
# *New insights about rearing conditions of* Homarus gammarus *larvae*

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## Resumo:

O lavagante europeu, *Homarus gammarus*, é considerado um dos recursos pesqueiros mais importante na Europa, bastante apreciado e com alto valor económico. No entanto, no último século as suas capturas tem vindo a diminuir drasticamente. Diversos programas de repovoamento têm vindo a ser implementados a nível mundial para recuperar os stocks, mas o seu desenvolvimento larvar é bastante exigente, dificultando o seu cultivo em cativeiro.

O presente estudo teve como objetivo contribuir para o aumento do conhecimento sobre o desenvolvimento e crescimento larvar de *H. gammarus*, por forma a otimizar protocolos de cultivo em condições de cativeiro. Para tal, foram realizadas três ensaios distintos e complementares, nomeadamente: (1) avaliação do efeito de diferentes dietas liofilizadas e alimento vivo, (2) avaliação do efeito da adição de probiótico no cultivo larvar e, por fim, (3) definição da relação entre o efeito da temperatura e o desenvolvimento larvar.

Os efeitos dos fatores em análise nos diferentes ensaios foram estimados ao nível da taxa de sobrevivência, taxa de crescimento e período intermuda, tendo por base as respostas individuais das larvas. Cada indivíduo foi monitorizado a cada 12h até ao estágio de pós-larva (PL) ou até ao momento da sua morte.

O perfil de ácidos gordos mostrou que as dietas copépodes (COP), copépodes liofilizados (DC) e zooplâncton selvagem liofilizado (DWZ) apresentam elevadas quantidades em ácido palmítico, DHA e EPA, ácidos gordos essenciais, comparativamente com as dietas náuplios de artémia vivos (LAN) e náuplios de artémia liofilizados (DAN). Paralelamente, estas dietas mostraram um perfil bastante semelhante ao das larvas Zoea I, demonstrando ser, novamente, os alimentos mais adequados para o cultivo larvar de *H. gammarus*.

O ensaio onde se avalia o efeito da dieta, mostrou que o alimento liofilizado, nas condições em que foi oferecido, não é adequado para o cultivo larvar de *H. gammarus*, traduzindo-se 100% de mortalidade antes de atingirem Zoea II, contrastando com os resultados obtidos com náuplios de artémia vivos, com 4,17% de sobrevivência até ao estágio PL. A adição de probiótico na água de cultivo, de acordo com a metodologia aplicada, mostrou-se igualmente ineficiente, não havendo

diferenças nas taxas de sobrevivência, período intermuda e taxas de crescimento comparativamente com as larvas cultivadas sem adição do probiótico.

As taxas sobrevivência larvar no ensaio onde se avalia o efeito da temperatura mostrou que esta decresce com o desenvolvimento larvar, excetuando para a temperatura de 23ºC, demonstrando mais variabilidade. Seria espectável que as taxas de sobrevivência acompanhassem o aumento da temperatura, no entanto, isso não se verificou, resultando em taxas de sobrevivência mais elevadas a 19ºC. Relativamente às taxas de crescimento, os resultados mostraram que estas decrescem com o desenvolvimento larvar para todas as temperaturas testadas, e que o aumento da temperatura não se traduziu num aumento das taxas de crescimento.

O aumento da temperatura de cultivo traduziu-se na diminuição do período intermuda em todas as fases de desenvolvimento larvar testadas e para todas as temperaturas, variando de 4.77 (Z1) a 16.5 dias (Z3) a 16ºC, paralelamente, a 23ºC, variou entre 3.02 (Z1) e 9.75 dias (Z3). Os resultados obtidos, são uma indicação extremamente útil para otimização futura de protocolos relativos ao desenvolvimento larvar de *H. gammarus*.

**Palavras-chave:** *Homarus gammarus*, condições de cultivo larvar, alimento vivo e seco, probiótico, efeito da temperatura.

## Abstract:

The European lobster, *Homarus gammarus*, is considered one of the most important fishery resources in Europe, with high economic value and very appreciated seafood. However, in the last century their catch has been decreasing dramatically. Several restocking programs have been developed, but their larval development is quite demanding, hindering its cultivation.

The present study aimed to contribute to increased knowledge about the larval development and growth of *H. gammarus* in order to optimize the protocols of captive cultivation. To fulfill these objectives, three complementary and distinct tests were performed, namely: (1) evaluate the effect of different lyophilized diets and live food, (2) evaluate the probiotic effect added to larval rearing and, finally, (3) define the relationship between the temperature effect and the larval development.

The effects of the different factors analyzed in experiments were estimated by survival rates, specific growth rates and intermoult period, based on the individual responses of larvae. Each subject was monitored every 12 hours until the post-larvae (PL) stage or until its death.

The fatty acid profile showed that the copepods (COP), dry copepods (DC) and dry wild zooplankton (DWZ) diets had high amounts of palmitic acid, DHA and EPA, essential fatty acids, compared with the live artemia nauplii (LAN) and dry artemia nauplii (DAN) diets. Simultaneously, these diets and larvae Zoea I showed similar fatty acid profile, showing once again, that they are the most suitable food for larval rearing of *H. gammarus*.

The diet effect experiment, showed that the dry food, for this type of larval rearing, it is not suitable for *H. gammarus* larvae, resulting in 100% of mortality before reaching Zoea II, contrasting with the obtained results using live brine shrimp nauplii, with 4.17% survival to PL stage. The addition of probiotics in seawater, proved to be ineffective, with no differences in survival and growth rates, and intermoult period, compared to larvae grown without addition of probiotic in rearing water.

In the temperature effect experiment, the larval survival rates decreased with larval development, except at 23°C, demonstrating wide variability. It would be expected that survival rates increased with temperature rising. However, this was not

observed in our experiment. Higher survival rates were obtained at the intermediate temperature, i.e. 19°C. With regard to growth rates, the results showed that they decrease with larval development for all tested temperatures, and the temperature increasing did not increased the growth rates.

The seawater rising temperature resulted in a decrease of intermoult period in all larval development stages and at all tested temperatures, ranging from 4.77 (Z1) to 16.5 days (Z3) at 16°C, whereas at 23°C, ranged from 3:02 (Z1) and 9.75 days (Z3). The results obtained are an extremely useful guide for future optimization of protocols on larval development of *H. gammarus*.

**Key-words:** *Homarus gammarus*, larval rearing conditions, live and dry diets, probiotic, temperature effect.

## Table of contents

## List of figures

## List of tables

## Symbols and abbreviations list

**ARA –** Arachidonic acid

**CL –** Cephalothorax Length

**COP -** Copepods

**DAN –** Dry Artemia Nauplii

**DC –** Dry Copepods

**DHA –** Docosahexaenoic Acid

**EPA –** Eicosapentaenoic Acid

**F/2** – Guillard medium

**GR –** Growth Rates

**HUFA's –** High Unsaturated Fatty Acids

**IP –** Intermoult Period

**LAN –** Live Artemia Nauplii

**LDT –** Larval Development Time

**MT –** Metric Tons

**PL –** Post-larvae

**PUFA –** Polyunsaturated Fatty Acids

**SGR –** Specific Growth Rates

**SR –** Survival Rates

# 1. Introduction

## 1.1. European Lobster – Biology and Ecology

Lobsters belong to a very large class of invertebrate animals, referred as crustaceans (Beard & McGregor 2004). The distribution of European lobster, *Homarus gammarus*, is quite wide, it occurs along the eastern European coast from the southwest of Sweden and Denmark to the Atlantic coast of Morocco. The species also extends though less abundantly throughout the coastline of the Mediterranean Sea, and has been reported from the westernmost end of the Black Sea in the straits of Bosporus (Beard et al. 1985; Prodöhl et al. 2007).

European lobster, *Homarus gammarus*, considered one of the most important clawed lobster, are usually located at the sub-littoral zone up to 150m deep (Holthuis 1991). It is typically found on rocky substrates with places where they can refuge from predators or threats (Kristiansen et al. 2004). They are primarily nocturnal animals which feed on shellfish such as mussels, crustaceans, such as hermit crabs and other species, and polychaetes (Beard & McGregor 2004; Kristiansen et al. 2004).

Like all decapod crustaceans, their growth is characterized by the occurrence of exoskeleton moulting, reaching marketable size in the wild after 26-30 moults, corresponding to 5-7 years (Beard & McGregor 2004). The growth rates, which are strongly dependent on water temperature, decrease in frequency during the later development stages (Beard & McGregor 2004).

In most of the regions sexual maturation of females, does not occur before 5 to 8 years of age, although like many other lobster species, this is largely dependent on the water temperature (Prodöhl et al. 2007; Browne et al. 2009). Spawning occurs from March to September, with two evident peaks, the first between March and April, and the second from August to September (Beard & Wickins 1992; Browne et al. 2009). The reproduction usually takes place during the summer and is related with the moulting cycle of adult individuals (Browne et al. 2009). After extrusion, the eggs are laid on the female pleopods and remain there between approximately 10 to 12 months, until hatching the following summer (Browne et al. 2009).

Remarkable morphological structures of *H. gammarus* are the two big claws on the front of the body, which in turn are morphological different (Fig. 1). The smallest is

elongated and has sharper edge, called cutting claw, being used to fragment and hold prey.

The largest claw, called crushing claw, features more rounded edges and wide. The position of claws is very variable, being observed in the left or right side of the animals.

The claws lost during fights, for example, can grow again but several moults are required to be proportional to the animal's body (Holthuis 1991; Beard & McGregor 2004).

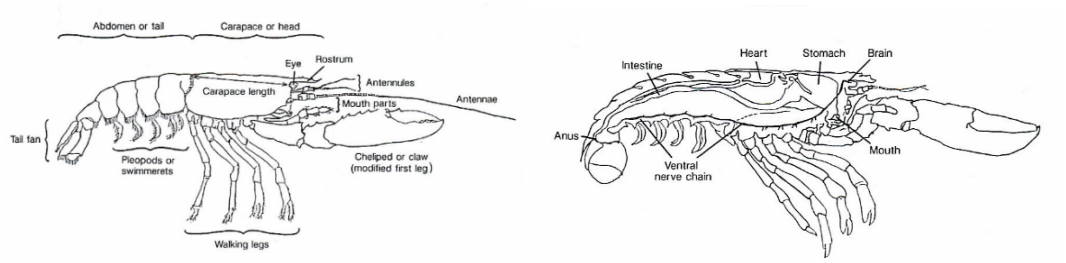


Figure 1 – The external (left) and internal (right) anatomy of a mature *H. gammarus* (adapted from The National Lobster Hatchery).

Females and males can be distinguished by examining the first pair of pleopods situated beneath the tail, immediately behind the legs (Fig. 2). In females pleopods arise separate, the males are united, forming a sturdy rod (Beard & McGregor 2004).

