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INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR

MESTRADO EM CIÊNCIAS DO MAR - RECURSOS MARINHOS

On the use of live grass shrimp (*Palaemonetes varians*) as the only prey for cuttlefish (*Sepia officinalis*) culture throughout the life cycle

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Dissertação apresentada ao
Instituto de Ciências Biomédicas Abel Salazar
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especialidade de Aquacultura

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Aknowledegements

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Abstract

In this thesis, general methodologies used for the pilot-scale production of the cuttlefish *Sepia officinalis* are described.

The life cycle of cuttlefish fed exclusively on live grass shrimp was studied during 2 consecutive generations (winter and summer). A total of 27 and 30 cuttlefish were used for the winter and summer experiments, respectively. Both populations were cultured in a 250 L flow-through seawater system. Water temperature was of 15±3 ºC (winter cycle) and of 23±2 ºC (summer cycle). Salinity was 35±2 PSU during the winter cycle and 37±2 PSU during the summer cycle. Both generations were fed live grass shrimp (*Palaemonetes varians*) *ad libitum* during the whole life cycle. Cuttlefish from the winter and the summer experiments started spawning at 220 and 90 days old, respectively. Mean growth rates were of 3.6 and 13.8 %body weight.day^{-1} for the winter and summer cycles, respectively. After 20 days, cuttlefish from the summer cycle grew significantly larger ($p<0.05$) than cuttlefish from the winter cycle. At the start of spawning, cuttlefish from the winter and summer cycles weighed 201.76 ± 34.40 g and 45.51 ± 8.45 g, respectively. Survival at the end of the winter and summer cycles was of 82% and 77%, respectively. The fatty acid profile of grass shrimp was similar to the fatty acid profile of the cuttlefish (Navarro & Villanueva, 2000). Nutritional profiles and the suitability of the grass shrimp as a new diet is discussed. Grass shrimp appears to be a good diet for the culture of cuttlefish throughout the life cycle.

The culture of cuttlefish hatchlings using different densities and enriched environments was studied for 20 days. This study was made in order to try to determine the optimal values of density for large scale culture in the near future, and to clarify the open question of the use or not of a substrate to enhance growth and survival during the culture of *S. officinalis*. Both experiments were performed in a flow-through sea-water system with mean water temperature of 23±2ºC and mean salinity of 37±2 PSU. Grass shrimp was supplied *ad libitum* as food. The density experiment studied the effects on growth and survival of 3 different cuttlefish densities (52, 515 and 1544 hatchlings.m^{-2}, referred as density 1, 10 and 30, respectively) while the enriched environment experiment studied the effects of using a sandy with shelters substrate on cuttlefish growth and survival.

In the density experiment, growth was different between the 3 densities ($p<0.05$), with a better final growth in weight for density 10 (0.603±0.141g). Mean IGR was of 8.8%, 9.6% and 9.2% body weight.day^{-1} for the densities 1, 10 and 30, respectively. Survival at the end of experiment was of 77.8%, 42.5% and 57.8% for the densities 1, 10 and 30, respectively.

In the enriched environment experiment, no significant differences ($p>0.05$) were found in growth between the two groups. IGR was of 10.1% and 9.7% body weight.day^{-1} for enriched and non-enriched environments, respectively. Survival was higher in non-enriched environments (70%

versus 60%).

These results indicate that the first stage of cuttlefish culture (at mean temperatures of 23±2ºC) should be done in a non-enriched environment, using maximum densities of about 500 hatchlings.m^{-2}, in a minimum area of about 600 cm^2.

Two groups of 100 cuttlefish hatchlings each were used to determine the duration of the yolk reserves, during which growth can be obtained with no food supply. One group was fed live grass shrimp *ad libitum* from the third day of life onwards, while the other was starved during the all experiment. The experiment lasted 7 days, because this was the first day where negative growth was obtained. Determination of nutritional condition was adapted from the methodologies of Clemmesen (1988, 1990), Clarke *et al.* (1989) and Caldarone & Buckley (1991) to determine DNA and RNA content of both groups. RNA/DNA condition ratios and instantaneous growth rates were used as a way to describe their condition and when all the yolk reserves would be exhausted.

RNA/DNA ratios described clearly the differences ($p<0.05$) between fed and starved hatchlings cultured at 23±2ºC, especially from day 4 onwards. Correlation between IGR and RNA/DNA ratios was only attained for starved population ($R=0.90$; $p=0.037386$). DNA concentration was identical ($p>0.05$) between groups and did not displayed a clear relation with increasing age. Values of RNA concentration were of about 8.5 $\mu g.g^{-1}$ during the 3 final days of experiment. RNA concentration values for starved hatchlings decreased between day 5 and the end of the experiment, when they reached 0 $\mu g.g^{-1}$. Both values should describe maximum and minimum rate of protein synthesis in fed cuttlefish hatchlings, at a temperature of 23±2ºC. Although literature consulted indicated that *S. officinalis* depletes yolk reserves in 3 days, RNA concentration was also similar ($p>0.05$) for the fourth and fifth days.

IGR seems to be the most accurate way to describe hatchlings growth and condition.

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Chapter 1

Introduction

1. Introduction

Cephalopods are dynamic predators and occupy a dominant position in the marine food webs (Boletzky & Hanlon, 1983). They have short life cycles, fast growth rates and high conversion efficiencies (Forsythe & Van Heukelem, 1987; Lee, 1994). Cephalopod are known to be unique and versatile models for investigation (Boletzky & Hanlon, 1983; Boucaud-Camou, 1990; Koueta *et al.*, 1998; Lee *et al.*, 1998). On the other hand, cephalopod species represent an important seafood supply for human consumption worldwide. According to FAO (www.fao.org), cephalopods contribute to approximately 14% of the world fisheries. The fast decline of the worldwide fish stocks as well as the technological advances over the last 13 years, makes profitable and necessary the development of technology for the culture of new species, in aquaculture. The high commercial value of the cephalopods, particularly in the Asian and Mediterranean markets, and aspects of biology and physiology mentioned above make them interesting for large-scale aquaculture (Caddy & Rodhouse, 1998; Lee *et al.*, 1998; Kunisaki, 2000). Its production for humans consumption could therefore be foreseen, since short life cycles and fast growth rates imply lower production periods and costs associated. Statistical values of landings and extensive aquaculture from FAO and DGPA (Portuguese Fisheries Statistics) for the last 20 years confirm an increasing human consumption and a market yet to explore. The main reasons for this incresing demand is that they are a good source of protein and essential lipids, and that they can be consumed in a variety of forms: eaten raw as *sashimi* or *sushi*, cooked as *tempura* or deep-fried, boiled as *nimono*, or processed into delicacies like *surume* (dried squid), smoked squid, *saki-ika* (shredded dried squid) (Kunisaki, 2000).

Experimental culture of cephalopod started in the 1960's, by the korean and japanese, on some species of the Sepiidae family. These pioneer studies were then followed and complemented by european and north-american scientists in the following years (Gonçalves, 1989).

Richard (1971) and Pascual (1978) were some of the first researchers who succeeded in culturing the european cuttlefish *Sepia officinalis* (Linnaeus, 1758) in the laboratory.

This species has several characteristics that make it one of the most prom-

ising cephalopod species for aquaculture. It has large eggs, which are easily transported and maintained. Hatchlings resemble miniature adults in many habits and behaviour, and feed on relatively large live prey. Survival of hatchlings is usually high, compared to other marine species. This species is resistant to disease (Forsythe *et al.*, 1994), as well as handling, and can be easily shipped. It also tolerates crowding and accepts non-living food (Lee *et al.*, 1998). Since it has a life cycle, animals are normally available within short periods of time and in some cases, two generations can be obtained every year (Domingues *et al.* in press-a). These reasons, associated to the fact that culture and reproduction are easy in a captive environment, the capacity of the species to tolerate high densities in culture, high survival rates and food conversions, as well as the increased human consumption makes this species the one eligible for being profitable in intensive culture (Coelho & Martins, 1989; Henry & Boucaud-Camou, 1989; Lee *et al.*, 1991). It's nutritional profile, as a high protein and EPA/DHA lipid source, makes it one of more suitable and healthy forms of human food (Boucaud-Camou, 1990; Kunisaki, 2000; Navarro & Villanueva, 2000).

Research concerning the possibility of using cuttlefish as a candidate species for aquaculture started in the 1980's, with experiments carried out on the possibility of using this species in coastal lagoons (Coelho *et al.*, 1989; D'Apote & Palmegiano, 1982; Gonçalves, 1989; Palmegiano & Sequi, 1981, 1984; Sequi & Palmegiano, 1984). In the 1990's, initial research on pilot large-scale was carried out, in order to obtain animals for biomedical research (Forsythe *et al.*, 1991), at the NRCC, in Galveston (Texas, USA). This work showed good results until the 7^{th} generation (Forsythe *et al.*, 1994), after which, cuttlefish laid eggs but all were infertile and did not hatch.

Cuttlefish is being grown in extensive aquaculture for a number of years. Eggs are caught and left in ponds for 3 months, when adults are captured and sold for human consumption. According to DGPA data, until 1996, this production did not exceed 1 ton.year^{-1} of cuttlefish biomass produced. According to Rodger & Davies (2000), Tunisia has also been producing cuttlefish since 1990. Production worldwide has been low, generally not exceeding 30 animals per culture cycle, and results have been mainly used to characterize the life cycle of local populations. Only the National Resource Centre for Cephalopods (NRCC) in Gal-

veston (Texas, USA) has been producing cuttlefish in relative large numbers that can reach over 2000 individuals each year. A great number of these cuttlefish are sold for biomedical research. There is also a great potential to be explored, concerning the production of cuttlefish of smaller than the minimum capture sizes allowed by DGPA, which would reduce the impact of illegal fishing (undersized individuals) on the natural environment.

According to Lee (1994), growth rates for young cephalopods cultured at 23ºC can be as high as 13.5% $BW.d^{-1}$ (body weight per day). Hanlon *et al.* (1991) obtained daily growth rates of 3-4% $BW.d^{-1}$, with an weight increase from 500 to 1400 g, at temperatures which varied between 20-24ºC, over 10 months. Domingues *et al.* (2001b) cultured *S. officinalis* at 24-30ºC and obtained daily IGR's of 11.8% $BW.d^{-1}$ for hatchlings and mean IGR of 2.5-8.5% $BW.d^{-1}$, throughout the life cycle (Domingues *et al.*, 2001c).

Since the problems with water quality have been minimized during the past few years, the main problem that kept this species out of the commercial aquaculture business is the adequate food supply and cost associated. A large amount of different diets have been tested but only live food has attained good results in growth, condition and survival (Toll & Strain, 1988; DeRusha *et al.*, 1989; Lee *et al.*, 1991; Castro *et al.*, 1993; Domingues, 1999; Koueta & Boucaud-Camou, 1999; Domingues *et al.*, 2001b). Until now, good growth rates and low mortality have been obtained feeding live mysid shrimp during the first 10 to 20 days of the life cycle (Domingues *et al.*, 2001b and 2001c; Koueta *et al.*, 2002). Nevertheless, costs associated with mysid production and their low fecundity (Domingues *et al.*, 1998; Domingues *et al.*, 2000) are a bottleneck in the first stage culture of the cuttlefish. In addition, mysid abundance in the wild varies considerably throughout the year, with periods of very low abundance. Experiments using *Artemia* sp. as first food promoted poor growth (DeRusha *et al.*, 1989; Domingues *et al.*, 2001c) and high mortality (Pascual, 1978; Navarro & Villanueva, 2000). This is probably due to the lack of some lipids required to growth and survival of the cuttlefish (Navarro & Villanueva, 2000).

Grass shrimp, *Palaemonetes varians*, could possibly be used as an alternative first diet and throughout the life cycle, since it has been used in post-hatchling culture of the cuttlefish with good results (Richard, 1971; DeRusha *et*

al., 1989; Domingues *et al.*, 2001b and 2001c). This species is much more abundant and can be obtained from the wild throughout the year in in the Ria Formosa lagoon (South Portugal).

The main objective of this dissertation research was to determine if the culture of *S. officinalis* throughout the life cycle could be accomplished using the grass shrimp *P. varians* as the only prey during the life cycle. Additionally, the effects of the prey on growth, survival, condition of individuals and other aspects of the life cycle were to be investigated.

Secondary objectives were: 1) to determine both the best densities and the best abiotic conditions for culture, when using grass shrimp as the only prey; 2)to determine, using the RNA/DNA ratio, when hatchlings of cuttlefish had used all of their yolk reserves and could start to feed and 3) to determine the biochemical composition of the grass shrimp.

Chapter 2

Grass shrimp as the only source of food

2. THE USE OF LIVE GRASS SHRIMP (*P. varians*) AS THE ONLY PREY FOR THE CULTURE OF CUTTLEFISH, *S. officinalis* THROUGHOUT THE LIFECYCLE.

2.1 Introduction

Although cephalopod culture methods and system designs have been developed (Boletzky & Hanlon, 1983; Boyle, 1991), the limited dietary alternatives for growing cephalopods still remain a problem. The range of prey species of cephalopods are quite broad and are described for several species in their habitat (Boletzky, 1983; Castro & Guerra, 1990; Guerra & Castro, 1988), as well as the range of live and dead marine food accepted (DeRusha *et al.*, 1989). In *S. officinalis*, acceptable growth rates and low mortality have been obtained feeding live mysid shrimp during the first few weeks of their life. Nevertheless, costs associated with mysid production and their low fecundity (Domingues *et al.*, 1998; Domingues *et al.*, 2000) are a bottleneck in the first stage culture of the cuttlefish. Also, mysid abundance in the wild varies immensely throughout the year, being at times extremely low. Experimental work using *Artemia* sp. as first food promoted poor growth (DeRusha *et al.*, 1989; Domingues *et al.*, 2001c) and high mortality (Pascual, 1978; Navarro & Villanueva, 2000). This is probably due to the lack of some lipids required for growth and survival of the cuttlefish (Navarro & Villanueva, 2000; Koueta *et al.*, 2002). Live grass shrimp (*P. varians*) might be used as an alternative first diet, since it has been used in post-hatchling culture of the cuttlefish with good results (Richard, 1971; Pascual, 1978; Domingues *et al.*, 2001b and 2001c). The advantages of using only a prey species to culture *S. officinalis* could be the difference in costs and labour that is keeping this species out of commercial large-scale culture. Grass shrimp is much more abundant than mysids and can be obtained from the wild throughout the year in ponds near the culture facilities (Ramalhete Field Station for Experimental Aquaculture), Ria Formosa Lagoon (South Portugal) (annex).

Very little in known about cuttlefish and prey nutritional contents. Only few results have been published (Navarro & Villanueva, 2000; Koueta *et al.*, 2002). If large-scale culture of this species is to be attained, there is a need to investigate

and understand nutritional contents of both in order to understand prey selection, growth rates and mortalities associated. Once most of the work is done in methodologies to keep, rear or culture *S. officinalis* (Forsythe *et al.*, 1994) the next step will be understanding it's nutritional needs in every culture phase (hatchling, juvenile, adult).

