Effects of two feeding regimens on the growth rate of the colonial hydroid *Eudendrium carneum* (Clarke, 1882)

TRABAJO FIN DE MÁSTER

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Abstract

Hydrozoa is a group of invertebrates belonging to the phylum Cnidaria. Conformed by more than 3.800 species, they are widely represented in all marine environments. Through the last decades the number of studies related to this group have increased significantly, with particular attention to taxonomy and phylogeny. These have led to a solid base that serves as starting point for new studies to explore aspects of physiology, an area where few steps have been taken; one of these aspects is related to feeding. Hydroid feeding behavior begins to be of interest now that multiple species are intended to be kept in captivity, in order to improve knowledge of their biology. Hydrozoa are generally assumed to be carnivorous, capturing preys with tentacles full of nematocysts, but now we know that they also often exhibit a filter-feeding strategy. This, together with the opportunistic behavior opens the possibility to experiment with new feeding alternatives not necessarily based on live prey. In the present study, we deepen in this possibility by supporting us in the techniques of the aquaculture. After building a system that allows the survival of colonies, we established an experimental design in order to test the effect of the type of food on colony development. The different types of food used in the study were: frozen *Artemia* nauplii, commercial food for gilthead seabream and live *Artemia* nauplii as a control. Interestingly our results suggest that the commercial gilthead seabream food favors a significantly higher development in colonies of *Eudendrium carneum* than that with both live and frozen nauplii of *Artemia*.

Resumen

Los hidrozoos constituyen un grupo de invertebrados marinos pertenecientes al filo Cnidaria. Actualmente lo conforman más de 3.800 especies, convirtiéndolo en el grupo más diverso del filo, y estando representados mundialmente en todos los ambientes marinos. En las últimas décadas el número de estudios relacionados con el taxón se ha incrementado significativamente, especialmente aquellos centrados en la taxonomía y filogenia. De esta forma se ha establecido una sólida base a partir de la cual han empezado a desarrollarse nuevos estudios que exploran otros aspectos, como la fisiología, área en la cual sólo se han dado los primeros pasos; uno de estos aspectos es el relacionado con la alimentación. La alimentación de cnidarios empieza a ser de interés ahora que se intenta mantener en cautividad a muchas especies para aumentar el conocimiento sobre su biología. En general se ha asumido que los pólipos de los hidrozoos son carnívoros que capturan sus presas usando sus tentáculos llenos de nematocistos, pero ahora se sabe que, además, pueden exhibir una estrategia de alimentación basada en la filtración. La existencia de este comportamiento abre una ventana de posibilidades para experimentar con nuevas alternativas que no estén basadas en el uso de presas vivas como alimento. En el presente estudio profundizamos en esta posibilidad apoyándonos en las técnicas de la acuicultura. Tras construir un sistema que permitiera la supervivencia de colonias de hidrozoos, se estableció un diseño experimental para determinar el efecto del tipo de alimento en su desarrollo. Los diferentes tipos de alimento utilizados en el estudio fueron: nauplios de *Artemia* congelados, pienso comercial de dorada y nauplios vivos de *Artemia* como control. De manera muy interesante nuestros resultados sugieren que la alimentación con pienso comercial de dorada favorece el desarrollo de las colonias de *Eudendrium carneum*, que es significativamente mayor que el obtenido con nauplios de *Artemia*, vivos o congelados.

1. Introduction

Cnidarians are an ancient and diverse phylum with a fossil history dating back to the mid-Cambrian (Cartwright *et al*., 2007). The name of the group comes from the latin “cnida” whose meaning is "nettle" and the suffix “ria” that indicates "conforms to" (Brusca and Brusca, 2005). This name refers to cells called cnidocytes that characterize the phylum. In these cells when the cnidocilium comes into contact with some foreign surface it triggers the rapid deployment of the rolled filament to nail and inoculate the venom (Maldonado, 2004).

Currently in the phylum are recognized seven classes, within which for the present work we will focus on Hydrozoa. This is a group of invertebrates conformed by more than 3.800 species, widely represented in all marine environments, especially in benthic communities (Gili, 1982; Boero, 1984). Hydroids generally form colonial structures, made up of either individual or multiple polyps that usually remain attached to the substrate by a framework of hollow tubes, stolons or a hydrorhiza from which they emerge vertically (Bouillon *et al*., 2006). In the group, there are multiple reproductive strategies that can range from asexual reproduction by budding to sexual reproduction by releasing gametes from gonophores. Some species only have the polyp phase, while in others there is an alternation of generations between the polyp and the medusa stage.

In the last decades, studies related to the taxon have increased significantly. This has increased our knowledge about its biology, ecology and also its diversity. Only in the Mediterranean, the number of registered species has doubled in the last 40 years and continues ascending (Boero *et al*., 1997). Most studies have focused on taxonomy and distribution, providing a solid foundation of knowledge that serves as a starting point for new developing branches, such as the study of physiology and life cycles. Feeding behavior is one of the areas that is now becoming more important. During the last years there has been an increasing interest in culturing cnidarians under controlled conditions; this trend has been marked by jellyfish exhibits in aquariums. But, regardless of the phylum, the successful rearing of larval and juvenile stages of aquatic organisms is a challenge (Léger *et al*., 1986).

