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# *Broodstock conditioning of the Portuguese oyster Crassostrea angulata (Lamarck, 1819): influence of diets*

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2014

escola superior de turismo
e tecnologia do mar
instituto politécnico de leiria

# *Broodstock conditioning of the Portuguese oyster Crassostrea angulata (Lamarck, 1819): influence of diets*

**Catarina Miranda Castilho dos Anjos**

Dissertação para obtenção do Grau de Mestre em Aquacultura

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2014

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Escola Superior de Turismo e Tecnologia do Mar – Peniche, Instituto Politécnico de Leiria 2014

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**Resumo**

*Crassostrea angulata* comummente denominada por ostra portuguesa apresenta um enorme potencial para a indústria ostreícola. Na Europa, as populações puras desta espécie encontram-se restringidas ao sul de Portugal e Espanha, nomeadamente no Rio Sado, Rio Mira e Rio Guadalquivir. A conservação destas populações é importante para diversificar a produção e preservar a biodiversidade. Assim, é extremamente importante o desenvolvimento de técnicas de produção artificial de semente. O acondicionamento de reprodutores é uma fase fundamental na produção de bivalves em maternidade. Diversos fatores regulam o ciclo reprodutivo, sendo o alimento o mais importante. No entanto, a influência da qualidade nutricional do alimento (microalgas) no sucesso reprodutivo tem sido pouco explorado. De forma a avaliar os efeitos das diversas dietas no desempenho reprodutivo de *C. angulata*, os reprodutores foram acondicionados com diferentes regimes alimentares: Dieta 1: combinação bi-específica de *Pavlova lutheri* e *Isochrysis galbana* clone T-ISO (1:1); Dieta 2: combinação tri-específica de *P. lutheri, I. galbana* clone T-ISO e *Skeletonema costatum* (1:1:1); Dieta 3: combinação bi-específica de *S. costatum* e *Chaetoceros calcitrans* (1:1) e Dieta 4: combinação tri-específica de *P. lutheri, S. costatum* e *C. calcitrans* (1:1:1). Ao longo do acondicionamento foram recolhidas amostras de ostras para avaliar o índice de condição, o estado de desenvolvimento gonadal e a composição bioquímica. No final do período de acondicionamento, foi induzida a desova, visando a avaliação da fecundidade, taxa de fecundação e taxa de eclosão larva D. Os resultados mostraram heterogeneidade entre os reprodutores acondicionados com as diferentes dietas, tendo sido evidente o efeito das características nutricionais destas. As dietas tiveram impacto no processo de gametogénese e armazenamento de energia, sendo que os melhores resultados foram obtidos em reprodutores alimentados com dietas predominantemente constituídas por diatomáceas (Dieta 3 e Dieta 4). Os reprodutores que foram alimentados com dietas maioritariamente constituídas por flagelados (Dieta 1 e Dieta 2) demonstraram uma fraca performance reprodutiva. Na resposta à estimulação da postura, a fecundidade mais elevada foi observada no tratamento em que se utilizou a Dieta 4. No entanto, a maior taxa de eclosão larvar ocorreu no tratamento com a Dieta 3. O menor desempenho foi registado nos reprodutores alimentados com a Dieta 1, que não desovaram. Uma abordagem integral incorporando os resultados obtidos neste estudo, evidenciam e

reforçam a ideia de que o grupo das microalgas diatomáceas corresponde às necessidades nutricionais de *C. angulata*, sendo essencial para o seu acondicionamento.

**Palavras-chave:** *Crassostrea angulata*; Produção artificial; Acondicionamento dos reprodutores; Composição bioquímica; Desova; Dietas microalgais.

**Abstract**

The Portuguese oyster *Crassostrea angulata* shows great potential in oyster farming. In Europe, pure populations of this species were observed only in the southern coasts of Portugal and Spain, namely in Rio Sado, Rio Mira and Rio Guadalquivir. The conservation of *C. angulata* populations is important in the context of production diversification and biodiversity preservation. In this way the zootechnological development for seed hatchery production is extremely important. Broodstock conditioning is a key step in the process of rearing bivalves in hatchery. Many factors regulate the reproductive cycle, being food the most important. However the influence of the nutritional quality of different phytoplankton on reproduction success has been poorly explored. To evaluate the effect of different diets on *C. angulata* reproductive performance, broodstock were conditioned with different food regimes: Diet 1: bi-specific combination of *Pavlova lutheri* and *Isochrysis galbana* clone T-ISO (1:1); Diet 2: tri-specific combination of *P. lutheri*, *I. galbana* clone T-ISO and *Skeletonema costatum* (1:1:1); Diet 3: bi-specific combination of *S. costatum* and *Chaetoceros calcitrans* (1:1) and Diet 4: tri-specific combination of *P. lutheri*, *S. costatum* and *C. calcitrans* (1:1:1). During conditioning, samples of oysters were collected to evaluate condition index, the gonadal development and biochemical composition. At the end of the conditioning period oysters were induced to spawn to evaluate fecundity, fertilization and hatching rate. Results showed heterogeneity on the reproductive performance of *C. angulata* fed with different diets. The diets had an impact on the gametogenesis process and energy storage, being the best results obtained in broodstock fed with the diets predominantly diatoms (Diet 3 and Diet 4). Whereas those fed with diets majority flagellates (Diet 1 and Diet 2) had an unsuccessfully performance. The highest fecundity was observed with females that fed Diet 4. However the highest hatching rate was obtained with the Diet 3. The lowest reproductive output performance was recorded for broodstock fed with the Diet 1, which did not spawn. Holistic approaches incorporating all results in this study reveal and reinforce the idea that the diatom microalgae group presented the nutritional requirements to *C. angulata* broodstock, being essential in the conditioning phase.

**Keywords:** *Crassostrea angulata*; Hatchery production; Broodstock conditioning; Biochemical composition; Spawning; Microalgae diets.

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## List of abbreviations and symbols

| | |
|---|---|
| AFDW | Ash free dry weight |
| ANOVA | Analysis of variance between groups method |
| ARA | Arachidonic acid |
| C.cal | *Chaetoceros calcitrans* |
| CI | Condition index |
| *Df* | Degrees of freedom |
| DGRM | Directorate-General for Natural Resources, Safety and Maritime Services |
| DHA | Docosahexaenoic acid |
| DW | Dry weight |
| EFAs | Essential fatty acids |
| EPA | Eicosapentaenoic acid |
| FAO | Food and agriculture organization |
| Fas | Fatty acids |
| GC | Gas chromatography |
| K-W | Kruskal-Wallis |
| NaOH | Sodium hydroxide |
| *P* | *P* value |
| Pav | *Pavlova lutheri* |
| PUFA | Polyunsaturated fatty acids |
| SD | Standard error |
| Skt | *Skeletonema costatum* |
| TCA | Trichloroacetic acid |
| T-ISO | *Isochrysis galbana* clone T-ISO |
| UV | Ultra violet |

## 1. Introduction

Fish food supply has grown dramatically (FAO, 2014), leading to an increase on the overexploited marine resources, with the increasing of world population. Aquaculture is defined as "the art of increasing and rearing aquatic animals and plants" (Barnabé, 1994) and seems to be the obvious way out of this problem. Nonetheless, according to Naylor et al. (2000) aquaculture is a possible solution, but also a contributing factor, to the collapse of fisheries stocks worldwide, depending on the type of aquaculture activity. There are four main areas of aquaculture production: algae, molluscs, crustaceans and fish (Barnabé, 1994; FAO, 2014). In the molluscs aquaculture, which are main filter feeders, the net contribution to global fish supplies and food security is great, because ecological impacts are small, relative to other forms of aquaculture (Naylor et al., 2000). This type of culture is the oldest form of aquaculture (Lubet, 1994; Knauer and Southgate, 1999), naturally with an extensive history. The production of bivalve molluscs is a strategic activity since it contributes significantly to the preservation of local economies and generates capital and employment on the littoral areas (Cardoso et al., 2013). Being an important area and rapidly expanding (Cyrus and Pelot, 1998; Hilgerloh et al., 2001; Helm et al., 2004). In 2012, molluscs accounted for 22.8% of world aquaculture production and the two major bivalve species groups produced were clams and oysters (FAO, 2014).

In Portugal, the production of bivalves is one of the most important social and economic activities, with a great growing potential as a fisheries subsector, due to the edaphic-climatic and geographic conditions. Artisanal production of bivalve molluscs is mainly based on the culture of the European clam (*Ruditapes decussatus*) and oysters (*Crassostrea* sp.). The main production areas of these species are the Ria de Aveiro (40º42'N 08º40'W), the Rio Sado estuary (37º43'N 08º17'W), the Ria de Alvor (37º07'N 08º36'W) and the lagoon system of the Ria Formosa (36°59'N 7°55'W). The Portuguese producers usually use three methods to rearing bivalves in an extensive way, these include: bottom culture and tray or bag culture, both undertaken in sheltered intertidal areas, pond culture in old fish farming and floating suspended cultivation in longlines used for suspended lantern net and raft (Schuller, 1998). The production of oysters represents 7.5% of the national annual marine aquaculture production and 18.8% of shellfish production (DGRM, 2014), with a crescent tendency. Until the 1970s, in Portugal and also in France *C. angulata*, Portuguese oyster was a major species for the shellfish industry, however this

species started to become affected by a viral disease in the late 1960s, and its exploitation collapsed. To overcome this situation the French producer introduced the Japanese oyster (*C. gigas*) to support local farming production (Grizel and Héral, 1991; Boudry et al., 1998; Batista et al., 2005). Nevertheless in Portugal, the *C. gigas* was illegally introduced in the 90s, since it is considered an exotic species. In the last four decades, it was been observed a slight recovery of the natural beds of *C. angulata*, especially in the Rio Sado estuary.

At the present, severe episodes of *C. gigas* mortality have been observed, especially in France, leading to a need to diversify oyster species production, in this way the Portuguese oyster *C. angulata*, could be a promising species in European aquaculture. Moreover, due to the fact that in Europe, pure populations of *C. angulata* were observed only in the southern coasts of Portugal and Spain (Boudry et al., 1998; Fabioux et al., 2002), namely in Rio Sado estuary, Rio Mira estuary **(**Fabioux et al., 2002**)** and Rio Guadalquivir (Michinina and Rebordinos, 1997), the conservation of pure populations of this species is also important in the context of biodiversity preservation (Batista et al., 2005).

The bivalve aquaculture industry depends on the availability of high quality of seed , which will grow rapidly to commercial size (Caers et al., 1999; Ojea et al., 2004). The zootechnological development for seed production on hatchery is extremely important (Marshall et al., 2010), to provide juveniles for commercial production, restoration projects and research (Wallace et al., 2008). The advantages of bivalve hatchery are numerous, and have been regarded as a "safety net" for the industry. Permits contradict the low availability of natural seed organisms that are unable to support commercial mass cultures, also allows the restoring of exhausted natural beds (Robert and Gérard, 1999; Velasco and Barros, 2007; Marshall et al., 2010; Prado et al., 2010). It also allows that larvae and spat are reared under controlled conditions, and the supply of genetic strains with improved biological characteristics, through breeding programs in broodstock (Robert and Gérard, 1999).

There are four essential phases in the operation of oyster seed production: broodstock conditioning, larval production, spat production and algal culture (Breese and Malouf, 1975; Utting and Spencer, 1991; Robert and Gérard, 1999; Helm et al., 2004; Joaquim et

al., 2008). Broodstock conditioning is a key step in the hatchery process (Helm et al., 2004; González-Araya et al., 2012a) and is intended to maximize fertility of the broodstock, while maintaining oocytes quality and larvae viability (Utting and Millican, 1997). The conditioning required to bring broodstock to the optimum stage of gonadal development.

According to Kennedy et al. (1996), the gonadal development depends on the synergetic effect of both internal and external factors. Specific endogenous rhythms synchronized by external factors, such as temperature, nutrition, animal health and others regulate the reproductive cycle (Breese and Malouf, 1975; Robert and Gérard, 1999; Chávez-Villalba et al., 2002; Joaquim et al., 2008), being food the most important (Utting and Millican, 1997; Marshall et al., 2010). Therefore, in the hatchery, a way to induce sexual maturation in bivalves is the manipulation of their physical and nutritional environments (Gallager and Mann, 1986; Helm et al., 2004).

The diet provided to adults can affect the biochemical composition of their gonads and the quality of eggs and larval viability. Until now no commercial formulated diet for bivalves is available and hatcheries mostly rely on the use of microalgae (Muller-Fuega, 2000; Brown, 2002; Pronker et al., 2008). Feeding bivalves with a mixture of microalgae has become a common practice in hatcheries since microalgae's species vary considerably in their nutritional value and seems that optimal food conditions can only be obtained by mixing species (Benemann, 1992**;** Utting and Millican, 1997; Knauer and Southgate, 1999; Brown, 2002; Helm et al., 2004; Spolaore et al., 2006; Joaquim et al., 2008).

Cultured marine algae species are used as sole food supply during conditioning period (Utting and Spencer, 1991; Robert and Gérard, 1999; Helm et al., 2004). They must have an appropriate size and shape for ingestion, high nutritional qualities, be readily digested, need to be easily cultured and absence of toxins (Brown, 2002; Spolaore et al., 2006). The most desirable microalgae in a hatchery are *Isochrysis galbana*, *I. galbana* clone (T-ISO), *Tetraselmis suecica* (Benemann, 1992; Brown, 2002; Spolaore et al., 2006), *Pavlova lutheri, Chaetoceros calcitrans, Skeletonema costatum* (Brown, 2002; Spolaore et al., 2006). Broodstock requires 2-6% of the oyster dry meat weight in dry weight of microalgae (Utting and Spencer, 1991; Utting and Millican, 1997; Helm et al., 2004) of food ration when being conditioned and a certain nutritional qualities are necessary to ensure proper gametogenesis (Robert and Gérard, 1999). The biochemical composition of

the diet influences the physiology of bivalves, particularly if a minor component is lacking. However, the composition of the specific forms of those major components (proteins, carbohydrates and lipids) can also be of influence (Matias et al., 2009; Joaquim et al., 2011). Lipids are usually used as an energy source during gametogenesis (Holland, 1978; Martínez et al., 2000; Delgado et al., 2004), and constitute the principal nutritional reserve in eggs and larvae, conditioning their viability (Matias et al., 2011). Despite the lack of studies in *C. angulata*, it is clear that the best diets for bivalve conditioning are those with high essential fatty acids (EFAs), particularly eicosapentaenoic acid, 20:5(n-3) (EPA), docosahexaenoic acid, 22-6(n-3) (DHA) and arachidonic acid 20:4(n-6) (ARA), since the adults bivalves are limited or unable to produce these de *novo* from shorter chain pre-cursors (Utting and Millican, 1997; Utting and Millican, 1998; Brown, 2002; Spolaore et al., 2006). Due to this reason, they must be provided exogenously since the quantity and quality of lipid in microalgae diet will influence the polyunsaturated fatty acid (PUFA) composition of the broodstock gonad and eggs (Utting and Millican, 1997; Utting and Millican, 1998).

The present study was designed to evaluate the effect of different diets on the reproductive output of *C. angulata* and express the evolution of the different biochemical composition (proteins, glycogen, lipid and fatty acids) during sexual maturation, aiming to find a broodstock conditioning diet that maximizes fecundity and egg quality being suitable to be used in commercial hatcheries.

## 2. Materials and methods

### 2.1. Experimental design

Six hundred and twenty-two adult oysters, *C. angulata*, were collected in Ria de Alvor (37º07'N 08º36'W) (Portugal). However, these animals were initially collect from the Monte da Pedra, Alcácer channel in Rio Sado estuary (37º43'N 08º17'W). This is one of the few pure natural beds of oyster *C. angulata* (Batista, 2007). The oysters were transferred one year before harvesting to Ria de Alvor, aiming to obtained optimal biological condition.