The objective of this research was to determine if *S. officinalis* could be cultured through the life cycle exclusively on a diet of live grass shrimp, and the consequences on growth, mortality, age at spawning, fecundity and fertility. Additionally, grass shrimp nutritional content (total lipids and protein; amino acid and fatty acid content) was to be determined and compared with cuttlefish nutritional profile.

2.2 Material and methods

2.2.1 Growth Experiments

A total of 27 and 30 newly born cuttlefish hatchlings were used for the winter and summer cycles, respectively. All cuttlefish used in each experiment were born on the same day and from the same egg clusters. Eggs were obtained by reproduction in captivity (fig. 1). After copulation, female cuttlefish laid eggs on plastic net (fig. 2). Eggs were then removed from these collectors, separated in single units and placed in hatching baskets (fig. 3). Hatching baskets used ensured maximum hatching efficiency with very strong and thin aeration (fig. 4). After hatching, hatchlings were placed in small baskets (16.9 L water volume; 1 mm mesh size) (fig. 5) in a indoor flow-through system with 250 L tanks (annex and

Fig. 1 - Male and female adult cuttlefish copulating and laying eggs, in the laboratory.

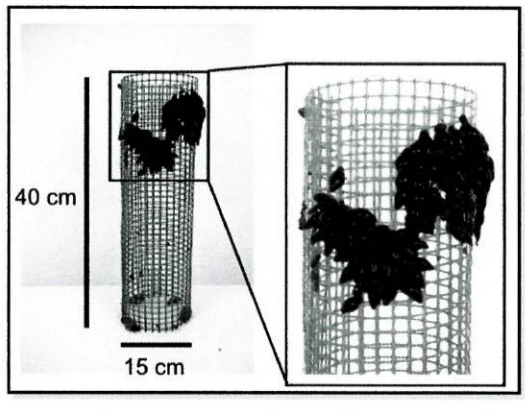

Fig. 2 - Eggs layed by cuttlefish and removed for embryonic development in hatching baskets.

Fig. 3 - Eggs in hatching baskets, with strong aeration

fig. 6). When cuttlefish reached 30 days, they were removed from these baskets and to the larger tanks mentioned above. Water flow of this system was of 12 Lh^{-1}. Water was filtered through an anti-bacterial apparatus (pre-filter of 500µm, plus 55µm and a ultra-violet set) to reduce contamination and improve water quality before reaching the culture tanks (fig. 6). This ensured an excellent water quality from a physical, chemical and bacteriological point of view. Water temperature was 15±3 °C during the winter cycle and 23±2 °C during the summer cycle. Salinity was 35±2 PSU during the winter cycle and 37±2 during the summer cycle; lights were running on a 14 hour per day basis in both experiments.

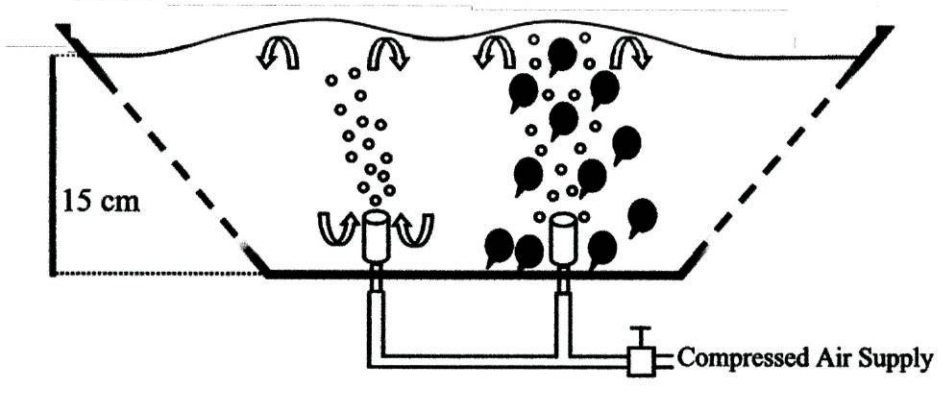

Fig. 4 - Schematic representation of hatching baskets. Water and egg flow are descibed by the arrows.

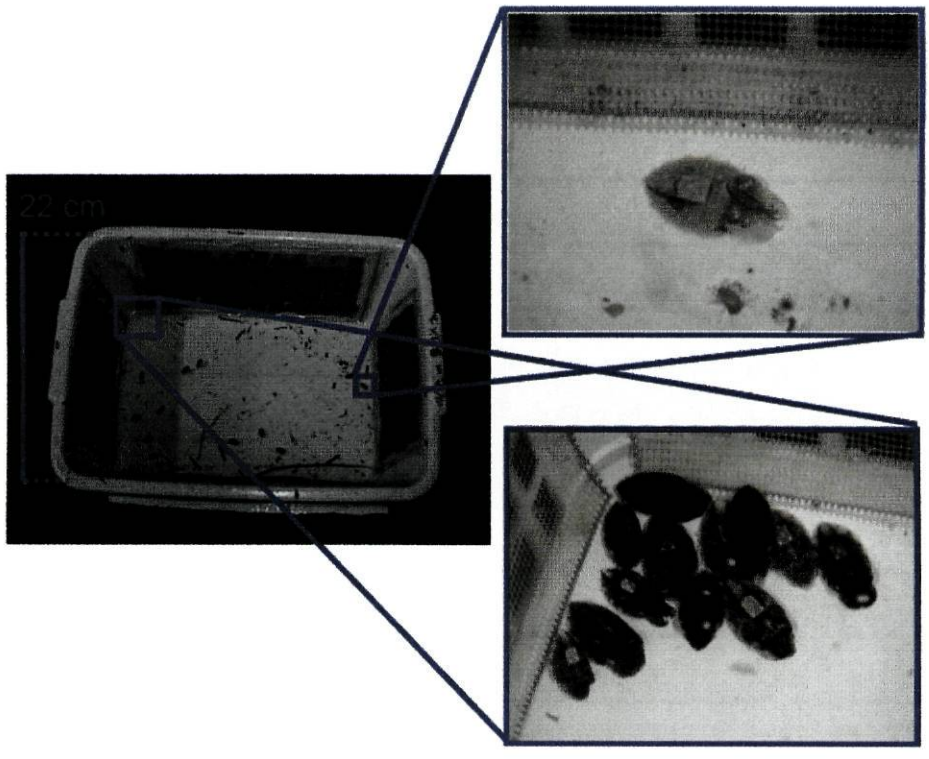

Fig. 5 - Rearing baskets used for the first stage culture of *S. officinalis*.

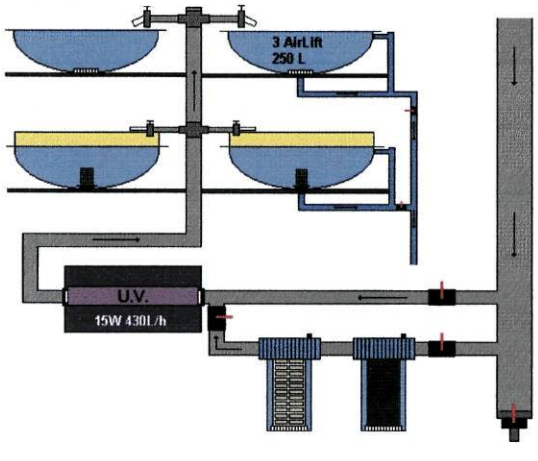

Fig. 6 - Schematic representation of indo- or flow through system.

Cuttlefish were fed live grass shrimp (*P. varians*) of increasing size throughout both experiments. Food was supplied *ad libitum* and captured from nature. During the first 20 days, both populations were fed juvenile *P. varians* (0,011±0,0006g; n=200). After that, cuttlefish were fed live adult *P. varians* of two different increasing sizes (0,035±0,007g ; n=200 and 0,090±0,022g ; n=200).

Cuttlefish were weighed every 10 days. Data collected was used to calculate: 1) Mean Weight; 2)Mean Instantaneous Growth Rate (IGR) (%BW.d^{-1})=(LnW2-LnW1)/t*100, where W2 and W1 are the final and initial weight, respectively, Ln

the natural logarithm and t the number of days of the time period and 3) Survival rates.

When egg laying started in each experiment, eggs were collected on a daily basis, and total egg numbers and weight were determined. Egg laying periods were determined for both experiments. About 50% of the eggs were individually weighed. From all the newly born hatchlings, about 30% were individually weighed to determine mean hatchling weight. Fertility (number of hatchlings born from the total of eggs laid) was also determined.

All adult cuttlefish were weighed after death and examined for sex determination, to determine differences in weight between males and females, as well as sex ratio within each group.

Student t-test (Zar, 1984) were performed to determine differences in growth between the two groups until the start of the spawning periods.

Mann-Whitney tests (Zar, 1984) were used to determine differences in weight between males and females in each group as well as between the two groups. Student t-test (Zar, 1984) was performed to determine differences in egg and hatchling weight between the summer and winter cycles.

2.2.2 Biochemichal Nutritional Essays

Grass shrimp samples were taken and stored at -80ºC. Later, samples were used to determine humidity, total protein, total lipid, amino acids and fatty acid profiles.

All biochemical nutritional essays were carried out in the laboratory, by Prof. Doutor Emídio Gomes, at the University of Porto.

Humidity was determined by drying at 105ºC, in a heat chamber with forced ventilation, until a constant weight was obtained.

Total protein (N x 6,25) was determined in a conventional macrojeldalh machine, using 1g samples, according to ISO rules [ISO 5983-1979 (E)].

Total lipids were determined by the method of Folch *et al.* (1957), by continuous extraction with petrol ether, during 6 hours, in a Soxhlet extractor.

Amino acid profile was determined according to Hogan *et al.* (1982), in a high performance liquid chromatographer (HPLC) Varian 5000, after acid hydrolysis (HCl 6N, 110ºC, 24 h).

Fatty acid profile was determined using a gaseous phase chromatographer Varian 3400. Muscle lipids were extracted according to the methodology of Folch *et al.* (1957). Separation of fatty acid methyl esters and dimethyl-acetols was made with baron fluoride-methanol, according to the methodology of Morisson & Smith (1964). A silica capillary column of 20m x 0,25mm was used. Temperature was programmed from 180 to 250ºC, with an increase of 4ºC per minute. Fatty acids were identified by comparison with a standard mixture (Sigma). Quantification was made in a previously programmed integrator, Hewlett Packard 4270.

Since the biochemical profile of both cuttlefish hatchlings and eggs have been described elsewhere (Bouchaud & Galois, 1990; Navarro & Villanueva, 2000), analytical essays of the hatchlings were not carried out. However, published and unpublished data was used to compare cuttlefish and grass shrimp fatty acid profiles.

2.3 Results

2.3.1 Growth Experiments

Figure 7 shows the growth of cuttlefish during both cycles. Hatchlings from the winter cycle weighed 0.079±0.012 g while those from the summer cycle weighed 0.092±0.015 g, and were significantly larger (t=-3.5328; p=0.0084) at the start of the experiment. Cuttlefish from the summer cycle were always significantly larger ($p<0.05$) throughout the experiment. At 90 days old, cuttlefish from the winter cycle weighed 7.74±1.51 g and were significantly smaller (t=-21.0659; p=0.0000) than those from the summer cycle, which weighed 45.51±8.45 g. At the start of spawning (90 days for the summer cycle and 220 days for the winter cycle), cuttlefish from the winter cycle weighed 201.76±34.40g and were significantly larger (t=16.5963; p=0.0000). Both generations had exponential growth until 60 days old. For the summer generation this growth was described by the equation $y=0.0443e^{0.867x}$ (r^2 = 0.9931), while for the winter generation, growth was described by the equation $y=0.0476e^{0.5839x}$ (r^2 = 0.994).

Mean Instantaneous growth rate (IGR) was of 3.6% and 13.8 % $BW.d^{-1}$ for the winter and summer cycles, respectively. Growth rates for both cycles are shown in figure 8. IGR was always higher during the summer cycle, except for the day 80. For both cycles, maximum IGR was obtained at 20 days old (8.37% and

22.37% BW.d^{-1}, for the winter and summer cycles, respectively). From day 20 onwards, IGR gradually decreased with age in both cycles. In the summer cycle, this decrease was faster until the age of 80 days, reaching a similar value of the winter cycle. For the winter cycle, this decrease was more gradual, with values between approximately 5 and 2% BW.d^{-1}.

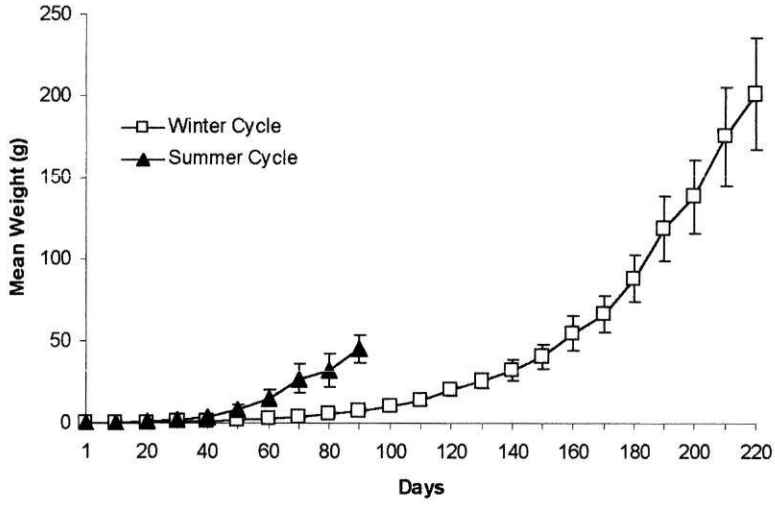

Fig. 7 - Variation of growth, in mean weight (g) during winter and summer cycles.

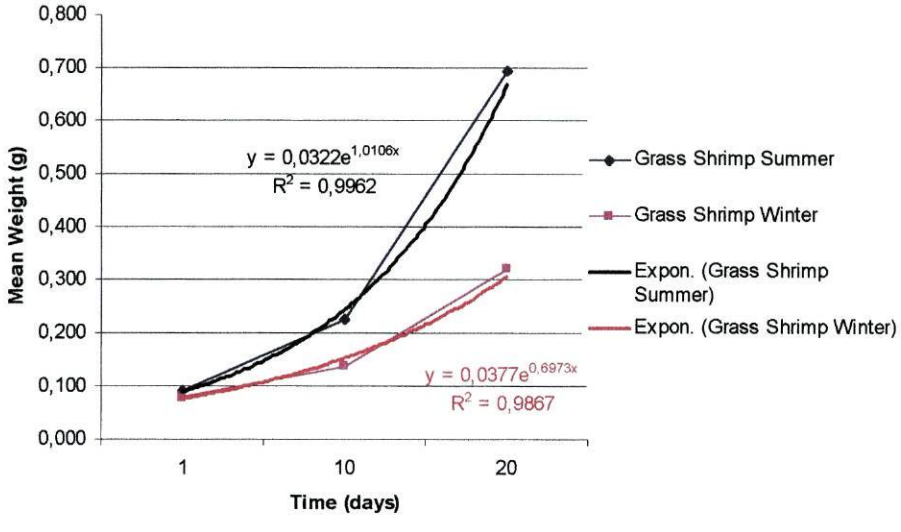

Fig. 8 - Growth exponential models of cuttlefish fed grass shrimp during the first 20 days of life.

