FACULTAD DE CIENCIAS DEL MAR

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Effect of different rotifers enrichment formula feed on growth and survival of sea bream larvae (*Sparus aurata*)

Isabel María Fernández Artiles

Degree in Marine Science (ULPGC)

isabel.fernandez102@alu.ulpgc.es

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Tutors: Carmen María Hernández-Cruz

Lidia Robaina Robaina

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ABBREVIATIONS

FAO	Food and Agriculture Organization of the United Nations
APROMAR	Asociación Empresarial de Productores de Cultivos Marinos de España
PUFAs	Polyunsaturated fatty acids
EPA	Eicosapentanoic acid 20:5 (n-3)
DHA	Docosahexanoic acid 22:6 (n-3)
UV	Ultraviolet
GIA	Aquaculture Research Group
ANOVA	Analysis of variance
DPH	Days post hatch

1. Introduction

1.1 Global situation of aquaculture

Since the beginning of civilization, the extraction of marine resources through fisheries and aquaculture has been present. Some examples of this are found in Egypt or China where, since ancient times, fishes have been breeding naturally. Even now, you can find people taking care of fish in small pounds caused by the overflow of the Nile River in Egypt or in Chinese rice fields, where carps are also farmed with the harvest. (Rueda, 2011)

Actually, the origin of the marine resources that we use is not only from fisheries, also aquaculture plays a crucial role. According to reports from the FAO in 2012, the increasing of fisheries has made that, about 30% of the fish stocks were found fully exploited. Also, the consumption per capita of seafood (excluding algae) has increased from 9.9 kg in 1960 to 19 kg in 2010. This has favored the boost of the aquaculture in the past three decades, having as a consequence an increase of the fish global consumption with 8.8% annual rate. This growth also is related with an increase of its global fish contribution from 9 - 49% in the last 30 years (APROMAR, 2013). Figure 1.

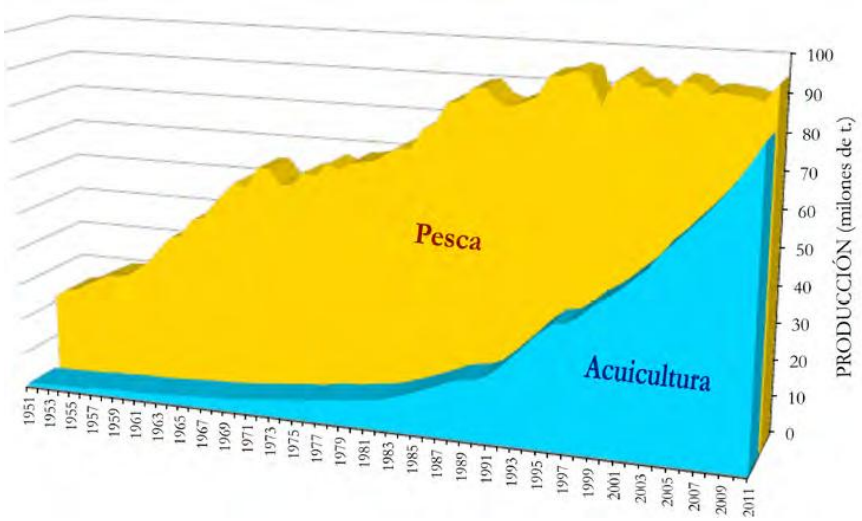

Figure 1. Development of global aquatic production (aquaculture and fisheries) from 1951 to 2011. APROMAR 2013

Currently, considering only fish, rainbow trout (*Oncorhynchus mykiss*), Atlantic salmon (*Salmo salar*) and sea bream (*Sparus aurata*) are, in that order, the fish species widely cultured in Europe (APROMAR, 2013).

1.2 Larviculture

Sea bream (*Sparus aurata*) is one of the most produced fish in Spain (APROMAR 2013). It is mainly due for its ease of cultivation and also by its high growth rate (between 1.5 and 2 years to reach commercial size). However, and despite the high gamete production of this specie during the three months of breeding, just 90% of the eggs are fertilized and around 70% of these hatch successfully. Moreover, only among 20 - 35% of these larvae percentage survives (Ortega, 2009). Most of this high mortality rate occurs during transition among endogenous to exogenous feeding.