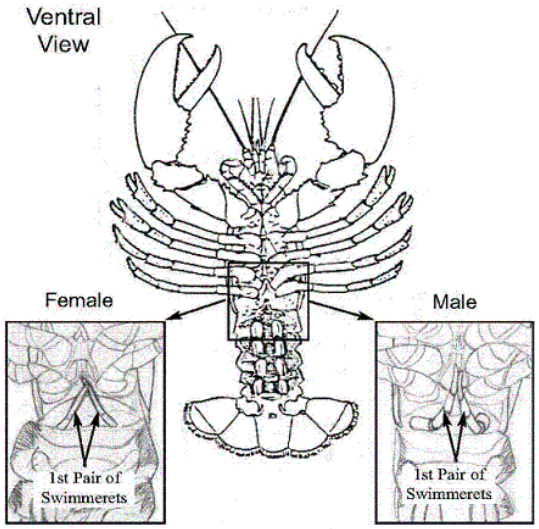


Figure 2 – Difference between a female and a male of clawed lobster (adapted from Maine Department of Marine Resources, 2013).

Females reach sexual maturity at 5-8 years of age, and when they reach a cephalothorax length (CL) between 80 and 85 mm (Holthuis 1991). Usually, males reach sexual maturity slightly smaller. Mating usually occurs between a male with hard exoskeleton and female that recently moulted, with soft exoskeleton, releasing a hormone preventing being eated by male (Beard & McGregor 2004; Skog 2008).

During the period that the eggs are in pleopods (Fig. 1 and 3B) progenitor gently agitate these structures, increasing egg oxygenation and proceeding to its cleaning (Uglem et al. 2006). The color of eggs gradually changes, from a deep green to a black, through a red color and finally transduced blue (Fig. 3A), when the embryo development is complete and yolk reserves depleted (Beard & McGregor 2004).

Figure 3 – Digital photography from eggs (A) close to hatch and female (B) with eggs in the pleopods, from *H. gammarus*.

Spawning occurs during several nights, between 3 and 5, while females carapace slant down and elevate the abdomen, stirring quickly the swimmers and releasing the larvae into the water column (Beard & McGregor 2004; Browne et al. 2009).

The larvae are planktonic, feeding on zooplankton. The planktonic phase lasts from 10 to 15 days in controlled environmental conditions (captive rearing) and 15 to 35 days in the wild. The development time is highly dependent on temperature (Browne et al. 2009).

The larvae undergo three development stages until metamorphosis to stage IV (post-larvae or decapodite), when they settles to the bottom surfaces (see detailed description below). The juvenile lobsters, which now assume miniature adults, sink and seek hiding places. Despite significant and widespread investigations (Mercer et al. 2001) little is known of the habits of these animals at this stage of development, given that they are rarely found or observed in the natural habitat (Agnalt et al. 2009).

## 1.2. Larval stages of *Homarus gammarus*

### 1.2.1. Zoea I

The individual hatch from the egg is designated as the first larval stage or Zoea I (Fig. 4A and B). At this stage, the larvae respond positively to light (positive phototaxia) and are exclusively planktonic. Shortly after its hatching, they move to the water surface and have a highly variable coloration during the first 24 hours. The red and yellow

coloration is produced in chromatophores, which are distributed in defined regions of the body. At this stage, the larvae are segmented and their cuticle is transparent, with internal organs very visible, after 24 hours this cuticle darkens to a dark color (blue / black), becoming opaque (Browne et al. 2009).

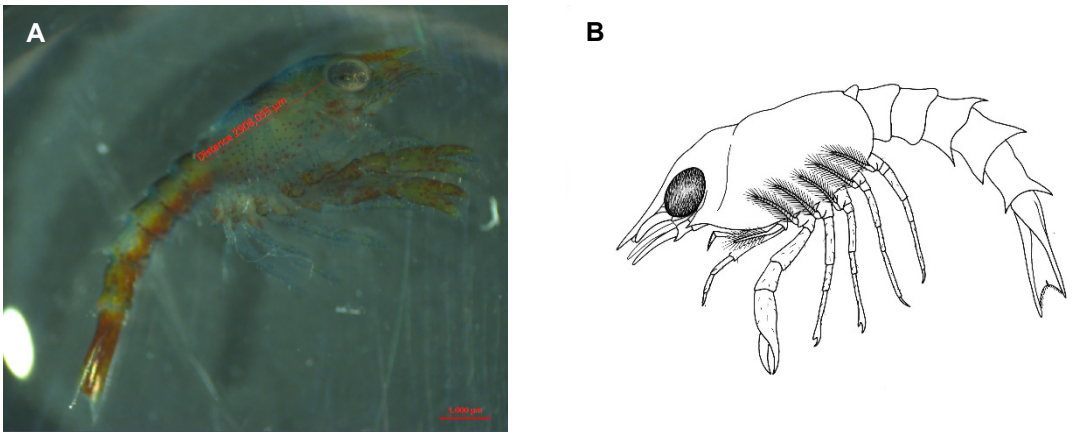


Figure 4 - Stereo microscope digital photography (A) and illustration (B) of the first larval stage, Zoea I, of *Homarus gammarus*.

### 1.2.2. Zoea II

The larvae at this stage show a behavior and coloration very similar to the previous stage (Fig. 5A and B). However, there is a greater development of the first pair of legs and pleopods, becoming forked. The uropods at the end of the telson are not divided (Browne et al. 2009).

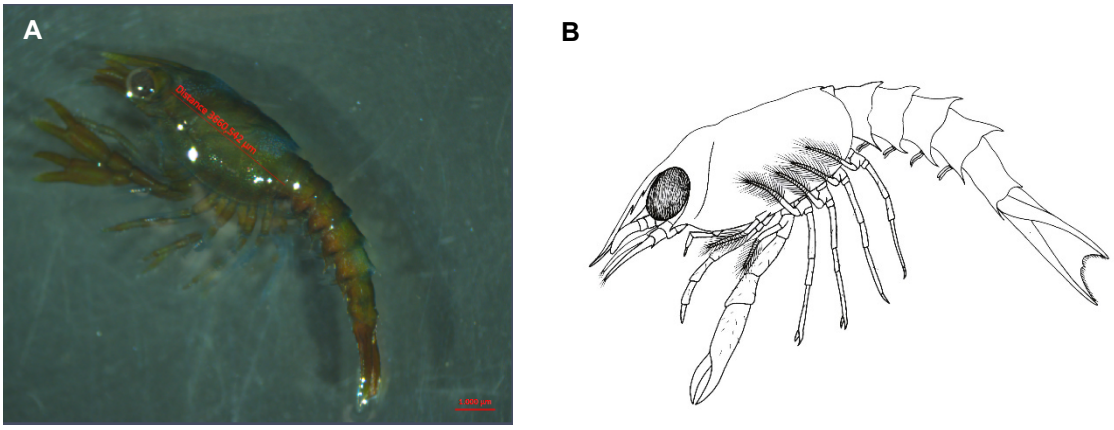


Figure 5 - Stereo microscope digital photography (A) and illustration (B) of the second larval stage, Zoea II, of *Homarus gammarus*.

### 1.2.3. Zoea III

At this stage of larval development, pleopods and claws present a larger size compared to the previous stage (Fig. 6A and B). At Zoea III, the larvae in the absence of upwelling currents in tanks have a strong tendency to sink to the bottom. It is also possible to observe the separation of the first pair of uropods (Browne et al. 2009).

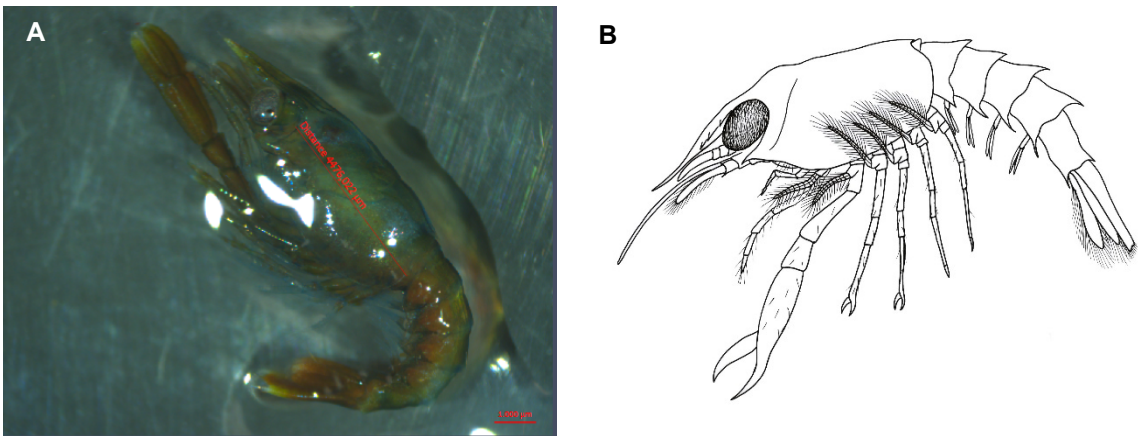


Figure 6 - Stereo microscope digital photography (A) and illustration (B) of the third larval stage, Zoea III, of *Homarus gammarus*.

### 1.2.4. Post-larvae (PL) or decapodite

This stage is referred to post-larval or as decapodite (Fig. 7A and B). The individual morphology differs greatly from the previous stages, which is very similar to the adults. At this stage, the organisms are very active swimmers, showing a well-defined swimming behavior aided by pleopods. The pereiopods are used for locomotion in bottom surfaces. In the first 4 to 5 days after settling, they are strongly planktonic and progressively change to benthic behavior (Browne et al. 2009).

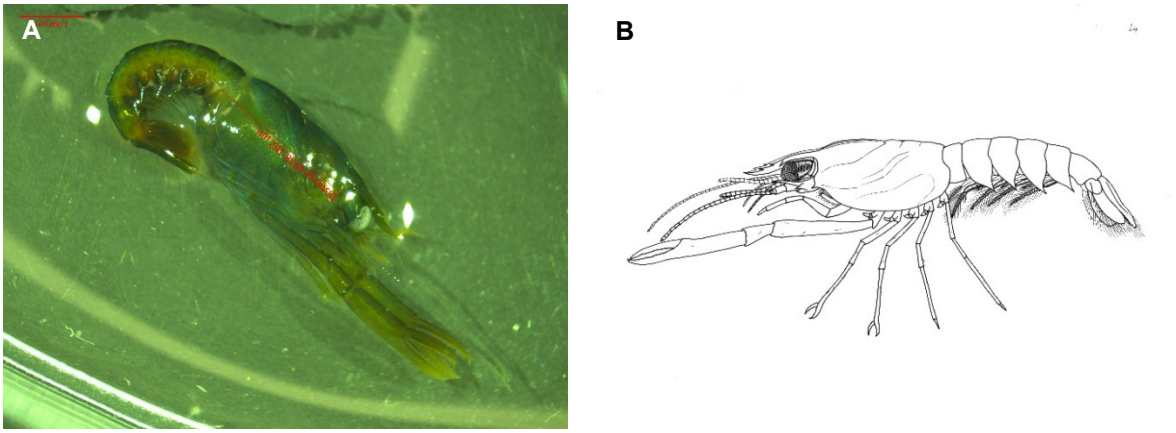


Figure 7 - Stereo microscope digital photography (A) and illustartion (B) of the first juvenile stage, post-larvae, of *Homarus gammarus*.

