Universidade do Algarve

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# EFFECT OF TEMPERATURE ON EMBRYONIC DEVELOPMENT, LARVAL VIABILITY AND BIOMARKERS IN THE FIRST STAGES OF LIFE OF *OCTOPUS VULGARIS* (CUVIER, 1797)

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# Effect of temperature on embryonic development, larval viability and biomarkers in the first stages of life of *Octopus vulgaris* (Cuvier, 1797)

Master in Aquaculture and Fisheries

Specialization in Aquaculture

Master´s Thesis Co-Ordinated by:

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Faro, 2015

## DECLARAÇÃO DE AUTORIA DE TRABALHO

Declaro ser o autor deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

Universidade do Algarve, 29 de setembro de 2015

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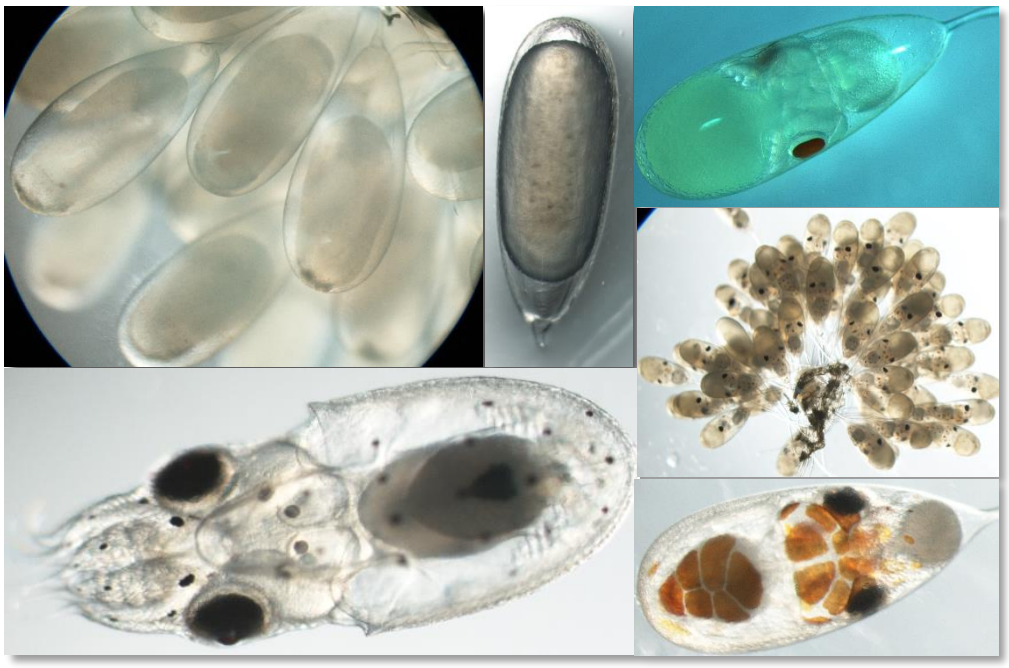

"Success is getting what you want. Happiness is liking what you get."
— H. Jackson Brown Jr.

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# 1. Abstract

*Octopus vulgaris*, rearing/production presents several problems, mainly during the first stages, from the incubation of the eggs to the rearing of paralarvae. In the last years, several research groups have been doing an effort to produce *O. vulgaris* in aquaculture (large scale), focused on larval rearing phase, in order to solve these bottlenecks.

In this sense, this study aims to analyse the effect of different temperatures on common octopus embryonic development from one single female brood; as well as, larval viability and biomarkers of growth, physiological stress, antioxidant defences and neuronal activity (RNA/DNA, HSP70, GST and AChE) respectively, in the first stages of *Octopus vulgaris* life.

*O. vulgaris* eggs from the same female brood were incubated from stage XV to hatchlings at two different temperatures (19ºC and 22ºC), and then they were maintained at room temperature (22ºC ± 1) for 14 days. Survival, specific growth rate, biomass and dry weight were measured over time at the two temperatures. Samples from 0 and 14 days were taken in order to analyse biomarkers individually. Only HSP70 was analysed using a pool of 7-8 paralarvae.

The results showed differences between temperatures in terms of survival and specific growth rate. RNA/DNA ratios and GST (0 and 14 days), and HSP70 results also showed differences between temperatures and ages (0, 7 and 14 days). No significant differences were found in AChE activity between groups (age and temperatures). The results point out at the RNA/DNA ratio, GST and HSP70 as sensitive biomarkers for growth, thermal stress and antioxidant defences in paralarvae. However, growth and temperature did not alter the neurotransmission system of the paralarvae.

**Key Words**: *Octopus vulgaris*, paralarval rearing, Temperature, Egg incubation, HSP70, RNA/DNA ratio, AChE, GST.

## 2. Resumo

O cultivo de *Octopus vulgaris*, apresenta diversos problemas, principalmente durante os primeiros estádios de desenvolvimento. Nas últimas décadas, muitos grupos de investigação têm feito um esforço no sentido de conseguir perceber quais as soluções para os problemas do cultivo desta espécie. O presente estudo tem como objetivo analisar o efeito de diferentes temperaturas de incubação (19ºC e 22ºC) no desenvolvimento embrionário da postura de apenas uma fêmea. Foram analisados biomarcadores de crescimento, stresse fisiológico, defesas antioxidantes e atividade neuronal (RNA/DNA, HSP70, GST e AChE, respetivamente). Os ovos foram incubados a partir do estádio XV até à eclosão e posteriormente foram mantidos à temperatura ambiente (22±1ºC) durante 14 dias. Sobrevivência, crescimento específico, biomassa e peso seco foram medidos nesse período. Foram recolhidas amostras com o intuito de analisar os diferentes biomarcadores por individuo. Para analisar HSP70, utilizaram-se "pools" de paralarvas (7-8). Os resultados apresentam diferenças significativas relativamente à sobrevivência e crescimento específico entre temperaturas. Os resultados do rácio de RNA/DNA e GST (0 e 14 dias) e HSP70 (0,7 e 14 dias) apresentam diferenças significativas entre diferentes idades e temperaturas. Na análise efetuada à AChE não foram encontradas diferenças significativas de atividade entre os diferentes grupos (idades e temperaturas). É notável a ampla variabilidade de resposta aos biomarcadores das paralarvas provenientes da mesma postura. Os resultados demonstram que o rácio RNA/DNA, GST e HSP70 como biomarcadores sensíveis para o crescimento, stress térmico e defesas antioxidantes em paralarvas. No entanto, o crescimento e a temperatura parece não alterar o sistema neurotransmissor dos indivíduos.

**Termos Chave**: *Octopus vulgaris*, cultura larvar, Temperatura, Incubação dos ovos, HSP70, RNA/DNA rácio, AChE, GST.

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# 3. Introduction

## 3.1 – *Fisheries Problems and Aquaculture Emergence*

Nowadays, it has been observed that marine fisheries all over the world are widely perceived to be in crisis. The fish stocks are dwindling year after year and, it is clear that many fish stocks are overexploited even with the catch restrictions and gear modifications. Aiming to continue to meet the food needs of the world population and also preserving fish stocks, aquaculture has emerged and may present a viable solution to the increase of protein demands (Branch *et al*., 2010; Worm *et al*., 2009). Aquaculture industry produces aquatic organisms inshore, onshore or in offshore areas implying interventions since broodstock management until rearing process to enhance production. This is probably the fastest growing food-producing global sector. There are several groups of aquatic species produced at the present in the globe namely finfish, mollusks, crustaceans, amphibians, reptiles, aquatic invertebrates, marine and freshwater algae (FAO, 2014).

Although it was practiced in ancient times, the real development of aquaculture took place after the 2nd World War. It was due to an exponential increase in world demand for fish, motivated by the reduction of fisheries by depleting natural stocks. This fact, increased attention on the species of cephalopods. Cephalopod catches have grown steadily in the last 40 years, from about 1 million metric tonnes in 1970 to around 3.6 million metric tonnes in 2010 (FAO, 2012).

Many cephalopod species have a very high fisheries profile and value, worth more than $US1.5 billion in annual trade. In the last decade, a number of aquaculture trials for octopuses have been undertaken in many countries around the world.

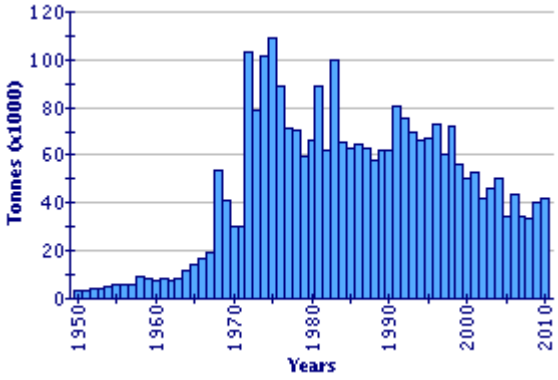


Figure 3.1 - Global capture production of *O. vulgaris* (FAO Fishery Statistics)

This group of mollusks requires considerable further research, particularly into diversity, roles in ecosystems, reproductive biology, fisheries impacts and management, and conservation status. The biggest challenge for octopus aquaculture are the resolution of several problems associated to high mortality rates when stocking densities are high (including the prevalence of cannibalism), requirements for low cost and high quality feed, and raising the earliest life stages (particularly for species with planktonic phase) (FAO, 2014).

### 3.2 - *General Characteristics*

The group of mollusks specifically the class of cephalopods are very complex creatures and have attracted great interest for aquaculture research. Cephalopods have a short life cycle ranging from 6 months to 2 years (Boyle, 1987; André *et al*., 2009). Therefore, variations in annual recruitment cause large, and to date unpredictable, fluctuations in the stocks, and the understanding of the causes represent a key element for proper management (Koueta *et al*., 2000).