According to different authors (Pascual & Yúfera, 1987; Rivera & Botero, 2009), the mortality occurrence is mainly due to larvae morphological limitation, involving mouth size, and also physiological restrictions as a result of an incomplete development of the digestive system. Through the years, a direct relationship between the type and quality of food provided and the extent of larval survival has been demonstrated (Lazo, 2000; Conceição *et al.*, 2010; Hamre *et al.*, 2013).

The simplicity of digestive tract in hatched larvae, being little more than a straight tube without differentiation and with a very low enzyme activity (Govoni *et al*., 1986), makes elaborated diets inefficient at this stage of development (Lazo, 2000; Izquierdo *et al.*, 2000; Conceição *et al.*, 2010).

Some authors such as Conceição *et al.* in 2010, not only point out to the digestive system of the larvae as the responsible of a poor diet. They also qualifies them as visual predators, being the movements of living prey the trigger of their feeding response. This predatory instinct in the stages of larval development supports zooplanckton living preys as the most appropriate diet for larval feeding.

According to Cerecedo-Civera *et al.* in 2004, to ensure the supply of larvae by living prey; they must have certain requirements such as: having the right size to the larvae mouth, a slow movement, a high availability and also, they have to stimulate larvae hunting instinct. Besides, the preys must reach all minimum nutritional requirements and, therefore, with easily digestion for the larvae. Few species of zooplankton reach these requirements; among them we can find rotifers (*Brachionus sp.*), brine shrimp and copepods, being the first two species the most widely cultivated for the nutrition purpose (Lazo, 2000; Conceição *et al.*, 2010).

Some reasons make rotifers an excellent source of living prey for larvae during its first weeks of exogenous feeding: they have a small size, between 70 - 350 μm, a high growth rate, the capability to resist high population densities and an elevated tolerance range to growing conditions and management. The rotifers also have an elevated content of protein, between 29 - 63% of its dry weight (Jeeja *et al.*, 2011). Nevertheless, only between 9 - 28% of its dry weight is lipid composition, which can vary according the diet supplied to the rotifers (Conceição *et al.*, 2010).

These lipids are polyunsaturated fatty acids (PUFAs), belonging to the group of phospholipids, which serve as the main energy source for marine fish larvae in their early stages (Rodriguez *et al.*, 1997; Lazo, 2000; Bell *et al.*, 2003; Hamre *et al.*, 2013.). Among these fatty acids, eicosapentaenoic acid (EPA) 20:5 (n-3) and docosahexaenoic acid (DHA) 22:6 (n-3) plays a very important role. These PUFAs not only just provide energy to larvae; they are also essential for the cell membranes integrity and functions (Sargent *et al.*, 1997; Rivera & Botero, 2009).

Despite that, some studies show the limited capacity of fish larvae to convert EPA into DHA. Sargent *et al.* (1997) have demonstrated an important deficiency of the enzyme delta-5-desaturase, responsible for enlongation and desaturation of PUFAs. Besides, rotifers are usually fed with baker's yeast, which gives them a deficient composition of PUFAs that will be transmitted to the larvae by prey ingestion. So, to improve the uptake of these fatty acids, larvae are feed with PUFAs high enriched rotifers through microalgae or commercial products (Hamre *et al.*, 2008; Conceição *et al.*, 2010). This enrichment must have a 1:2 ratio of EPA:DHA according to Lazo (2000), who proved that, in excess, these PUFAs generate harmful effects on neuronal and optical system of the larvae.

Along with a diet rich in PUFAs, an appropriate culture medium also promotes larval survival. The technique called "green water", which is based on the addition of microalgae directly to larval culture tanks, has achieved great results in terms of increased survival, growth and ingestion rate of larvae (Øie *et al.*, 1997; Reitan *et al.*, 1997). Rocha *et al.* (2008) also proved that the presence of microalgae in tanks increases the feeding response of larvae of sea bream significantly. Furthermore, the “green water” technique contribute to preserve the nutritional value of prey (Makridis &

Olsen, 1999) and even, to muddy the water, making the movement of living prey more perceptible for larvae (Naas *et al.*, 1992 & 1996).