Five hundred and forty oysters were randomly distributed into four conditioning groups. *C. angulata* broodstock were conditioned with four nutritional regimes: Diet 1 - bi-specific combination of *Pavlova lutheri* (Pav; size:4×6 μm; dry weight:102.3 pg) and *I. galbana* clone T-ISO (T-ISO; size:3×5 μm; dry weight: 30.5 pg); Diet 2 - tri-specific combination of Pav, T-ISO and *Skeletonema costatum* (Skt; size:10×5 μm; dry weight: 52.2pg); Diet 3 - bi-specific combination of Skt and *Chaetoceros calcitrans* (C.cal; size:3-6 μm; dry weight: 11.3 pg) and Diet 4 - tri-specific combination of Skt, C.cal and Pav. The mixed diets were constituted in a proportion of 1:1 or 1:1:1 dry weight of respective microalgae species. The dry weight and size are reported in Brown et al. (1997).

Each group of adults oysters (66.1 g ± 9.2 mean whole weight, 0.6 ± 0.2 mean meat dry weight, 79.2 ± 8.1 mean length) were distributed homogeneously in triplicate tank (25 l) per experimental condition. The tanks containing natural seawater filtered through 1 μm, in a flow-through circuit at a rate of 0.8 l $min^{-1}$. Seawater temperature was maintained at 21±1 ºC by a heat exchanger with titanium plates. The food was added to the circulation water by means of a variable-flow peristaltic pump, in a ratio of 4% of the oyster dry meat weight (g) in dry weight of microalgae (mg) (Utting and Millican, 1997; Helm et al., 2004). In order to maintain constant the food ration in each condition, total food varied as the condition progressed and oyster were removed in successive samplings. *C. angulata* broodstock conditioning was held over eleven weeks-period from November 2013 to February 2014. During conditioning, the first sample was obtained at the beginning (week 0) and at the weeks 5 and 9 and at the end of the conditioning period (week 11). At each sampling time, three groups of ten oysters were randomly selected for histological study of

gonadal development, to determination of condition index and biochemical composition (proteins, glycogen, lipids and fatty acids). These were then stored at – 20 ºC until prior analysis.

Microalgae were batch cultured in 80 l plastic bag. The filtered (1 µm), UV-treated seawater (salinity 35) was chlorinated for 24 h, neutralized with thiosulfate and enriched with $F_2$ medium before inoculation. Continuous aeration was provided to enhance growth and prevent algae from settling. Microalgae were grown under continuous light at an intensity of 9900 lux at the culture surface, with a temperature of 20±2 ºC and was harvested daily in the exponential growth phase. Before being used as food, algal densities were determined daily by standard algal cell counts (Büker chamber). During the conditioning period, each diet was collected for biochemical characterization (proteins, carbohydrates, lipids and fatty acids). These samples were centrifuged, resuspended with 0.5M ammonium formate and stored at – 20 ºC.

### 2.2. Spawning and larval rearing

At the end of the experimental period, mature bivalves taken from each treatment were placed in a spawning tank (36±2 specimens). Spawning induction was triggered by thermal shock, through a rapid increase of temperature from 15 °C to 30±2°C at each interval of 2 h. Individual that responded to the stimulus for the release of sperm or eggs were separated into individual receptacles, to avoid self-fertilization. When spawning is completed, the oocytes from each female were passed through a 100 µm nylon mesh sieve and retaining on 20 µm mesh sieve and resuspended with filtered seawater (0.45 µm) in a known volume. To evaluate fecundity, three 50 µl samples of each oocyte suspension by female were taken and counted. The same procedure was done for males; however spermatozoids passed only through a 100 µm nylon mesh sieve. The sexual product from all males of the same nutritional regime was mixed together.

Fertilization of oocyte from each female by nutritional regime was carried out keeping an oocyte/spermatozoid ratio of 1:10 (Matias et al., 2009). After 1 h of fertilization, three 50 µl samples were taken to evaluate fertilization rate. Embryos from each female and from each diet were incubated in triplicate 500 ml containers, with 0.45 µm filtered and UV-irradiated seawater, maintained at 20±2°C, at a density of 100 eggs per ml. After 48 h, the containers were emptied, and D-larvae were recovered by sieving on a

30 μm mesh screen. Three 100 μl aliquots from each treatment were taken to calculate the veliger rate (% of D-larvae) relative to the initial number of eggs.

## 2.3. Analytical procedures

### 2.3.1. Histology

Each oyster was carefully opened and visceral mass was excised. They were thereafter fixed in Davidson´s solution (Shaw and Battle, 1957) for at least 48h. Samples were dehydrated with a series of ethanol treatments of increasing concentration, cleared in toluene and embedded in paraffin. Finally, 5-6 μm sections were cut, mounted on glass slides and stained with haematoxylin-eosin (Martoja and Martoja, 1967). Sections were examined under a microscope for sex determination (male, female or hermaphrodite) and to evaluate gonadal development stage. The gonadal stages were classified according to Mann (1979), which was as follows in Table I.

Table I. Reproductive scale for *Crassostrea angulata* development based on Mann (1979).

| Stages | Histologic Description |
|---|---|
| Resting stage | |
| **0** | There is no trace of sexuality; follicles are non-existent or elongated and consist of undifferentiated germinal epithelium. |
| Early growth stage | |
| **I** | Follicles are small and isolated with numerous spermatogonia or oogonia. |
| Late growth stage | |
| **II** | Follicles actively develop with primary gametes and some free (secondary) oocytes and spermatozoa. |
| Maturation stage | |
| **III** | Near ripe or ripe follicles are densely packed with matured gametes; presence of oocytes with distinct nucleus and nucleolus, spermatozoa are oriented with tails toward the follicle lumen. |
| Spawning and reabsorbing stage | |
| **IV** | Follicles are distended and some are broken; however, numerous gametes may still remain. In some cases, redevelopment takes place with increased number of primary oocytes and spermatocytes. In other cases, gametes are refractory, re-development is not obvious, and phagocytes are present. |

### 2.3.2. Condition index

Condition index of oysters was calculated as outlined by Walne and Mann (1975): [ash free dry weight (AFDW) of meat (g)/dry shell weight (g)] × 100.

Each individual was carefully opened, all the flesh removed from the shells and placed on absorbent paper, to drain for 5 min. The soft tissues and the shell were dried at 80°C for 24 h and then weighted. Then the meat was ashed in a muffle furnace at 450°C for 24h and re-weighted.

### 2.3.3. Biochemical composition

Hundred and thirty oyster specimens were unfrozen, opened carefully and the entire soft body was removed from the shell. The body was homogenized in an ice bath and four aliquots were stored at – 20ºC for use in biochemical analysis. Proteins, glycogen, total lipids and fatty acids were determined by standard methods. Proteins were determined using the modified Lowry method (Shakir et al., 1994), after extraction with normal sodium hydroxide. Glycogen content was determined from dried flesh (80°C for 24 h) homogenate using the anthrone reagent (Viles and Silverman, 1949). Total lipids were extracted from fresh homogenized material, following Bligh and Dyer (1959) and were estimated spectrophotometrically on a microplate reader at 375 nm using tripalmitin as a standard, after charring with concentrated sulphuric acid (Marsh and Weinstein, 1966). Biochemical composition results are the mean of duplicate determination and expressed as a total organic ash free dry weight (µg $mg^{-1}$ of AFDW).

Each microalgae diet biochemical composition was evaluated by a micro-analytical fractionated extraction scheme developed by Holland and Gabbott (1971) and Holland and Hannant (1973). Lyophilized diets samples were homogenized in 500 µl distilled water using a sonicator. Each homogenate was separate in two samples (200 µl). One of these samples were taken for determine proteins and carbohydrates and the other for the analysis of total lipids. Protein concentration was assayed by the method of Lowry et al. (1951), modified by Bensadoun and Weinstein (1976) and Hess et al. (1978). Proteins were precipitated by cold 5% trichloroacetic acid (TCA) and the precipitate washed in warm 1.0 N NaOH. This was read spectrophotometrically at 750 nm using serum albumin as a standard. Hydrolysed samples of TCA supernatant were used for the determination of carbohydrates by a

modification of the method of Folin and Malmros (1929). This element was quantified with a ferricyanate reduction reaction at 420 nm using glucose as a standard. Total lipid content was extracted by the method of Bligh and Dyer (1959), using tripalmitin as a standard and the absorbance determined at 375 nm. Results are the mean of duplicate determination and expressed as a percentage of total organic dry weight (% of DW).

The extraction and analysis of fatty acids (FAs) were performed according to a modified Lepage and Roy (1986) procedure as described by Masood et al. (2005). This method, for the oyster sample, was carried out in duplicate on pooled material of ten individuals and for diets done in duplicate for each microalgal diet sample. The sample was combined with a mixture of methanol:acetyl chloride (20:1) and heated at 80ºC for one hour. Heptane was added and centrifuge. The upper organic phase was collected and transferred to GC vials and was injected into the chromatograph. After transesterification, the separation on FAs was carried on a Finnigan TRACE GC Ultra gas chromatograph, with a TR-FAME column (30mx0.25mm IDx0.25m).The carrier gas was helium and the device operates with a temperature program. FAs were detected by flame ionization and identified by comparing their retention time with standards [PUFA-1 Marine Source e PUFA-3 Menhaden oil (Supelco Analytical)]. The FAs compositions were expressed as the absolute (μg $mg^{-1}$) and relative contents (% of total FAs) of the total fatty acids of each lipid fraction.

### 2.4. Statistical analysis

Results are expressed as mean±standard error of the mean. Differences in condition index, biochemical composition (diets and broodstock), number of oocytes released, fertilization and veliger rate were tested by analyses of variance (ANOVA) or Kruskal–Wallis ANOVA on ranks, whenever the assumptions of ANOVA failed among sampling times of the same nutritional regime and between regimes. Multiple pairwise comparisons were performed using the post-hoc parametric Tukey test or the non-parametric Dunn's test. Percentage data were arcsine transformed to normalize variance (Sokal and Rohlf, 1981). *P* value being set at 0.05. Statistical analyses of the data were carried out using the SIGMASTAT 3.11 statistical package.

# 3. Results

## 3.1. Biochemical composition of the diets

The biochemical composition of the diets, specifically proteins, carbohydrates and total lipids are presented in Table II and fatty acids listed in Table III. Proteins were the predominant constituent of the microalgae followed by total lipids and carbohydrates. Lowest values of proteins and carbohydrates were observed in Diet 3 and the lowest values of total lipids were registed in the Diet 2. The diets formulated with the majority of diatoms (Diet 3 and Diet 4) presented lowest values of proteins comparatively with the diets constituted fundamentally by flagellates (Diet 1 and Diet 2). Significant differences were observed among diets, between flagellate diet (Diet 1), predominantly diatoms diets (Diet 3 and Diet 4) and between Diet 2 and Diet 3 (K-W, $H$=17.51, $df$=3, $P \leq 0.001$). The Diet 4 showed higher proportions of carbohydrates relatively to the other diets, however significant differences were only detected between Diets 3 and 4 (K-W., $H$=10.78, $df$=3, $P$=0.013). Also, the Diet 4 showed the highest level of total lipids, but no significant differences among diets were observed (ANOVA, $F$=1.08, $df$=3, $P$=0.38).

Table II. Biochemical composition (mean±SD, $n$=3) of the different food regimes: Diet 1 (Pav and T-ISO); Diet 2 (Pav, T-ISO and Skt); Diet 3 (Skt and C.cal); Diet 4 (Skt, C.cal and Pav). The values were expressed in relative contents (% of dry weight).

| | **Diets biochemical composition** | | |
|---|---|---|---|
| | **Proteins (%)** | **Carbohydrates (%)** | **Total Lipids (%)** |
| Diet 1 (Pav and T-ISO) | 26.79 ± 5.32 | 1.21 ± 0.77 | 11.58 ± 2.05 |
| Diet 2 (Pav, T-ISO and Skt) | 24.24 ± 2.06 | 2.61 ± 0.71 | 9.70 ± 1.26 |
| Diet 3 (Skt and C.cal) | 17.11 ± 0.85 | 0.56 ± 0.14 | 11.32 ± 3.33 |
| Diet 4 (Skt, C.cal and Pav) | 17.37 ± 1.06 | 3.30 ± 0.51 | 12.27 ± 2.77 |

The % of total FAs in each diet differed according with the predominance of the species found in each food regimes (Table III). The species of Diet 1 were only constituted by flagellates. This diet was rich in 14:0, 16:0, 18:1(n-9), 18:2(n-6), 18:3(n-3), 18:4(n-3) and 22:6(n-3) FAs (9.76±0.45%, 10.96±0.06%, 8.33±0.03%, 6.80±0.06%, 7.13±0.01%, 24.50±0.01%, 8.69±0.33%, respectively) and poor in 20:5(n-3) (0.80±0.01%). Also Diet 2 (predominantly formulated by flagellates) showed similar composition with Diet 1, but the content of 18:2(n-6) (4.17±0.01%) was lower, contrary the 16:1(n-7) (14.65±0.06%) and

20:5(n-3) FAs (6.23±0.04%) values were higher. The diets constituted fundamentally by diatoms (Diet 3 and Diet 4) were richer in 16:1(n-7) (Diet 3 – 33.13±0.02%, Diet 4 – 30.46±0.06%) and 20:5(n-3) FAs (Diet 3 – 9.36±0.14%, Diet 4 – 10.77±0.01%) than the diets formulated fundamentally by flagellates (Diet 1 and Diet 2). Diet 3, constituted only by diatoms also presented high value content of 16:0 FA (19.43±0.52%) and low levels of 18:2(n-6) (0.86±0.00%) and 22:6(n-3) FAs (0.41±0.01%). Relatively to Diet 4 formulated with two diatoms and one flagellate, the values are similar to the Diet 3 however the value level of 22:6(n-3) FA (0.90±0.01%) was higher in Diet 4. Therefore the Diet 1 and Diet 2 were characterized with high 22:6/20:5 ratio (Diet 1 – 10.85±0.22%, Diet 2 – 0.84±0.00%) and with low (n-3)/(n-6) ratio (Diet 1 – 4.09±0.03%, Diet 2 – 4.59±0.05%). In the contrary, Diet 3 and Diet 4 were characterized with low 22:6/20:5 ratio (Diet 3 – 0.04±0.00%, Diet 4 – 0.08±0.00%) and high (n-3)/(n-6) ratio (Diet 3 – 7.55±0.10%, Diet 4 – 7.92±0.39%). Statistically the differences were not evident ($P>0.05$).