## 1.3. Economic importance of European lobster, *Homarus gammarus*

The European clawed lobster, *Homarus gammarus*, is a very appreciated shellfish all over the world, with high market value, three times the price of its American counterpart (Barrento et al. 2009), making this seafood one of the most important resource in European fisheries (Lizárraga-Cubedo et al. 2003; Uglem et al. 2006; Agnalt et al. 2009; Barrento et al. 2009; Schmalenbach et al. 2009; Benavente et al. 2010 and references therein).

In 2011, the annual European landings of clawed lobster was 5,169 MT (Metric Tons) of live weight, corresponding to 59.4 million euros The United Kingdom were the

major producers with 3,195 MT, followed by France with 774 MT and Ireland with 756 MT (Fishery Statistic, 2013).

During the last century there has been a dramatic catches decline and reduction of total landings in Europe, with pronounced depletions between 1960 and 1980, showing evident signs over-exploitation of stock (Kristiansen et al. 2004; Rosa et al. 2005; Uglem et al. 2006; Agnalt et al. 2009; Schmalenbach et al. 2009; Benavente et al. 2010).

Along this period, the landings in Norway declined more than 90% to only around 50 MT per year (Kristiansen et al. 2004; Agnalt et al. 2009), and, on the German coast, landings decreased from 80,000 animals per year to a few hundred (Schmalenbach et al. 2009). However, between 2007 and 2011, European landings showed signs of growth, with higher landings than the previous year (Fishery Statistic 2013).

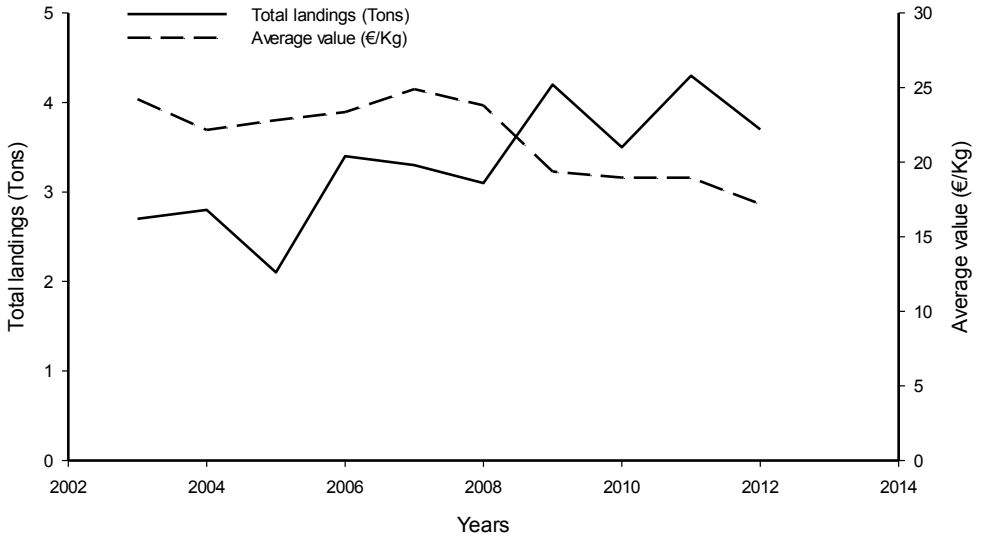


Figure 8 – Variations of total landings, in tons, of clawed lobster in Portugal between 2003 and 2012 and the average value (€) per kilogram (Source: Direcção-Geral das Pescas e Aquicultura).

In Portugal, in the last ten years, European lobsters landings have slightly increased, while its price have decreased, as shown in figure 8.

### 1.4. Captive larval rearing of *Homarus gammarus* – state of the art

With the evident decline of European lobster stocks throughout Europe proven by an overall reduction in total landings in the last century (Contarini et al. 2008; Benavente et al. 2010), became preponderant to adopt appropriate management mechanisms and socks protection (Nicosia & Lavalli 1999; Browne et al. 2009).

These pressures on lobster populations did not decreased in the last century and it is unlikely to decrease in the future, thus, restocking programs have been implemented in several European countries (Beard & Wickins 1992; Van Der Meeren 2005; Uglem et al. 2006; Benavente et al. 2010; Schmalenbach et al. 2011). The leading countries in these programs are Norway, Ireland, United Kingdom and France (Van Der Meeren 2005; Uglem et al. 2006; Schmalenbach et al. 2011; Scolding et al. 2012).

Restocking programs consist in larval rearing and mass production of juvenile lobsters for further release in areas previously identified as being suitable for juvenile lobsters (Beard & Wickins 1992; Contarini et al. 2008; Scolding et al. 2012). These programs are based in two different strategies: production of large number of small juveniles (stage IV or V) or smaller number of bigger lobster, reared for longer time in hatcheries (Contarini et al. 2008).

In Norway, between 1990 and 1997, a large scale program was made, releasing of 128.000 cultured juvenile lobster (14 mm CL) to increase recruitment, and proved that it is possible to strengthen a depleted population with restocking programs (Van Der Meeren 2005; Schmalenbach et al. 2011). This releasing study showed that juveniles survived to be captured in the commercial fishery 5 to 10 years after release and that they add to the natural recruitment in the same way as wild lobsters (Van Der Meeren 2005).

As pointed out by Browne et al. 2009, the demand for fisheries resources is increasing with the world population and the sustainability of all fisheries depends on the acceptance and application of appropriate management controls, which protect the breeding stock and maintains the environment they live in. Enlightened management measures are fundamental to any lobster fisheries self-sustainability and should always be the first choice when considering methods for increasing or preserving stock levels.

Different rearing conditions and diets have been used for larval development of *Homarus gammarus* (Table 1). Briefly, regarding rearing tanks,individual containers (e.g. Kurmaly et al. 1990) or cylindrical conical tanks have been used with water recirculation (e.g. Kristiansen et al. 2004) or water renewal (e.g. Uglem et al 2006).

The rearing temperatures varied from 11ºC (e.g. Schmalenbach & Franke 2010) to 25ºC (e.g. Kurmaly et al. 1990) and the dominant diets tested were brine shrimp (e.g. Schmalenbach et al. 2009) and Mysis (e.g. Beard et al 1985) (frozen or not) frequently complemented with microalgae (e.g. Contarini et al. 2008).

Table 1 – Summary of the different rearing conditions and diets used on larval development of *Homarus gammarus*. Survival rate (SR, %), larval development time (LDT, days) and growth rate (GR, mm $d^{-1}$) indicated when available.

| Rearing conditions | Diet | Results | Reference |
|---|---|---|---|
| 40L cylindrical conical tanks<br>Upstream water flow<br>1500-2000 larvae/tank<br>T=20±2ºC, Salinity 30±2 | Frozen Mysis (*ad libitum*) | SR: 10<br>LDT : Approximately 16<br>GR: n.d. | Beard et al. 1985 |
| Individual containers (125 mL) with bottom net<br>T=25ºC, Salinity 32,5 | Twice/day<br>Diets: frozen lobster larvae(1), frozen mysis(2), BML(3), kanazawa(4), CD434a(5) and CD434b(6) | SR: (1) 76,6±2,67, (2) 79,99±1,52, (3) 62,93±2,21, (4) 73,66±2,26, (5) 36,25±2,88, (6) 57,92±1,16<br>LDT: n.d.<br>GR (): (1) 0,076, (2) 0,114, (3) 0,039, (4) 0,049, (5) 0,032, (6) 0,056 | Kurmaly et al. 1990 |
| 40L(37 larvae/L) and 100L(25 Larvae/L) cylindrical conical tanks<br>T=14-16ºC, Salinity 28-32 | Three times per week<br>Frozen Mysis (50g 1st day to 410g in the last) and brine shrimp nauplii (5 nauplii/mL) | SR: n.d.<br>LDT: 11-17 days<br>GR: n.d. | Beard & Wickins 1992 |
| 40L cylindrical conical tanks<br>Upstream water flow and bottom aeration<br>Recirculation water system (RAS)<br>T=18-20ºC<br>1500 larvae/tank (maximum) | Twice per day<br>Frozen brine shrimp and frozen Mysis | SR: n.d.<br>LDT: approximately 12<br>GR: n.d. | Kristiansen et al. 2004 |
| 90L cylindrical conical tanks<br>Bottom aeration<br>T=18-20ºC<br>Water renewal every two day | Renewal every two day<br>6-8g brine shrimp cysts/tank plus microalgae (*I. galbana* and T-ISO, 150 cells/µL) | SR: 29,5<br>LDT: 14-16<br>GR: n.d. | Uglem et al. 2006 |
| 60L cylindrical conical tanks<br>T=18,8±1,05ºC<br>Larval density/tank: 1000(a), 1001-2000(b), 2001-3000(c), 3001-4000(d), 4001-5000(e) and >5000(f) | Between 110 to 1500 brine shrimp nauplii fed/larvae/day plus *T. suecica* and *I. galbana* (50-150x$10^6$ cells/L) | SR: (a)77,86; (b)50,01; (c)43,96; (d)26,67; (e)28,23; (f)17,98<br>LDT: approximately 12<br>GR: n.d. | Contarini et al. 2008 |
| 80L cylindrical conical tanks<br>Bottom aeration<br>T=18-21ºC<br>600-1000 larvae/tank<br>Water renewal every two day | Brine shrimp nauplii (15-25 nauplii/mL) plus *Chaetoceros* and *Isochrysis* | SR: approximately 40<br>LDT12-14<br>GR: n.d. | Browne et al. 2009 |
| Specific semi-flow tanks<br>T=17-19ºC | Brine shrimp nauplii and every other day with minced crabs | SR: n.d<br>LDT: approximately 17<br>GR: n.d. | Schmalenbach et al. 2009 |
| 90L cylindrical conical tanks<br>Bottom aeration<br>T=18-20ºC<br>500-1000 larvae/tank<br>Water renewal every two day | Brine shrimp nauplii (6-8 g/tank) plus *I. galbana* (150 cells/µL) | SR: 29,5<br>LDT: 12-15<br>GR: n.d. | Benavente et al. 2010 |
| Individual closed cups of 80 mL<br>T=11ºC(a), 12 ºC(b), 13 ºC(c), 14 ºC(d), 15 ºC(e), 16 ºC(f), 20 ºC(g) and 22 ºC(h)<br>Salinity 31<br>Water change every day | Brine shrimp nauplii (30 nauplii/larvae) | SR: (d) 10 (e) 10 (f) 68 (g) 55 (h) 80<br>LDT: (d) 26.3±3.8; (e) 35.3±3.5 (f) 19.7±2.0 (g) 14.4±1.5 (h) 13.5±2.8<br>GR: n.d. | Schmalenbach & Franke 2010 |
| 90L cylindrical conical tanks<br>Upwelling water circulation (RAS)<br>Bottom vigorous aeration<br>15-20 larvae/L<br>T=17-19ºC, Salinity 31 | Brine shrimp nauplii (3-5 nauplii/mL enriched with 0,2g/L DHA Selco® and 0,007mL/L Bio-Moss) plus 100 mL/L of *C. muelleri* and *I. galbana* in exponential phase | SR: n.d.<br>LDT: approximately 18 days<br>GR: n.d. | Scolding et al. 2012 |

## 1.5. Major constrains on *Homarus gammarus* larval development

A major problem associated with the production of this species in captivity relates to cannibalism (Debuse et al. 1999; Kristiansen et al. 2004; Prodöhl et al. 2007; Browne et al. 2009). However, this cannibal behaviour is not intentional. The animals do not seek individuals of the same species for feeding, but only when they meet and are in starvation (Skog 2008).