Survival rate at the start of spawning was 82% for the winter cycle and 77% for the summer cycle. Higher mortality was registered for the first 20 days, in both cycles.

Differences in several aspects of the winter and summer cycles are shown in Table I. Males were larger than females in both the winter (U=12.00; Z=-2.9399; p=0.003) and the summer (U=20.00; Z=-2.2667; p=0.023) cycles. Males from the winter cycle grew larger than those from the summer cycle (U=0.00; Z=3.8129; p=0.000). Similarly, females from the winter cycle grew larger than those from the summer cycle (U=0.00; Z=-3.5386; p=0.000).

Females from the winter cycle laid more eggs and had higher individual fecundity than those from the summer cycle (Table I). Eggs laid by females from the winter cycle were larger (t=-36.7700; p=0.000) than those laid by females from the summer cycle. Hatchlings born from eggs laid by females from the winter cycle were also bigger (t=-2.3714; p=0.019).

Hatchling percentage within each cycle could not be compared due to partial sample loss. Massive mortality of winter cycle hatchlings was due to problems with water quality in the main water captation filter.

Table I - Results from the winter and summer life cycles for *S. officinalis* cultured in the laboratory with live grass shrimp (*Palaemonetes varians*) as the only prey.

	Grass Shrimp Winter Cycle	**Grass Shrimp Summer Cycle**
Duration of life cycle (days)	262	126
Duration of spawning period (days)	42	36
Eggs laid (n)	5916	1808
Total egg weight (g)	3448,5	236,8
Eggs (n) / Female	370	301
Eggs sampled (n)	2939	848
Mean egg weight (g)	0,56 ± 0,17	0,25 ± 0,08
Largest egg weighed (g) Smallest egg weighed (g)	2,464 0,114	0,622 0,142
Hatchlings sampled (n)	93	204
Mean weight hatchlings (g)	0,076 ± 0,013	0,081 ± 0,016
Mean weight females (g); (n)	193,3 ± 30,8 (16)	64,7 ± 12,7 (6)
Mean weight males (g); (n)	310 ± 89,6 (7)	77,6 ± 22,3 (18)
Mean temperature; Time to hatch	25ºC ; 25 days	20ºC ; 28days
Hatching percentage	-	16%

2.3.2 Biochemichal Nutrional Essays

Figure 9 presents the amino acid profile (in $g.kg^{-1}$ of sample) for the grass shrimp. Arginine, leucine, lysine and valine values were above 1.5 $g.kg^{-1}$ of sample. Cysteine, histidine, isoleucine, methionine, phenylalanine, threonine and tyrosine values varied between 1.5 and 0.5 $g.kg^{-1}$ of sample. Tryptophan value was below 0.5 $g.kg^{-1}$ of sample. All the other essential amino acids were not present in the grass shrimp.

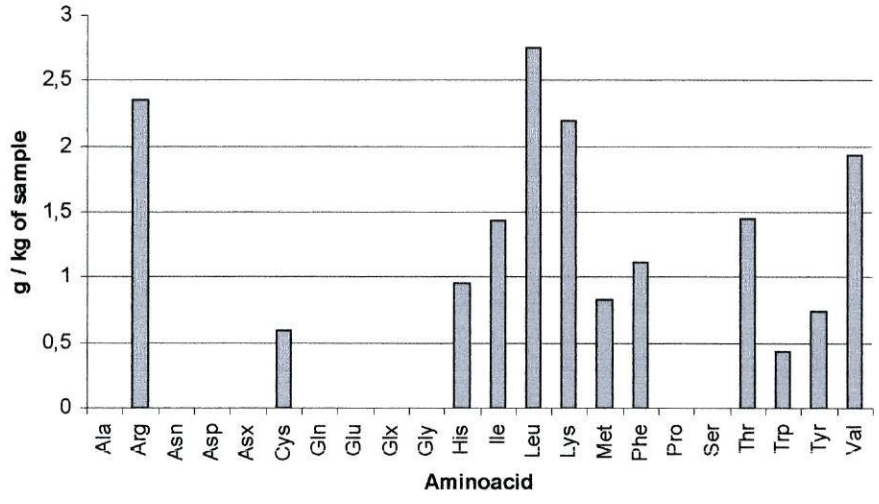

Fig. 9 - Amino acid profile of the grass shrimp (*P. varians*).

Figure 10 presents the fatty acid profile in chains of percent of total fatty acid. The main fatty acids identified were 16:0, 18:1 and 20:5.

Table II shows the total lipid, total protein, humidity and ashes content in percentage of dry weight. The value calculated for the lipid to protein ratio was 7.89% of dry weight (Table II).

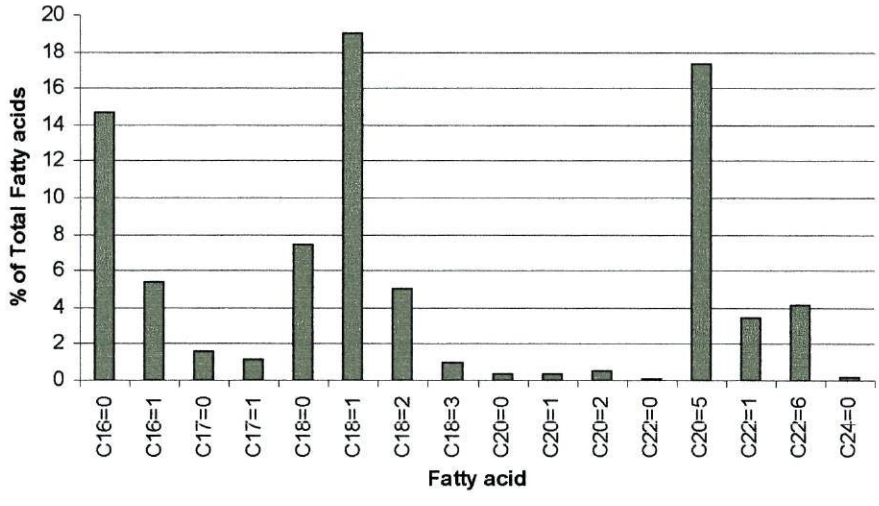

Fig 10 - Fatty acid profile of grass shrimp (*P. varians*).

Table II - Grass shrimp nutritional profile in total lipid, total protein, humidity and ashes.

	% of Dry weight
Total Lipid	5,58±0,19
Total Protein	70,75±4,08
Ashes	17,42
Humidity	79,95±0,78
Lipid-Protein ratio	7,89

2.4 Discussion

In this study, cuttlefish culture for two consecutive generations using live grass shrimp as the only prey was acomplished. Hatchlings easily preyed upon grass shrimp with sizes that could be twice as big as their mantle length, in both cycles. Cephalopods have high metabolic ratios, which results in short life cycles of one to two years in nature (Forsythe & Van Heukelem, 1987; Lee, 1994). Life cycles as short as 6 months old have already been obtained in captivity (Domingues *et al.,* 2001c and in press-a). Since they are poikilothermic, temperature has a great effect on growth and spromote higher feeding and growth rates (Richard, 1971; Forsythe & van Heukelem, 1987; Forsythe *et al.*, 1991, 1994; Lee, 1994; Domingues *et al.*, 2001b and in press-a).

Temperature was the most important factor to explain the great differences between the winter and summer cycles. Growth was considerably higher during the summer and life span was shorter, due to the much higher temperatures (23±2 ºC compared to 15±3 ºC). The variation in standard deviation gradually increased with age, in both cycles. This difference became higher when sexual maturity was attained (fig. 7), because females invest much more energy in reproductive efforts than males (Boletzky, 1983). Life span during the winter cycle was about 50% longer, and cuttlefish grew almost 5 times larger (201.76±34.40g compared to 45.51±8.45g). Both growth curves showed exponential growth for the initial part of the life cycle. During both cycles, exponential growth ended at day 60. Although cuttlefish from the winter generation were much smaller after 60 days old, exponential growth ended at that age, as for the summer generation. This species shows exponential growth rates during the early parts of the life cycle (Forsythe & Van Heukelem, 1987). During the present experiment this exponential phase had the same duration, and was independent from the water temperature.

Age at the start of spawning in the present study (3 months old at 23ºC) was much shorter when compared with results of Forsythe *et al.* (1991), who reported ages of 9 to 10 months old at temperatures ranging between 21ºC and 24ºC. At 15ºC, cuttlefish in the present study started spawning at 9 months old, while cuttlefish cultured by Forsythe *et al.* (1991), at temperatures between 16ºC and

18ºC, started spawning between 13 to 14 months old.

According to Forsythe *et al.* (1994), egg laying period represents 25% of the life cycle for this species. We obtained values of 28% for the summer cycle and of 16% for the winter cycle. This difference can be explained by the much shorter life cycle during the summer. The fast increase in water temperature towards the end of the life cycle of the winter population (from 16,3ºC to 22,3ºC – a 6ºC difference in two weeks) triggered spawning. Cuttlefish from the summer cycle, cultured at average water temperatures of 23ºC, started spawning at 90 days old, which is 30 days sooner than reported by Domingues *et al.* (in press-a), cultured at much higher temperatures (27ºC). Spawning in the summer cycle also started much sooner when compared to the results reported by Forsythe *et al.* (1991; 1994), at similar temperatures. The group used in the winter cycle was from the second generation cultured in captivity, while the summer group was from the third generation. It is possible that inbreeding plays a major factor explaining the increasing shorter life cycles, when comparing summer populations of the first generation cultured by Domingues *et al.* (in press-a) and the third generation used in the summer cycle. Nevertheless, results obtained here suggest that water temperature can act as life cycle limit in both cycles, having a maximum and a minimum temperature for the start of spawning period for the winter and summer cycles, respectively. The knowledge of the spawning temperature limits is particularly important when managing brood stocks. With this information, eggs can be obtained whenever necessary, without using any other physical or chemical stimulae to induce spawning. Similarly, this knowledge could possibly be used to better understand the patterns of wild cuttlefish life cycle in the Ria Formosa Lagoon (South Portugal).

The individual fecundity was higher for the winter cycle (370 eggs female^{-1}) than for the summer cycle (301 eggs female^{-1}). This is most likely due to differences in female body weight. The rates obtained here fall within the range reported by Mangold-Wirz (1963) of 200 to 550 eggs female^{-1}, by Richard (1971) with fecundities between 150 and 500 eggs female^{-1}, and also by Villa (1998), who estimated fecundity values of *S. officinalis* from the Ria Formosa varying between 98 and 385 eggs female^{-1}. Nevertheless, other authors found much higher individual fecundities. Forsythe *et al.* (1994) reported fecundities above 3000

eggs female^{-1} in larger cuttlefish cultured at similar lower temperatures. Hanley *et al.* (1998) indicates 2000 eggs female^{-1} and Boletzky (1979) reported more 1000 eggs female^{-1}. The individual fecundity varied considerably between cycles (Table I) and was higher than that reported by Domingues *et al.* (in press-a). Egg weight and individual fecundity is directly related to female size (Boletzky, 1983), and larger females lay larger eggs (Boletzky, 1987). Although the number of egg sampled during the winter cycle was lower than during the summer cycle, eggs from the winter cycle showed a wider range in weight (Table I). The fact that there were more females in the population from the winter cycle (inverted sex ratios were obtained between both cycles; with more females than males during the winter cycle), which also had a wider size range could possibly explain the higher standard deviation in egg weight in this sample.

Embryonic development is faster when water temperature is higher (Bouchaud & Daguzan, 1989; Caverivière *et al.*, 1999). During the winter cycle embryonic development was of 25 days (25ºC) while during summer was of 28 days (20ºC). These results confirmed the findings of the authors mentioned above and are within the curve for embryonic development reported by Boletzky (1987). Richard (1971) obtained a longer development time (38 days) also at 20ºC. Nevertheless, eggs used by Richard (1971) were larger than those used in the present study, and originated bigger hatchlings (average weight of 0.111 g).

Although eggs laid by females from the winter cycle were bigger (0.56±0.17g) when compared to those from the summer cycle (0.25±0.08g), hatchlings from the winter cycle were smaller (0.076±0.013g) when compared to those from the summer cycle (0.081±0.016g). This could possibly be explained by the longer embryonic development of eggs during the summer cycle. Bouchaud (1991) reported that hatchlings born from eggs kept at 24ºC were two times smaller than those born from eggs kept at 15ºC. Bouchaud & Daguzan (1990) indicated that at higher temperatures, yolk absorption by the hatchling is lower and therefore they are born smaller. A higher number of hatchlings born with external yolk sac from eggs kept at higher temperatures was observed, supporting these findings. According to Bouchaud & Galois (1990), faster embryonic development induced by higher temperatures not only originates smaller hatchlings but also hatchlings with lower lipid content. These authors also stated that embryos consume only

26% of their egg-yolk at 24ºC, while egg-yolk consumption was close to 78% at 15ºC.

Forsythe *et al.* (1991) reported that *S. officinalis* cultured in the laboratory displayed below-normal fertility (< 40%). Nevertheless, the same authors also stated that the hatchling viability was good (> 90%). Forsythe *et al.* (1994) also reported low fertility rates, not higher than 20%, for *S. officinalis* cultured in captivity, while Hanley *et al.* (1998) indicated average hatching rates of 48%. Hatching rates of 50% were reported by Domingues *et al.* (2001c) for the first generation of cuttlefish cultured in captivity. This rate was higher than that obtained for the second generation during the winter cycle (41%) by Domingues *et al.* (in press-a). During the present study, fertility rates for the summer cycle (third generation) were of only 16%. These results are supported by the findings of Forsythe *et al.* (1994), who also reported a decrease in hatching rate for the latest generations, when culturing *S. officinalis* for 7 consecutive generations.