Among the most efficient microalgae for the "green water" technique in larval fish culture, there is the class Prymnesiophyceae with the microalgae *Isochrysis galbana*. For crustacean larvae culture the classes Prasinophyceae with *Tetraselmis sp*. and Chlorophyceae with *Dunaliella tertiolecta* are the most far used (Conceição *et al.*, 2010).

The three microalgae mentioned above have been widely studied by Nevejan *et al.* (2003) and Huerlimann *et al.* (2010), showing a special interest in their unsaturated fatty acids. In their studies, *Tetraselmis sp.* has a dry weight composition between 0.7 - 3.8% of EPA; *Isochrysis sp.* oscillates between 0.3 - 0.9% of EPA and 8.2 - 15% for DHA, being the lowest amount for *Dunaliella sp.* with 0 - 0.4% and 0.2 - 0.4% for EPA and DHA repectively.

From the three previously described species of microalgae, *Tetraselmis sp.* and *Isochrysis galbana* have been widely used for rotifers enrichment, with better results reported according to their increased PUFAs levels content (Conceição *et al.*, 2010).

1.3 Objetives

To optimize the larval rearing trough adequate feeding and therefore achieve higher survival, it is necessary to better known the nutritional requirements to be supplied in the diet.

An experiment has being developed to test the effectiveness of different rotifers enrichment formula feed prepared from three PUFAs rich microalgae: *Tetraselmis sp.*, *Isochrysis sp.* and *Dunaliella sp.* during the larvae development of sea bream (*Sparus aurata*). Efficacy on larval growth and survival will be evaluated and compared with the current commercial enrichment (DHA Protein SELCO) and with rotifers fed only with baker's yeast.

2. Material and methods

The experiment with gildhead sea bream (*Sparus aurata*) larvae was carried out during March - April 2014 in the Experimental Station of the Aquaculture Research Group (GIA) at the Marine Scientist and Technological Park, belonging to the University of Las Palmas de Gran Canaria in Taliarte (Telde, Gran Canaria).

The larvae for this experiment were obtained from natural spawning from the guildhead sea bream broodstock. Eggs were collected on a special net and then, incubated for 24 hours with a smooth water flow. The dead and unfertilized eggs sank in the net, being fertilized eggs (floating fraction) volumetrically counted and separated into fractions of 20 000 eggs $tank^{-1}$. For this experiment, 15 fiberglass tanks of 200L were used. The seawater provided to the tanks were previously filtered and sterilized with ultraviolet (UV).

The tanks were always provided with central aeration and open water circuit with a bottom input and surface output. The renewal of water was at 25% during the first 3 days until larvae opened their mouths and at 5% during the rest of the experiment. The tanks drains were provided with nets of 315 μm mesh size, allowing the outflow of organic waste but not larvae, getting so the renewal of the culture medium.

Temperature (°C) and oxygen (mg ml^{-1}) were daily measured (Oxy Guard-Guard-handy beta, Zeigler Bros, Gardners, USA) at the same hour of the day, obtaining a mean of 19.32 ± 0.35 ºC and 7.14 ± 0.45 mg ml^{-1} for temperature and oxygen respectively. The culture was maintained under controlled photoperiod 12 hours of light and dark with artificial illumination of 1700 lux (Digital Lux Tester YF-1065, Powertech Rentals, Western Australia, Australia), being water salinity around 37 ‰.

On the fourth day post hatching, the larvae had their mouths opened and started the exogenous feeding. To maintain a suitable culture conditions for the larvae and rotifers, 0.5L of *Nannochloropsis sp.* were added once per day during the experiment. The rotifers used in this experiment were cultivated in the GIA facilities and enriched with the different treatments, always the day before larval feeding.