Consequently, increasing the cultivation density intensifies this behaviour, resulting in huge production losses. To overcome this problem, captive rearing of lobsters requires reduced stocking densities, or even individual rearing, resulting in high initial investments and intensive maintenance procedures. (Meeren 2005; Uglem et al. 2006; Browne et al. 2009; Schmalenbach et al. 2009; Benavente et al. 2010).

Currently, the efforts of lobster hatcheries are focused on the developing of elaborate technical solutions for keeping the animals in single compartments and feed the juveniles in a fast way ( Uglem et al. 2006; Drengstig & Bergheim 2013).

Another bottleneck for commercial culture of lobsters, lies in the lack of high quality dry feed, specifically designed for lobsters larvae (Drengstig & Bergheim 2013). The nutritional value of the diet offered is extremely important that must be adjusted to the requirements for this species, as well as the way that the feed is offered to the larvae.

## 1.6. Diets and larval rearing of crustaceans decapods

### 1.6.1. Traditional diets

Currently, brine shrimp and rotifers remain the most widely used live food for the first larval stages. This fact is explained by high densities and biomasses obtained in its production which satisfies the needs inherent in larval production. (Marcus & Murray 2001; Camus & Zeng 2009; Drillet 2010; Ohs et al. 2010; Drillet et al. 2011; Ribeiro & Souza-Santos 2011).

Brine shrimp and rotifers are easily produced, but they are not nutritionally valuable, as first feed for larvae stages of several decapod species. (Camus et al. 2009).

Such characteristic makes necessary to proceed enrichment strategies of those live diets that could be done with the addition of microalgae.

In addition, digestibility and particle size are unsuitable to the mouth and digestive tract that is immature in early stages. For many species, their swimming behaviour is also a major obstacle to capture food particles, (Payne & Rippingale 2001; Marcus & Wilcox 2007; Camus & Zeng 2009; Ohs et al. 2010; Ribeiro & Souza-Santos 2011).

These inadequate features often result in mortalities during the transition to exogenous feeding, resulting in low growth rates, reducing size of individuals, and inability to rear some species (Payne & Rippingale 2000; Payne & Rippingale 2001; Camus & Zeng 2009; Ribeiro & Souza-Santos 2011).

### 1.6.2. Copepods as a potential diet – strengths and weakness

Several studies, all over the globe, have been carried out on these aquatic organisms (Carotenuto et al. 2012). This group of animals represents about 80% of the zooplankton, playing an important role in aquatic ecosystems, being a fundamental part of the food chain, actively participating in energetic nutrient cycle and ecosystems (Camus & Zeng 2009; Camus et al. 2009; Drillet 2010; Gonçalves et al. 2012).

In marine and estuarine ecosystems, this importance lies not only in its huge biomass and species diversity, but also due to the fact that they are primary consumers, being the link between primary producers, phytoplankton, and higher trophic levels (Lee et al. 2006; Dahms et al. 2007; Camus & Zeng 2009; Camus et al. 2009; Ohs et al. 2010; Carotenuto et al. 2012; Gonçalves et al. 2012).

The higher trophic levels consist mainly of larvae and juveniles of vertebrates and invertebrates, belonging to numerous species, reinforcing its preponderance in ecosystems (McKinnon et al. 2003; Lee et al. 2006; Dahms et al. 2007; Camus et al. 2009; Ohs et al. 2010). Since, copepods are preponderant in fish and crustaceans biology, their production is fundamental in large scale, as well as their integration in techniques and methodologies of aquaculture production (Matias-Peralta et al. 2005; Lee et al. 2006; Camus et al. 2009; Drillet et al. 2011).

Copepods are presented as a solution to production disabilities meeting the larval needs, believing it to be the future of the larval feeding, maximizing the production of species already cultivated and introducing new species in commercial production (Drillet 2010).

The characteristics that are at the origin of this belief are: (1) copepods are natural food present in ecosystems; (2) larvae are prepared to feed them or very similar preys; (3) nutritional value is similar to the needs avoiding enrichment, high levels of HUFA's (DHA, ARA and EPA); (4) great range of sizes during the various stages of their life cycle; (5) high digestibility; (6) source of antioxidants, astaxanthin, vitamins C and E, provide better pigmentation; (7) its swimming behaviour, stimulates predation, increasing food intake, providing healthy and faster development, higher survival and resistance to stress (McKinnon et al. 2003; Piasecki et al. 2004; VanderLugt & Lenz 2008; Puello-Cruz et al. 2009).

However, copepods haven't only advantages over rotifers and brine shrimp, some of the biological characteristics are also constraints as regards their use on a large scale production of fish and crustaceans.

Its richness is an asset in its use as live food, but also a constraint, since this diversity makes each species unmatched, having singular characteristics, hindering the creation of a productive standard and simple methodology (Marcus & Wilcox 2007; Drillet 2010). Thus, it is necessary to formulate particular methodological approaches in order to achieve and ensure great and continuous productivity (Ribeiro & Souza-Santos 2011).

Nowadays it is possible the captive production of copepods, however mass production, compared with the biomasses obtained in the production of brine shrimp and rotifers, it is not possible, especially due to harmful effects of high densities on crops (Payne & Rippingale 2000; Payne & Rippingale 2001; Murray & Marcus 2002; Camus & Zeng 2009; Drillet 2010).

To suppress the needs of these organisms in larval production, you can get them from the natural environment, however, this activity raises some issues, including environmental impact, since we are removing this aquatic animals that integrate aquatic food chains, their availability and abundance is seasonal, and the introduction of unwanted organisms and parasitic copepods in cultivation (Schipp 2006).

These organisms are influenced by exogenous factors, including temperature, salinity, photoperiod, cultivation density, quantity and quality of available food, and endogenous factors such as age and development stage (Støttrup & Norsker 1997; Camus et al. 2009; Devreker et al. 2009; Gonçalves et al. 2012; Neila et al. 2012).

These factors, in turn have a profound effect on the productivity obtained in a culture, therefore, may influence time before maturation of individuals, percentage of females producing eggs, embryonic development, quality and quantity of eggs and therefore the quantity and quality of nauplii, numbers of postures, time between postures and survival ( Payne & Rippingale 2000; Ohs et al. 2010; Beyrend-Dur et al. 2011; Drillet et al. 2011).

It is reported that small amounts of food delays the development of the copepods until adulthood, increases competition for food among individuals, and as a last resort, increases mortality (Drillet 2010). Stocking densities too high can lead to very intense cannibalistic behaviour, even with other food sources available, females may even feed on their own released nauplii (Drillet 2010).

### 1.6.3. Use of probiotics on crustaceans larval rearing

With its fast growth, aquaculture has become an important economic activity in many countries all over the globe (Balcázar et al. 2006; Qi et al. 2009). Consequently, in large scale production, animals are exposed to adverse and stressful rearing conditions, frequently leading to diseases, resulting in major losses (Gullian et al. 2004; Kesarcodi-Watson et al. 2008; Wang et al. 2008; Al-Dohail et al. 2011; Sivakumar et al. 2012).

Since diseases are one of the major constraints to aquaculture production (Gomez-Gil et al. 2000; Castex et al. 2009; Dhanasekaran et al. 2010), particularly in the early development stages (Gomez-Gil et al. 2000), and traditional disease control methodology, such as disinfectants and antimicrobial drugs, are becoming ineffective (Gomez-Gil et al. 2000; Verschuere et al. 2000; Panigrahi et al. 2004; Dhanasekaran et al. 2010; Soundarapandian & Babu 2010), it is urgent to find new solutions, more effective and environmentally friendlier (Farzanfar 2006; Wang et al. 2008; Castex et al. 2009; Dhanasekaran et al. 2010).

The massive use of antimicrobials for disease control and growth promotion in animals increases the selective pressure exerted on the microbial world and encourages the natural emergence of bacterial resistance (Verschuere et al. 2000). In addition, there are environmental problems associated with the chemical additives (Wang et al. 2008).

Therefore, probiotics have been the subject of several studies and has been shown to be a protective barrier against pathogenic bacteria in the most economic and environment-friendly manner (Al-Dohail et al. 2011).

Gatesoupe (1999) defines probiotics as “microbial cells that are administered in such a way as to enter the gastrointestinal tract and to be kept alive, with the aim of improving health”. Dhanasekaran et al. (2010) defined probiotics more precisely, describing it as “mono or mixed cultures of live microorganisms which, when applied to animal, beneficially affect the host by improving the properties of the indigenous micro flora”.

Probiotics, as production supplements, exhibit several benefits to its host, these improvements include better feed utilization and digestibility by enhancement of enzymatic activity, inhibition of a pathogen via production of antagonistic compounds, competition for attachment sites, competition for nutrients, better immunostimulatory functions, antimutagenic and anticarcinogenic activity and growth promoting factors (Verschuere et al. 2000; Bomba et al. 2002; Panigrahi et al. 2004; Kesarcodi-Watson et al. 2008).

Tseng et al. (2009) tested shrimp, *Litopenaeus vannamei*, feed with supplementation of *B. subtilis* E20 during 98 days, and obtained survival rates significantly higher compared with control, without probiotic, and challenged with *Vibrio alginolyticus*, showing immunologic adaptation with increases of phenoloxidase and phagocytic activity.

Additionally, water quality improvement using probiotics, have been reported (Verschuere et al. 2000; Panigrahi et al. 2004; Wang et al. 2008; Al-Dohail et al. 2011). Dalmin et al. (2001) showed that the use of *Bacillus sp*. improved water quality, survival and growth rates and the health status of juvenile *Penaeus monodon* and reduced the pathogenic vibrios.

