spanish aquaculture production is the largest within all European countries, it reached over 266,000 tonnes in 2009, of which around 250,000 corresponded to the production of marine species (96% of the total production) and the rest to inland aquaculture (4% of the total production). Around 190,000 tonnes of this production corresponds to the production of mussels mainly in Galicia. The total value of the production of aquaculture in Spain was 413 million euros in 2009 (APROMAR, 2011). In 2010 however, the production of marine finfish aquaculture in Spain suffered a reduction of 9.4% over the previous year, occurring in all finfish species reared: sea bass, sea bream and turbot (APROMAR, 2011) (Figure 4).

Aquaculture in Spain, as in the rest of Europe, is facing a series of constraints mainly related to the evolution of markets, site availability, diseases, planning, infrastructures and human resources. The sea bass and sea bream industry could be described as a sector entering a mature phase, in which competition has increased, and prices and margins have significantly diminished, demanding additional efficiency, productivity and economies of scale (Basurco and Lovatelli, 2003). However, better

knowledge on the reared species may allow the creation of more advanced methods for rearing which, together with the increasing diversification, may certainly reinforce the competitiveness of the sector.

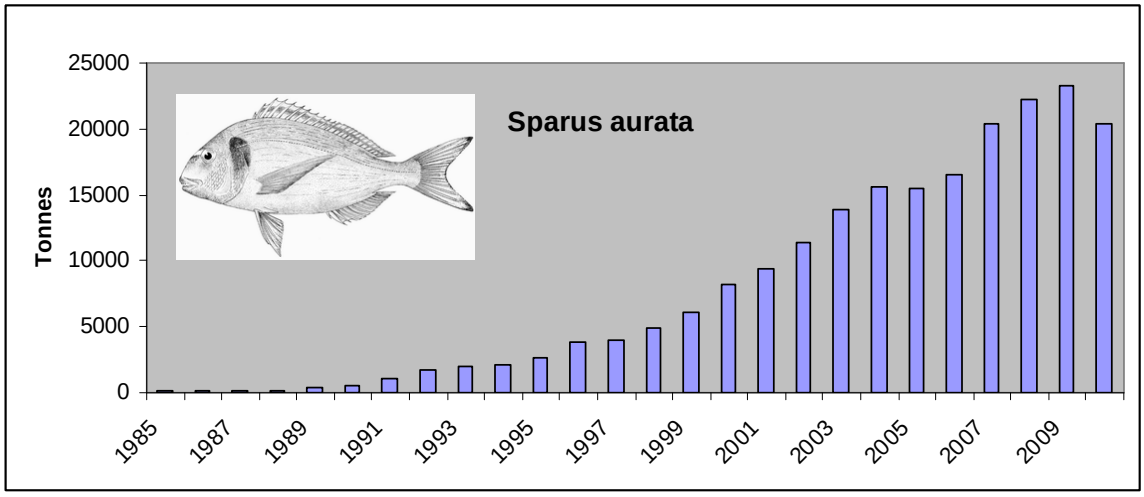


Figure 4: Spanish production of sea bream (*Sparus aurata*) (FAO, 2010)

1.4. Sparidae

Sparidae, commonly called breams and porgies, is a family of the order Perciformes and includes about 115 species classified in 33 genera (Nelson, 2006). They have a wide distribution, from tropical to temperate waters, and are found in the Atlantic, Indian and Pacific Oceans, and the Mediterranean Sea. This family is composed mainly of marine coastal fish, of high economic value, exploited and farmed for human consumption, as well as for recreational purposes. The total Sparidae production was around 700,000 tonnes in 2009 (FAO, 2010), with around 37% (261,000 tonnes) of the production coming from aquaculture (Figure 5).

Sparidae are physically characterized by an oblong body, moderately deep and compressed, with a large head profile often with a steep upper profile, regularly curved with a maxilla hidden by a sheath when the mouth is closed (Carpenter and Niem, 2001). They have 24 vertebrae and a single dorsal fin, usually with 10–15 spines, and three spines in the anal fin. Their overall colour is highly variable, from pinkish or reddish to yellowish or greyish, often with silvery or golden reflections, and dark or coloured spots, stripes, or bars. Most of them are carnivorous and feed on benthic

invertebrates. Many species have been found to be hermaphroditic: some have male and female gonads simultaneously, while others change sex as they get larger.

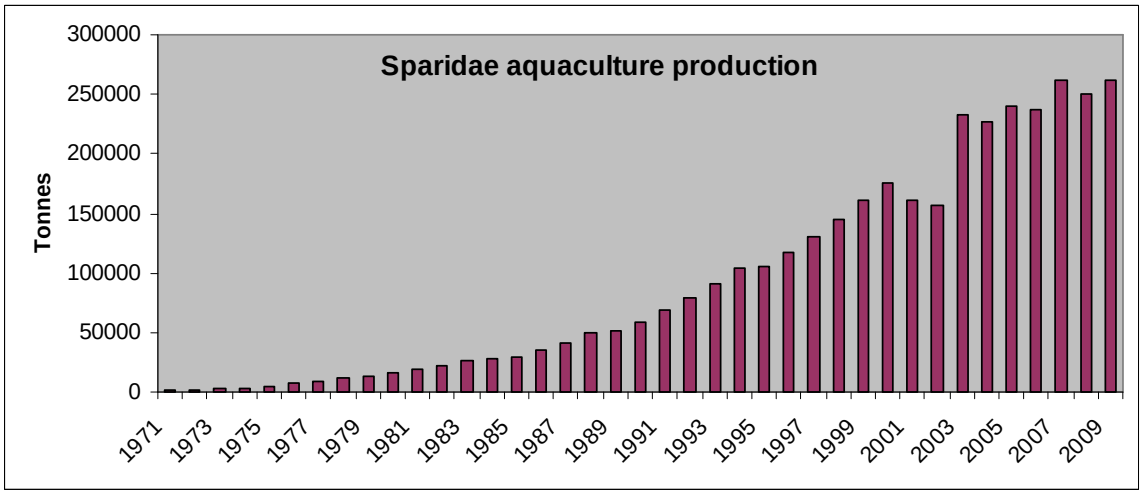


Figure 5: Global aquaculture production of Sparidae (FAO, 2010)

The gilthead seabream has an oval body, rather deep and compressed, with a head profile regularly curved with small eyes (FAO 2005-2009). The overall colour is silvery-gray; a large black blotch at the origin of the lateral line extends on the upper margin of the operculum; a golden frontal band exists between the eyes. The gilthead seabream is commonly found throughout the Mediterranean; it is also found in the Atlantic Ocean from the British Isles to Cape Verde and around the Canary Islands. It is a benthopelagic (demersal behaviour) specie, found in coastal environments to depths of about 30 meters (adults may be found up to 150 meters deep). The species is mainly carnivorous (molluscs, crustaceans and fish), but also herbivorous. As regards it reproductive biology, this species is a protandrous hermaphrodite, with the majority of individuals being functional males in the first two years (20-30 cm) and then turn into females (33-40 cm). Spawning typically occurs from December to April (Cataudella et al., 1995).

Although the UN Food and Agriculture Organization aquaculture statistics compiles data for about 20 species, more than 75% of the production is referred to just two species, the gilthead seabream (*Sparus aurata*, 136000 tonnes) cultured mainly in the Mediterranean region (Figure 6), and the red sea bream (*Pagrus major*) cultured in the Asia-Pacific region.

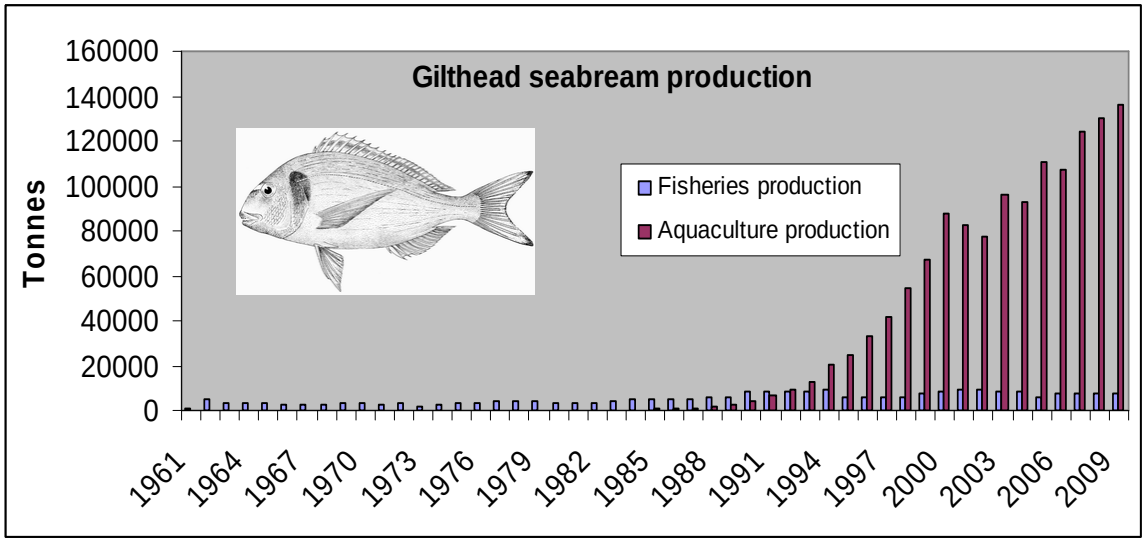


Figure 6: Global production of gilthead sea bream (FAO, 2010)

When analyzing the evolution of gilthead sea bream production, the first FAO production statistics recorded for gilthead sea bream are those from Italy in 1970 with 10 tonnes. Ten years later, in 1980, eight countries reported production outputs for a total of 775 tonnes. Since then production grew rapidly and in 2009 statistics include more than 20 countries, with Greece being the major producer and Spain being the third. The main reason for the slow development of the gilthead sea bream industry at the early stages was the initial difficulty in the production of large quantities of quality juveniles. However, the establishment of better hatchery techniques in the 80 - 90s, including adequate broodstock management, larval culture, and feeding, proper hygiene measures and enabled the supply of the required juveniles (Basurco et al., 2011).

The availability of controlled reproduction techniques for gilthead sea bream (*Sparus aurata*), as well as for European sea bass (*Dicentrarchus labrax*), generated a production scheme based on a reliable and consistent supply of juveniles, and it opened the door towards the industrialization of marine aquaculture in the Mediterranean region (Basurco et al., 2011).

Hatcheries have evolved and increased their production capacity as the demand for seed has expanded over the years. Thus, in the early days hatcheries producing yearly outputs of 1 million juveniles were considered to be large, while currently the production of 10 million or more juveniles from a single facility is the norm. At present, about 100 commercial hatcheries are in operation in Europe, with production capacities ranging from 5 million to 20 million fingerlings or more (Basurco et al, 2011).

The total gilthead sea bream harvest in 2009 was 144,130 tonnes, with about 94% coming from aquaculture (Figure 6) and the remaining 6% (8,600 tonnes) coming from capture fisheries from Mediterranean countries (FAO, 2010).

Most of the life cycle takes place in sea cages at average densities of 15-25 $kg \cdot m^{-3}$ with a food conversion ratio (FCR) of 1.5-2. The culture period varies with location and water temperature, but usually it takes between 18 and 24 months for a specimen to reach 400 g from hatched larvae. Commercial size can vary from 250 g to 1.5 kg (APROMAR, 2011).

Gilthead sea bream eggs are produced in land-based hatcheries from selected broodstock of various age groups, from 1-year old male to 10-year old females. A single female can produce more than 1 million eggs in a reproductive season and the normal fertilization rate is 90-95% (Sola et al., 2007). Larval may be cultured under controlled conditions in small (2-6 meters) circular tanks. Between 3 and 4 days post hatching, larvae start exogenous feeding and are offered live food (rotifers *Brachionus plicatilis*, followed by *Artemia* spp.) and then commercial dry food until they complete metamorphosis. Larval rearing is explained with more detail in the next section.

1.5. Larval rearing

Fish larvae is a transitional life form that develops from the fertilized egg through various embryonic stages with yolk as its only nutrient and energy supply to the stage of metamorphosis through exogenous feeding phase in which it is capable of detect, capture and digest live prey. In nature, survival and success of fish larvae depend mainly on food supply and the avoidance of predators, with only a few larvae surviving through metamorphosis to become juvenile fish that can be recruited into the fish stock. All organs and biological systems that develop during the embryonic and larval stages and how these systems are established during early development will influence how the fish performs later in life. As opposed to farm animals, in which the most sensitive life stages occur inside the mother in a constant environment and with a steady supply of nutrition, fish in their early stages directly contend with a fluctuating, harsh environment.

Under culture conditions, the success rate for fish larvae is much higher than in nature due to regulated food supply and absence of predators, but even under such conditions the mortality rate is high and can vary greatly between different batches.

Therefore, aquaculture and especially intensive and hyperintensive cultures need a strict control of the parameters of the rearing, trying to avoid fast or big changes in temperature, pH, oxygen and water currents.

Larval rearing of sea bream (*Sparus aurata*) is performed using several methodologies ranging from extensive to hyperintensive in presence or absence of phytoplankton. Divanach et al. (1999) used a scheme to classify European larviculture techniques for sea bream (Figure 7), incorporating measures of tank volume, larval stocking density, source of microalgae and prey (endogenous/exogenous) and nature of water supply (open/closed circuit). According to this scheme, the "intensity" of the rearing method is based entirely on larval stocking density and tank volume. Within each intensity category, there can be variation in water source, extent of trophic autonomy, prey type and presence/absence of phytoplankton. Although the last decade has seen an increase in the production of juveniles, this is not due to a total control of the intensive technologies applied but rather as a result of repeated applications of rearing and to progress on the control of reproduction (Divanach et al., 1999).

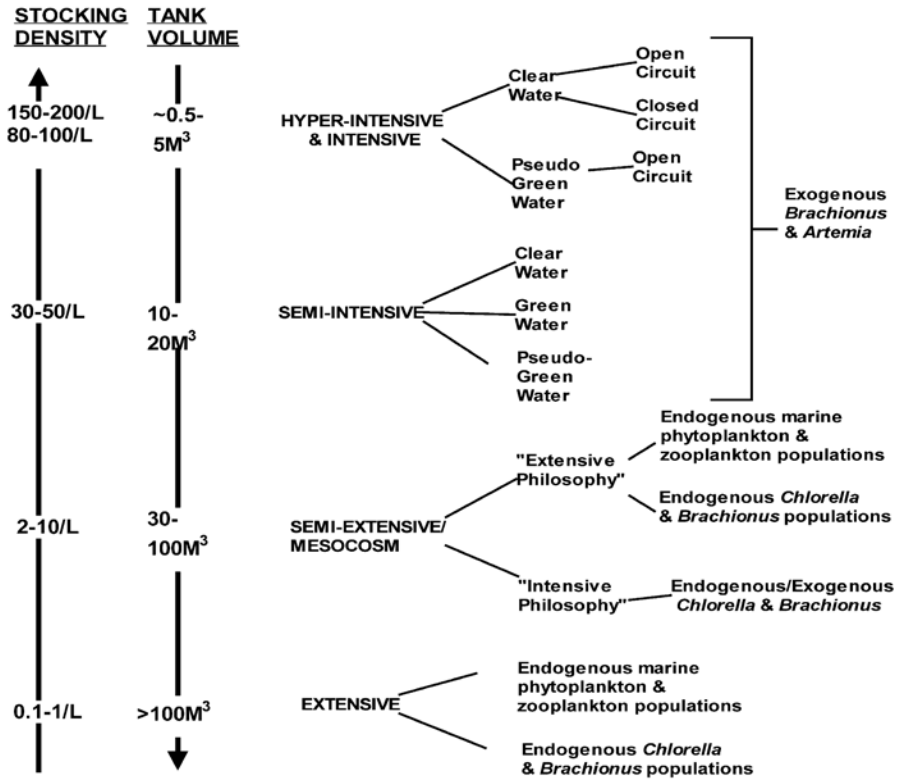


Figure 7: Classification of larviculture methods for gilthead sea bream (Divanach et al., 1999)

With the extensive methodology, high quality fry can be produced but just at low densities (Figure 7) and this is a problem for the industrial development. On the other hand are the two intensive rearing techniques widely applied: “clear water” and “green water”, both have some constraints. The “clear water” technique (Coves and Gaset, 1993; Kentouri et al., 1993) is characterized by high mortality at the early stages of development, skeletal deformities associated with the absence of swim-bladder, unconformity with wild fish (Divanach et al., 1996), low growth rate and unbalanced size distribution of the population. The “green water” technique is based on the establishment of optimum conditions for endogenous phytoplankton bloom of specific organisms (Saroglia et al., 1989); its problems are the slow and season-dependent development of the endogenous food chain deterioration that can lead to a complete failure and larval mortality (Divanach and Kentouri, 1983; Dhert et al., 1998).

The pseudo-green water methodology (Papandroulakis et al., 2001) is characterized by daily addition of microalgae and zooplankton to the larval rearing tanks (Figure 7) to keep the desired concentrations in the tanks. With this methodology, microalgae and live prey are produced in separate facilities and not in the larval rearing tanks as in the green water technique, in this way the maintenance and the quality of the food chain could be guaranteed.

In finfish mariculture, seed production and larval rearing still is one of the major bottlenecks for the industrial development with high mortality rates that can vary greatly between batches. The control of the environment, particularly the bacterial one, seems to be of particular importance during the rearing of the early developmental stages (Rekecki et al., 2009). The addition of microalgae to the system has been shown to improve the survival in some species (Naas et al., 1992; Hernández-Cruz et al., 1994; Gulbrandsen et al., 1996; Cahu et al., 1998; Lazo et al., 2000; Al-Abdul-Elah et al., 2001; Papandroulakis et al., 2001; Skiftesvik et al., 2003; Faulk and Holt, 2005; Makridis et al., 2009). In sea bream, the use of phytoplankton is a pre-request for successful larvae rearing improving the survival and accelerating the development. The precise mechanism remains unknown, the main hypothesis are explained in the next section.

1.6. The use of microalgae in larval rearing

There is a growing international body of evidence for significant advantages from adding phytoplankton to larval fish rearing systems (Hernandez-Cruz et al., 1994; Liao et al., 2001; Papandroulakis et al., 2001, 2002; Faulk and Holt, 2005). The most recent hypothesis for the better performance of fish larvae reared under controlled green-water systems are that microalgae:

- provide a direct and indirect nutritional value for larvae
- act as chemical and digestive stimulants
- enhance the non-specific immune system
- enhance environmental conditions for feeding from increased turbidity, light scattering and visual contrast enhancement
- improve water quality due to stripping of nitrogenous substances and increased oxygenation rates
- possess antimicrobial and antiviral properties
- possess detoxifying properties (Palmer et al., 2007; Makridis et al., 2009)

Some of these hypotheses are presented in more detail below.

1.6.1 Nutritional value:

Marine fishes are unable to convert shorter chain fatty acids such as linolenic acid (18:3n-3) and linoleic acid (18:2n-6) to longer chain highly unsaturated fatty acids (HUFAs) due to low activity of the necessary enzymes, thus making it necessary to provide these fatty acids through the diet (Mourente and Tocher, 1993; Ghioni et al., 1999). As rotifers and *Artemia* are naturally deficient in HUFAs it is necessary to enrich these live feeds with essential fatty acids prior to offering them to the larvae (Sargent et al., 1997). Marine microalgae are widely used as enrichment in first-feeding of marine fish larvae. With the enrichment with microalgae, the protein level can be improved as well (Reitan et al., 1997).

The algae are also added to the larval tanks in order to modify and stabilize the nutritional quality of the rotifers in the period before they are consumed by the larvae (Reitan et al., 1993, 1997). Reitan et al. (1993) found that the nutritional quality of

rotifers decreased more rapidly in “clear water” than in “green water” culture systems and that, over time, the fatty acid profile of the rotifers began to reflect that of the microalgae added to the systems. Without any algae present, the rotifers may starve and lose both their lipid content and individual protein weight, which may be important for their use in first-feeding of marine fish larvae (Reitan et al., 1997; Makridis and Olsen, 1999).

Moreover, the ingestion of unicellular algae by some marine fish larvae is well documented (Moffat, 1981; Van der Meeren, 1991; Holmefjord et al., 1993; Reitan et al., 1991, 1993, 1994, 1998; Tytler et al., 1997). So, a direct nutritional value for fish larvae is also possible. Additionally, it has been suggested that algae, by releasing attractant compounds, may stimulate the appetite of larvae (Stottrup et al., 1995).

In short, the algae may act as a food source for the early stages of fish larvae and also for the live feed, the rotifers, maintaining their nutritional value in the larval rearing tanks.

1.6.2 Immune system:

Several studies have demonstrated that the specific immune system in marine fish develops several weeks post hatching and therefore at the very early stages, fish are solely based in the non-specific immune system. Microalgae have also possible immunostimulant effect, as their incorporation in the diet increased disease resistance as shown in other studies (Austin et al., 1992; Makridis et al., 2009). The mechanism is probably related to the active uptake of polysaccharides present in algal cell walls by the fish larvae stimulating the non specific immune system (Lavens and Sorgeloos, 1996).

1.6.3 Light conditions and feeding behaviour:

Most fish species depend on vision in their search for prey. In the family Sparidae, the normal development of the visual system is essential for successful prey capture and predator avoidance, leading to increased larval growth and survival (Roo et al., 1999). Visual ability or visual range of fish has been measured in various ways by the use of psychophysical (Anthony, 1981a) and behavioural studies (Confer et al., 1978; Gregory and Northcote, 1993). In most instances visual range is manifested through behaviour. A common measure is the reaction distance, which is the distance at which an animal

reacts to an object in its environment. The probability of prey detection in fish is proportional to reaction distance (Confer and Blades, 1975). Light intensity, spectral quality and turbidity are known to affect larval feeding capabilities by altering prey search behaviour, reaction distances and ultimately feeding success (Huse, 1994; Link and Edsall, 1996; Utne-Palm, 1999; Cobcroft et al., 2001).

Visibility of a prey depends upon the ability of the fish to detect contrast between prey and background. Contrast may be detected by fish in the form of brightness or colour. The relative importance of colour and brightness contrast is determined by the fish's visual pigments, the reflectance characteristics of the prey, the radiance level and the spectral distribution of the ambient light (Anthony, 1981b) and the visual sensitivity to these properties (Lythgoe, 1968; Douglas and Hawryshyn, 1990). Spectral composition of a fish's cone pigments is often related to the water colour at ambient depth and the visual tasks of the species (Lythgoe, 1980, 1984).

The use of algal cell-induced turbidity in larval culture has been shown to improve visual feeding responses of larvae, including the timing of first-feeding, the proportion of larvae feeding and feeding intensity (Naas et al., 1992; Reitan et al., 1997; Cahu et al., 1998; Lazo et al., 2000; Al-Abdul-Elah et al., 2001; Skiftesvik et al., 2003; Faulk and Holt, 2005; Rocha et al., 2008). The hypothesis is that suspended particles a) enhance visual contrast and b) scatters and disperse light allowing larvae to better visualize the prey. In fact, in turbid media, a high level of scattered lights occurs where particle size is greater than the light wavelength, light intensity is reduced with depth and spectral quality may also be altered (Lythgoe, 1988). Some authors have demonstrated that the feeding performance of fishes is adversely affected in turbid environments and suggested that this is the general case (Benfield and Minello, 1996; Utne, 1997; Carton 2005). However, other studies have produced conflicting results; Boehlert and Morgan (1985) found an enhanced feeding rate at moderate to high turbidities (500-1000 ppm) in a study of larval pacific herring *Clupea harengus pallasi*. Miner and Stein (1993) found the same increase in feeding rate in a study of larval bluegill *Lepomis macrochirus*. One explanation for these findings is the physical effect hypothesis, that turbidity increases the contrast between the prey and its surroundings and thereby increases the ability of the fish to detect the prey (Boehlert and Morgan, 1985; Miner and Stein, 1993; Utne, 1997).

In turbid water, light scattering will function to illuminate the preys from all directions, decreasing apparent transparency and increasing contrast by increasing

backlighting (Boehlert and Morgan, 1985). Increased visual contrast will therefore result in enhanced prey detection at close range in intermediate turbid waters.