Until now, mysids promoted better overall results in culturing the early stages of *S. officinalis* (Pascual, 1978; DeRusha *et al.*, 1989; Forsythe *et al.*, 1994; Koueta & Boucaud-Camou, 1999; Koueta, Castro & Boucaud-Camou, 2000; Koueta *et al.*, 2002; Domingues *et al.*, 2001c and in press-a). Values calculated in this study for growth and mean IGR for the winter cycle showed a similar pattern to that described by Domingues *et al.* (in press-a) when feeding cuttlefish with live mysids for the first 20 days. Although they showed different growth patterns during the first 20 days, cuttlefish from the winter cycle in the present experiment recovered the weight later during the life cycle and both Domingues *et al.* (in press-a) and grass shrimp groups showed similar weight at the start of the spawning period (mean weight ~200 gr.). The life cycle and survival were similar for both winter groups (~260 days, ~82%). Apparently, the effects of feeding mysids during the first 20 days instead of grass shrimp were not very beneficial when comparing to the use of grass shrimp. Temperatures in both experiments were similar and cuttlefish fed mysids grew better for the first 20 days of the life cycle. Therefore, larger sizes at the end of the life cycle would be expected for the cuttlefish fed mysids initially (Domingues *et al.*, in press-a). This was not observed, indicating that grass shrimp has a good nutritional profile, allowing cuttlefish to recover weight during the last part of the life cycle. These findings are supported by the

fact that both groups were sharing the same physical, chemical and bacteriologic conditions, and being fed *ad libitum*. Considering the fatty acid profile of the grass shrimp and the findings of Navarro & Villanueva (2000) on the fatty acid needs of cephalopods, this could be better explained. According to these authors, the main fatty acid profile of cephalopods is composed of high values of 16:0, 20:5n-3 and 22:6n-3 chains. These authors also stated that palmitic acid (16:0), 20:5n-3 and 22:6n-3 were the most abundant fatty acids found in the lipids of many cephalopod species, not only in the muscle but also in the digestive glands, the central nervous system and in the photoreceptors. These components are less abundant when embryos grow faster and, therefore, hatchlings will have to find some food before consuming all the lipid reserves. Grass shrimp profile is highly rich in 16:0, 18:1 and 20:5 chains. When comparing the results obtained here with those of Navarro & Villanueva (2000) and Domingues *et al.* (unpublished results), a match between the main fatty acid chains available both in the cuttlefish and grass shrimp is evident (fig. 11). Since cephalopods are known to have a predominant amino acid metabolism (Lee, 1994), which is showed in their high growth rates, lipid metabolism has somehow been neglected. Not only amino acid and protein profiles are important. Lipid and fatty acid profiles could possibly have great importance in growth and mortality, especially during the first weeks of life. This is supported by the analyses of the lipid-protein ratio presented in table II.

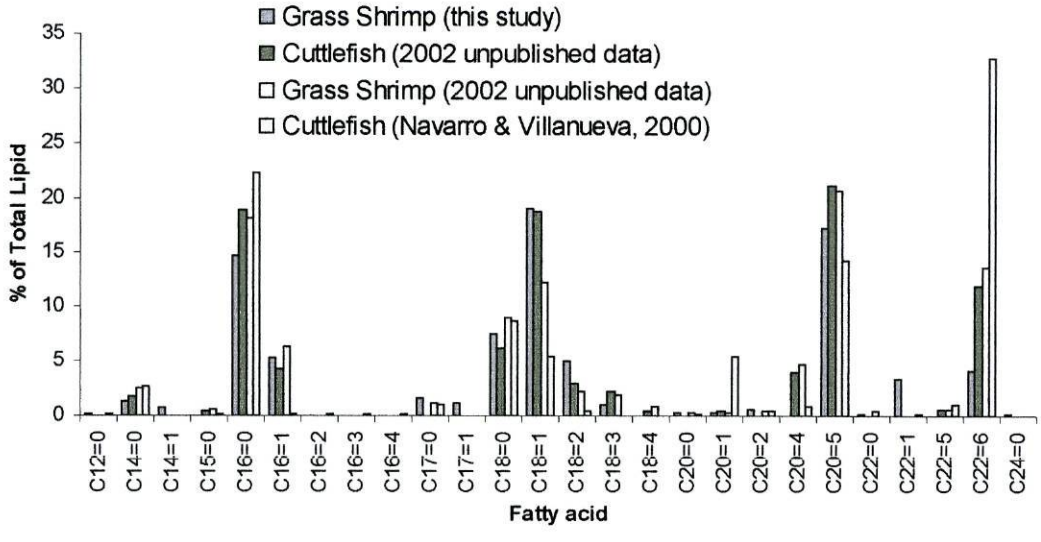

Fig. 11 - Comparisation between fatty acid profiles of cuttlefish (Navarro & Villanueva, 2000; Domingues *et al.*, unpublished data) and grass shrimp (this study and Domingues *et al.*, unpublished data).

Sargent *et al.* (1989) stated that the precise form of the lipid used during embryonic and early larval development could vary between species. According to these authors, a significant amount of the phospholipids and cholesterol is retained in the form of the developing fish larval body and so phospholipids may only be a significant energy source in phospholipids-rich eggs. Navarro & Villanueva (2000), reported that cephalopod para-larvae and juvenile must require a food rich in polyunsaturated fatty acids (PUFA), phospholipids and cholesterol, and a moderated content in neutral lipids. This is supported by the cephalopod lipid composition and the one of their natural food. Although further studies are necessary, it is known that shrimp profiles fall within these needs (Navarro & Villanueva, 2000). Therefore the advantages of using only this type of prey to culture the cuttlefish during the whole life cycle are considerable.

When trying to culture adult cuttlefish with artificial diets supplemented with amino acids, Domingues (1999) obtained significant growth only with those with high levels of methionine and lysine. Lee (1994), stated that the predominant free amino acids present in cephalopod tissues (>100mg/100g tissue) are octopine, praline and arginine while the predominant amino acids present in tissue protein (>1.0g/100g tissue) are glutamate, aspartate, leucine, alanine, lysine and isoleucine. These amino acids were present in significant quantities in the grass shrimp nutritional profile, except for octopine. This could be one of the many aspects that makes grass shrimp a suitable diet for the culture of cuttlefish throughout the life cycle. Additional studies on amino acid profile of cuttlefish hatchlings are necessary in order to extend the knowledge on amino acid metabolic requirements for *S. officinalis*.

Duration of spawning period in this experiment was longer (table I) than reported by Domingues *et al.* (in press-a). This was not expected, since both females and males in the present experiment were smaller when compared to those use by Domingues *et al.* (in press-a). Nevertheless, females fed mysids initially laid larger eggs that delivered larger hatchlings. This fact might indicate that mysids are still a better initial food for cuttlefish, when compared to grass shrimp.

Grass shrimp seems to be a good food for cuttlefish culture. It replaced mysids as first food with similar results in body weight at time of spawning. Nevertheless, female body weight at time of death (248.0±33.1g) was considerably

higher in the population fed mysid (Domingues *et al.*, in press-a) than weight of females used in this study during the winter cycle (193.3±30.8g), at similar temperatures.

Grass shrimp abundance in the Ria Formosa lagoon decreases the costs and labour associated with fishing, when compared to those related to mysids collection. Results for summer growth, amount of food supply per day, nutritional content and production of grass shrimp need to be more thoroughly investigated in future experiments so cuttlefish culture at a commercial large-scale can been foreseen.

Chapter 3

Density culture when using grass shrimp

3. THE INFLUENCE OF CULTURE DENSITY AND ENRICHED ENVIRONMENTS ON THE FIRST STAGE CULTURE OF YOUNG CUTTLEFISH, *S. officinalis*.

3.1 Introduction

The cuttlefish (*S. officinalis*) is one of the most well known cephalopods (Boletzky, 1983) and its culture has been attained with success throughout the life cycle in many places around the world (Pascual, 1978; Forsythe *et al.*, 1994). However, it is known that the first stage culture of this species is the most problematic and when most mortality occurs (Richard, 1971; 1975; Domingues *et al.*, 2001a). Recent studies showed that grass shrimp (*P. varians*) can and should be considered as an adequate prey for the culture of the early stages of cuttlefish culture. It promotes very similar results in life span, survival, and growth, when compared with the use of mysids for the first part of their culture (see Chapter 2). Furthermore, grass shrimp has been used successfully to culture cuttlefish throughout the life cycle (Domingues *et al.*, 2001c and in press-a).

One of the most important aspects in commercial culture of marine animals is culture density. These studies are important in order to maximize the amount of animals cultured in each tank with optimal growth and survival, in order to reduce production costs. Culture densities in fish are now well studied for a number of commercial aquaculture species such as sea bream, sea bass and common sole. However, little has been published about culture densities for *S. officinalis* during several stages of the life cycle. It is known that cuttlefish handles crowding well (Forsythe *et al.*, 1994; 2002) but more specific studies about this subject are necessary.

When studying density, behavioural aspects of this species have to be taken into consideration. Behaviour studies conducted by Boal *et al.* (1999) showed that adult cuttlefish avoid each other, as well as close proximity. Mather (1986) studied feeding in captive cuttlefish and found a female dominating hierarchy. It is known that in nature, cuttlefish are semi-solitary. Exception to this solitary behaviour is the reproductive period, when individuals migrate to locations closer to shore to find a mate and spawn. During our last 2 years of research that we have been culturing cuttlefish (4 complete life cycles achieved), this female dominance

has never been observed. The only dominance observed by us during feeding periods was exclusively associated to size and not sex, at all stages of the life cycle. Male dominance has been observed only during mating, when males force females to copulate. This behaviour induces female stress and cuttlefish start banging against the tank walls, which causes skin damage and open doors to infections (Hanlon & Forsythe, 1990). The same kind of behaviour and a solution to improve long-term rearing health of cuttlefish is described by Hanley *et al.* (1999) by introducing soft materials in the sides of the tanks. During the early stages of the life cycle, we have observed cuttlefish congregating and leaving most of the tank empty (fig. 5), especially at early ages (< 1 month old). Avoidance behaviour described above by these authors has not been observed in cuttlefish hatchlings or juvenile cultured in our facilities during the past 2 years. Only when sexual maturation is attained, avoidance by females towards males is frequently observed.

Some authors have studied the importance of enriched environments in the culture tanks as a way to stimulate cuttlefish feeding and subsequent growth (Dickel, 1999). Previous studies by Boletzky (1996) showed that culturing cuttlefish with a substrate of shelters and/or sand did not affected growth and survival, although the animals were more sensitive and launched more ink when sand and/or shelters were not present. These results suggest that, without a hiding place in the tanks, cuttlefish were more stressed. In fact, Dickel *et al.* (2000) obtained better growth for cuttlefish hatchlings and juveniles when cultured in enriched environments. However these were behaviour studies and did not imply direct correlations with density and the main question of using enriched environments when culturing cuttlefish at high densities, leaving this as an open subject of study.

The objective of this study was to determine how culture density and the use of enriched environments affect growth and survival of young cuttlefish during the first part of their life cycle. Another objective was to establish a methodology for the most effective culture of the first stages of cuttlefish, when using grass shrimp as prey.

3.2 Material and Methods

3.2.1 General Methodology

Two experiments were conducted in order to clarify the importance of densities and the use of enriched environments (sand with shelters) in growth and survival of the early stages of the cuttlefish. Both experiments were conducted using plastic baskets (5.4 L water volume) with 1mm plastic mesh all around. Baskets for density experiment were divided in 3 equal parts (1.8 L water volume) (fig. 12), while those of enriched environment experiment were not divided (fig. 13). These baskets were placed in 500 L tanks of a flow-through system described by Domingues *et al.* (2001a) (12 $L.h^{-1}$ water flow).

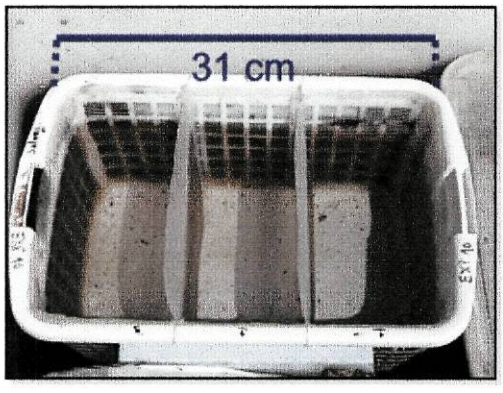

Fig. 12 - Baskets used in density experiment.

Fig. 13 - Baskets used in enriched environments experiment.

Water temperature was of 23±2 ºC and salinity was of 37±2 PSU; photoperiod was supplied by natural light (about 14h light per day).

Food was supplied *ad libitum* (to prevent cannibalism and enhance maximum growth) and captured from nature. All density and enriched baskets sets were fed *P. varians* (0.011±0.0006g; n=200) of similar hatchling mantle length.

All individuals used in each experiment were born on the same day and from the same egg clusters laid in our facilities. This methodology was used because, until now, there is no definition on hatchlings selection at hatching and how long yolk reserves support hatchling life for a specific culture temperature.

Cuttlefish were weighed every 5 days. Data collected was used to calculate: 1) Mean Weight; 2)Mean Instantaneous Growth Rate (IGR) (%$BW.d^{-1}$)=(LnW2-LnW1)/t*100, where W1 and W2 are the initial and final weight, respectively, Ln the natural logarithm and t the number of days of the time period and 3) Survival

rates.

3.2.2 Density experiment

Three different densities where studied during 20 days. Newly born cuttlefish hatchlings were used for the 3 densities tested. The first density was of one cuttlefish hatchling per 1/3 of basket (52 hatchlings.m^{-2}), using a total of 18 hatchlings. The second was of 10 cuttlefish hatchlings per 1/3 of basket (515 hatchlings.m^{-2}), using a total of 60 hatchlings. Third was of 30 cuttlefish hatchlings per 1/3 basket (1544 hatchlings.m^{-2}), using 90 hatchlings. Eighteen, six and three replicates were used for densities 1, 10 and 30, respectively.

ANOVA tests (Zar, 1984) were performed to determine differences in growth between the 3 different densities.

3.2.3 Enriched environments experiment

A total of 40 cuttlefish were used for each different basket setup (the first with sand and 3 PVC shelters, and the second on empty white baskets). Two baskets, with a volume of 5.4 L water volume and a total area of 583.cm^{-2}, were used for each variable. Twenty cuttlefish were placed in each replicate during the 20 days trial. Density for the two different environments was of 343 hatchlings.m^{-2}.

Student t-test (Zar, 1984) was performed to determine differences in growth between the two groups.

3.3 Results

3.3.1 Density Experiment

The three different densities promoted different weight gain, growth rates and survival. Figure 14 shows growth of cuttlefish hatchlings cultured in 3 different densities. Hatchlings weighed 0.105±0.0316g, 0.088±0.0175g and 0.087±0.0200g at day 1, for densities 1, 10 and 30, respectively. Overall growth was different among the 3 densities ($p<0.05$). However, at day 10, the 3 different densities groups were equal in weight ($F=0.201656$; $p=0.817655$). Final weights were of 0.510±0.13896g, 0.603±0.14143g and 0.547±0.06018g, for densities 1, 10 and 30, respectively.

All experiment groups showed exponential growth until 20 days old. Density 1 group growth is described by the equation $y=0.0597e^{0.4226x}$ (r^2 = 0.9655), while for density 10 group growth is described by the equation $y=0.028e^{0.5125x}$ (r^2 = 0.9968), and for density 30 group growth is described by the equation $y=0.053e^{0.468x}$ (r^2 = 0.9975). This patterns of overall growth in weight gain clarifies how different growth was between the 3 densities studied.

