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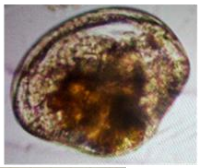
Deliverable 7.1

Settling culture procedures at hatchery and outdoor stages



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Contents

1. EXECUTIVE SUMARY

Cerastoderma edule is widely exploited and supports important commercial bivalve fisheries throughout its geographical range, particularly in Atlantic Area (AA) of Europe. However, the synergistic action of fishing pressure coupled with the rapid growth rate, short lifespan and huge mortality by disease (*e.g.* marteliosis) of *C. edule* leads to large inter-annual fluctuations in stock abundance and periodic recruitment failure. Within COCKLES project an objective was intended to contribute to restore and increase cockle populations. The development of restocking programs supported by advances in *C. edule* aquaculture could be an efficient fishery management strategy to rebuild stocks. In this action, we intended to develop efficient culture procedures in hatchery and outdoors stages. To achieve this purpose, in the context of **hatchery research**, studies were developed aiming to contribute to find the best diet for cockles conditioning and larval culture phase and, the most economically viable technique for larval production. Concerning, **outdoors stage research**, two different culture systems for cockle's growth were tested: a suspended culture in a suspended structure in earthen ponds tanks used for fish production in polyculture system and in a shellfish plot in Ria Formosa lagoon.

Hatchery research - The evaluation of effect of different diets during sexual maturation and spawning success provided detailed information on hatchery broodstock performance of *C. edule*. The results showed that nutritional value of diet supplied to broodstock during conditioning clearly influences the gametogenesis process as was evidenced by the differences in the condition index between the *Isochrysis aff galbana* (T-iso) 25% + *Chaetoceros calcitrans* (C.cal) 75% and the other diets tested. In this way we can define the most suitable diet for the conditioning phase this bi-specific diet. Concerning larval phase, the effect of two common microalgae used in bivalve hatcheries, *I. aff galbana* and *C. calcitrans* used as monospecific and bispecific diet, on the survival, growth and settlement of *C. edule* larvae was evaluated. This study clearly showed that *C. edule* larvae cannot use *C. calcitrans* with the same efficiency than *I. aff galbana*. The use of a monospecific diet during hatchery production could greatly facilitate routine management, a specific recommendation, based on results, to achieve optimal larval growth, would be to use *I. aff galbana* monospecific diet during all larval phases. The larval culture of cockles in a high-density culture system, such as the RAS system, can be a good opportunity to minimizing operative cost. The comparison of *C. edule* larval development between RAS and Batch

systems was evaluated. Results showed that the mean survival in RAS was the double of the traditional Batch system and growth was also higher. Also, larval rearing at different densities in RAS system was evaluated, indicating that larvae culture in RAS can be performed until an initial density of 30 larvae per mL, without significant losses in survival rate. The *C. edule* larval rearing performed at high stocking densities in RAS present a reduction in the operating costs. In general, the holistic results contribute to improve the global technology in hatchery to produce *C. edule*.

Outdoors stage research - The on-growing phase is the ultimate phase of the production and aims to grow seeds to commercial size as quickly as possible to make the operation economically attractive. The viability of cockles on-growing phase performed in suspended culture, in a suspended structure in earthen ponds tanks used for fish production in polyculture, and in an intertidal shellfish plot was evaluated. Results showed that attaining market size in few months with high densities was possible with both rearing systems. In conclusion, rearing cockles in intertidal shellfish plots and in earthen pond is viable.

These hatchery and outdoors stage research findings provided valuable information on the biology and zoothecnhical aspect of *C. edule*, information that is essential to assess its potential for aquaculture. This basic, but crucial information could be a useful tool for aquaculture producers and also constitute an important instrument for develop restocking and selective breeding programs.

2. INTRODUCTION

The common cockle (*Cerastoderma edule*, Cardiidae) is an intertidal and subtidal species which lives in temperate climate waters and its area of distribution extends along the north-eastern Atlantic coastline from the western region of the Barents Sea and the Baltic to the Iberian Peninsula, and south along the coast of West Africa to Senegal (Hayward & Ryland 1995). Cockles are particularly abundant in sandy substrates such as estuaries and bays (Dabouineau & Ponsero 2009), where population densities can reach several thousand individuals per square meter (Coosen et al. 1994, Tyler-Walters 2007).

Cerastoderma edule is widely exploited and supports important commercial bivalve fisheries throughout its geographical range (Dare et al., 2004; Freire et al., 2010; Malham et al., 2012; Martinez-Castro &

Vázquez, 2012), particularly in Atlantic Area (AA). However, according to FAO (2017) a sharp decrease in cockles' catch was observed in last years. The synergistic action of fishing pressure coupled with the rapid growth rate, short lifespan and huge mortality by disease (*e.g.* marteliosis) of *C. edule* leads to large inter-annual fluctuations in stock abundance and periodic recruitment failure (Ramón, 2003). In some years, the abundance of this species decreases dramatically, threatening the biological and eventually, the economic sustainability of this fishery. The development of restocking programs supported by advances in *C. edule* aquaculture could be an efficient fishery management strategy to rebuild stocks (Joaquim et al., 2014). Also, producing marteiliosis-resistant cockle strains appears to be a promising approach to overcome this disease in endemic areas, considering the difficulties to fight against marteiliosis in an open sea context, and because selective breeding programs have been successful to increase mollusk resistance against various diseases (Smits et al., 2020).

The production of bivalve mollusks is a strategic activity since it contributes significantly to the preservation of local economies and generates capital and employment on the littoral areas. The bivalve aquaculture industry depends indeed on the availability of high quality juveniles, which will grow rapidly to commercial size and which does not depend on environmental conditions and the fluctuations of the natural populations' recruitments (Ojea et al., 2004). It also permits to select the size and weight of seed most suitable to initiate the growth production phase during all year long. The control of the zootecnhical conditions allows the production of juveniles with certain features of interest such as optimum survival and growth, disease resistance, etc. In this sense, the artificial production of bivalve juveniles appears as a way to satisfy the needs of the aquaculture industry, since it will allow obtaining a controlled product. For hatcheries to consistently produce spat it is essential to develop broodstock conditioning, larval and post-larval culture techniques (Pernet et al., 2004; Pronker et al., 2013). 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 spat cost in hatchery (Rico-Villa et al., 2006). Achieving optimal algal composition to feed bivalve which will allow optimum performances, has been the object of extensive nutritional studies for many aquaculture bivalve species (*e.g.* Liu et al., 2009; Pettersen et al., 2010), however such information was scarce for *C. edule* (Pronker et al., 2013). Effectively, the effect of microalgal diet on different life stage performance

is difficult to generalize and seems to be species-specific and sensitive to algal culture conditions (Pernet & Tremblay, 2004).

Hatchery production of juveniles can be a major contribution to improve aquaculture if it complies the assumption that the economic return should far surpasses the investment in seed for restocking. In this context, the culture of cockles in a high-density culture system, such as the Recirculating Aquaculture System, can be a good opportunity to minimizing operative cost. This system could improve productivity since is possible to rearing larvae in higher density (densities can be trebled) than in traditional Batch System and to make better and more efficient use of available resources. Time can be saved in raising the density of larvae without the need to drain statically operated tanks 3 or 4 times each week. On the other hand, it has great potential within physical space constraints or to reduce labor costs and time spent in larval husbandry (Kamermans et al., 2016).

The on-growing phase is the ultimate phase of the production and aims to grow seeds to commercial size as quickly as possible to make the operation economically attractive. This phase of bivalve aquaculture industry depends also on the availability of ground plots with adequate environmental conditions to organisms' growth. The seasonal environmental changes and the location of the ground plots have a great influence on bivalves, namely in survival, growth and reproduction. Indeed, the quality and food availably are factors of great importance to the bivalve ground plot productivity. For this phase it was possible to use juveniles produced in hatchery or from natural cockle beds. The on-growing phase begins with the sowing of seed of an appropriate size to maximize the result of the growing when legal market size is reached. However, the size of seed produced in hatchery or wild juveniles must comply the assumption that the economic return should far surpasses the investment in seed for restocking.