The common octopus, *O. vulgaris,* is a coastal, benthic and sedentary species, living between 0 and 200 meters depth, mostly founded at 100 meters depth (Belcari *et al*., 2002), which adopts a reproduction strategy with a production of numerous small eggs that they puts normally attached to a solid surfaces in protected shelters (rocks, shells), infrequent found in man-made objects (fishing gears) normally in shallow coastal waters with low light (Naef, 1928; Wodinsky, 1972; Mangold and Boletzky, 1973; Boletzky, 1977). This species have a sexual dimorphism evident in juveniles (Boletzky, 1989). Males of *O. vulgaris* have one of those arms modified in a copulatory organ, the hectocotylus (Hanlon and Messenger, 1998; Iglesias et al., 2000) through which transfer spermatophores into the mantle cavity of female (Mangold, 1987). This species, is distributed around the world (Figure 3.2), mainly in Atlantic Sea and Indico-Pacific (Mangold, 1983).

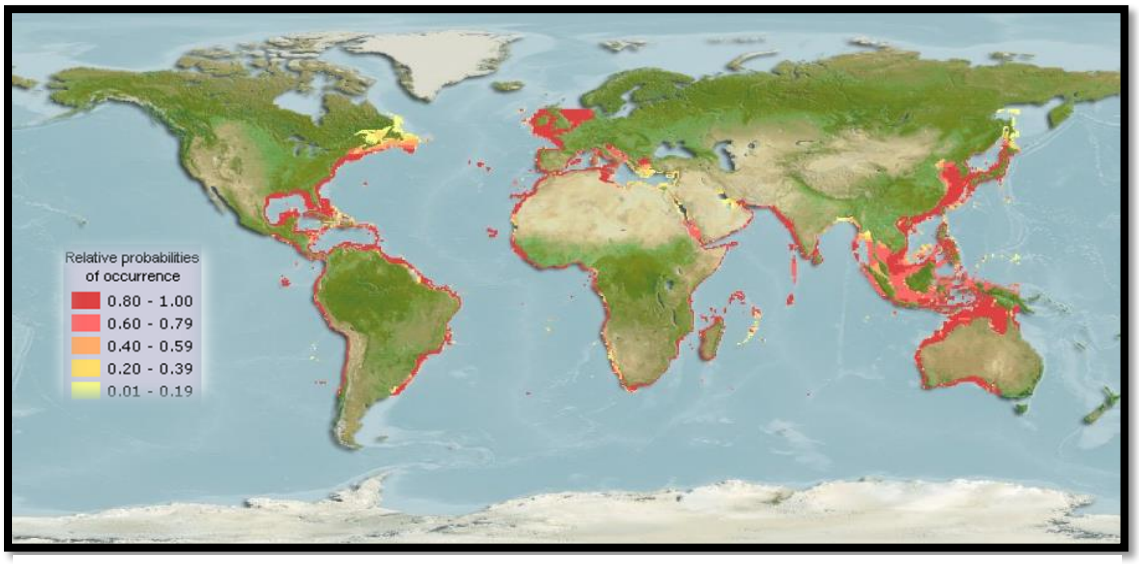


Figure 3.2 - Worldwide distribution of *Octopus vulgaris* (Source: AquaMaps)

In the last years, several research groups have been doing an effort to produce *O. vulgaris* in aquaculture (large scale), focused on larval rearing phase, in order to solve the bottlenecks from the incubation of the eggs to the rearing of paralarvae, mainly during the first stages due to non-availability of adequate living diet, lack of standardization of the culture technique, high mortality and unpredictable results on these early stages as a result of the limited knowledge of the biology of this species (Moxica *et al*., 2002; Seixas *et al*., 2010; Iglesias *et al*., 2006, 2007; Morillo-Velarde, 2013). This is definitely the best known and more studied species of cephalopod from culture perspective (Cagnetta and Sublimi, 2000; Vaz-Pires *et al*., 2004; Villanueva and Norman, 2008).

This cephalopod adapts easily to captivity and is considered a serious candidate due to biological and physiological characteristics (Vaz-Pires *et al*., 2004; Miliou *et al*., 2005; Iglesias *et al*., 2007), such as, high growth rates achieving 8 % of body weight per day with high feed conversion, or by incorporating 30-70% of their own weight of food ingested (Mangold and Boletzky, 1973; Mangold, 1983; Lee, 1994). This high conversion efficiency is definitely dependent on food quality (Mangold, 1983). It is observed a high protein content in their body composition, representing 70 to 90% of dry weight accepted as food of all kinds of marine animals (fish and shellfish) (Lee, 1994; Domingues *et al*., 2010) and high consumption and commercial value in the world (Iglesias *et al*., 1997, 2004; Vaz-Pires *et al*., 2004; Miliou *et al*., 2005).

According to Brown *et al*., (2006) *O. vulgaris* prefer nocturnal activity in aquaculture conditions and it was observed by Villanueva (personal observation) that most hatchlings occur during night. This happens probably due to the fact that paralarvae have positive phototaxis at hatching which was describe by Villanueva (1995) and Nixon and Mangold (1998), until some later paralarval stages which diminish, dissipate or inverse near or after the settlement. (Villanueva, 1995; Villanueva and Norman, 2008). It is possible that hatchings occur during the night so the paralarvae are more protected from the predators, however, this trend of the octopus paralarvae hatch during off-hours light must be confirmed and quantified taking into account the lunar rhythms and tides (Villanueva and Norman, 2008).

This species shows a high fertility, each female can produce 100 to 500 thousand elongate eggs (Naef, 1928; Mangold, 1983; Iglesias *et al*., 1997), arranged in clusters and caring them (cleaning, oxygenating with water flushes and protecting from predators) throughout the incubation period, after which the females dies (Naef, 1928; Nixon and Mangold, 1996). At the beginning, shortly after being placed, the eggs measure about 2 - 2.5 mm in total length (Mangold, 1983; Nixon and Mangold, 1998; Iglesias *et al*., 2000) and almost 1 mm of width (Mangold, 1983).

It was known that incubation time period appears to be inversely related with temperature, low temperatures require more days of incubation and *vice versa* (Naef, 1928; Itami *et al*., 1963; Mangold and Boletzky, 1973; Villanueva, 1995; Caverière, 1999). It is also known that embryonic development in octopus depends on temperatures of each considered geographical and bathymetric zones (Mangold and Boletzky, 1973). Concerning this, it is very important to study the tolerance limits during embryonic development and how different temperatures can affect the paralarvae rearing in order to ascertain patterns of growth and welfare.

According to this, biomarkers as RNA/DNA for growth patterns, HSP70 to understand thermal tolerance (physiological stress), GST (oxidative stress) and AChE (neuronal activity) were analyzed.

### 3.3 - *From egg to paralarvae*

The common octopus (*O. vulgaris),* is a poikilothermic so their metabolism is regulated by temperature (Vidal *et al*., 2014). Temperature is the main factor that control the life cycle of this species because it directly influences egg development (embryo), yolk absorption rate, growth rate, feeding rate (food conversion efficiencies) and lifespan. At high temperatures development, metabolism is accelerated and at low temperatures

development suffers slower progress (Naef, 1928; Mangold and Boletzky, 1973; Boletzky, 1987; Hamasaki and Morioka, 2002; Vidal *et al*., 2002). In early stages, as meroplanktonic organisms they strongly dependent on temperature (Katsanevakis and Verriopoulos, 2006).

At different temperatures, growth could be influenced by activity or stress as a result of increased energy demand. Though, growth rates should increase up to an optimum (depending on temperature), above which energy requirements are too high to maintain feed conversion, and metabolic processes and growth rates are reduced (Vidal and Boletzky, 2014). Hamasaki and Morioka (2002) observed that embryo takes about 69 days at 16.5ºC to hatch and about 21 days at 25.5ºC. According to Mangold (1983), the embryo takes 22-25 days at 25ºC to hatch. In fact, these effects were observed also in other cephalopods species (Forsythe, 1993). For planktonic paralarvae, Katsanevakis and Verriopoulos (2006) observed that temperature modulates planktonic periods which are shorter when temperature is increasing and longer at low temperatures.

During the incubation period at 23ºC, the eggs suffer a strong metamorphosis, passing through different embryonic stages (I - XX) until the hatching. These changes occurs immediately after laying and the stage I begins after almost 2 days with a complete blastoderm. At the second day, the endomesoderm formation starts (stage I-II) and continuous to form until stage III (84 hours). Normally at stage IV (day fourth) the eggs are with the germinal disc covering the animal pole of the egg. At stage V (five days old) the mesoderm are better developed and the germinal disc covers a small portion of the yolk mass. At stage VI (day six), the different mesoderm portions can be distinguish (arm crown, head, gill area, mantle rudiment) and the germinal disk covers at this time like 50% of the yolk mass. The stage VII, with seven days, the embryo shows a similar aspect with more specific development of the arms, the buccal area is now more visible and the yolk are almost covered (70%). In the stage VIII (eight days old), the ocular and buccal edge are more evident, the arm elements become uniformly outstanding, the siphon become clearly visible in both sides and finally the yolk envelope is practically complete at this point. With nine days old (stage IX) occurs the closing of the yolk and in this stage it is possible to see the divided yolk sac and the embryonic body. The funnel are now totally define, the ocular vesicles come more closed, and the buccal invagination and the statocysts goes more profound. At stage X (ten days), it is possible to see that eye vesicles and statocysts are closed, the funnel are now completely formed and the mantle cavity

formation begins. Stage XI (eleven days), when the embryonic fin differentiation achieves the maximum, the eye stalks are not formed but the corresponding tissue are clearly visible in both sides. Stage XII (twelve days old), the arm crown shows some evolution, the funnel lobes band together to structure the funnel tube and the tips of the gills goes increasingly in anterior direction. At stage XIII (thirteen days old) the arms present already the three suckers with different sizes, the funnel completed the metamorphosis, the mantle continuing growing to close the mantle cavity and the gills have a branchial lamella. Until this stage the development evolves almost one stage per day and then slows down. At stage XIV the embryonic evolution is fifteen days old, the arms are now fused with the mantle, the ocular edges cover more the eye, the mouth are more deeper and the mantle are bigger also covering the funnel pouches. At stage XV (eighteen days) the mouth starts to be surround by the dorsal arms, the suckers presents a depression characterizing a future suction cell, it is possible to observe the olfactory tubercle and also the inner yolk sac (energetic reserves) that starts in this stage. During stage XVI (twenty-one days) the embryo have the primary "cap" practically integrated with the junction of the posterior extremities of the edges with the integumental ridge, with this movement the olfactory tubercle are at this point detached from de eye and the first appearance of chromatophores can be observed. At stage XVII-XVIII (twenty-four days of development), the olfactory tubercle are in its natural position and at this point the embryo presents a distinct paralarval form with visible chromatophores. At stage XVII-XVIII (twenty-four days of development), the olfactory tubercle are in its natural position and at this point the embryo presents a distinct paralarval form with visible chromatophores. In the final two stages XIX-XX (twenty-eight days old) it is very clearly to see a complete developed paralarvae with the yolk sac that could be absorbed inside the envelope or outside when stimulated to hatch (Naef, 1928) (Figure 3.3 A and B).