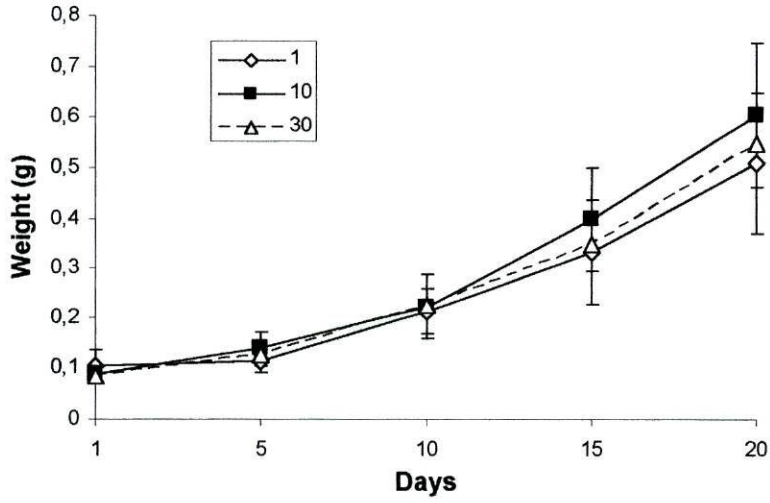

Fig. 14 - Growth of cuttlefish hatchlings cultured in 3 different densities for the first 20 days of the life cycle.

Mean instantaneous growth rate (IGR) was of 8.8±3.45, 9.6±5.44 and 9.2±1.06 % BW.day^{-1} for the densities 1, 10 and 30, respectively. Growth rates for each density were not significantly different ($p<0.05$) and are shown in figure 15. Maximum values of IGR were of 11.9±3.19, 11.6±10.42 and 11.3±1.16 % BW.day^{-1} for each density, respectively. Minimum values of IGR were of 5.0±3.42, 8.3±0,22 and 7.7±2.11 % BW.day^{-1} for each density, respectively.

Figure 16 shows the evolution of cumulative mortality during time of experiment. Survival at the end of experiment was of 77.8% in density 1, 42.5% in density 10 and 57.8% in density 30. Mortality increased with weight gain for both 3 density groups.

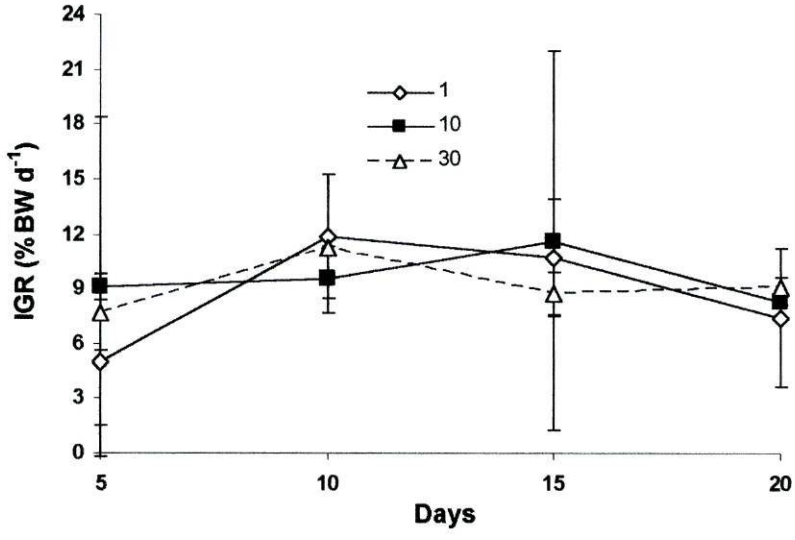

Fig. 15 - Growth rates (% BW.d^{-1}) of cuttlefish hatchlings cultured in 3 different densities, dor the first 20 days of the life cycle.

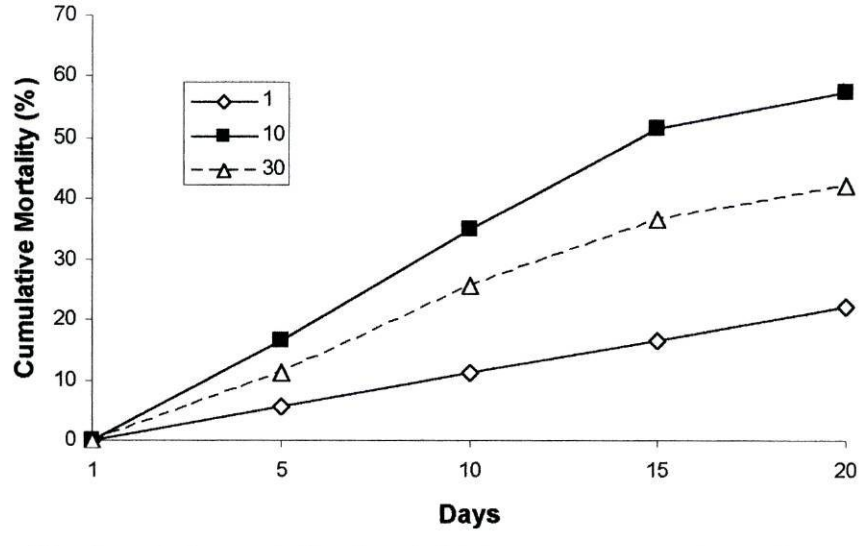

Fig. 16 - Cumulative mortality of cuttlefish hatchlings cultured in 3 different densities, for the first 20 days of the life cycle.

3.3.2 Enriched environment experiment

Figure 17 shows growth in weight of both different experiment groups. At the start of the experiment, hatchlings from the enriched environment weighed 0.077±0.0104g while those from the clean baskets weighed 0.073±0.0100g and were not significantly different ($p<0.05$). From beginning to the end of the experiment both groups showed similar growth ($p<0.05$), in weight gain.

Individuals in both experiment had exponential growth until 20 days old. For the enriched environment group this growth can be described by the equation

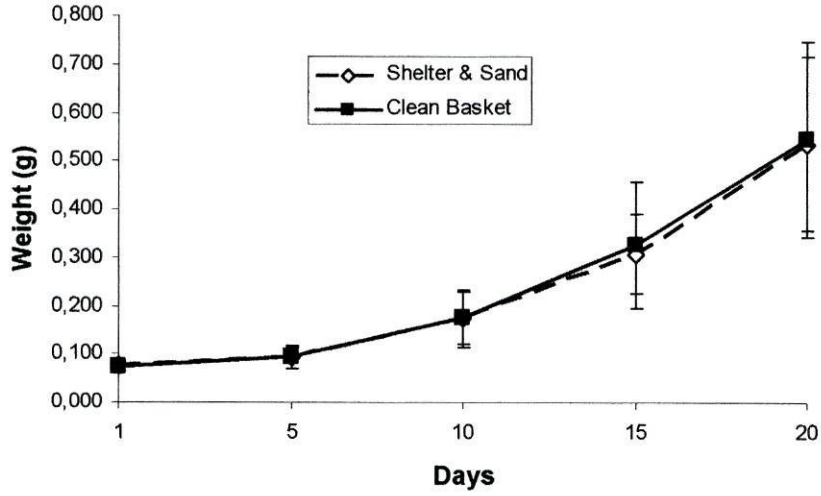

Fig. 17 - Growth of cuttlefish hatchlings cultured with or without enriched environments, for the first 20 days of the life cycle.

$y=0.04e^{0.5069x}$ (r^2 = 0.978), while for the clean basket group, growth can be described by the equation $y=0.0378e^{0.527x}$ (r^2 = 0.985).

Mean instantaneous growth rate (IGR) was of 10.1% and 9.7 % $BW.d^{-1}$ for the groups of enriched environments and clean basket, respectively. Growth rates for both groups are shown in figure 18. Both IGR curves show a similar pattern and maximum values of about 12.5% $BW.d^{-1}$ at day 10. Minimum values of 3.7% and 5.1% $BW.d^{-1}$ were observed in enriched and non-enriched environments, respectively, at day 5.

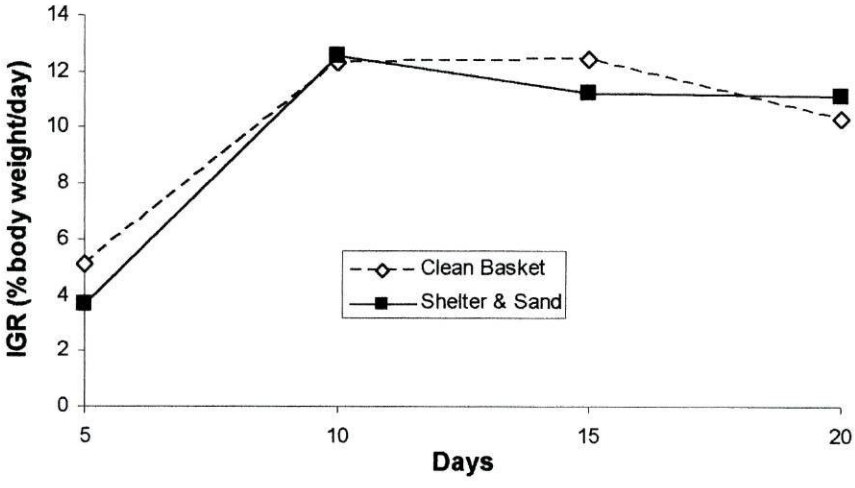

Fig. 18 - Growth rates (%$BW.d^{-1}$) of cuttlefish hatchlings cultured with or without enriched environments, for the first 20 days of the life cycle.

Figure 19 shows the variation of cumulative mortality during the experiment. The non-enriched environments showed a 70% survival while enriched environments showed 60%.

Resuming, both groups showed similar growth in weight gain, similar IGR's and survival.

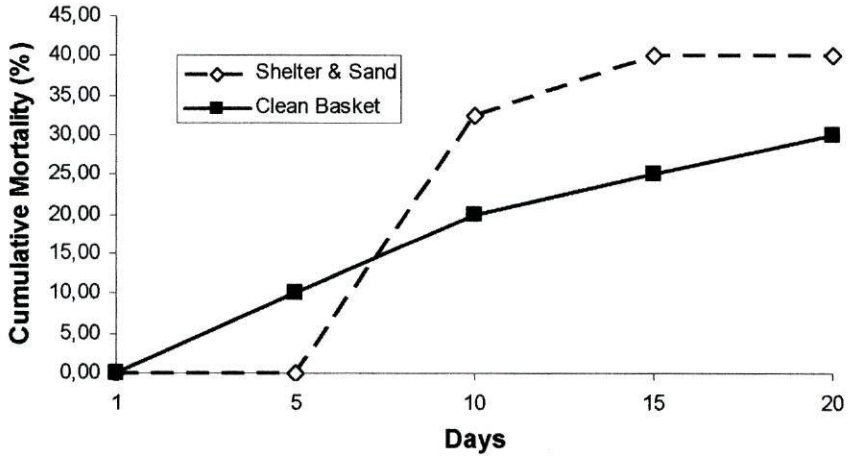

Fig. 19 - Cumulative mortality of cuttlefish hatchlings cultured with or without enriched environments, for the first 20 days of the life cycle.

3.4 Discussion

Density seems to affect growth of cuttlefish hatchlings. During this experiment, hatchlings from density 10 (515 hatchlings.m^{-2}) grew larger. Nevertheless, hatchlings cultured at this density had the worst survival percentage (fig. 16). Forsythe *et al.* (1994) suggested maximum culture densities of 250-300 hatchlings.m^{-2}, while Nabhitabhata (1995) suggested an optimal culture density of 500 hatchlings.m^{-3} for *Sepia pharaonis*. Forsythe *et al.* (2002), indicated densities as high as 400 juvenile cuttlefish.m^{-2} (average weight of 1.5g) for temperatures between 17ºC and 25ºC, without impact on feeding and growth. Since *S. officinalis* is a benthic species and does not require a large water column, the density suggested by Nabhitabhata (1995) can not be compared to density 10 (515 hatchlings.m^{-2}; 5417 hatchlings.m^{-3}). Values of culture density for this species should be considered in area and not volume or discrepancies of values can be as high as these ones. Cuttlefish can be cultured in tanks with very near to the ground water column as long as the total water volume of the system is high (e.g. race-

way system) (Boletzky & Hanlon, 1983). As benthic animals, they will compete for the bottom area in the tank, and not for the volume of water available. The water volume used in cuttlefish culture is more important when using recirculating sea-water systems than when using flow through systems. Importance is further increased when the total water volume of the recirculating system is low, because of the nitrogenous compounds and others.

Density 1 (52 hatchlings.m^{-2}) had the lower growth and mean IGR (fig 14 and 15), but the best survival percentage. Lower growth could possibly be caused by stress due to isolation. DeRusha *et al.* (1989) also reported that *S. officinalis* was not very tolerant to long-term isolation, during a 2 month period, but contrary to results obtained here, reported lower survival, compared to group reared cuttlefish. This could possibly explain the lower growth obtained in density 1. This author used larger animals, weighing between 4-40g. Nevertheless, recent results by Domingues *et al.* (in press-b), using newly born hatchlings (mean weight of 0.200g with densities of 125 hatchlings.m^{-2}), as in the present study did not show differences in growth between hatchlings placed in isolation and others cultured at several densities in smaller bottom areas (80 cm^2). Also, it has to be taken into consideration the amount of energy used by the hatchlings to capture the prey and the amount of space available (bottom area), both higher in the present study.

Density 30 (1544 hatchlings.m^{-2}) had an intermediate value of growth, mean IGR and survival, suggesting that most probably there were too many cuttlefish in the basket, and thus result in increased stress while hunting for prey. Boal *et al.* (1999) reported results of stress when cuttlefish were cultured in overcrowded groups. In density 30, competition for food and place were the most probable cause of a slower growth.

Results obtained for cuttlefish culture throughout the life cycle fed on grass shrimp (Chapter 2) for the first 20 days, at similar temperatures and a density of 515 hatchlings.m^{-2} (similar to density 10), showed similar values for growth (exponential: $y=0.0524e^{0.5071x}$; $r^2=0.9964$) and mean IGR (10.11% BW.d^{-1}). Nevertheless, survival was much higher (83%) than that obtained in this experiment. This indicates that not only density is important. Culture area should also be taken in consideration. Although culture densities where similar, space available

was smaller in the present experiment and this may be reflected in the final survival percentage. If this is extended to the percentage of survival in the other densities of the density experiment, then this could explain the lower values of survival for densities 10 and 30.

Warnke (1994) reported that when rearing cuttlefish in groups, attack on prey can be about 3 times faster, which reflects inter-individual influence. This author also reported that group cultured cuttlefish also ingested more prey (with values of up to twice) than individually reared animals. According to Forsythe & Van Heukelem (1987), captive cephalopods interact with conspecifics, competitors and prey over the course of their life. These authors also reported that aspects of each of these interactions can influence growth either directly by affecting food intake or indirectly by altering energy use and diverting it from somatic growth. This could explain feeding hierarchies described by Forsythe & Van Heukelem (1987), Warnke (1994) and Mather (1986) and the results obtained in the density experiment, especially for density 1.