Wang et al. (2005) demonstrate that the use of commercial probiotics in *Penaeus vannamei* juveniles can reduce dissolved concentrations of nitrogen and phosphorus,

increasing shrimp production. The water quality improvement, using probiotics, were shrimp is cultured has been received considerable attention (Verschuere et al. 2000).

Several approaches have been adopted to evaluate the effect of probiotics in crustaceans rearing (Table 2). The strains used in the studies belong to the genus *Bacillus*, being isolated from several shrimp species, evaluating their effect on growth, survival, protection against pathogenic bacteria and production of digestive compounds.

Table 2 – Experimental studies made in last few years using probiotics in crustaceans rearing (adapted from Ninawe & Selvin 2009).

| Strain | Source | Evaluated for | Effective dose/mode of application | Reference |
|---|---|---|---|---|
| *Bacillus* S11 | Black tiger shrimp | Growth and survival of black tiger shrimp *Penaeus monodon* | 1 kg wet wt (~100 g dry wt) of BS11 (~$10^{10}$ CFU $g^{-1}$) in 3 kg of feed (2,5% BS11/3 kg) | Rengpipat et al. 2003 |
| *Bacillus subtilis* BT23 | Shrimp culture ponds | Against the growth of *Vibrio harveyi* isolated by agar antagonism assay from *Penaeus monodon* | $10^6$-$10^8$ CFU $ml^{-1}$ for 6 d | Vaseeharan and Ramasamy 2003 |
| *Pseudomonas sp*. PM11 *Vibrio fluvialis* PM17 | Gut of farm reared sub-adult shrimp | Immunity indicators of *Penaeus monodon* | *Pseudomonas sp*. PM 11 @ $10^3$ bacterial cells $ml^{-1}$ for 3 days and *V. fluvialis* PM 17 @ $10^3$ bacterial cells $ml^{-1}$ for 7 days | Alvandi et al. 2004 |
| *Arthrobacter* XE-7 | Isolated from *Penaeus chinensis* | Protection of *Penaeus chinensis* post-larvae from pathogenic vibrios such as *Vibrio parahaemolyticus*, *Vibrio anguillarum* and *Vibrio nereis* | $10^6$ CFU/ml | Li et al. 2006 |
| *Bacillus subtilis* and *B. megaterium* | Marine environment | Production of digestive enzymes, proteases, carbohydrolases and lipases | Potential application in shrimp feeds | Solano and Soto 2006 |
| *Paenibacillus spp*., *B. cereus* and *P. polymyxa* | Seawater, sediment and marine fish gut samples | Against pathogenic Vibrios | $10^4$ and $10^5$ CFU $ml^{-1}$ | Ravi et al. 2007 |
| *Synechocystis* MCCB 114 and 115 | Seawater | Antagonism angainst *V. harveyi* | Post-larvae fed on the cyanobacterial cultures | Preetha et al. 2007 |
| *Bacillus licheniformis* | Shrimp pond | Intestinal microbiota and immunity of the white shrimp *Litopenaeus vannamei* | *B. licheniformis* suspension of $10^5$ CFU $ml^{-1}$ for 40 days | Li et al. 2007 |
| Lactic-acid bacteria | Shrimp gut | Survival of marine shrimp, Litopenaeus vannamei challenged with V. harveyi | Liquid diet supplemented with B6 strain at $10^8$ CFU/ml | Vieira et al. 2007 |
| *Lactobacillus planctarum* | Shrimp isolate | Immune response and microbiota of shrimp digestive tract of *Litopenaeus vannamei* challenged with *V. alginolyticus* and *V. harveyi* | $10^{10}$ CFU/kg diet and $10^8$ CFU/kg feed | Chiu et al. 2007 |
| *Vibrio alginolyticus* UTM 102, *Bacillus subtilis* UTM 126, *Roseobacter gallaeciensis* SLV03, and *Pseudomonas aestumarina* SLV22 | Gastrointestinal tract of adult shrimp *Litopenaeus vannamei* | Antagonism against the shrimp-pathogenic bacterium, *Vibrio parahaemolyticus* PS-017 | Feed supplement | Balcázar et al. 2007 |
| *Bacillus subtilis* UTM 126 | Shrimp culture pond | Protection against vibriosis in juvenile *Litopenaeus vannamei* | $10^5$ CFU/g | Balcázar and Rojas-Luna 2007 |
| *Pediococcus acidilactici* | Strain MA 18/5M, CNCM | Survival of *Litopenaeus stylirostris* against against Vibriosis caused by *Vibrio nigripulchritudo* | Probiotic-coated pellet feed | Mathieu et al. 2008 |
| *B. subtilis*, *B. natto* and *B. licheniformis* | Not available | Growth and digestive enzyme activity of *Litopenaeus vannamei* | 1.5 to 7.5% supplemented to the feed | Gómez and Shen 2008 |

Table 3 presents authorized probiotics to feed inclusion. Several probiotics have been proposed as biological control agents in aquaculture, although the most used belong to the lactic acid bacteria (*Lactobacillus* and *Carnobacterium*), to the genus *Vibrio*, to the genus *Bacillus* and genus *Pseudomonas*, however other species or genera have been used, such as *Aeromonas* and *Flavobacterium* (Balcázar et al. 2006).

Table 3 - List of microorganism authorized as probiotics in feeding stuffs under Council Directive 70/524/EEC (adapted from Balcázar et al. 2006)

| **Probiotics** | |
|---|---|
| *Bacillus cereus var. toyoi* | *Lactobacillus plantarum* |
| *Bacillus licheniformis* | *Lactobacillus rhamnosus* |
| *Bacillus subtilis* | *Pediococcus acidilactici* |
| *Enterococcus faecium* | *Saccharomyces cerevisiae* |
| *Lactobacillus casei* | *Streptococcus infantarius* |
| *Lactobacillus farciminis* | |

## 2. Objectives

The aim of the present study was to evaluate survival rate, growth rate and intermoult period during the larval development of *Homarus gammarus* subjected to different conditions of food and temperature.

The specific objectives were:

(1) To estimate the effect of live and dry diet on survival rate and intermoult period of larval stages of *H. gammarus*;
(2) To evaluate the effect of adding probiotic to the water on the survival rate, specific growth-rate and intermoult period during the larval development of *H. gammarus*;
(3) To estimate the effect of different temperatures on the larval development of *H. gammarus*;

To fulfill the specific objectives previously enumerated, all experiments were based on individual responses of larvae subjected to different conditions of food and temperature. Each single larva was followed from hatch until reach the post larvae stage or until death. This methodology allows us the incorporation of individual variability on the estimate of demographic parameters (survival rate, specific-growth rate and intermoult period).

# 3. Materials and methods

## 3.1. Adult females maintenance and larvae collection

Gravid adult female European lobsters *Homarus gammarus* were captured in the northeast Atlantic (exact capture date and location unknown), obtained from an importer of live crustaceans in May 2013 and transported to the laboratory on an insulated box.

The animals were maintained on a recirculating water system, System 2500/5000 Filtration Unit (TMC®), in a 500 L tank with refuge, under natural light/dark cycle, at temperature of 16°C, salinity 35 and dissolved oxygen (DO) levels close to saturation. The amounts of nitrites, nitrates and phosphates were kept always under 0.25 mg/L. Its diet consisting of frozen shrimp, squid and mussels, was administrated twice a day, morning and evening.

The egg maturation rate was calculated using the formula present in appendix 1, in order to predict the approximate time until egg hatching. Spawning was allowed to occur naturally overnight in darkness, and pelagic larvae were collected by nets the following morning. The larvae were then quantified and distributed by the different assays (see 3.4, 3.5 and 3.6).

## 3.2. Diets preparation

### 3.2.1. Dry copepod biomass – *Acartia tonsa*

Dry copepods were obtained from *Acartia tonsa* cultures. An initial stock copepod culture was started using cold stored (4ºC) *A. tonsa* eggs obtained from cultures maintained on the aquaculture laboratory of the School of Tourism and Sea Technology (ESTM – IPLeiria).

The eggs were incubated in rectangular polyethylene tanks containing 40 L of filtered seawater (0.45 µm, 35 PSU) at 23±1°C with gentle permanent aeration (air filtered through 0.2 µm). The hatched organisms were fed *ad libitum* a mixture of *Isochrysis galbana* and *Rhodomonas lens*.

Microalgae were obtained from batch cultures at exponential growth phase. Microalgae cultures were grown in 5 L plastic carboys containing enriched seawater (35 PSU) with f/2 medium, at 21°C under a 12:12 h light:dark cycle, with aeration (air filtered through 0.2 µm). This microalgae were selected because its use in mass production of several calanoid copepods resulted in high development rates and survival (Carotenuto et al. 2012), such as *A. tonsa* (Ismar et al. 2008) and *G. imparipes* (Payne & Rippingale 2001).

Every two days, 10% of the seawater volume was changed, the eggs were collected (if existed) and food concentration adjusted to 10000 cells $mL^{-1}$ at 2:1 ratio (*I. galbana*:*R. lens*). The water was sieved at 200 µm, collecting adults and copepodites, and 64 µm, collecting eggs, nauplii and smaller copepodites.

After seawater renewal, food concentration was adjusted and determined using the equations, one for each microalgae specie, in appendix 2, that relates cell counting and optical concentration.

For the production of copepod biomass, growth cultures of *A. tonsa* were done on four cylindrical 15 L fiberglass tanks. In each tank, was incubated 15000 eggs (previously collected from stock copepod culture), at the same conditions described above.

At the end of generation development time (aprox. 10 days), adults of *A. tonsa* were collected from by sieving through 200 µm mesh. After collection, the organims were washed with distilled water, transferred to 15 mL falcon tubes and then frozen at -80°C (Thermo Electro Corporation, Forma -86C ULT freezer). After complete freezing, they were lyophilized (Coolsafe Scanvac) between 24 and 48 hours and stored until its use, sealed and in cold 4°C (Beko).

### 3.2.2. Dry wild zooplankton

Wild zooplankton samples were collected near the coastline of Peniche (39º20’24.94’’N; 9º23’00.17’’W) on June 2013 by oblique tows made with a double zooplankton net (200 µm and 500 µm).

The samples were concentrated by sieving through a 200 µm mesh net, washed with distilled water, transferred to 50 mL falcon tubes and frozen at -80°C (Thermo Electro Corporation, Forma -86C ULT freezer).

After complete freezing, the samples were lyophilized (Coolsafe Scanvac) for a period of time between 24h and 48 h and stored at 4°C (Beko).

### 3.2.3. Live and dry *Artemia* nauplii

*Artemia* nauplli was offered to *H. gammarus* larvae as live or dry food. The brine shrimp were obtained from commercial cysts (Ocean Nutrition®), decapsulated and placed for hatching according to the protocol described in appendix 3.