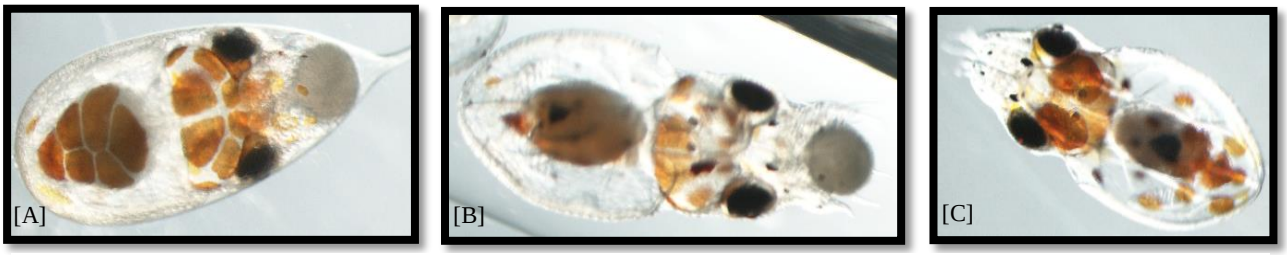


Figure 3.3 - [A] Embryo of O. vulgaris at XIX – XX stage; [B] Premature paralarvae (stimulated to hatch); [C] Recently completed developed hatched paralarvae. Photographs by the author.

When the embryo finished this metamorphosis process and is fully develop inside the eggs, hatch into paralarvae, termed proposed by Young and Harman (1988), for a planktonic phase in the first stages (days), moving later to a benthic phase (Hamasaki and Morioka, 2002). This paralarvae stages differ from the adults in their morphology (transparent musculature, simple chromatophores, short arms, few suckers, normally three per arm), physiology (all organs are well separated at hatch excluding the reproductive system), ecology (water circulation, light, temperature, chemical parameters) and behavior (free-swimming by a water propulsion jet) (Boletzky, 1977, 1992; Iglesias and Fuentes, 2014; Villanueva and Norman, 2008). According to Villanueva and Norman (2008), the musculature transparency is correlated with the planktonic life phase, reducing their shape and visibility to predators (Figure 3.3 C).

The mechanisms that stimulate the hatching operation are unknown (Villanueva and Norman, 2008). It was observed by Sarvesan (1969) that females in the course of hatching frequently expel water with the funnel to the eggs to stimulate the hatching.

Although the better results until settlement were obtained feeding the *O. vulgaris* paralarvae with different crustaceans species encompassing zoeas of *Palaemon serrifer* (Itami *et al*., 1963), zoeas of *Liocarcinus depurator*, *Pagurus prideaux* (Villanueva, 1995) and *Maja squinado* as a complementary diet with *Artemia* (Iglesias *et al*., 2004). According to Carrasco (2006), the ideal diet for paralarvae culture was crab zoeae supplemented with *Artemia* in alternating days at least during the first 45 days. Hamasaki *et al*., (1991) demonstrated that it is possible to feed *O. vulgaris* paralarvae only with enriched (*Nannochloropsis* sp.) *Artemia* during planktonic phase until settlement. Afterwards, Fuentes *et al*., (2011) reported that, within different feeds, *Artemia* enriched with *Nannochloropsis* sp. was the best result in terms of developed, higher paralarvae dry weight and a good survival rate until 30$^{th}$ day (almost 50%).

The adverse effects caused by extremely environmental conditions, in this case, incubation temperature, influence at certain point the larval development. The optimum temperatures for growth could be achieved through the identification and quantification of cellular stress (physiological stress) responses and RNA/DNA quantitative analysis both used as indicators (biomarkers) of larval quality.

3.4 - *Physiological stress – Heat Shock Protein (HSP70)*

Stress proteins as HSP70, are produced in response to non-lethal stress to protect organisms from ensuing harsh stress that would in any other way be lethal (Whitley *et al*., 1999). In this study, the levels of HSP70 could be very useful as a stress biomarker, to understand the range of temperatures that *O. vulgaris* species develop better.

Temperature is related with survival and life cycle of *O. vulgaris* from eggs incubation, embryo development, paralarvae growth, to settlement until adults (final stage) (Naef, 1928; Mangold and Boletzky, 1973; Caverivière *et al*., 1999) According to Naef (1928) and Caverivière *et al*., (1999), different temperatures affect embryogenesis ranging from 2-3 weeks to 2-3 months. Incubation temperature should not be too high (above 27ºC), since it damages the gelatinous layer of the egg capsule and consequently affects metabolic functions (energy consumption rates) and may even end up killing the embryo (Bouchaud, 1991; Villanueva *et al*., 1995), inducing high early mortality (Hamasaki and Morioka, 2002). As was mentioned above, temperature is known to be the abiotic factor that modulate organisms physiology and varies across the different depths and ecosystems (Rosa and Seibel, 2008). Its change can cause an environmental disturbance, stimulating the production of heat shock proteins (HSPs) obtained from digestive gland, muscle, and mantle (Wang *et al*., 2013).

There are a lot of cells in almost all organisms that produce a great quantities of a highly conserved and universally synthetized heat shock proteins, particularly the HSP70. This family of proteins is present in specific cellular areas such as cytoplasm, mitochondria and endoplasmatic reticulum. These proteins are involved in different processes, one of those is stress responses (protective mechanism) (Pelham, 1984; Parsell and Lindquist, 1993; Samples *et al*., 1999; Agarraberes and Dice, 2001; Mayer and Bukau, 2005).

Extreme temperatures reflect stress conditions for the organism. As a response, organisms induce the synthesis of heat shock proteins when the temperature exceeds their normal values. When this factor occur the synthesis of these proteins (HSP70) extremely increases (Malyshev, 2013).

These HSP´s produces a heat shock response (HSR) caused by thermal stress. Osovitz and Hofmann (2005) observed that in sea urchins thermal events modified the expression of HSP70, showing that HSP70 extraordinarily increased in response to high

temperatures. Identically was observed during the last embryo stages of *O. vulgaris* where it was detected higher levels of HSP70 at 18ºC than at 21ºC, but when the embryo hatched into paralarvae the levels of HSP70 at 21ºC dramatically increased almost 8 times more than at 18ºC. These results revealed a great response to increasing temperature (Repolho *et al*., 2014) and indicate different termo-regulation during development.

Moreover, Feder and Hofmann (1999) reported that it is possible to know the thermal history of the organism by analyzing the expression of HSP70, and therefore this parameter could represent a good biomarker of temperature stress response in organisms.

3.5 - *Growth – Nucleic acids (RNA/DNA and Proteins)*

There are different forms to study growth, through morphometric measurements (size and weight) or biochemical approach, such as RNA concentration or the RNA/DNA ratio (Clarke *et al*., 1989). This ratio is considered a good indicator and commonly used for determining nutritional and growth condition because is directly related to ongoing tissue in aquatic organisms (Holm-Hansen *et al*., 1968; Buckley, 1979, 1980; Clemmensen, 1988, 1993). The use of RNA/DNA ratio is based on the principle that DNA concentrations is constant in cells of a given species (Regnault and Luquet, 1974) and maintain unaltered by environmental conditions (Richard *et al*., 1991) as water temperature (Foster *et al*., 1992). The RNA is directly involved in protein synthesis concentrating in tissues related with growth rate (Sutcliffe, 1965), which is known to vary with age, life-stage, organism size, and state of disease and with changing environmental conditions, like food availability and temperature (Buckley, 1984). In fact RNA/DNA ratios have been used as a biomarker in quantitative analysis on a wide range of aquatic organisms, for instance zooplankton (Wagner *et al*., 1998), fish species (Buckley, 1984; Clemmensen, 1989, 1993, 1994; Bergeron, 1997; Buckley *et al*., 1999; Clemmensen *et al*., 2003), cephalopods (Clarke *et al*., 1989; Houlihan *et al*., 1990, 1998; Pierce et al., 1999; Sykes *et al*., 2004) and also in other groups.

In cephalopod species with short life cycle and fast growth such as octopus, research on different development stages is needed to improve the understanding of octopus biology (Pierce, 1999). The RNA/DNA ratio is an index of presumed cellular protein synthesis capacity (Roark *et al*., 2009). According to Pierce *et al*., (1999) immature cephalopods have higher RNA/protein ratios than mature individuals. It was

observed by Houlihan *et al*., (1990) that RNA/Proteins ratios were correlated with *O. vulgaris* growth rates.

Temperature can be used as a second independent variable enhancing the estimation of growth (Buckley *et al*., 1984). Organisms in good condition tend to have higher RNA/DNA ratios, faster growth, than those in poor condition (Buckley, 1979; Vidal *et al*., 2006).