Hydroids are generally assumed to be carnivorous, capturing their prey with tentacles full of nematocysts, but now we know that they also often exhibit a filter-feeding strategy where may use particulate matter suspended in the water column and it depends on the particle concentration and water movement intensity (Puce *et al*., 2002). Although zooplankton makes up a large proportion of the diet of most hydroids, several species also exploit benthic prey (Christensen, 1967; Bavestrello *et al.,* 2000; Cerrano *et al.,* 2000). Studies on the feeding behavior of hydroids have been carried out by observing the reactions of polyps to the administration of chemical compounds such as amino-acids and GSH (Reduced Glutathion). The feeding response observed in these studies was a contraction of the hydranth, an increase in tentacle movements, and the enlarging of the mouth (Loomis, 1955; Fulton, 1963; Blanquet and Lenhoff, 1968; Pardy and Lenhoff, 1968; Rushforth, 1969).

Many studies have been limited because of the difficulty of maintaining hydroids alive long enough to be able to experiment under controlled conditions. In the present study, we explore the possibility of using food different than *Artemia* nauplii as a source of nutrients necessary for the correct development of hydroid colonies. For this, we rely on the use of aquaculture techniques, creating an aquarium system that allows us to keep the colonies in optimal conditions for the necessary time.

Aquaculture is defined as the set of techniques related to the production and cultivation of aquatic organisms (FAO, 2003). Nowadays it presents itself as one of the branches of science with greater applications and rapid growth, from its implementation in the production of food to its role in the conservation of species. It has been gaining ground until has been established and recognized as an important and sustainable way to advance in different areas of knowledge.

The techniques of aquaculture are also applied in two areas of great interest, the business of aquariums and aquariology. The first one has been growing 14% annually since the 70s, only talking about freshwater fish; in 2014 more than one billion of them were traded worldwide, including about 5,300 species (Hulme, 2009; Collins and Minteer, 2013). In general, for the scientific community aquariums hobby represent only a source of problems that seriously threaten biodiversity conservation due to the multiple escapes and release of future invasive species in diverse ecosystems (Maceda-Veiga *et al*., 2014). But aquarists and scientists are beginning to work as a team looking for the use of techniques to open new windows to research, allowing the observation *in vivo*.

Regarding hydrozoans, the role of aquaculture has been restricted and poorly applied in the right way. Multiple investigations are carried out without applying the basic concepts of aquaculture. Often the mistake is made of thinking about aquaculture and just imagining large concrete tanks in gigantic installations that depend on natural water flows or expensive recirculation systems. But the truth is that just wanting to keep 500-microns hydroids alive for a week in the laboratory, we also need to think about aquaculture. It is necessary that we begin to consider, among other aspects, the installation, the flow, the current, the water quality, the photoperiod, at the moment of establishing the experimental design of our investigations with hydroids.

The genus *Eudendrium* (Ehrenberg, 1834) has served as a model for multiple trials (Barange and Gili, 1988; Bavestrello and Arillo, 1992; Puce *et al*., 2002). This genus comprises marine colonial hydroids frequently found in the Mediterranean, with a life cycle characterized by, the absence of jellyfish generation. During the fertile season, male and female colonies release gametes from which, after fertilization, a planula larva will be generated, which will live in the water column until its attachment on the substrate. Once established, the planula will go through a series of metamorphosis processes that will result in a first polyp from which a new colony will be generated (Sommer, 1990). Species of the genus in the Mediterranean have been extensively studied in order to understand their life cycle (Boero *et al*., 1986), reproduction (Wasserthal, 1973; Bavestrello and Cerrano, 1992; Zega *et al*., 2007), associated fauna (Bavestrello *et al*., 1996) and feeding (Barange and Gili, 1988; Puce *et* a*l*., 2009).

Regarding food, studies for the group have always been using live *Artemia* as food (Piraino, 1991; Martell *et al*., 2016). However, it is well known that the nutritional value of *Artemia* is very low and for proper use in fish larvae it is always necessary to carry out an enrichment that allows the incorporation of essential fatty acids such as docosahexaenoic acid (DHA) (Léger *et al.*, 1986; Navarro *et al*., 1993; Izquierdo, 1996). Although, studies on the composition of animals in terms of fatty acids are not numerous in Hydrozoa, the presence of DHA has been verified in corals, and possible metabolic pathways continue to be evaluated in order to determine if it is an essential fatty acid or not for these organisms (Imbs, 2013).

Taking into account the above, it is possible that a food rich in fatty acids represents a better option to feed hydroids in order to keep them alive for a long period of time. To assess this possibility, the main objective of this work is to evaluate the effect of two diets, one based on *Artemia* and another based on commercial food for gilthead seabream, a food rich in fatty acids due to the inclusion of fish oils in their composition (Fountoulaki *et al*., 2003; Cahu and Infante, 2001; Montero *et al*., 2008), on the colony development of *Eudendrium carneum* (Clarke, 1882).