Table III. Total fatty acid composition (mean±SD, *n*=2) of the different food regimes: Diet 1 (Pav and T-ISO); Diet 2 (Pav, T-ISO and Skt); Diet 3 (Skt and C.cal); Diet 4 (Skt, C.cal and Pav). The values were expressed in mean absolute (µg $mg^{-1}$) and relative contents (% of total FAs).

| | **Diet 1** (Pav and T-ISO) | | **Diet 2** (Pav, T-ISO and Skt) | | **Diet 3** (Skt and C. cal) | | **Diet 4** (Skt, C. cal and Pav) | |
|---|---|---|---|---|---|---|---|---|
| **Fatty acids** | **Contents** (µg $mg^{-1}$) | **Rel. Contents** (%) | **Contents** (µg $mg^{-1}$) | **Rel. Contents** (%) | **Contents** (µg $mg^{-1}$) | **Rel. Contents** (%) | **Contents** (µg $mg^{-1}$) | **Rel. Contents** (%) |
| 12:0 | 0.19 ± 0.02 | 0.16 ± 0.01 | 0.16 ± 0.01 | 0.17 ± 0.01 | 0.63 ± 0.00 | 0.56 ± 0.00 | 0.69 ± 0.02 | 0.56 ± 0.01 |
| 14:0 | 11.29 ± 0.52 | 9.76 ± 0.45 | 8.10 ± 0.05 | 8.35 ± 0.05 | 9.14 ± 0.20 | 8.08 ± 0.18 | 11.16 ± 0.06 | 9.10 ± 0.05 |
| 16:0 | 12.68 ± 0.07 | 10.96 ± 0.06 | 12.61 ± 0.04 | 13.00 ± 0.04 | 22.00 ± 0.58 | 19.43 ± 0.52 | 18.41 ± 0.09 | 15.00 ± 0.07 |
| 16:1 (n-9) | 0.17 ± 0.01 | 0.15 ± 0.01 | 0.06 ± 0.00 | 0.06 ± 0.00 | 0.01 ± 0.02 | 0.01 ± 0.01 | 0.02 ± 0.03 | 0.02 ± 0.02 |
| 16:1 (n-7) | 5.96 ± 0.07 | 5.15 ± 0.06 | 14.21 ± 0.06 | 14.65 ± 0.06 | 37.51 ± 0.02 | 33.13 ± 0.02 | 37.38 ± 0.08 | 30.46 ± 0.06 |
| 16:2 (n-7) | 0.14 ± 0.01 | 0.13 ± 0.01 | 0.11 ± 0.01 | 0.12 ± 0.01 | 0.10 ± 0.01 | 0.09 ± 0.01 | 0.07 ± 0.00 | 0.06 ± 0.00 |
| 16:2 (n-4) | 1.23 ± 0.01 | 1.07 ± 0.01 | 1.77 ± 0.03 | 1.82 ± 0.03 | 2.67 ± 0.02 | 2.36 ± 0.01 | 3.52 ± 0.01 | 2.87 ± 0.01 |
| 18:0 | 2.30 ± 0.02 | 1.99 ± 0.01 | 3.41 ± 0.01 | 3.52 ± 0.01 | 5.27 ± 0.06 | 4.66 ± 0.05 | 4.03 ± 0.03 | 3.28 ± 0.03 |
| 18:1 (n-9) | 9.64 ± 0.03 | 8.33 ± 0.03 | 5.31 ± 0.06 | 5.48 ± 0.06 | 1.52 ± 0.02 | 1.34 ± 0.01 | 1.52 ± 0.04 | 1.24 ± 0.04 |
| 18:1 (n-7) | 2.40 ± 0.07 | 2.07 ± 0.06 | 1.32 ± 0.01 | 1.37 ± 0.01 | 0.29 ± 0.02 | 0.26 ± 0.01 | 0.71 ± 0.02 | 0.58 ± 0.01 |
| 18:2 (n-6) | 7.87 ± 0.07 | 6.80 ± 0.06 | 4.05 ± 0.01 | 4.17 ± 0.01 | 0.97 ± 0.00 | 0.86 ± 0.00 | 1.31 ± 0.00 | 1.07 ± 0.00 |
| 18:3 (n-6) | 0.42 ± 0.01 | 0.37 ± 0.01 | 0.45 ± 0.00 | 0.46 ± 0.00 | 0.20 ± 0.00 | 0.18 ± 0.00 | 0.39 ± 0.00 | 0.32 ± 0.00 |
| 18:3 (n-3) | 8.25 ± 0.02 | 7.13 ± 0.01 | 4.08 ± 0.01 | 4.21 ± 0.01 | 0.07 ± 0.01 | 0.07 ± 0.01 | 0.41 ± 0.01 | 0.34 ± 0.01 |
| 18:4 (n-3) | 28.35 ± 0.01 | 24.50 ± 0.01 | 13.46 ± 0.10 | 13.87 ± 0.10 | 0.25 ± 0.01 | 0.23 ± 0.01 | 0.96 ± 0.02 | 0.78 ± 0.01 |
| 20:0 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.38 ± 0.01 | 0.40 ± 0.01 | 0.29 ± 0.02 | 0.26 ± 0.01 | 0.57 ± 0.01 | 0.47 ± 0.01 |
| 20:2 (n-6) | 0.01 ± 0.02 | 0.01 ± 0.01 | 0.05 ± 0.00 | 0.05 ± 0.00 | 0.07 ± 0.01 | 0.07 ± 0.01 | 0.07 ± 0.01 | 0.06 ± 0.01 |
| 20:4 (n-6) | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 |
| 20:5 (n-3) | 0.93 ± 0.02 | 0.80 ± 0.01 | 6.04 ± 0.03 | 6.23 ± 0.04 | 10.60 ± 0.16 | 9.36 ± 0.14 | 13.22 ± 0.02 | 10.77 ± 0.01 |
| 22:0 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.16 ± 0.18 | 0.16 ± 0.18 | 0.32 ± 0.45 | 0.28 ± 0.40 | 0.28 ± 0.40 | 0.23 ± 0.33 |
| 22:2 (n-6) | 0.07 ± 0.00 | 0.06 ± 0.00 | 0.03 ± 0.00 | 0.03 ± 0.00 | 0.02 ± 0.00 | 0.02 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 |
| 22:4 (n-6) | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.07 ± 0.01 | 0.08 ± 0.01 | 0.19 ± 0.02 | 0.17 ± 0.01 | 0.17 ± 0.00 | 0.14 ± 0.00 |
| 22:5 (n-6) | 1.57 ± 0.11 | 1.36 ± 0.09 | 0.81 ± 0.00 | 0.84 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.11 ± 0.00 | 0.09 ± 0.00 |
| 22:5 (n-3) | 0.06 ± 0.01 | 0.06 ± 0.01 | 0.05 ± 0.01 | 0.06 ± 0.01 | 0.05 ± 0.01 | 0.05 ± 0.01 | 0.18 ± 0.02 | 0.15 ± 0.01 |
| 22:6 (n-3) | 10.05 ± 0.38 | 8.69 ± 0.33 | 5.06 ± 0.02 | 5.22 ± 0.02 | 0.46 ± 0.02 | 0.41 ± 0.01 | 1.10 ± 0.01 | 0.90 ± 0.01 |
| 24:0 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.01 | 0.01 ± 0.01 | 0.01 ± 0.01 | 0.01 ± 0.01 | 0.00 ± 0.00 | 0.00 ± 0.00 |
| Total saturated | 27.40 ± 0.61 | 23.67 ± 0.52 | 27.70 ± 0.16 | 28.55 ± 0.17 | 42.41 ± 0.80 | 37.45 ± 0.71 | 42.12 ± 0.39 | 34.32 ± 0.32 |
| Total mono | 32.16 ± 0.26 | 27.78 ± 0.23 | 34.33 ± 0.07 | 35.38 ± 0.07 | 68.55 ± 0.65 | 60.54 ± 0.57 | 67.36 ± 0.02 | 54.88 ± 0.01 |
| Total poly | 59.72 ± 0.63 | 51.60 ± 0.54 | 37.55 ± 0.01 | 38.71 ± 0.01 | 20.41 ± 0.18 | 18.03 ± 0.16 | 26.96 ± 0.09 | 21.96 ± 0.07 |
| (n-3) | 48.30 ± 0.46 | 41.73 ± 0.40 | 30.14 ± 0.05 | 31.07 ± 0.05 | 16.15 ± 0.22 | 14.27 ± 0.19 | 21.21 ± 0.02 | 17.28 ± 0.01 |
| (n-6) | 11.82 ± 0.20 | 10.22 ± 0.18 | 6.56 ± 0.06 | 6.77 ± 0.06 | 2.14 ± 0.00 | 1.89 ± 0.00 | 2.68 ± 0.13 | 2.19 ± 0.11 |
| (n-3)/(n-6) | 4.73 ± 0.04 | 4.09 ± 0.03 | 4.46 ± 0.05 | 4.59 ± 0.05 | 8.55 ± 0.11 | 7.55 ± 0.10 | 9.72 ± 0.48 | 7.92 ± 0.39 |
| 22:6/20:5 | 12.56 ± 0.26 | 10.85 ± 0.22 | 0.81 ± 0.00 | 0.84 ± 0.00 | 0.05 ± 0.00 | 0.04 ± 0.00 | 0.10 ± 0.00 | 0.08 ± 0.00 |
| 22:5/20:4 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 |

### 3.2. Broodstock gonadal development and condition index

The gonad development advanced under the experimental conditions, however higher heterogeneity among diets was observed, during broodstock conditioning (Figures 1, 2 and 3). At the beginning of conditioning 60% of individuals were in resting (stage 0), 30% were males in early gametogenesis (stage I) and 10% were females in mature stage (stage III). After 5 weeks of conditioning, a regression of gonadal development was observed in broodstock that fed Diet 1, with 90% of the individuals at resting stage (stage 0). Oyster fed with Diet 2 had a slow evolution, with 40% in stage 0 (resting), 30% in initial phase of gametogenesis (stage I) and 30% in late growth (stage II). In relation to progenitors fed with Diet 3 and Diet 4, it was detected some heterogeneity among individuals at the same diet, which is evident by the co-occurrence of 10% in resting stage, 20% to 30% in early growth stage and 50% to 70% in late growth stage. Also in Diet 4, one oyster exhibited male and female gametes at the same time, this animal is considered hermaphrodite. At 9 weeks of conditioning, the proportion of sexually mature females

(stage III) varied between 10% and 30% for Diet 4 and Diet 3, respectively. In both diets (Diet 3 and Diet 4) the majority of individuals were in late development (stage II) (Diet 3 – 70%; Diet 4 – 80%). *C. angulata* broodstock fed with Diet 1 have a sluggish gonadal progress, with 70% in resting (stage 0), 20% of the animals in early gonadal development (stage I) and 10% late growth (stage II). At the end of the conditioning (11 weeks), the most effective diet was the Diet 3, with 60% of mature oysters. Whereas those fed with Diet 1 had an unsuccessfully gonadic development, with 80% of individuals in resting stage.

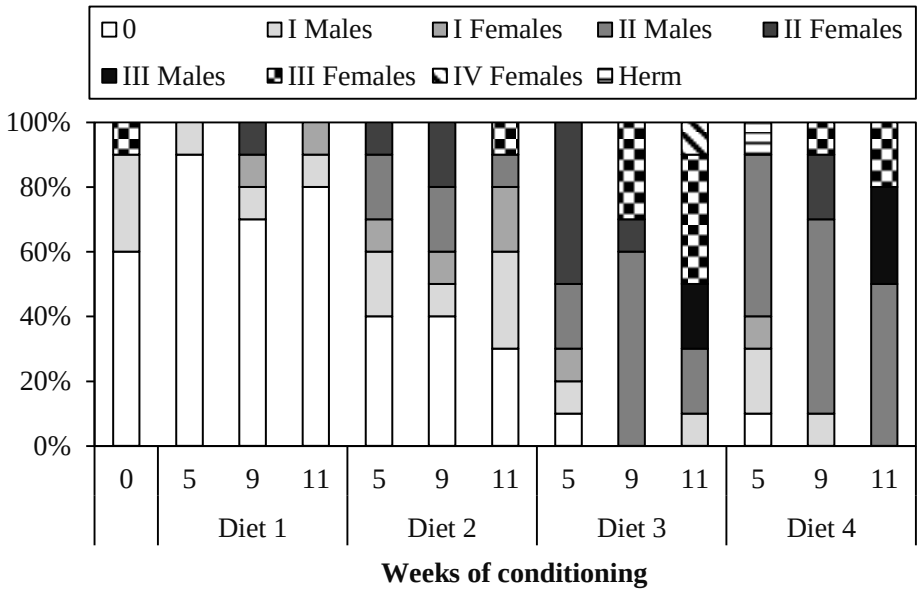


Figure 1. Gonad development (%) in *Crassostrea angulata* broodstock conditioned with different food regimes: Diet 1 (Pav and T-ISO); Diet 2 (Pav, T-ISO and Skt); Diet 3 (Skt and C.cal); Diet 4 (Skt, C.cal and Pav). Stage 0: Resting. I: Early growth. II: Late growth. III: Maturation. IV: Spawning and reabsorbing Herm: Hermaphrodite.

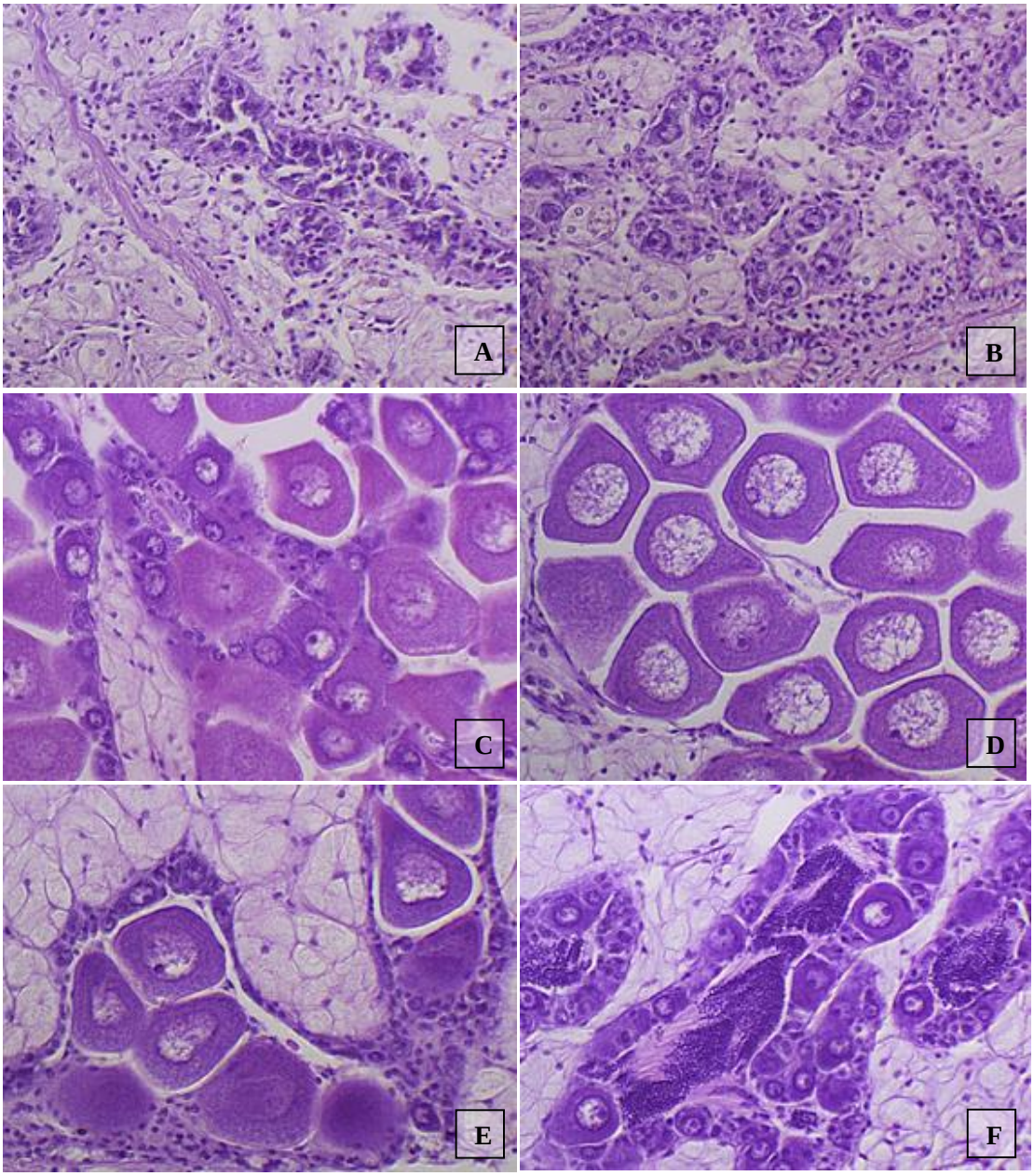


Figure 2. Microphotographs of histological sections of *Crassostrea angulata* broodstock female gonad conditioned with different food regimes: Diet 1 (Pav and T-ISO); Diet 2 (Pav, T-ISO and Skt); Diet 3 (Skt and C.cal); Diet 4 (Skt, C.cal and Pav). A – Resting (stage 0). B – Early growth (I). C – Late growth (II). D – Maturation (III). E – Spawning and reabsorbing (IV). F – Hermaphrodite (Herm). Magnification – 40×. Coloration - haematoxylin-eosin.