Due to high social and economic importance of *C. edule* it seems essential to optimize the production conditions of this species. The culture of cockles both in hatchery or shellfish plots is not a common practice as for other bivalves, such as clams and oysters. Usually, in addition to fishing this species, bivalve producers collect specimens that grow naturally in their licensed areas in the intertidal zone. However, due to the poor availability of cockle populations the interest of the industry in developing hatchery production and land-based grow-out techniques for cockles has increased considerably. At the

moment, there is no commercial hatchery that can provide cockle seed in a continuous way for growing out. Moreover, research in cockle culture is very scarce and only three articles were found on this subject. Pronker et al. (2013) have described for the first time the production of *C. edule* on a commercial scale. They concluded that the best diet for broodstock conditioning is 100% of the microalgae *Tetraselmis suecica,* that induced 12% of female to spawn 3,380,000 eggs. A mixed diet of *Phaeodactilum tricornutum* and *Skeletonema costatum*, fulfilled the dietary requirements of spat to obtain an average growth rate of 168 µm day^{-1} and an average final size of 19.0±1.9 mm. Also, theses authors demonstrated that hatchery produced cockles (F1) can act as broodstock, thereby closing the production cycle. It was possible to obtain several millions of eggs and larvae at each spawning event, though this part of the production cycle still needs further improvement. This information suggests that large scale of cockle cultivation in hatchery seems technically feasible, but still many questions must be answered before this culture becomes economically viable. Ferreira et al. (2015) also found progress in the gametogenic cycle when broodstock was fed with FAO diet enriched with *Rhodomonas lens*. According to the authors, this diet increased gonadal condition index and also extended the stages of gonad maturation and spawning. Finally, Fuentes et al. (2015) also reported good results in inducing sexual maturation of cockles, however without specifying which microalgae species were supplied in conditioning.

The studies developed in COCKLES project intended to contribute to restore and increase cockle's production. COCKLES **Deliverable 7.1** aims to provide information for developing efficient culture procedures in hatchery and outdoors stages. To achieve this deliverable, in the context of **hatchery** research, studies were developed aiming to contribute to find the best diet for cockles conditioning and larval culture phase and, the most economically viable technique for larval production. Concerning, **outdoors** stage research, two different culture systems for cockle's growth were tested: a suspended culture in a suspended structure in earthen ponds used for fish production in polyculture and in a shellfish plot in Ria Formosa lagoon.

3. MATERIALS AND METHODS

Settling culture procedures at hatchery and outdoor stages of cockle *C. edule* were investigated. For juvenile's production, culture procedures, including **hatchery** protocols for conditioning of broodstock and larval rearing were developed in the Tavira Experimental Shellfish Aquaculture Station of the Portuguese Institute for Sea and Atmosphere, I. P. (IPMA, IP). Posteriorly, on-growing experiments were carried out to test different possibilities of **outdoor** culture of cockles: - under shellfish plots in intertidal zones of Ria Formosa; and - in suspended structure in earthen ponds tanks used for fish production in polyculture in the Olhão Pilot Fish Farming Station of IPMA, IP.

3.1 Hatchery culture procedures

As the work on the production of cockle in hatchery is scarce and the bibliographic resources found included partial information on this subject, COCKLES experiments were essentially focused to test diets and nutritional regimes on broodstock conditioning and larval rearing.

3.1.1 Broodstock conditioning

Broodstock conditioning is a key step in the process of rearing bivalve in hatchery aiming to induce gametogenesis completion of individuals out of his natural reproductive season. This process consists in maintaining breeders under conditions were different parameters such as water temperature and food addition are controlled in order to provide the complete sexual development of gonads or the extension of the spawning season.

3.1.1.1 Broodstock pre-treatment

Adult cockles were collected in natural banks and dry transported to hatchery at a temperature between 10 and 15ºC. Once in hatchery, cockles were rinsed to eliminate some detritus and placed in quarantine tanks with seawater at 2 to 4°C below the environmental temperature for at least one week, before conditioning. During this period, the minimum amount of food was administered for their survival.

3.1.1.2 Tank systems and water treatment

The conditioning was performed in 25 to 60-L plastic tanks in a flow-through system (Figure 1) at a flow rate of 0.8 L min^{-1} with natural sand filtered and aerated (0.5 L min^{-1}) seawater. Adult cockles were placed in the tanks inside perforated trays, to allow the passage of faeces and debris at a density of 340 ind.m^{-}

^{2} per tank, that corresponding 90 individuals per tank. Sieves of 20 and 100 µm were kept at the end of the tanks for eggs collection. Survival of progenitors and spontaneous spawning were checked daily.

The maintenance of the adequate conditions of cultivation, hygiene and animal health was a compromise during the conditioning. Tanks were sniffed to remove faeces every two days and a complete cleaning for tanks disinfection was performed once a week. The remaining system components, such as filters, water and/or food pipes, etc., had a cleaning schedule that ensured water and food quality.

Figure 1- Conditioning tanks.

3.1.1.3 Broodstock feeding

Similarly, to other bivalve's species, microalgae in the exponential growth phase were continuously pumped, at a daily ratio of 4% of cockle's dry weight (g) in microalgae dry weight (mg). Microalgae concentration and the proportion of the different species (diet formulation) was calculated daily according to the number of individuals in each tank.

Aiming to identify the most adequate diet for cockles conditioning phase, three different food regimes, previously tested for conditioning other bivalve species, were tested:

- Diet 1 – *Isochrysis aff galbana* (T-iso) 75%+ *Chaetoceros calcitrans* (C.cal) 25%
- Diet 2 – *Isochrysis aff galbana* (T-iso) 25%+ *Chaetoceros calcitrans* (C.cal) 75%
- Diet 3 – *Tetraselmis suecica* 100%

The Diet 1 and Diet 2 with different proportions of each microalgae were normally used for clams (Matias et al., 2016) and, Diet 3 constituted only by the microalgae *Tetraselmis suecica*, was chosen with the objective to compare with published data, that suggest this diet as the most adequate for conditioning this species (Pronker et al., 2013). A fourth treatment of unfed cockles was included as a reference.

Food flows were regulated to 0.05 L min^{-1} to ensure a continuous and adequate concentration of microalgae in each tank during all day, allowing a proper feeding of individuals.

Each treatment was performed in triplicate at density of 340 $ind.m^{-2}$ per tank, that corresponding 90 individuals per tank.

3.1.1.4 Temperature regime

Temperature was recorded and controlled daily to keep it constant at 21±1ºC (Matias et al., 2009).

3.1.1.5 Photoperiod

The effect of photoperiod is important in the success of gonadal development of the bivalves (Joyce et al., 2013). An alternated combination of temperature and photoperiod according to natural conditions reveals a clear development of sexual products and spawns out of for natural periods. In conditioning experiments, the photoperiod was maintained at 15:9 h (light/dark) (Utting & Millican, 1997).

3.1.1.6 Gonadal and gamete quality evaluation

The gonadal condition of breeders was determined by estimating the condition index and examining gonads smear. Samples were taken at the beginning, middle and at the end of conditioning period. At each sampling time 10 individuals were taken to evaluate condition index and gamete development.

To estimate the condition index, the dry weight of the whole soft tissues and that of the shell from each cockle were determined after oven drying at 80°C for 24 h. Soft tissue were then ashed at 450°C in a muffle furnace and the ash weight was determined. The condition index was calculated according to Walne and Mann (1975): CI = ash free dry weight (mg) × 100 /dry shell weight (mg).

For the evaluation of gamete development, examination of gonad smears was performed with microscope at 40× magnification. Each gonad sample was classified according to the following scale:

resting, developing, ripe, spawned and reabsorbing gonad. Ripe gonad condition in most cockle broodstock was searched for. It occurred in females when the oocytes occupy the centre of gonad follicles after their peduncle linking to the follicle wall has ruptured; in males, when gonad follicles are filled with spermatozoa.

3.1.2 Spawning and fertilization

Spawning induction allows to obtain gametes when individuals are sexually mature. Breeders were stimulated to release their gametes in response to thermal shocks (Figure 2) after broodstock conditioning. Those shocks consisted of rapid temperature increase, from 20 ºC to 28 ºC, over a 6 h interval.

Figure 2 – Cockles in a bath for thermal shock-spawning induction.

If no success occurred, animals were maintained in conditioning tanks for spontaneous spawning. Fertilized eggs and embryos were retained on a 20 µm sieve after passing through a 100 µm sieve to remove debris (Figure 3).

Figure 3 - Sieves for collecting eggs and embryos.

3.1.3 Embryonic phase

C. edule fertilized eggs and embryos were collected on a sieve, washed with filtered (0.35 µm) and UV-treated seawater and redistributed in a known volume of filtered seawater. Subsamples were taken and counted aiming to determine the total number of fertilized eggs and embryos.