For the experiments that used live artemia, nauplii were collected in less than 6 hours post hatch, quantified and directly added to *H. gammarus* larvae. For the experiments that uses dry food, the *Artemia* nauplii were collected at the same time previously referred, sieved through 200 µm mesh, washed with distilled water, transferred to 15 mL falcon tubes and then frozen at -80°C (Thermo Electro Corporation, Forma -86C ULT freezer).

After complete freezing, the samples were lyophilized (Coolsafe Scanvac) for a period of time between 24h and 48 h and stored at 4°C (Beko).

### 3.2.4. Probiotic preparation

*Lactobacillus plantarum* (Strain designation ITQB M 32 A, DSM No. 10492, DSMZ®) were inoculated in MRS broth (Merk®), previously prepared (see appendix 4). The bacteria was incubated, orbital (Incobator 1000 with Vnimax 1010, Heidolph®), during 24 hours at 33°C. After 24 hours, the cellular concentration was estimated using spectrophotometry (Thermo Electro Corporation, Helios Aquamate), at λ=595 nanometers (nm), using the equation in appendix 5.

The concentration obtained was $3.22 \times 10^8$ UFC's/mL. After incubation, the cells were harvested by centrifugation (Centrifuge 5804 R, Eppendorf®), at 5000 rotations per minute (rpm) during 15 minutes at 15°C, and washed twice with autoclaved (Trade

Raypa, Steam Sterilizer) seawater, 15 minutes at 121°C. Aliquots were frozen, at -80°C (Thermo Electro Corporation, Forma -86C ULT freezer), until its use.

3.2.5. Evaluation of nutritional profile by fatty acid analysis

Fatty acid quantification was performed in all diets tested (brine shrimp nauplii, wild zooplankton and copepods), as well as for *Homarus gammarus* larvae recently hatched. The biomass used for fatty acid quantification varied from 30 mg (dry copepods) to 200 mg (wet *Artemia* or *H.gammarus larvae*).

The weight for each diet was: 100 milligrams of brine shrimp (dry weight) and 200 milligrams of brine shrimp (wet weight), 100 milligrams of zooplankton (dry weight), and 30 milligrams of copepods (dry weight) and 50 milligrams of copepods (wet weight). 200 milligrams (wet weight) of European lobster larvae were used for the same procedure. All the samples were analyzed according to the modified method of Bligh and Dyer (1959) (see appendix 6).

### 3.3. Assay 1: Evaluation of the effect of different diets Lyophilized vs. live food

In this experiment it was tested four different diets: (1) Live Artemia nauplii (LAN), (2) Dry Artemia nauplii (DAN), (3) Dry wild zooplankton (DWZ) and (4) Dry copepods (DC) – plus one control (CT), without food.

In each treatment, it was used 24 larvae obtained from the broodstock tank (see 3.1.) and individualized in a 100 mL plastic container. For each vessel/larvae was administrated 200-300 µg of dry food and approximately 100 live artemia nauplii at a final density of 1 prey/mL. Seawater (35 PSU) temperature was maintained at 21ºC, dissolved oxygen levels close to saturation and ammonia levels always kept under 0.25 mg/L.

Every 12h, each individual was checked for larval stage and live/death condition. Seawater change and food adjust was daily performed. The two previously procedures were performed until the individual reach post larvae stage (PL).

For each larval stage/treatment, 5 individuals recently hatched were randomly selected for cephalothorax length measuring (CL, from the beginning of cephalothorax until back of the eye). Measurement were done through digital image analysis (software Zen 2011, Blue edition) based on digital photography obtained with the use of a camera (Zeiss Axio Cam MRc) attached to a stereo microscopy (Zeiss Stemi 2000-C).

### 3.4. Assay 2: Effect of probiotic adding in the larval rearing

In order to test the effect of probiotic on the culture medium of *H. gammarus* larvae, it was chosen the bacteria *Lactobacillus plantarum*. This assay consisted on two different treatments: (1) live Artemia nauplii plus *L. plantarum* and (2) live Artemia nauplli without *L. plantarum*. The prey density used on both treatments was 1 prey/ml and the probiotic concentration of $1.45x10^5$ UFC/ml. The experimental design applied to evaluate the response of *H. gammarus* larvae was equal to the previous one described on section 3.4.

### 3.5. Assay 3: Relation between temperature and larval development

This experiment was divided in four trials corresponding to four different temperatures: 16, 19, 21 and 23ºC. For a given temperature, it was prepared 40 culture vessel containing one recently hatched larvae of *H. gammarus* and Artemia nauplii at a final density of 2 prey/mL. The intermoult period (IP) for each development stage was obtained by averaging the time between successive stages of a single larva.

Again, the experimental design applied to evaluate the response of *H. gammarus* larvae was equal to the previous one described on section 3.4.

## 3.6. Data analysis

### 3.6.1. Statistical analysis

For each larval stage (this is, Zoea I, II and III), a one-way analysis of variance (ANOVA) or Kruskal-Wallis non-parametric tests (depending on the violation or not of the normality and homogeneity of variance assumptions) (Zar, 2009) were applied to assess the differences between the four levels of temperature (16$^{o}$C, 19$^{o}$C, 21$^{o}$C and 23$^{o}$C) for intermoult period (IP) and specific growth rate (SGR).

Whenever applicable, Tukey multiple-comparison test was performed after the analysis of variance to detect differences between temperatures.

Additionally, t-test was employed to determine significant differences relatively to the effect of the presence of probiotic in larval rearing on IP and SGR (Zar, 2009). Also, this analysis was performed for each larval stage.

For all statistical tests, the significance level was set at p-value≤0.05. All calculations were performed with Sigma Plot (Version 12.0) and IBM SPSS Statistics 21. Where applicable, results are presented as mean ± standard deviation (SD).

### 3.6.2. Belehradek's equation

The functional relationship between temperature and development time was then estimated by the Belehradek's function:

$$D = a \times (T + c)^{b}$$

where D is the development time between stages (IP in days), T is temperature (°C) and *a* and *c* are coefficients, estimated using the program SigmaPlot (Version 12.0) (McLaren, 1963 & 1965). The values of b, indicative of curvilinearity in the stage duration-temperature equation, used in data analysis were the same obtained in Mackenzie (1988). The effects of temperature and development stage on stage duration and size were analyzed with a two-way factorial analysis of variance (ANOVA).

# 4. Results

## 4.1. Fatty acids profile

Fatty acid profile of diets and Homarus gammarus larvae are shown in table 4, showing that palmitic acid (C 16:0), oleic acid (C 18:1 n9) and octadecatetranoic acid (parinaric) (C 18:4 n3) presented the highest percentages in brine shrimp diets, LAN and DAN, however in DAN diet palmitoleic acid (C 16:1), vaccenic acid (C 18:1 n7) and ecosapentanoic acid (EPA) (C20:5 n3) were present in percentages above 10% (Table 4). In diets DC, COP and DWZ the dominant fat acids (FA) were palmitic acid, EPA and docosahexaenoic acid (DHA) (C 22:6 n3).

European lobster Zoea I larvae showed four abundant FA, palmitic acid, oleic acid, EPA and DHA. COP diet had the highest percentage of polyunsaturated fat acids (PUFA's) and n3 FA, contrasting with the lowest percentage of n6 FA, and the highest percentage was in DAN diet. It is important to notice that Zoea I has a percentage of arachidonic acid (ARA, 20:4n6) much higher that remain samples tested. All GC chromatograms obtained are available at appendix 7.

Table 4 – Percentage of the most abundant fatty acids found in the diets tested on assay 1, and in *H. gammarus* Zoea I. Samples of live Artemia nauplii (LAN), copepods (COP) and Zoea I were not dried before lipid extraction. All the remain samples were dried before analyses. N.d.: not detected.

| | **Diets** | | | | | ***H. gammarus*** |
|---|---|---|---|---|---|---|
| **Fatty Acid** | **LAN** | **DAN** | **DC** | **COP** | **DWZ** | **Zoea I** |
| C 12:0 | 0.30 | 0.14 | 2.17 | 0.13 | 7.19 | 0.47 |
| C 14:0 | 1.75 | 1.89 | 5.87 | 3.30 | 7.30 | 0.80 |
| C 14:1 | 0.70 | 1.44 | 0.69 | 0.28 | 0.38 | N.d. |
| C 16:0 | 16.03 | 12.61 | 19.14 | 21.11 | 16.08 | 17.47 |
| C 16:1 | 1.73 | 10.68 | 4.39 | 2.73 | 4.34 | 2.66 |
| C 18:0 | 6.89 | 3.72 | 7.96 | 7.22 | 5.78 | 7.01 |
| C 18:1 n9 | 15.48 | 15.16 | 2.75 | 3.24 | 2.50 | 11.49 |
| C 18:1 n7 | 5.84 | 10.28 | 3.28 | 2.44 | 2.15 | 8.66 |
| C 18:2 n6 | 5.21 | 4.73 | 0.30 | 1.25 | 1.65 | 0.89 |
| C 18:3 n3 | 0.61 | 0.52 | 0.24 | 0.18 | 0.23 | 0.47 |
| C 18:4 n3 | 24.55 | 13.33 | 1.46 | 2.01 | 1.18 | 0.34 |
| C 20:1 n9 | 8.77 | 2.96 | 2.91 | 2.33 | 3.81 | 1.17 |
| C 20:4 n6 | 0.35 | 1.75 | 0.57 | 0.39 | 0.31 | 4.74 |
| C 20:5 n3 | 1.28 | 11.53 | 17.99 | 20.07 | 19.37 | 1984 |
| C 22:6 n3 | 0.16 | 0.09 | 22.19 | 26.72 | 20.81 | 16.47 |
| C 24:1 n9 | 0.02 | 0.01 | 2.11 | 2.65 | 0.07 | N.d. |
| Σ %n3 | 27.93 | 25.64 | 42.82 | 49.64 | 42.66 | 38.44 |
| Σ %n6 | 5.60 | 6.49 | 2.66 | 1.82 | 2.75 | 5.85 |
| PUFA's | 33.53 | 32.13 | 45.48 | 51.46 | 45.41 | 44.29 |

The PCA analysis (Fig. 9) showed that the parinaric acid, oleic acid and palmitic acid (C 16:0) are dominant in both diets of brine shrimp, LAN and DAN. For Zoea I larval stage, COP, DP and DWZ the dominant FA were the palmitic acid, EPA and DHA, revealing their richness in PUFA's.