Tree complete microalgae enrichments were prepared with a mix of krill oil, vitamin E, vitamin C, antioxidants, one of the tested algae (*Isocrysis sp.*, *Tetraselmis sp.* or *Dunaliella sp.*) and baker's yeast. Two more enrichments were used: a negative control prepared based in only baker's yeast and a positive one (commercial DHA Protein SELCO)

The above five daily enrichments were prepared according to subsequent proportions: 0.25 g L^{-1} for the commercial product (DHA Protein SELCO); 0.2 g/million of rotifers from microalgae tested diets and 0.4 g/million of rotifers for baker's yeast. Each day, 12 million of rotifers were enriched for approximately 17 hours. These rotifers were then supplied to their respective tanks to achieve a concentration of 10 rotifers ml^{-1}. All treatments were tested in triplicate.

During the 15 days of the experiment, three size and weight samples were done. The first one at 4^{th} DPH with a sample of 30 larvae collected randomly from tanks. The other two samples were taken at 11^{th} and 14^{th} DPH, in these cases, with a number of 30 larvae per tank.

At the end of the experiment, survival larvae were analyzed for each treatment by counting the remaining live larvae in each tank. The activity test was conducted by handling 20 larvae per tank out of the water in a scoop net for 30 seconds and, subsequently, allocating them in another tank supplied with clean seawater to determine survival after 24 hours. The biomass was obtained multiplying the dry weight of the larvae by the number of animals which survived.

All obtained data were treated using the statistical software SPSS. Firstly, a Nonparametric Kolmogorov-Smirnov test was performed in order to determine if the samples have a normal distribution.

All samples which showed a normal distribution were proceeded for the analysis of variance (ANOVA) to compare significant differences according to the diets provided.

As the data of weight were so little (10 larvae group getting only 3 data per tank), the nonparametric Kolmogorov-Smirnov test accepted the normality of the data. Then, a Levene test was used to prove the homogeneity of variances and also an ANOVA to see if the differences in weight between diets were significant.

For biomass and survival rate, it was necessary to transform the obtained data from samplings into logarithms due its wide variation. After that, we proceeded with a Kolmogorov-Smirnov test which accepted a normal distribution again for both cases due the little data we performed. Then, it was done again a Levenne test and an ANOVA with a Tukey test when the differences found with ANOVA were significant.

3. Results

3.1 Growth

The differences found after performing the statistical analysis on the size and weight of larvae sampled were not significant (Table I).

As can be seen in the 11th DPH size sampling, the highest average size (4.52 ± 0.39 mm) and the lowest (4.39 ± 0.42 mm) correspond to larvae fed with diets 5 (baker's yeast) and 3 (*Isochrysis sp.*) respectively (Figure 2).

Nonetheless the 14th DPH size sampling, figure 3, again shows how the diet 3 has the lowest mean (4.63 ± 0.56 mm) but, in this case, the highest (4.83 ± 0.55 mm) belongs to the larvae fed with diet 1 (DHA Protein SELCO).

In terms of weight growth, the data collected during 14th DPH sampling gave anomalous values, so we only consider as valid the data collected during 11th DPH sampling. That group of data were analyzed, without found significant differences between diets, Figure 4, but there is a trend toward higher weight in larvae fed with diet 5 (0.22 ± 0.07 mg) and towards lower weight in those larvae fed with diet 2 (0.15± 0.09 mg).

TABLE I. Larvae size, weight, biomass, survival rate and activity test along the experiment.

	DPH 4		DPH 11		DPH 14		Survival rate (%)	Activity test (%)
	Size (mm)	Weight (µg)	Size (mm)	Weight (µg)	Size (mm)	Biomass (µg)		
Initial	3.53 ± 0.23	0.10 ± 0.01	-	-	-	-	-	-
Diet 1 (DHA SELCO)	-	-	4.43 ± 0.33 [a]	0.17 ± 0.08 [a]	4.83 ± 0.55 [a]	8.74 [a]	0.29 [b]	8.33
Diet 2 (*Tetraselmis sp.*)	-	-	4.47 ± 0.40 [a]	0.15 ± 0.09 [a]	4.78 ± 0.45 [a]	84.07 [a]	2.88 [a]	70.00
Diet 3 (*Isochrysis sp.*)	-	-	4.39 ± 0.42 [a]	0.17 ± 0.07 [a]	4.63 ± 0.56 [a]	167.57 [a]	7.36 [a]	86.67
Diet 4 (*Dunaliella sp.*)	-	-	4.47 ± 0.32 [a]	0.19 ± 0.08 [a]	4.66 ± 0.34 [a]	4.31 [a]	0.29 [b]	15.00
Diet 5 (Baker's yeast)	-	-	4.52 ± 0.39 [a]	0.22 ± 0.07 [a]	4.72 ± 0.49 [a]	43.78 [a]	1.89 [a]	25.00