Incubation of eggs and embryos was carried out in incubate 250 L fibreglass cylinder-conical tanks, at a density of 100 embryos ml^{-1}, in volume of 220 L, with seawater filtered at 0.35 µm cartridge filter and ultraviolet treatment, at 21º C with very slight aeration. In general, aeration during this early stage is not recommended. The mechanical effects of the disturbance can lead to abnormal development. However, incubation in the cylinder-conical tanks, a very slight aeration should be used to avoid the aggregation of embryos at the bottom of the tank, due to its immobility. During incubation embryos transformed into trocophora larvae and then into veliger larvae; incubation lasted two days.

3.1.4 Larval culture

Once embryos transformed into veliger larvae, larval culture was performed using two different systems, batch rearing system and recirculating aquaculture system (RAS).

3.1.4.1 Batch rearing system

Batch rearing system has been traditionally used for bivalve larvae culture. Larval culture tanks and all equipment used were thoroughly cleaned and then rinsed with either freshwater. After incubation, larvae were transferred to the same cylinder-conical tanks as those used for embryo incubation (Figure 5), but in this case with a volume of 20 L. Tanks were provided with seawater, filtered (0.35 µm cartridge filter), UV- treated and aerated (0.5 L min^{-1}), at 21º C.

Figure 5 – Batch system. Cylinder-conical tanks for larval rearing.

During larval culture, seawater was changed three times a week. Tanks were emptied by a bottom drain, delivering the discharge flow into a sieve battery with different mesh size gradient; the top sieve with larger mesh gradient, appropriate to allow larvae passing through and to retain large debris, and the bottom sieve with smaller mesh size appropriate to retain larvae. The mesh sizes in the sieve battery were different as larvae culture progressed and larval size increased.

3.1.4.2 Recirculating Aquaculture System (RAS)

RAS system used was an experimental system of IPMA composed by culture and sump unities (Figure 6). The sump consists in a reservoir which contains the biofilter. Seawater from the culture unit was collected in the inlet section of the sump and flowed upwards into the decanting cabinet. Thereafter, seawater flowed from the top to the biofilter section. The filtered water passed to the resting cabinet whence was pumped to the culture unity at 1000 mL per min. after going through a U.V. system. The

culture unities contained a filter whose mesh size varied according to size larvae. In this system only 10% of water was changed daily, essentially to avoid salinity rises.

Figure 6 – Recirculating aquaculture system (RAS) for larval rearing.

3.1.4.3 Evaluation of different diets for larval rearing in Batch System

Larvae were now at the stage where they need feeding with unicellular cultured microalgae. Usually, progressive diet is used to meet growing biomass. Normally in first days, bivalve larvae are mixotrophic, that means that yolk reserves can contribute also to the maintenance of larvae, so the input of phytoplankton was low.

Aiming to provide crucial information on its nutritional requirements and to identify the most adequate diet for this stage of cockle life, four diets were tested: two progressive monospecific diet (flagellate *Isocrhysis aff galbana* (Clone T-iso) and the diatom *Chaetoceros calcitrans)*, one progressive bispecific diet (flagellate *I. aff galbana* (Clone T-iso) plus the diatom *C. calcitrans*) and without food (unfed that gives the knowledge of the reach of the yolk reserves) (Figure 7):

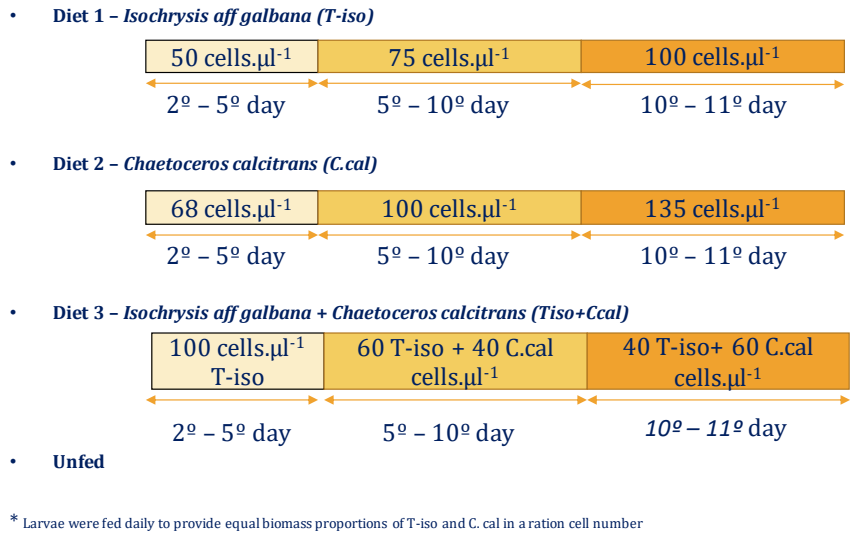

Figure 7 – Different feed regimes used in *Cerastoderma edule* larval rearing.

These diets were based on other nutritional regimes normally used for bivalves rearing (Matias et al., 2014). Larvae were reared at a Batch system (at the same condition describe above), with an initial density of 7 larvae ml^{-1}, in triplicate. Larvae were fed daily at a concentration according to the established nutritional regimes.

Percent larval survival was determined at each water renewal. Antero-posterior shell length (antero-posterior axis, expressed in µm) was measured for 50 randomly sampled larvae from each replicate using an ocular micrometer. The presence of a foot was scored to determine larval development status. Larvae that showed a clearly visible foot bulging out of the shell (pediveligers) was considered metamorphosed.

3.1.4.4 Larval rearing in Batch System *vs* Recirculating Aquaculture System (RAS)

The cockle larvae rearing performance was compared in two systems: a laboratory-scale closed recirculating system – RAS and the traditional larval rearing system – Batch. The same methodology described above for both system was used. Larval were reared at an initial density of 7 larvae ml^{-1}, in triplicate. Food was added daily and the diet was constituted by *I. aff galbana* at a progressive concentration, as describe above (Diet 1).

At each water renewal, three 1 ml aliquots were taken to evaluate the survival (relative to initial number of larvae). Also, larvae samples from each treatment were taken to evaluate shell length, based on measured of 50 randomly larvae an ocular micrometer. The presence of a foot was scored to determine larval development status; larvae that showed a clearly visible foot bulging out of the shell (pediveligers) and lost its velum were considered metamorphosed.

During experimental period, environmental parameters such temperature, salinity, pH and dissolved oxygen were monitored, using multiple parametric probe.

3.1.4.5 The effect of density on larval rearing in a recirculating aquaculture system (RAS)

At the same time that was evaluated the comparison of larvae rearing in batch system *vs* RAS, another experiment was done, aiming to evaluate the effect of different larval density in RAS. The early D-larvae collected were dispensed at an initial density of 10 and 30 larvae ml^{-1} in 5 L. Food (*I. aff galbana* at a progressive concentration) was added daily to each tank and water was renewed every 2-3 days. Over the larval rearing period and at each water renewal, larvae were recovered by draining the tanks. They were counted to estimate survival and to detect presence of foot and/or any morphological alterations in the velum. The presence of a foot was scored to determine larval development status; larvae that showed a clearly visible foot bulging out of the shell (pediveligers) and lost its velum were considered metamorphosed. At each water renewal, a sample of larvae was taken in order to estimate mean shell length. Antero-posterior shell length was measured for 50 randomly sampled larvae from each replicate using an ocular micrometer.

Temperature, salinity, pH and dissolved oxygen were monitored, using multiple parametric probe, during larval rearing.

3.2 Outdoor culture procedures

Two different outdoor culture systems were tested: in intertidal shellfish plots in a bivalve production park in Ria Formosa lagoon (37°01'25''N; 07°48'41''W) and in a suspended culture in a suspended structure in earthen ponds tanks used for fish production in polyculture (37°01'54''N; 07°49'17''W). These systems were both located in Olhão region, Algarve, Portugal (Figure 8).

Figure 8 – Location of the intertidal plots in Ria Formosa and earthen pond in the Pilot Fish Farming Station of IPMA (EPPO).

Survival, growth in length (mm), width (mm), thickness (mm), weight (g) and condition index (Walne & Mann, 1975) was determined to evaluate the success of on-growing culture in the different culture conditions tested.