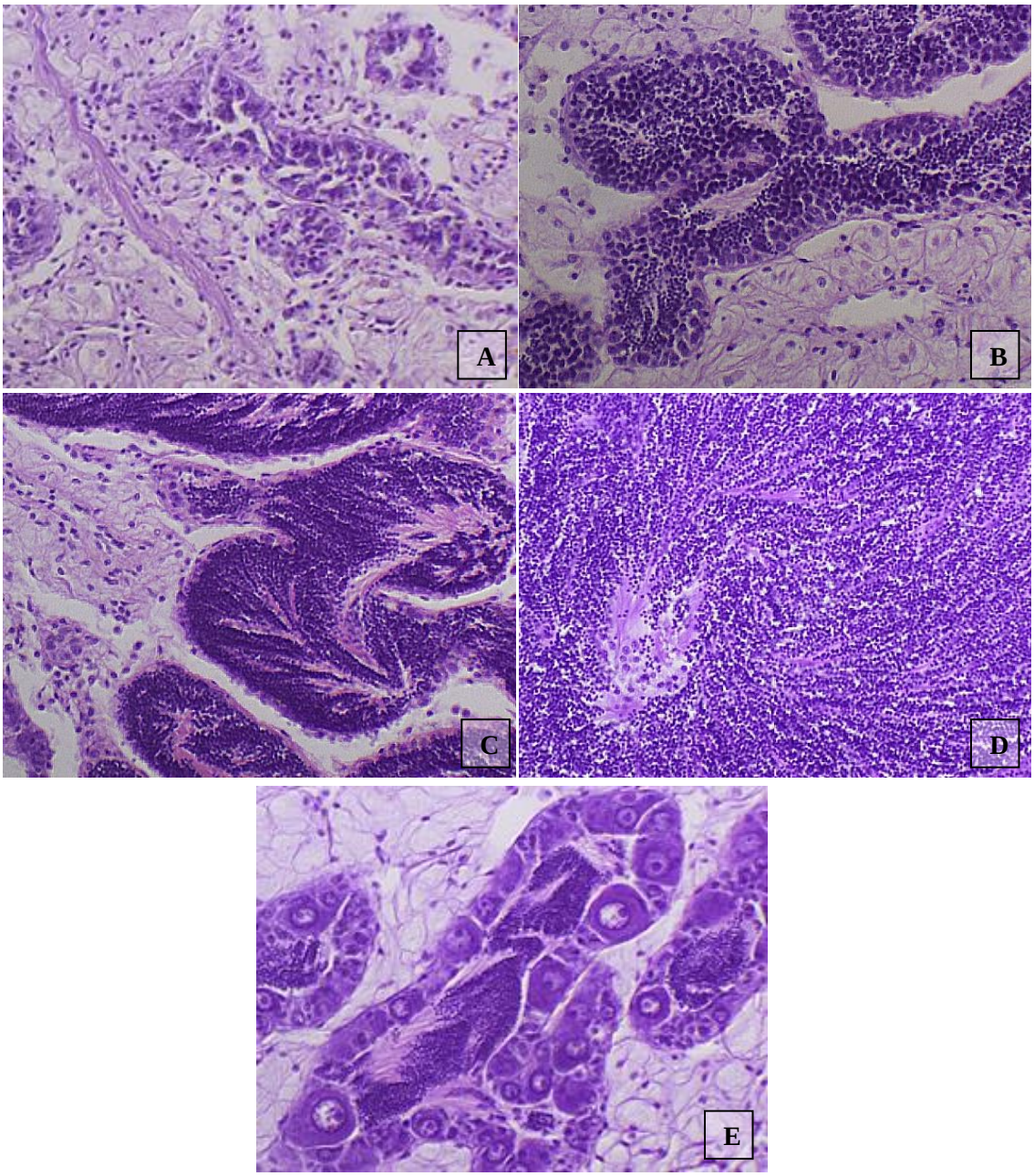


Figure 3. Microphotographs of histological sections of *Crassostrea angulata* broodstock male gonad conditioned with different food regimes: Diet 1 (Pav and T-ISO); Diet 2 (Pav, T-ISO and Skt); Diet 3 (Skt and C.cal); Diet 4 (Skt, C.cal and Pav). A – Resting (stage 0). B – Early growth (I). C – Late growth (II). D – Maturation (III). E – Hermaphrodite (Herm). Magnification – 40×. Coloration - haematoxylin-eosin.

The condition index (Figure 4) reflects the physiologic status of broodstock and was related to the stage of gonadal development (see Figures 1, 2 and 3). The conditioned index values of oysters fed with Diet 1 and Diet 2, decreased during experimental period ($T_0$ – 1.03±0.41; $T_5$: Diet 1 – 0.75±0.32, Diet 2 – 0.87±0.60; $T_9$: Diet 1 – 0.60±0.18; $T_{11}$: Diet 1 – 0.56±0.27), however a slight increase was observed between weeks 9 to 11, in broodstock fed Diet 2 ($T_9$ – 0.79±0.41, $T_{11}$ – 1.08±0.55). This was in agreement with gonadal development observed in Figure 1 for Diet 1 and Diet 2. An increased of the condition index was observed in the individuals fed with Diet 3, during all conditioning period ($T_5$ – 1.62±0.40, $T_9$ – 1.97±1.00, $T_{11}$ – 2.83±0.95). However condition index of broodstock fed with Diet 4 increased from the beginning of the conditioning until week 9 ($T_0$ – 1.03±0.41; $T_5$ – 1.09±0.42; $T_9$ – 2.20±1.10) and after that a slight decrease ($T_{11}$ – 2.02±0.79) were observed. So the highest values of condition index were obtained with broodstock fed with Diet 3 at week 11 and Diet 4 at week 9, being correlated with successfully gonadic development. In contrary the condition index of oysters conditioned with Diet 1 and Diet 2 generally decreased along the conditioning period. At the end of the experimental period, significant differences of broodstock condition index were detected among the diets Diet 1 and Diet 2 with Diet 3 and Diet 4 (ANOVA, $F$=28.36, $df$=3, $P \leq 0.001$).

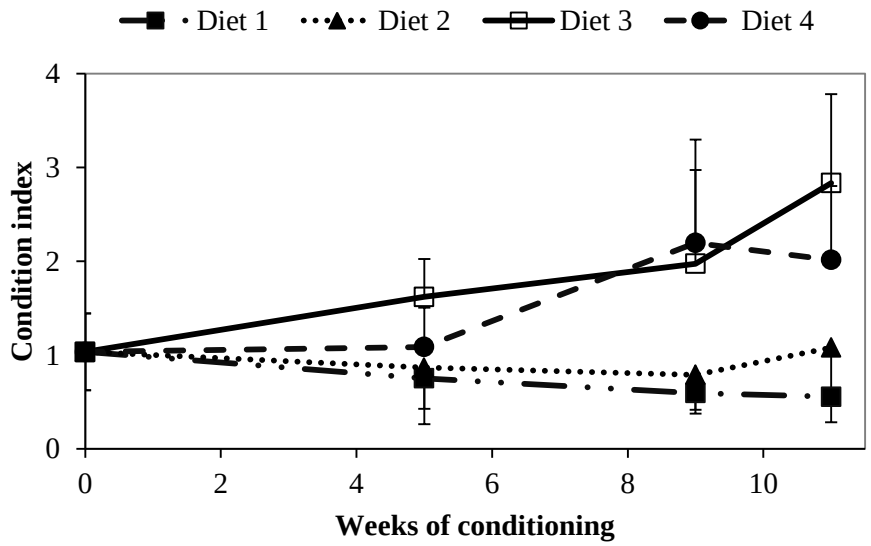


Figure 4. Condition index (mean±SD, $n$=10) in *Crassostrea angulata* broodstock conditioned with different food regimes: Diet 1 (Pav and T-ISO); Diet 2 (Pav, T-ISO and Skt); Diet 3 (Skt and C.cal); Diet 4 (Skt, C.cal and Pav).

### 3.3. Broodstock biochemical composition

In relation to the biochemical compounds dynamics, the broodstock showed very heterogeneous responses to the different nutritional regimes. Protein contents of the oysters ranged from 242.21±64.00 µg $mg^{-1}$ AFDW to 521.80±161.87 µg $mg^{-1}$ AFDW (Figure 5). During the first five weeks of conditioning it was observed an increase in progenitors protein content, in all diets ($T_0$ – 242.21±64.00 µg $mg^{-1}$ AFDW; $T_5$: Diet 1 – 521.80±161.87 µg $mg^{-1}$ AFDW, Diet 2 – 426.63±112.45 µg $mg^{-1}$ AFDW, Diet 3 – 371.92±36.09 µg $mg^{-1}$ AFDW, Diet 4 – 407.47±74.45 µg $mg^{-1}$ AFDW). Between weeks 5 to 9 a decline in proteins was recorded ($T_9$: Diet 1 – 438.49±92.21 µg $mg^{-1}$ AFDW, Diet 3 – 367.91±84.84 µg $mg^{-1}$ AFDW, Diet 4 – 363.12±125.57 µg $mg^{-1}$ AFDW), followed by a slight increase ($T_{11}$: Diet 1 – 515.81±63.79 µg $mg^{-1}$ AFDW, Diet 3 – 392.68±58.45 µg$mg^{-1}$ AFDW, Diet 4 – 435.37±87.02 µg $mg^{-1}$ AFDW), until the end of experimental period. Moreover, the levels of broodstock proteins fed with Diet 2 remained practically constant ($T_9$ – 467.48±154.06 µg $mg^{-1}$ AFDW, $T_{11}$ – 459.38±91.20 µg $mg^{-1}$ AFDW). At the end of the experimental period, significant differences were observed in protein content between broodstock fed with flagellate diet (Diet 1), and predominantly diatoms diets (Diet 3 and Diet 4), also between Diet 2 and Diet 3 (ANOVA, $F$=8.40, $df$=3, $P \leq 0.001$).

Glycogen and total lipids are considered the main energy reserves for the gametogenesis. So during the conditioning time, variations are very notable. The mean glycogen value in oysters fluctuated from 18.00±7.24 µg $mg^{-1}$ AFDW to 51.08±28.93 µg $mg^{-1}$ AFDW (Figure 6). Oyster accumulated glycogen from the beginning of the conditioning ($T_0$ – 22.46±9.02 µg $mg^{-1}$ AFDW) until the week 5 in all diets tested ($T_5$: Diet 1 – 27.59±11.88 µg $mg^{-1}$ AFDW, Diet 2 – 46.68±28.90 µg $mg^{-1}$ AFDW, Diet 3 – 47.96±11.03 µg $mg^{-1}$ AFDW, Diet 4 – 33.21±5.59 µg $mg^{-1}$ AFDW), however this accumulation was more pronounced in Diet 2 and Diet 3. The glycogen content of broodstock fed with Diet 1 remained practically constant during conditioning ($T_9$ – 24.28±10.22 µg $mg^{-1}$ AFDW, $T_{11}$ – 18.00±7.24 µg $mg^{-1}$ AFDW) in contrast, glycogen of the oysters fed with Diet 4 rise during this period ($T_9$ – 43.40±18.47 µg $mg^{-1}$ AFDW, $T_{11}$ – 46.79±22.00 µg $mg^{-1}$ AFDW). In the broodstock fed on Diet 2 and Diet 3, the glycogen contents present a decrease from the second sampling time to third one ($T_9$: Diet 2 – 23.47±10.75 µg $mg^{-1}$ AFDW, Diet 3 – 41.59±17.90 µg $mg^{-1}$ AFDW), with a subsequent increase until the end of conditioning ($T_{11}$: Diet 2 – 28.47±9.49 µg $mg^{-1}$ AFDW, Diet 3 –

51.08±28.93 µg mg$^{-1}$ AFDW). At the end of conditioning, significant differences were observed in glycogen contents of broodstock fed predominantly flagellate diets (Diet 1 and Diet 2) and broodstock fed predominantly diatoms diets (Diet 3 and Diet 4)(K-W., $H$=43.16, $df$=3, $P$<0.001).

The levels of broodstock total lipid content ranged between 34.24±8.23 µg mg$^{-1}$ AFDW and 82.92±28.40 µg mg$^{-1}$ AFDW (Figure 7). In the oysters fed with Diet 1 and Diet 2, a slight increase from the beginning to week 9 ($T_0$ – 34.24±8.23 µg mg$^{-1}$ AFDW; $T_5$: Diet 1 – 44.20±13.60 µg mg$^{-1}$ AFDW, Diet 2 – 55.02±10.82 µg mg$^{-1}$ AFDW; $T_9$: Diet 1 – 54.28±23.90 µg mg$^{-1}$ AFDW, Diet 2 – 58.66±24.30 µg mg$^{-1}$ AFDW) was observed with a subsequent decline until the end of conditioning ($T_{11}$: Diet 1 – 52.69±14.58 µg mg$^{-1}$ AFDW, Diet 2 – 49.30±18.62 µg mg$^{-1}$ AFDW). Total lipids contents in oyster that fed Diet 3 and Diet 4 presented an increase from the beginning to the week 5 of conditioning ($T_5$: Diet 3 – 66.28±21.96 µg mg$^{-1}$ AFDW, Diet 4 – 82.92±28.40 µg mg$^{-1}$ AFDW), followed by a decrease until week 9 ($T_9$: Diet 3 – 65.68±20.76 µg mg$^{-1}$ AFDW, Diet 4 – 72.15±35.80 µg mg$^{-1}$ AFDW), with a subsequent increase ($T_{11}$: Diet 3 – 75.23±35.44 µg mg$^{-1}$ AFDW, Diet 4 – 72.82±37.50 µg mg$^{-1}$ AFDW). The total lipids contents of oysters presented significant differences among broodstock fed predominantly with flagellate diets (Diet 1 and Diet 2) and broodstock fed predominantly with diatoms diets (Diet 3 and Diet 4)(K-W., $H$=30.40, $df$=3, $P$≤0.001).

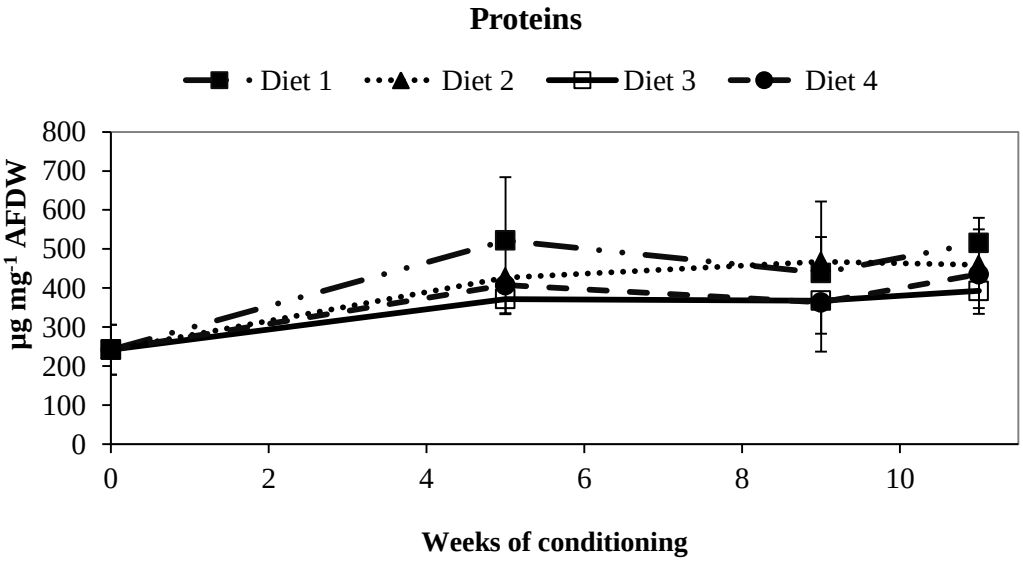


Figure 5. Protein contents (mean±SD, $n$=10) in *Crassostrea angulata* broodstock conditioned with different food regimes: Diet 1 (Pav and T-ISO); Diet 2 (Pav, T-ISO and Skt); Diet 3 (Skt and C.cal); Diet 4 (Skt, C.cal and Pav). The values were expressed in mean absolute (µg mg$^{-1}$ of AFDW).