Blanc *et al.* (1998) reported that young cuttlefish, in nature, live on a heterogeneous substratum of stone, shell debris, sand and stone. When composing the enriched environment baskets a substrate of sand with 3 PVC shelters was used (fig. 13). The composition of the substrata can be very important because cuttlefish usually lies buried during the day (Boletzky, 1996) and hunts in the water column during the night (Hanlon & Messenger, 1988; Budelman *et al.*, 1991).

Another aspect which needs clarification is the use of enriched environments when culturing *S. officinalis*. Cuttlefish are more relaxed, ink less and grow larger when sand and shelter is available, compared to clear tanks (Dickel *et al.*, 2000). This did not happen in the present experiment, since growth was similar among groups. The benefits for commercial large-scale culture are even less clear. When using enriched environments, precautions need to be taken in order to minimize ammonia peaks within the baskets. Time dedicated to cleaning the sand or even to setup the baskets makes it not economically viable and could even affect growth by causing stress to cuttlefish. Based on the results of this study the use of enriched environments should not be beneficial for pilot or large-scale culture of this species.

Mean IGR was similar between the 2 groups (around 10% $BW.d^{-1}$) and

higher when compared with the density experiment. This is possibly due to the lower density used in the enriched environment experience (343 hatchlings.m^{-2}).

Survival was higher in the non-enriched environment groups, indicating that this could be the more suitable environment for the culture of *S. officinalis* hatchlings.

When comparing growth in the non-enriched environment groups and groups of densities 10 and 30 (both non-enriched), better results were obtained in the non-enriched environment groups. In this case, a bigger bottom surface was used. Again, as mentioned before, this strongly indicates that not only culture density but also culture area is of great importance.

Results from both experiments indicate that the first stage of cuttlefish culture (at mean temperatures of 23±2ºC) should be done in a non-enriched environment, using maximum densities of about 500 hatchlings.m^{-2}. As for bottom area, a minimum area of about 600 cm^2 is suggested here, regardless of the densities tested, to reduce stress and increase growth and survival. This suggested value was based on both growth and survival data and the bottom area available during the enriched environments experiment and the experiment from Chapter 2.

Chapter 4

Maximum starvation period in cuttlefish hatchlings

4.DETERMINATION OF MAXIMUM STARVATION PERIOD FOR CUTTLEFISH (*S. officinalis*) HATCHLINGS, USING A NUTRITIONAL CONDITION INDEX

4.1 Introduction

The nutritional condition of the larval stages plays an important role in the knowledge of recruitment of marine species. The nutritional status of fish larvae can be evaluated using several methods; morphometric, histological and biochemical (Chícharo, 1993). Biochemical indices are more sensitive and accurate to detect first signs of starving.

According to Holl & Webb (1989), fluorescence methods for the detection of nucleic acids have been known since the pioneer work of Le Peck & Paoletti (1966). Strongly fluorescent dyes whose fluorescence is enhanced upon binding to nucleic acids have been used to quantitatively estimate nucleic acid contents. These fluorescence methods offer the potential for high sensivity using non-radioactive reactants, and they are quick, quantitative and simple to use.

One of the most commonly used methods for determining the nutritional condition and growth is using the RNA/DNA ratio (Buckley, 1979; 1980; Buckley & Lough, 1987; Clemmesen, 1988; 1990; Robinson & Ware, 1988). The use of this methodology is based on the assumption that DNA is present in constant concentrations, under changing environmental conditions or during starvation (Richard *et al.*, 1991), while RNA varies, decreasing in starving animals (Buckley., 1984; Buckley & Lough, 1987). According to Bulow (1987), RNA concentrations are higher in tissues with faster growth rates or with a higher rate of protein synthesis. The RNA/DNA ratio indicates quantities/concentrations of RNA per cell and is the most accurate when estimating tecidular proteossynthetic activity (Bulow, 1987; Buckley, 1981; 1984). The use of RNA concentration in a tissue as an indicator of growth is based on the assumption that RNA is related to the potential for protein synthesis (Houlihan, 1991). However, large variation in the RNA/DNA ratio may occur in fed larvae (Clemmesen, 1988; Raae *et al.*, 1988).

Determination of the nutritional condition using this ratio has been con-

ducted on a wide range of marine organisms, but mainly on fish (Bullow, 1970; Buckley, 1984; Robinson & Ware, 1988), crustaceans (Anger & Hirche, 1990) and bivalve molluscs (Chícharo & Chícharo, 1995). RNA content and the RNA/DNA ratio have proven to be reliable indices of the nutritional condition of larval fish (Buckley, 1984; Wright & Martin, 1985; Buckley & Lough, 1987; Clemmesen, 1987, 1988; Robinson & Ware, 1988; Canino *et al.*, 1991, Richard *et al.*, 1991; Chícharo, 1993; Canino, 1994), especially when conditions of growth are known. Recent studies have suggested that the RNA/DNA ratio is one of the best indicators of the nutritional condition of several marine organisms (Clemmesen, 1994, Bailey *et al.*, 1995; Chícharo, 1997).

This nutritional condition ratio has also been applied to cephalopods. RNA concentrations in the muscle were shown to be directly correlated with growth in *Octopus vulgaris* (Houlihan *et al.*, 1990) and *Sepia officinalis* (Clarke *et al.*, 1989; Castro & Lee, 1994). According to Clarke *et al.* (1989), RNA/DNA ratios of cuttlefish with low growth rates were similar to those reported for fish larvae; nucleic acid ratios at higher growth rates were higher than those reported so far. These authors also stated that higher values of RNA/DNA ratio could be related to the very high growth rates showned by cephalopods. Similar results were reported more recently by Koueta *et al.* (2000), where higher concentrations of RNA was related to higher daily growth rates.

Results reported by Anger & Hirche (1990), Hovenkamp & White (1991) and Bergeron & Bolhic (1994) suggested that there is a correlation between RNA/DNA ratios and growth rate. Mathers *et al.* (1994) found that an increases in protein content was not associated with similar increase in RNA concentration. Clarke *et al.* (1989) reported that the percentage of DNA/dry weight was much more stable in fed larvae than the RNA/DNA ratio. However, the work of Castro & Lee (1994) showed that the RNA contents in mantle muscle could be used as short-term indicator of instantaneous growth rate and condition of cuttlefish.

When culturing any species in intensive aquaculture, time of first feeding is of extreme importance because of the work and cost involved in the production. Food should be available when larvae or para-larvae are finishing the absorption of the inner yolk reserves and starting to feed externally, preying on an adequate species provided by nature or by man. Cuttlefish are known to be voracious

throughout their life cycle and accept a wide range of prey (Guerra, 1985; Castro & Guerra; 1989; Pinczon du Sel & Dagusan, 1992). However, prey items should be of adequate size and with a nutritional profile that will supply all the needs in protein and lipids. This nutritional profile will be reflected in growth rates throughout the life cycle, since growth is exponential during the first part of their life cycle (Forsythe & Van Heukelem, 1987; Lee, 1994).

Some authors (Wells, 1958; Richard, 1971; 1975; Boletzky, 1983; 1987; Nixon, 1985) have studied the embryonic development and the early life history of cuttlefish and set the end of the inner yolk reserves around the third day of life. However, according to Domingues *et al.* (2001c), cuttlefish in unfed or poor nutritional condition (as result of prey with poor nutritional profile) last as much as 20 days.

The objective of this research was to determine:

1) how long cuttlefish hatchlings could survive on inner yolk reserves, after hatching, using RNA/DNA condition ratios and instantaneous growth rates as a way to describe their condition;
2) the duration of the inner yolk reserves would be exhausted.

4.2 Material and Methods

4.2.1 Experiments

A group of 100 cuttlefish hatchlings was used to determine the maximum starvation period, while another group of 100 hatchlings (control) were fed live grass shrimp (*P. varians*) captured from nature. The control group was fed *ad libitum* from day 3 to 7. Both groups were placed in baskets (5,4 L water volume, 1mm mesh) which were in 250 litter tanks of a flow-through system with a U.V. unit, described in Chapter 2. Water flow was of 12 Lh^{-1}. Water temperature was of 23±2ºC and salinity was of 37±2 PSU; lights were running on a 12 hour per day basis. This system ensured an excellent water quality from a physical, chemical and bacteriological point of view.

Since several authors reported that cuttlefish hatchlings start external feeding on the third day of life (Richard, 1971; 1975; Boletzky, 1983), this was the starting period for collection of data on growth, survival and condition. However, two samples of 20 newly born hatchlings were taken to determine RNA and DNA

concentrations and ratio at time of hatching. The experiment lasted until the 7th day. The experiment was terminated on the 7th day because, at this time, negative growth rates for the starved animal started to be obtained. Each day, 20 hatchlings were collected from each group, weighed, frozen and stored (-80ºC) for later nutritional condition analysis. Data collected were used to calculate: 1) Mean Weight; 2)Mean Instantaneous Growth Rate (IGR) (%BW.d^{-1})=(LnW2-LnW1)/t*100, where W2 and W1 are the final and initial weight, respectively, Ln the natural logarithm and t the number of days of the time period and 3) Survival rates.

4.2.2 RNA/DNA Analysis

Methods for the determination of nutritional condition were adapted from Clemmesen (1988, 1990), Clarke *et al.* (1989) and Caldarone & Buckley (1991).

Purification of tissues was achieved by homogenising each cuttlefish in 2,5ml TRIS-HCl solution for 5 seconds with an ultra-turrax unit (IKA Labortechnik) and 10 seconds in an ultrasonic homogenizer unit (4710 Series, Cole Parmer Instruments Co.). This methodology was taken in order to obtain maximum homogeneity of tissues and disruption of cells, as well as a total destruction of the cuttlebone. After that, homogenate was washed with 50µl 3% sarcosine Tris-HCl and 1.35 mlTris-HCl (Trizma, pH=8) solutions and centrifuged during 5 minutes, at 9860 RPM and 4ºC. Subsequent fluorescence-photometric measurements were done. DNA+RNA measurements were done combining 0.2ml of supernatant with 0.4ml of Tris-NaCl (Trizma, pH=7.5) and 0.05ml ethidium bromide (EB) (0.1mg.ml^{-1}), a nucleic flurochrome dye specifically designed. DNA measurements were done combining 0.2ml of supernatant with 0.35ml of Tris-NaCl (Trizma, pH=7.5) and 0.05ml of ribonuclease A (Type-II A, Sigma, 0.12µg.ml^{-1}). After that, this mixture was incubated in a water bath for 30 min at 37ºC and allowed to reach room temperature for 20 min. Same quantities of ethidium bromide (EB) (0.1mg.ml^{-1}) were then used to stain the DNA and DNA+RNA in mixture samples. Each cuttlefish sample was analysed in triplicates. Values of DNA and RNA where then estimated calculating the mean value of the 3 replicates. Methodology used is shown in figure 20.

Determinations of nucleic acid concentration were identical for both

DNA+RNA and DNA measurements. The fluorescence due to total RNA was then calculated using the difference between total fluorescence (RNA+DNA) and the fluorescence after the appliance of ribonuclease A. This second fluorescence is assumed to be due to DNA, after the subtraction of the self-fluorescence by the enzyme. Both fluorescences were determined by exciting at 365nm and reading at 590nm in a Hitachi spectrofluorometer (model 650-10). Concentrations of both DNA and RNA were determined using standard curves of known concentrations of calf DNA and yeast RNA, after carrying out the same methodology as for the samples (annex). Negative values of RNA were discarded since they were out of the calibration curve.

Mann-Whitney test (Zar, 1984) was used to determine differences in RNA/DNA, [DNA]/g larvae and [RNA]/g larvae between fed and unfed para-larvae.

Spearman Rank Order Correlation test (Zar, 1984) was used to determine correlations between IGR's and RNA/DNA of fed and unfed para-larvae.

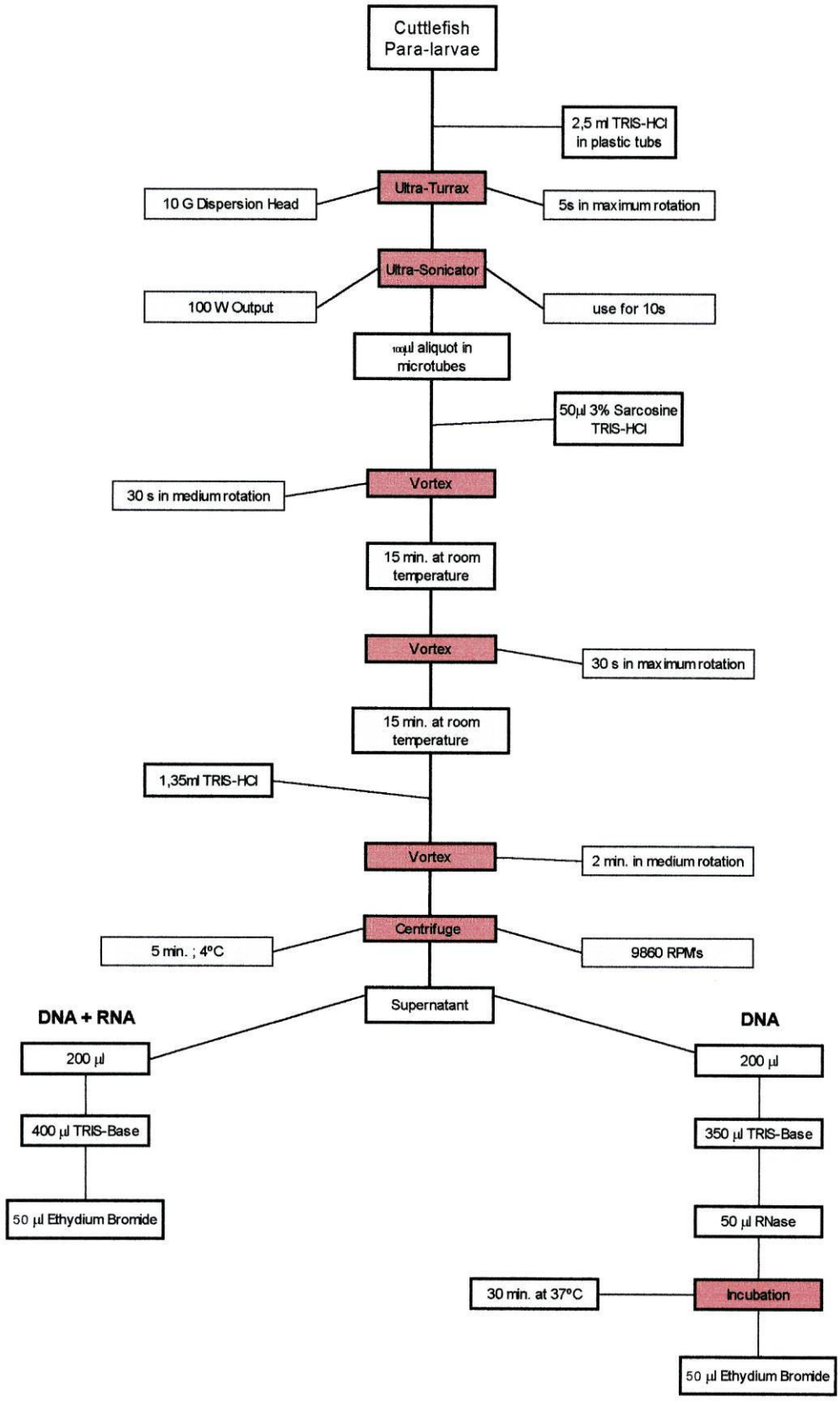

Fig. 20 - Flow-chart of sarcosine extraction procedure adapted from Clemmesen (1988, 1990), Clarke *et al.* (1989) and Caldarone & Buckley (1991).