It is also possible that the visibility of the prey could be improved by algal pigments in the guts (Naas et al., 1992). Dendrinos et al. (1984) observed increased feeding efficiency of sole larvae when *Artemia* was stained with colours which contrasted with the background.

Otherwise, the presence of algae in the rearing tanks stimulated feeding behaviour in red drum larvae (Lazo et al., 2000). Many compunds such as betaine, inosine 5-monophosphate and amino acids have been shown to stimulate feeding in fish (Metailler et al., 1983; Mearns, 1986; Knutsen, 1992; Kolkovski et al., 1997; Rocha et al., 2008) and are natural constituents of phytoplankton and/or zooplankton. It is of particular interest to note that β-dimethylsulfoniopropionate (DMSP) is a naturally occurring analogue to betaine, which many marine microalgae utilize as osmolyte (Kiene et al., 1998). Since betaine is considered to be one of the most effective feeding stimulants, DMSP might act as an agonist to betaine in the chemical stimulation of feeding behaviour in fish (Lazo et al., 2000). Microalgae contain significant fractions of compounds such as soluble polypeptides, free amino acids, and polyamines which are commonly considered chemoattractants in larval feeds (Hamana and Niitsu, 2006).

1.6.4 Water quality:

The presence of microalgae in the larval tanks has positive effects on the water quality in the rearing system by removing metabolic by-products excreted by the fishes and producing oxygen (Houde 1975, 1978; Naas et al., 1992).

1.6.5 Microbial and viral community:

Phytoplankton may reduce the microbial load and the number of pathogenic bacteria. Antimicrobial activity has been detected in extracts of microalgae (Duff and Bruce 1966; Austin and Day 1990; Austin et al., 1992; Tendencia and de la Peña 2003; Kokou et al., 2011) and in bacteria isolated from microalgae (Makridis et al., 2006). This antimicrobial activity can be caused by a) associated microbiota (Makridis et al., 2006), b) antimicrobial proteins or fatty acids produced by the microalgae cells (Kokou et al., 2007) or c) free oxygen radicals produced due to the photosynthetic activity of the

microalgae cells (Marshall et al., 2005). In microalgal cultures, culturable members of the *Vibrio* group were absent or present in very low numbers (Salvesen et al. 2000; Eddy and Jones, 2002; Tendencia and de la Peña 2003; Sainz-Hernandez and Maeda-Martinez 2005; Makridis et al. 2006; Conceiçao et al., 2010)

Gut microflora in fish affects nutrition, growth, and susceptibility to disease (Conway, 1989; Nicolas et al., 1989; Hansen et al., 1992; Olsson et al., 1992; Gatesoupe, 1993; Vadstein et al., 1993; Ringo et al., 1995). Many bacterial diseases of marine fish larvae probably begin in the gut from normal flora or opportunistic pathogens and are more likely after consumption of infected food organisms (Muroga et al., 1987; Tanasomwang and Muroga, 1988; Conway, 1989; Nicolas et al., 1989; Hansen et al., 1992; Olsson et al., 1992; Gatesoupe, 1993; Vadstein et al., 1993; Ringo et al., 1995). The larval gut flora may be dependent on the bacterial flora both of the water and of the prey (Rogers et al., 1980; Nicolas et al., 1989); bacteria ingested by larvae can contribute to establishment of the primary intestinal microflora. In this regard, rotifers fed on algae have a better microbial quality for the larval fish than those fed on baker's yeast. The microalgae in larval feeding tanks contributed to the establishment of an early gut microbial flora in Atlantic halibut larvae (Skjermo and Vadstein, 1993; Olsen et al., 2000). Skjermo and Vadstein (1993) found that the addition of microalgae to Atlantic halibut larval rearing tanks increased bacterial density by 45%. Selection of bacteria in the gut of larvae in green water was more active than for those in clear water, indicating that the microalgae produced substances that enhanced the ability of certain bacteria to grow in the gut (e.g., lectins, taxins). In green water, the gut flora contained a small fraction of opportunistic bacteria and consisted of mostly slow-growing bacteria. Vadstein et al. (1993) also found that matured green water had a more stable microflora than clear water (e.g., less change in bacteria with variations in organic matter).

It is reported that marine microalgae possess antimicrobial properties active against various microorganisms, including bacteria, fungi and viruses (Fabregas et al., 1999). According to this study, the antiviral effect of microalgae is due to the existence of sulphated polysaccharides, which can inhibit viral infection and/or replication. *Chlorella minutissima* baths of juvenile groupers (*Epinephelus marginatus*) infected with nodavirus resulted in zero mortality opposed to 35 % mortality of the control group and 60 % mortality of antibiotic treated group (Katharios et al., 2005). The result of this study suggests that *Chlorella minutissima* has strong antiviral properties.

1.6.6 Activation of digestive enzymes:

Microalgae can act as an exogenous source of enzymes needed for the digestion at early stages, since endogenous proteolytic capacity is considered rather low (Walford and Lam, 1993).

A triggering effect of microalgae on digestive enzyme production has been suggested (Reitan et al., 1993) but under-researched. Cahu et al. (1998) showed that algal addition affected pancreatic and intestinal digestive functions during larval development; they found an increase in trypsin activity and a strong increase in enzymatic activities of brush border membrane (earlier enterocite maturation) in larvae reared in presence of microalgae. Lazo et al. (2000) suggested that the higher trypsin and aminopeptidase activity detected in presence of algae in red drum (*Sciaenops ocellatus*) larvae may have influenced the digestion of the tested microdiet. An enhancement of trypsin has been clearly demonstrated in marine shrimp larvae fed microparticles in the presence of algae (Le Vay et al., 1993). Trypsin and chymotrypsin are digestive proteases grouped into the serine protease family, they are synthesized and secreted as inactive zymogen precursors by pancreatic cells. Trypsin and chymotrypsin are structurally very similar, although they recognise different substrates. Trypsin acts on lysine and arginine residues, while chymotrypsin acts on large hydrophobic residues such as tryptophan, tyrosine and phenylalanine.

Many compounds present in phytoplankton could potentially influence digestive enzyme activity in fish larvae. Polyamides, algae growth regulators, have been shown to stimulate cholecystokinin release in rats, which mediates the release of pancreatic enzymes (Fioramonti et al., 1994). Amino acids may increase secretion of certain hormones, such as somatostatin and bombasin, which stimulate the secretion of pancreatic enzymes (Chey, 1993; Kolkovski et al., 1997; Koven et al., 2001). Cahu and Zambonino-Infante (1995) observed increased trypsin secretion in European sea bass larvae fed a mixture of free amino acids in their diets. Koven et al. (2002) demonstrated that Atlantic herring (*Clupea harengus*) larvae responded with increased trypsin and cholecystokinin (hormone that controls the pancreatic enzyme secretion) when tube fed solutions containing bovine serum albumin and/or free amino acids compared to fish that were fed the saline carrier only.

Here we have introduced some hypothesis to explain the mechanism by which marine fish larvae reared in presence of microalgae show a better growth performance

and survival, but up to my knowledge any study investigated the effects on the endogenous antioxidant system and the oxidative stress. In the next section these issues are explained.

1.7. Oxidative stress

1.7.1 Reactive oxygen species (ROS)

Like all aerobic organisms, fish depend on oxygen presence in the environment, using it primarily for energy generation via oxidative phosphorylation. This process is associated with the reduction of oxygen molecules to water by cytochrome oxidase which uses over 90% of oxygen consumed by an organism (Storey, 1996). The generation of various by-products of oxygen metabolism that can damage biological molecules, called reactive oxygen species (ROS), is the other side of the coin. This situation of needing oxygen to live but at the same time undergoing the danger of oxidation is known as the "oxygen paradox" (Davies, 2000). The oxygen paradox derives from the chemical nature of oxygen, which in its atomic form (O) is a free radical and in its molecular form (O_2) is a free bi-radical (Davies, 2000). The valence layer of atomic oxygen contains an unpaired electron. When two oxygen atoms combine to form molecular oxygen, its electrons remain as two unpaired electrons. A radical is defined as any atom or molecule with one or more unpaired electrons. Radicals are formed by the loss or gain of an electron from a non-radical. The bi-radical nature of the oxygen molecule allows oxidation/reduction reactions.

The tetravalent reduction of oxygen, catalyzed by cytochrome oxidase at the end of the mitochondrial electron transport chain, produces water. However, as a result of electron leakage from the electron transport chain and in oxidase reactions like those catalyzed by xanthine oxidase, cytochrome P450 reductase, glucose oxidase, etc., several reactive intermediates (ROS) are produced (Winston and Di Giulio, 1991; Kelly et al., 1998). Respiratory chain has been reported to be a main intracellular source of ROS (Ramasarma, 1982; Lenaz, 1998; Fridovich, 2004), since under physiological conditions approximately 0.1% of all oxygen entering the mitochondrial electron transport chain is incompletely reduced to superoxide radical (Fridovich, 2004). Therefore, under a situation that enhances oxidative metabolism, it may be expected that an increase in both ROS generation and ROS-scavenging mechanisms occurs.

Low levels of ROS are indispensable in many biochemical processes, including intracellular messaging in the cell differentiation and cell progression or the arrest of growth, apoptosis (Ghosh and Myers, 1998), immunity (Yin et al., 1995), and defense against microorganisms (Bae et al., 1997; Lee et al., 1998). In contrast, high doses and/or inadequate removal of ROS result in oxidative stress, which may damage cellular components and tissues (Czene et al., 1997; Chopra and Wallace, 1998). Oxidative stress arises if ROS generation prevails over their degradation (Sies, 1991). ROS include superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (•OH), singlet oxygen, ozone, lipid peroxides, nitric oxide (NO) and peroxynitrite ($ONOO^-$, also classified as a reactive nitrogen species (RNS) formed by the reaction of NO and O_2^- species) (Halliwell and Gutteridge, 1999). Singlet oxygen, hydroxyl radical and $ONOO^-$ are the most relevant chemical agents in the direction induction of oxidative damage in biological systems.

ROS can modify all cellular macromolecules including proteins, lipids, and DNA. Their attack on proteins can lead to peptide bond cleavage, modifications of amino acid residues, reactions of peptides with lipids and carbohydrate oxidation products, oxidation of sulfhydryl groups, formation of carbonyl derivatives of proteins, etc. (Stadtman, 1993). Accumulation of oxidatively damaged proteins leads to inactivation of enzymes and, in turn, metabolism perturbations (Szweda and Stadtman, 1992; Cabiscol and Levine, 1995). Lipid peroxidation, specifically polyunsaturated fatty acid (PUFA) oxidation is acknowledged as being highly deleterious, resulting in damage to cellular biomembranes, particularly to those of subcellular organelles, which contain relatively large amounts of PUFA (Halliwell and Gutteridge, 1999; Matés et al., 1999). Tissue lipid PUFA content and unsaturation index are critical factors in lipid peroxidation, and as fish, particularly marine fish, tissues contain large quantities of n-3 highly unsaturated fatty acids (HUFA) (Sargent et al., 1999), they may be more at risk from peroxidative attack than are mammals (Bell and Cowey, 1985). Therefore, although HUFA are extremely important in the synthesis of biomembranes during cellular differentiation and organogenesis in fish, they also impose a significant peroxidation risk. In fish, *in vivo* lipid peroxidation caused by oxygen radicals is a principal cause of several diseases such as jaundice (Sakai et al., 1989), nutritional muscular dystrophy (Watanabe et al., 1970; Murai and Andrews, 1974) and haemolysis (Kawatsu, 1969). Most of the products produced in lipid peroxidation are toxic and mutagenic. They may form DNA adducts, giving rise to mutations and altering gene

expression (Martnett, 1999). Peroxidized membranes become rigid and change permeability and integrity. At nucleotides, DNA is one more cellular target for ROS action. ROS, especially •OH, react with nucleotides and the sugar-phosphate backbone leading to point mutations (Buxton et al., 1988).

1.7.2 Antioxidant defenses

Biological oxidation is a primitive process and, in the face of the inevitable consequences of O_2 toxicity, evolution has provided appropriate defensive strategies. The antioxidant defence system of an aerobic organism can suppress the generation of free radicals, neutralizing them or repairing the damage that they cause.

This antioxidant system is formed by a group of low-molecular weight compounds (glutathione, ascorbic and uric acid, tocopherols, etc.) and a set of enzymes with antioxidant activity. The last are superoxide dismutase (SOD) and catalase which decompose O_2^- and H_2O_2, respectively, and glutathione-dependent enzymes (glutathione peroxidase, GPx; glutathione S-transferase, GST; glutathione reductase, GR) (Halliwell and Gutteridge, 1999; Hermes-Lima, 2004). The basic biochemistry of this antioxidant system is well documented and is described below.

Antioxidant defenses play an important role in providing protection from oxidative assault during larval development and metamorphosis. These periods are highly demanding in energy and oxygen uptake, thus, the influence of a poor nutritional status or other unfavourable conditions, can enhance oxidative stress in larvae. The antioxidant defenses can be enhanced under stressful situations and during development in order to provide cell protection (Peters and Livingstone, 1996; Mourente et al., 1999a; Stephensen et al., 2000; Livingstone, 2001; Dandapat et al., 2003; Rueda-Jasso et al., 2004; Solé et al., 2004). Moreover, most of the fish species studied showed strong enzymatic changes in antioxidant defenses when their larvae changed the metabolic energy source from endogenous lipids to exogenous food (Peters and Livingstone, 1996; Mourente et al., 1999b; Rudneva, 1999; Luckenbach et al., 2003; Kalaimani et al., 2008); this phase is considered a highly energy demanding phase in the life cycle, with a high requirement for oxygen (Solé et al., 2004; Kalaimani et al., 2008). Rudneva (1999) studied the antioxidant system of some Black Sea animals in early development and found that during marine animal embryogenesis the activities of most of the examined antioxidant enzymes tended to increase in eggs and especially in hatching

larvae, while the contents of low molecular weight were decreased. High correlations between antioxidant enzyme activities, content of low molecular weight antioxidants and developmental stages of examined marine animals were established (Rudneva, 1999).

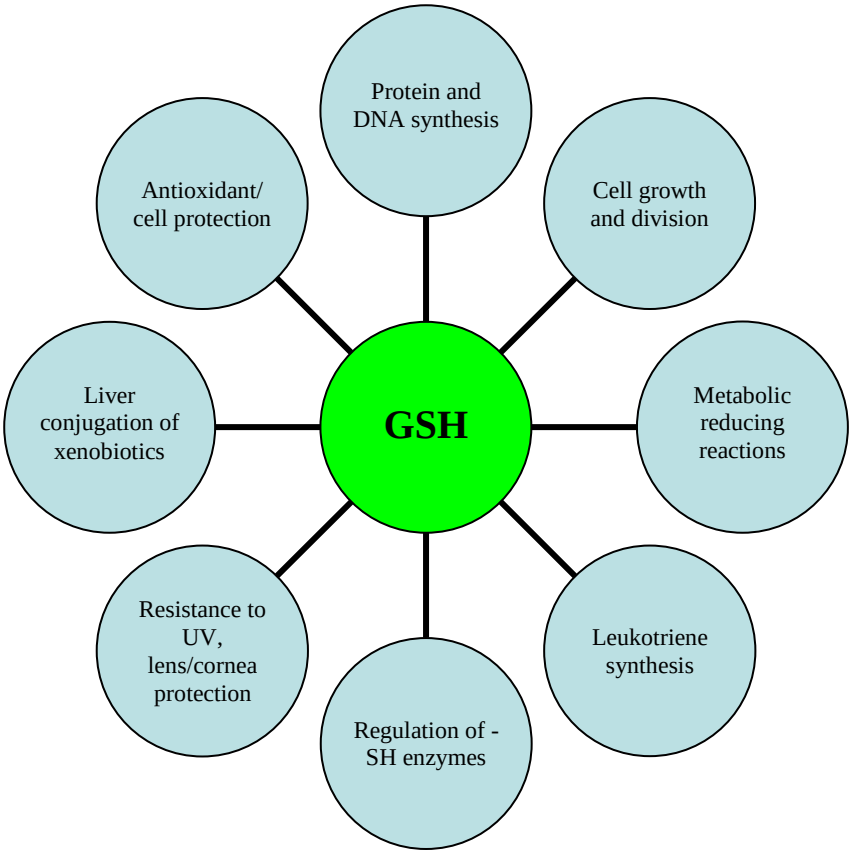


Figure 8: Cellular functions linked to the reducing power of GSH (Kidd, 1997).

Glutathione (GSH, L-γ-glutamyl-L-cysteinylglycine) is ubiquitous in nature, is a water-soluble antioxidant tripeptide found in the cytosol and the mitochondria (Halliwell and Gutteridge, 1999) of virtually all types of living cells. GSH has two characteristic structural features: a sulfhydryl (SH) group and a γ-glutamyl linkage. All the biological functions ascribed to this tripeptide are related to both of these features. Among the several important functions of GSH (Figure 8), glutathione participates in detoxification at several different levels, may scavenge free radicals, be conjugated with potentially harmful electrophilic compounds and reduce peroxides directly or in a enzyme catalyzed-reaction through the oxidation of two molecules of GSH to a molecule of glutathione disulfide (GSSG) (Kidd, 1997). Thus, GSH provides the cell

with multiple defenses not only against ROS but also against their toxic products (Hayes and McLellan, 1999). GSH acts either non enzimatically or as a cofactor for glutathione peroxidases and transferases (Figure 9). These enzymes are described below.

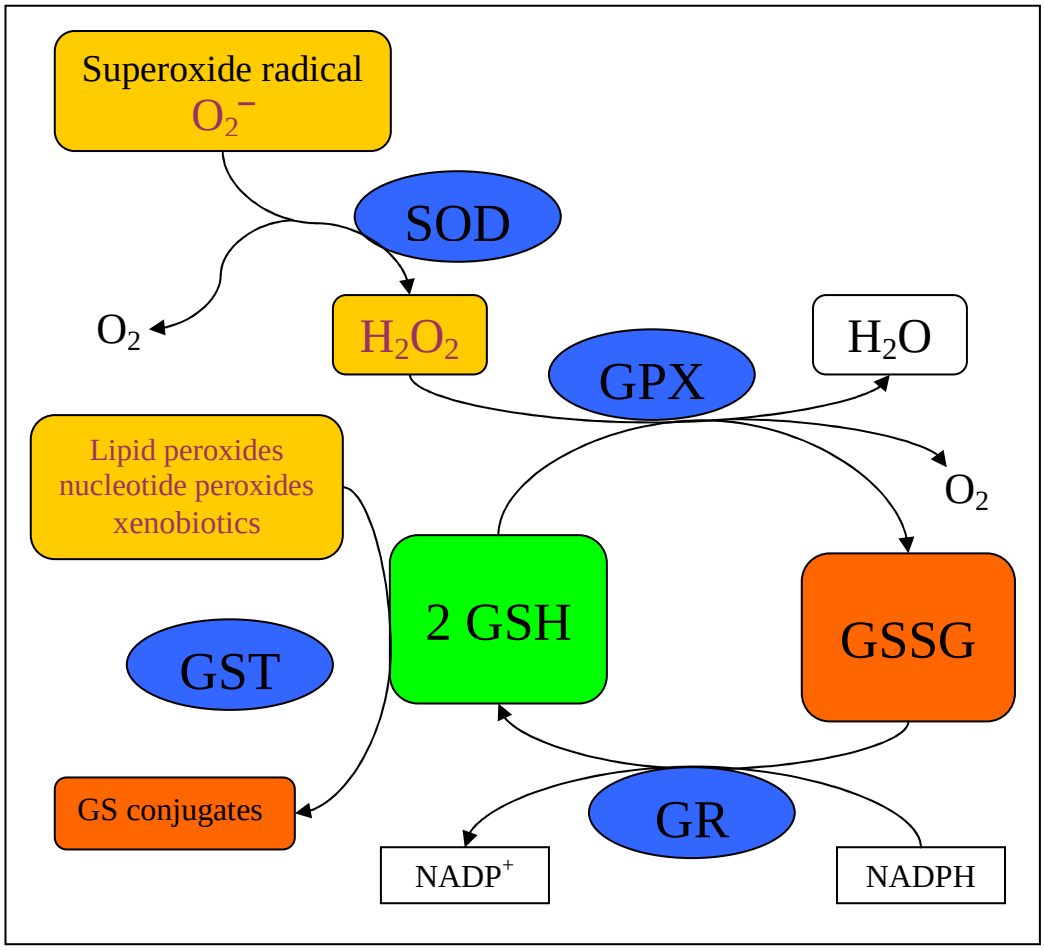


Figure 9: Summary of the pathways for the generation of ROS and actions of some enzymes involved in antioxidant defenses in the cell.

Glutathione peroxidases (GPX) catalyze the reduction of H_2O_2 generated by superoxide dismutase or from UV radiation of water, and organic hydroperoxides (lipid peroxides, nucleotide peroxides) produced by the reaction of organic molecules with ROS, to water or the corresponding alcohols (Arthur, 2000; Stephensen et al., 2002; Halliwell, 2006). This reaction needs reduced GSH (Figure 9). GPX was first discovered in 1957 as an antioxidant enzyme in erythrocytes (Mills, 1957). Later, several additional isoforms of mammalian GPX were identified, but the presence of different isoforms has not been investigated in fish (Stephensen et al., 2002).

Glutathione-S transferases (GST) conjugate GSH to electrophiles (Ketterer et al., 1983). GST also functions as an antioxidant enzyme by conjugating breakdown products of lipid peroxides to GSH (Figure 9). Some GST isozymes display peroxidase activity (Ketterer et al., 1983; Hayes and Pulford, 1995). In this reaction GSH is oxidized to GSH disulfide (GSSG). Based on the peroxidase activity displayed by michrosomal GST it was proposed that the physiological role of such enzyme could be to protect membranes from lipid peroxidation (Stephensen et al., 2002).

Glutathione reductase (GR) catalyzes the reaction by which GSSG is reduced back to GSH using NADPH (Figure 9) to maintain the reduction potential of the cell (Winston and Di Giulio, 1991; Halliwell and Gutteridge, 1999; Stephensen et al., 2002).

Superoxide dismutases (SOD) are metalloenzymes playing a key role in the defence against the toxic effects of ROS by scavenging superoxide radical (O_2^-) and converting it into hydrogen peroxide and oxygen (Figure 9).

1.7.3 Factors affecting oxidative stress

Most studies on ROS production and oxidative stress have been conducted in mammalian systems, but some of adverse effects of ROS have also been studied in aquatic organisms. Information in relation to in vivo lipid peroxidation and the endogenous antioxidant defenses either in wild or cultured fish species is still limited (Murata and Yamuchi, 1989; Sekiya et al., 1991; Sakai et al., 1992; Murata et al., 1996). In addition, most of the studies have been done in salmonids and freshwater (Bell et al., 1984, 1985b, 1987; Cowey et al., 1985; Radi et al., 1987; Roy et al., 1995; Fontagné et al., 2006; Elena-Díaz et al, 2010) with few studies in marine fish (Stéphan et al., 1995; Murata et al., 1996; Mourente et al., 2000; Tocher et al., 2002, 2003; Morales et al., 2004; Solé et al., 2004; Fernández-Díaz et al., 2006; Kalaimani et al., 2008) and less studies specifically investigating the activity of the antioxidant system in larval stages (Aceto et al., 1994; Peters et al., 1994; Peters and Livingstone, 1996; Solé et al., 2004; Fernández-Díaz et al., 2006; Kalaimani et al., 2008; Tovar-Ramírez et al., 2010).

It is reported that oxidative stress in aquatic organisms is more profound during nutritional deficiency (Mourente et al., 1999a; Avanzo et al., 2002; Hidalgo et al., 2002; Tocher et al., 2003; Morales et al., 2004), elevated temperature (Hwang and Lin, 2002), hypoxia (Kolkovski et al., 2000) and exposure to xenobiotics (Pedrajas et al., 1995;

Dandapat et al., 1999; Roméo et al., 2000; Peña-Llopis et al., 2003; Rudneva and Zalevskaya, 2004).