2. Material and methods

2.1 *Animals*

Colonies of genus *Eudendrium* (Figure 1) were collected manually in the port of Valencia and kept in sea-water during transport to the Marine Biology Laboratory of University of Valencia. Once there, the colonies were identified and all associated fauna was removed.

Figure 1. Part of a colony of *Eudendrium*.

2.2 *Experimental design*

2.2.1 *Design of an aquarium system for Hydrozoa*

For the maintenance of the colonies in the laboratory, a system was set up taking into consideration characteristics as water circulation, water quality, lighting and temperature. A container with a capacity of 5 liters and measurements of 30x18x12 cm was modified making two holes to generate a water inlet and outlet (Figure 2). By means of hoses, an adjustable power pump was placed outside the container in order to generate water movement through the system. Inside the container a raised grid (Figure 2) was placed to keep the colonies separated from the bottom, where sediments are usually deposited. The system was maintained in a room with regulated temperature (26°Cº) and exposed to natural light. For the experiments, artificial salt-water was prepared from the salt of the "Tropical Marin" signature. To maintain a good water quality, the total volume of water was replaced every two days.

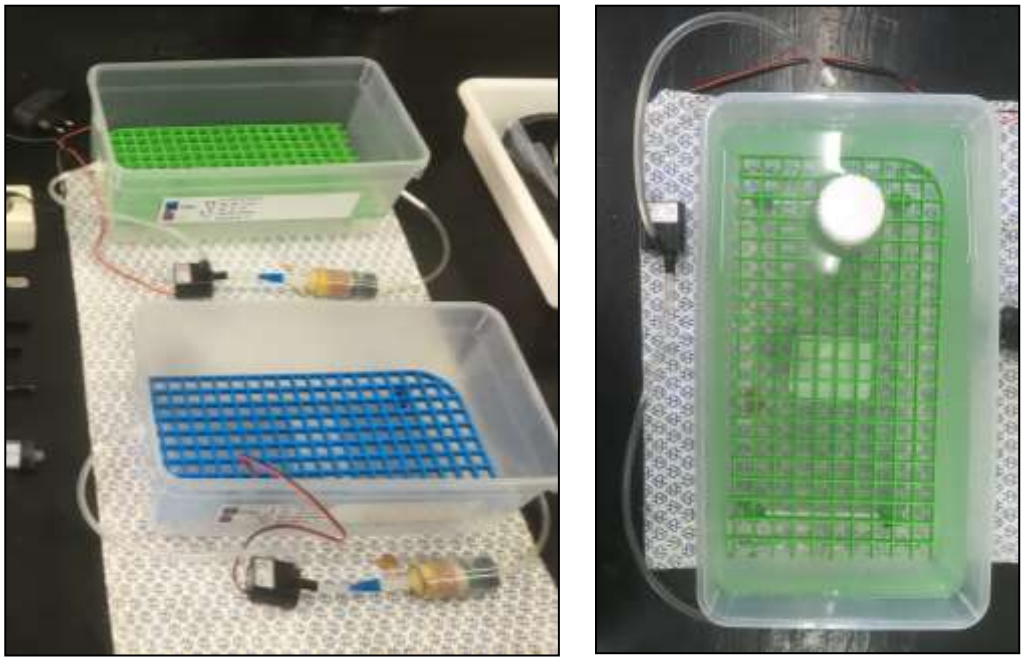

Figure 2. System designed for maintenance of hydrozoan colonies

2.2.2 *Determination of the experimental unit*

In order to test the effect of different types of food in the development of the colonies it was necessary to employ colony segments with the same number of polyps. The minimum number of polyps that allowed the development of new colonies was calculated. For this, two experimental groups were established, each with three replicates of segments with 1, 5, 10, 15, 20, 25 and 30 polyps by segment. They were independently attached to pieces of PVC with nylon (Figure 3), without knotting to avoid damaging the stolon, and fed with live *Artemia* nauplii every two days. The survival of the polyps was evaluated over a period of 15 days.