RNA concentrations and protein synthesis should be higher in fast growth tissues and it is not clear if water temperature affect these relationships (Foster *et al*., 1992). RNA content in mantle tissue were lower in bigger individuals. It was observed that in common octopus the analysis of different tissues could give different results regarding this parameter. It might be expected that results from mantle tissue could be more consistent than tissues from arm (this tissues may show higher growth rates when regenerating after damage). Regarding this problem, it is important to standardize the site from which tissue is taken (Pierce, 1999). However, a limitation to calculate RNA/DNA ratio for larval growth is the weight. In fish, if each larvae has less than 0.8 mg of dry weight, pools of larvae must be done to provide sufficient tissue for analysis using classical methods (Buckley, 1984).

In this work, to test these hypothesis (if water temperature could positively influence embryonic development from the stage XV; if the weight is a limitation in cephalopods, *O. vulgaris* for nucleic acids analysis), a protocol was adapted in order to use an individually paralarvae as sample without weighting them and to observe differences between individuals.

### 3.6 - *Antioxidant defenses - Glutathione S-transferase (GST)*

Glutathione S-transferases (GSTs) are a multigene superfamily of multifunctional soluble enzymes. Apart of their essential functions in intracellular transport, they play a critical role in defense against oxidative damage and peroxidative stress products of DNA and lipids (Hayes and Pulford, 1995; Goto *et al*., 2009). In estuarine fish, Madeira *et al*. (2013) found that differences in GST activity could be species-specific.

Fluctuation of oxygen levels in the environment origin changes in GST activity in invertebrates (Oliveira *et al*., 2005). Graham (1949) described in one salmonid species (*Salvelinus fontinalis*), that exists a relationship between temperature and lethal oxygen

levels. Also Schurmann and Steffensen (1992) determined the levels of oxygen saturation and the reason that cod, *Gadus morhua*, move to cold water improving the survival chances at low oxygen levels. Lower temperatures means a decreased in metabolic rate as a result of slower running of biochemical reactions, as enzymatic variations.

According to this hypothesis, GST activity was investigated in *O. vulgaris* eggs incubated at different temperatures.

### 3.7 - *Neuronal activity - Acetylcholinesterase (AChE)*

Acetylcholinesterase (AChE) is a very important enzyme to the nervous system and therefore it is commonly used as a biomarker for neurotoxicity. It was found that the activity of this enzyme is strongly related to water temperature (Durieux *et al*., 2011).

Neurons receive both excitatory and inhibitory inputs by neurotransmitters. The presence of phosphoric esters, carbamates, sulfonate esters and other compounds in the environment causes an inhibition of AChE in the neurons (Tim-Tim *et al*., 2009). According to Talesa *et al*. (1995) the molecular forms of AChE are more restrict in invertebrates than in vertebrates, consequence of no asymmetric forms. Thus, cephalopods are prosperous of AChE and possibly the animals with the fastest cholinergic neuromuscular transmission (Wächtler, 1988). The AChE is responsible for acetylcholine neurotransmitter degradation in synapses (Varó *et al*., 2007).

During the early determinative phases of growth the evolution can be mostly related with the nerves. One of the main enzymes which have important functions in neurotransmission and regenerative process is the AChE (Fossati *et al*., 2013). In this work, the determination of AChE was tested in paralarvae individually in order to predict and understand species vulnerability to stressful environmental temperature.

## 4. Objectives

This study aims to analyse the effect of different temperatures on common octopus embryonic development from one single female brood; as well as, larval viability and biomarkers of growth, physiological stress, antioxidant defences and neuronal activity (RNA/DNA, HSP70, GST and AChE, respectively).

# 5. Materials and Methods

The first part of this study was conducted at the Instituto Español de Oceanografia (IEO), in Tenerife, (Canarian Islands, Spain), and handing of the octopus (adults and paralarvae) was done according to the policies of the Spanish legislation (Royal Decree RD53/2013) and the European Directive 2010/63/EU.

## 5.1 - *O. vulgaris broodstock (IEO)*

Figure 5.1 - Adult of O. vulgaris

The capture of wild *O. vulgaris* broodstock individuals (Figure 5.1) was performed by professional artisanal fishermen on the Tenerife island coast (Canary Islands, Spain). After being caught, broodstock individuals were kept in 1000 L quadrangular fiberglass tanks, where individuals of similar weight were placed with a sex ratio of 2 females per male (2:1). Sex determination was performed by verifying the existence of the hectocotilized arm in putative males. The tanks were maintained in natural photoperiod, with natural water temperature and salinity (around 23-24ºC and 33-35 ‰), respectively. Each tank surface was covered with 50% of a shady net. The tanks had an open seawater system with a flow of 6 L/min which entered the tank by the top of the water column and exited through a filter mesh (1 cm) located on the bottom. A mix of frozen squid (*Loligo opalescens*), mussels (*Mytilus edulis*) and prawns (*Parapenaeus longirostris*) were added to the on-growing adults *ad libitum*. PVC pipes and clay pots were placed inside the tanks to provide dens.

5.2 - *Collection of the eggs (IEO)*

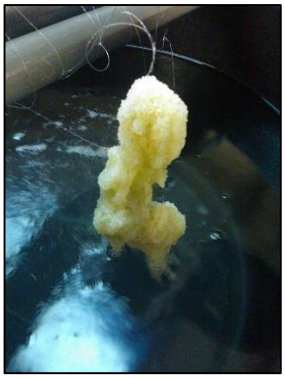

Figure 5.2 - Cluster of eggs

The presence of eggs was verified once a week to avoid disturbing the breeders, and also it was possible to see when the breeders were feeding. When an egg mass was observed, the remaining individuals were removed and placed in a different tank, leaving the fertilized female alone with the egg mass. Samples were taken from that egg cluster, a line with around 1 cm (Figure 5.2), to identify the development stage. When the embryos achieved the XV stage, some eggs (4 lines with 4-5 cm each) were transferred to an acclimatized room (constantly 22ºC) and then they were placed at different temperatures (19ºC and 22ºC) in six fiberglass tanks (10 L). The eggs were taken only when the embryos achieved the stage XV because no differences by temperature have been found between stage XV and XX in outer yolk sac (growing) (Nande *et al*., 2014).

5.3- *Eggs incubation to hatched paralarvae (IEO)*

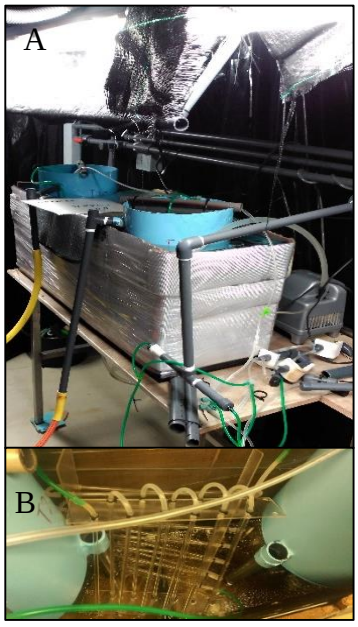


Figure 5.3 - (A) Tanks for 19ºC experiment; (B) serpentines used to refrigerate de water.

To incubate the eggs at 19ºC an open water system was used, but water passed through 3 glass serpentines (4 meters each) immersed in a cold (16ºC) bath water in order to obtain 19ºC (Figure 5.3). The system consisted in a 200 L aquarium that was used to place 2 small 10 L cylindrical-conical fiberglass blue tanks. These small tanks also have an entry of aeration with a wood stone. The water leaves the two tanks through 360 µm mesh (mechanical filtration). At 22ºC it was used an open water system. The water entered directly into the two 10 L cylindrical-conical fiberglass blue tanks and left through a 360 µm mesh. Each tank was equipped in the same way with the same aeration and water entrance. All small fiberglass tanks were partially covered with a shade net in order to avoid too much light intensity.

After hatching, the paralarvae were reared in a three 100 L fiberglass cylinder-conical black tanks at 22ºC (environmental temperature) with soft aeration until 14 days old.

In this experiment green water was not used due to logistical problems. Water quality was promoted through the use of a filtration system, consisting of three inline mesh filters (with a porosity of 20, 5 and 1 μm) and of an UV filter, prior water entering in each tank of the culture system. Water was supplied through the top of the tanks, with a flow of 70 mL/min, ensuring at the least 100% water renovation per day. Dissolved oxygen was provided through the use of moderate aeration (by one porous plastic aeration stone - 3 cm in length), placed in the lateral side of tanks. The tanks were under a light regime of 200 lux of intensity and a photoperiod of 12L:12D. Water temperature and dissolved oxygen were measured with a DO METER PRO ODO device. Ammonia ($NH_3$), nitrites ($NO_2$) and pH were measured with a TETRA test aquarium kits for $NH_3$ and $NO_2$ and with a Hanna-HI-98107 pH Metter.

5.4 - *Artemia culture and Enrichment (IEO)*

Paralarvae were fed with enriched *Artemia* (*A. franciscana*) in naupliar stages. The *Artemia* (0.3 *Art*/ml) was enriched with T-ISO (lyophilized *Isochrisys affinity galbana*) considering this type of feed the best option to cultivate octopus paralarvae at massive scale without having availability problems (Iglesias *et al*., 2004).

5.5 - *Collection of the samples for morphometric measurements (IEO)*

Survival and growth were obtained from cultured paralarvae. A sample of 15 paralarvae was taken from each experimental temperature group (19ºC and 22ºC) at 0 and 14 days and the dry weight (DW) was determined by weighing each paralarvae after anesthetized and killed in ice-cold-chilled water, washing individuals with distilled water and drying them in a stove at 100ºC±1ºC for 24 h.