In recent years, oxidative stress has been deeply studied in mammals and related to a broad variety of processes, diseases, and syndromes, such as mutagenesis, cell transformation, cancer, arteriosclerosis, heart attacks, chronic inflammatory diseases, photooxidative eye stress, disorders of the central nervous system, and a wide array of age-related disfunctions, the latter leading to theories proposing ROS as the cause of ageing (Harman, 1956; Beckman and Ames, 1998; Halliwell and Gutteridge, 1999; Sohal and Orr, 2012).

1.8. Microbial control

Cultured fishes are constantly threatened by microbial attacks. Moreover, the incidence of microbial diseases has increased as has intensified aquaculture (Verpraet et al., 1992; LeBreton, 1996; Rodgers and Furones, 1998), causing major economic losses worldwide. Vibriosis and photobacteriosis are primarily diseases of marine and estuarine fish, both in natural and commercial production systems throughout the world, occurring only occasionally in freshwater fish. Vibriosis and photobacteriosis diseases can cause significant mortality in fish, reaching values of up to 100% in infected facilities, being currently responsible for the most outbreaks of fish farming plants (Almeida et al., 2009). The vibriosis and photobacteriosis are caused by bacteria from the family Vibrionaceae. Starting from 1990, European countries (especially Southern European countries) were confronted with these pathogens. Since then, several countries from the Mediterranean area have been dealing with high mortality rates in cultured populations of sea bass (*Dicentrarchus labrax*) and sea bream (*Sparus aurata*). Vibrionaceae species are also known to cause disease in humans, most often following to the consumption of contaminated aquaculture products (Almeida et al., 2009).

Diseases are more frequent in farmed fish than in the wild. Farmed fish live at greater density than wild fish which enhances the transference of pathogens between individuals. Overfeeding, high temperature and fast growth to cultivate fish as soon as possible in fish farming plants create unfavourable conditions. Moreover, overfeeding causes accumulation of organic wastes which are feed for microorganisms increasing the risk of disease outbreaks. Sick, moribund and dead fish increase also the risk of pathogens when they are not properly removed from the farming area, namely if water

renewal is low. Also, opportunistic pathogens may become more aggressive in a polluted environment (Almeida et al., 2009).

Often, when a bacterial infection appears, the first reaction is to administer antibiotics either by bathing in therapeutic solutions or by feeding with medicated feeds. The use (and especially the abuse) of antibiotics in aquaculture has been growing concern in recent years, particularly with regard to the environmental impact and food safety.

Antibiotics ending in the environment (either as uneaten medicated feed or directly) can alter the composition of the marine bacterial flora promoting the selection of antibiotic-resistant bacteria. Resistance to antibiotics can potentially be transmitted horizontally by gene transfer to terrestrial bacteria including pathogenic bacteria to humans and other animals (Cabello, 2006)

The antibiotic resistance of bacteria pathogenic to fish is also a concern for the aquaculture industry as it reduces the therapeutic value of the few antibiotics allowed for use in fish (Sorum et al., 1992; Rigos and Troisi, 2005). In addition, antibiotic residues in fish for human consumption can alter the bacterial flora of consumer and/or cause allergic or toxic reactions (Cabello, 2006).

For all these reasons it is very important to have alternative treatments to antibiotics in combating bacterial infections. Recently, progress has been made in areas such as preventive vaccination, prebiotics and probiotics and more recently in phage therapy which involves the use of virus.

Based on the new classification system proposed by Raoult and Forterre for viruses, a prokaryotic virus can be defined as a capsid-encoding organism that is composed by proteins and nucleic acids, self assembles in a nucleocapsid that uses a ribosome-encoding prokaryotic organism for the completion of its life cycle (Raoult and Forterre, 2008).

Bacteriophages are kind of virus that infect only bacteria and are usually specific to a single strain of bacteria. The phages used for therapy have a lytic cycle of life (infect the host bacteria and use its cellular machinery to replicate in mass until the bacteria is lysed and all the phage progeny goes out ready to infect other bacteria). The phages have some advantages over antibiotics: phages are self-replicating and strain-specific; they are supposed to increase in number as long as the infection persists and attack only the target bacteria and not the beneficial flora; moreover, phage therapy display limited resistance development, bacteria will certainly develop resistance to

phages too, but since phages have a higher mutation and replication rate, they can outcompete the adaptation of the bacteria and development of resistance is therefore limited (Almeida et al., 2009). Another advantage is the high resistance of phages to environmental conditions, phages are found within the same environment as their bacterial hosts, indicating the ability to survive in the same surrounding as their host bacteria (Almeida et al., 2009). The high specificity of the phages can be considered also as a disadvantage because it requires prior identification of the bacterial strain in order to select the appropriate phage to combat it. Moreover, the occurrence of side effects such as allergic reactions or toxicity is much lower in phage therapy than in antibiotic therapy (Housby and Mann, 2009).

1.9. Objectives of the study

The aim of this study was to evaluate whether the microalgae addition to the larval rearing tanks (pseudo-green water system) affect the endogenous antioxidant system and the digestive system during the first 60 dph of sea bream larvae when compared with the clear water system aiming the understanding of the underlying mechanism by which microalgae improve the performance of the larval rearing.

In addition, the effect of the addition of the bacteriophage KVP40 to the larval rearing tanks on the growth, performance, quality and survival of sea bream larvae were evaluated comparing the clear water plus phages system with the clear water system as a pilot study attempting to establish the phage therapy to the fish larval rearing systems.

2. **MATERIAL AND METHODS**:

2.1. Technology applied:

The experiment was performed in triplicates (3 tanks, one of them for sampling) for each of the tested conditions, that is the clear water technique (noted as CW), the pseudo-green water technique (noted as PGW) and clear water with the phages treatment (noted as PH).

The pseudo green water technique was realized following the method described by Papandroulakis et al. (2001). This technique is based on the frequent addition of phytoplankton and zooplankton to the larval rearing tanks. It integrates the principles of clear water and green water and minimizes some of their problems and constraints. The main difference from the classical green water is that phytoplankton is not produced in the rearing tank but its concentration remains constant through daily addition at a concentration of about $4 \pm 2 \times 10^5$ cells ml^{-1}. The pseudo green water methodology was applied only during the critical phase of the larval development (4-30 dph), while the larvae are extremely weak and sensitive to changes in the environment, easily stressed and feed with difficulty. After this time, clear water methodology was applied.

The PH treatment was realized adding the bacteriophage KVP40 which is a broad host range lytic vibriophage isolated from sea water in 1992 (Matsuzaki et al., 1992). Previously we tested that this phage can infect different vibrios isolated from the HCMR facilities. Phages were produced in mass by inoculating 1.5 litres of a liquid *Vibrio anguillarum* culture in early exponential phase and incubating at 25º C with shaking; after that the culture was centrifuged at 5,000 x *g* for 10 minutes and the supernatant was filtered through 0.2 μm filter and stored at 4º C. The produced phage was then precipitated with 10% polyethylene glycol (PEG) and then resuspended in 150 ml of sterile sea water. 50 ml of phages were added to the PH tanks just before the first live feed at 4 days post hatching, and then at days 8 and 20 after hatching; the phage concentration added (concentration in 50 ml tubes) was $1.20*10^9$; $9.10*10^9$ and $1.50*10^9$ PFU/ml respectively.

After 60 days post hatching (dph) larvae were transferred for pregrowing. Individuals from each treatment were selected in two groups according to their size and reared for an additional period of 60 days. At the end of this period, an estimation of the

larvae quality was performed in terms of developmental deformities (operculum, swim bladder and jaw) and the individual wet weight was estimated.

2.2. Rearing system:

The larval rearing trials were carried out at the intensive hatchery Unit of the Institute of Aquaculture, Hellenic Center for Marine Research.

The rearing systems comprised of 500-l cylindro-conical tanks grouped in pairs. Each pair of tanks was connected to a biofilter where water was filtered mechanically and biologically (Figure 10). The system were filled with natural seawater of 40‰ salinity and remained closed with a daily renewal rate of ~ 5% until the end of the larval rearing.

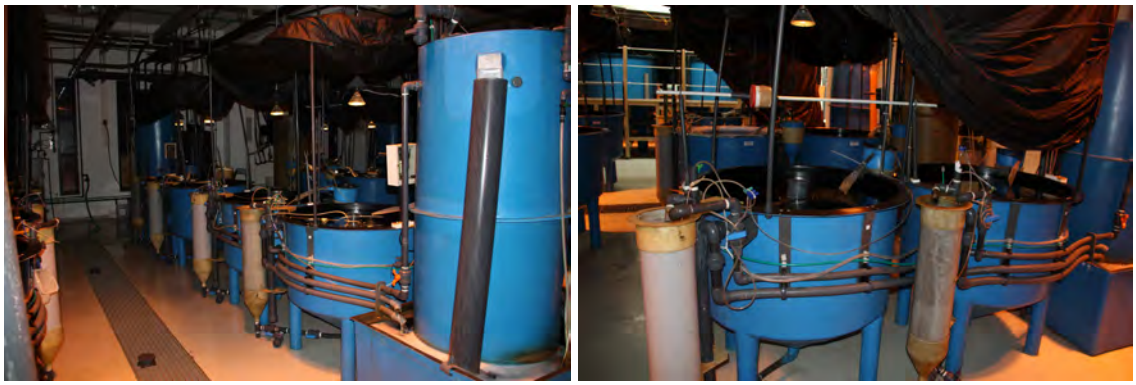


Figure 10: Larval rearing facilities at the Institute of Aquaculture (HCMR)

Water circulation was achieved in two ways according to the stage of rearing. During embryogenesis, egg hatching and the autotrophic larval stage, water circulated in the tanks through the biofilter. After first feeding, water circulation was autonomous for each tank at a rate of 10-20% per hour. A daily renewal from the biological filter was maintained at 3% daily. After 20 dph water was circulated again from the biological filter at rate of 10-15% daily and increased gradually to 100% daily at the end of the larval rearing phase. Aeration was provided in the tanks through a wooden diffuser during the autotrophic stage and was minimized or even stopped after first feeding and until the end of the experiment (due to the low biomass in the tanks, the oxygen was not a problem). A cooling system helped to maintain the temperature in the tanks at 18.3º ± 2ºC. Light was provided in each tank with a lamp with an intensity of 100-500 lux at the surface of the water. The photoperiod was established at 24 h Light –

0 h Dark since the first feeding onwards. A skimmer was installed between day 6 and 20 to keep the surface free from lipids, a requisite for good swimbladder inflation.

Feeding was performed using an automatic food distributing system composed of a peristaltic pump, stocking silos (Figure 11), one feeding tank per rearing tank and a series of electric solenoid valves operated by a computer program. With this system feeding can be executed continuously during the desired time and with the desired amount (Papandroulakis et al., 2002).

First feeding with rotifers was at 4 dph for all tanks. However, the last feeding with rotifers was at different time for each condition, when larvae reached 6.5 mm in total length (TL). First feeding with *Artemia* was also at different time for each treatment, when larvae achieved 5 mm in TL. First feeding with commercial dry food (a mix of Grow-S, 200-300 μm; and Proton 2/3, 300-500 μm) was also size-dependent and therefore was at different time for each condition (Figure 12).

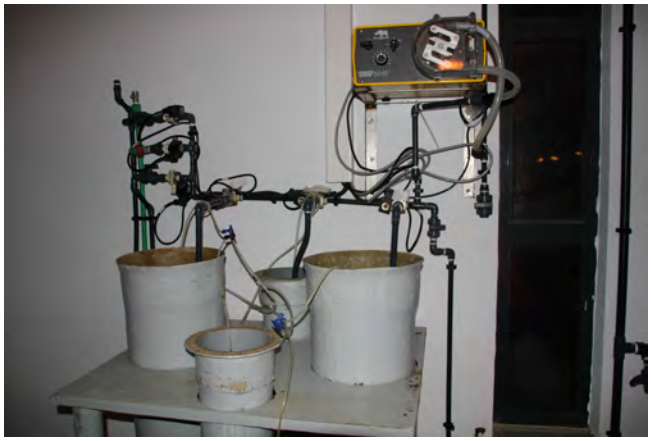


Figure 11: Automatic feeding system (stocking silos and pump)

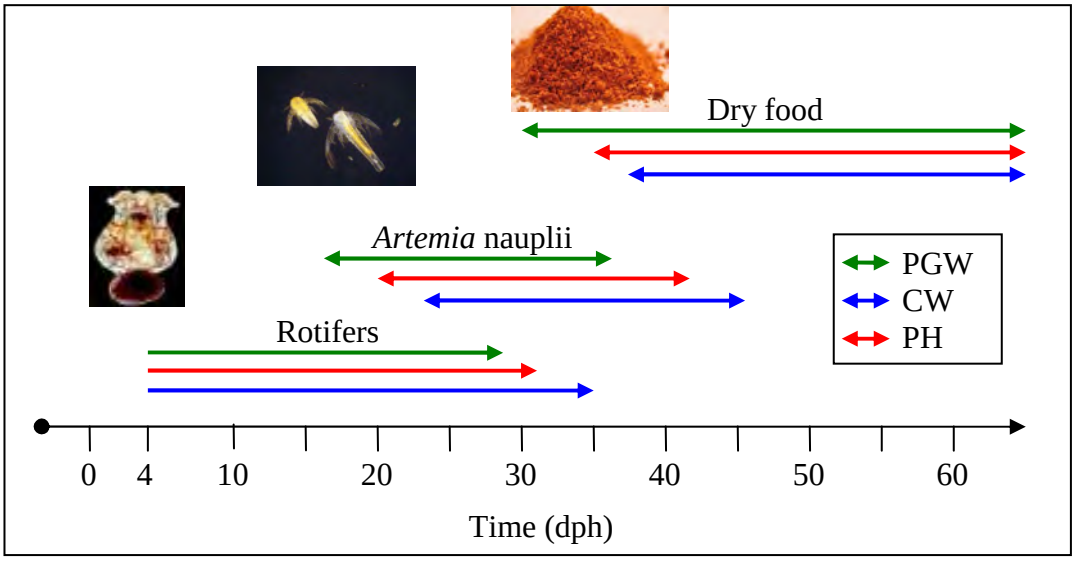


Figure 12: Larval feeding sequence for the different treatments.

2.3. Fish larvae:

Two egg batches were used. The sea bream (*Sparus aurata*) eggs from the first batch were obtained from Andromeda S.A. and the eggs from the second batch were obtained from Forkys S.A. The transport of the eggs was done in plastic bags with oxygen and seawater. Eggs were incubated in 500 l tanks at a stocking density of 94-120 eggs/l.

2.4. Auxiliary cultures:

The phytoplankton organism used was *Chlorella minutissima* (strain isolated from Heraklion bay in Crete in 1992), which was grown in photobioreactors at Institute of Marine Biology of Crete (IMBC) as semicontinuous unialgal culture (Figure 13). About 1-4 litres of phytoplankton, according to the density of the phytoplankton culture, were added daily to the pseudo-green water larval rearing tanks.

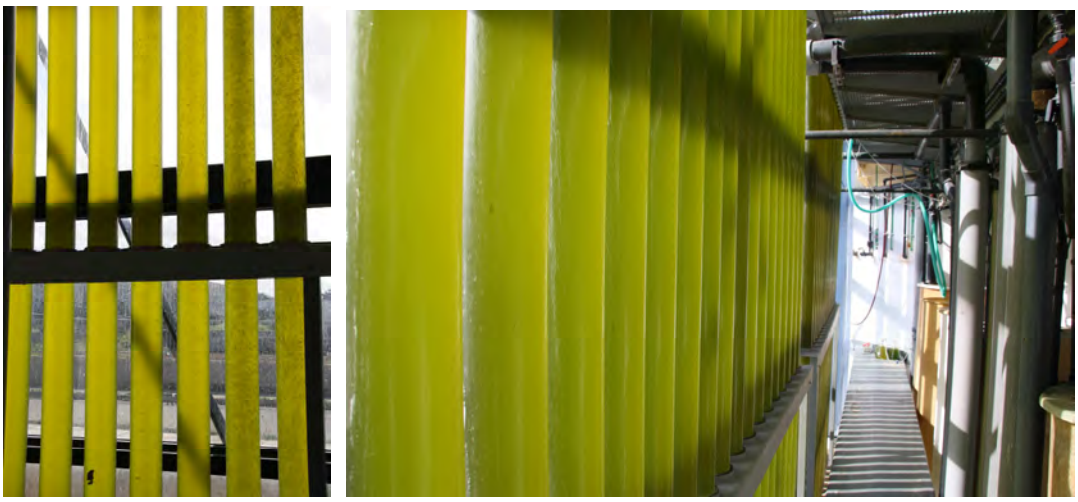


Figure 13: Photobioreactors for *Chlorella minutissima* culture

The rotifers (*Brachionus plicatilis*) were semicontinuously cultured in 25 ‰ seawater at 25ºC in 1600 litres tanks (Figure 14) with aeration. A commercial product (S. Parkle, Selco, INVE) was provided daily as food for the rotifers. Rotifers were enriched with *Chlorella minutissima* and commercial products (DHA Protein Selco, INVE) according to the specifications of the provider.

Instar II *Artemia* nauplii were produced in seawater at >25ºC with aeration. *Artemia* nauplii were also enriched with commercial products (S.presso Selco, INVE) according to the specifications of the provider.

Figure 14: Tanks with automatic feeders for the rotifer (*B. plicatilis*) culture.

2.5. Growth performance:

Samples for total length and wet weight were taken during the first 60 days post hatching to identify potential differences on the growth between treatments.

For the TL, 10 fishes per tank every 2nd day were measured under a stereo microscope Leica type MZ 125 or Olympus Optical co., Ltd., type SZH-ILLK. Deformed specimens were omitted from the data. Additionally, some characteristics (i.e. presence of food in the stomach, size of the yolk sac and oil droplet, liver condition, swimbladder inflation, notochord bending, anomalies or malformations) were recorded to have an overview of the rearing performance and to identify potential problems as soon as possible.

For the WW, 10 fishes per tank every 6 days were weighted using a precision electronic balance Mettler AT201.

In addition, samples for wet weight were taken at 120 days post hatching to test if there was a compensated growth after the experiment.

2.6. Samples for enzyme extracts and activity assays:

Whole body samples were taken on day 0, 4, 10, 20, 30, 40, 50 and 60 after hatching for biochemical analysis of the endogenous antioxidant system and the digestive enzymes trypsin and chymotrypsin. The samples were taken at specific developmental stages: recently hatched larvae were taken on 0 dph; first feeding stage of larvae was studied at 4 dph, when the mouth of the larvae opened and the larvae were fed with the rotifer *B. plicatilis*; to observe the changes in the antioxidant levels corresponding to the rotifer feeding, samples were taken on 10 dph; antioxidant status at 20 dph was observed after the inclusion of *Artemia* nauplii in the diet; additionally, samples were taken on 30, 40, 50 and 60 dph to monitor the antioxidant status during larval development and metamorphosis and to compare between treatments. For each sample, 2 grams of fish were collected, dried on paper, frozen on -80ºC, freeze-dried and stored at -80ºC until analysis. Larval extracts were assayed for the determination of some digestive (trypsin and chymotrypsin) and antioxidant (superoxide dismutase, glutathione peroxidase, glutathione reductase and glutathione S-transferase) enzyme activities and the concentration of glutathione.

2.7. Data analysis:

For comparison of the growth rate between the different conditions, regression analysis was used. The general model used was of the form $Y=a_0+a_1 \cdot t+a_2 \cdot D+a_3 \cdot t \cdot D$; where Y is the dependent variable, t the time, D a dummy variable (with values 0 and 1 for each condition tested) and a_i (i=1,2,3) constants. This method tests the hypothesis that the constants a_2 and a_3 are zero. Time series have the same slope when constant a_3 is zero and the same initial value when a_2 is zero. When both constants are zero, time series describe similar dependent variables (Sokal and Rohlf, 2012).

Biochemical data are presented as mean ± standard error (n = 3). The biochemical results were subjected to statistical evaluation with Student's *t*-test. Significant limits were set at $P<0.05$. In the figures, different letters indicate where significance lies.

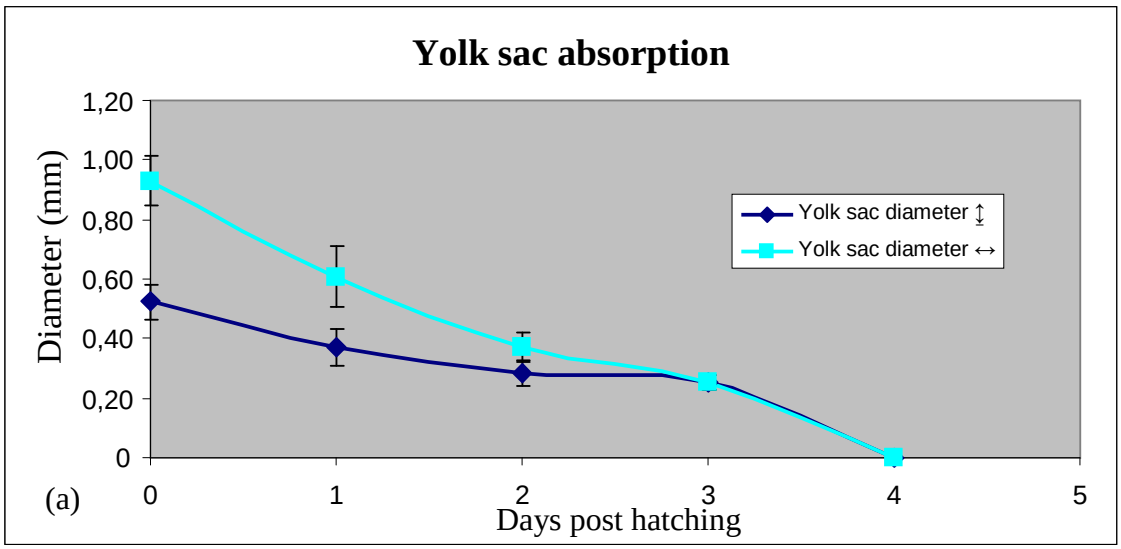
Survival values were also analyzed with Student's *t*-test, significant limits were set at $P<0.05$.

All statistics were operated by the software SPSS for Windows (v 15.0, SPSS Inc.).

3. **RESULTS**:

3.1 Growth:

The yolk sac absorption was completed at 4 dph (Figure 15a) and the oil droplet absorption was finished at 10 dph (Figure 15b).



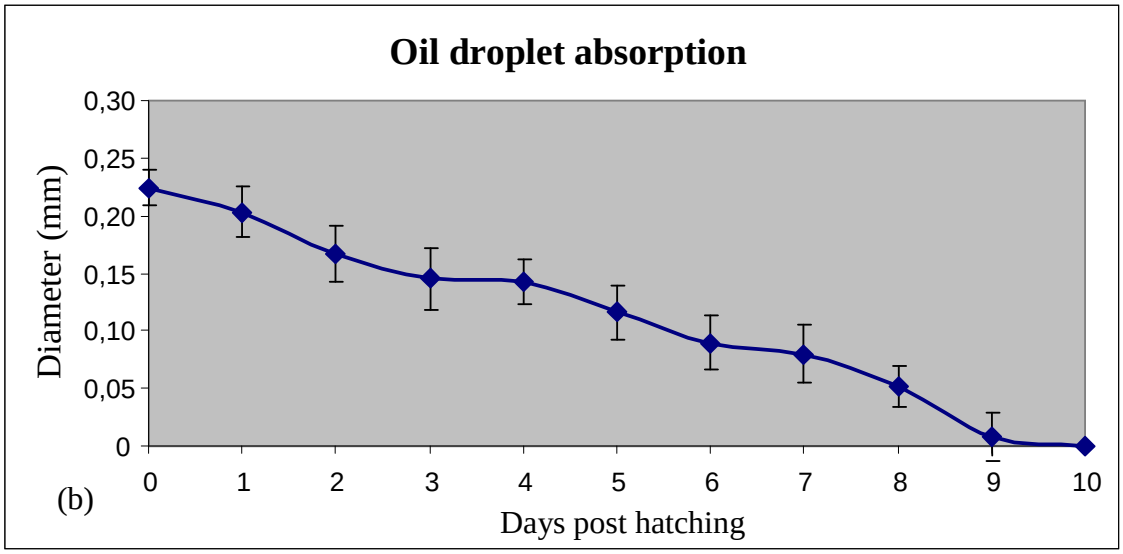


Figure 15: Evolution of the (a) yolk sac and (b) oil droplet absorption.