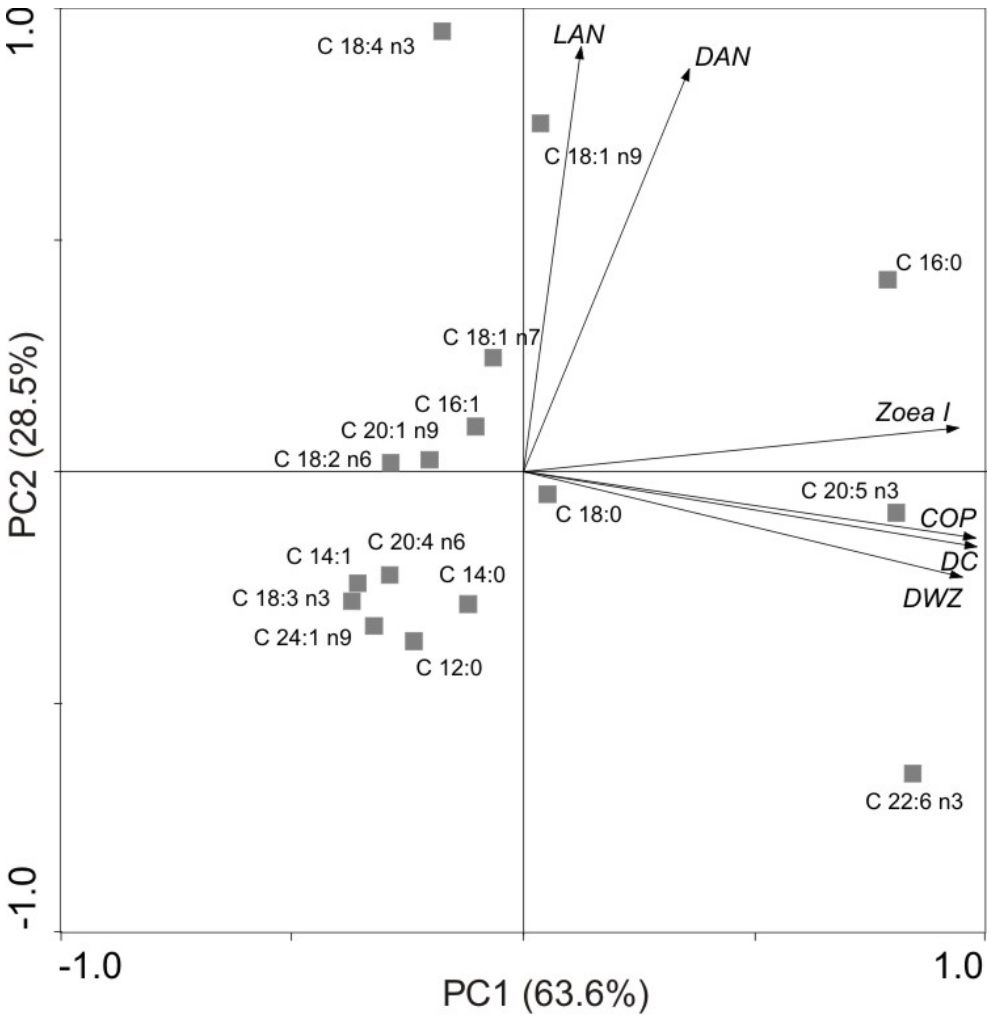


Figure 9 – PCA analysis of the fatty acid profile of the tested diets and Zoea I of *Homarus gammarus.* LAN refers to live artemia nauplii, DAN dry artemia nauplii, COP to copepods, DC dry copepods and DWZ to dry wild zooplankton.

### 4.2. Assay 1: Evaluation of the effect of different diets Lyophilized vs. live food

This assay showed that the LAN diet provided the best survival rates, with 16 larvae reaching Zoea II, 10 reaching Zoea III and 1 reaching PL, corresponding to 66.67%, 41.67% and 4.17%, respectively, for the remaining diets, DAN, DC, DWZ and CT, all larvae died before reaching Zoea II (Fig. 10A).

The IP is shown in Fig. 10B, revealing a greater IP in Zoea III (7.5 days), comparing with Zoea I and II (3.41±0.38 days and 3.83±0.25 days, respectively), with IP's very similar, but with tendency to IP increase with the larval development.

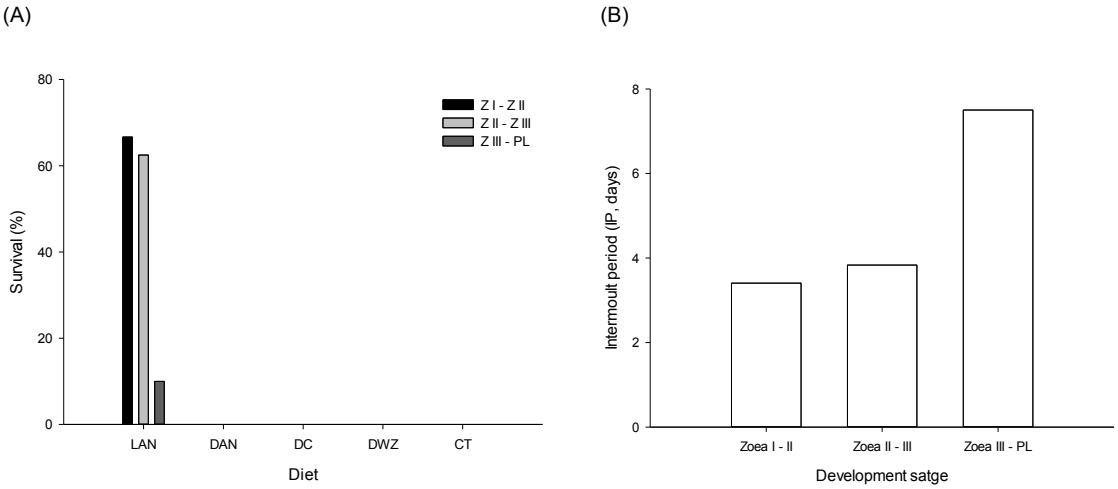


Figure 10 – Larval survival rate (%) (A) obtained for the different tested diets (LAN, DAN, DC, DWZ and CT) and Intermoult period (IP, days) (B) for LAN diet. Vertical bar corresponds to SD.

Cephalothorax length (CL, mm) of the different larval stages (Zoea I to III) and post-larva of *Homarus gammarus*, of LAN diet is summarized on Table 5.

Table 5 - Carapace length (mm) of the different larval stages (Zoea I to III) and post-larva of *Homarus gammarus* fed with LAN.

| | Zoea I | | | Zoea II | | | Zoea III | | | PL | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Diet | N | Mean | SD | N | Mean | SD | N | Mean | SD | N | Mean | SD |
| LAN | 5 | 3.05 ± | 0.07 | 5 | 3.86 ± | 0.25 | 5 | 4.63 ± | 0.17 | 1 | 5.22 ± | |

Among the several diets proposed, LAN demonstrated to be the best diet for use on the following assays, namely probiotic and temperature effect.

### 4.3. Assay 2: Effect of probiotic adding in the larval rearing

The larval survival in this trial shown no differences between treatments. However, it was observed slightly low survival for all larval stages subjected to probiotic (Fig. 11A).

For LAN treatment, survival rates were equal to 70.83% (Zoea I), 70.58% (Zoea II) and 8.33% (Zoea III, with just only one larvae reaching PL stage). These results were some different from the ones obtained with the diet LAN+P: 58.33%, 57.14% and 12.5% of survival between Zoea I and II, II and III and between Zoea III and PL, with just one larvae reaching PL stage.

The intermoult period (IP) was very similar between treatments in all development stages, with a tendency to increase in last developmental stages (Fig. 11B). For LAN, the IP was 3.47±0.45 days, 3.96±0.33 days and 7.50 days for Zoea I, II and III, respectively. Concerning to LAN+P, IP was equal to 3.86±1.06 days, 3.88±0.35 days and 8.00 days for Zoea I, II and III, respectively.

Cephalothorax length (CL, mm) of the different larval stages (Zoea I to III) and post-larva of *Homarus gammarus* is summarized on Table 6. Generally, the CL showed no differences between treatments.

Table 6 - Carapace length (mm) of the different larval stages (Zoea I to III) and post-larva of *Homarus gammarus* in both treatments.

| | Zoea I | | | Zoea II | | | Zoea III | | | PL | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Treatment | N | Mean | SD | N | Mean | SD | N | Mean | SD | N | Mean | SD |
| LAN | 5 | 3.04 ± | 0.04 | 5 | 3.87 ± | 0.14 | 5 | 4.52 ± | 0.11 | 1 | 5.23 ± | |
| LAN+P | 5 | 3.07 ± | 0.08 | 5 | 3.93 ± | 0.07 | 3 | 4.55 ± | 0.20 | 1 | 5.20 ± | |

For the specific growth rate (SGR, % $d^{-1}$), the results showed no significant differences between treatments (Fig. 11C). However, it was noted a tendency to decrease with larval development.

LAN treatment showed a SGR of 7.02±0.86 % $d^{-1}$ in Zoea I, 4.02±0.34 % $d^{-1}$ in Zoea II and 2.05 % $d^{-1}$ in Zoea III, comparing with 6.73±1.38 % $d^{-1}$ in Zoea I, 3.83±0.34 % $d^{-1}$ in Zoea II and 1.68 % $d^{-1}$ in Zoea III registered for the diet LAN+P.