Values (mean and standard deviation) with the same letters are not significantly different (p-value > 0.05)

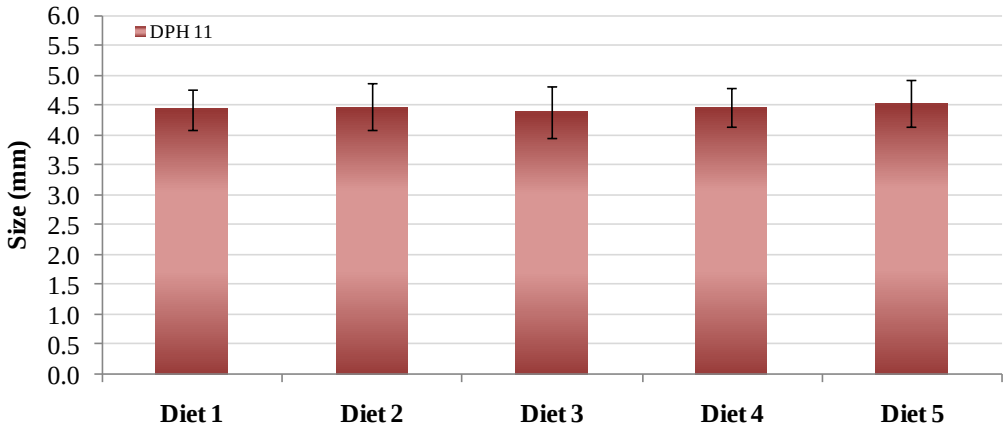

Figure 2. Average size of 11th DPH sea bream larvae fed with the different diets.

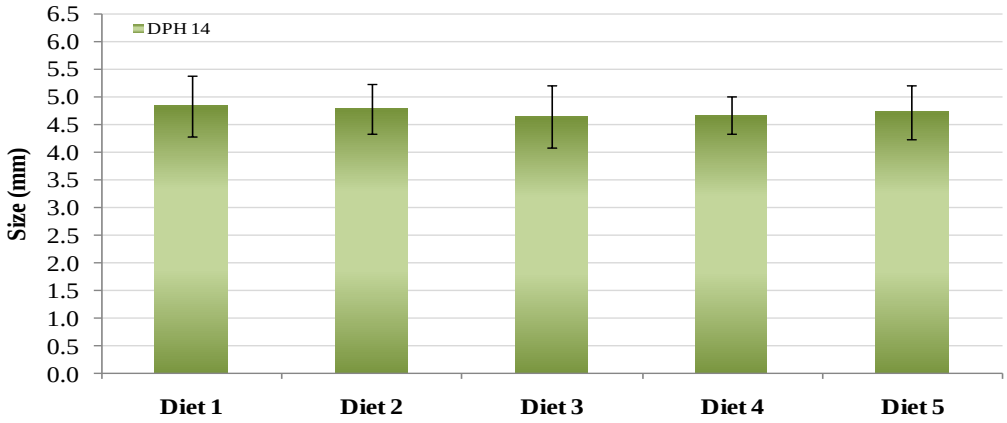

Figure 3. Average size of 14th DPH sea bream larvae fed with the different diets.