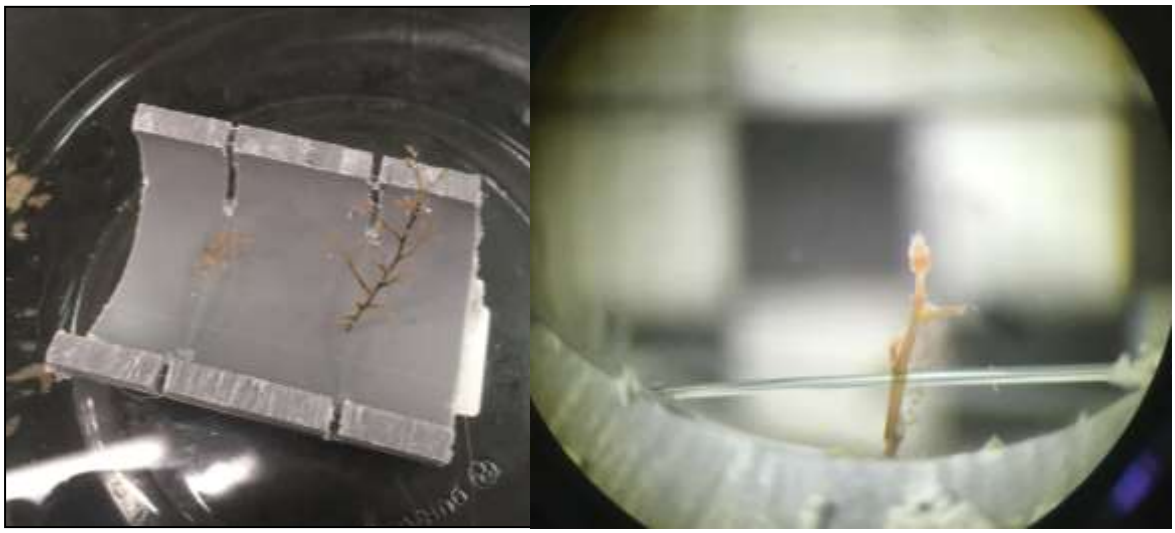

Figure 3. Colony segments attached to the PVC pieces

2.2.3 *Determination of the sample size*

A pilot feeding experiment was carried out during ten days to elaborate a preliminary ANOVA and to be able to estimate the size effect. Then, the data obtained were tested using the software G*Power (Version 3.1.9.2) in order to estimate the sample size necessary to obtain statistically representative results.

2.3 *Feeding experiment*

Once the experimental unit and the sample size were defined, we extracted 39 colony segments, with 22 polyps each, from colonies of *Eudendrium carneum* collected in the port of Valencia. The segments were divided into three groups and maintained during 39 days. The control group (Group V) was fed with nauplii of 48-hour hatched *Artemia* whereas the other two groups (N and P) were fed with frozen nauplii and commercial gilthead seabream pellets, respectively. These three groups were maintained in three water systems (Figure 4) with identical conditions (container of 30x18x12 cm, four liters of artificial sea-water, and water flow generated by a 9V pump). Each container had the same number of colony segments off each group, for a total of 13. To avoid pseudoreplication (Hulbert, 1984) the individual position of the segments within the container was randomly varied after each feeding event.

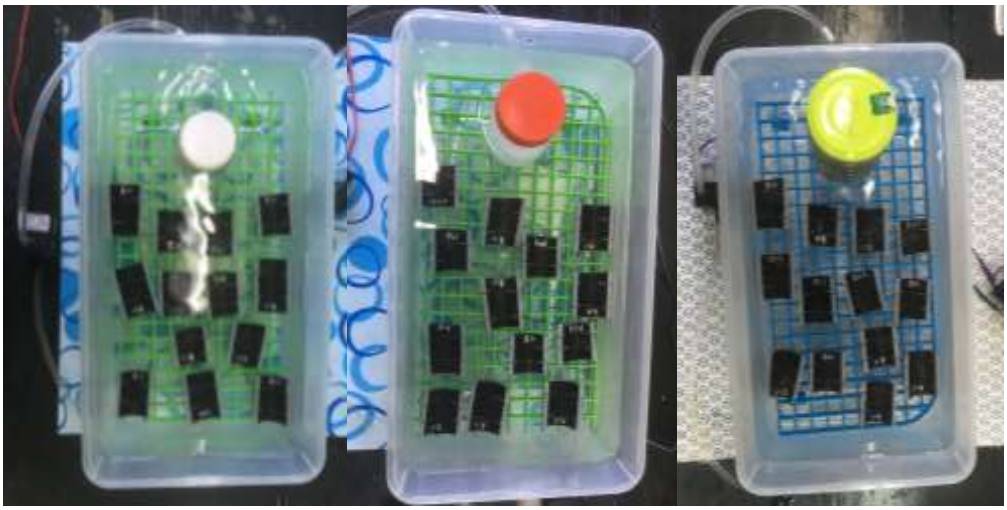

Figure 4. Layout of the colony segments during the feeding experiment

The colony segments were fed every two days at the same hour during the experiment. For feeding, each group was separated into different containers, in which the colonies were sprayed with the corresponding type of food. For groups N and P an aerator was connected to the feeding containers to keep the particles in suspension and in contact with the colonies.

To test the effect of the type of food in the growth rate of the colonies, the number of polyps in each segment was counted every two days.

2.4 *Diets*

The commercial gilthead seabream food was provided by the Aquaculture and biodiversity research group from the Institute of Animal Science and Technology of the Polytechnic University of Valencia (Spain). The composition of this food is shown in Table 1.

Table 1. Proximate composition (expressed as percentage of wet weight) of commercial gilthead seabream food, and live *Artemia* from San Francisco bay (SFB) (Gallagher and Brown, 1975).