3.1.1 Total length:

Larvae present an exponential growth rate in all conditions tested during the experimental period. The PGW group measured 15.89 ± 2.06 mm at 60 days post hatching, while the PH and CW groups reached only 13.26 ± 1.77 mm and 13.05 ± 1.57 mm respectively at 60 days after hatching. At 17 dph, PH group was 10% smaller (4.53 ± 0.22 mm) and CW group was 19% smaller (4.10 ± 0.26 mm) than the PGW group (5.05 ± 0.32 mm). This size difference persists until the end of the experiment.

No significant differences (P>0.05) in growth rate in terms of total length were observed within similar treatments (Table I), therefore the data were pooled together to compare between treatments. Total length data were $\log_e$-transformed before the statistical analysis.

Table I: Results of the regression analysis for the comparison of growth in terms of total length within treatments (level of significance at 95%).

Compared tanks	*P* value of the coefficient $\alpha 1$	*P* value of the coefficient $\alpha 2$	*P* value of the coefficient $\alpha 3$	Difference between conditions
PGW 1-PGW 2	<0.005	0.704	0.978	Not significant
CW 1-CW 2	<0.005	0.601	0.456	Not significant
PH 1-PH 2	<0.005	0.809	0.009	Not significant

The growth performance in terms of total length in the PGW, CW and PH treatments compared in pairs is shown in Figure 16. CW treatment showed the lowest growth rate within all treatments with an exponential rate of 0.0222 mm/day, PH had an exponential rate of 0.0233 mm/day and PGW performed the best rate with an exponential rate of 0.0268 mm/day.

Significant differences in the growth rate were found when comparing the PGW treatment with the CW and the PH treatments, but no significant differences were found comparing PH with CW (Table II).

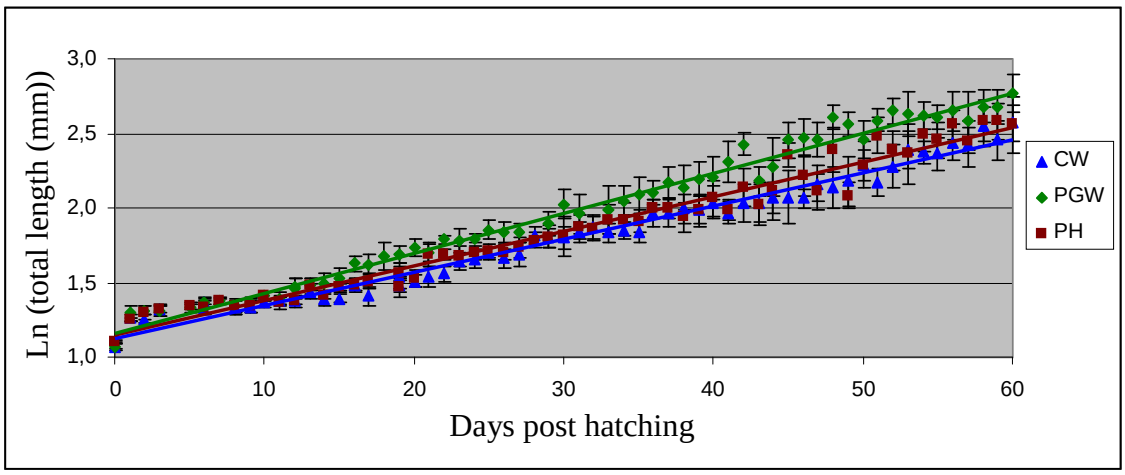


Figure 16: Growth performance in terms of total length until 60 days post hatching in the PGW, CW and PH groups (n=10; ±standard deviation).

Table II: Results of the regression analysis for the comparison of growth in terms of total length between treatments (level of significance at 95%).

Compared conditions	*P* value of the coefficient $\alpha 1$	*P* value of the coefficient $\alpha 2$	*P* value of the coefficient $\alpha 3$	Difference between conditions
PGW-CW	<0.005	0.108	<0.005	Significant
PGW-PH	<0.005	0.411	<0.005	Significant
CW-PH	<0.005	0.537	0.121	Not significant

3.1.2 Wet weight:

Larvae present an exponential growth rate in all conditions tested during the experimental period in terms of wet weight as well. The PGW group weighted 31.96 ± 18.75 mg at 60 days post hatching, while the PH and CW groups reached only 15.60 ± 7.35 mg and 14.34 ± 5.08 mg respectively. The difference in wet weight between groups appears at around day 13. At 16 dph, PH group was 46% lighter (0.243 ± 0.06 mg) and CW group was 47% lighter (0.239 ± 0.09) than the PGW group (0.452 ± 0.14 mg).

As in total length, there were no significant differences (P>0.05) in growth rate in terms of wet weight within treatments (Table III), therefore the data were pooled for statistical analysis between treatments. Weight data were $\log_e$-transformed before the statistical analysis.

Table III: Results of the regression analysis for the comparison of growth in terms of wet weight within treatments (level of significance at 95%).

Compared tanks	*P* value of the coefficient $\alpha 1$	*P* value of the coefficient $\alpha 2$	*P* value of the coefficient $\alpha 3$	Difference between conditions
PGW 1-PGW 2	<0.005	0.325	0.092	Not significant
CW 1-CW 2	<0.005	0.304	0.282	Not significant
PH 1-PH 2	<0.005	0.545	0.755	Not significant

The growth development in terms of wet weight in the PGW, CW and PH treatments is shown in Figure 17. Significant differences in growth rate in terms of wet weight were found when comparing PGW with CW and PH treatments, but no significant differences were found when comparing CW with PH treatment (Table IV).

Table IV: Results of the regression analysis for the comparison of growth in terms of wet weight between treatments (level of significance at 95%).

Compared conditions	*P* value of the coefficient $\alpha 1$	*P* value of the coefficient $\alpha 2$	*P* value of the coefficient $\alpha 3$	Difference between conditions
PGW-CW	<0.005	<0.005	<0.005	Significant
PGW-PH	<0.005	0.207	<0.005	Significant
CW-PH	<0.005	0.015	0.558	Not significant

The larvae from the PGW showed the highest growth rate with an exponential rate of 0.0975 mg/day, PH had en exponential rate of 0.0862 mg/day and CW treatment had the lowest growth rate with a rate of 0.0846 mg/day.

Table V: Average wet weight at 120 days post hatching.

	PGW	CW	PH
Big-size group	1.04±0.36 mg	0.59±0.23 mg	0.67±0.35 mg
Small-size group	0.55±0.2 mg	0.32±0.17 mg	0.42±0.19 mg

At 120 days post hatching, the difference in size was still more evident (Table V), with the larvae from PGW treatment being around 70% heavier than CW and between 55% and 30% heavier than the larvae from the PH treatment.

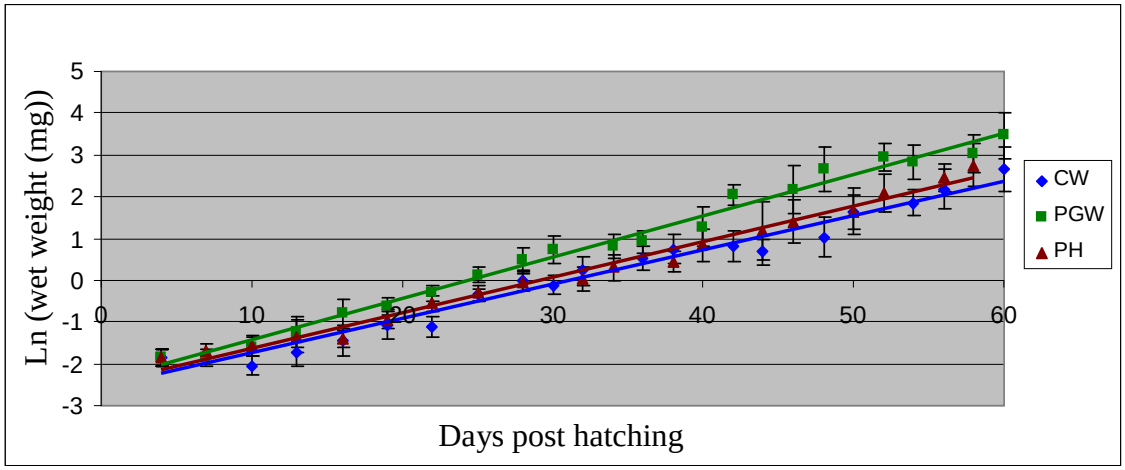


Figure 17: Growth performance in terms of wet weight until 60 days post hatching in the PGW, CW and PH groups (n=10; ±standard deviation).

3.2 Survival:

The survival at 60 days after hatching was slightly higher, but not significantly different, in the PGW treatment than in any other treatment, from a total of 116,000 eggs initially incubated in PGW, 14,481 larvae survived until 60 days post hatching (12.5% survival). This difference in survival became greater between 60 and 120 days post hatching, when 78% survival was recorded for the PGW group and the CW and PH groups displayed the same survival, 43% (Table VI).

Table VI: Survival (%) at 60 and 120 dph.

Treatment	% survival 0 - 60 dph	% survival 60 - 120 dph
PGW	12.5	78
CW	11.6	43
PH	9.5	43

3.3 Quality of the larvae:

The larvae from the PGW treatment had the best quality at the end of the experiment (120 dph) in terms of developmental deformities with only 11% of operculum deformities, 7% of swim-bladder malformations and 1% of jaw anomalies (Figure 18).

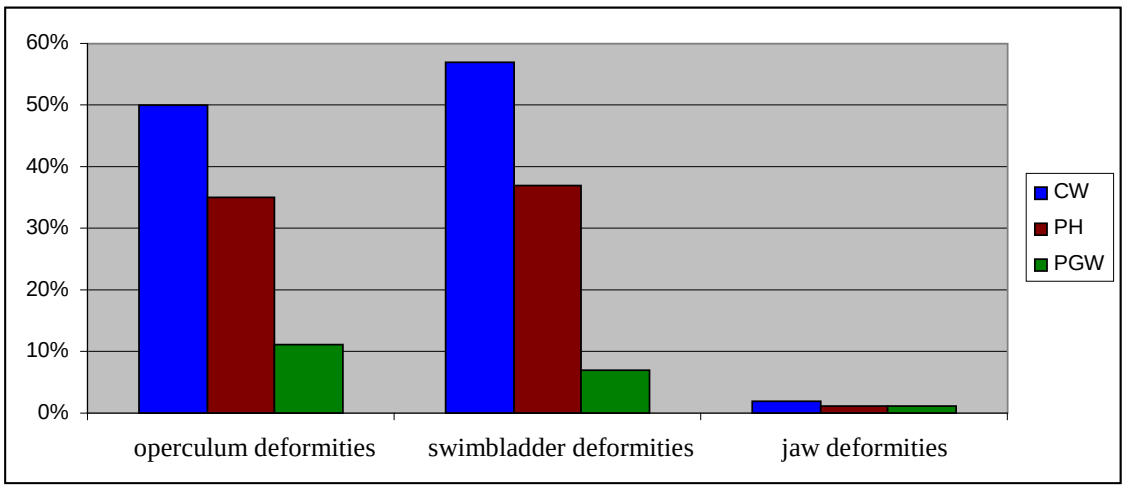


Figure 18: Estimation of larval quality at 120 days after hatching.

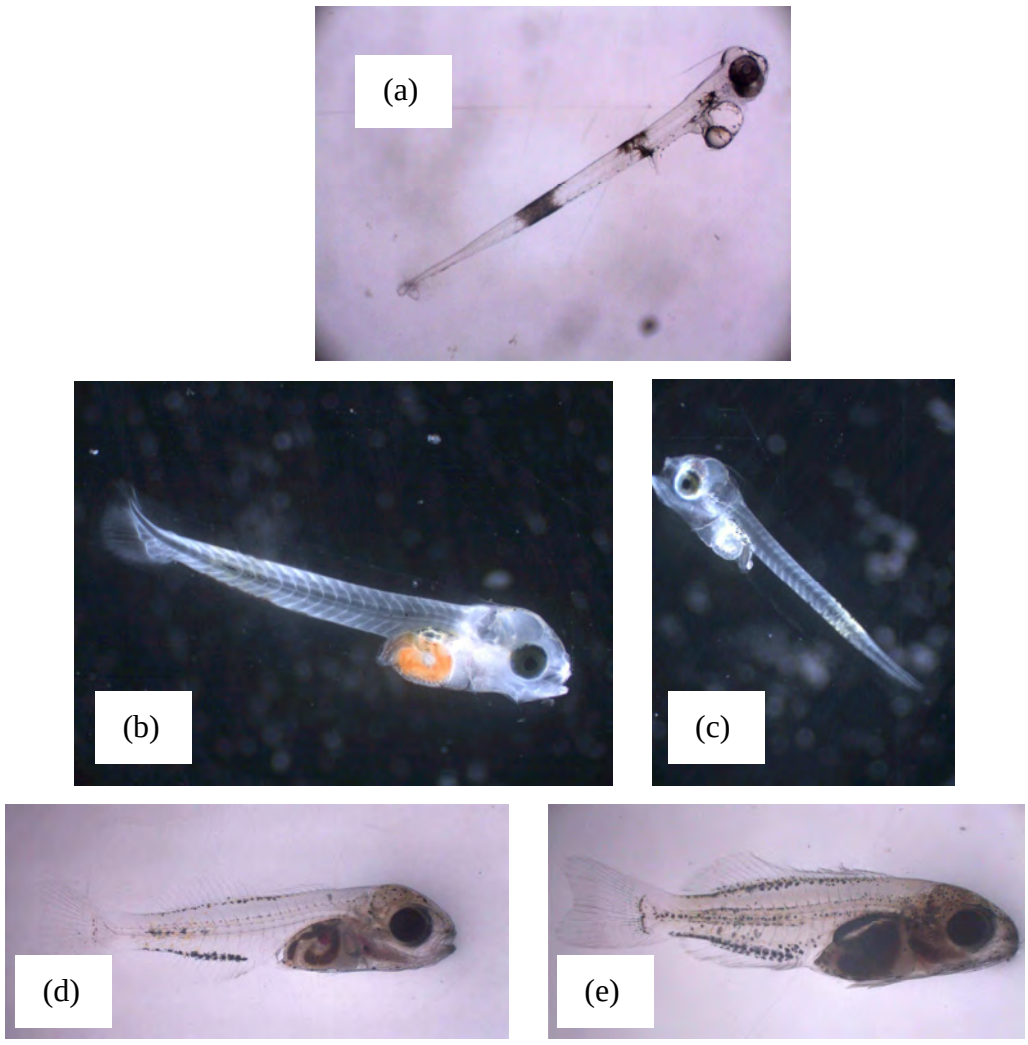


Figure 19: Photographs of sea bream larvae reared under CW and PGW conditions at different developmental stages (a) 2 dph, (b) 22 dph in PGW, (c) 22 dph in CW, (d) 56 dph in CW, (e) and 54 dph in PGW.

4. **DISCUSSION**:

Present results showed that the addition of microalgae to the rearing system enhanced the survival and improved significantly the growth performance of *S. aurata* larvae in terms of total length, wet weight and quality of the larvae, as reported for sea bream and other fish species (Naas et al., 1992; Reitan et al., 1993; Hernández-Cruz et al., 1994; Stottrup et al., 1995; Gulbrandsen et al., 1996; Cahu et al., 1998; Lazo et al., 2000; Al-Abdul-Elah et al., 2001; Papandroulakis et al., 2001; Skiftesvik et al., 2003; Faulk and Holt, 2005; Makridis et al., 2009). In addition, our experiment showed that algal addition affected both the endogenous antioxidant system and the pancreatic digestive function during larval development.

Antioxidant defences play an important role in providing protection from oxidative assault during larval development and metamorphosis. These periods are highly demanding in energy and oxygen uptake, thus, the influence of a poor nutritional status or other unfavourable conditions, can enhance oxidative stress in larvae (Peters and Livingstone, 1996; Mourente et al., 1999a; Dandapat et al., 2003; Rueda-Jasso et al., 2004; Solé et al., 2004). In our study, a significant influence of the presence of algae was detected among all the enzymes assayed.

Significant differences in terms of antioxidant specific activities were detected between the CW and the PGW treatments especially during the first month, but also differences in the activity during the second month were appreciated in some enzymes. The results of each molecule assayed are discussed below.

Contradicting results for SOD activity during early development of fish have been reported in other studies. While this enzymatic activity decreased in *Scophthalmus maximus* (Peters and Livingstone, 1996) and in *Dentex dentex* (Mourente et al., 1999a), an increase in the activity was reported during larval development in other fish species (Aceto et al., 1994; Rudneva, 1999). Nevertheless, it must be taken into account that the study with *D. dentex* corresponded only to the endogenous phase, when the fish uses the oil globule reserve and *S. maximus* is a cold climate flat-fish species with different lipid metabolic behaviour.

In this study, a significantly higher SOD activity was observed at 10 dph in the CW group; this may be a response to strong metabolic changes, such as the change from endogenous to exogenous feeding (rotifer feeding stage), as this phase is considered as a highly energy demanding phase in the larval life-cycle, with a high requirement for

oxygen (Solé et al., 2004; Fernández-Díaz et al., 2006) and the consequent need to metabolize hydrogen peroxide (H_2O_2) and organic peroxides. In the present study, larvae from the PGW treatment showed no differences in SOD specific activity between 4 and 10 dph but a significant increase at 20 dph, which corresponds with the *Artemia* feeding stage. It is important to consider that total soluble protein concentration measured in 10 days post hatch larvae was more than twice in the larvae from the PGW group (1.136 mg protein/ml) than in the larvae from the CW group (0.543 mg protein/ml) and that complicates the comparison between treatments. In fact, specific activity is the ratio activity per mg of body protein and does not reflect a lowering in digestive capacity (Ma et al., 2005).

GSH showed significantly higher concentration at hatching than at 4 dph which suggest the important role of this low molecular weight antioxidant during the egg and embryo stage against oxygen damage, as suggested in previous works (Rudneva, 1999; Mourente et al., 1999a; Kalaimani et al., 2008; Elena-Díaz et al., 2010). It is also noticeable that, except at 50 dph and at 60 dph, GSH concentration was always significantly higher in the PGW group than in the CW group, suggesting a higher capacity to cope with oxidative stress in the PGW group.

GPX activity profile in larvae from the PGW group showed a minimum at 10 dph. This may be related with the oil droplet absorption, which was completed at this stage, and the consequent reduction in lipid content, as one of the GPX functions is to reduce lipid peroxides. Nevertheless, CW group showed no statistical differences between 0, 4 and 10 dph suggesting the need to face with a higher oxidative stress in this group. After this, at 20 dph, both CW and PGW groups displayed a significant increase in GPX activity, with the activity registered in the CW group being significantly higher than the activity measured in the PGW group. This increase may be related with the adaptation to exogenous feeding; it has been observed in other studies that larvae antioxidant enzyme profiles exhibit variations from endogenous to exogenous feeding (Mourente et al., 1999a; Peters and Livingstone, 1996; Rudneva, 1999). Then, GPX specific activity decreased to low levels in both groups, but after that great differences were noticed again, as larvae from the PGW group showed a significant increase to display high values at 40 and 50 dph and decrease to low levels at 60 dph, whereas larvae from the CW group showed low levels with small but significant variations until the end of the experiment. The high values detected during the second month in the PGW group and

not in the CW group suggested the need to face with high oxidative stress and may be related with the metamorphosis stage.

GST specific activity showed in general terms a similar pattern in the CW and the PGW groups, with a significant increase at the beginning, to diminish later and increase progressively again until the end of the experiment. However, the time of these changes is different in each group, meanwhile larvae from CW group displayed the maximum at 10 dph and the minimum at 20 dph, in the PGW group the maximum was at 20 dph and the following minimum was at 30 dph. These time differences in GST activity suggest that the oxidative stress was induced at different stages in the CW and PGW groups, probably related with the time at which diet was changed.

GR activity increased gradually from 4 dph to 20 dph in the CW group and from 10 dph to 20 dph in the PGW group. This may be related with the observation of increased GPX and GST activities, since these enzymes produce oxidized glutathione (GSSG) which is reconverted to reduced GSH by the GR enzyme.

In general terms, we can highlight the changes seen in antioxidant enzymes activities at 10 dph, when endogenous reserves are finished and larvae started to feed on rotifers, and at 20 dph, when the *Artemia* feeding was initiated. Important enzymatic changes have also been reported in other fish species when the metabolic energy source in larvae changes from endogenous lipids to exogenous food (Peters and Livingstone, 1996), at this stage the general metabolic rate could be enhanced, and consequently, more damage to proteins could occur (Peters and Livingstone, 1996; Luckenbach et al., 2003). The activity profile of antioxidant enzymes during larval development under *Artemia* diet has been demonstrated to be a stage specific compensatory mechanism to neutralize peroxides (Dandapat et al., 2003; Fernández-Díaz et al., 2006).

It has been demonstrated that digestive tract and digestion process undergoes major developmental changes during the first weeks of life in fish (Govoni et al., 1986; Walford and Lam, 1993; Sarasquete et al., 1993, 1995; Moyano et al., 1996; Zambonino-Infante and Cahu, 1994, 2001; Cara et al., 2003; Yúfera et al., 2004; Falk-Petersen, 2005; Pérez-Casanova et al., 2006; Faulk et al., 2007; Lazo et al., 2007; Yúfera and Darias, 2007; He et al., 2012). Enhanced digestive capacities are suggested to improve growth and survival of fish larvae (Pedersen et al., 1990; Abi-Ayad and Kestemont, 1994; Cahu and Zambonino-Infante, 1995; Cahu et al., 1998; Lazo et al., 2000; Cara et al., 2003), since the rate of digestion in the intestinal system limits the uptake of nutrients to the blood stream and can potentially limit the growth of the whole

organism (Lemieux et al., 1999). Pancreas secretion is the first step of maturation process of digestive function (Govoni et al., 1986; Sarasquete et al., 1993; Zambonino-Infante and Cahu, 2001; Yúfera et al., 2004; Ma et al., 2005; Lazo et al., 2007; Yúfera and Darias, 2007; He et al., 2012) with trypsin and chymotrypsin being the principal enzymes involved in protein digestion at early stages, when stomach is not yet fully developed.

Cahu et al. (1998) studied the effects on digestive enzymes of algal addition in sea bass larvae; they found an increase in trypsin specific activity in the larvae reared with microalgae from day 8 to day 16 post hatching whereas chymotrypsin was not affected, and they concluded that algae improve growth performance and survival by triggering digestive enzyme production at both pancreatic and intestinal level. Lazo et al. (2000) in a similar study in 3- to 14-days post hatch red drum (*Sciaenops ocellatus*) larvae detected significantly higher trypsin activity in presence of algae.

In the present work, the effect of microalgae addition was shown also by differences in the activities of both trypsin and chymotrypsin, but the result obtained are different from those registered by Cahu et al. (1998). In this study, a significant increase in trypsin specific activity was detected from hatching until 20 days post hatching in the CW group, while the PGW group showed a decrease in trypsin specific activity between 4 and 10 days post hatching. This decrease is converted in no statistical differences if we consider the activity per larvae as used in other studies (Moyano et al., 1996; Lazo et al., 2000, 2007; Faulk et al., 2007). Indeed, this is a way to standardize the data and eliminate the possible effect of different weights derived from both treatments for the same age (Moyano, personal communication). However, the values of activity per larvae are still significantly lower than those registered for the CW group; this may be explained because of the major prey ingestion in the PGW group (data not showed) during the first weeks of life that may have contributed to digestion by addition of exogenous enzymes as suggested in other studies (Kolkovski et al., 1997; Pérez-Casanova et al., 2006) and hence diminished the need of the larvae to produce their own enzymes. A decrease in trypsin activity was detected during the second month in both CW and PGW groups with the values registered for the CW group being significantly lower than those for the PGW group. The decrease in both groups may be related with the stomach functionality, which in previous studies was established at 40 dph in *S. aurata* (Moyano et al., 1996), and the acidic proteases replacing the alkaline proteases; the significant differences in trypsin activity detected between groups during the second

month suggested a higher digestive capacity in larvae from the PGW group which agrees with the growth performance in this group.