Specific growth rate (SGR% per day) was calculated as follows according to Ricker (1979):

$$\text{Specific growth rate} = \left[\frac{\ln DWf - \ln DWi}{t}\right] \times 100$$

where DWi and DWf represent the initial and final paralarvae dry weights respectively, ln the natural logarithm and t the cultivation period (14 days). (BI%) represents the increment of biomass during cultivation period per day and was calculated as follows:

$$\text{Biomass} = \frac{\left[\left(\text{nºparalarvae} \times \left(\frac{\text{survival}}{100}\right) \times \text{DWf}\right) - (\text{nºparalarvae} \times \text{DWi})\right]}{(\text{nºparalarvae} \times \text{DWi}) \times \text{Age}} \times 100$$

5.6 - *Collection of the samples for biochemicals indices (IEO)*

In order to analyze RNA/DNA and total proteins, a sample of 16 paralarvae was taken from each experimental temperature group (19ºC and 22ºC) at 0 and 14 days (n=64). The paralarvae were individual selected, taken out from the tank, anesthetized and killed in ice-cold-chilled water, washed with distillated water, introduced in an identified Eppendorf tube and rapidly frozen at -80ºC until they were analyzed. Also pools of eggs samples at stage XV (n=6) were collected for the same analysis using the same procedure described above.

To perform the HSP70 analysis, 3 samples were collected with pools of 7-8 paralarvae from each experimental temperature group and from different ages (0, 7 and 14 days) using the same procedure described above. All samples were stored for further biomarkers analyses at the Institute of Aquaculture of Torre la Sal (IATS-CSIC, Castellón, Spain).

5.7 - *Biomarkers analysis (IATS-CSIC)*

This part of the work was developed at IATS-CSIC (Castellón, Spain). The biomarkers assays carried out were: RNA/DNA ratio (growth), proteins and heat shock proteins (HSP70) (stress), antioxidant defenses (GST) and neuronal activity (*AChE*) in order to ascertain patterns of growth and welfare.

5.7.1 - *Nucleic acid and protein assays*

Nucleic acids concentrations were determined using two extractions kits (Picogreen® for DNA and Ribogreen® for RNA) following the protocol described in Varó *et al*., (2007, 2013) and adapted to one paralarvae. Briefly, for each sample (0 and 14 days) 200 µl of TE 1x assay buffer was added (10 mM Tris-HCL; 1 mM EDTA, pH= 7.5) and was homogenized in cold and centrifuged at 10000 g, 5 minutes at 4ºC. Then,

180 µl of supernatant was collected and aliquoted in different eppendorfs (150 µl for DNA and protein determination, and 30 µl for RNA). The samples were kept at – 20ºC.

For DNA assay the samples were diluted (1/8). A high range curve (standard curve) was prepared with a known DNA concentration (2µg/ml) with points between 0-2 µg/ml. Samples were placed on black 96-well plate. First, were added 10 µl of RNase A (diluted 400x) to each well and then 50 µl of each sample (3x) was introduced. These samples were incubated for 1 hour at 37ºC. After this incubation period, it was added to each sample 50 µl of 1x TE and 100 µl of PicoGreen® (200x diluted), getting a total of 200 µl per sample. Then, the samples were incubated during 5 minutes at room temperature protected from the light. Finally, samples fluorescence were read in the microplate reader (485nm excitation, 535 nm emission).

For the RNA assay the samples were diluted (1/40) with a 1x TE buffer. A high range curve was prepared with a known RNA concentration (2µg/ml) with points between 0-2 µg/ml. For preparing the 96-well black plate 1 µl of DNase I buffer at 1x was used (enzymatically digestion) provided by the RiboGreen® kit. After that, 50 µl of the sample was added in each well (3x) and incubated at 37ºC for 1 hour. Later, it was added 50 µl of TE 1x and 100 µl of RiboGreen® (200x diluted) getting a total of 200 µl per sample. Then, the samples were incubated during 5 minutes at room temperature protected from the light. Finally, samples fluorescence were read in the microplate reader (485nm excitation, 535 nm emission).

Total protein concentration of each sample, previously diluted (1/2), was evaluated using a DC Lowry kit (Bio-Rad) assay and bovine serum albumin stock (BSA), as standard (0-1,4 mg/ml). The 96-well transparent plate was prepared using the diluted samples which were pippeted in each independent well and were added 10 µl of solution A plus 80 µl of solution B. The plate was incubated for 15 min at room temperature in darkness (covered with aluminum paper) and finally read at 700nm absorbance in the microplate reader. All determinations were carried out in triplicate.

### 5.7.2 - *HSP70*

The content of HSP70 was assessed by immunodetection technique. For that, samples were homogenized in ice-cold calcium-magnesium free saline buffer containing 20 mM Hepes, 500 mM NaCl and 12.5 mM KCl (pH = 7.3) and complemented freshly with: 1 mM dithiothreitol (DTT), 1 mM phenymethysulfonyfluoride (PMSF), 1% Igepal

and 1% protease inhibitor cocktail (Complete-Mini, EDTA-free, ROCHE). Then samples were centrifuged at 15.000 g (4ºC) for 20 min and the supernatant obtained was kept at -80ºC. Equals amounts of protein from each sample were separated using Bio-Rad Mini-Protean TGX Precast gels (4-20% resolving gel) in a Mini-Protean Tetra cell system (Bio-Rad), for 10 min at 120 V and 16 min at 300 V, and then transferred onto PVDF membranes (0-2µm, Trans-Blot® Turbo™ Mini PVDF Transfer Packs) at 1.3 A, 25 V for 10 min in a Trans-Blot® Turbo™ Blotting System (Bio-Rad). Membranes were blocked 1 h at room temperature in 5% non-fat dry milk (Bio-Rad, Blotting-Grade Blocker) in 1x TBS. The immunodetection was performed using HSP70 mouse monoclonal antibody (Sigma). Blots were incubated overnight at 4ºC with the primary antibody diluted 1:2500 in 3% non-fat dry milk in 1x TBS, after rinsed twice and followed by four washes in 1x TBS with 0.1% Tween-20. After incubated for 2 hours in anti-mouse IgG secondary antibody conjugated with peroxidase (Sigma) diluted 1:50.000 in 3% non-fat dry milk in 1x TBS, thereafter rinsed and washes were repeated as before. Blocking, immunodetection and washes were done under constant agitation at room temperature. Blots were developed using Pierce ECL 2, Western Blotting Substrate kit as recommend by manufacturer, and visualized on a VERSADOC Imaging System (Bio-Rad). The intensity of the HSP70 bands in the blots were semi-quantified by densitometry using the Quantity One software (Bio-Rad v 4.3.1). The density of each band was normalized to the density of the HSP70 band of a commercial standard (Sigma) in each blot (Varó *et al*., 2007), and HSP70 levels were expressed as arbitrary units HSP70/ng protein.

5.7.3 - *Enzimatic assays (AChE and GST)*

In order to analyze these two enzymes, individual paralarvae from 0 day and 14 days were used for each experimental temperature group (n= 24). Each sample was homogenized in cold with the addition of 600µl phosphate buffer (100mM phosphate buffer, pH 7,4 containing 150mM of KCL) and centrifuged at 10.000 g (4ºC) for 15 minutes. The supernatant was collected and separated in different aliquots for the different enzymatic analyses.

5.7.4 - *AChE assay*

AChE activity was determined by the Ellman method (Ellman *et al*., 1961) and adapted to microplate according to Varó *et al*. (2002 and 2015), using acetilthiocholine ATC as substrate. Briefly, 0.2 ml of the reaction solution [30 mL of phosphate buffer (0.1 M, pH = 7.4 containing 150 mM of KCL, 1 mL of 5,50-dithiobis(2-nitrobenzoic acid) (DTNB) 10 mM, and 0.2 mL of acetylthiocholine iodide 75 mM] were added to 0.1 ml of diluted (1/2) sample.

Determinations were performed immediately after putting the reaction solution, following the reaction during 5 minutes at 415 nm ($\varepsilon$= 13.6 $mM^{-1}cm^{-1}$) using a microplate reader TECAN® Ultra Evolution. AChE activity calculated based on Lambert-Beer law as follows below and expressed in nmol/min/mg prot.

$$AChE = \left(\left(\frac{A}{t}\right) \times \left(\frac{1}{\varepsilon \times I}\right) \times \left(\frac{Total\ Vol.Exp.}{Sample\ Vol.}\right) \times DF \times \left(\frac{1}{Prot.total}\right)\right) \times 1000$$

Where:

A/t= Slope (Absorbance/min);

$\varepsilon$= 13.6 mM/cm (DTNB);

*I*= 0.9 (length path);

Total volume experiment= 300 (µl)

Sample volume= 100 (µl)

DF= Dilution factor

Protein total (mg/ml)

The reaction which allows activity determination,

Acetilcoline + $H_2O$ $\xrightarrow{AChE}$ tiocoline + acetate

Butiriltiocoline + $H_2O$ $\xrightarrow{BChE}$ tiocoline + butirate

5,5'-ditio-bis(2-dinitrobenzoic) ⟶ 5-mercapto-2-nitrobenzoic (Yellow color)

5.7.5 - *GST assay*

The activity of GST was determined by the Habig method (Habig *et al*., 1974), adapted to microplate by Solé *et al*. (2015), using 1-chloro-2,4-dinitrobenzene (CDNB)

as substrate. Briefly, 0.2 ml of the reaction solution [19 ml phosphate buffer (100mM, pH = 7,4-7,2), 0,5 ml of GSH 47 mM and 0,5 ml of CDNB 47 mM] were added to 25 µl of homogenate supernatant. Enzymatic activity was determined by monitoring changes in absorbance, using a TECAN® Ultra Evolution microplate reader, every 60 s for 15 min, at a wavelength of 340 nm ($\varepsilon$= 9.6$mM^{-1}cm^{-1}$). GST activity was calculated based on Lambert-Beer law as follows below and expressed in nmol/min/mg prot.

$$\text{GST} = \left(\left(\frac{A}{t}\right) \times \left(\frac{1}{\varepsilon \times I}\right) \times \left(\frac{Total\ \ Vol.Exp.}{Sample\ \ Vol.}\right) \times DF \times \left(\frac{1}{Prot.total}\right)\right) \times 1000$$

Where:

A/t= Slope (Absorbance/min);

$\varepsilon$= 9.6 mM/cm (CDNB);

*I*= 0.8 (length path);

Total volume assay= 225 µl

Sample volume= 25 µl

DF= Dilution factor

Protein total (mg/ml)

5.8 - *Statistical Analyses*

Data analyses were performed using the software SPSS (IBM SPSS Statistics 22 Inc.). To test the effect of temperature and different development stages on growth and metabolism, RNA/DNA, RNA/PROT and DNA/PROT ratios, HSP70 levels, AChE and GST activities, normality and homogeneity of variances were verified by Shapiro-Wilk and Levene's tests, respectively. Then, One-way ANOVAs, with Brown Forsythe test when need, were applied for each biomarker followed by Tukey HSD test (homogenous variances) or Games-Howell test (heterogeneous variances) for post-hoc multiple comparisons. A significance level of $p \leq 0.05$ was used in all statistical tests.