	Crude protein (CP)	*Crude lipid (CL)*	*Ash (As)*	*Crude fibre (CF)*	*Nitrogen free (NFE)*
Sea bream commercial food	48	23	11	2,2	14
Live Artemia from SBB	43	19,3	20,6	3,5	-

The *Artemia* cysts used were from the commercial firm "Ocean Nutrition", settled in San Francisco. According to the manufacturer, the cysts do not correspond to a single species of *Artemia*, so they present it as *Artemia* sp. The reported composition for this type of food is shown in Table 1.

2.5 *Parameters calculated*

The 48-hour variation in the mean specific growth rate, *K*, was calculated with an adapted Radford growth equation (Stebbing, 1981; Piraino, 1991):

$$K = \Delta \ln W / \Delta t, \text{ where } W = \text{number of polyps}$$

In addition, it was studied if there were significant differences in the number of polyps after the 39-day experimental period due to food type as an indicator of the growth rate.

2.6 *Statistical analysis*

The effect of the different feeding regimens was analyzed by one-way ANOVA using the above parameters and the significance of the differences in mean values was analyzed by Neuman-Keuls test. The statistical assays were carried out with Statgraphics (Statgraphics Centurion XVI version 16.2.04) taking P-value of 5%.

3. Results

3.1 *Experimental unit determination*

From the first day of the experiment the number of polyps of all the colony segments decreased. For the segments with 1 and 5 polyps the survival was always zero. In the segments with 10 polyps there was a low survival until day 9, when all the polyps were dead, similarly occurred in segments with 15 polyps, where all polyps had disappeared by day 13. For segments with 20, 25 and 30 polyps, these survived during the 15 days of experiment. For the last day, the greatest survival was observed in colony segments with 20 polyps, with a value of 25% (Figure 5).

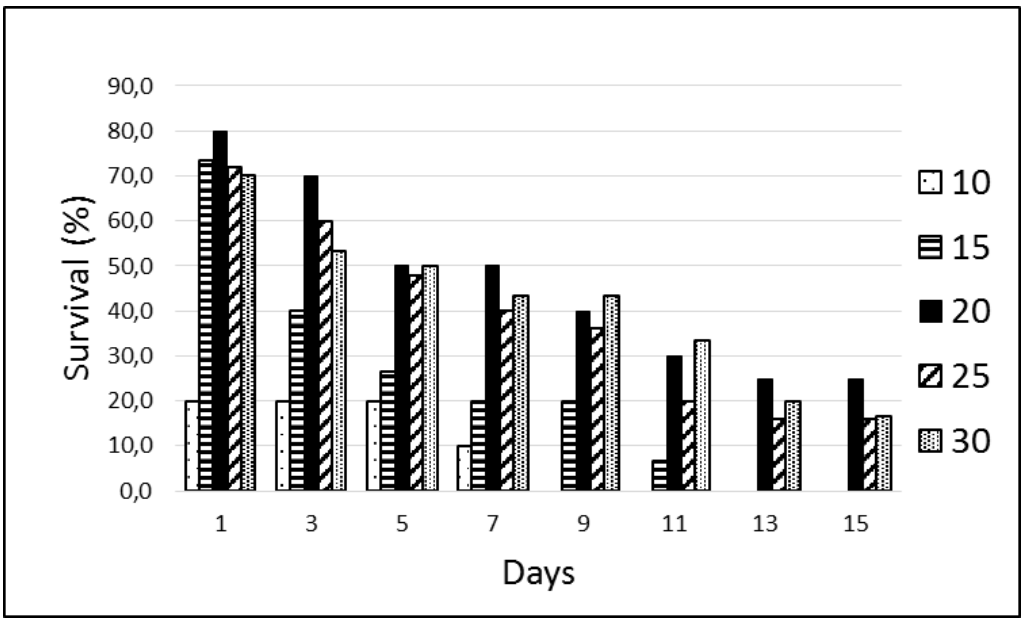

Figure 5. Survival variation in *E. carneum* colony segments with different initial number of polyps.

3.2 *Sample size determination*

For the pilot feeding experiment the estimated effect size was 0.677. Using this result we determined the sample size, which was estimated in 39. It means that at least 39 colony segments were necessary to perform the feeding experiment correctly.

3.2 *Feeding experiment*

At the beginning of the experimental process, the number of polyps decreased in all groups reaching a minimum at day 15 (Figure 6). For groups N and V, minimum values of zero polyps per colony were observed, while for group P the minimum value observed was two polyps.

After day 15 the number of polyps in the colonies began to increase, reaching maximum values at day 35 with colonies with five polyps in group N, seven in group V and 20 polyps in

group P. After day 35 the growth rate stabilized for group P and began to decrease in the groups N and V (Figure 6).