3.2.1 Culture in intertidal shellfish plots

Transplant of juveniles from natural cockle beds to shellfish plots in Ria Formosa, in an effort to rebuild relatively high-density patches was tested. Substrate in on-growing areas was conditioned prior in order to facilitate the sowing of cockle seed and eliminate predators. This procedure required the removal of macroalgae, dead individuals and predator shelters, and the treatment of the substrate by adding clean and coarse sand. The bivalve production park in Ria Formosa used in the experiment has a total area of 7444m^2.

The experimental area was composed by 12 squares 1x1m in 2 rows of 6. Half of the squares were seeded for a wild density - 1200g.m^{-2} (expected final density – 2 kg.m^{-2}) and the other half with 1800 g.m^{-2} (expected final density – 3 kg.m^{-2}). For each density, half of the squares were protected with a plastic net with a grid 5x5mm to avoid predation (Figure 9 - 11).

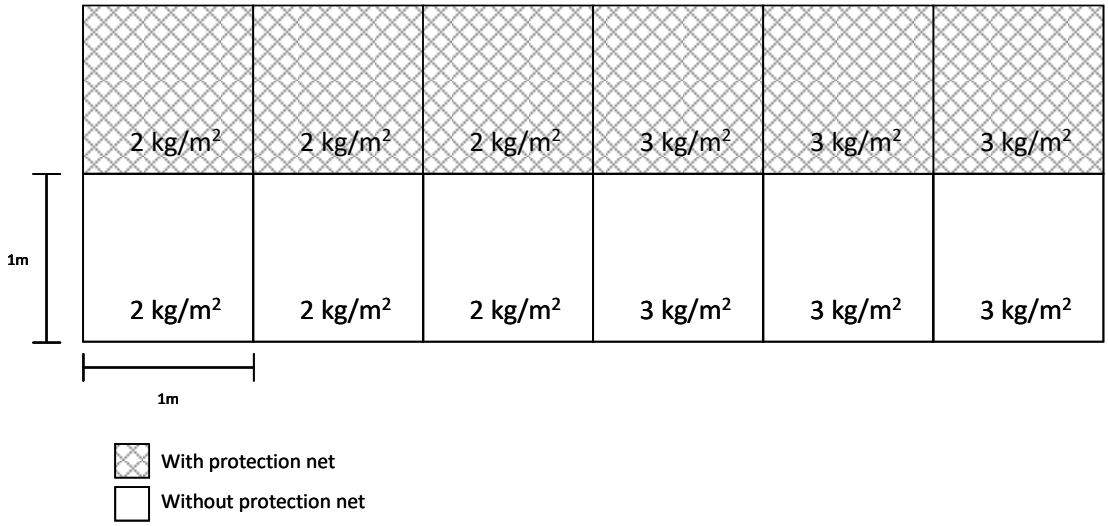

Figure 9 – Scheme of the Production Park experimental area.

Figure 10 – Seeding in a production park

Figure 11 – Protection net.

On a monthly base, five cockles from each replicate, density and treatment (with and without net) were sampling for biometric measures (length, width, thickness and weight) and condition index determination.

3.2.2 Culture in suspended structures

Two different containers for suspended cockle culture were tested: rigid mesh bags (Figure 12) and circular perforated (1 cm mesh) trays. Each tray holds four perforated (5 mm mesh) containers arranged as pie pieces within the tray (Figures 12 and 13); the trays (40 cm in diameter and 10 cm in height) were piled in stacks of four trays and only the second (downwards) tray and the bottom tray of each stack hold cockles, thus allowing comparison of culture at two depths (20 and 40 cm) (Figure 12). Two densities were tested in each container type, 2kg/m^2 and 3kg/m^2.

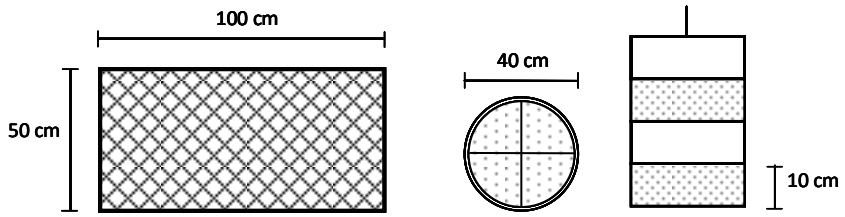

Figure 12 – Scheme of rigid mesh bag and circular trays.

Figure 13 – Circular trays.

The culture system was composed by 6 rigid mesh bags and 6 stacks of trays hanging from a cable (long-line) with floaters (Figures 14 and 15), set side by side alternating the two different densities. This system also had on both line extremes, for purpose of replacement of the sampled individuals, 2 supplementary rigid mesh bags and 2 stacks of trays with the different densities.

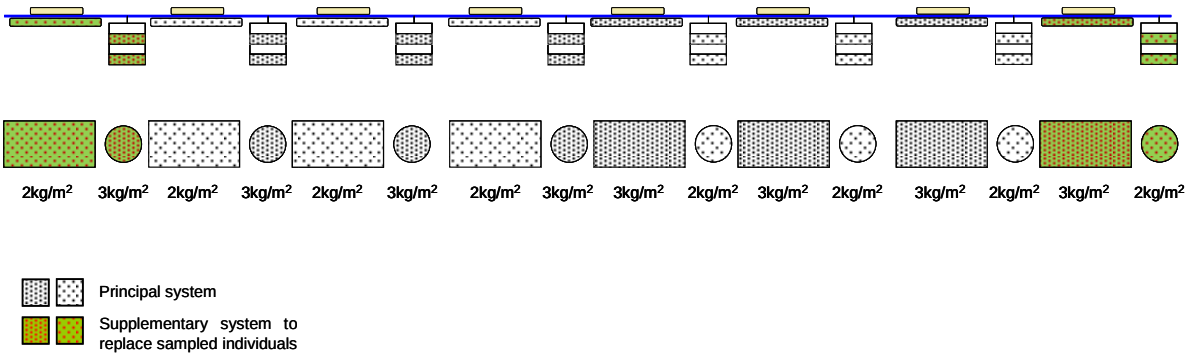

Figure 14 – Scheme of the culture procedure in the earthen pond.

Figure 15 – Culture device in the earthen pond.

These structures required cleaning labor once a month or once every fifteen days, depending on the level of encrustation of macroalgae and other fouling organisms. Concerning, rigid mesh bags cleaning, it is only necessary to turn them over to maintain the water circulation. However, circular trays needed to be replaced and washed with fresh water or leave them to dry in the sun to later remove the crusting organisms.

On a monthly base, five cockles from each replicate, density and structure (rigid mesh bag and circular tray) were sampling for biometric measures (length, width, thickness and weight) and condition index determination.

4. RESULTS AND DISCUSSION

4.1. Hatchery culture

4.1.1 Comparison of different diets for broodstock conditioning

Broodstock conditioning is a key step in the process of rearing bivalve in hatchery and is directly connected with the nutritional criteria of the species aiming to sexually mature individuals out of his natural reproductive season (Anjos et al., 2017). This process consists in maintain breeders under conditions were different parameters such as water temperature and food addition are controlled in order to provide the complete sexual evolution of gonads or the extension of the spawning season. Normally, the conditioning period can vary between two or ten weeks, depending on the initial maturation stage of individuals. In bivalves, best results are obtained when broodstock conditioning started during the sexual rest, however, it is also possible to develop gonads up to one month after conditioning started with adequate diet. In general, the onset of gametogenesis took place in early autumn, progressed throughout the winter, and the mature stage was finally reached in spring with the occurrence of the spawning period during spring and summer (Maia et al., 2021).

The manipulation of the gonadal cycle and spawning period is indeed crucial to adult's spawn earlier or later than occurs in the natural environment. As mentioned earlier in broodstock conditioning temperature and food availability are key external factors. The biochemical composition of the diet influences the physiology of bivalves. In general, changes in each biochemical component are closely linked to the state of sexual maturity of bivalves and are related to energy supply either directly from the ingested food or from previously stored reserves (Navarro et al., 2000; Pérez-Camacho et al., 2003).