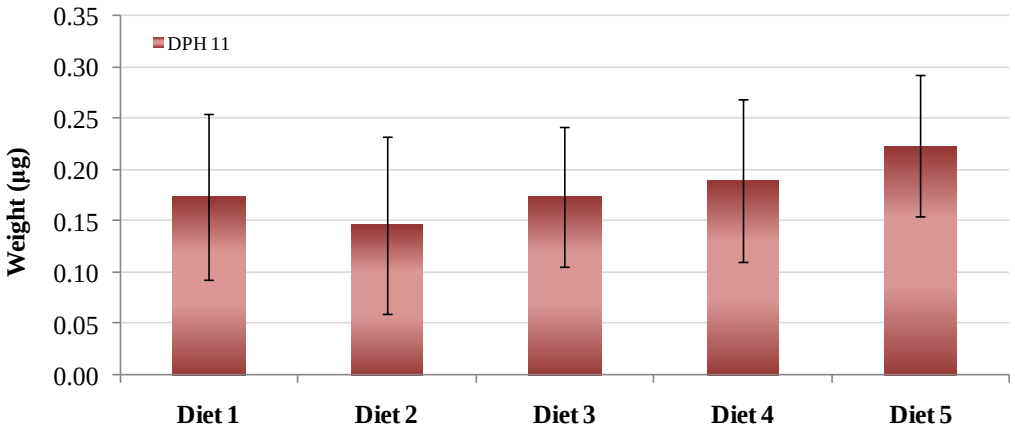

Figure 4. Average weight of 11th DPH sea bream larvae fed with the different diets.

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3.2 Survival rate

Regarding survival rates, the data obtained at the end of the experiment showed significant differences after perform statistical analysis (p-value = 0.013). These differences were found between diet 3 (*Isochrysis sp.*), which had the highest survival rate, and diets 1 (DHA Protein SELCO) and 4 (*Dunaliella sp.*), both with the lowest survival rate as is shown in figure 5.

However, due to the abnormality of the diet 1 data, which should have been one of the best survival rates, it is thought that it could be a problem with the product.

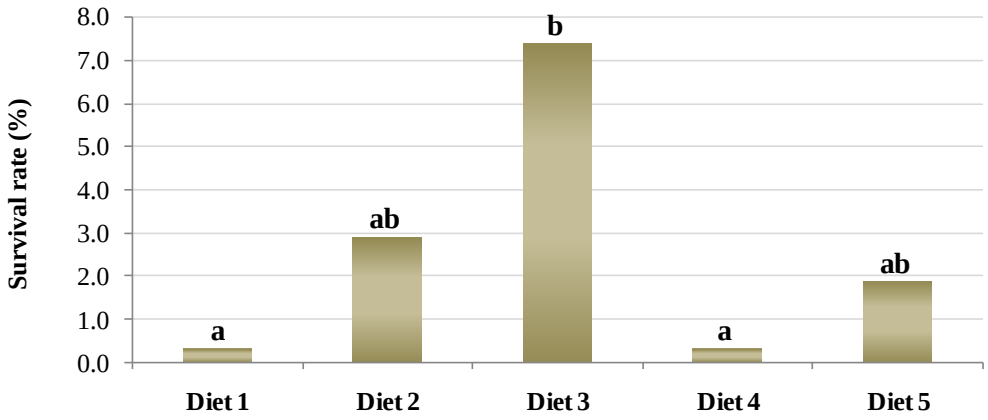

Figure 5. Survival rate (%) of sea bream larvae at the end of the experiment according to different diets.
The diets with the same letters are not significantly different (p-value > 0.05)

3.3 Biomass

Once the experiment ended, all the tanks had a variable number of survival larvae for the count of the biomass and its subsequent statistical analysis. As we can see in the figure 6, its distribution is similar to the obtained for survival rate except that, in this case, the differences of larvae biomass found between diets were not significant.

It was also shown that the highest biomass was found in the tanks where larvae were fed with diet 3 (167.57 mg) while the lowest were again in tanks whose larvae were fed with diets 1 (DHA Protein SELCO) and 4 (*Dunaliella sp.*), being the last one the diet with the lowest obtained biomass (Figure 6).