Chymotrypsin enzyme is an alkaline protease activated by trypsin, hence a temporal correlation between trypsin and chymotrypsin activities could be expected, however the results obtained in this study did not follow this rule at least in the CW group, in which chymotrypsin specific activity showed the maximum specific activity at 40 dph coinciding with a very low trypsin activity. On the other hand, larvae from the PGW group showed a relative peak in chymotrypsin activity at 30 dph coinciding with the highest trypsin activity in this group.

A general trend have been previously found in fish larvae, with the activity of the trypsin generally increasing first, and then this activity being gradually replaced by that of chymotrypsin (Cahu et al., 1998; Lazo et al., 2007; He et al., 2012). In general terms our data supports this idea.

The ratio trypsin/chymotrypsin have been previously utilized in fish larvae as indicator of the nutritional status (Walford and Lam, 1993; Uberschär, 1995; Moyano et al., 1996; Ribeiro et al., 1999; Cara et al., 2003, 2007; Rungruangsak-Torrisen et al., 2006; Suzer et al., 2007; Cara et al., 2007; Shan et al., 2009; He et al., 2012). However, in this study such correlation between this enzymatic ratio and the nutritional status could not be made, at least during the first month, since this ratio was higher in the CW group than in the PGW group and larvae from the CW group had the worst growth performance.

A direct cause and effect relationship can not be elucidated from this study. Further and more detailed research is needed to identify the mechanism through which algae modified the production and of antioxidant and digestive enzymes, in addition of increasing growth, survival and quality of sea bream larvae. Besides, other possible effects of algae, such as an action on microbial flora, a nutritional effect or an improvement of growth by the release of a growth enhancing factor, cannot be disregarded.

Otherwise, the experiment with bacteriophages showed only slight differences in terms of growth performance (wet weight and total length), survival and quality when compared with the CW group. The results could appear disappointing; however we have to take into account the pilot approximation of the experiment. Future investigations should study the permanence of the phages in the closed system and/or the possible

application of various strains of bacteriophages grouped that may balance the high host-specificity of the viruses.

5. **CONCLUSIONS**:

1. The addition of microalgae to the rearing system enhanced the survival and improved significantly the growth performance of *S. aurata* larvae in terms of total length, wet weight, quality and survival of the larvae.

2. The addition of microalgae to the sea bream larval rearing system resulted in a clear modification of the profile of antioxidant defenses (SOD, GSH, GPX, GST and GR) during the first two months, but especially during the period of changes in the diet; when endogenous reserves are finished and larvae started to feed on rotifers and when the *Artemia* feeding was initiated.

3. Microalgae addition and developmental stage have been shown to modify the activities of both trypsin and chymotrypsin during the first 60 days post hatching.

4. The beneficial effect of adding microalgae to the sea bream larval rearing system (PGW) has been related to a decrease in the antioxidant defenses.

5. Addition of bacteriophages, (as performed) resulted in a slight effect in terms of growth performance (total length and wet weight), survival and quality when compared with the CW group.

6. Bibliography:

ABI-AYAD A. and KESTEMONT P. 1994. Comparison of the nutritional status of goldfish (*Carassius auratus*) larvae fed with live, mixed or dry diet. Aquaculture, 128, 163-173.

ACETO A., AMICARELLI F., SACCHETTA P., DRAGANI B., BUCCIARELLI T., MASCIOCCO L., MIRANDA M. and DI ILIO C. 1994. Developmental aspects of detoxifying enzymes in fish (*Salmo iridaeus*). Free Radical Research, 21, 285-294.

AKERBOOM T.P. and SIES H. 1981. Assay of glutathione, glutathione disulfide, and glutathione mixed disulfides in biological samples. Methods in Enzymology, 77, 373-382.

AL-ABDUL-ELAH K.M., ALMATAR S., ABU-REZQ T. and JAMES C.M. 2001. Development of hatchery technology for the silver pomfret *Pampus argenteus* (Euphrasen): Effect of microalgal species on larval survival. Aquaculture Research, 32, 849-860.

ALMEIDA A., CUNHA Â., GOMES N.C.M., ALVES E., COSTA L. and FAUSTINO M.A.F. 2009. Phage Therapy and Photodynamic Therapy: Low Environmanetal Impact Appraches to Inactivate Microorganisms in Fish Farming Plants. Marine Drugs, 7, 268-313.

ANTHONY P.D. 1981a. Visual contrast threshold in the cod *Gadus morhua* L. Journal of Fish Biology, 19, 87-103.

ANTHONY P.D. 1981b. Fish behaviour studies. In: Triennal Review of Research 1979-1981 (Anon ed.), pp. 23-27. Edinburgh: Department of Agriculture and Fisheries for Scotland.

ARTHUR J.R. 2000. The glutathione peroxidases. Cellular and Molecular Life Sciences,57, 1825-1835.

ASOCIACIÓN EMPRESARIAL DE PRODUCTORES DE CULTIVOS MARINOS (APROMAR). 2011. La Acuicultura Marina de Peces en España.

AUSTIN B. and DAY J.G. 1990. Inhibition of prawn pathogenic *Vibrio* spp. by a commercial spray-dried preparation of *Tetraselmis suecica*. Aquaculture, 90, 389-392.

AUSTIN B., BAUDET E. and STOBIE M. 1992. Inhibition of bacterial fish pathogens by *Tetraselmis suecica*. Journal of Fish Diseases, 15, 55-61.

AVANZO J.L., MENDONCA JUNIOR C.X.DE and CESAR M.D. 2002. Role of antioxidant system in induced nutritional pancreatic atrophy in chicken. Comparative Biochemistry and Physiology Part B, 131, 815-823.

BAE Y.S., KANG S.W., SEO M.S., BAINES I.C., TEKLE E., CHOCK P.B. and RHEE S.G. 1997. Journal of Biological Chemistry, 272, 217-221.

BASURCO B. and LOVATELLI A. 2003. The aquaculture situation in the Mediterranean Sea. Predictions for the future. Available at: http://hdl.handle.net/1834/543

BASURCO B., LOVATELLI A. and GARCÍA B. 2011. Current Status of Sparidae Aquaculture. In: M.A. Pavlidis and C.C. Mylonas (Eds.). Sparidae: Biology and Aquaculture of Gilthead Sea Bream and Other Species, First Edition. 1-50 p.

BECKMAN K.B. and AMES B.N. 1998. The free radical theory of aging matures. Physiological Reviews, 78, 547-581.

BELL J.G. and COWEY C.B. 1985. Roles of vitamin E and selenium in the prevention of pathologies related to fatty acid oxidation in salmonids. In: Cowey C.B., Mackie A.M., Bell J.G. (Eds.), Nutrition and Feeding in Fish. Academic Press, London. 333-347 p.

BELL J.G., COWEY C.B. and YOUNGSON A. 1984. Rainbow trout liver microsomal lipid peroxidation. The effect of purified glutathione peroxidase, glutathione S-transferase and other factors. Biochimica et Biophysica Acta, 795, 91-99.

BELL J.G., COWEY C.B., ADRON J.W. and SHANKS A.M. 1985. Some effects of vitamin E and selenium deprivation on tissue enzyme levels and indices of tissue peroxidation in rainbow trout (*Salmo gairdneri*). British Journal of Nutrition, 53, 149-157.

BELL J.G., COWEY C.B., ADRON J.W. and PIRIE B.J.S. 1987. Some effects of selenium deficiency on enzyme activities and indices of tissue peroxidation in Atlantic salmon parr (*Salmo salar*). Aquaculture, 65, 43-54.

BENFIELD M.C. and MINELLO T.J. 1996. Relative effects of turbidity and light intensity on reactive distance and feeding of an estuarine fish. Environmental Biology of Fishes, 46, 211-216.

BERGMEYER H.U., GAWEHN K. and GRASSL M. 1974. In: Methods of Enzymatic Analysis, Bergmeyer H.U. (Ed.), vol. 1, 2nd ed., pp 515-516, Academic Press, Inc., New York (NY).

BOEHLERT G.W. and MORGAN J.B. 1985. Turbidity enhances feeding abilities of larval Pacific herring, *Clupea harengus pallasi*. Hydrobiologia, 123, 161-170.

BRADFORD M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical biochemistry, 72, 248-254.

BUXTON G.V., GREENSTOCK C.L., HELMAN W.P. and ROSS A.B. 1988. Critical review of rate constants for reactions of hydrated electrons, hydrogen atoms and hydroxyl radicals. Journal of Physical and Chemical Reference Data, 17, 513-517.

CABELLO F.C. 2006. Heavy use of prophylactic antibiotics in aquaculture: A growing problem for human and animal health and for the environment. Environmental Microbiology, 8, 1137-1144.

CABISCOL E. and LEVINE R.L. 1995. Carbonic anhydrase: III. Oxidative modification in vivo and loss of phosphatise activity during aging. Journal of Biological Chemistry, 270, 14742-14747.

CAHU C.L. and ZAMBONINO-INFANTE J.L. 1995. Effect of the molecular form of dietary nitrogen supply in sea bass larvae: Response of pancreatic enzymes and intestinal peptidases. Fish Physiology and Biochemistry, 14, 431-437.

CAHU C.L., ZAMBONINO-INFANTE J.L., PÉRES A., QUAZUGUEL P. and LE GALL M.M. 1998. Algal addition in sea bass (*Dicentrarchus labrax*) larvae rearing: effect on digestive enzymes. Aquaculture, 161, 479-489.

CARA B., MOYANO F.J., CÁRDENAS S., FERNÁNDEZ-DÍAZ C. and YÚFERA M. 2003. Assessment of digestive enzyme activities during larval development of white bream. Journal of Fish Biology, 63, 48-58.

CARA B., MOYANO F.J., ZAMBONINO J.L. and FAUVEL C. 2007. Trypsin and chymotrypsin as indicators of nutritional status of post-weaned sea bass larvae. Journal of Fish Biology, 70, 1798-1808.

CARPENTER K.E. and NIEM V.H. 2001. FAO Species Identification Guide for Fishery Purposes. The Living Marine Resources of the Western Central Pacific, Vol. 5, 2791-3380.

CARTON A.G. 2005. The impact of light intensity and algal-induced turbidity on first-feeding *Seriola lalandi* larvae. Aquaculture Research, 36, 1588- 1594.

CATAUDELLA S., CROSETTI D. and MARINO G. 1995. The sea breams. Production of aquatic animals, 289-303. Fishes, Elsevier, Amsterdam.

CHEY W.Y. 1993. Hormonal control of pancreatic exocrine secretion. In: Go, V.L.W, Gardner, J.D., Brooks, F.P., Lebenthal, E., Di Magno, E.P., Sheele, G.A. Eds., The Pancreas; Biology, Pathology and Disease. Raven Press, New York, pp. 403-424.

CHOPRA S. and WALLACE H.M. 1998. Induction of spermidine/spermine N1-acetyltransferase in human cancer cells in response to increased production of reactive oxygen species. Biochemical Pharmacology, 55, 1119-1123.

COBCROFT J.M., PANKHURST P.M., HART P.R and BATTAGLENE S.C. 2001. The effects of light intensity and algae-induced turbidity on feeding behaviour of larval striped trumpeter. Journal of Fish Biology, 59, 1181-1197.

CONCEIÇAO L.E.C., YÚFERA M., MAKRIDIS P., MORAIS S. and DINIS M.T. 2010. Review Article. Live feeds for early stages of fish rearing. Aquaculture Research, 41, 613-640.

CONFER J.L. and BLADES P.I. 1975. Omnivorous zooplankton and planktivorous fish. Limnology and Oceanography, 20 (4), 571-579.

CONFER J.L., HOWICK G.L., CORZETTE M.H., KRAMER S.L., FITZGIBBON S. and LANDESBERG R. 1978. Visual predation by planktivores. Oikos, 31, 27-37.

CONWAY P. 1989. Lactobacilli: fact and fiction. In: The regulatory and protective role of the normal microflora. Proceeding 5th Bengt E. Gustaffson Symposium Held at the Wenner-Green Center, Stockholm, Sweden. pp. 263-281.

COVES D. and GASSET E. 1993. Gilthead seabream (*Sparus aurata*) intensive larval rearing in closed systems. In: Proceeding of the World Aquaculture ’93. European Aquaculture Society Special Publication, 19, 342.

COWEY C.B., BELL J.G., KNOX D., FRASER A. and YOUNGSON A. 1985. Lipids and antioxidant systems in developing eggs of salmon (*Salmo salar*). Lipids, 20, 567-572.

CZENE S., TIBAECK M. and HARMS–RINGDAHL M. 1997. pH-dependent DNA cleavage in permeabilized human fibroblast. Biochemical Journal, 323, 337-341.

DANDAPAT J., RAO K.J. and CHAINY G.B.N. 1999. An in vitro study of metal ion-induced lipid peroxidation in giant freshwater prawn, *Macrobrachium rosenbergii* (de Man). Biometals, 12, 89-97.

DANDAPAT J., CHAINY G.B.N and RAO K.J. 2003. Lipid peroxidation and antioxidant defence status during larval development and metamorphosis of giant prawn, *Macrobrachium rosenbergii*. Comparative Biochemistry and Physiology Part C, 135, 221–233.

DAVIES K.J.A. 2000. Oxidative stress: the paradox of aerobic life. Biochemical Society Symposia, 61, 1-31.

DENDRINOS P., DEWAN S. and THORPE J.P. 1984. Improvement in the feeding efficiency of larval, post larval and juvenile Dover sole (*Solea solea* L.) by the use of staining to improve the visibility of *Artemia* used as food. Aquaculture, 38, 137-144.

DHERT P., DIVANACH P., KENTOURI M. and SORGELOOS P. 1998. Rearing techniques of difficult marine fish larvae. World Aquaculture, 48-55.

DIVANACH P. and KENTOURI M. 1983. Influence des conditions trophiques initiales sur la résorption des réserves lipidiques, la croissance et la survie des larves de daurade *Sparus aurata* en elevage extensif. Aquaculture, 35, 43-55.

DIVANACH P., BOGLIONE C., MENU B., KOUMOUNDOUROS G., KENTOURI M. and CATAUDELLA S. 1996. Abnormalities in finfish mariculture: An overview of the problem, causes and solutions. In: Seabass and seabream culture: problems and prospects. International Workshop, Verona, Italy, October 16-18, 1996. E.A.S., Belgium, 45-66.

DIVANACH P., PAPANDROULAKIS N., KENTOURI M. 1999. Bases de techniques de l'elevage larvaire de la daurade (*Sparus aurata*). La pisciculture française, 134, 61-69.

DOUGLAS R.H. and HAWRYSHYN C.W. 1990. Behavioural studies of fish vision: an analysis of visual capabilities. In: The Visual System of Fish (Douglas, R.H. & Djamgoz, M.B.A., eds.), pp. 373-418. London: Chapman & Hall.

DUFF D.C.B., BRUCE D.L. and ANITA N.D. 1966. The antibacterial activity of marine planktonic algae. Canadian Journal of Microbiology, 12, 877-884.

EDDY S. and JONES S. 2002. Microbiology of summer flounder *Paralichtys adspersus* fingerling production at a marine fish hatchery. Aquaculture, 211, 9-28.

ELENA-DÍAZ M., FURNÉ M., TRENZADO C.E., GARCÍA-GALLEGO M., DOMEZAIN A. and SANZ A. 2010. Antioxidant defences in the first life phases of the sturgeon *Acipenser naccarii*. Aquaculture, 307, 123-129.

FABREGAS J., GARCÍA D., FERNANDEZ-ALONSO M., ROCHA A.I., GÓMEZ-PUERTAS P., ESCRIBANO J.M., OTERO A. and COLL J.M. 1999. In vitro inhibition of the replication of haemorrhagic septicaemia virus (VHSV) and African swine fever virus (ASFV) by extracts of marine microalgae. Antiviral Research, 44, 67-73.

FALK-PETERSEN I.B. 2005. Comparative organ differentiation during early life stages of marine fish. Fish and Shellfish Immunology, 19, 397-412.

FAO (Food and Agriculture Organization of the United Nations). 2005–2009. Cultured Aquatic Species Information Programme. Sparus aurata. In: FAO Fisheries and Aquaculture Department [online] (eds. F. Colloca and S. Cerasi). Rome. Available at: http://www.fao.org/fishery/culturedspecies/Sparus aurata/en

FAO (Food and Agriculture Organization of the United Nations), 2010. The State of World Fisheries and Aquaculture, 2010. FAO, Rome. 219 pp.

FAULK C.K. and HOLT G.J. 2005. Advances in rearing cobia *Rachycentron canadum* larvae in recirculating aquaculture systems: Live prey enrichment and greenwater culture. Aquaculture, 249, 231-243.

FAULK C.K., BENNINGHOFF A.D. and HOLT G.J. 2007. Ontogeny of the gastrointestinal tract and selected digestive enzymes in cobia *Rachycentron canadum* (L.). Journal of Fish Biology, 70, 567-583.

FERNÁNDEZ-DÍAZ C., KOPECKA J., CAÑAVATE J.P., SARASQUETE C. and SOLÉ M. 2006. Variations on development and stress defences in *Solea senegalensis* larvae fed on live and microencapsulated diets. Aquaculture, 251, 573-584.

FIORAMONTI J., FARGEAS M.J., BERTRAND V., PRADAYROL L. and BUENO L. 1994. Induction of postprandial intestinal motility and release of cholecystikinin by polyamines in rats. American Journal of Physiology, 267, 960-965.

FONTAGNÉ S., BAZIN D., BRÈQUE J., VACHOT C., BERNARDE C., ROUAULT T. and BERGOT P. 2006. Effects of dietary oxidized lipid and vitamin A on the early development and antioxidant status of Siberian sturgeon (*Acipenser baeri*) larvae. Aquaculture, 257, 400-411.

FRIDOVICH I. 2004. Mitochondria: are they the seat of senescence? Aging Cell, 3, 13-16.

GATESOUPE F.J. 1993. *Bacillus* sp. spores as food additive for the rotifer *Brachionus plicatilis*: improvement of their bacterial environment and their dietary value for larval turbot, *Scophthalmus maximus* L. In: Fish nutrition in practice, 4th International Symposium on Fish Nutrition and Feeding, Biarritz, France. Institut National de la Recherche Agronomique, Paris. pp. 561-568.

GHIONI C., TOCHER D.R., BELL M.V., DICK J.R. and SARGENT J.R. 1999. Low C_{18} to C_{20} fatty acid elongase activity and limited conversion of stearidonic acid, 18:4(n-3), to eicosapentanoic acid, 20:5(n-3), in a cell line from the turbot, *Scophthalmus maximus*. Biochimica et Biophysica Acta, 1437, 170-181.

GHOSH J. and MYERS E. 1998. Inhibition of arachidonate 5-lipoxygenase triggers massive apoptosis in human prostate cancer cells. Proceedings of the National Academy of Sciences of the United States of America, 22, 13182-13187

GOVONI J.J., BOEHLERT G.W. and WATANABE Y. 1986. The physiology of digestion in fish larvae. Environmental Biology of Fishes, Vol. 16, No. 1-3, 59-77.

GREGORY R.S. and NOTHCOTE T.G. 1993. Surface, planktonic, and benthic foraging by juvenile Chinook salmon (*Oncorhynchus tshawytscha*) in turbid laboratory conditions. Canadian Journal of Fisheries and Aquatic Sciences, 50, 233-240.

GULBRANDSEN J., LEIN I. and HOLMEFJORD I. 1996. Effects of light administration and algae on first feeding of Atlantic halibut larvae, *Hippoglossus hippoglossus* (L.). Aquaculture Research, 27, 101-106.

HALLIWELL B. 2006. Reactive species and antioxidants. Redox Biology is a fundamental theme of aerobic life. Plant Physiology, 141, 312-322.

HALLIWELL B. and GUTTERIDGE J.M.C. 1999. Free Radicals in Biology and Medicine. 3er ed. Clarendon Press, Oxford.

HAMANA K. and NIITSU M. 2006. Cellular polyamines of lower eukaryotes belonging to the phyla Glaucophyta, Rhodophyta, Cryptophyta, Haptophyta and Percolozoa. The Journal of General and Applied Microbiology, 52(4), 235-240.

HANSEN G.H., STRØM E. and OLAFSEN J.A. 1992. Effect of different holding regimens on the intestinal microflora of herring (*Clupea harengus*) larvae. Applied and Environmental Microbiology, 58, 461-470.

HARMAN D. 1956. A theory based on free radical and radiation chemistry. The Journals of Gerontology, 11, 298-300.

HAYES J.D. and PULFORD D.J. 1995. The glutathione S-transferase supergene family: regulation of GST and the contribution of the isoenzymes to cancer chemoprotection and drug resistance. Critical Reviews in Biochemistry and Molecular Biology, 30, 445-600.

HAYES J.D. and MCLELLAN L.I. 1999. Glutathione and glutathione-dependent enzymes represent a co-ordinately regulated defence against oxidative stress. Free Radical Research, 31, 273-300.

HE T., XIAO Z., LIU Q., MA D., XU S., XIAO Y. and LI J. 2012. Ontogeny of the digestive tract and enzymes in rock bream *Oplegnathus fasciatus* (Temminck et Schlegel 1844) larvae. Fish Physiology and Biochemistry, 38, 297-308.

HERMES-LIMA M. 2004. Oxygen in biology and biochemistry: role or free radicals. In: Storey K.B. (Ed.), Functional Metabolism: Regulation and Adaptation. Wiley-Liss, Hoboken, 319-368.

HERNÁNDEZ-CRUZ C.M., SALHI M., FERNÁNDEZ-PALACIOS H. and IZQUIERDO M.S. 1994. Improvements in the culture of *Sparus aurata* L. larvae in relation to the use of antibiotics, phytoplankton and rearing system. Aquaculture, 124, 269-274.

HIDALGO M.C., EXPÓSITO A., PALMA J.M. and HIGUERA DE LA M. 2002. Oxidative stress generated by dietary Zn-deficiency: studies in rainbow trout (*Oncorhynchus mykiss*). International Journal of Biochemistry and Cell Biology, 34, 183-193.

HOLMEFJORD I., GULBRANDSEN J., LEIN I., REFSTIE T., LÉGER P., HUSE I., HARBOE T., SORGELOOS P., OLSEN Y., BOLLA S., REITAN K.I., VADSTEIN O., OIE G. and DANIELSBERG A. 1993. An intensive approach to Atlantic halibut fry production. Journal of the World Aquaculture Society, 24, 275-284.

HOUDE E.D. 1975. Effects of stocking density and food density on survival, growth and yield of laboratory-reared larvae of sea bream *Archosargus rhomboidalis* (L.) (Sparidae). Journal of Fish Biology, 7, 115-127.

HOUDE E.D. 1978. Critical food concentrations for larvae of three species of subtropical marine fishes. Bulletin of Marine Science, 28, 395-411.

HOUSBY J.N. and MANN N.H. 2009. Phage therapy. Drug Discovery Today, 14, 536-540.

HUSE I. 1994. Feeding at different illumination levels in larvae of three marine teleost species: cod, *Gadus morhua* L., plaice, *Pleuronectes platessa* L., and turbot, *Scophthalmus maximus* L. Aquaculture and Fisheries Management, 25, 687-695.

HWANG D.F. and LIN T.S. 2002. Effects of temperature on dietary vitamin C requirement and lipid in common carp. Comparative Biochemistry and Physiology Part B, 131, 1-7.

KALAIMANI N., CHAKRAVARTHY N., SHANMUGHAM R., THIRUNAVUKKARASU A.R., ALAVANDI S.V. and SANTIAGO T.C. 2008. Anti-oxidant status in embryonic, post-hatch and larval stages of Asian seabass (*Lates calcarifer*). Fish Physiology and Biochemistry, 34, 151-158.

KATHARIOS P., PAPADAKIS I.E., PRAPAS A., DERMON C.R., AMPATZIS K. and DIVANACH P. 2005. Mortality control of viral encephalopathy and retinopathy in 0+ grouper *Epinephelus marginatus* after prolonged bath in dense *Chlorella minutissima* culture. Bulletin of the European Association of Fish Pathologists, 25 (1), 28.