# 6. Results

6.1 - *Survival*

The values of weight, specific growth rate (SGR%), survival and percentage of biomass (BI%) for each experimental temperature (19ºC and 22ºC) are presented in table 6.1. Paralarvae at lower temperatures showed a better development, evident comparing the dry weights (DWi and DWf of each temperature). These two experimental groups showed different SGR % (5.88 *vs* 2.90) respectively. However, differences in survival were inversely proportional per temperature during 14 days. The percentage of biomass (BI%) is related with the specific growth rate and survival. Despite of the high SGR values at 19ºC there was also a low survival (26%) resulting in a higher loss of biomass (-2.91) meaning that the increase of weight did not compensate the mortality. In the other hand, at 22ºC the SGR values were lower but with a higher survival (58%) resulting in higher biomass values (-0.93) than at 19ºC.

Table 6.1 - General parameters of the two experimental incubation temperatures of *O. vulgaris* and reared paralarvae from hatchling to 14 days post hatch (n=300 per temperature) at room temperature (22ºC).

| **Incubation temperatures** | **19ºC** | **22ºC** |
|---|---|---|
| **Initial paralarvea individuals** | 300 | 300 |
| **Age (days)** | 14 | 14 |
| **Initial DW (mg) 0 days paralarvae** | 0.18 ± 0.02 (n=30) | 0.2 ± 0.02 (n=30) |
| **Final DW (mg) 14 days paralarvae** | 0.41 ± 0.06 (n=30) | 0.3 ± 0.08 (n=30) |
| **SGR %** | 5.88 (n=30) | 2.90 (n=30) |
| **Survival (%)** | 26 | 58 |
| **BI%** | -2.91 | -0.93 |

6.2 - *Nucleic acid and protein indices*

The impact of different temperatures during incubation period from eggs at stage XV to paralarvae at 0 and 14 days on growth is shown in Fig. 1. These three ratios RNA/DNA (index of nutritional condition and growth rate), RNA/PROT (cell protein synthesis capacity) and DNA/PROT (protein content per cell) are directly associated to growth. At 19ºC the minimum and maximum values obtained of RNA/DNA ratios were 5.8 at 0 days and 26.5 at 14 days, respectively. At 22ºC the minimum and maximum values were 7.2 at 0 days and 21.1 at 14 days, respectively. Despite of no significant differences between temperatures at the same age (Figure 7), at higher temperatures (22ºC) the embryonic development was higher than at lower temperatures (19ºC) showed by difference of 7 days between hatchlings (first at 22ºC and after at 19ºC).

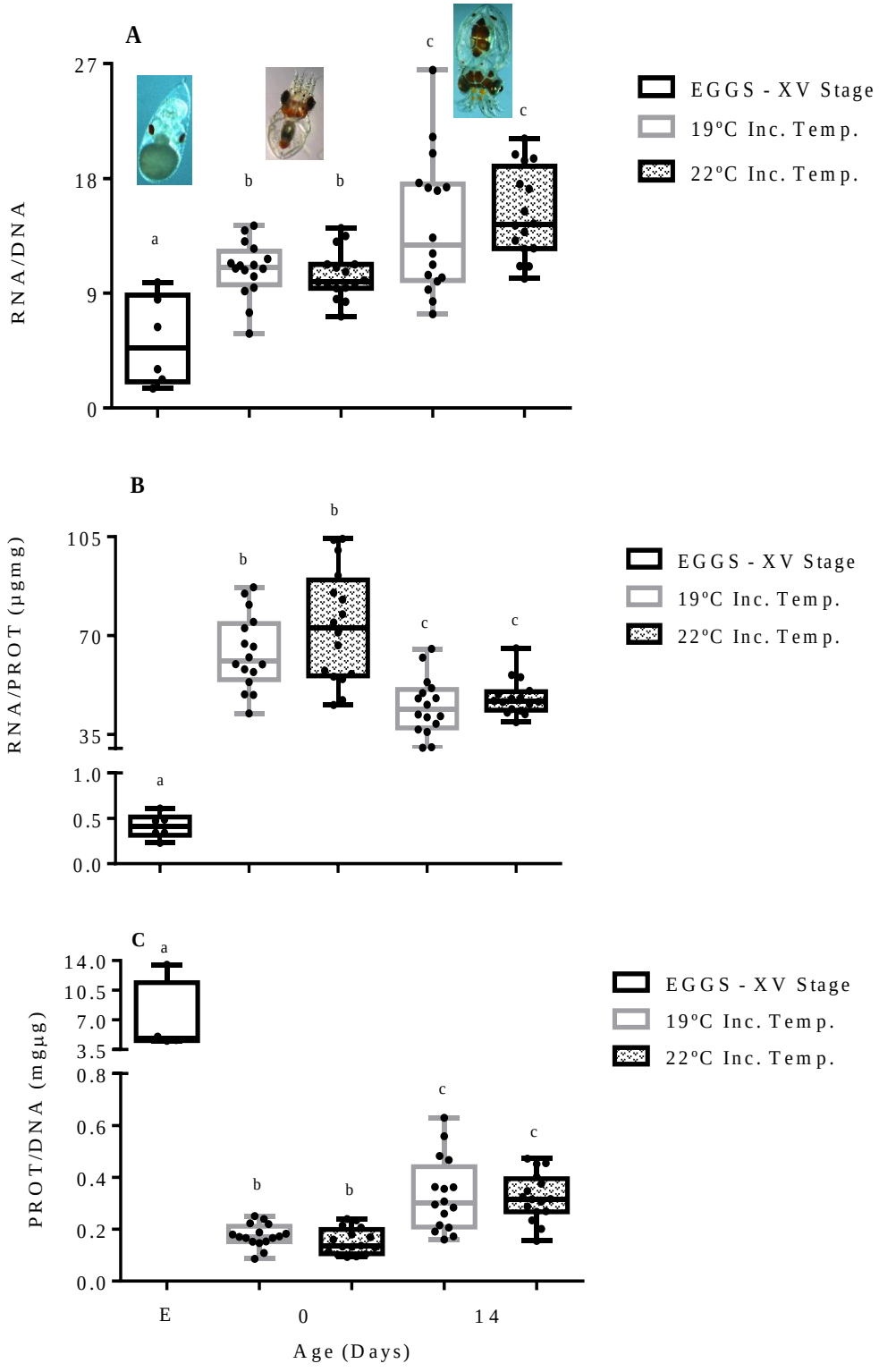


Figure 6.2 – **A**: RNA/DNA, **B**: RNA/PROT, and **C**: PROT/DNA ratios at different temperatures among different ages (embryos at stage XV, 0 and 14 days) of *Octopus vulgaris* paralarvae respectively. Values are represented by each paralarvae (0 and 14 days) and pool of eggs (ww 0.02 – 0.04g). Different letters denote significant differences ($p \leq 0.05$) between ages and temperatures.

Regarding RNA/DNA and PROT/DNA ratios, these ratios were similar in both groups and showed a significant increase between 0 and 14 days post-hatch, regardless of the incubation temperature. (Fig 6.2 A and C). The opposite occurred in relation of RNA/PROT ratios (Fig 6.2 B).

6.3 - *HSP 70*

Thermal stress was analyzed and the levels of HSP70 were evaluated as represented below in Figure 6.4. HSP70 expression was similar at newly hatched paralarvae in both groups, increasing from 0 to 7 days post-hatch, but the increase in 22ºC group was significantly higher than the value observed in the 19ºC group (12.49 *vs* 4.96 AU/ng prot respectively). However, at 14 days post-hatch the expression level in 22ºC group was reduced, reaching similar values to the 19ºC group (3.58 and 4.57 AU/ng prot) (Figure 6.4).

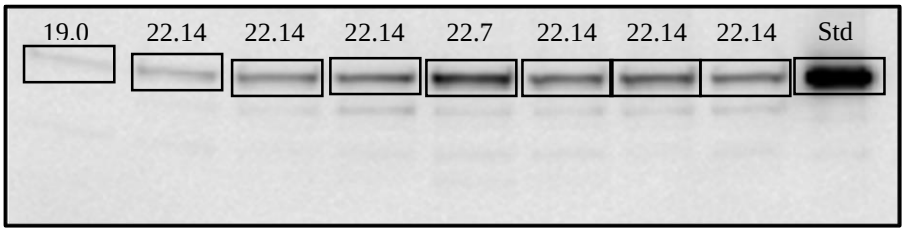


Figure 6.3 - Western blot analysis of O. vulgaris paralarvae. Std (HSP70), standard used to compare different blots (19.0 – 19ºC (0 days); 22.14 – 22ºC (14 days) and 22.7 – 22ºC (7 days)).