4.3 Results

4.3.1 Growth

Both starved and fed cuttlefish para-larvae showed different IGR's (fig. 21). Starved cuttlefish group showed a steady decline in IGR from values of about 6% $BW.d^{-1}$ (day 4) to negative values of approximately -1.4% $BW.d^{-1}$. Fed para-larvae showed similar values of IGR (minimum of 3.6% and maximum of 8.4% $BW.d^{-1}$) to the ones obtained in Chapter 2.

Mortality was low and only 6 out of 100 para-larvae (6%) died in each group being tested.

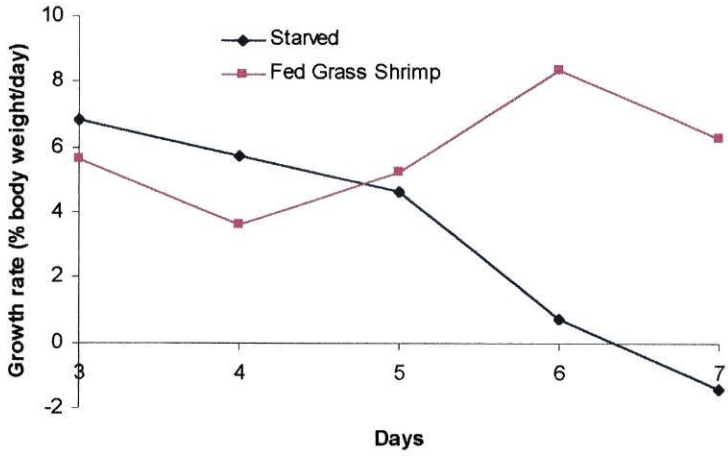

Fig. 21 - Growth rates of starved and fed hatchlings.

4.3.2 RNA/DNA Analysis

Standard calibration curves were obtained for both DNA and RNA. DNA calibration can be described using linear regression analysis by the equation $y=13.961x+3.7498$ ($r^2 = 0.996$; n=588). RNA calibration can be described using linear regression analysis by the equation $y=4.3829x+3.9095$ ($r^2 = 0.992$; n=315).

Differences between the RNA/DNA ratio of starved and fed hatchlings during the experiment is shown in figure 22. Hatchling condition was significantly different between both fed and unfed para-larvae ($p<0.05$). RNA/DNA ratio of fed hatchlings increased, while starved hatchlings decreased, throughout the experiment. Maximum values of RNA/DNA ratio (1.60 at day 7) were obtained in fed hatchlings while minimum values (0.00 at day 7) were obtained in starved hatchlings. Standard deviation of RNA/DNA ratio was high for both groups.

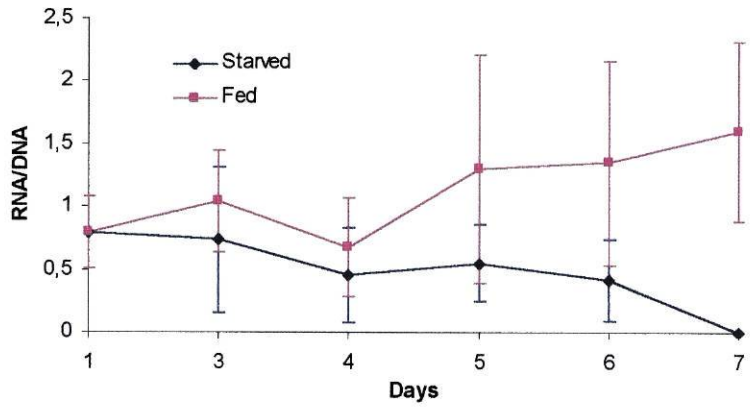

Fig. 22 - RNA/DNA ratios of starved and fed hatchlings.

DNA concentration per g of larvae for both groups during the experiment is shown in fig. 23. This index showed significant identical ($p>0.05$) concentrations until day 4. From day 5 until day 7, DNA concentrations were significantly different ($p<0.05$) between both groups. DNA concentration was higher in starved than in fed hatchlings. Maximum values were obtained in starved hatchlings (13.04 $\mu g.g^{-1}$ at day 5) while minimum values were obtained in fed hatchlings (5.23 $\mu g.g^{-1}$ at day 7). Standard deviation was higher in starved than in fed hatchlings.

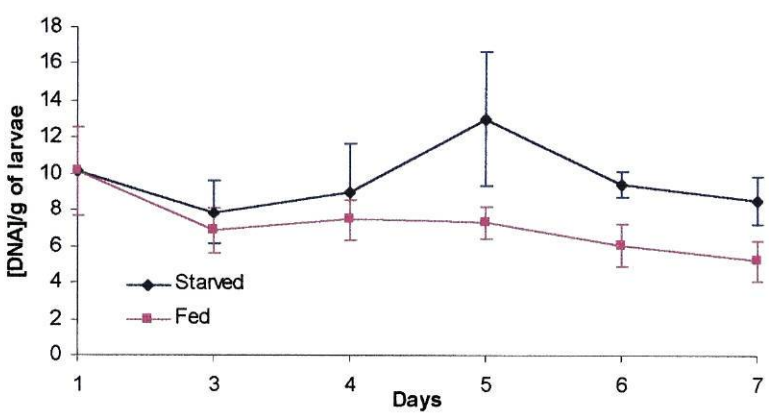

Fig. 23 - [DNA]/g of larvae of starved and fed hatchlings.

RNA concentration per g of larvae for both groups during the experiment is shown in fig. 24. This index showed identical ($p>0.05$) concentrations for day 4 and 5 only. RNA concentration was higher in fed than in starved hatchlings. Maximum values were obtained in fed hatchlings (9.05 $\mu g.g^{-1}$ at day 5) while minimum values were obtained in starved hatchlings (0.00 $\mu g.g^{-1}$ at day 7). Standard deviation was high in both groups.

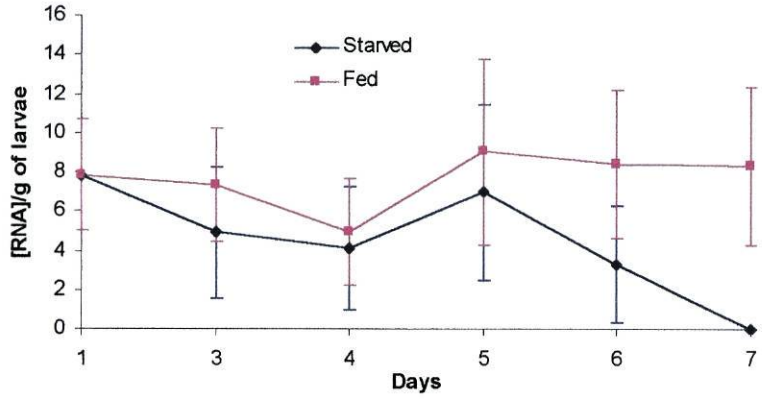

Fig. 24 - [RNA]/g of larvae of starved and fed hatchlings.

Correlation of IGR and RNA/DNA for both fed and unfed hatchlings is shown in figures 25 and 26. Spearman Rank Order Correlation showed correlation between IGR and RNA/DNA for the starved group ($R=0.90$; $p=0.037386$), but did not show correlation for the fed grass shrimp group ($R=0.80$; $p=0.104088$). Both IGR and RNA/DNA decreased with increasing age for the starved group while the opposite showed for the fed group.

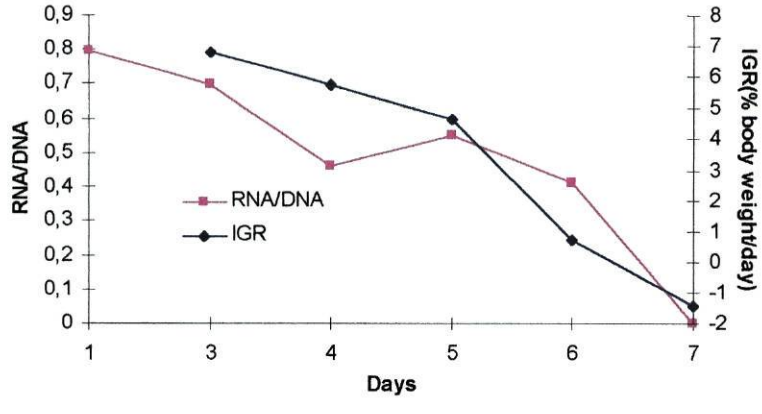

Fig. 25 - RNA/DNA ratios and IGR for starved hatchlings.

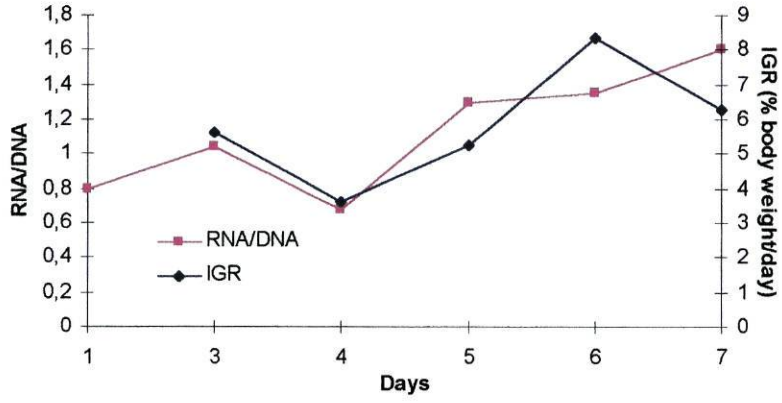

Fig. 26 - RNA/DNA ratios and IGR for fed hatchlings.

4.4 Discussion

A methodology for the extraction and purification of DNA and RNA present in cuttlefish hatchling tissues was adapted with success.

Condition is a measure of the physical status or well-being of an animal and may be used used to evaluate the growth or survival rates (Bolger & Connoly, 1989; Ferron & Leggett, 1994).

According to Berrigan & Charnov (1994), both low temperature and below normal feeding rates will reduce growth rates in most ectotherms. Forsythe *et al.* (1991) stated that both temperature and ration levels are the main factors determining variations in cephalopod growth rates. Since temperature was the same for both groups, differences in growth are directly related to feeding or starving.

This study showed that growth rates increased in fed hatchlings and decreased in starved hatchlings. Previous studies showed that growth rates have been the most accurate way of measuring cuttlefish growth and condition for the first weeks of life. Instantaneous growth rates have been calculated for a number of cephalopods (Forsythe, 1993; Lee, 1994) and can give some indication of cuttlefish condition over time. Unfed or underfed animals will display negative or very slight variations in IGR during time of survey, respectively. Contrary, well fed animals will display positive IGR. In the present experiment, fed and starved hatchlings showed these patterns.

Clarke *et al.* (1989) reported that, in juvenile cuttlefish, the RNA/DNA ratio was positively correlated with growth in individuals kept at 17,5ºC, but no correla-

tion was found on those kept at 12ºC. In this study, RNA/DNA ratios described clearly the differences between fed and starved hatchlings cultured at 23±2ºC, especially from day 4 onwards. Nevertheless, the methodology used showed some flaws, especially at the RNA determination. RNA determination was made by subtraction of DNA+RNA by DNA. Some samples of both groups did not show any values for RNA, providing negative values. This problem increased in starved hatchlings reaching 7 days old. These negative values were not used, except on day 7 for starved samples (empirically considered as 0). RNA/DNA ratio seems to show variation in samples. In fact, Clemmesen (1988) and Raae *et al.* (1988) reported large variations in the RNA/DNA ratio in their samples, especially in fed larvae.

According to Bullow (1987) and Buckley (1979), RNA/DNA ratios are extremely sensitive to variations in food availability. This can be correlated with the nutritional status and growth rates of many animals. When trying to establish a correlation between IGR and RNA/DNA ratios for both fed and starved groups, results were not conclusive. Although the results showed a similar pattern, a statistical correlation was only observed for the starved group. According to Clarke *et al.* (1989), if the RNA/DNA ratio is to be used as a tool in ecological studies, there is a need for this relationship of RNA/DNA ratio with nutritional status and growth rates to be precise. These authors also add that RNA/DNA ratios would only allow distinction between zero, average and maximal growth.

Fed cuttlefish hatchlings readily accepted grass shrimp from the first day of feeding (day 3) onwards. Results obtained lead us to believe that inner yolk reserves should last at least to the fourth day after hatching, at a temperature of 23±2ºC. This assumption is made by the decline in both patterns of IGR and RNA/DNA ratios for fed hatchlings on the fourth day, and the fast decline in starved hatchlings from the fifth day onwards.

Chícharo (1993) stated that DNA concentration was especially sensitive to starving conditions, after complete depletion of yolk reserves. Bergeron & Person Le-Ruyet (1997) suggested that DNA concentration would be a better and simpler index of larval nutritional status than the RNA/DNA ratios, since it was more stable in fed larvae and the methodology used to measure DNA only would be easier and more sensitive. Although DNA concentration was supposed to be con-

stant, this was not observed in the present experiment, since values of both groups varied considerably ($p<0.05$). Values of DNA concentration were higher in starved than in fed hatchlings and did not display a clear relation with increasing age. These results indicate that DNA concentration should not be of great importance when describing nutritional condition of cuttlefish hatchlings.

According to Pierce *et al.* (1999) there is no direct relation between RNA concentration and growth rates in cephalopod species like *Loligo forbesi*, *Eledone cirrhosa* and *Octopus vulgaris*. However, Koueta *et al.* (2000) found that changes in RNA content of muscle were related to growth in *S. officinalis* hatchlings. Considering that in the present study, the whole animal was used for analysis, RNA content of the hatchlings should describe growth. RNA concentration of fed hatchlings increased from day 5 and then remained constant until the end of the experiment. Values of RNA concentration were 8.5 $\mu g.g^{-1}$ in mean value during the 3 final days of experiment and should describe maximum rate of protein synthesis in fed cuttlefish hatchlings, at 23±2ºC. RNA concentration of starved hatchlings decreased from day 5 until the end of the experiment, when they reached the minimum of 0 $\mu g.g^{-1}$. These values should describe starved hatchling condition and minimum or non existent rate of protein synthesis, at 23±2ºC. Koueta *et al.* (2000) stated that RNA content of the muscle increased significantly between underfed (low ration) and maintenance fed (medium ration) groups. Results from the present study fall within the results obtained by these authors, after the fifth day, with a clear distinction between fed and starved animals.