KAWATSU H. 1969. Studies on the anemia of fish-III. An example of macrocytic anemia found on brook trout, *Salvelinus fontinalis*. Bulletin of Freshwater Fisheries Research Laboratory, 19, 161-167.

KELLY S.A., HAVRILLA C.M., BRADY T.C., ABRAMO K.H. and LEVIN E.D. 1998. Oxidative stress in toxicology: established mammalian and emerging piscine model systems. Environmental Health Perspectives, 106, 375-384.

KENTOURI M., DIVANACH P., PAPANDROULAKIS N. and STEFANAKIS S. 1993. Biological performance of intensively reared sea bream (*Sparus aurata*) larvae in a pilot closed system. Proceeding of the World Aquaculture ’93. European Aquaculture Special Publication, 19, 396.

KETTERER B., COLES B. and MEYER D.J. 1983. The role of glutathione in detoxication. Environmental Health Perspectives, 49, 59-69.

KIDD P.M. 1997. Glutathione: Systemic Protectant Against Oxidative and Free Radical Damage. Alternative Medicine Review, 3, 155-176.

KIENE R.P., HOFFMAN W.L.P. and WALKER J.E. 1998. Seawater microorganisms have a high affinity glycine uptake system which also recognizes dimethylsulfoniopropionate. Aquatic Microbial Ecology, 15, 39-51.

KNUTSEN J.A. 1992. Feeding behaviour of North Sea turbot *Scophthalmus maximus* and Dover sole *Solea solea* larvae elicited by chemical stimuli. Marine Biology, 113, 543-548.

KOKOU F., FERREIRA T., TSIGENOPOULOS C.S., MAKRIDIS P., KOTOULAS G., MAGOULAS A. and DIVANACH P. 2007. Identification of bacteria growing in association with bath cultures of the microalgae *Chlorella minutissima*. In: 8th International Marine Biotechnology Conference, pp. 105. Eilat, Israel.

KOKOU F., MAKRIDIS P., KENTOURI M. and DIVANACH P. 2011. Antibacterial activity in microalgae cultures. Aquaculture Research, 2011, 1-8.

KOLKOVSKI S., TANDLER A. and IZQUIERDO M.S. 1997. Effects of live food and dietary digestive enzymes on the efficiency of microdiets for seabass (*Dicentrarchus labrax*) larvae. Aquaculture, 148, 313-322.

KOLKOVSKI S., CZESNY S., YACKEY C., MOREAU R., CIHLA F., MAHAN D. and DABROWSKI K. 2000. The effect of vitamins C and E in (n-3) highly unsaturated fatty acids-enriched *Artemia* nauplii on growth, survival, and stress resistance of fresh water walleye *Stizostedion vitreum* larvae. Aquaculture Nutrition, 6, 199-206.

KOVEN W., KOLKOVSKI S., HADAS S., GAMSIZ K. and TANDLER A. 2001. Advances in the development of microdiets for gilthead seabream, *Sparus aurata*: a review. Aquaculture, 194, 107-121.

KOVEN W. 2002. Stimulatory effect of ingested protein and/or free amino acids on the secretion of the gastro-endocrine hormone cholecystokinin and on tryptic activity, in early-feeding herring larvae, *Clupea harengus*. Marine Biology, 140 (6), 1241-1247.

LAVENS P. and SORGELOOS P. 1996. Manual on the production and use of live food for aquaculture. FAO Fisheries Technical Paper No. 361. Rome, FAO. 295 p.

LAZO J.P., DINIS M.T., HOLT G.J., FAULK C. and ARNOLD C.R. 2000. Co-feeding microparticulate diets with algae: toward eliminating the need of

zooplankton at first feeding in larval red drum (*Sciaenops ocellatus*). Aquaculture, 188, 339-351.

LAZO J.P., MENDOZA R., HOLT G.J., AGUILERA C. and ARNOLD C.R. 2007. Characterization of digestive enzymes during larval development of red drum (*Sciaenops ocellatus*). Aquaculture, 265, 194-205.

LE BRETON A. 1996. An overview of the main infectious problems in cultured seabass, *Dicentrarchus labrax* and sea bream, *Sparus aurata*: solutions? In: Chatain B., Saroglia M., Sweetman J. and Lavens P. (Eds.), Seabass and Seabream Culture: Problems and Prospects. Contributions and Short Comunications Presented at an International Workshop, 16-18 October 1996, Verona, Italy. European Aquaculture Society, Oostende, Belgium, 67-86 p.

LE VAY L., RODRIGUEZ A., KAMARUDIN M.S. and JONES D.A. 1993. Influence of live and artificial diets on tissue composition and trypsin activity in *Penaeus japonicus* larvae. Aquaculture, 118, 287-297.

LEE Y.J., GALOFORO S.S., BERNS C.M., CHEN J.C., DAVIS B.H., SIM J.E., CORRY P.M. and SPITZ D.R. 1998. Glucose deprivation-induced cytotoxicity and alterations in mitogen-activated protein kinase activation are mediated by oxidative stress in multidrug-resistant human breast carcinoma cells. Journal of Biological Chemistry, 273, 5294-5299.

LEMIEUX H., BLIER P.U. and DUTIL J.D. 1999. Do digestive enzymes set a physiological limit on growth rate and food conversion efficiency in the Atlantic cod (*Gadus morhua*)? Fish Physiology and Biochemistry, 20, 293-303.

LENAZ G. 1998. Role of mitochondria in oxidative stress and aging. Biochimica et Biophysica Acta, 1366, 53-67.

LIAO I.C., SU H.M. and CHANG E.Y. 2001. Techniques in finfish larviculture in Taiwan. Aquaculture, 200, 1-31.

LINK J. and EDSALL T.A. 1996. The effect of light on Lake Herring (*Coregonus artedi*) reactive volume. Hydrobiologia, 332, 131-140.

LIVINGSTONE D.R. 2001. Contaminant reactive oxygen species production and oxidative damage in aquatic organisms. Marine Pollution Bulletin, 42, 656-666.

LUCKENBACH T., FERLING H., GERNHÖFER M., KÖHLER H.R., NEGELE R.D., PFEFFERLE E. and TRIEBSKORN R. 2003. Developmental and subcellular effects of chronic exposure to sub-lethal concentrations of ammonia,

PAH and PCP mixtures in brown trout (*Salmo truta* f. *fario* L.) early life stages. Aquatic Toxicology, 65, 39-54.

LYTHGOE J.N. 1968. Visual pigments and visual range underwater. Vision Research, 8, 997-1012.

LYTHGOE J.N. 1980. Vision in fishes: ecological adaptations. In: Environmental Physiology of Fishes (Ali, M.A. ed.), pp. 431-446.

LYTHGOE J.N. 1984. Visual pigments and environmental light. Vision Research, 24, 1539- 1550.

LYTHGOE J.N. 1988. Light and vision in the aquatic environment. In: Sensory Biology of Aquatic Animals (Atema, J., Fay, R.R., Popper A.N. & Tavolga W., eds.) pp. 57-82. Berlin: Springer Verlag.

MA H., CAHU C., ZAMBONINO-INFANTE J.L., YU H., DUAN Q., LE GALL M.M. and MAI K. 2005. Activities of selected digestive enzymes during larval development of large yellow croacker (*Pseudosciaena crocea*). Aquaculture, 245, 239-248.

MAKRIDIS P. and OLSEN Y. 1999. Protein depletion of the rotifer *Brachionus plicatilis* during starvation. Aquaculture, 174, 343-353.

MAKRIDIS P., ALVES R. and DINIS M.T. 2006. Microbial conditions and antimicrobial activity in cultures of two microalgae species, *Tetraselmis chuii* and *Chlorella minutissima*, and effect on bacterial load of enriched *Artemia* metanauplii. Aquaculture, 255, 76-81.

MAKRIDIS P., MOREIRA C., ALVES COSTA R., RODRIGUES P., DINIS M.T. 2009. Use of microalgae bioencapsulated in *Artemia* during the weaning of Senegalese sole (*Solea senegalensis* Kaup). Aquaculture, 292, 153-157.

MARNETT L.J. 1999. Lipid peroxidation-DNA damage by malondialdehyde. Mutation Research, 424, 83-95.

MARSHALL J.A., DE SALAS M. ODA T. and HALLEGRÄF G. 2005. Superoxide production by marine microalgae. I. Survey of 37 species from 6 classes. Marine Biology, 147, 533-540.

MATÉS J.M., PÉREZ-GÓMEZ C. and NÚÑEZ DE CASTRO I. 1999. Antioxidant Enzymes and Human Diseases. Clinical Biochemistry, 32, 595-603.

MATSUZAKI S., TANAKA S., KOGA T. and KAWATA T. 1992. A broad-host-range vibriophage, KVP40, isolated from sea water. Microbiology and Immunology, 36, 93-97.

MAVIS R.D. and STELLWAGEN E. 1968. Purification and Subunit Structure of Glutathione Reductase from Baker's Yeast. The Journal of Biological Chemistry, 243, 809-814.

MEARNS K.J. 1986. Sensitivity of brown trout (*Salmo trutta* L.) and Atlantic salmon (*Salmo salar* L.) fry to amino acids at the start of exogenous feeding. Aquaculture, 55, 191-200.

METAILLER R., CADENA-ROA M. and PERSON-LE RUYET J. 1983. Attractive chemical substances for the weaning of Dover sole (*Solea vulgaris*): qualitative and quantitative approach. Journal of the World Mariculture Society, 14, 679-684.

MILLS G.C. 1957. Hemoglobin catabolism. I. Glutathione peroxidase, an erythrocyte enzyme which protects haemoglobin from oxidative breakdown. The Journal of Biological Chemistry, 229, 189-197.

MINER J.G. and STEIN R.A. 1993. Interactive influence of turbidity and light on larval bluegill (*Lepomis macrochirus*) foraging. Canadian Journal of Fisheries and Aquatic Sciences, 50, 781-788.

MOFFAT N.M. 1981. Survival and growth of northern anchovy larvae on low zooplankton densities as affected by the presence of a *Chlorella* bloom. Rapp. P.-V. R&N. Cons. Bit. Explor. Mer, 178, 475-480.

MORALES A.E., PÉREZ-JIMÉNEZ A., HIDALGO M.C. ABELLÁN E. and CARDENETE G. 2004. Oxidative stress and antioxidant defenses after prolonged starvation in *Dentex dentex* liver. Comparative Biochemistry and Physiology Part C, 139, 153-161.

MOURENTE G. and TOCHER D.R. 1993. Incorporation and metabolism of ^{14}C-labelled polyunsaturated fatty acids in juvenile gilthead sea bream *Sparus aurata* L. *in vivo*. Fish Physiology and Biochemistry, 10, 443-453.

MOURENTE G., TOCHER D.R., DIAZ E., GRAU A. and PASTOR E. 1999a. Relationships between antioxidants, antioxidant enzyme activities and lipid peroxidation products during early development in *Dentex dentex* eggs and larvae. Aquaculture, 179, 309-324.

MOURENTE G., TOCHER D.R., DIAZ-SALVAGO E., GRAU A. And PASTOR E. 1999b. Study of the n-3 highly unsaturated fatty acids requirement and antioxidant status of *Dentex dentex* larvae at the *Artemia* feeding stage. Aquaculture, 179, 291-307.

MOURENTE G., DÍAZ-SALVAGO E., TOCHER D.R. and BELL J.G. 2000. Effects of dietary polyunsaturated fatty acids/vitamin E (PUFA/tocopherol) ratio on antioxidant defense mechanisms of juvenile gilthead sea bream (*Sparus aurata* L., Osteichthyes, Sparidae). Fish Physiology and Biochemistry, 23, 337-351.

MOYANO F.J., DÍAZ M., ALARCÓN F.J. and SARASQUETE M.C. 1996. Characterization of digestive enzyme activity during larval development of gilthead seabream (*Sparus aurata*). Fish Physiology and Biochemistry, Vol. 15, 2, 121-130.

MURAI T. and ANDREWS J.W. 1974. Interaction of dietary α-tocopherol, oxidized menhanden oil and ethoxyquin on channel catfish (*Ictalurus punctatus*). Journal of Nutrition, 104, 1416-1431.

MURATA H. and YAMAUCHI K. 1989. Relationships between the 2-thiobarbituric acid values of some tissues from cultured red sea bream and its dietary α-tocopherol. Nippon Suisan Gakkaishi, 55, 1435-1439.

MURATA H., SAKAI T., YAMAUCHI K., ITO T., TSUDA T., YOSHIDA T and FUKUDOME M. 1996. In vivo lipid peroxidation levels and antioxidant activities of cultured and wild yellowtail. Fisheries Science, 62, 46-68.

MUROGA K., HIGASHI M. and KEITOKU H. 1987. The isolation of intestinal microflora of farmed red seabream (*Pagrus major*) and black seabream (*Acanthopagrus schlegeli*) at larval and juvenile stages. Aquaculture, 65, 79-88.

NAAS K.E., NÆSS T. and HARBOE T. 1992. Enhanced first feeding of halibut larvae (*Hipoglossus hippoglossus* L.) in green water. Aquaculture, 105, 143-156.

NANDI A. and CHATTERJEE I.B. 1988. Assay of superoxide dismutase activity in animal tissues. Journal of Biosciences, 13, 305-315.

NELSON J.S. 2006. Fishes of the World, 4th Ed. John Wiley & Sons, Inc., New York. 601 p.

NICOLAS J.L., ROBIC E. and ANSQUER D. 1989. Bacterial flora associated with a trophic chain consisting of microalgae, rotifers and turbot larvae: Influence of bacteria on larval survival. Aquaculture, 83, 237-248.

OLSEN A.I., OLSEN Y., ATTRAMADAL Y., CHRISTIE K., BIRKBECK T.H., SKJERMO J. and VADSTEIN O. 2000. Effects of short term feeding of microalgae on the bacterial flora associated with juvenile *Artemia franciscana*. Aquaculture, 190, 11-25.

OLSSON J.C., WESTERDAHL A., CONWAY P.L. and KJELLEBERG S. 1992. Intestinal colonization potential of turbot (*Scophthalmus maximus*)- and dab (*Limanda limanda*)-associated bacteria with inhibitory effects against *Vibrio anguillarum*. Applied and Environmental Microbiology, 58, 551-556.

PALMER P.J., BURKE M., PALMER C., BURKE J. 2007. Review Article. Developments in controlled green-water larval culture technologies for estuarine fishes in Queensland, Australia and elsewhere. Aquaculture, 272, 1-21.

PAPANDROULAKIS N., DIVANACH P., ANASTASIADIS P. and KENTOURI M. 2001. The pseudo-green water technique for intensive rearing of sea bream (*Sparus aurata*) larvae. Aquaculture International, 9, 205-216.

PAPANDROULAKIS N., PAPAIOANNOU D., DIVANACH P. 2002. An automated feeding system for intensive hatcheries. Aquacultural Engineering, 26, 13-26.

PAPANDROULAKIS N., KENTOURI M., MAINGOT E. and DIVANACH P. 2004. Mesocosm: a reliable technology for larval rearing of *Diplodus puntazzo* and *Diplodus sargus sargus*. Aquaculture International, 12, 345-355.

PEDERSEN B.H., UGELSTAD I. and HJELMELAND K. 1990. Effects of a transitory, low food supply in the early life of larval herring (*Clupea harengus*) on mortality, growth and digestive capacity. Marine Biology, 107, 61-66.

PEDRAJAS J.R., PEINADO J. and LÓPEZ-BAREA J. 1995. Oxidative stress in fish exposed to model xenobiotics. Oxidatively modified forms of Cu,Zn-superoxide dismutase as potential biomarkers. Chemico-Biological Interactions, 98, 267-282.

PEÑA-LLOPIS S., DOLORES FERRANDO M. and PEÑA J.B. 2003. Fish tolerance to organophosphate-induced oxidative stress is dependent on the glutathione metabolism and enhanced by *N*-acetylcysteine. Aquatic Toxicology, 65, 337-360.

PÉREZ-CASANOVA J.C., MURRAY H.M., GALLANT J.W., ROSS N.W., DOUGLAS S.E. and JOHNSON S.C. 2006. Development of the digestive capacity in larvae of haddock (*Melanogrammus aeglefinus*) and Atlantic cod (*Gadus morhua*). Aquaculture, 251, 377-401.

PETERS L.D., PORTE C. and LIVINGSTONE D.R. 1994. 7-ethoxyresorufin O-deethylase (EROD) and antioxidant enzyme activities in larvae of sardine

(*Sardina pilchardus*) from the North coast of Spain. Marine Pollution Bulletin, 28, 299-304.

PETERS L.D. and LIVINGSTONE D.R. 1996. Antioxidant enzyme activities in embryologic and early larval stages of turbot. Journal of Fish Biology, 49, 986-997.

RADI A.A.R., MATKOVICS B. and CSENGERI I. 1987. Comparative studies of the phospholipid fatty acids and the antioxidant enzyme activities in fish with different feeding habits. Comparative Biochemistry and Physiology, 87B, 49-54.

RAMASARMA T. 1982. Generation of H_2O_2 in biomembranes. Biochimica et Biophysica Acta, 694, 69-93.

RAOULT D. and FORTERRE P. 2008. Redefining viruses: lessons from Mimivirus. Nature Reviews Microbiology, 6, 315-319.

REITAN K.I., BOLLA S. and OLSEN Y. 1991. Ingestion and assimilation of microalgae in yolk sac larvae of halibut, *Hipoglossus hippoglossus* (L.). In: Larvi '91 – Fish and Crustacean Larviculture Symposium (ed. by P. Lavens, P. Sorgeloos, E. Jaspers & F. Ollevier), pp. 332-334. European Aquaculture Society, Special Publication 15, Gent, Belgium.

REITAN K.I., RAINUZZO J.R. OIE G. and OLSEN Y. 1993. Nutritional effects of algal addition in first-feeding of turbot (*Scophthalmus maximus* L.) larvae. Aquaculture, 118, 257-275.

REITAN K.I., BOLLA S. and OLSEN Y. 1994. A study of the mechanism of algal uptake in yolk-sac larvae of Atlantic halibut (*Hippoglossus hippoglossus*). Journal of Fish Biology, 44, 303-310.

REITAN K.I., RAINUZZO J.R., OIE G. and OLSEN Y. 1997. A review of the nutritional effects of algae in marine fish larvae. Aquaculture, 155, 207-221.

REITAN K.I., NATVIK C.M. and VADSTEIN O. 1998. Drinking rate, uptake of bacteria and microalgae in turbot larvae. Journal of Fish Biology, 53, 1145-1154.

REKECKI A., DIERCKENS K., LAUREAU S., BOON N., BOSSIER P. and VAN DEN BROECK W. 2009. Effect of germ-free rearing environment on gut development of larval sea bass (*Dicentrarchus labrax* L.). Aquaculture, 293, 8-15.

RIBEIRO L., ZAMBONINO-INFANTE J.L., CAHU C., DINIS M.T. 1999. Development of digestive enzymes in larvae of *Solea senegalensis*, Kaup 1858. Aquaculture, 179, 465-473.

RIGOS G. and TROISI G.M. 2005. Antibacterial agents in Mediterranean finfish farming: A synopsis of drug pharmatokinetics in important euryhaline fish species and possible environmental implications. Reviews in Fish Biology and Fisheries, 15, 53-73.

RINGO E., STRØM E. and TABACHEK J.-A. 1995. Intestinal microflora of salmonids: a review. Aquaculture Research, 26, 773-789.

RINGO E. and BIRKBECK T.H. 1999. Intestinal microflora of fish larvae and fry. Aquaculture Research, 30, 73-93.

ROCHA R.J., RIBEIRO L., COSTA R. and DINIS M.T. 2008. Does the presence of microalgae influence fish larvae prey capture?. Aquaculture Research, 39, 362-369.

RODGERS C.J. and FURONES M.D. 1998. Disease problems in cultured marine fish in the Mediterranean. Fish Pathology, 33, 157-164.

ROGERS P.A., GLENN A.R. and POTTER I.C. 1980. The bacterial flora of the gut contents and environment of larval lampreys. Acta Zoologica, 61, 23-27.

ROMÉO M., BENNANI N., GNASSIA-BARELLI M., LAFAURIE M. and GIRARD J.P. 2000. Cadmium and copper display different responses in the kidney of the sea bass *Dicentrarchus labrax*. Aquatic Toxicology, 48, 185-194.

ROO F.J., SOCORRO J., IZQUIERDO M.S., CABALLERO M.J., HERNÁNDEZ-CRUZ C.M., FERNÁNDEZ A. and FERNÁNDEZ-PALACIOS H. 1999. Development of red porgy *Pagrus pagrus* visual system in relation with changes in the digestive tract and larval feeding habits. Aquaculture, 179, 499-512.

ROY W.J., MATTY A.J. and BELL J.G. 1995. Tissue vitamin E concentrations during smoltification and seawater transfer in farmed Atlantic salmon, *Salmo salar* L. Aquaculture Nutrition, 1, 51-57.

RUDNEVA I.I. 1999. Antioxidant system of Black Sea animals in early development. Comparative Biochemistry and Physiology Part C, 122, 265–271.

RUDNEVA I.I. and ZALEVSKAYA I.N. 2004. Larvae of sand smelts (*Atherina hepsetus* L.) as a bioindicator of pollution in the Black Sea coastal waters. Russian Journal of Ecology, 35, 86-90.

RUEDA-JASSO R., CONCEIÇAO L.E.C., DIAS J.W., DE COEN E., GOMES E., REES J.F. SOARES F., DINIS M.T. and SORGELOOS P. 2004. Effect of dietary non-protein energy levels on condition and oxidative status of Senegalese sole (*Solea senegalensis*) juveniles. Aquaculture, 231, 417-433.

RUNGRUANGSAK-TORRISEN K., MOSS R., ANDRESEN L.H., BERG A. and WAAGBØ. 2006. Fish Physiology and Biochemistry, 32, 7-23.

SAINZ-HERNÁNDEZ J.C. and MAEDA-MARTÍNEZ A.N. 2005. Sources of *Vibrio* bacteria in mollusc hatcheries and control methods: a case study. Aquaculture Research, 36, 1611-1618.

SAKAI T., MURATA H., ENDO M., YAMAUCHI K., TABATA N. And FUKUDOME M. 1989. 2-Thiobarbituric acid values and contents of α-tocopherol and bile pigments in the liver and muscle of jaundiced yellowtail *Seriola aquiqueradiata*. Agricultural Biology and Chemistry, 53, 1739-1740.

SAKAI T., MURATA H., YAMAUCHI K., SEKIYA T. and UKAWA M. 1992. Effects of dietary lipid peroxides contents on in vivo lipid peroxidation, α-tocopherol contents, and superoxide dismutase and glutathione peroxidase activities in the liver of yellowtail. Nippon Suisan Gakkaishi, 58, 1483-1486.

SALVESEN I., REITAN K.I., SKJERMO J. and OIE G. 2000. Microbial environments in marine larviculture: impacts of algal growth rates on the bacterial load in six microalgae. Aquaculture International, 8, 275-287.

SARASQUETE M.C., POLO A. and GONZÁLEZ DE CANALES M.L. 1993. A histochemical and immunohistochemical study of digestive enzymes and hormones during the larval development of the sea bream, *Sparus aurata* L. Histochemical Journal, 25, 430-437.

SARASQUETE M.C., POLO A. and YÚFERA M. 1995. Histology and histochemistry of the development of the digestive system of larval gilthead seabream, *Sparus aurata*, L. Aquaculture, 130, 79-92.

SARGENT J.R., MCEVOY L.A. and BELL J.G. 1997. Requirements, presentation and sources of polyunsaturated fatty acids in marine fish larval feeds. Aquaculture, 155, 117-127.

SARGENT J.R., BELL J.G., MCEVOY L.A., TOCHER D.R. and ESTÉVEZ A. 1999. Recent developments in the essential fatty acid nutrition of fish. Aquaculture, 177, 191-199.