In our study, broodstock conditioning started in March 2019, when individuals were already in an advanced stage of gonadal development (Maia et al., 2021). This fact, imply a reduction on conditioning period being possible to detect gonad in ripe stage one month after conditioning started. Condition index of bivalves has been considered as the key parameter of the sexual maturation process (*e.g.*

Matias et al., 2009). Two of the four diets contributed to increase the condition index of the broodstock, in other words, to their gonad development. The highest mean condition index value was obtained with the bi-specific diet formulated with higher content of a diatom microalgae (*Isochrysis aff galbana* 25% + *Chaetoceros calcitrans* 75%), that after one month of conditioning, followed by the other bi-specific diet (*Isochrysis aff galbana* (T-iso) 75%+ *Chaetoceros calcitrans* (C.cal) 25%) (Figure 16). At this time, the smears showed that cockles fed with the former diet had ripe gonad. One month later, the condition index of cockles feed bi-specific diets had dropped, which it possible associated to spontaneous spawning, that was not possible to detected due to absence of sieves to collect eggs. The mean condition index of cockles fed with the diet 100% *Tetraselmis suecica* remained stable during all conditioning period (Figure 16) and there was no progression in gonad condition. The mean condition index of the cockles of the unfed treatment even had failed after one month since the beginning of the experiment. Mortality rate was significantly high in all treatments, reaching 100% in the unfed treatment (Figure 16).

Thermal shocks failed to induce spawning every time it was assayed. However, successful spawning occurred spontaneous, in the conditioning tanks, at the end of the experimental period (2 month of conditioning).

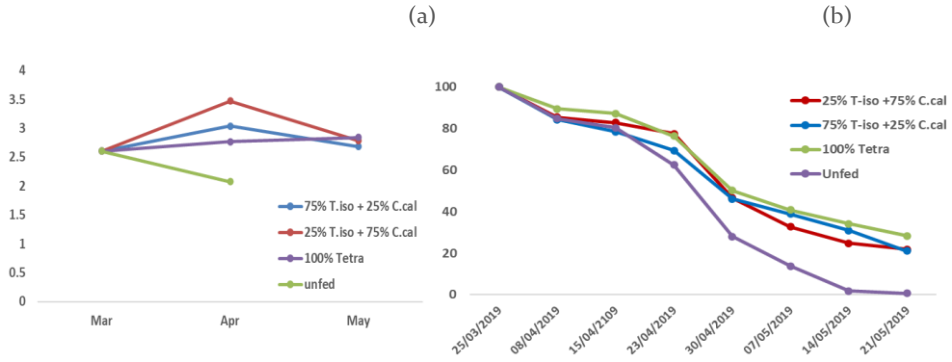

Figure 16 – Condition Index (a) and survival rates (%) (b), by diets, during *Cerastoderma edule* broodstock conditioning.

Fully shelly D-larvae were recovered 24 h after starting incubation and tanks containing newly developed D-larvae were drained to a 20 µm mesh screen and subsequently draining to a graduate beaker to

quantify the total number of D-larvae, by taken 1 mL aliquots (Figure 17). Before D-larvae recovered (incubation), no food was added because bivalve embryonic phase and trocophora larvae do not have the ability to feed. During this period, energy came from reserves laid down during egg development (oogenesis) by the maturing females.

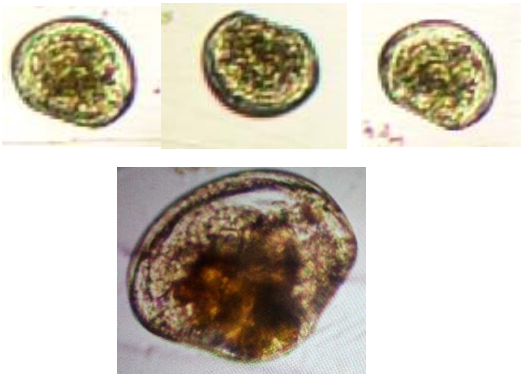

Figure 17 – *Cerastoderma edule* larvae.

4.1.2 The effect of different diets in larval rearing

Larval rearing is the second phase in hatchery husbandry. Successful bivalve larval growth and development depends on the energy available during the endotrophic and the subsequent exotrophic developmental phases (Matias et al., 2011). Moreover, during the early larval development, larvae rely essentially on existing reserves from the female gametes. The subsequent exotrophic phase that lasts until larval metamorphosis depends on the nutritional value of the diet provided to promote larval growth. The duration of these periods is species specific and strongly dependent on rearing temperature and food (Rico-Villa et al., 2009). It was then essential to identify feeding regimes that result in maximum growth, survival and settlement but which in turn also reduce hatchery operating costs.

Experiment developed in this study was carried out to improve hatchery efficiency of *C. edule* by assessing the nutritional value of two different common microalgae used in bivalve hatcheries, *I. aff galbana* and *C. calcitrans* used as monospecific and bispecific diet.

Survival is an important parameter in evaluating culture conditions and consequently the availability of bivalve larvae for further aquaculture operations. The choice of microalgae used as food strongly affects the survival of planktothrophic bivalve larvae (*e.g.* Gouda et al., 2006). In the present study, the survival rate (Figure 18) was low for all the tested diets, as reported in other works published on this species (Pronker et al., 2013). However, the mono-specific diet constituted by flagellate microalgae (T-iso) proved to be the most suitable diet for larval rearing (about 30% survival at 11th day of larval culture). The settlement was initiated at 11th days of culture only with diet 1. The bi-specific diet is normally the best diet for larval stage in other bivalve species, such as clams and oysters (*e.g.* Utting & Millican, 1997), however in our study, the survival rate decreased greatly between the day 8 and the day 11, when metamorphosis began. The introduction of diatom diet (*C. calcitrans*) did not produce better results.

The high survival rate observed on the unfed larvae until day 8 after fertilization confirm that the yolk reserves have a long reach in larval development.

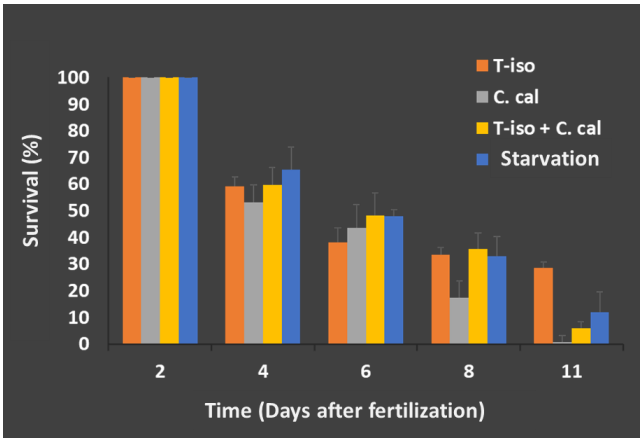

Figure 18 – Survival rate (%; mean ± SD) of *Cerastoderma edule* larvae fed with different diets.

Larvae reared under the four nutritional regimes all presented an increase in shell length growth over time. The larval growth results (Figure 19) showed the same tendency than the survival rate, however with more evidence of T-iso diet (diet 1) benefit from day 6. With this diet the larval growth rate was about 6 µm/day. The shell length growth observed in unfed larvae suggests that the biosynthesis of the shell is a priority in the distribution of energy resources. In extreme nutritional stress, energy seems to be provided by body reserves and/or the catabolism of tissues with a loss of organic matter.

In this study the larval growth results clearly indicated that the diets constituted by *I. aff galbana* are highly nutritional balanced for *C.edule*. Indeed, even the larvae fed the flagellate monospecific diet (T-iso) showed best larval performance than bispecific diets with the diatom in equal proportions.

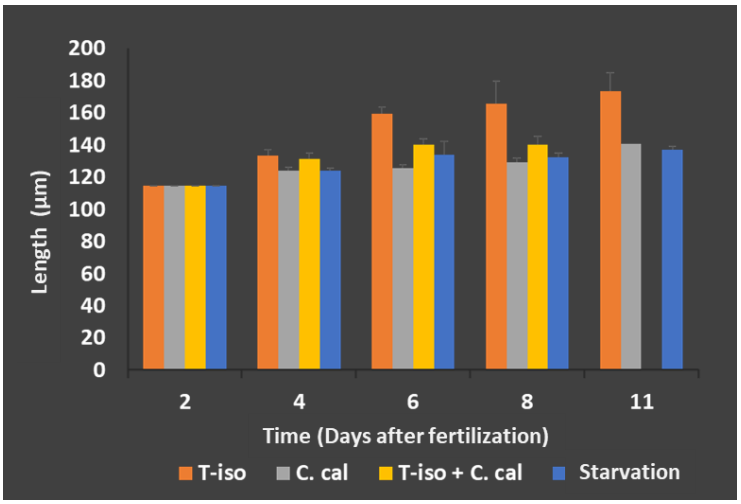

Figure 19 – Growth in length (µm; mean ±SD) of *Cerastoderma edule* larvae fed with different diets.