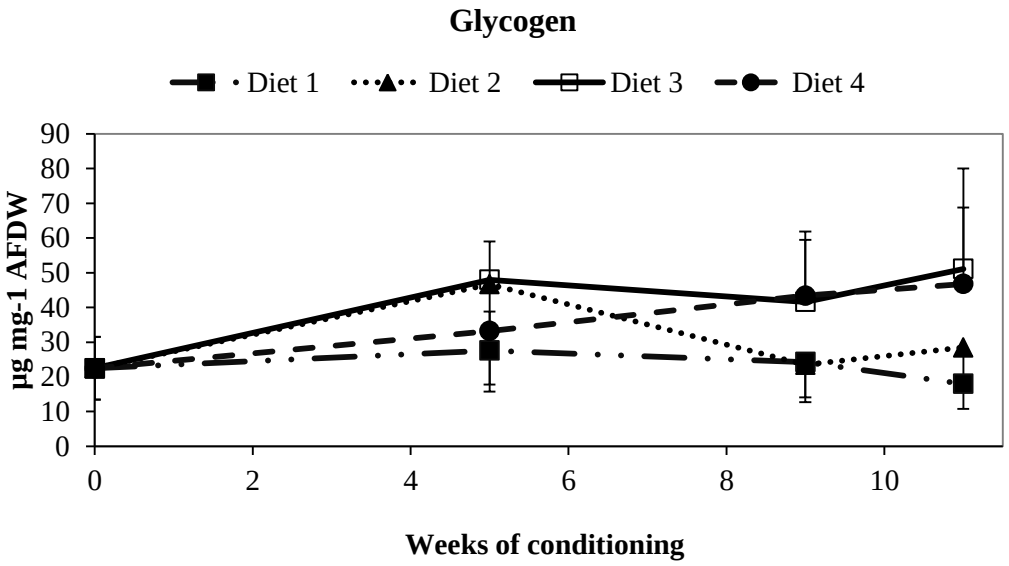


Figure 6. Glycogen contents (mean±SD, *n*=10) in *Crassostrea angulata* broodstock conditioned with different food regimes: Diet 1 (Pav and T-ISO); Diet 2 (Pav, T-ISO and Skt); Diet 3 (Skt and C.cal); Diet 4 (Skt, C.cal and Pav). The values were expressed in mean absolute (µg $mg^{-1}$ of AFDW).

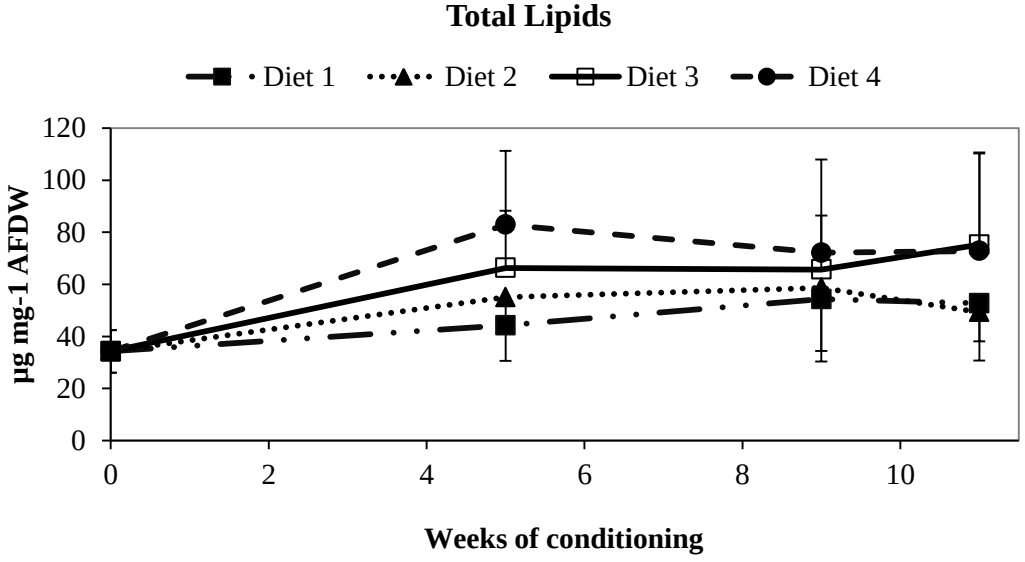


Figure 7. Total lipids contents (mean±SD, *n*=10) in *Crassostrea angulata* broodstock conditioned with different food regimes: Diet 1 (Pav and T-ISO); Diet 2 (Pav, T-ISO and Skt); Diet 3 (Skt and C.cal); Diet 4 (Skt, C.cal and Pav). The values were expressed in mean absolute (µg $mg^{-1}$ of AFDW).

The FAs composition of the broodstock conditioned with different food regimes are listed in Tables IVa and IVb. There was identified 53 FAs in total, which signified 85.38±1.85% of total FAs. From these FAs only 25 was presented in Tables IVa and IVb and the FAs not showed represented only 13.27±2.15% of total FAs. Most prominent FAs

found over all diets and sampling times were 16:0, 16:1(n-7), 18:0, 20:5(n-3) and 22:6(n-3), which represented 48.38±8.69% of total FAs. For all diets and sampling times, 16:0 was one of the main FAs existing in the adult oyster, where the value of your content remains constant along the conditioning time (10.79±0.87% - 15.45±0.28% of total FAs). Other fatty acid with remarkable expression was 16:1(n-7), that presented high values in oysters fed with Diet 3 (9.63±0.11% - 13.77±0.03% of total FAs) and Diet 4 (8.76±0.14% - 12.45±0.16% of total FAs), in contrast with oysters fed with Diet 1 (0.59±0.04% - 0.97±0.04% of total FAs) and Diet 2 (3.14±0.12% - 4.53±0.12% of total FAs) that presented lower values.Contrary, the progenitors fed with Diet 3 and Diet 4 showed the highest levels of 20:5(n-3) (Diet 3 – 15.44±0.10% - 17.29±1.07%, Diet 4 – 16.62±0.16% - 17.75±0.42% of total FAs) and the lowest levels of 22:6(n-3) (Diet 3 – 2.32±0.07% - 3.23±0.12%, Diet 4 – 3.33±0.00% - 4.17±0.11% of total FAs), that imply the low ratio of 22:6/20:5 (Diet 3 – 0.14±0.01% - 0.19±0.00%, Diet 4 – 0.20±0.01% -0.25±0.01% of total FAs). Moreover, oysters fed with Diet 1 and Diet 2 presented the 22:6(n-3) a dominant fatty acid (Diet 1 – 9.42±0.37% - 10.04±0.03%, Diet 2 – 7.69±0.20% - 8.68±0.07% of total FAs) and showed moderate levels of 20:5(n-3) (Diet 1 – 6.76±0.02% - 11.05±0.01%, Diet 2 – 11.50±0.11% - 11.84±0.21% of total FAs), however, a high ratio 22:6/20:5 was achieved in oysters conditioned with both diets (Diet 1 – 0.85±0.03% - 1.49±0.00%, Diet 2 – 0.65±0.01% - 0.76±0.01% of total FAs). Overall data showed that the profile and amount of fatty acids varied widely between the diets constituted predominantly by flagellates (Diet 1 and Diet 2) and the diets formulated mainly by diatoms (Diet 3 and Diet 4), however significant differences were not evident ($P>0.05$).

Table IVa. Total fatty acid composition (mean±SD, *n*=10) in *Crassostrea angulata* broodstock conditioned with different food regimes: Diet 1 (Pav and T-ISO); Diet 2 (Pav, T-ISO and Skt). The values are expressed in mean absolute (µg $mg^{-1}$) and relative contents (weight % of total FAs). Sampled at the beginning (0) and after 5, 9 and 11 weeks of conditioning.