SAROGLIA M., INGLE E., MARIANO G., DEL VECCHIO M.L. and PORRELLO S. 1989. Produzione commerciale di avanotti di spigola in Italia. Contributo della tecnologia acque verdi. Nova Thalassia, 10, 309-317.

SEKIYA T., MURATA H., SAKAI T., YAMAUCHI K., YAMASHITA K., UKAWA M., KANAI M. and SHIMADA M. 1991. An attempt to control lipid

peroxidation in the tissues of yellowtails and to enhance their biological protective ability by feeding high α-tocopherol brown meal. Nippon Suisan Gakkaishi, 57, 287-292.

SHAN X.J., HUANG W., CAO L., XIAO Z.Z. and DOU S.Z. 2009. Ontogenetic development of digestive enzymes and effect of starvation in miiuy croaker *Miichthys miiuy* larvae. Fish Physiology and Biochemistry, 35, 385-398.

SIES H. 1991. Oxidative stress: introduction. In: Oxidative Stress: Oxidants and Antioxidants. (Ed. Sies, H.) Academic Press, San Diego. 21-48 p.

SKIFTESVIK A.B., BROWMAN H.I. and ST-PIERRE J.-F. 2003. Life in green water: the effect of microalgae on the behaviour of Atlantic cod (*Gadus morhua*) larvae. In: The Big Fish Bang Proceedings of the 26th Annual Larval Fish Conference (ed. by H.I. Browman & A.B. Skiftesvik), pp. 97-103. Institute of Marine Research, Bergen, Norway.

SKJERMO J. and VADSTEIN O. 1993. The effect of microalgae on skin and gut bacterial flora of halibut larvae. In: Proceedings of the First International Conference on Fish Farming Technology, Trondheim, Norway, pp. 61-67.

SKJERMO J. and VADSTEIN O. 1999. Techniques for microbial control in the intensive rearing of marine larvae. Aquaculture, 177, 333-343.

SKOPELITOU K., DHAVALA P., PAPAGEORGIOU A.C. and LABROU N.E. 2012. A Glutathione Transferase from *Agrobacterium tumefaciens* Reveals a Novel Class of Bacterial GST Superfamily. PLoS One, 7(4), e34263. doi:10.1371/journal.pone.0034263

SOHAL R.S. and ORR W.C. 2012. The redox stress hypothesis of aging. Free Radical Biology and Medicine, 52, 539-555.

SOKAL R.R. and ROHLF F.J. 2012. Biometry: the principles and practice of statistics in biological research. 4th ed. W.H. Freeman and Co.: New York. 937 pp.

SOLA L., MORETTI A., CROSETTI D. et al. 2007. Genetic effects of domestication, culture and breeding of fish and shellfish, and their impacts on wild populations. Gilthead seabream -*Sparus aurata*. Genetic Impact of Aquaculture Activities on Native Populations (eds. T. Svasand, D. Crosetti, E. Garcia-Vazquez, & E. Verspoor), pp. 47–54. Genimpact final scientific report. Available at: http://genimpact.imr.no/

SOLÉ M., POTRYKUS J., FERNÁNDEZ-DÍAZ C. and BLASCO J. 2004. Variations on stress defences and metallothionein levels in the Senegal sole, *Solea*

senegalensis during early larval stages. Fish Physiology and Biochemistry, 30, 57-66.

SORUM H., ROBERTS M.C. and CROSA J.H. 1992. Identification and cloning of a tetracycline resistance gene from the fish pathogen *Vibrio salmonicida*. Antimicrobial Agents and Chemotherapy, 36, 611-615.

STADTMAN E.R. 1993. Oxidation of free amino acids residues in proteins by radiolysis and metal-catalyzed reactions. Annual Review of Biochemistry, 62, 797-821.

STÉPHAN G., GUILLAUME J. and LAMOUR F. 1995. Lipid peroxidation in turbot (*Scophthalmus maximus*) tissue: effect of dietary vitamin E and dietary n-6 and n-3 polyunsaturated fatty acids. Aquaculture, 130, 251-268.

STEPHENSEN E., STURVE J. and FÖRLIN L. 2002. Effects of redox cycling compounds on glutathione content and activity of glutathione-related enzymes in rainbow trout liver. Comparative Biochemistry and Physiology Part C, 133, 435-442.

STOREY K.B. 1996. Oxidative stress: adaptations in nature. Brazilian Journal of Medical and Biological Research, 29, 1715-1733.

STOTTRUP J.G., GRAVNINGEN K. and NORSKER N.H. 1995. The role of different algae in the growth and survival of turbot larvae (*Scophthalmus maximus* L.) in intensive rearing systems. ICES Marine Science Symposium, 201, 173-186.

SUZER C., KAMACI O., ÇOBAN D., SAKA Ş., FIRAT K., ÖZKARA B. and ÖZKARA A. 2007. Digestive enzyme activity of the red porgy (Pagrus pagrus, L.) during larval development under culture conditions. Aquaculture research, 38, 1778-1785.

SZWEDA L.I. and STADTMAN E.R. 1992. Iron-catalyzed oxidative modification of glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides*. Journal of Biological Chemistry, 267, 3096-3100.

TANASOMWANG V. and MUROGA K. 1988. Intestinal microflora of larval and juvenile stages in Japanese flounder (*Paralichthys olivaceus*). Fish Pathology, 23, 77-83.

TENDENCIA E.A. and DE LA PEÑA M. 2003. Investigation of some components of the greenwater system which makes it effective in the initial control of luminous bacteria. Aquaculture, 218, 115-119.

TOCHER D.R., MOURENTE G., VAN DER EEKEN A., EVJEMO J.O., DÍAZ E., BELL J.G., GEURDEN I., LAVENS P. and OLSEN Y. 2002. Effects of dietary vitamin E on antioxidant defense mechanisms of juvenile turbot (*Scophthalmus maximus* L.), halibut (*Hippoglossus hippoglossus* L.) and sea bream (*Sparus aurata* L.). Aquaculture Nutrition, 8, 195-207.

TOCHER D.R., MOURENTE G., VAN DER EEKEN A., EVJEMO J.O., DÍAZ E., WILLE M., BELL J.G. and OLSEN Y. 2003. Comparative study of antioxidant defense mechanisms in marine fish fed variable levels of oxidized oil and vitamin E. Aquaculture International, 11, 195-216.

TOVAR-RAMÍREZ D., MAZURAIS D., GATESOUPE J.F., QUAZUGUEL P., CAHU C.L. and ZAMBONINO-INFANTE J.L. 2010. Dietary probiotic live yeast modulates antioxidant enzyme activities and gene expression of sea bass (*Dicentrarchus labrax*) larvae. Aquaculture, 300, 142-147.

TYTLER P., IRELAND J. and MURRAY L. 1997. A study of the assimilation of fluorescent pigments of microalgae *Isochrysis galbana* by the early larval stages of turbot and herring. Journal of Fish Biology, 50, 999-1009.

UBERSCHÄR B. 1995. The use of tryptic enzyme activity measurements as a nutritional condition index: laboratory calibration data and field application. ICES Marine Science Symposia, 201, 119-129.

UTNE A.C.W. 1997. The effect of turbidity and illumination on the reaction distance and search time of the marine planktivore *Gobiusculus flavescens*. Journal of Fish Biology, 50, 926-938.

UTNE-PALM A.C. 1999. The effect of prey mobility, prey contrast, turbidity and spectral composition on the reaction distance of *Gobiusculus flavescens* to its planktonic prey. Journal of Fish Biology, 54, 1244-1258.

VADSTEIN O., OIE G., OLSEN Y., SALVESEN I., SKJERMO J. and SKJÅK-BRÆK G. 1993. A strategy to obtain microbial control during larval development of marine fish. In: Proceedings of the First International Conference on Fish Farming Technology, Trondheim, Norway, pp. 69-75.

VAN DER MEEREN T. 1991. Algae as first food for cod larvae, *Gadus morhua* L.: Filter feeding or ingestion by accident?. Journal of Fish Biology, 39 (2), 225-237.

VERPRAET R., CHAIR M., LEGER P., NELIS H., SORGELOOS P., DE LEENHEER A. 1992. Live-food mediated drug delivery as a tool for disease

treatment in larviculture. The enrichment of therapeutics in Rotifers and *Artemia nauplii*. Aquacultural Engineering, 11, 133-139.

WALFORD J. and LAM T.J. 1993. Development of digestive tract and proteolytic enzyme activity in seabass (*Lates calcarifer*) larvae and juveniles. Aquaculture, 109, 187-205.

WATANABE T., TAKASHIMA F., OGINO C. and HIBIYA T. 1970. Effect of α-tocopherol on carp. Nippon Suisan Gakkaishi, 36, 623-630.

WENDEL A. 1980. Glutathione Peroxidase. In: Jakoby W.B. (Ed.). Enzymatic Basis of Detoxication, Academic Press, New York, 333-353.

WINSTON G.W. and DI GIULIO R.T. 1991. Prooxidant and antioxidant mechanisms in aquatic organisms. Aquatic toxicology, 19, 137-161.

WIRNT R. and BERGMEYER H.U. 1974. Chymotrypsin. In: Methods of Enzymatic Analysis, Bergmeyer H.U. (Ed.), vol. 2, pp 1009-1012, New York (NY).

YIN G.Y., YIN Y.F. and HE X.F. 1995. Effect of zhuchun pill on immunity and endocrine function of elderly with kidney-yang deficiency. Chung Kuo Chung Hsi I Chieh Ho Tsa Chih, 15, 601-603.

YÚFERA M., FERNÁNDEZ-DÍAZ C., VIDAURRETA A., CARA J.B. and MOYANO F.J. 2004. Gastrointestinal pH and development of the acid digestion in larvae and early juveniles of Sparus aurata (Pisces: Teleostei). Marine Biology, 144, 863-869.

YÚFERA M. and DARIAS M.J. 2007. The onset of exogenous feeding in marine fish larvae. Aquaculture, 268, 53-63.

ZAMBONINO-INFANTE J.L. and CAHU C.L. 1994. Development and response to a diet change of some digestive enzymes in sea bass (*Dicentrarchus labrax*) larvae. Fish Physiology and Biochemistry, 12, 399-408.

ZAMBONINO-INFANTE J.L. and CAHU C.L. 2001. Ontogeny of the gastrointestinal tract of marine fish larvae. Comparative Biochemistry and Physiology, 130, 477-487.

ZOR T. and SELINGER Z. 1996. Linearization of the Bradford Protein Assay Increases Its Sensitivity: Theoretical and Experimental Studies. Analytical Biochemistry, 236, 302-308.

7. ANNEX:

"Bioexplore" Project

Report: Enzymatic analysis of fish larvae samples.

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Sample pretreatment and enzyme assays were performed in the frame of the "Bioexplore" research project and were collaborative work of the teams of the Laboratory of Enzyme Technology, Department of Agricultural Biotechnology, Agricultural University of Athens, and the Hellenic Centre for Marine Research.

The biochemical analyses of the antioxidant system were carried out in the Department of Agricultural Biotechnology at the Agricultural University of Athens following the next procedure:

Enzyme extracts were prepared by homogenization of pooled whole larvae in cold 0.1 M KH_2PO_4 extraction buffer pH = 6 containing 1 mM EDTA, followed by sonication (5 times x 6 seconds) and centrifugation at 10,000 rpm during 7 minutes. Supernatants were collected and directly assayed for enzyme activity.

Larval extracts were used for the determination of enzyme activity. Measurements of digestive (trypsin and chymotrypsin) and antioxidant enzyme activities (glutathione transferase, glutathione peroxidase, glutathione reductase and superoxide dismutase) were carried out according to standard protocols (Mavis and Stellwagen, 1968; Bergmeyer et al., 1974; Wirnt and Bergmeyer, 1974; Wendel, 1980; Nandi and Chatterjee, 1988; Skopelitou et al., 2012).

The activity of trypsin was determined by a continuous spectrophotometric assay using N-benzoyl-L-arginine ethyl ester (BAEE) as substrate (Bergmeyer et al., 1974). Trypsin activity was expressed in BAEE units. One BAEE unit corresponds to the amount of enzyme that produces a ΔA_{253} of 0.001 per minute at pH 7.6 and 25ºC using BAEE as substrate. The activity of chymotrypsin was determined by a continuous rate spectrophotometric assay using N-benzoyl-L-tyrosin ethyl ester (BTEE) as substrate as

(Wirnt and Bergmeyer, 1974). One unit of chymotrypsin is the amount of enzyme that hydrolyzes 1.0 μmol of BTEE per minute at pH 7.8 and 25ºC.

The level of glutathione (GSSG + GSH) in samples was determined using the continuous reduction of 5,5'-dithiobis-(2-nitrobenzoic acid) or DTNB to TNB and glutathione reductase/NADPH system (Akerboom and Sies, 1981). One unit of glutathione transferase, glutathione peroxidase, glutathione reductase is defined as the amount of enzyme that catalyzes the turnover of 1 μmol of substrate per minute. One unit of SOD activity was defined as the amount of enzymes necessary to produce a 50% inhibition of the pyrogallol autoxidation method.

Observed reaction velocities were corrected for spontaneous reaction rates when necessary. All initial velocities were determined in triplicate in buffers equilibrated at constant temperature. Turnover numbers were calculated in the basis of one active site per subunit. Specific activity is expressed in μmol • min^{-1} per mg of protein.

Concentration of soluble protein extracts was determined by the Bradford Method (Bradford, 1976), using serum albumin (0.2 mg/ml, fraction V) as standard (Bradford, 1976; Zor and Selinger, 1996).

All reagents for enzyme assays were purchased from Sigma (St Louis, USA).

The enzymatic activities (units) are presented as means of triplicate measurements with the standard error of the mean.

For the activities of antioxidant enzymes, standardization to protein content seem to be more appropriate because the antioxidative status of the cells is linked to cellular targets (e.g. lipids, proteins, DNA) and not to the size, mass or water content of the animal. This standardization procedure indicates priorities for the organism in the synthesis of antioxidative proteins within the context of their global protein synthesis. Accordingly, expressing activity with respect to protein content appears to be most appropriate for comparing antioxidant enzyme activities.

In addition, for the activities of digestive enzymes, standardization to the weight of the fish was also performed. These activities were estimated by using the activity units per ml of extract, the weight of the sample and the average weight of the larvae at the time of sampling. The weight was estimated using the growth rates resulted in section 3.1.2. The actual equations for each group were for the PGW (1) and CW (2).

(1) $W_t = W_0 * e^{a \bullet t}$, where W_0 = 0.0881, a = 0.0975.

(2) $W_t = W_0 * e^{a \bullet t}$, where W_0 = 0.0788, a = 0.0846.

7.1 Oxidative stress:

7.1.1 Superoxide dismutase assay

The determination of the enzymatic activity of SOD at early stages of development of the gilthead sea bream (*Sparus aurata*) showed this activity at all the stages in the first two months after hatching. A significant decrease was detected between 0 and 4 dph. At 10 dph, which corresponds with the rotifer-feeding stage, a very high specific activity of SOD was registered in the CW group, being significantly greater than on any other day (Figure 20). At 20 dph SOD activity decreased again in the CW group and remained relatively constant until the end of the experiment. However, the PGW group showed this increase in the activity at 20 dph, which corresponds with the *Artemia* feeding stage, and the decrease thereafter was less pronounced. At 60 dph, the SOD activity increased again in the PGW group whereas in the CW group a significant decrease was detected.

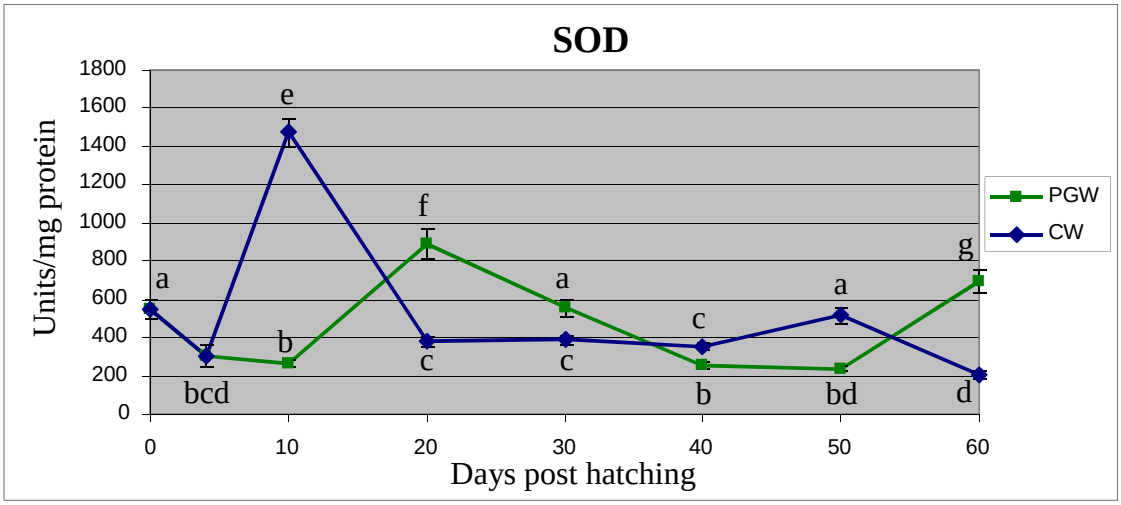

Figure 20: Superoxide dismutase specific activity in whole *S. aurata* larvae reared under PGW and CW conditions until 60 days post hatching.

7.1.2 Glutathione assay

Glutathione was also detected at all developmental stages studied. The maximum value registered was at hatching (1.5 μmol/mg protein), with this concentration being

significantly greater than on any other day. At 4 dph the glutathione concentration was half of the activity detected at hatching. Then, GSH concentration stopped decreasing at 10 dph (rotifer feeding stage) in both groups, but the concentration was significantly greater for the PGW group than for the CW group. At 20 dph, the activity increased again in the PGW group, but in the CW group, GSH values were not significantly different than at 10 dph. The trend in the CW group between 20 and 50 dph was slowly but significant increase to diminish again at 60 dph, while in the PGW group GSH concentration was continuously decreasing until 60 dph (Figure 21).

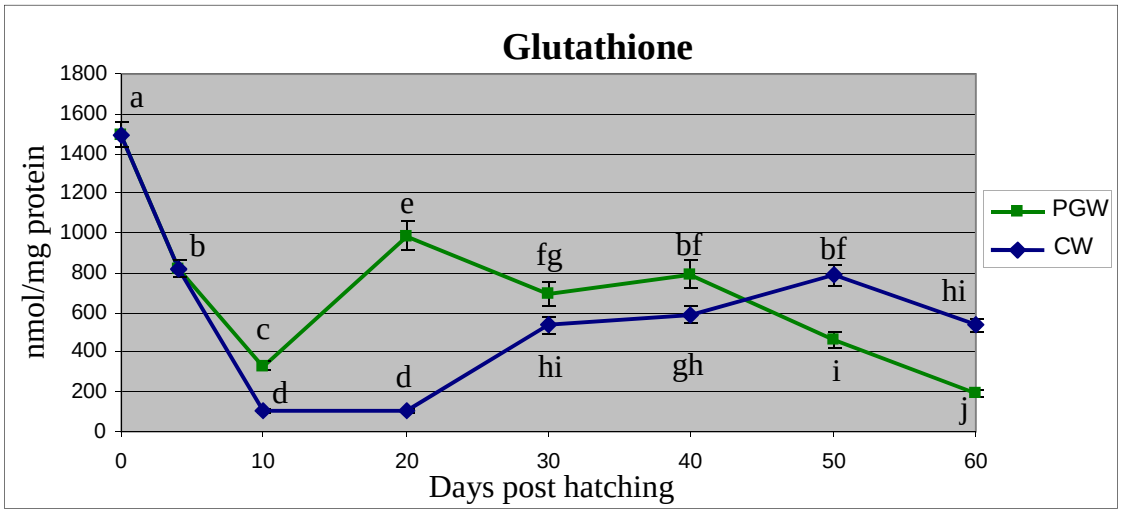

Figure 21: Glutathione content (nmol/mg protein) in whole *S. aurata* larvae reared under PGW and CW conditions 60 days post hatching.

7.1.3 Glutathione reductase assay

The activity of the GR was also detected at all the developmental stages studied. On can emphasize the high GR activity registered at 20 dph in the CW group, which was significantly greater than on any other day. This peak in GR activity at 20 dph was also detected in the PGW group, but the values registered were significantly lower than in the CW group. GR activity decreased significantly at 30 dph in both groups. After this, there is a small but significant trend to increase in the PGW group until 60 dph whereas in the CW group GR activity remains constant with small fluctuations (Figure 22).

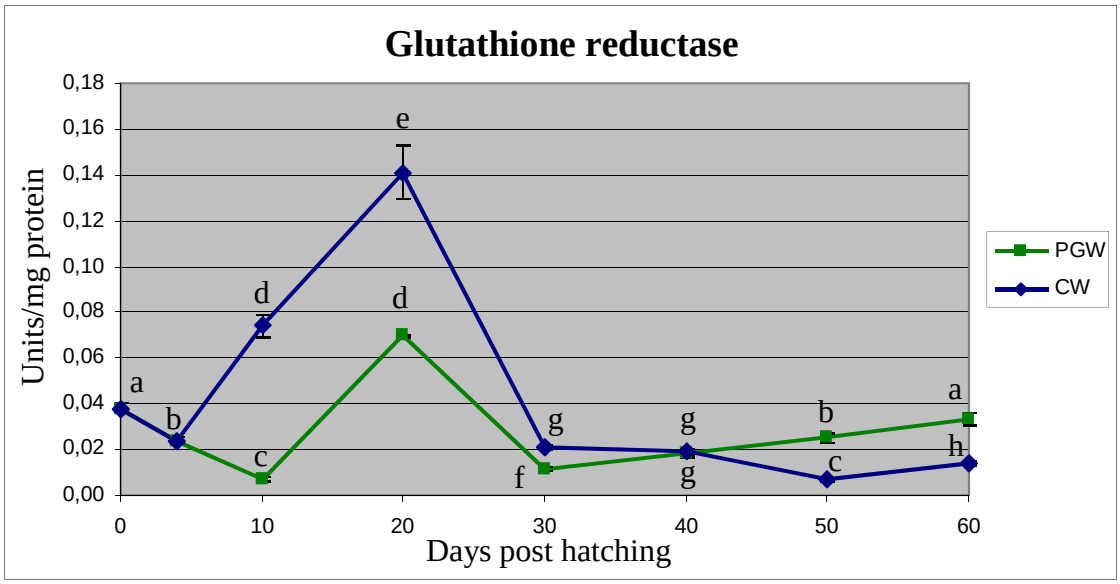

Figure 22: Glutathione reductase specific activity in whole *S. aurata* larvae reared under PGW and CW conditions until 60 days post hatching.

7.1.4 Glutathione peroxidase assay

In the specific activity of the enzyme GPX in the CW group, a significantly higher value was observed at 20 dph; GPX activity in the CW group decreased again at 30 dph and remained at this level until the end of the experiment. On the other hand, PGW group showed a significant decrease at the rotifer feeding stage (10 dph) which increased again at 20 dph. After this, GPX specific activity in the PGW group suffered a significant decrease and thereafter rose again to reach significantly higher values at 40 and 50 dph. At 60 dph, GPX activity declined significantly in PGW group (Figure 23).