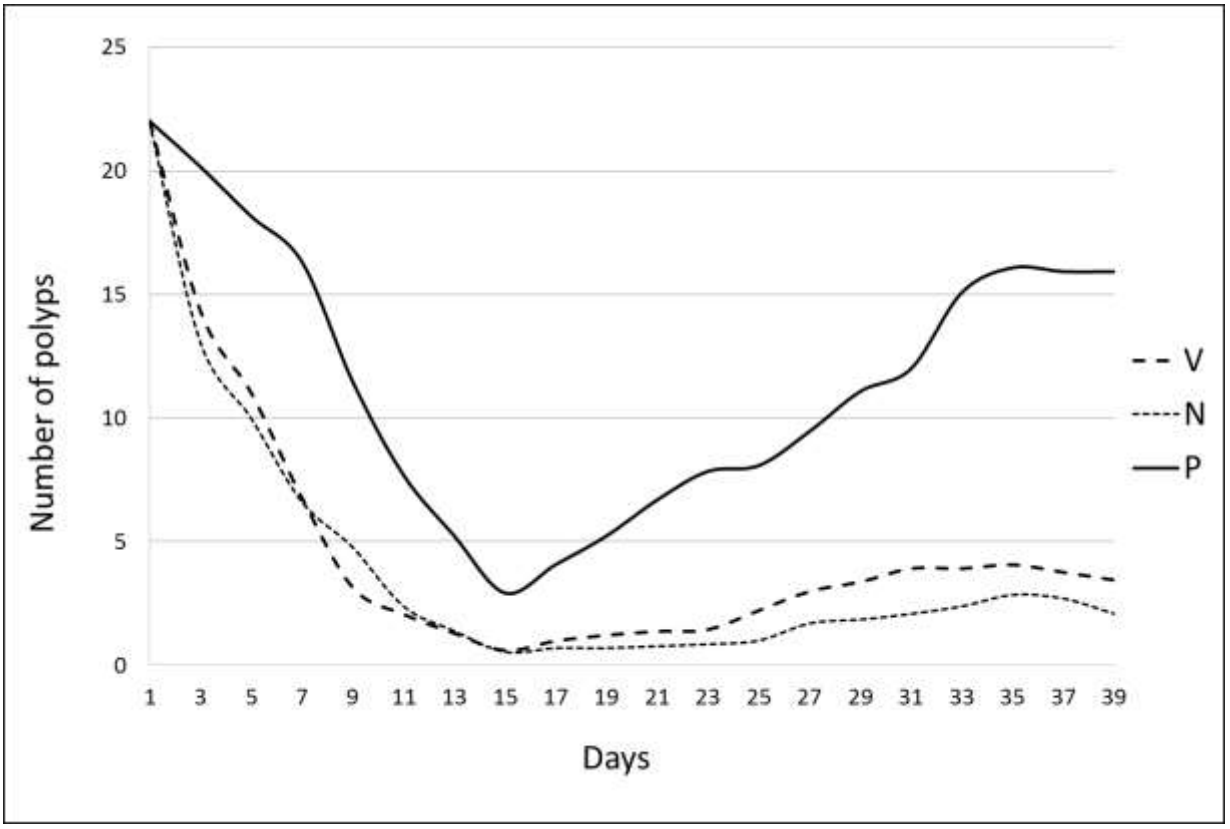

Figure 6. Variation in the number of polyps per colony segment in *E. carneum* during the 39 days of experiment (N correspond to the group feed with frozen *Artemia* nauplii, P correspond to the group feed with commercial gilthead seabream food, and V correspond to the control group feed with live *Artemia* nauplii)

Regarding the final number of polyps per colony segment, significant differences ($P \leq 0.05$) were observed in the group fed with gilthead seabream commercial food respect to both the N group and the control group. There were no significant differences between groups N and V (Tables 2-3).

Table 2. ANOVA statistical summary

Font	*F-ratio*	*P-value*	*Middle square*
Between groups	155.45	0.0000	756.0
Inside groups	-	-	4.86235

Table 3. ANOVA Multi-range testing

Contrast	*Sig.*	*Difference*
N-P	*	-13.8462
N-V		-1.38462
P-V	*	12.4615

The growth rate *K* was estimated and expressed in terms of percentage. Values above zero represent net growth while negative values correspond to net decrease. Net decrease was observed for all groups from day 1 to 15, when minimum values were reached. During these days the group P was always the one that showed smaller decrease. At day 39, groups V and N showed again net decrease while in group P there was no increase or decrease respect to day 38. The maximum value for net growth was observed for group V on day 17, reaching almost 25%, meanwhile the maximum decrease was also observed for group V on day 9, with a net decrease value of 38% (Figure 7).