| | Oyster Diets | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Diet 1 | | | | | | | | Diet 2 | | | | | | | | | |
| | Weeks | | | | | | | | | | | | | | | | | |
| | 0 | | 5 | | 9 | | 11 | | 5 | | 9 | | 11 | | | | | |
| Fatty acids | Contents (µg $mg^{-1}$) | Rel. Contents (%) | Contents (µg $mg^{-1}$) | Rel. Contents (%) | Contents (µg $mg^{-1}$) | Rel. Contents (%) | Contents (µg $mg^{-1}$) | Rel. Contents (%) | Contents (µg $mg^{-1}$) | Rel. Contents (%) | Contents (µg $mg^{-1}$) | Rel. Contents (%) | Contents (µg $mg^{-1}$) | Rel. Contents (%) | Contents (µg $mg^{-1}$) | Rel. Contents (%) | Contents (µg $mg^{-1}$) | Rel. Contents (%) |
| 12:0 | 0.03 ± 0.00 | 0.09 ± 0.01 | 0.02 ± 0.00 | 0.04 ± 0.00 | 0.01 ± 0.02 | 0.02 ± 0.03 | 0.02 ± 0.00 | 0.03 ± 0.00 | 0.01 ± 0.00 | 0.02 ± 0.00 | 0.05 ± 0.01 | 0.09 ± 0.01 | 0.00 ± 0.00 | 0.01 ± 0.00 | | | | |
| 14:0 | 0.40 ± 0.00 | 1.18 ± 0.00 | 0.41 ± 0.00 | 0.92 ± 0.00 | 0.87 ± 0.03 | 1.60 ± 0.05 | 0.32 ± 0.05 | 0.61 ± 0.10 | 1.05 ± 0.02 | 1.92 ± 0.04 | 0.99 ± 0.05 | 1.70 ± 0.09 | 0.85 ± 0.07 | 1.72 ± 0.14 | | | | |
| 16:0 | 5.29 ± 0.10 | 15.45 ± 0.28 | 5.55 ± 0.10 | 12.55 ± 0.23 | 7.37 ± 0.00 | 13.57 ± 0.00 | 5.68 ± 0.46 | 10.79 ± 0.87 | 7.61 ± 0.12 | 13.84 ± 0.21 | 7.64 ± 0.12 | 13.03 ± 0.20 | 5.93 ± 0.04 | 12.03 ± 0.08 | | | | |
| 16:1 (n-9) | 0.16 ± 0.00 | 0.46 ± 0.00 | 0.26 ± 0.01 | 0.59 ± 0.01 | 0.39 ± 0.00 | 0.73 ± 0.01 | 0.27 ± 0.01 | 0.51 ± 0.03 | 0.28 ± 0.01 | 0.50 ± 0.01 | 0.35 ± 0.00 | 0.59 ± 0.00 | 0.20 ± 0.00 | 0.41 ± 0.01 | | | | |
| 16:1 (n-7) | 0.76 ± 0.02 | 2.22 ± 0.07 | 0.40 ± 0.01 | 0.90 ± 0.01 | 0.52 ± 0.02 | 0.97 ± 0.04 | 0.31 ± 0.02 | 0.59 ± 0.04 | 2.49 ± 0.07 | 4.53 ± 0.12 | 1.84 ± 0.07 | 3.14 ± 0.12 | 1.85 ± 0.09 | 3.75 ± 0.18 | | | | |
| 16:2 (n-7) | 0.83 ± 0.02 | 2.42 ± 0.06 | 0.85 ± 0.02 | 1.92 ± 0.05 | 0.91 ± 0.01 | 1.68 ± 0.01 | 1.23 ± 0.06 | 2.33 ± 0.11 | 0.73 ± 0.03 | 1.34 ± 0.05 | 0.89 ± 0.00 | 1.53 ± 0.01 | 0.78 ± 0.02 | 1.58 ± 0.05 | | | | |
| 16:2 (n-4) | 0.03 ± 0.00 | 0.10 ± 0.01 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.01 ± 0.02 | 0.02 ± 0.03 | 0.00 ± 0.00 | 0.01 ± 0.01 | 0.13 ± 0.01 | 0.23 ± 0.01 | 0.09 ± 0.00 | 0.16 ± 0.01 | 0.10 ± 0.01 | 0.20 ± 0.01 | | | | |
| 18:0 | 2.77 ± 0.05 | 8.09 ± 0.16 | 3.26 ± 0.08 | 7.38 ± 0.18 | 3.36 ± 0.01 | 6.19 ± 0.02 | 4.26 ± 0.09 | 8.09 ± 0.18 | 3.08 ± 0.11 | 5.61 ± 0.19 | 3.77 ± 0.04 | 6.43 ± 0.06 | 2.64 ± 0.04 | 5.35 ± 0.08 | | | | |
| 18:1 (n-9) | 1.29 ± 0.01 | 3.77 ± 0.02 | 1.73 ± 0.00 | 3.92 ± 0.01 | 2.91 ± 0.00 | 5.36 ± 0.00 | 1.63 ± 0.18 | 3.10 ± 0.34 | 1.68 ± 0.03 | 3.06 ± 0.05 | 2.07 ± 0.02 | 3.53 ± 0.04 | 1.31 ± 0.14 | 2.66 ± 0.29 | | | | |
| 18:1 (n-7) | 2.15 ± 0.03 | 6.28 ± 0.08 | 2.34 ± 0.00 | 5.29 ± 0.00 | 3.28 ± 0.00 | 6.05 ± 0.01 | 2.79 ± 0.12 | 5.30 ± 0.22 | 4.61 ± 0.00 | 8.37 ± 0.00 | 4.63 ± 0.01 | 7.90 ± 0.01 | 3.81 ± 0.05 | 7.73 ± 0.09 | | | | |
| 18:2 (n-6) | 0.55 ± 0.02 | 1.61 ± 0.06 | 0.74 ± 0.03 | 1.67 ± 0.06 | 1.33 ± 0.01 | 2.46 ± 0.02 | 0.58 ± 0.01 | 1.11 ± 0.03 | 0.96 ± 0.00 | 1.74 ± 0.00 | 1.03 ± 0.02 | 1.75 ± 0.03 | 1.05 ± 0.03 | 2.13 ± 0.06 | | | | |
| 18:3 (n-6) | 0.05 ± 0.00 | 0.14 ± 0.01 | 0.04 ± 0.00 | 0.10 ± 0.00 | 0.05 ± 0.00 | 0.10 ± 0.00 | 0.06 ± 0.00 | 0.11 ± 0.01 | 0.10 ± 0.00 | 0.18 ± 0.01 | 0.11 ± 0.00 | 0.19 ± 0.00 | 0.09 ± 0.00 | 0.19 ± 0.01 | | | | |
| 18:3 (n-3) | 0.39 ± 0.01 | 1.13 ± 0.04 | 0.37 ± 0.01 | 0.85 ± 0.02 | 0.70 ± 0.01 | 1.29 ± 0.02 | 0.28 ± 0.01 | 0.53 ± 0.01 | 0.53 ± 0.01 | 0.96 ± 0.02 | 0.51 ± 0.01 | 0.87 ± 0.02 | 0.54 ± 0.02 | 1.11 ± 0.04 | | | | |
| 18:4 (n-3) | 2.75 ± 0.10 | 8.04 ± 0.30 | 3.30 ± 0.07 | 7.47 ± 0.16 | 4.05 ± 0.13 | 7.46 ± 0.25 | 3.81 ± 0.16 | 7.23 ± 0.31 | 3.81 ± 0.05 | 6.92 ± 0.10 | 4.33 ± 0.00 | 7.38 ± 0.01 | 4.32 ± 0.06 | 8.76 ± 0.11 | | | | |
| 20:0 | 0.01 ± 0.00 | 0.04 ± 0.00 | 0.00 ± 0.00 | 0.01 ± 0.01 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | 1.59 ± 0.02 | 2.89 ± 0.04 | 0.07 ± 0.00 | 0.12 ± 0.00 | 0.07 ± 0.00 | 0.14 ± 0.00 | | | | |
| 20:2 (n-6) | 0.11 ± 0.00 | 0.34 ± 0.01 | 0.13 ± 0.00 | 0.30 ± 0.01 | 0.17 ± 0.01 | 0.31 ± 0.02 | 0.15 ± 0.00 | 0.29 ± 0.01 | 0.17 ± 0.00 | 0.30 ± 0.00 | 0.19 ± 0.00 | 0.33 ± 0.00 | 0.20 ± 0.01 | 0.42 ± 0.02 | | | | |
| 20:4 (n-6) | 0.03 ± 0.03 | 0.08 ± 0.08 | 0.04 ± 0.04 | 0.09 ± 0.10 | 0.20 ± 0.07 | 0.37 ± 0.13 | 0.08 ± 0.01 | 0.15 ± 0.01 | 0.06 ± 0.00 | 0.12 ± 0.01 | 0.10 ± 0.01 | 0.17 ± 0.02 | 0.03 ± 0.02 | 0.06 ± 0.04 | | | | |
| 20:5 (n-3) | 3.68 ± 0.14 | 10.76 ± 0.40 | 4.71 ± 0.10 | 10.67 ± 0.22 | 3.67 ± 0.01 | 6.76 ± 0.02 | 5.82 ± 0.01 | 11.05 ± 0.01 | 6.50 ± 0.01 | 11.82 ± 0.02 | 6.94 ± 0.12 | 11.84 ± 0.21 | 5.67 ± 0.05 | 11.50 ± 0.11 | | | | |
| 22:0 | 0.01 ± 0.00 | 0.04 ± 0.01 | 0.01 ± 0.00 | 0.03 ± 0.00 | 0.03 ± 0.00 | 0.06 ± 0.01 | 0.01 ± 0.00 | 0.02 ± 0.00 | 0.04 ± 0.01 | 0.07 ± 0.01 | 0.04 ± 0.00 | 0.08 ± 0.01 | 0.03 ± 0.00 | 0.07 ± 0.00 | | | | |
| 22:2 (n-6) | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.35 ± 0.03 | 0.66 ± 0.05 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.12 ± 0.17 | 0.21 ± 0.30 | 0.19 ± 0.00 | 0.40 ± 0.01 | | | | |
| 22:4 (n-6) | 0.01 ± 0.01 | 0.02 ± 0.03 | 0.01 ± 0.00 | 0.03 ± 0.01 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.01 ± 0.00 | 0.03 ± 0.01 | 0.01 ± 0.01 | 0.01 ± 0.01 | 0.01 ± 0.00 | 0.03 ± 0.01 | 0.01 ± 0.00 | 0.02 ± 0.00 | | | | |
| 22:5 (n-6) | 0.18 ± 0.00 | 0.53 ± 0.01 | 0.53 ± 0.01 | 1.20 ± 0.03 | 1.02 ± 0.00 | 1.88 ± 0.01 | 0.69 ± 0.04 | 1.31 ± 0.08 | 0.63 ± 0.01 | 1.14 ± 0.01 | 0.72 ± 0.02 | 1.23 ± 0.03 | 0.84 ± 0.01 | 1.70 ± 0.02 | | | | |
| 22:5 (n-3) | 0.40 ± 0.00 | 1.17 ± 0.01 | 0.56 ± 0.01 | 1.26 ± 0.01 | 0.45 ± 0.01 | 0.84 ± 0.02 | 0.67 ± 0.06 | 1.28 ± 0.11 | 0.48 ± 0.01 | 0.88 ± 0.02 | 0.45 ± 0.01 | 0.77 ± 0.01 | 0.46 ± 0.00 | 0.93 ± 0.01 | | | | |
| 22:6 (n-3) | 2.44 ± 0.05 | 7.12 ± 0.13 | 4.33 ± 0.06 | 9.80 ± 0.14 | 5.45 ± 0.02 | 10.04 ± 0.03 | 4.96 ± 0.19 | 9.42 ± 0.37 | 4.38 ± 0.04 | 7.96 ± 0.06 | 4.51 ± 0.12 | 7.69 ± 0.20 | 4.28 ± 0.03 | 8.68 ± 0.07 | | | | |
| 24:0 | 0.00 ± 0.00 | 0.01 ± 0.00 | 0.00 ± 0.01 | 0.01 ± 0.01 | 0.01 ± 0.01 | 0.02 ± 0.01 | 0.01 ± 0.00 | 0.02 ± 0.00 | 0.00 ± 0.00 | 0.01 ± 0.01 | 0.01 ± 0.00 | 0.02 ± 0.01 | 0.01 ± 0.00 | 0.02 ± 0.01 | | | | |
| Total saturated | 8.88 ± 0.15 | 25.94 ± 0.45 | 9.60 ± 0.11 | 21.73 ± 0.24 | 12.13 ± 0.00 | 22.35 ± 0.01 | 10.78 ± 0.62 | 20.46 ± 1.17 | 13.97 ± 0.22 | 25.40 ± 0.40 | 13.11 ± 0.22 | 22.35 ± 0.37 | 10.08 ± 0.19 | 20.46 ± 0.39 | | | | |
| Total mono | 11.54 ± 0.12 | 33.72 ± 0.36 | 12.95 ± 0.18 | 29.29 ± 0.40 | 17.63 ± 0.04 | 32.48 ± 0.08 | 14.23 ± 0.66 | 27.00 ± 1.26 | 13.08 ± 0.32 | 23.78 ± 0.57 | 19.30 ± 0.27 | 32.90 ± 0.45 | 13.84 ± 2.69 | 28.08 ± 5.47 | | | | |
| Total poly | 13.82 ± 0.38 | 40.36 ± 1.12 | 19.47 ± 0.34 | 44.05 ± 0.76 | 22.16 ± 0.14 | 40.83 ± 0.26 | 24.24 ± 0.46 | 46.02 ± 0.87 | 22.21 ± 0.20 | 40.37 ± 0.36 | 24.37 ± 0.49 | 41.55 ± 0.83 | 22.65 ± 0.15 | 45.94 ± 0.30 | | | | |
| (n-3) | 11.70 ± 0.36 | 34.16 ± 1.06 | 16.62 ± 0.30 | 37.61 ± 0.69 | 17.85 ± 0.08 | 32.89 ± 0.15 | 20.43 ± 0.43 | 38.78 ± 0.81 | 18.98 ± 0.17 | 34.50 ± 0.31 | 20.62 ± 0.28 | 35.15 ± 0.48 | 18.88 ± 0.14 | 38.30 ± 0.28 | | | | |
| (n-6) | 1.36 ± 0.00 | 3.99 ± 0.01 | 2.14 ± 0.02 | 4.84 ± 0.04 | 3.61 ± 0.07 | 6.66 ± 0.13 | 2.75 ± 0.07 | 5.21 ± 0.14 | 2.58 ± 0.00 | 4.69 ± 0.01 | 2.97 ± 0.19 | 5.06 ± 0.33 | 3.13 ± 0.01 | 6.35 ± 0.01 | | | | |
| (n-3)/(n-6) | 2.93 ± 0.09 | 8.57 ± 0.25 | 3.43 ± 0.03 | 7.77 ± 0.07 | 2.68 ± 0.04 | 4.94 ± 0.08 | 3.92 ± 0.02 | 7.44 ± 0.05 | 4.05 ± 0.03 | 7.36 ± 0.06 | 4.08 ± 0.21 | 6.96 ± 0.35 | 2.97 ± 0.03 | 6.03 ± 0.06 | | | | |
| 22:6/20:5 | 0.23 ± 0.00 | 0.66 ± 0.01 | 0.41 ± 0.00 | 0.92 ± 0.01 | 0.81 ± 0.00 | 1.49 ± 0.00 | 0.45 ± 0.02 | 0.85 ± 0.03 | 0.37 ± 0.00 | 0.67 ± 0.00 | 0.38 ± 0.00 | 0.65 ± 0.01 | 0.37 ± 0.01 | 0.76 ± 0.01 | | | | |

Table IVb. Total fatty acid composition (mean±SD, *n*=10) in *Crassostrea angulata* broodstock conditioned with different food regimes: Diet 3 (Skt and C.cal); Diet 4 (Skt, C.cal and Pav). The values are expressed in mean absolute (µg $mg^{-1}$) and relative contents (weight % of total FAs). Sampled after 5, 9 and 11 weeks of conditioning.