According to Richard *et al.* (1991), all methods for condition analysis have their limits, some are too time-consuming, others require large samples or are valid only for particular larval stages. In spite of the lack of correlation between IGR and RNA/DNA ratios in fed hatchlings, IGR still seems to be the most appropriate indicator for cuttlefish condition since it can be applied throughout the life cycle, and is cheaper and less time consuming. Forsythe (1993) stated that exponential growth patterns of some temperate cephalopods means that very small changes in the growth rates of juveniles will translate into large differences in the adults. These small changes can not be measured by RNA/DNA ratios because of the variation associated with this method and described earlier. Castro & Lee (1994), also stated that RNA/DNA ratio may not always be the best growth indica-

tor for *S. officinalis*.

Nevertheless, it is possible that RNA/DNA ratios or RNA concentration could be used to evaluate condition of cuttlefish hatchlings for ecological and fisheries research, as tools to evaluate recruitment and wild larval condition. Thus, the present work could be seen as a new approach to the determination of standard nutritional condition patterns to be used when studying condition of wild cuttlefish hatchlings. According to Jackson & Choat (1992), short lifespan species display exponential or linear growth. Because of this, these animals spend about half of their life as small juveniles. Moltschaniwskyj & Martinez (1998) added that this may not be a disadvantage to their survivorship and that condition of these juvenile marine organisms, rather than size, may play an important role in population dynamics. According to Van Heukelem (1979), slow growth by under-nutrition results in individuals maturing at smaller sizes, while if growth rates are reduced by lower temperatures then maturation will occur at larger sizes. Based on this assumption, Moltschaniwskyj & Martinez (1998) added that the effects of temperature and nutrition on growth differ. Taking all this into account, laboratory determination of RNA/DNA ratio and RNA concentration patterns could provide some answers and clues about cuttlefish condition in the wild, and the recruitment associated. Nevertheless, they can not be used as precise tools to evaluate cuttlefish condition during the first stages of their life, as it is not accurate enough.

Chapter 5

Conclusions

5. Conclusions

S. officinalis has been cultured in the laboratory throughout the life cycle during the four or five last decades. Usually, several types of live or frozen natural prey have been used. During the first weeks of the life cycle, live prey of adequate size and nutritional value have to be available to achieve cuttlefish culture. Live mysids have promoted better growth and survival rates during this part of the life cycle. Other live prey used such as adult *Artemia* sp. have resulted in poor growth and survival. After this first and critical phase of the life cycle, cuttlefish can be cultured using other live or frozen prey, such as fish, crabs or shrimp.

Several species from the genus *Palaemonetes*, including the grass shrimp *P. varians,* have been used, both live or frozen, to culture *S. officinalis*, after the first weeks of the life cycle. During the present study, cuttlefish culture was attained with good results using exclusively grass shrimp. Initial growth was lower than when using mysids but final weight and other aspects of the life cycle were not affected by the exclusive use of grass shrimp. Survival rates were similar to the ones reported by other authors. The fact that cuttlefish culture was successful has considerably reduced labour and production costs. Although, there is a need to carry out more research on grass shrimp nutritional profile, namely on lipid classes (e.g. Cholesterol and phospholipids). Results obtained in this work showed that, at least in the fatty acid profile, cuttlefish and grass shrimp are similar, which could explain the good results obtained here. Future nutritional research should be done on more specific profiles of the grass shrimp, cuttlefish eggs, hatchlings, juveniles and adults. These results would be important for the possible elaboration of a commercial pelleted diet for cuttlefish. Until now these artificial diets have been based on amino acid requirements (since cephalopods have a protein-based metabolism) and they promoted very poor growth and survival. Therefore, it is possible that essential lipids used for structural purposes (polar lipids) are of vital importance, and should also be considered when elaborating these artificial diets.

The knowledge of optimal density are of vital importance for successful commercial aquaculture. This is especially important for solitary and semi-solitary species in nature, which is the case of *S. officinalis.*

Density that promoted better results in this study was density 10, the intermediate one. This indicates that this was a good base to work with and future studies on hatchling density could be conducted with slightly higher or lower densities, compared to this one, to more accurately determine optimal culture density. Attention must also be paid when culturing this species at different temperatures as optimal culture densities probably will vary.

Optimal densities for hatchlings suggested here are higher than those suggested by other authors in previous experiments. Nevertheless, future research should also be done with juvenile and adult cuttlefish, since space requirements may change with age. Densities should also be tested with higher numbers and larger tanks before commercial culture is attempted.

The use of enriched environments have problems associated to labour, increasing costs. Also, in the present study, the use of enriched environments was not beneficial for cuttlefish culture. Nevertheless, it recreates in a small extent conditions in nature. This study indicates that tanks without substrate clearly are the best solution for maternity purposes, before placing juveniles in the grow out tanks and/or ponds.

The use of inner yolk reserves and cuttlefish growth associated were not clearly explained. This would very important for the determination of time for first feeding. Additionally, the use of condition indexes to describe growth and condition on cephalopod species are unclear. The present work showed that IGR is still the most accurate method to describe growth and condition, since in aquaculture animals are always available and IGR can be determined at any time. Nevertheless, RNA/DNA ratios and RNA concentration can be useful to determine condition of cuttlefish in nature, since we do not have access to IGR. Therefore they can be a useful tool in ecological and fisheries biology studies. These methods were not satisfactory in describing growth since their accuracy was not good enough. Also, they are time consuming and expensive. Nevertheless, peaks of RNA concentration for well fed and starved hatchlings were determined and could be of some use in the ecological and fisheries biology studies mentioned above.

Chapter 6

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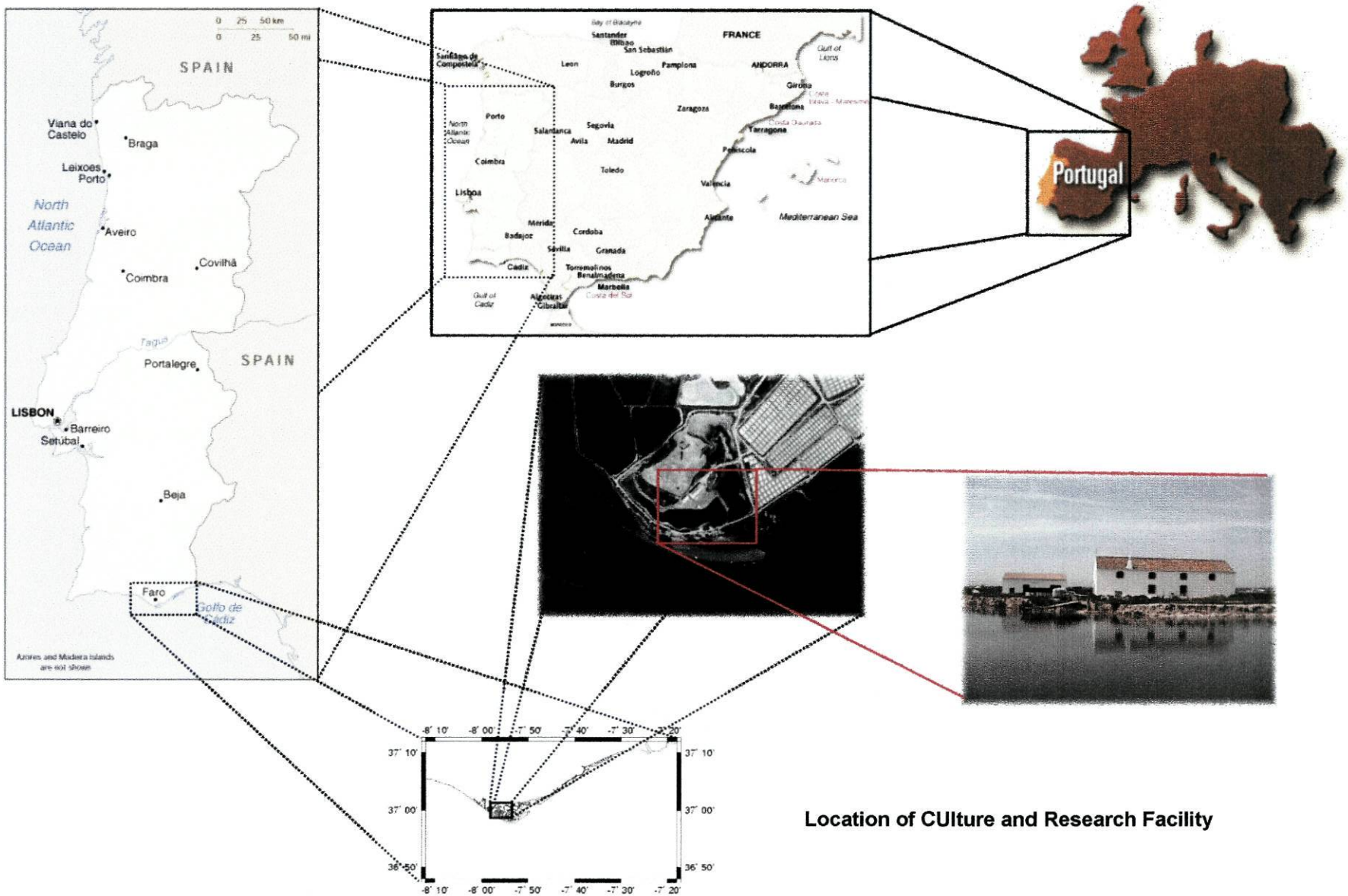
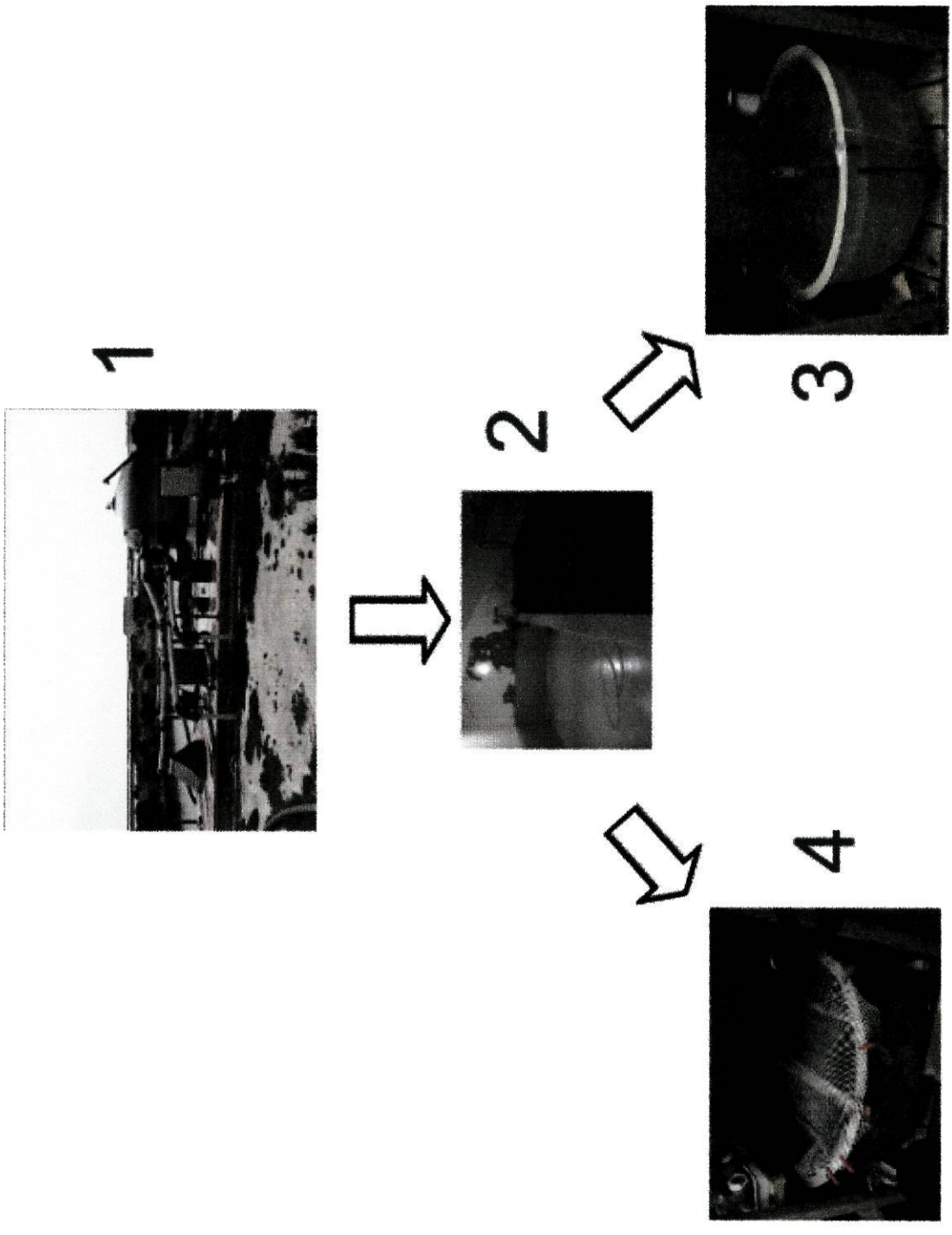
Annex



Location of CUlture and Research Facility



Photographic Flow Chart of water flow from captation to culture tanks

1- Industrial sand mechanical filter;
2- Main water reservatory inside;
3- 250 L culture tank
4- 250 L culture tank covered during mating period

ANNEX III

Solution Preparation Procedures

- **Tris-HCl** (pH=8,0) – final volume of 0,5 L
  = 2,22g Trizma HCl + 1,32g Trizma Base + 500mL Distilled Water
- **Tris-Base** (pH=7,5) – final volume of 0,5 L
  = 0,635g Trizma HCl + 0,118g Trizma Base + 500 Distilled Water
- **Ethydium Bromide Solution** (0,1mg/mL)
  = 1 mg Ethydium Bromide + 10 mL Distilled Water
- **Sarcosine Solution** (3% in Tris-HCl)
  = 3g of N-Laurilsarcosinate + 100 mL Tris-HCl
- **Ribonuclease A (RNase)** (0,01mg/mL)
  = 12mg RNase + 1 mL Tris-Base + 15 min. at 100ºC (Main Solution)

  10 x Dilutions for Storage:

  = 0,1 mL Main Solution + 0,9 mL Tris-Base (Storage Solution at -20ºC)

  10 x Dilutions for Analysis

  = 0,1 mL Storage Solution + 0,9 mL Tris-Base (Analysis Solution)

ANNEX IV

RNA/DNA Standard Flowchart