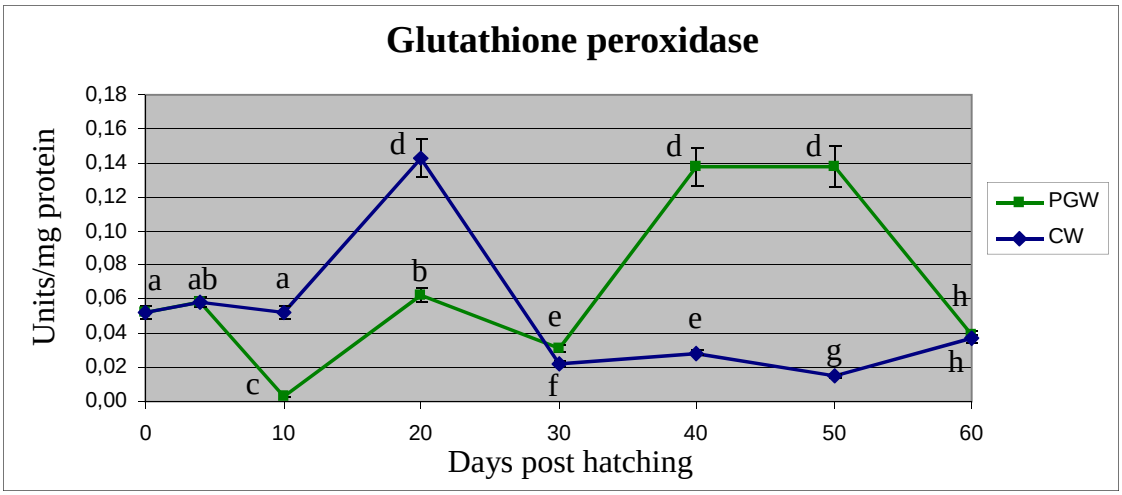

Figure 23: Glutathione peroxidase specific activity in whole *S. aurata* larvae reared under PGW and CW conditions until 60 days post hatching.

7.1.5 Glutathione S-transferase assay

Specific activity of the GST enzyme was also detected in all samples assayed. The activity was significantly lower at hatching and then augmented progressively until 10 dph in the CW group and until 20 dph in the PGW group. After that, GST specific activity showed a significant decrease in both groups (at 20 dph in the CW group and at 30 dph in the PGW group) and then GST activity increased again gradually. At 60 dph, a significant decline in GST activity was registered for the PGW group (Figure 24).

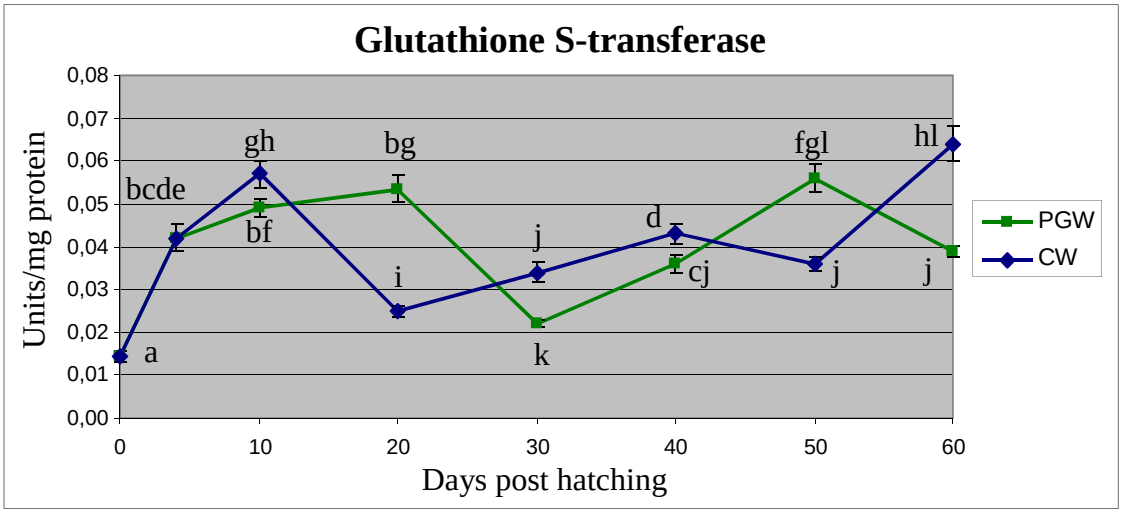

Figure 24: Glutathione S-transferase specific activity in whole *S. aurata* larvae reared under PGW and CW conditions until 60 days post hatching.

7.2 Digestive system:

7.2.1 Trypsin

The levels of trypsin specific activity detected at 4 dph were significantly higher than those measured in the larvae at hatching. Trypsin specific activity showed significant differences when comparing the CW and the PGW groups. While in the CW group the trend was to increase significantly until 20 dph and then decrease progressively until 60 dph, in the PGW group the highest activity recorded was at 30 dph and then the specific activity decreased, as in the CW group, until the end of the experiment (Figure 25).

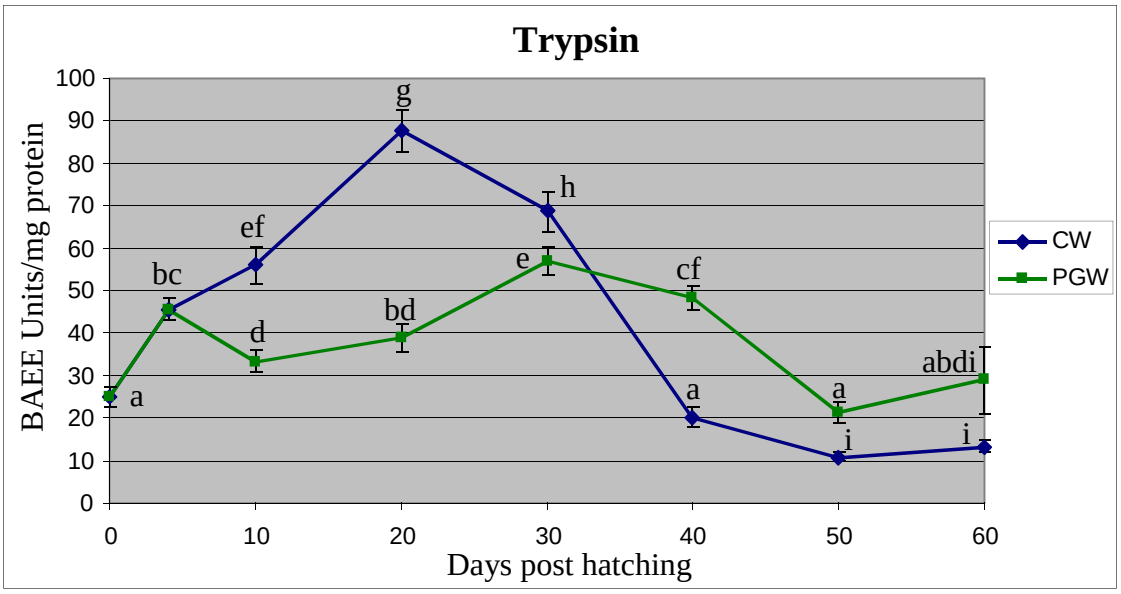

Figure 25: Trypsin specific activity in whole *S. aurata* larvae reared under PGW and CW conditions until 60 days post hatching.

The pattern observed presents some differences if we look at the trypsin activity in terms of activity per larvae (Figure 26), instead of activity per mg of protein. The lowest values of trypsin activity per larvae were registered at hatching. At 4 dph a significant increase was detected. Then, larvae from the CW group continued with this progressively and significant increase until 20 dph, whereas larvae from the PGW group showed the same increase starting from 10 dph to reach maximum levels at 30 and 40 dph. At 40 dph larvae from the CW group displayed a significant decrease in trypsin

activity. Thereafter, the values registered were lower than that of the PGW group, despite the significant decrease noticed in the PGW group at 50 dph (Figure 26).

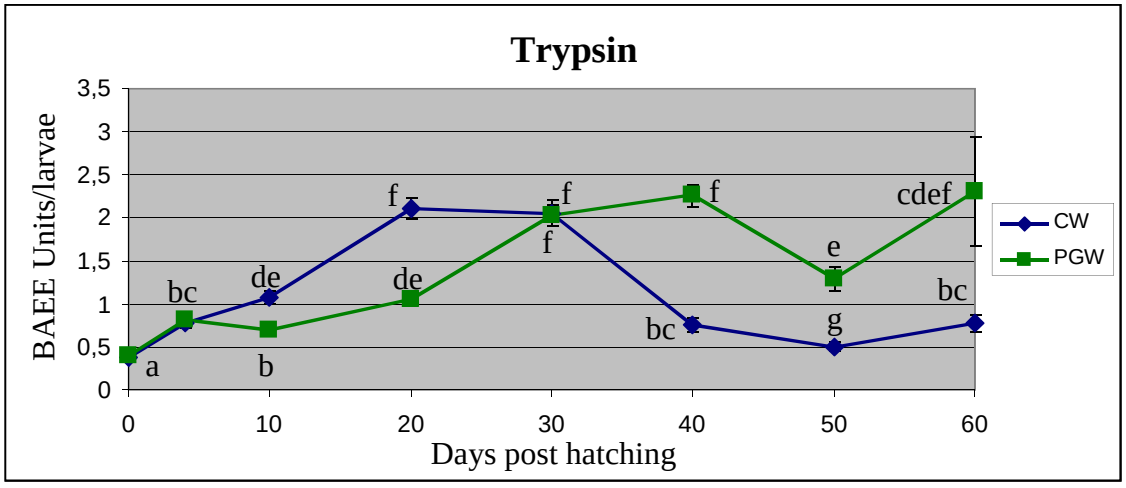

Figure 26: Total trypsin activity (units per min and individual) in whole *S. aurata* larvae reared under PGW and CW conditions until 60 days post hatching.

7.2.2 Chymotrypsin

Chymotrypsin specific activity was significantly higher at hatching compared with the following sample (4 dph). After that, the trend in chymotrypsin activity was to decrease in both groups. At 30 dph a significantly higher activity was registered in the PGW group followed by a sudden fall until 50 dph; an increase was also detected in the CW group but now, the higher value was detected at 40 dph and the respective decrease was registered until 50 dph. At 60 dph, both groups showed a significant increase in chymotrypsin specific activity (Figure 27).

When looking at the chymotrypsin total activity per larvae (Figure 28) it is still noticed a significant decrease at 4 dph, but the values registered at hatching are not the highest anymore. Larvae from the PGW group showed no differences in chymotrypsin activity per larvae until 20 dph, when the values were significantly higher than the correspondent in the CW group; after that, at 30 dph, a significant increase was detected in the PGW group to diminish and show low values again at 40 dph and 50 dph. At 60 dph were registered the highest chymotrypsin activity values in larvae from the PGW group. On the other hand, larvae from the CW group showed a small but significant increase in chymotrypsin activity per larvae at 10 dph, which decreased and showed

again low values at 20 dph and 30 dph. Next CW sample, at 40 dph, showed the highest values recorded for this group, with this values being significantly higher than that measured for the PGW group the same day. At 50 dph the levels suffered a significant decrease but still the values in the CW group are significantly higher than the corresponding in the PGW group. At 60 dph an increase was observed as in the PGW group, but the increase was much less in the CW group (Figure 28).

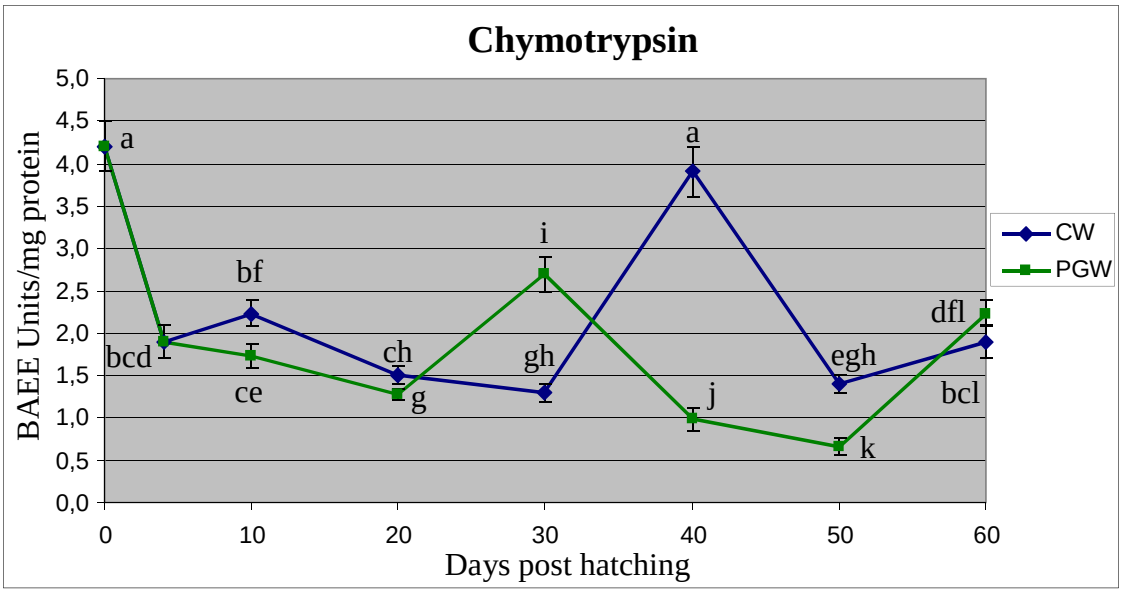

Figure 27: Chymotrypsin specific activity in whole *S. aurata* larvae reared under PGW and CW conditions until 60 days post hatching.

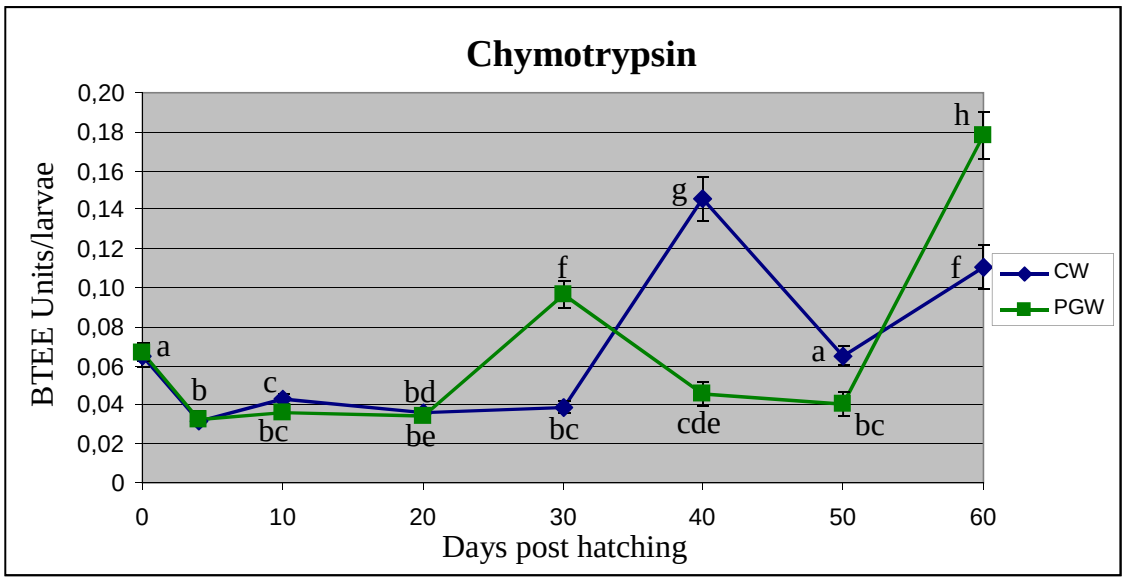

Figure 28: Total chymotrypsin activity (units per min and individual) in whole *S. aurata* larvae reared under PGW and CW conditions until 60 days post hatching.

7.2.3 Trypsin/chymotrypsin ratio

Larvae from the CW group showed a higher trypsin/chymotrypsin ratio than larvae from the PGW group during the first 30 days post hatching. However, during the second month, this trend is reversed with the differences between groups shrinking until 60 dph (Figure 29).

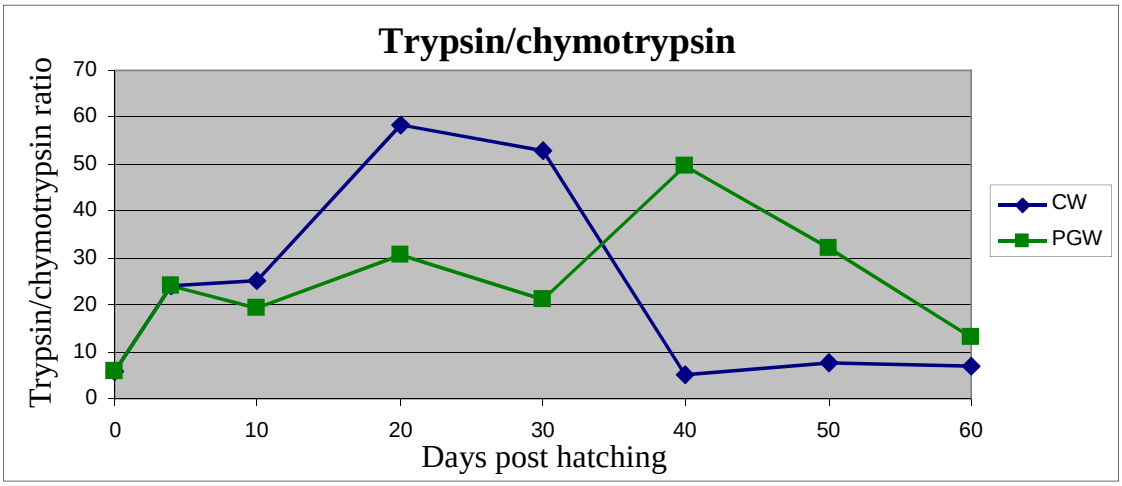

Figure 29: Trypsin/chymotrypsin ratio in whole *S. aurata* larvae reared under PGW and CW conditions until 60 days post hatching.

Table VII: Total and specific superoxide dismutase activities measurements.

Sample	Units/µl	mg protein/ml	Units/mg protein	Standard deviation
0 dph (hatch)	0.4	0.724	552.5	49
4 dph	0.3	1.166	300.6	19
CW – 10 dph	0.8	0.543	1495	75
CW – 20 dph	0.3	0.796	376.5	26
CW – 30 dph	0.3	0.776	386.5	22
CW – 40 dph	0.3	0.846	354.6	21
CW – 50 dph	0.6	1.164	515.3	42
CW – 60 dph	0.2	0.975	205.5	16
PGW – 10 dph	0.3	1.136	264	16
PGW – 20 dph	0.5	0.562	889.8	81
PGW – 30 dph	0.6	1.08	554.7	43
PGW – 40 dph	0.4	1.58	253.2	17
PGW – 50 dph	0.3	1.26	238.1	15
PGW – 60 dph	1.08	0.864	694.4	62

Table VIII: Total and relative glutathione concentrations.

Sample	Units/ml	mg protein/ml	Units/mg protein	Standard deviation
0 dph (hatch)	1083.3	0.724	1495	59.2
4 dph	958.3	1.166	821.9	45.6
CW – 10 dph	55.6	0.543	102.3	8.82
CW – 20 dph	83.3	0.796	104.4	7.61
CW – 30 dph	416.7	0.776	536.7	41.5
CW – 40 dph	500	0.846	591.3	42.5
CW – 50 dph	916.7	1.164	787.7	52.7
CW – 60 dph	522	0.975	535.2	36.8
PGW – 10 dph	375	1.136	330.3	22
PGW – 20 dph	554.1	0.562	985.6	68
PGW – 30 dph	750	1.08	694.3	56
PGW – 40 dph	1250	1.58	791.3	71
PGW – 50 dph	583.3	1.26	462.9	39
PGW – 60 dph	166.7	0.864	192.9	18

Table IX: Total and specific glutathione reductase activities measurements.

Sample	Units/ml	mg protein/ml	Units/mg protein	Standard deviation
0 dph (hatch)	0,027	0,724	0,038	0,2
4 dph	0,028	1,166	0,024	0,13
CW – 10 dph	0,04	0,543	0,074	0,5
CW – 20 dph	0,1125	0,796	0,141	1,2
CW – 30 dph	0,0161	0,776	0,021	0,12
CW – 40 dph	0,0161	0,846	0,019	0,13
CW – 50 dph	0,008	1,164	0,0069	0,04
CW – 60 dph	0,013	0,975	0,014	0,12
PGW – 10 dph	0,008	1,136	0,007	0,08
PGW – 20 dph	0,03925	0,562	0,070	0,04
PGW – 30 dph	0,012	1,08	0,011	0,08
PGW – 40 dph	0,028	1,58	0,018	0,12
PGW – 50 dph	0,032	1,26	0,025	0,21
PGW – 60 dph	0,028	0,864	0,033	0,27

Table X: Total and specific glutathione peroxidase activities measurements.

Sample	Units/ml	mg protein/ml	Units/mg protein	Standard deviation
0 dph (hatch)	0.0375	0.724	0.0520	0.4
4 dph	0.0677	1.166	0.0581	0.3
CW – 10 dph	0.0280	0.543	0.0520	0.39
CW – 20 dph	0.1138	0.796	0.1430	1.1
CW – 30 dph	0.0170	0.776	0.0221	0.2
CW – 40 dph	0.0242	0.846	0.0286	0.19
CW – 50 dph	0.0242	1.164	0.0150	0.09
CW – 60 dph	0.0360	0.975	0.0370	0.25
PGW – 10 dph	0.034	1.136	0.0030	0.04
PGW – 20 dph	0.036	0.562	0.0620	0.40
PGW – 30 dph	0.034	1.080	0.0310	0.17
PGW – 40 dph	0.0218	1.580	0.1375	1.10
PGW – 50 dph	0.0169	1.260	0.1381	1.20
PGW – 60 dph	0.034	0.864	0.0397	0.22

Table XI: Total and specific glutathione S-transferase activities measurements.

Sample	Units/ml	mg protein/ml	Units/mg protein	Standard deviation
0 dph (hatch)	0.0104	0.724	0.0143	0.11
4 dph	0.0487	1.166	0.0420	0.32
CW – 10 dph	0.059	0.543	0.0571	0.31
CW – 20 dph	0.0198	0.796	0.0250	0.13
CW – 30 dph	0.026	0.776	0.0340	0.24
CW – 40 dph	0.0365	0.846	0.0430	0.22
CW – 50 dph	0.042	1.164	0.0360	0.16
CW – 60 dph	0.0625	0.975	0.0640	0.41
PGW – 10 dph	0.056	1.136	0.0490	0.21
PGW – 20 dph	0.03	0.562	0.0535	0.31
PGW – 30 dph	0.023	1.08	0.0220	0.08
PGW – 40 dph	0.057	1.58	0.0360	0.22
PGW – 50 dph	0.07	1.26	0.0560	0.33
PGW – 60 dph	0.034	0.864	0.0390	0.14

Table XII: Total and specific trypsin activities measurements.

Sample	BAEE Units/ml	mg protein/ml	BAEE Units/mg protein	Standard deviation
0 dph (hatch)	18.1	0.724	25.01	2.63
4 dph	53.3	1.166	45.67	2.76
CW – 10 dph	30.5	0.543	56.08	4.27
CW – 20 dph	69.8	0.796	87.73	4.85
CW – 30 dph	53.4	0.776	68.76	4.80
CW – 40 dph	17.1	0.846	20.22	2.21
CW – 50 dph	12.6	1.164	10.80	0.95
CW – 60 dph	12.9	0.975	13.31	1.61
PGW – 10 dph	37.9	1.136	33.34	2.55
PGW – 20 dph	21.9	0.562	38.89	3.30
PGW – 30 dph	61.7	1.08	57.10	3.33
PGW – 40 dph	76.6	1.58	48.49	2.85
PGW – 50 dph	26.9	1.26	21.33	2.31
PGW – 60 dph	25	0.864	28.95	7.99

Table XIII: Total and specific chymotrypsin activities measurements.

Sample	BTEE Units/ml	mg protein/ml	Units/mg protein	Standard deviation
0 dph (hatch)	3.04	0.724	4.2	0.3
4 dph	2.21	1.166	1.9	0.2
CW – 10 dph	1.21	0.543	2.23	0.15
CW – 20 dph	1.19	0.796	1.5	0.1
CW – 30 dph	1.01	0.776	1.3	0.1
CW – 40 dph	3.3	0.846	3.9	0.3
CW – 50 dph	1.62	1.164	1.4	0.1
CW – 60 dph	1.85	0.975	1.9	0.2
PGW – 10 dph	1.96	1.136	1.73	0.15
PGW – 20 dph	0.71	0.562	1.27	0.06
PGW – 30 dph	2.9	1.08	2.7	0.2
PGW – 40 dph	1.54	1.58	0.98	0.13
PGW – 50 dph	0.83	1.26	0.66	0.11
PGW – 60 dph	1.92	0.864	2.23	0.15