| | **Oyster Diets** | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **Diet 3** | | | | | | **Diet 4** | | | | | |
| | **Weeks** | | | | | | | | | | | |
| | **5** | | **9** | | **11** | | **5** | | **9** | | **11** | |
| **Fatty acids** | **Contents (µg $mg^{-1}$)** | **Rel. Contents (%)** | **Contents (µg $mg^{-1}$)** | **Rel. Contents (%)** | **Contents (µg $mg^{-1}$)** | **Rel. Contents (%)** | **Contents (µg $mg^{-1}$)** | **Rel. Contents (%)** | **Contents (µg $mg^{-1}$)** | **Rel. Contents (%)** | **Contents (µg $mg^{-1}$)** | **Rel. Contents (%)** |
| 12:0 | 0.01 ± 0.00 | 0.02 ± 0.00 | 0.01 ± 0.00 | 0.02 ± 0.01 | 0.04 ± 0.01 | 0.06 ± 0.01 | 0.02 ± 0.01 | 0.03 ± 0.01 | 0.04 ± 0.01 | 0.06 ± 0.01 | 0.04 ± 0.01 | 0.05 ± 0.01 |
| 14:0 | 1.87 ± 0.01 | 2.82 ± 0.01 | 1.47 ± 0.11 | 2.25 ± 0.16 | 1.92 ± 0.02 | 2.55 ± 0.03 | 1.98 ± 0.06 | 2.39 ± 0.07 | 1.63 ± 0.04 | 2.26 ± 0.06 | 1.59 ± 0.02 | 2.19 ± 0.02 |
| 16:0 | 9.17 ± 0.03 | 13.84 ± 0.05 | 9.11 ± 0.31 | 13.87 ± 0.47 | 10.07 ± 0.04 | 13.39 ± 0.06 | 11.07 ± 0.25 | 13.36 ± 0.30 | 10.39 ± 0.08 | 14.40 ± 0.11 | 9.23 ± 0.09 | 12.68 ± 0.13 |
| 16:1 (n-9) | 0.17 ± 0.01 | 0.26 ± 0.01 | 0.17 ± 0.00 | 0.27 ± 0.01 | 0.17 ± 0.00 | 0.22 ± 0.00 | 0.23 ± 0.01 | 0.28 ± 0.01 | 0.19 ± 0.00 | 0.26 ± 0.00 | 0.17 ± 0.02 | 0.23 ± 0.03 |
| 16:1 (n-7) | 9.13 ± 0.02 | 13.77 ± 0.03 | 6.77 ± 0.18 | 10.31 ± 0.28 | 7.25 ± 0.09 | 9.63 ± 0.11 | 10.32 ± 0.13 | 12.45 ± 0.16 | 7.19 ± 0.07 | 9.97 ± 0.09 | 6.38 ± 0.10 | 8.76 ± 0.14 |
| 16:2 (n-7) | 0.49 ± 0.00 | 0.75 ± 0.01 | 0.49 ± 0.02 | 0.75 ± 0.03 | 0.70 ± 0.03 | 0.93 ± 0.04 | 0.64 ± 0.01 | 0.78 ± 0.01 | 0.57 ± 0.03 | 0.80 ± 0.04 | 0.72 ± 0.03 | 0.99 ± 0.04 |
| 16:2 (n-4) | 0.67 ± 0.01 | 1.01 ± 0.02 | 0.43 ± 0.02 | 0.66 ± 0.03 | 0.55 ± 0.02 | 0.73 ± 0.03 | 0.73 ± 0.01 | 0.88 ± 0.01 | 0.46 ± 0.02 | 0.64 ± 0.02 | 0.46 ± 0.02 | 0.63 ± 0.02 |
| 18:0 | 3.23 ± 0.03 | 4.88 ± 0.05 | 3.57 ± 0.03 | 5.44 ± 0.05 | 3.92 ± 0.13 | 5.21 ± 0.17 | 3.59 ± 0.03 | 4.34 ± 0.04 | 3.56 ± 0.01 | 4.94 ± 0.01 | 3.58 ± 0.03 | 4.91 ± 0.04 |
| 18:1 (n-9) | 0.60 ± 0.00 | 0.91 ± 0.01 | 0.50 ± 0.02 | 0.76 ± 0.03 | 0.46 ± 0.01 | 0.61 ± 0.01 | 0.85 ± 0.00 | 1.03 ± 0.00 | 0.80 ± 0.01 | 1.11 ± 0.01 | 0.46 ± 0.07 | 0.63 ± 0.09 |
| 18:1 (n-7) | 8.34 ± 0.14 | 12.59 ± 0.21 | 7.94 ± 0.26 | 12.09 ± 0.39 | 8.07 ± 0.12 | 10.73 ± 0.16 | 9.81 ± 0.01 | 11.83 ± 0.01 | 8.15 ± 0.01 | 11.29 ± 0.01 | 7.49 ± 0.07 | 10.29 ± 0.09 |
| 18:2 (n-6) | 0.34 ± 0.00 | 0.52 ± 0.01 | 0.26 ± 0.00 | 0.40 ± 0.01 | 0.35 ± 0.02 | 0.47 ± 0.02 | 0.59 ± 0.00 | 0.71 ± 0.00 | 0.55 ± 0.03 | 0.76 ± 0.04 | 0.49 ± 0.02 | 0.68 ± 0.02 |
| 18:3 (n-6) | 0.27 ± 0.00 | 0.41 ± 0.01 | 0.27 ± 0.00 | 0.41 ± 0.01 | 0.28 ± 0.02 | 0.37 ± 0.03 | 0.32 ± 0.00 | 0.38 ± 0.00 | 0.26 ± 0.02 | 0.36 ± 0.03 | 0.25 ± 0.01 | 0.35 ± 0.01 |
| 18:3 (n-3) | 0.07 ± 0.00 | 0.11 ± 0.00 | 0.02 ± 0.00 | 0.03 ± 0.00 | 0.03 ± 0.03 | 0.04 ± 0.04 | 0.21 ± 0.00 | 0.25 ± 0.00 | 0.27 ± 0.01 | 0.37 ± 0.01 | 0.19 ± 0.01 | 0.27 ± 0.01 |
| 18:4 (n-3) | 3.05 ± 0.03 | 4.60 ± 0.04 | 3.50 ± 0.28 | 5.33 ± 0.42 | 4.12 ± 0.17 | 5.48 ± 0.23 | 3.69 ± 0.04 | 4.45 ± 0.05 | 3.52 ± 0.30 | 4.89 ± 0.42 | 4.34 ± 0.38 | 5.96 ± 0.52 |
| 20:0 | 0.18 ± 0.00 | 0.28 ± 0.01 | 0.20 ± 0.00 | 0.30 ± 0.00 | 0.24 ± 0.01 | 0.32 ± 0.01 | 0.24 ± 0.00 | 0.29 ± 0.00 | 0.21 ± 0.01 | 0.29 ± 0.01 | 0.24 ± 0.01 | 0.33 ± 0.01 |
| 20:2 (n-6) | 0.26 ± 0.00 | 0.40 ± 0.01 | 0.28 ± 0.01 | 0.42 ± 0.01 | 0.37 ± 0.02 | 0.49 ± 0.03 | 0.32 ± 0.00 | 0.38 ± 0.00 | 0.30 ± 0.02 | 0.42 ± 0.03 | 0.47 ± 0.02 | 0.65 ± 0.03 |
| 20:4 (n-6) | 0.02 ± 0.00 | 0.03 ± 0.01 | 0.05 ± 0.03 | 0.07 ± 0.04 | 0.03 ± 0.01 | 0.04 ± 0.01 | 0.07 ± 0.02 | 0.09 ± 0.03 | 0.04 ± 0.02 | 0.06 ± 0.03 | 0.03 ± 0.01 | 0.05 ± 0.01 |
| 20:5 (n-3) | 10.23 ± 0.07 | 15.44 ± 0.10 | 11.10 ± 0.38 | 16.90 ± 0.58 | 13.00 ± 0.80 | 17.29 ± 1.07 | 13.89 ± 0.04 | 16.75 ± 0.04 | 12.81 ± 0.31 | 17.75 ± 0.42 | 12.10 ± 0.11 | 16.62 ± 0.16 |
| 22:0 | 0.05 ± 0.00 | 0.08 ± 0.00 | 0.10 ± 0.02 | 0.16 ± 0.04 | 0.09 ± 0.01 | 0.13 ± 0.01 | 0.07 ± 0.00 | 0.09 ± 0.00 | 0.04 ± 0.06 | 0.06 ± 0.08 | 0.09 ± 0.01 | 0.13 ± 0.01 |
| 22:2 (n-6) | 0.05 ± 0.07 | 0.08 ± 0.11 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.20 ± 0.27 | 0.27 ± 0.35 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.04 ± 0.06 | 0.06 ± 0.08 | 0.11 ± 0.01 | 0.16 ± 0.01 |
| 22:4 (n-6) | 0.03 ± 0.00 | 0.05 ± 0.01 | 0.06 ± 0.00 | 0.09 ± 0.01 | 0.12 ± 0.00 | 0.16 ± 0.00 | 0.02 ± 0.00 | 0.03 ± 0.00 | 0.04 ± 0.00 | 0.06 ± 0.00 | 0.08 ± 0.01 | 0.12 ± 0.01 |
| 22:5 (n-6) | 0.10 ± 0.00 | 0.16 ± 0.01 | 0.07 ± 0.00 | 0.11 ± 0.00 | 0.09 ± 0.01 | 0.13 ± 0.01 | 0.22 ± 0.01 | 0.27 ± 0.01 | 0.17 ± 0.01 | 0.24 ± 0.01 | 0.19 ± 0.01 | 0.27 ± 0.01 |
| 22:5 (n-3) | 0.43 ± 0.00 | 0.66 ± 0.01 | 0.51 ± 0.03 | 0.77 ± 0.04 | 0.81 ± 0.01 | 1.07 ± 0.01 | 0.60 ± 0.00 | 0.72 ± 0.00 | 0.60 ± 0.04 | 0.83 ± 0.05 | 0.84 ± 0.02 | 1.15 ± 0.03 |
| 22:6 (n-3) | 1.75 ± 0.01 | 2.64 ± 0.01 | 1.52 ± 0.05 | 2.32 ± 0.07 | 2.43 ± 0.09 | 3.23 ± 0.12 | 2.76 ± 0.00 | 3.33 ± 0.00 | 2.53 ± 0.09 | 3.51 ± 0.12 | 3.03 ± 0.08 | 4.17 ± 0.11 |
| 24:0 | 0.01 ± 0.01 | 0.01 ± 0.01 | 0.01 ± 0.00 | 0.01 ± 0.00 | 0.00 ± 0.01 | 0.01 ± 0.01 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.01 | 0.01 ± 0.01 | 0.01 ± 0.01 | 0.02 ± 0.01 |
| Total saturated | 15.87 ± 0.07 | 23.94 ± 0.11 | 15.44 ± 0.45 | 23.51 ± 0.69 | 17.47 ± 0.22 | 23.22 ± 0.29 | 18.62 ± 0.33 | 22.45 ± 0.40 | 16.94 ± 0.02 | 23.48 ± 0.03 | 15.82 ± 0.09 | 21.73 ± 0.13 |
| Total mono | 30.34 ± 0.07 | 45.78 ± 0.10 | 27.30 ± 0.52 | 41.57 ± 0.79 | 29.48 ± 0.14 | 39.19 ± 0.19 | 36.03 ± 0.29 | 43.46 ± 0.35 | 29.69 ± 0.03 | 41.15 ± 0.04 | 27.55 ± 0.26 | 37.83 ± 0.35 |
| Total poly | 21.18 ± 0.05 | 31.95 ± 0.07 | 22.36 ± 0.05 | 34.05 ± 0.07 | 27.80 ± 0.64 | 36.95 ± 0.86 | 29.18 ± 0.12 | 35.19 ± 0.15 | 26.71 ± 0.36 | 37.03 ± 0.50 | 28.77 ± 0.16 | 39.52 ± 0.22 |
| (n-3) | 18.69 ± 0.05 | 28.21 ± 0.08 | 20.24 ± 0.03 | 30.82 ± 0.05 | 24.83 ± 1.02 | 33.00 ± 1.36 | 25.92 ± 0.11 | 31.26 ± 0.13 | 24.01 ± 0.24 | 33.28 ± 0.33 | 25.61 ± 0.27 | 35.17 ± 0.37 |
| (n-6) | 1.58 ± 0.07 | 2.39 ± 0.11 | 1.41 ± 0.07 | 2.15 ± 0.10 | 2.02 ± 0.35 | 2.69 ± 0.46 | 2.22 ± 0.02 | 2.68 ± 0.03 | 1.95 ± 0.09 | 2.70 ± 0.13 | 2.31 ± 0.08 | 3.17 ± 0.11 |
| (n-3)/(n-6) | 7.85 ± 0.37 | 11.84 ± 0.56 | 9.42 ± 0.42 | 14.35 ± 0.64 | 9.42 ± 1.99 | 12.52 ± 2.65 | 9.67 ± 0.06 | 11.66 ± 0.07 | 8.90 ± 0.33 | 12.33 ± 0.46 | 8.08 ± 0.37 | 11.10 ± 0.51 |
| 22:6/20:5 | 0.11 ± 0.00 | 0.17 ± 0.00 | 0.09 ± 0.01 | 0.14 ± 0.01 | 0.14 ± 0.00 | 0.19 ± 0.00 | 0.16 ± 0.00 | 0.20 ± 0.00 | 0.14 ± 0.01 | 0.20 ± 0.01 | 0.18 ± 0.01 | 0.25 ± 0.01 |

### 3.4. Spawning and larval rearing

Spawning characteristics and larval parameters are expressed in Table V. The spawning and larval rearing were related to broodstock nutrition, with 5%, 82% and 83% of spawners in Diet 2, Diet 3 and Diet 4, respectively. The lowest performance was recorded for broodstock fed with the Diet 1 that did not spawn. The number of females among the spawners was always lower than the males. The average number of eggs released by females ranged from 0.04 to 10.87 million and it was again the broodstock fed with the Diet 4 that released the highest number, followed by Diet 3. Although, no significant differences were detected among the Diet 2, Diet 3 and Diet 4 (ANOVA, $F$=2.70, $df$=1, $P$=0.116). Only, the eggs released from the broodstock fed the diets formulated fundamentally by diatoms (Diet 3 and Diet 4), were viable. The fertilization rate was similar between this two diets ($P$>0.05). However, significantly higher hatching rate was obtained with broodstock that fed only diatoms (Diet 3) (80±8%) when compared with Diet 4 (73±12%) (K-W., $H$=5.65, $df$=1, $P$=0.017).

Table V. Spawning characteristics in *Crassostrea angulata* broodstock conditioned with different food regimes: Diet 1 (Pav and T-ISO); Diet 2 (Pav, T-ISO and Skt); Diet 3 (Skt and C.cal); Diet 4 (Skt, C.cal and Pav).

| **Spawning and larval parameters** | **Nutritional regime** | | | |
|---|---|---|---|---|
| | Diet 1 | Diet 2 | Diet 3 | Diet 4 |
| No. of oysters | 36 | 38 | 34 | 36 |
| Spawners (%) | - | 5 | 82 | 83 |
| Female spawners (%) | - | 3 | 35 | 31 |
| Means no. eggs released ($10^6$) | - | 0.035 | 5.92 ± 6.08 | 10.87 ± 8.04 |
| Fertilization rate (%) | - | - | 90 ± 8 | 94 ± 5 |
| D larvae (%) | - | - | 80 ± 8 | 73 ± 12 |

## 4. Discussion

Bivalve production in hatchery is undeniably related to the quality of food provided (Helm et al., 2004). With the present hatchery techniques, microalgae production can represent 30% to 40% of the hatchery production costs (Coutteau and Sorgeloos, 1992; Robert and Gérard, 1999; Rico-Villa et al., 2006). Achieving optimal algal composition for broodstock bivalve feed which allow optimum reproductive performances has been the object of some nutritional studies for aquaculture bivalve species (e.g.; Utting and Millican, 1997; Pronker et al., 2008; González-Araya et al., 2011; González-Araya et al., 2012b), however such information was unavailable, until know, for *C. angulata* broodstock. The effect of microalgal diets on reproductive performance is difficult to generalize and seems to be species-specific (Wilson et al., 1996; Utting and Millican, 1998; González-Araya et al., 2012a; Martínez-Pita et al., 2012) and sensitive to algal culture conditions (Harrison et al., 1990; Brown et al., 1997; Brown, 2002).

Experiment developed in this study was carried out to improve hatchery efficiency of *C. angulata* by assessing the nutritional value of four different common microalgae used in bivalve hatcheries, *I. galbana* clone T-ISO, *P. lutheri*, *S. costatum* and *C. calcitrans*. Effectively, the results obtained put in evidence the effect of different nutritional contents of the food regimes used to feed broodstock of *C. angulata*, as verified by the differences in reproductive output observed among broodstock fed with diets formulated fundamentally by flagellates or by diatoms. The gametogenic process, energy accumulation, spawning success and embryonic development were influenced by the nutritional content of the diet supplied.

Fundamentally, the success of the hatchery phase, broodstock conditioning, depends among other factors such temperature, the quantity and the quality of food supplied (Robert and Gérard, 1999; Soudant et al., 1999). In this work the diets supplied to each treatment presented equal quantity in terms of organic weight, differing in the microalgae species that constitute the diets, which imply differences in nutritional value in terms of biochemical composition (proteins, carbohydrates, lipids and FAs) of the four diets, which in turn reflects in the biochemical content of broodstock. This is one of the few works that describe the proteins, carbohydrates, lipids and FAs of the diets (Diet 1 – Pav and T-ISO; Diet 2 – Pav, T-ISO and Skt; Diet 3 – Skt and C.cal; Diet 4 – Skt, C.cal and Pav) and not of the single species that constitute the food regimes. The characterization of the diets is

relevant once that a controlled diet constituted by more than one microalgae species seems to cause changes in the biochemical composition of this diet, as they coincide with fatty acid profile of each phytoplankton species (Martínez-Pita et al., 2012). Microalgae typically contain high levels of proteins, followed by lipids and carbohydrates (Brown et al., 1997; Brown, 2002; Spolaore et al., 2006). The microalgae diets used in our study also showed these proportions. Nevertheless, the biochemical composition of microalgae can vary with culture conditions and with nutritional value of each species (Brown et al., 1997).

According to Zhukova and Aizdaicher (1995), unusual FAs or group of FAs may serve as biochemical indicators, in spite of the variability of the FAs profile of microalgae, once their specific features were retained. Fatty acids are key micro-nutrients present in the lipids, especially eicosapentaenoic acid (EPA, 20:5(n-3)) and docosahexaenoic acid (DHA, 22:6(n-3)) for marine animals (Volkman et al., 1992; Brown et al., 1997; Knauer and Southgate, 1999; Spolaore et al., 2006; Zhicui et al., 2006). Microalgae species from the group of diatoms such as *Chaetoceros* sp. and *Skeletonema* sp. contains higher levels of 16:1(n-7) and 20:5(n-3) FAs than 18:2(n-6) and 22:6(n-3) FAs (Zhukova and Aizdaicher, 1995; Brown et al., 1997; Brown, 2002; González-Araya et al., 2011; Martínez-Pita et al., 2012). Flagellate groups represented by *Isochrysis* sp. have higher levels of 18:2(n-6) and 22:6(n-3) FAs than 16:1(n-7) and 20:5(n-3) FAs (Brown et al., 1997; Brown, 2002; González-Araya et al., 2011; Custódio et al., 2014). In the case of *Pavlova* sp. (flagellate group) Zhukova and Aizdaicher (1995), Brown et al. (1997), Brown (2002), Ponis et al. (2006), Milke et al. (2008) and González-Araya et al. (2012a) noted that fatty acid profile of this microalgae was similar to the diatoms, nonetheless this was not coincident with our results, in which the content of 20:5(n-3) FAs was higher than in T-ISO and lower than in diatoms. The fatty acid profiles of the diets used in this work were similar to those previously reported for each microalgae species constituent of the diets. Despite no significant differences were observed in the total lipids content and fatty acid profiles among diets, the relative contents of FAs presented detectable and distinct variations. Diet 1 (flagellate diet) was rich in 18:2(n-6) and 22:6(n-3) FAs and poor in 16:1(n-7) and 20:5(n-3) FAs, when added one diatom (Diet 2) the percentage of 18:2(n-6) and 22:6(n-3) FAs drooped and the percentage of 16:1(n-7) and 20:5(n-3) FAs rose. Diet 3 (diatoms diet) had higher content in 16:1(n-7) and 20:5(n-3) and low levels of 18:2(n-6) and 22:6(n-3).

However when *P. lutheri* (flagellate) was added (Diet 4) the percentage of 16:1(n-7) and 20:5(n-3) were similar to the Diet 3, due to the fact that fatty acid profile of *Pavlova* sp. was more similar to the diatoms than to T-ISO (Brown et al., 1997; Brown, 2002).

Marine bivalves undergo a seasonal cycle of energy storage and utilization (Sühnel et al., 2012) that is linked to the progress of sexual maturation, promoting a highly variable competition for energy which develops between somatic process (growth, metabolism, maintenance) and the reproductive process (Lubet, 1994). The gonadal development of bivalve was considered as the key parameter of the sexual maturation process (e.g. Walne and Mann, 1975; Ojea et al., 2004).