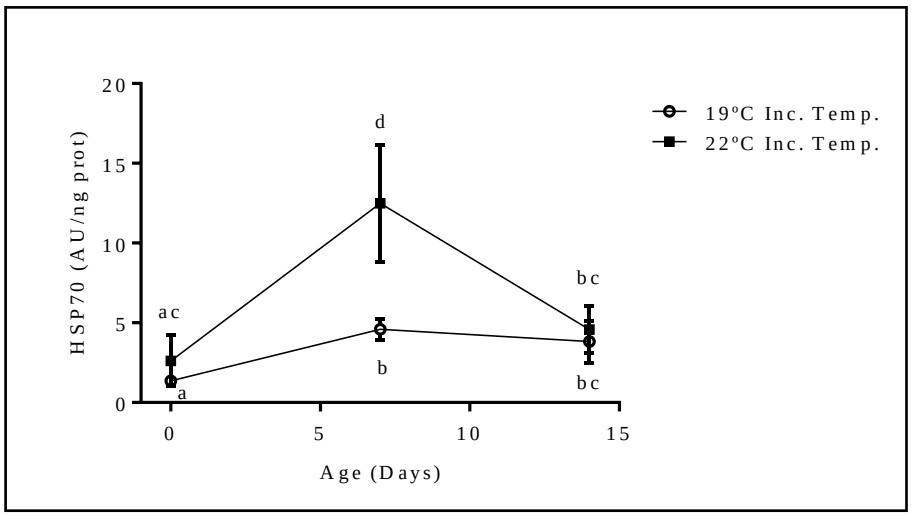


Figure 6.4 – Heat shock response (HSP70; HSP70 AU/ng protein) of the two experimental groups (19ºC and 22ºC incubation temperatures) at 0, 7 and 14 days. Values are means ± SD. Different letters represent significant differences ($p \leq 0.05$) between groups of ages and temperatures.

6.4 - *AChE and GST*

The higher value of AChE was reported in newly hatched paralarvae incubated at 22ºC (50.4 nmol/min/mg prot), but there were no significant differences among groups, probably due to the high variability obtained in AChE activity of paralarvae. In general terms, these analyses showed non-significant tendencies to higher levels of AChE in 22ºC group respect to 19ºC group as well as lower levels in 14 days post-hatch respect to newly hatched paralarvae (Figure 6.5 A).

GST activity showed a tendency to be higher in 19ºC group respect to 22ºC, but again the high variability obtained hindered significant differences between both groups. Only significant differences were found between the group of paralarvae with 14 days post-hatch at 19ºC and newly hatched paralarvae at 22ºC (32.26 *vs* 31.69 nmol/min/mg prot respectively) (Figure 6.5 B).

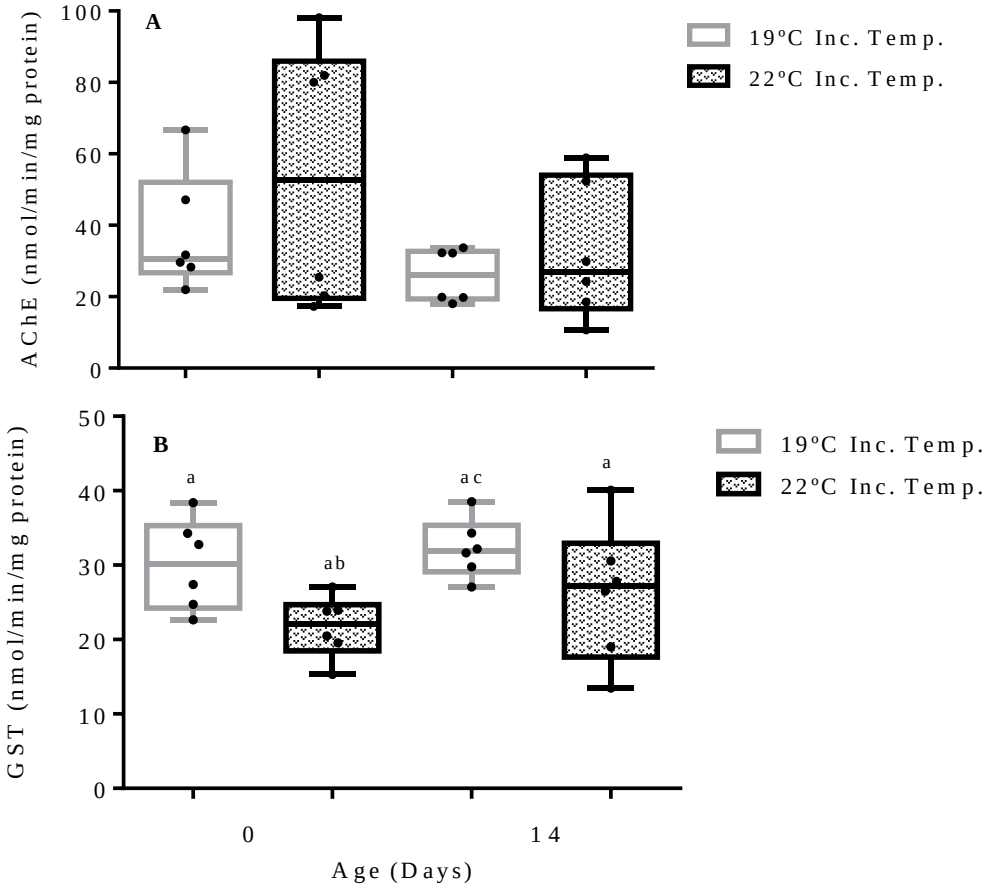


Figure 6.5 – **A**: Acetylcholinesterase (AChE; AChE/nmol/min/mg protein); **B**: Glutathione S-transferase (GST; GST/ nmol/min/mg protein) activity in octopus paralarvae with 0 and 14 days at 19ºC and 22ºC incubation temperatures (each point means an individual). Different letters denote statistical differences ($p \leq 0.05$) after one-way ANOVA and Tukey HSD post-hoc test.

# 7. Discussion

This study is the first that investigate the effect of temperature on egg incubation from a single female brood. Paralarvae viability was evaluated throughout classical biomarkers that are generally used in environmental quality assessment (Tim-Tim *et al*., 2009).

Temperature, as an abiotic factor plays an important role on the physiological process as in the ontogeny, especially during egg incubation and first life stages (Repolho *et al*. 2014). It is known that in cephalopods exists an inverse association between embryonic development time and temperature (Naef, 1928; Mangold and Boletsky, 1973; Villanueva *et al*., 1995 and Caverivière, 1999).

As expected, results obtained in the present study showed that temperature had a marked effect on *O. vulgaris* embryonic development. It was noted that embryos incubated at the lowest temperature 19ºC took one week more to hatch then eggs incubated at 22ºC. As observed by Repolho *et al*. (2014), in this study it was also observed that at 22ºC some percentage of hatched paralarvae was premature presenting furthermore an external yolk-sac (Figure 3 B). This phenomenon of reduction time of embryonic development was also described in this species by others authors as Mangold and Boletzky (1973), Caveriviére (1999) and Repolho *et al*. (2014). In this case, the differences between both incubations were not so prominent than differences described by these authors once the eggs were incubated from stage XV of embryonic development (end of organogenesis). Uriarte *et al*. (2012) observed in *O. mimus eggs* incubated at 14ºC and 18ºC that until stage XV, differences between temperature and embryonic development were bigger, as occurred in the present study in *O. vulgaris eggs*. Buckley *et al*. (1984) found effects of temperature in adding temperature as a second independent variable to the experiments with fish larvae of *Ammodytes americanus* implementing Buckley´s (1984) model, where temperature occupies an important place.

## 7.1 - *Growth and Survival*

Hamazaki *et al*. (1991) reported that weight of hatchlings is different between egg masses. On the contrary, in this study it was verified no differences in dry weights at hatchlings between different temperatures (0.18 ± 0.02 at 19ºC *vs* 0.2 ± 0.02 at 22ºC), in agreement with those recorded by Reis (2011) in two different experiments (0.25 ± 0.01

mg at 21.44 ± 0.27ºC and 0.17 ± 0.02 mg at 22.42 ± 0.26ºC). These values were lower comparing with other authors that had already reported 0.5 mg for octopus hatchlings from eggs incubated at 15.2ºC and between 13ºC and 19.5ºC (Navarro and Villanueva, 2000, Iglesias *et al*., 2000), respectively. At the end of the experiment (14 days), paralarvae incubated at 19ºC were heavier that paralarvae incubated at 22ºC (0.41 g *vs* 0.30 g, respectively). Paralarvae incubated at 19ºC appears to be physically more prepared to adverse conditions. Despite of a low survival (26%) it was verified a high SGR (5.88%). Vidal *et al*. (2002) shown in a squid species (*Loligo opalescens*) that incubation temperatures influences survival and growth rates, wherein incubation at lower temperatures would benefits hatchlings (largest and heaviest). Therefore these evidences could be associated with food availability, under conditions of insufficient food, variability would be reduced occurring the natural selection of the stronger and faster growing octopus paralarvae the "Superparalarvae". Vidal *et al*. (2006) also found this kind of event in a squid species (*Loligo opalescens*).

RNA/DNA ratio appears to be a sensitive reliable index of nutritional condition, food availability and growth rate in aquatic organisms that are associated to temperature variation (Buckley1979, 1980; Melzner *et al*., 2005; Vidal *et al*., 2006). In this study, only egg incubation temperature was different from stage XV to hatchling during the experiment. The amount of feed provided for paralarvae was the same for all tanks. It was verified that RNA/DNA ratio tend to increase with growth (develop). Although, Goolish *et al.* (1984) hypothesized in carp that at lower temperatures RNA content increased resulting as a compensatory mechanism to reduce levels of RNA activity. Later Foster *et al*. (1992) and Mathers *et al.* (1994) confirmed this hypothesis working on juvenile cod and larvae of herring. Finally, Buckley *et al.* (1990) studied the effect of temperature on embryonic and larval development of winter flounder (*Pseudopleuronectes americanus*) larvae at hatching and found that RNA content at hatching was inversely related to incubation temperature, which reflects probably a quite similar process.