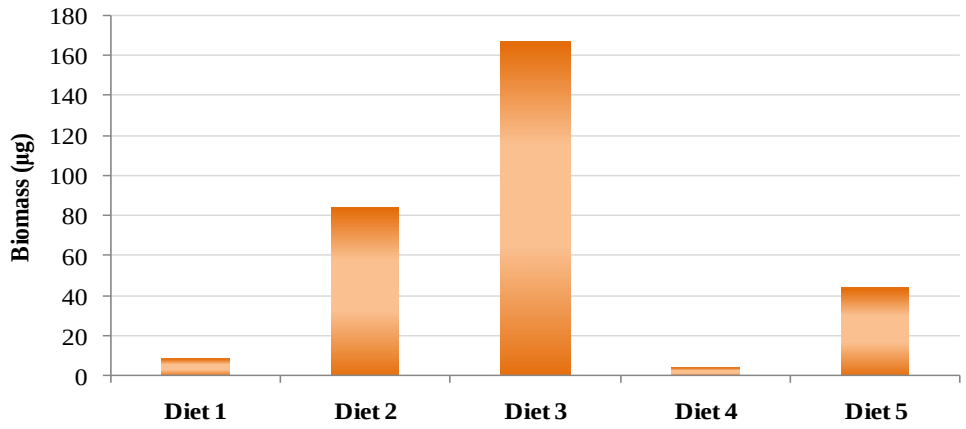

Figure 6. Biomass (µg) of sea bream larvae at the end of the experiment according to different diets.

3.4 Activity test

Observing the results of activity test (Figure 7), a similar distribution was found to those obtained for survival and biomass (Figures 5 and 6 respectively). It was moreover observed that larvae fed with diets 1 and 4 showed a decreasing survival rate after the applied stress, whereas larvae fed with diet 3 were the most are more resistant, with 86.67% of survival.

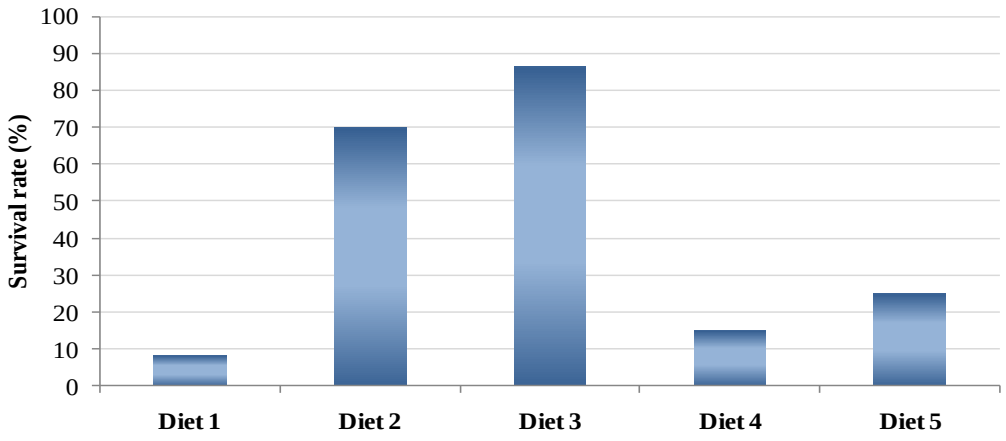

Figure 7. Larval survival rate after activity test at the end of the experiment according to different diets.

4. Discussion

Analyzing the results, we can't see any concluding response between tested diets regarding the size of sea bream larvae at the samplings of the experiment. But with weight data obtained, we can see a slight trend toward higher weight in diet 5 (baker's yeast). That trend might be related with low biomass levels found in the larvae tanks fed with that diet.

This increase in larval growth associated with decreased biomass has also been described by Roo *et al.* in 2010. They tested larval growth at different densities, resulting in a higher growth rate at low densities (> 50 larvae L^{-1}). So, they proved that high larval densities are associated with low rates of growth due to a decreased appetite, an increased aggressiveness of larvae and therefore an intense intraspecific competition for food resources. All this triggered a chronic stress situation in larvae, decreasing the biomass of the system and favoring the survival of larvae better adapted and more resistant (Hernandez-Cruz *et al.*, 1999).

However, the above described for biomass of diets 1 (DHA Protein SELCO) and 4 (*Dunaliella sp.*), where there are even less biomass in the tanks than in diet 5, does not correspond with the results obtained of average weight, which values should be higher.