Gonadal development in *Crassostrea* sp. generally includes a sequence of stages, from gametogenesis to degenerating stage (Mann, 1979; Enríquez-Díaz et al., 2009) and may be affected by manipulating temperature and nutrition, accelerating the gametogenesis or in retarding gonadal maturation (Gallager and Mann, 1986). In our study, the diet constituted only by diatoms (Diet 3) was the most effective to promote the gonad development of *C. angulata* broodstock. Contrary the broodstock that fed the diet formulated exclusively by flagellates (Diet 1) showed an unsuccessfully gonadic development. This fact could be explained by the different nutritional value of the species that composed the diets. According to González-Araya et al. (2013), *C. gracilis* and *S. marinoï* promote a better and faster *Ostrea edulis* gonadal development; however *P. lutheri* was considered a poor diet to conduce a faster gonadal development. The microalgae *I. galbana* clone T-ISO has been considered in an intermediate position; that is, when combined with a diatom could represent an efficient diet. Effectively, this is in agreement with our results, the unsuccessfully gonadic development observed in oysters that fed Diet 1 was related to the fact that this diet was composed only by *P. lutheri* and *I. galbana* clone T-ISO, since adding one diatom (*C. calcitrans*) as in Diet 2 broodstock gonadal performance was slightly improved. As referred, the best gonadal development performance was achieved in broodstock fed Diet 3 (*C. calcitrans* and *S. costatum*), nevertheless when it was added one flagellate to this diet as in Diet 4 (*P. lutheri*), the gonadal development of the broodstock was slightly slower. The low nutritional value of *P. lutheri* used in *C. angulata* maturation process as also reported by González-Araya et al. (2012a).

The physiologic status of the broodstock was evaluated by condition index. Beyond the gonadal development, condition index of bivalves has been considered as the key parameter of the sexual maturation process (e.g. Walne and Mann, 1975; Matias et al., 2009). During experimental period, the evolution of the condition index reflects the evolution of gonadal maturation. Similarly to the gonadal development the maximum performance of the condition index has been obtained in broodstock that fed Diet 3, followed by the progenitors fed with Diet 4 and Diet 2. The lowest broodstock physiologic status was observed in oysters that fed Diet 1. Thus it is assumed that broodstock fed Diet 3 has an optimal food regime that satisfies somatic and reproductive process. In contrast, the broodstock fed Diet 1 probably can make adjustments in metabolic adaption, indicating that possibly the nutritional value of the food regime was inadequate and energy was channeled essentially for maintenance of the basal metabolism. However, the evolution observed in the gonadal development and condition index of the individuals that were fed Diet 1, suggests that in case of nutritional stress, reproduction seems to be a priority and oysters allocate some available energy to this process for ensuring the viability of the species.

In marine bivalves, proteins are mainly used in structural function (Benninger and Lucas, 1984; Albentosa et al., 2007); glycogen (Fernandez-Castro and Vido-de-Mattio, 1987; Dridi et al., 2007) and total lipids are the central components for supplying energy demands (Martínez et al., 2000; Dridi et al., 2007). In situations of low glycogen levels or severe energy imbalance, proteins can be used as a source of energy for maintenance (Gabbot and Bayne, 1973; Benninger and Lucas, 1984; Ngo et al., 2006; Albentosa et al., 2007; Liu et al., 2008; Pogoda et al., 2013). In this study, broodstock fed Diet 1 during conditioning period, probably utilized the proteins as energy for maintenance and lipids were used as a source of energy for reproduction, once the glycogen content was practically constant and the values of proteins and total lipids decreased, while oyster's gonadal development slightly occurred. This occurrence indicates that Diet 1 did not ensure the sufficient nutritional requirements for the full gonadal development. Nevertheless, according to Pronker et al. (2008) and Pogoda et al. (2013), during gametogenesis, the oysters preferential form of energy reserve was the glycogen, after an initial period of storage glycogen this biochemical component was used simultaneously with food as energetic support (Zhicui et al., 2006; González-Araya et al., 2011) and the

lipids play an important role in the gamete formation (Gabbot, 1983; Martínez et al., 2000; Zhicui et al., 2006). The variations detected between glycogen and lipids may been attributed, to the conversion of glycogen to lipids biosynthesized during the formation of gametes (Gabbot, 1975; Gabbot, 1983; González-Araya et al., 2011; Pogoda et al., 2013), thus the drop in glycogen levels and the slight increase of total lipids, observed after the 5 weeks of conditioning in broodstock fed Diet 2, that reflected the late gonadal development, showing that the presence of one diatom improve the nutritional value of the diet. The results of this study show a clear dependence on lipid content on *C. angulata* broodstock sexual maturation evolution. *C. angulata* progenitors fed with diets constituted fundamentally by diatoms (Diet 3 and Diet 4), showed successful gonadal maturation and the high values of total lipids, the main reserve of oocytes (Utting and Millican, 1997; Soudant et al., 1999; Helm et al., 2004), although no significant differences were observed among total lipids of diets. After the 5 weeks of conditioning, a decrease in total lipids was observed in broodstock fed with Diet 4, this fact could be related with a partial spawning that was not detected, and this is characteristic of oysters (Lubet, 1994; Helm et al., 2004).

Not only the quantity, but also the quality of the lipids must be considered. Generally FAs profile of the oyster, contain high proportion of polyunsaturated fatty acids (PUFA) especially 20:5(n-3) and 22:6(n-3) (Utting and Millican, 1998; Knauer and Southgate, 1999; Zhicui et al., 2006; González-Araya et al., 2011), which appear to be a limiting factor on the optimum reproductive performances. In comparison to the flagellates, the diatoms used in this experiment was richer in 20:5(n-3) FA, which might have contributed to the better reproductive performance of the broodstock fed the diets constituted predominantly by diatoms (Diet 3 and Diet 4), comparatively to the broodstock that fed diets formulated by mostly flagellates (Diet 1 and Diet 2). These results indicate that either 20:5(n-3) or 22:6(n-3), but not necessarily both, were required to sustain the reproductive performance, reflecting a specific role of 20:5(n-3) in reproduction of *C. angulata* during the conditioning on the hatchery. This fundamental requirement for the 20:5(n-3) was previously reported for *C.gigas* in the studies undertaken by Helm and Laing (1987) and Soudant et al. (1999). Moreover, according to Soudant et al. (1999) the most suitable diets to feed broodstock in hatchery are those which mimic to the natural diets. Effectively, Pogoda et al. (2013) in a study with *O. edulis* and *C.gigas* reared in offshore, observed that the natural diet was mainly constituted by diatoms and the flagellates were not an

important component of the food regime. Thus it may be deduced that also a laboratory condition the most suitable diets are constituted preferentially by diatoms that were rich in 20:5(n-3) FAs.

The success in conditioning *C. angulata* in this study has been quantified by reproductive output, taking into account spawning success, fecundity, fertilization rate and hatching rate. There was an evident effect of the conditioning diet on the response of oysters to reproductive output. According to Utting and Millican (1998) and Nevejan et al. (2003), quality of the food given to broodstock during conditioning period has an important effect on their reproductive output, specially the level of the essential FAs 20:5(n-3) and 22:6(n-3) of the diet (Utting and Millican, 1997). The results achieved by Pronker et al. (2008), showed that for *M. edulis* the diet consisting mainly of diatoms gives better results than the diet dominated by flagellates. The present study also corroborates these observations, the best response to spawning induction and fecundity were obtained with broodstock fed Diet 3 and Diet 4 (predominantly diatoms). A possible explanation for these are the fact that diatoms diets were the most suitable for supplying energy to gametogenesis, once the diatoms species used were rich in lipids, especially in 20:5(n-3) FA. Although the Diet 4 had the highest glycogen and lipids levels comparing to the other diets, the oyster did not have the best reproductive output, one possible justification is the presence of the flagellate *P. lutheri*, that was not fully adequate to *C. angulata* broodstock conditioning. However, the change of this flagellate specie by other could improve the performance of this food regime, such as suggested by González-Araya et al. (2012a) and González-Araya et al. (2012b). The poor reproductive output performance observed with broodstock that fed Diet 1 and Diet 2 (mostly flagellates), reinforce the idea that these type of diets presented low nutritional value to conditioning *C. angulata*. In the bivalve hatcheries, more important than the number of eggs released was the success of fertilization and the survival of the D-larvae (Sühnel et al., 2012). Once again, the broodstock that fed Diet 3 conduce the highest values of D-larvae survival, indicating that this diet was the most suitable food regime to conditioning *C. angulata*.

## 5. Conclusion

The results of this study clearly emphasized the importance of the different feeding regimes on the *C. angulata* broodstock conditioning and the relationship among gametogenesis, physiological condition, reproductive output and biochemical composition of the Diets and broodstock. A holistic approach incorporating these results showed better reproductive response with the broodstock predominantly fed with diatoms (Diets 3 and 4) comparatively with the broodstock predominantly fed with flagellates (Diets 1 and 2). Also demonstrates that between broodstock predominantly fed with diatoms, Diet 3 is the highly suitable to maximize reproductive effort and output performance of *C. angulata*. This emphasizes the idea that the diatom microalgae group is essential in the conditioning of *C. angulata*. This work constitute an important first step in the hatchery *C. angulata* broodstock nutrition and a prerequisite for future work on improvement of broodstock conditioning and the optimization of feeding practices that will maximize the conditioning phase and minimize cost in aquaculture hatcheries.

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# Annex

**Annex:** Abstract for International Meeting on Marine Research 2014

# THE INFLUENCE OF DIFFERENT MICROALGAL DIETS ON *CRASSOSTREA ANGULATA* (LAMARCK, 1819) BROODSTOCK CONDITIONING

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## Abstract

The Portuguese oyster *Crassostrea angulata* shows great potential in oyster farming. In Europe, pure populations of this species were observed only in the southern coasts of Portugal and Spain, namely in Rio Sado, Rio Mira and Guadalquivir. The conservation of *C. angulata* populations is important in the context of production diversification and biodiversity preservation. In this way the zootechnological development for seed hatchery production is extremely important. Broodstock conditioning is a key step in the process of rearing bivalve in hatchery. Many factors regulate the reproductive cycle, being food the most important. However the influence of the nutritional quality of different phytoplankton on sexual maturation has been poorly explored. To evaluate the effects of different diets on *C. angulata* sexual maturity, broodstock were conditioned with different food regimes: Diet 1: bi-specific combination of *Pavlova lutheri* and *Isochrysis galbana clone T-ISO* (1:1); Diet 2: tri-specific combination of *P. lutheri*, *I galbana clone T-ISO* and *Skeletonema costatum* (1:1:1); Diet 3: bi-specific combination of *S. costatum* and *Chaetoceros calcitrans* (1:1) and Diet 4: tri-specific combination of *P. lutheri, S. costatum* and *C. calcitrans* (1:1:1). During conditioning, condition index and gonad histological analysis were performed. Results showed heterogeneity between diets. At the beginning of conditioning 60% of individuals were in resting (stage 0), 30% were males in early gametogenesis (stage I) and 10% were females in mature stage (stage III). At the end of the conditioning, the most effective diet was the Diet 3 (60 % of mature oysters with a mean condition index value of 2.83±0.95). Whereas those fed with Diet 1 have an unsuccessfully gonadic development, with 80% of individuals in resting stage. Indeed, the condition index, in Diet 1 decreased during the conditioning period. The results obtained in this study reinforce the idea that the diatom microalgae group is essential in the conditioning of *C. angulata* broodstock. This work constitute an important first step in the hatchery *C. angulata* broodstock nutrition and a prerequisite for future work on improvement of broodstock conditioning and the optimization of feeding practices that will maximize the conditioning phase and minimize cost in aquaculture hatcheries.

**Keywords:** Bivalves; *Crassostrea angulata*; Hatchery; Broodstock conditioning; Histology; Condition index; Algal diets.

## **Annex:** Abstract for Aquaculture Europe 2014

### HATCHERY BROODSTOCK CONDITIONING OF THE PORTUGUESE OYSTER *CRASSOSTREA ANGULATA* (LAMARCK, 1819): INFLUENCE OF DIFFERENT DIETS

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#### Introduction

The Portuguese oyster *Crassostrea angulata* could be a promising species in world aquaculture. The conservation of pure populations of this species is important in the context of production diversification and biodiversity preservation (Batista et al., 2005). In this way the zootechnological development for seed hatchery production is extremely important. Broodstock conditioning is a key step in the process of rearing bivalve seed in hatchery (González-Araya et al., 2012). Many factors regulate the reproductive cycle, being food the most important (Utting and Millican, 1997). However the influence of the nutritional quality of different phytoplankton on sexual maturation has been poorly explored. The present study was designed to evaluate the effects of different conditioning diets on sexual maturity and reproductive output of *C. angulata*.

#### Materials and methods

*C. angulata* broodstock were conditioned at 21±1°C, in flow-through system and fed with different diets: Diet 1 - *Pavlova lutheri* and *Isochrysis galbana clone T-ISO* (1:1); Diet 2 - *P. lutheri*, *T-ISO* and *Skeletonema costatum* (1:1:1); Diet 3 - *S. costatum* and *Chaetoceros calcitrans* (1:1); Diet 4 *S. costatum*, *C. calcitrans* and *P. lutheri* (1:1:1). During conditioning, samples of oysters were collected to evaluate condition index and perform histological analysis. At the end of the conditioning period, oysters were induce to spawn, by thermal stimulation and percentage of spawners, mean of eggs released and percentage of D larvae was evaluated.

#### Results

The gonad development advanced under the experimental conditions, however higher heterogeneity among diets was observed. At the beginning of conditioning 60% of individuals were in resting (stage 0), 30% were males in early gametogenesis (stage I) and 10% were females in mature stage (stage III). At the end of the conditioning, the most effective diet was the Diet 3, with 60% of mature oysters. Whereas those fed with Diet 1 have an unsuccessfully gonadic development, with 80% of individuals in resting stage.

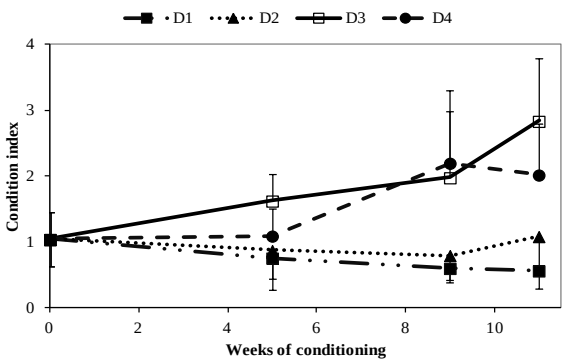


Fig. 1. Condition index (mean±SD, *n*=10) in *Crassostrea angulata* broodstock conditioned with different food regimes.

The highest values of condition index were obtained with the Diet 3 and Diet 4. Otherwise oysters conditioned with Diet 1 and Diet 2 the condition index generally decreased along the experimental period (Fig 1).

Table I. Spawning characteristics in *Crassostrea angulata* broodstock conditioned with different food regimes.

| Spawning and larval parameters | Nutritional regime | | | |
|---|---|---|---|---|
| | Diet 1 | Diet 2 | Diet 3 | Diet 4 |
| No. of oysters | 36 | 38 | 34 | 36 |
| Spawners (%) | - | 5 | 82 | 83 |
| Female spawners (%) | - | 3 | 35 | 31 |
| Means no. eggs released ($10^6$) | - | 0.035 | 5.92 ± 6.08 | 10.87 ± 8.04 |
| D larve (%) | - | - | 80 ± 8 | 73 ± 12 |

The greatest number of eggs released was obtained with diet 4. However the highest hatching rate was obtained with the diet 3. The lowest performance was recorded for broodstock fed with the diet 1 that did not spawn (Table I).

**Discussion and conclusion**

A holistic approach incorporating all results showed that the Diet 3 was the more adequate for *C. angulata* broodstock conditioning. Otherwise, the Diet 1 exhibit the lowest performance. González-Araya et al. (2012) and González-Araya et al. (2013) assumed that diatoms single diets are highly recommended for *Ostrea edulis* broodstock conditioning whereas *P. lutheri* should be excluded. Also, Pronker et al. (2008) reported for *Mytilus edulis* that the best diet for conditioning was constituted predominantly by diatoms. Our results were in agreement with these observations. The results obtained constitute an important first step in the conditioning *C. angulata* broodstock nutrition and a prerequisite for future work on improvement of broodstock conditioning and the optimization of feeding practices that will maximize the conditioning phase and minimize cost in aquaculture hatcheries.

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