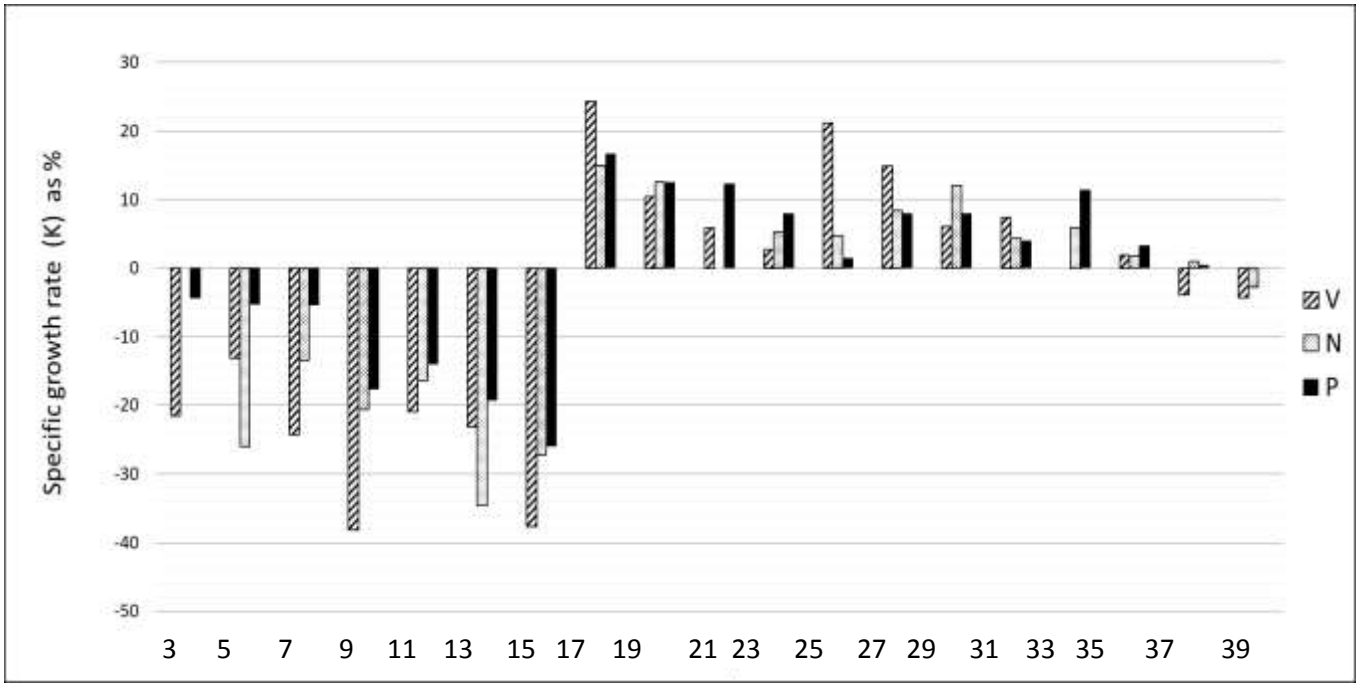

Figure 7. Pattern of specific growth rate (%) of colony segments exposed to two different diets and the control group. (**N** correspond to the group feed with frozen *Artemia* nauplii, **P** correspond to the group feed with commercial gilthead seabream food, and **V** correspond to the control group feed with live *Artemia* nauplii)

4. Discussion

When we look at the global picture of our feeding experiment, the first thing that stands out is the clear differentiation of three stages during the experimental period. A first stage of decrease during the first 15 days in which the number of polyps decreases until reaching zero in some cases (Figure 5), a following 20 days period of generalized growth, represented by the increase in the number of polyps in the colonies, and, finally, a terminal stage from day 35 on, where the growth begins to slow down until it stabilizes or decreases. This pattern agrees with observations done in other studies that evaluated, under natural conditions, colonies of *Eudendrium carneum*. These studies suggest that the abundance and vigor of the polyps decline during periods of stress, or in response to adverse conditions, but that, after these new polyps develop from dormant stolons and colonies are able to recover (Calder, 1990).

In 1973 Wyttenbach et al. studied the growth of stolons in *Eudendrium* colonies, finding significant differences between species regarding the form of stolon development, but with the existence of patterns of growth common to all. These cycles were constituted by a phase of decrease that lasted about 10 days, followed by one of growth of 15-20 days; after this the cycle was repeated. During our experiment, carried out under controlled conditions, we detected the same pattern described under natural conditions. This could suggest that changes in the net growth and decrease may be the result of the acclimatization to the new conditions and the effect of the type of food, while the duration of the cycle would be regulated by other biological factors and, as a consequence, has been the same for all the groups regardless of the type of food.

From the first stage of the cycle, it is pertinent to emphasize that polyps were always present when colony segments were fed with the commercial food for gilthead seabream. For some species of the genus *Eudendrium,* it has been described that the decrease in the number of polyps, during stress or adverse conditions, is because they are reabsorbed for establishing a resting hydrorizae that serves of latent structure (Bavestrello and Arillo, 1992). The fact that in group P the colonies never reach values of zero polyps, may indicate that these colonies do not need to reabsorb all polyps because they were in better nutritional conditions.