4.1.3 Batch System *vs* Recirculating Aquaculture System

The culture procedures to determine the feasibility of rearing *C. edule* larvae in a recirculating aquaculture system (RAS) was developed and compared with the traditional larval rearing methodology (Batch).

The temporal variation of the physical parameters of water was the expected in these types of system (Figure 20). Temperature (> 2 ºC) and salinity (> 2) were higher in the RAS system and dissolved oxygen was lower (approximately 2 mg/L) compared to the Batch system. The higher temperature in the RAS system is generally beneficial in larval rearing, since it normally speeds up larval growth and the sump unity of the system maintains water quality, so survival did not suffer much change because of different temperature.

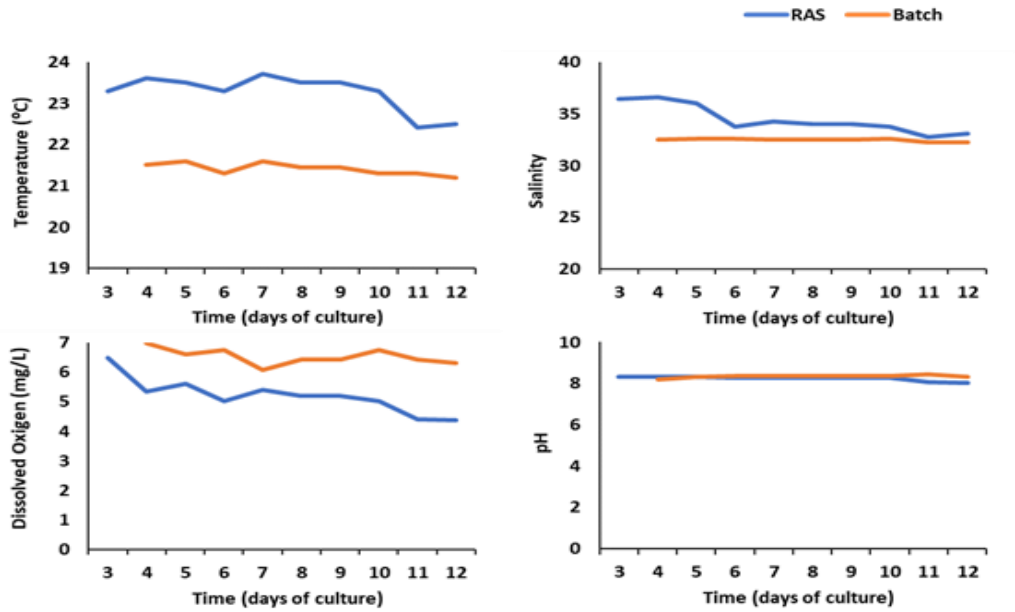

Figure 20 – Physical parameters of water in RAS and Batch systems during the *Cerastoderma edule* larval rearing.

Differences in larvae survival and growth between RAS and batch systems were not significant (Figure 21 and 22

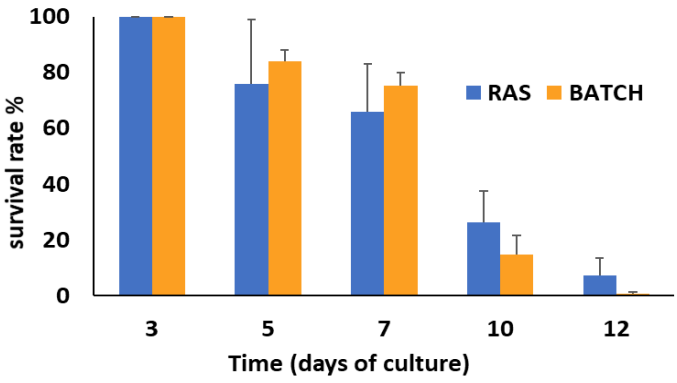

Figure 21 – Survival rate (%) of *Cerastoderma edule* larvae reared in RAS and Batch systems.

In RAS system more than 70% larvae were ready to start metamorphosis at day 12 while that percentage was lower than 50% in the batch system.

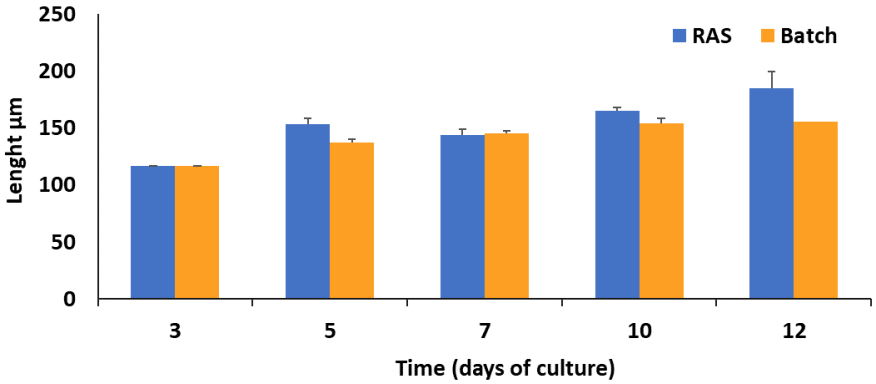

Figure 22 – Growth in length (µm) of *Cerastoderma edule* larvae reared in RAS and Batch systems.

4.1.4 The effect of density on larval rearing in a recirculating aquaculture system (RAS)

In the assay to test the effect of density on larval rearing in RAS, despite the variability between replicates (due to the clogging of one sieve), no significant differences were found between densities and practically all surviving larvae had foot at the end of the experiment (Figure 23).

As shown in the previous experiments, readiness to metamorphosis occurs around the 11th day after fertilization. So, larvae culture in RAS can be performed until an initial density of 30 larvae per mL without significant losses in survival rate, compared to the low density.

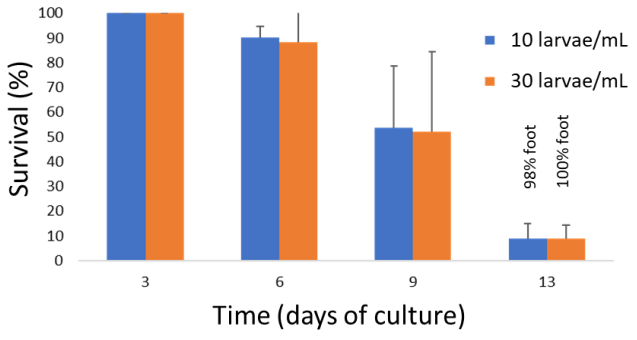

Figure 23 – Growth in length (µm) of *Cerastoderma edule* larvae reared in RAS and Batch systems.

4.2. Outdoor culture procedures

4.2.1 Culture in intertidal shellfish plots

In shellfish production the organisms' survival is dependent on cumulative mortality, which is often the sum of mortalities originated by various factors (the seed size, biological and physiological condition, environmental pathogens and predation) and the interaction between them. Effectively, the on-growing experiments carried out showed that net protection against predators increased survival between 15 and 20 % in seed with an initial size of 19 mm (2.2 g by weight), in the first month after restocking. After one month of culture, juveniles were robust enough and well adapted to the new habitat to dispense the net protection. After one month of culture, survival varied widely between replicates and between samples. The absence of barriers between replicates allowed the displacement of cockles within the plot area and consequently biased the results of survival.

The growth of bivalves varies with several factors: growing area, season of the year, temperature, quantity and quality of food and dissolved oxygen (Martinez., 1991) among others. While many of these factors are dependent on each other, some of them are dominant; indeed, food availability and temperature are the factors that have the major influence on growth (*e.g.* Paterson & Nell, 1998). The *C. edule* juvenile's length growth rate observed in intertidal shellfish plots was 60 µm per day in a mean period of 3.8 autumn/winter months, when the water temperature and food were lower. In general, the mean wild density - 1200g.m^{-2} (expected final density – 2 kg.m^{-2}) showed slightly better results than cockles reared at the high density – 1800 g.m^{-2} (expected final density – 3 kg.m^{-2}). Biomass yield was around 1400 g.m^{-2} and individuals reached maximum values of 25.71±2.94 mm of mean length and 5.31±1.38 g of mean fresh weight, on low density condition without net treatment (Figure 25).