However, it was difficult to understand a growth pattern in paralarvae because of the high dispersion of the results obtained in the ratios, even the "pools" of eggs presented a wide dispersion. Results showed no significant differences between temperatures at the same age due probably to the huge variability at individual level. According to Moltschaniwskyj (1994) one of the major fact that could possibly cause this variability in RNA/DNA ratios is the capacity of cephalopods to generate new muscle

cells throughout their lifecycle. Vidal *et al*. (2006) have shown in cephalopods paralarvae that there was a high variability related to RNA/DNA ratios in squid. The presence of food makes that exists a trend for a higher variability, conceding the possibility to survive of individuals with different growth rates. Regarding problems/limitations that can occur in similar methods. Buckley (1984) said that the weight of the sample could be a limiting factor to do this experiment. Although, the protocols were adapted using only one paralarvae and despite of no significant differences the results were completely reliable. Melzner *et al*. (2005) also found that depending on detergents concentrations used in extraction buffers and comparing different RNA/DNA assays in others cephalopod species that can produce in RNA/DNA values higher relative variances.

Respecting to results of RNA/PROT ratio (a measure of cell protein synthesis capacity), it was observed that paralarvae at 0 days presented a higher dispersion than paralarvae at 14 days. These results are related with RNA/DNA ratio, when RNA/DNA ratio was higher, RNA/PROT was lower and *vice-versa*. The high values of RNA/PROT observed at day 0 for both temperatures could reflect the later growth found at day 14. Meaning that after a great quantity of protein synthesis is followed by growth, as it was observed in *Mytilus edulis* (Hawkins, 1985). Houlihan *et al*. (1990) showed that high growth rates achieved by *O. vulgaris* species are not only related to high rates of protein synthesis but are consequently related with protein degradation. An increased of protein synthesis accompanied by particularly low rates of protein degradation and high efficiencies of retention of synthesized protein is a pattern of increasing growth commonly in animals.

It was expected that low temperatures had a higher protein synthesis, since organisms acclimated to low temperatures exhibit greater enzyme activity to compensate for lower reaction rates (Solé *et al*., 2015). Although, it was not verified any significant differences in RNA/PROT ratios between temperatures at the same age.

As expected, with more energetic reserves *O. vulagris* eggs showed higher amounts of protein content per cell according to PROT/DNA ratio (Roark *et al*., 2009).

In fish, PROT/DNA ratio seems to be related with temperature (Caldarone, 2005) and appears to be correlated with SGR (Carter et al., 1998). In this study, no significant differences were found between different temperatures at the same age and also any correlation between PROT/DNA ratio and SGR.

Thus, common standard method is needed in order to do better comparisons between research groups (Caldarone et al., 2001). Fluorescence sensitivity assays with a microplate format enables a rapid, quantitative readout and suitable analysis. In a microplate well is possible to input a huge number of samples and the fluorescent signal is generated within whole cells, analyzed by measuring fluorescence intensity from the well without the need for cellular imaging.

### 7.2 - *HSP70 – Thermal Stress*

Results of HSP70 levels showed significant differences between temperatures at the same ages.

According to what was expected, it was observed significant differences between recently hatched paralarvae at different temperatures and no significant differences in HSP70 levels between temperatures at 14 days, probably favorited by acclimatization period, since paralarvae were exposed to the same temperature (22ºC) as long as they hatched. Logan and Somero (2011), described in a fish species that acclimatization can occur during days.

Considering the results of Repolho *et al*., (2014), in this study it was used the eggs of only one female to analyze HSP70 and also RNA/DNA from an advance stage (XV) to a hatched paralarvae and at different temperatures (19ºC and 22ºC).

As unexpected, results of this heat shock response were very interesting showing an expression of HSP70 in paralarvae incubated and maintained at room temperature (22ºC) at 7$^{th}$ day followed by a huge drop until day 14$^{th}$. Pörtner (2002) found that HSP production responds preferably to short-term temperatures variations. Nonetheless, thermal stress could be induced also at high temperatures after long term exposures considering alterations in HSP levels found by Feidantsis *et al*. (2009) in *Sparus aurata* muscle tissue. The expression of HSP70 observed at this point was difficult to explain since these paralarvae were acclimatized at 22ºC and considering that were always incubated and maintained at this temperature during the experiment.

As a hypothesis to explain this event, that could be associated to zooplankton physical and behavioral characteristics. In the wild, paralarvae are planktonic, almost invisible and living in the water column in the early stages. Such as meroplankton (0.2mm – 2cm), *O. vulgaris* paralarvae do vertical migrations and that could be correlated with temperatures. In shallow waters, a temperature seems to be colder than upon to the

surface. In nature, *O. vulgaris* in planktonic phase were found in open sea over a depth of several hundred meters (Mangold, 1983). Takeda (1990) also observed that juveniles are nearly absent from the sea surface during daytime and present from the seafloor to the surface at night, with higher abundances of hatchlings found near the surface at night. This species at hatching present only 65 to 80 chromatophores (Mangold, 1983), ineffective to protect them from the sun light at water surface. It can be observed (Figure 7.1) that 14 days paralarvae did not present the body coated with chromatophores and the musculature are still very transparent.

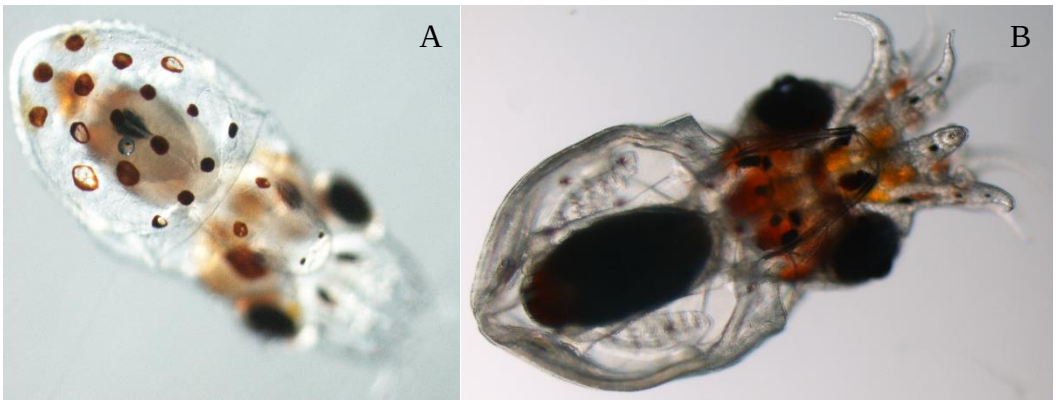


Figure 7.1 - (A) Paralarvae with 7 days; and (B) paralarvae with 14 days from eggs incubated at 22ºC. Photographs by the author

It was observed variations of HSP70 expression, represented by significant differences at 0, 7 and 14 days at 19ºC. Comparing with 22ºC, it was also found at 19ºC a peak of HSP70 expression at 7$^{th}$ day followed by a small drop until day 14$^{th}$, suggesting an acclimation of *O. vulgaris* paralarvae to room temperature.

## 7.3 - *Neuronal activity (AChE)*

The results showed a presence of a wide range of distribution values and no significant differences between ages and temperatures were registered in terms of AChE activity. Temperatures seemed to not directly affect AChE activity. It was noted different expressions of AChE in other studies. Durieux *et al*. (2011) and Solé *et al*. (2015) found a positive expression in juveniles of striped seabass *Morone saxatilis* and *Solea senegalensis*, respectively. In the other hand, Botté *et al*. (2013) verified a negative expression in a tropical reef fish, *Acanthochromis polyacanthus*, and in others, as *(LepomisMacrochirus)* the expression was not significant (Beauvais *et al*. 2002).

Beauvais *et al*. (2002) concluded that water temperature had no relation on AChE activity as it was evident in this study. However, it was shown that paralarvae at hatchlings had activity of this enzyme and that could be a useful tool for future works.

### 7.4 - *Antioxidant defences (GST)*

Considering the results of GST activity it was observed that hatchlings presented no significant differences between temperatures and the same occurred at 14 days old paralarvae, possibly due to the individual diffusion. In the other hand, it was noted a significant difference in GST activity between hatchling paralarvae at 22ºC and paralarvae with 14 days at 19ºC. Tang *et al*. (1994) analyzed GST in *O. vulgaris* digestive gland and one of the conclusions was that proteins concentration and temperature could help in GST characterization.

As expected, GST activity of paralarvae incubated at 19ºC was higher than paralarvae incubated at 22ºC. Comparing to Repolho *et al*. (2014) at 21ºC hatchling paralarvae expressed higher values than at 19ºC. The increase in GST may be a strategy to prepare for oxidative stress in an effort to protect tissues against oxidative damage. The activity of this enzyme appears to respond to variations in the concentration of oxygen in the environment (Oliveira, 2005). In spite of the attempt to maintain equal conditions among the tanks, differences in oxygen levels may have happened explaining different expression of antioxidant defenses.

# 8. Conclusions

In the present study, was verified that it is possible to analyse different biomarkers at individual level in recently hatched paralarvae due to high protein content. The incubation of the eggs from stage XV of development (final organogenesis) to the final stage (XX) at different temperatures (19ºC and 22ºC) seemed to not affect embryonic development as well as the paralarval viability. However, HSP70 expression at 7 post-hatch paralarvae was the only biomarker significantly affected by temperature. In addition, RNA/DNA ratios and HSP70 expression seemed to be also affected by the age of the paralarvae. The high variability obtained in GST and AChE determinations hampered potentials significant differences among treatments. In consequence further studies are necessary to clarify the influence of the incubation temperature in these biomarkers. The collection of more samples in future works would allow to draw more conclusions and to clarify some of the raised hypothesis.

Finally, results point out RNA/DNA ratio, HSP70 and GST as sensitive biomarkers for growth, thermal stress and antioxidant defences in paralarvae. However, growth and temperature did not alter the neurotransmission system of *O. vulgaris* paralarvae.

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