In aquaculture, when we refer to the nutritional value of food, one of the most important parameters is the composition of essential fatty acids (EFA). This is because many marine organisms need fatty acids, like eicosapentaenoic acid (EPA), arachidonic acid (ARA) and docosahexaenoic Acid (DHA), to perform physiological functions vital to their development (Léger *et al*, 1986; Navarro *et al*., 1993; Izquierdo, 1996). In our experiment three feeding regimens were applied, two of these based on *Artemia* nauplii (frozen and live) and other on commercial gilthead seabream food. By not applying enrichment processes, the *Artemia* nauplii used had a low nutritional value due to the absence of DHA and low levels of other

fatty acids like EPA and ARA (Monroig, *et al*., 2003, Monroig, *et al*., 2006; Viciano, *et al*., 2015). This can also be observed in the food composition shown in Tables 1, where we found a higher level of crude lipid in the gilthead seabream food compared to *Artemia* nauplii. In this way, the feeding of group P not only contributed more lipids, these were also of better quality (specifically because they contained DHA). About proteins, the diets do not exhibit a significant difference in terms of quantity, but it has been pointed out that the proteins present in *Artemia*, despite having a better digestibility because they are of low molecular weights, present an amino acid profile that does not usually meet the main needs of other marine organisms (Watanabe *et al*., 1983; Ortega, 2000).

Even taking into account all of the above, currently we have a lack of knowledge about the nutritional requirements of hydroids. For the group, the properties of some oxylipins are the chemotaxis and activation of cell differentiation. These components come from the oxidation of polyunsaturated fatty acids (PUFAs), and, therefore, these substances might be possible candidates as chemical mediators of hydroid typical biological processes like regeneration and asexual reproduction through bud formation, where cell movement and transdifferentiation play a major role (Bode *et al.,* 1986; Stanley-Samuelson, 1991; Gerwick *et al.,* 1993). For corals, the content of total lipids has been well studied and it varies with the season, depth, illumination, and even with the stage of development (Imbs, 2013). Regarding free fatty acids, it has been shown that these represent the major part of lipid composition in the toxin and nematocyst capsules of some cnidarians like *Physalia physalis* (Linnaeus, 1758). In general, organisms in a reproductive stage concentrated lipids in the gonads (5.5%-6.1%) and tentacles (4.1%). Of these fatty acids, DHA was the most abundant in almost all the species studied (Joseph, 1979).

All the studies carried out in this area lead to the same conclusion: in Cnidarians there is an extensive metabolism of lipids, which could suggest that these are important for their development, especially DHA, which cannot be found in *Artemia* nauplii. In contrast, commercial gilthead seabream food has high nutritional value due to its high content of fish oil, rich in essential fatty acids like DHA (Izquierdo *et al*., 2003; Izquierdo *et al*., 2005; Martínez-Llorens, *et al*., 2007; Montero *et al*., 2008). In the present study, the results show that in group P not only the decrease in number of polyps was smaller during the first 15 days, but also that, during the growth stage, it was the group with the greatest amount of polyps (20), and by the end of the experiment this group showed the higher growth rate until the beginning of the next cycle. Moreover, it is important to remark that the differences in the number of polyps in day 39 (Figure 6) between group P and the other two groups (N and V) were statistically significant, supporting the results obtained.

This evidence suggests that hydroids are not an exception and, as with the other marine groups, they benefit from a diet with a greater nutritional value.
The results obtained encourage us to think in new and better ways to keep hydroids in laboratory conditions, which will allow to improve knowledge in areas such as physiology, reproduction and life cycles that are not being studied in depth due to the difficulties of keeping them alive for long enough. Here we maintained colonies for 39 days with only one

event of feeding every two days. We suggest testing survival with one event of feeding by day to get closer to simulating natural conditions.

The future for studies related to hydroid physiology is promising, more now that aquaculture techniques allow us to establish long-term experiments to study different aspects *in vivo*. Specifically in relation to food, it will be pertinent to evaluate the effect of the gilthead seabream food on colony development in the development of colonies from other species in order to verify if it is actually a better feeding option for hydroids, in terms of nutritional value. On the other hand, it is also important to take into account the practical aspects of the feeding event itself. During the study it was necessary to grind the gilthead seabream food to obtain sufficiently small particles; it will be useful to determine exactly the optimal particle size and the best method to obtain them.

It is also advisable to incorporate elements to improve aspects of the maintenance system, one of the most important, the water quality. In this study we use water replacement to maintain water quality, but it will be prudent to use a filtration system and monitor parameters such pH, nitrites, nitrates and salinity.

Now that the first steps have been taken, the aquaculture of hydroids has only just begun. Multiple studies will be needed to determine the optimal parameters for each species of interest, a lot of work is still ahead, but it will be worthy. The possibilities are endless in terms of the knowledge that can be obtained from this type of studies. Feeding is only one of the applications, we can think of reproduction and life cycles, which certainly help us to solve taxonomy problems.

The aquaculture techniques are a powerful tool to hydroid studies. We must use them in the right way to take advantage of their potential in order to continue increasing our knowledge of this fascinating group.

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