During this work, it has been exposed at many times the great importance of the intake of these fatty acids in the larvae. Many authors have found in them the response to an increase or decrease of growth and survival in these early stages of larval development (Salhi, *1997*; Rodríguez *et al.*, 1994; Salhi *et al.*, 1994). The data obtained for diets 1 and 4, does not fit with the described by Hernández-Cruz *et al.* (1999) and Roo *et al.* (2010), showing then a poorly PUFAs enriched diet for larvae.

Yeast supplied to rotifers in the diet 5, had around 1.3% of PUFAs [1] while the diet 4, made from the microalga *Dunaliella sp.*, has only around 0.4 - 0.8% of these fatty acids (Nevejan *et al.*, 2003). This agrees with the data observed for us.

However, the data obtained for diet 1 do not correspond to expectations. A study by Silva in 1999 evaluated the content of PUFAs in rotifers enriched with DHA Protein SELCO, obtaining levels of EPA and DHA of 12.1 and 25.2 mg g^{-1} of dry weight of EPA and DHA respectively.

These levels cannot be compared with those obtained for rotifers enriched with yeast which, according to Watanabe *et al.* in 1983, ranging from 1 to 2 mg g^{-1} of dry weight of EPA. This is why it's thought that the anomalous data found in the diet 1 could be explained as a possible result of a fatty acid oxidation, perhaps due to a poor maintenance of the product. This would be clarified in part analyzing the biochemical composition of the diets, but that is not the objective of this experiment.

On the other hand, we also have high levels of PUFAs in diet 3, made from *Isochrysis sp.*, which do not show an increase in average larval growth compared with the other diets tested with lower content of these fatty acids. According Huerlimann *et al.* (2010), this microalgae has around 8.5 - 15.9% of dry weight of PUFAs. Silva in 1999 performed an experiment to analyze the level of PUFAs in *Isochrysis galbana* enriched rotifers, obtaining a composition between 15 - 20 mg g^{-1} of dry weight in EPA and DHA. These values are very similar to those obtained by this author in rotifers enriched with DHA Protein SELCO.

However, for the tanks fed with diet 3, high biomass was observed, which were also reported by Hernandez-Cruz *et al.* (1999), and latter on by Roo *et al.* (2010). They stated that an increase biomass is associated with reduced growth of the larvae. However, neither the survival rate nor the larvae resistance were not reduced by this high biomass due to the high PUFAs levels in the diet.

Similarly with the diet 3, the diet 2 (*Tetraselmis sp.*) exhibits low values of average weight and high in terms of biomass, survival and resistance. However, despite having relatively high levels of PUFAs (0.7 - 3.8% of dry weight of algae according Huerlimann *et al*. in 2010), this diet does not exceed the values achieved by diet 3 in any respect.

Several authors argue that this may be due to the high requirements of DHA compared to EPA in larvae as a result of the high rate of growth and development of tissues and structures in the larval stage (Takeuchi *et al.*, 1994; Rivera & Botero, 2009). DHA has been described as an important component of the tissue neural larvae and also improves vision and, consequently, better catch prey which leads to a better fed (Rodriguez *et al.*, 1997; Furuita *et al.*, 2000; Rivera & Botero, 2009). That is the reason why, despite that diet 3 has a lower content of EPA than diet 2, possessing a much higher value of the DHA improves larvae development.

Finally, larvae fed with diets 2 and 3 showed a higher resistance to stress caused by the activity test, but larvae fed with diet 3 obtained the best results. An experiment conducted by Takeuchi *et al.* (1991) with larvae of Japanese sea bream (*Pagrus major*), showed that those fed larvae with a DHA rich diets performed better survival rate before the test activity than whose larvae which were fed with diets rich in EPA.

5. Conclusions

The growth of the larvae is highly dependent on the tank larvae density, and thus higher intensity of larvae competition during feeding. A reduction of number of larvae per tank appears a possible reduction to this problem.

Among the tested diets, the use of microalgae *Dunaliella sp.* for rotifers enrichment does not provide acceptable results in larval growth and survival rate. By the other hand, the microalgae *Tetraselmis sp.*, and despite its high levels of EPA, does not provide enough PUFAs for larvae mainly by its low DHA content.

High DHA content diets, as that containing *Isocrhysis sp.* in present experiment, promote better survival and stress resistance of the larvae.

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