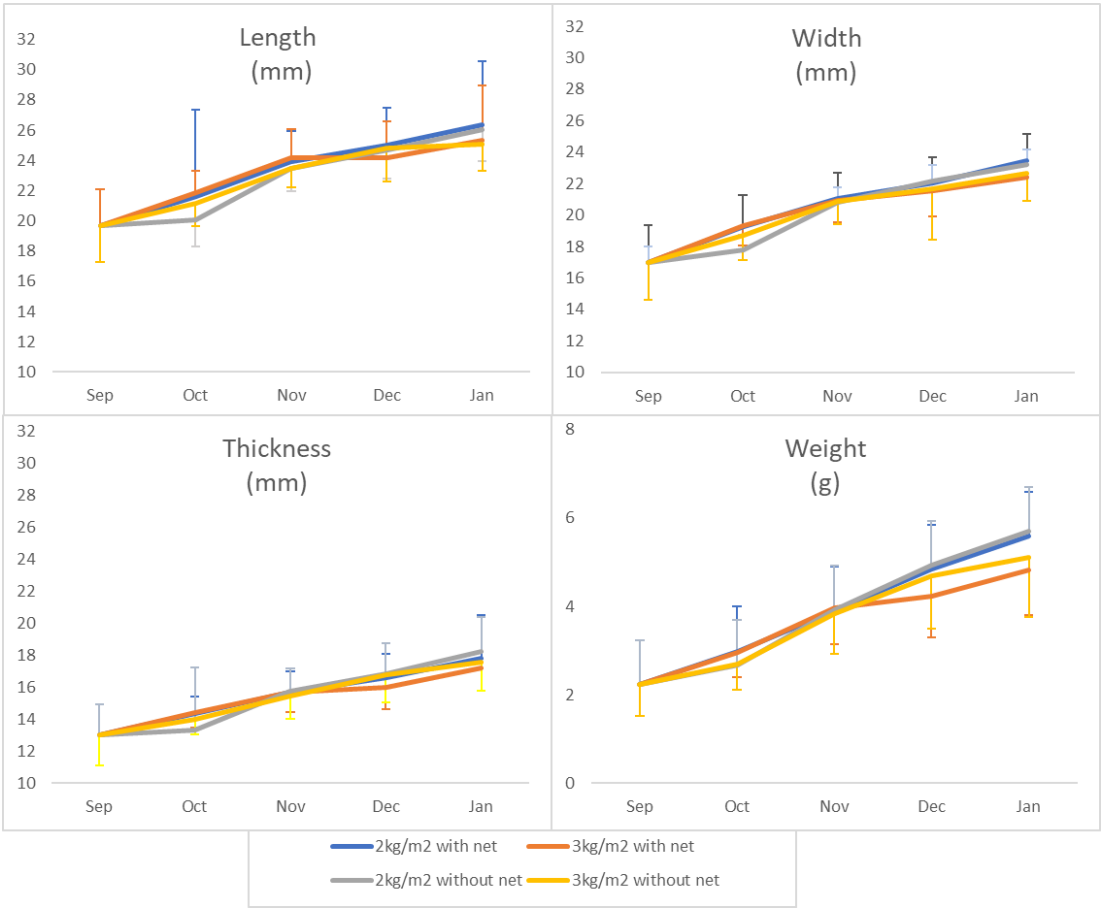

Figure 25 – Growth of *Cerastoderma edule* in intertidal shellfish plots located in Ria Formosa.

The cockles condition index showed similar values for all the rearing conditions tested, at the end of the experiment (Figure 26). Cockles with net protection exhibited a slight better condition in November and December, however its condition also decreased in the last sampling, as in non-protected cockles, reaching values obtained in the beginning of the experiment (4.55±0.97). Best results were obtained in October for cockles in condition 2kg.m^{-2} without net. This variation in cockle's condition index, during the sampling period, can be more related with environmental parameters than the rearing systems they were subjected.

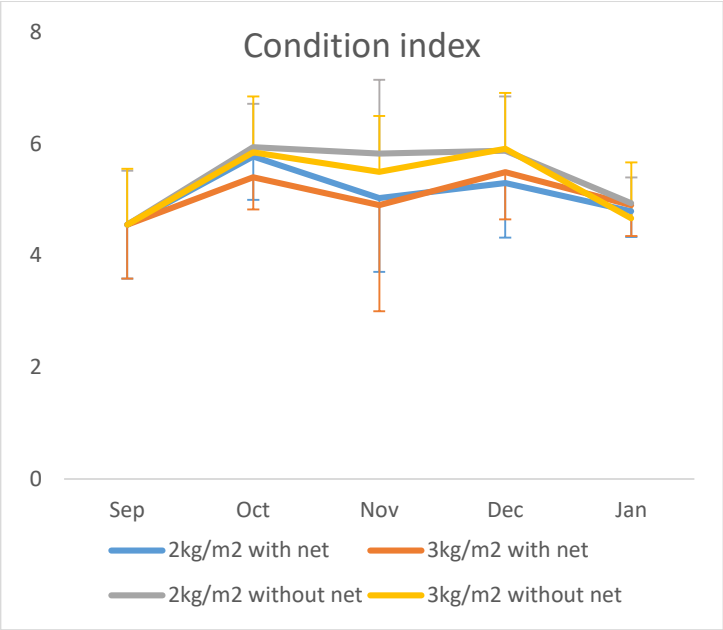

Figure 26 – Condition index of *Cerastoderma edule* in shellfish plots located in Ria Formosa.

4.2.2 Suspended culture

Growth of cockles reared in suspended culture was significantly better than the growth of cockles reared in intertidal shellfish plots located in Ria Formosa. In the same period (3.8 autumn/winter months), length growth rate of cockle reared in the circular trays at 3 $kg.m^{-2}$ can reach 85 µm per day (27.83±1.69 mm length and 6.92±1.30 g of weight at the end of the experiment). In rigid mesh bags, growth was slower (25.22±1.91 mm length and 5.29±1.05 g of weight in 3 $kg.m^{-2}$ density at the end of the experiment) (Figure 27). Biomass yield in circular trays and bags varied between 1300$g.m^{-2}$ and 1900 $g.m^{-2,}$ and the effect of density and depth of rearing were not substantially significant. This difference between cockles reared in intertidal shellfish plots *vs* the suspended system was specially, the reflection of the greater increase in weight.

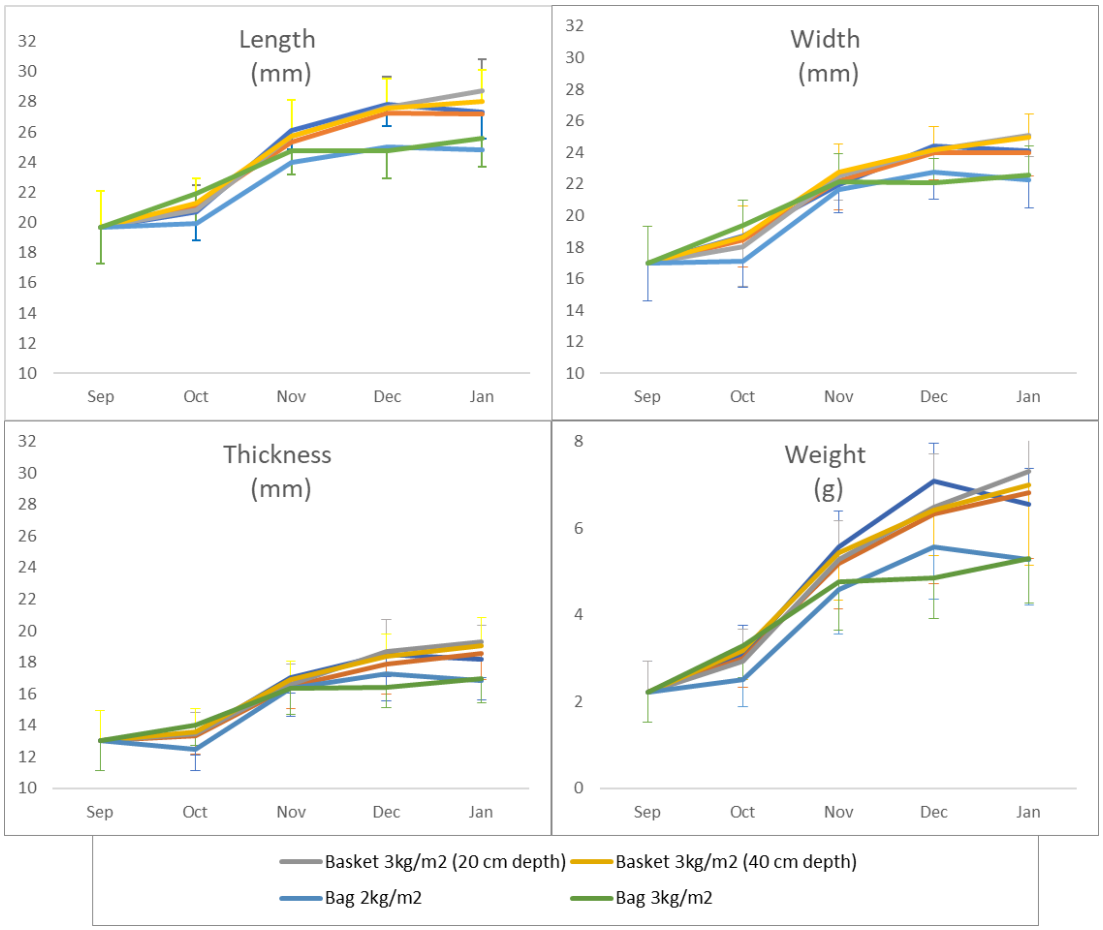

Figure 27 – Growth of *Cerastoderma edule* juveniles in suspended culture.

Condition index of cockles reared in suspended culture was substantially higher in cockles reared in the suspended systems (Figure 27). Best condition index was found in November for cockles that were reared in circular trays placed at 40 cm of depth with a density of 3 kg.m^{-2}. As for cockles reared in intertidal shellfish plots, condition index decreased substantially in December and January, reinforcing the thesis that this decrease can be more related with environmental parameters than the rearing systems they were subjected.

The differences between replicates did not allow to see significant differences in growth and condition index between densities.

The high cockle's growth found in suspended culture compared with intertidal shellfish plot culture may be explained by the highest apport of organic nutrient that can exist in polyculture ponds due to the addition of fish food. On the other hand, in this ecosystem, cockles were continuously immersed with continuous food availability which can be advantageous, compared with the intertidal plots where cockles are exposed to long periods of submersion.

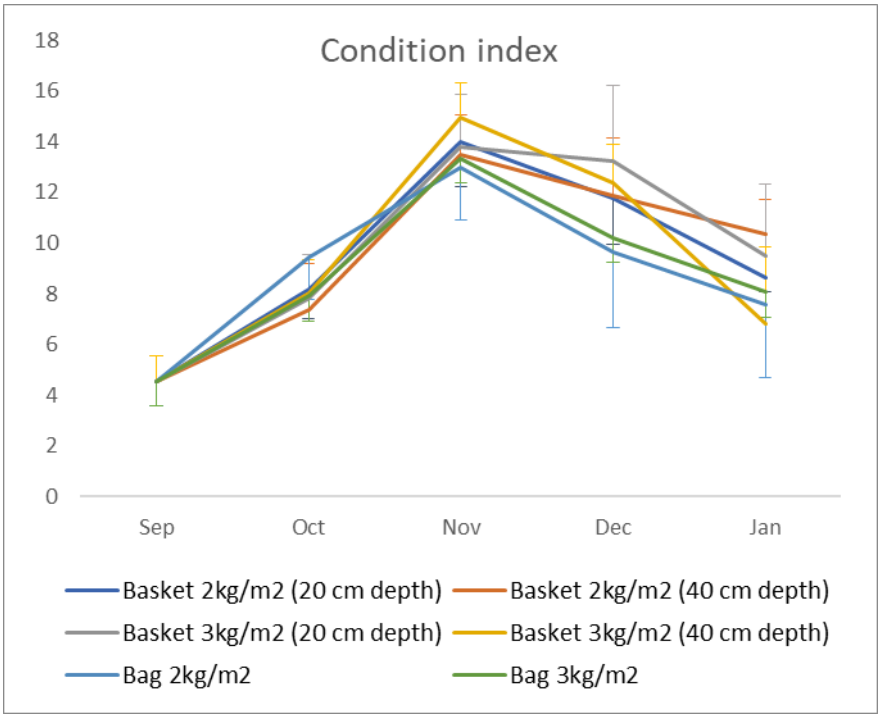

Figure 28 – Condition index of *Cerastoderma edule* in suspended culture.

In spite of achieving greater growth increments in the culture of cockles in earth ponds tanks compared to their culture in intertidal shellfish plots, the cockles produced in this system present shell deformations resulting from the lack of sediment in the culture structures. These shell deformations do not increase mortality but decrease the quality of the product and create obstacles to their sale directly

to the consumer as a fresh product; no problem would be expected to be sold to cannery industry or proceeded food without shell.

5. CONCLUSION

Bivalves are the main component of the benthic fauna of many marine and estuarine areas. In the last centuries, due to their high economic importance, there has been widely exploited and consequently large inter-annual fluctuations in stock abundance and periodic recruitment failure has been observed (Maia et al., 2021). This is the case of the cockle *Cerastoderma edule*, a species with a high social and economic importance in Atlantic Area (AA) in Europe. In order to contribute to overcome these situations, the development of *C. edule* rearing programs, and hatchery and outdoor growing technologies for the production of this species seems essential. However, until know only very few studies in cockle's aquaculture have been performed on this subject (Pronker et al., 2013; Ferreira et al., 2015; Fuentes et al, 2015).

The evaluation of the effect of different diets during sexual maturation and spawning success provided detailed information on hatchery broodstock performance of *C. edule*. The results obtained showed that nutritional value of the diet supplied to broodstock during conditioning clearly influences the gametogenesis process as was evidenced by the differences in the condition index between the diet composed by *Isochrysis aff galbana* (T-iso) 25%+ *Chaetoceros calcitrans* (C.cal) 75% and the other tested diets. In conclusion, these results can contribute to improve global hatchery technological development of *C. edule* and should be taken into consideration in *C. edule* production programs for broodstock conditioning and consequently high quality spat production.

The effect of the nutritional value of two different common microalgae used in bivalve hatcheries, *I. aff galbana* and *C. calcitrans* used as monospecific and bispecific diet, on the survival, growth and settlement of *C. edule* larvae was evaluated, allowing the achievement of a basic, but crucial information of nutritional requirements. This study clearly showed that *C. edule* larvae cannot use *C. calcitrans* with the same efficiency than *I. aff galbana* at early stages of development. In conclusion, taking into consideration that the use of a monospecific diet during hatchery production could greatly facilitate

routine management, a specific recommendation, based on results, to achieve optimal larval growth, would be the use of *I. aff galbana* monospecific diet during all larval phases. The establishment of the most adequate diet and ratio at each larval development stage is crucial information to define *C. edule* larval rearing programs. Nevertheless, the low survival rate attained with this monospecific diet suggest the need for further research looking for new diets or culture conditions contributing to increase larvae survival until metamorphosis.

The comparison of *C. edule* larval development between RAS system and Batch system was evaluated. Results showed that although the high mortality in both system, ten days after fertilization, the mean survival in RAS was the double of the traditional Batch system and growth was also higher. In conclusion, RAS constitute an efficient alternative methodology to culture of *C. edule* larvae.

The effect of larval density (10 and 30 larvae ml^{-1}) in RAS showed that larval culture could be performed until an initial density of 30 larvae per mL without significant losses in survival rate, compared to the low density. In conclusion, the *C. edule* larval rearing performed at high stocking densities in RAS present a reduction in the operating costs to produce this species.

The viability of cockles on-growing phase performed in suspended culture in an Integrated earthen tanks of fish culture and in an intertidal shellfish plot was evaluate. Results showed that was possible to attained commercial size in few months with high densities, in both rearing systems. In conclusion, juvenile's cockles reared in intertidal shellfish plots and in a suspended system in earthen ponds are both viable. The supremacy obtained of individual's growth in the suspended culture is undermined by deformations that can decrease the quality of the product and create obstacles to their sale directly to the consumer. So, this product can be forwarding to cannery industry or proceeded food without shell and cockles reared in inteltidal plot is more targeted to fresh consume.

In general, these studies provided valuable information on the biology and zoothecnhical aspect of *C. edule*, information that is essential to assess its potential for aquaculture. This basic but crucial information could be a useful tool for aquaculture producers and also constitute an important instrument for develop restocking and selective breeding programs. However, it is important to carried

out further research to improve culture protocols of *C. edule*, specially concerning metamorphosis and nursery stages.

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