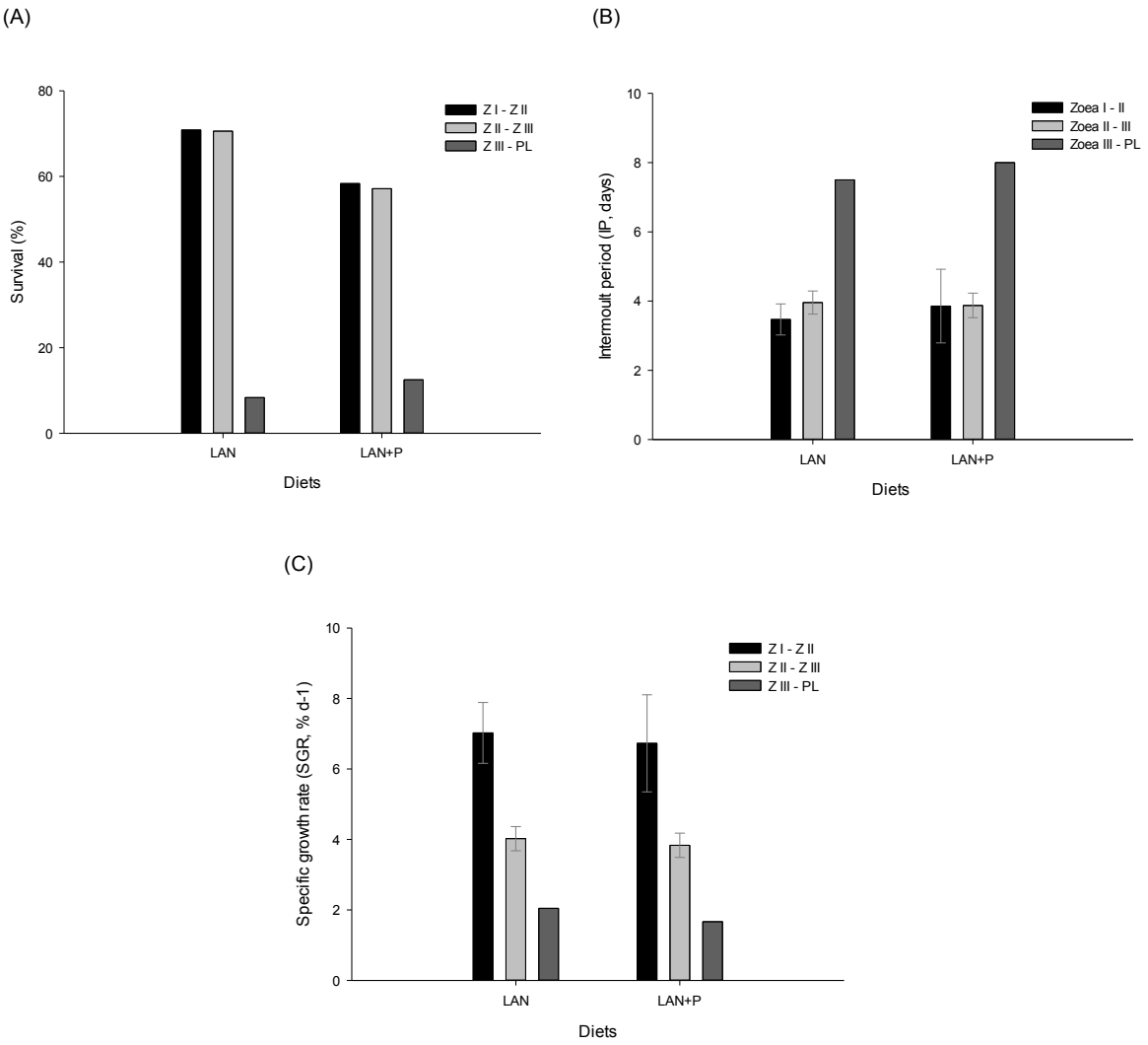


Figure 11 - Survival rate (%) (A), Intermoult period (IP, days) (B) and specific growth-rate (SGR, % $d^{-1}$) (c) for the different larvae stages subjected to the treatments LAN and LAN+P. The vertical lines corresponds to the SD.

The results obtained here have shown that the addition of this specific probiotic in larval rearing of European lobster doesn´t produce any advantage in all parameters evaluated. So, for the following trials, was not added probiotic to rearing water.

### 4.4. Assay 3: Relation between temperature and larval development

4.4.1. Temperature effect on survival rate

The figure 12 shows the larval survival for the different tested temperatures. Concerning to Zoea I, the highest survival rate were found at 23ºC (80%, n=32) and 19ºC (70%, n=28). In opposition, the lowest survival rates were noted at 21ºC (62.5%, n=25) and 16ºC (60.0%, n=24)).

Between Zoea II and Zoea III the temperature 19ºC demonstrated the highest survival (64.3%, n=18), being the lowest noted at 23ºC (9.4%, n=3). At 21ºC and 16ºC, the survival rates were respectively 60% (n=15) and 33.3% (n=8).

Between Zoea III and PL, at 23ºC, the survival rate was the highest, corresponding to 66.7%, with only 2 of the 40 larvae reaching PL stage. At 19ºC, five larvae reached the PL, corresponding to a survival rate of 27.8% between Zoea III and PL. At temperatures 16ºC and 21ºC, two larvae reached to PL, corresponding to 25% and 13.3%, respectively.

For temperatures 16ºC, 19ºC and 21ºC, the survival rate decreased with the larval development, contrasting with the results obtained in temperature 23ºC.

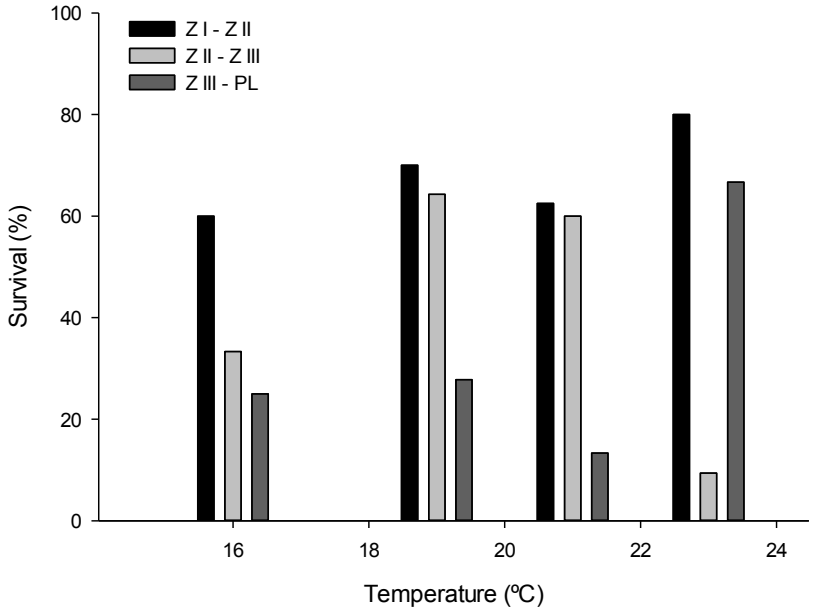


Figure 12 - Survival rate (%) of *Homarus gammarus* larval stages to different temperatures.

### 4.4.2. Specific growth rate (SGR)

Cephalothorax length (CL, mm) of the different larval stages (Zoea I to III) and post-larva of *Homarus gammarus* is summarized on Table 7. Generally, the carapace length of *H. gammarus* larvae tends to decrease with temperature increasing, except for PL with CL very similar between temperatures.

Table 7 – Carapace length (mm) of the diferente larval stages (Zoea I to III) and post-larva of *Homarus gammarus* reared at 16, 19, 21 and 23ºC (Mean value SD).

| | Zoea I | | | Zoea II | | | Zoea III | | | PL | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Temp. (ºC) | N | Mean | SD | N | Mean | SD | N | Mean | SD | N | Mean | SD |
| 16 | 5 | 3.04 ± | 0.07 | 5 | 3.75 ± | 0.19 | 5 | 4.71 ± | 0.18 | 2 | 4.91 ± | 0.01 |
| 19 | 5 | 3.10 ± | 0.08 | 5 | 3.66 ± | 0.08 | 5 | 4.59 ± | 0.12 | 5 | 5.13 ± | 0.23 |
| 21 | 5 | 3.08 ± | 0.06 | 5 | 3.64 ± | 0.09 | 5 | 4.12 ± | 0.14 | 2 | 4.90 ± | 0.23 |
| 23 | 5 | 3.00 ± | 0.07 | 5 | 3.57 ± | 0.10 | 3 | 4.27 ± | 0.22 | 2 | 4.93 ± | 0.09 |

For Zoea I, the highest SGR observed was 19ºC, 8.17 % $d^{-1}$, which presented significant differences from the others temperatures (p-value<0.05). One the other hand, the lowest value was set at 16ºC, 5.07 % $d^{-1}$, presenting significant differences (p-value<0.05) when contrasting to the remaining temperatures.

Finally, no differences (p-value>0.05) were observed between 21ºC and 23ºC (Fig. 13). In Zoea II temperature 23ºC provided the best SGR, 5.91 % $d^{-1}$, and significant differences (p-value<0.05) were detected when comparing with the other temperatures. The lowest value was set at 19ºC, 2.56 % $d^{-1}$, with significant differences (p-value<0.05) from the remain treatments.

Concerning to the temperatures 16ºC and 21ºC, no significant differences were detected (p-value>0.05). The last larval stage evaluated, namely Zoea III, revealed no statistical significant differences (p-value>0.05) between 16ºC, 19ºC and 23ºC, and also between 19ºC, 21ºC and 23ºC (p-value>0.05). The highest SGR in Zoea III was set at 21ºC, 2.12 % $d^{-1}$, contrasting with 16ºC, 0.40 % $d^{-1}$, which presented the lowest value. Moreover, when comparing both temperatures (i.e., 21ºC and 23ºC), significant statistical differences were observed (p-value<0.05; Fig. 13).

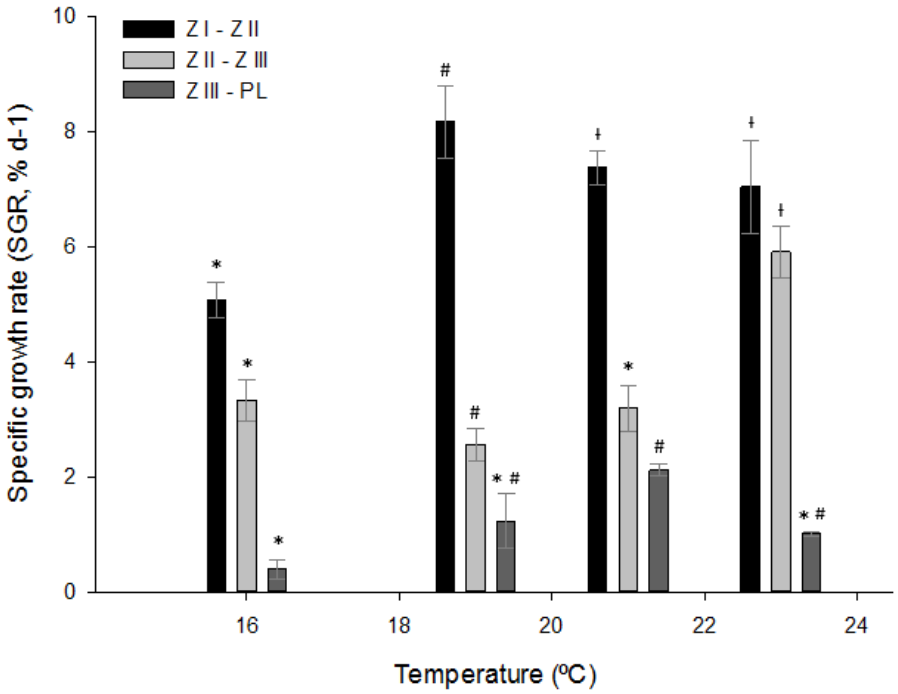


Figure 13 – Specific growth rate (SGR, % $d^{-1}$) for the tested temperatures of the different larval stages (mean values and respective SD represented by vertical lines). The symbols *, # and □ represent statistical differences between temperatures for a given larval stage.

### 4.4.3. Intermoult Period (IP) – Comparison between larval stages

For Zoea I, the results shows significant differences, when compared the temperature 16ºC with the others (p-value<0.05, Fig. 14).

At this temperature, the IP was equal to 4.77 days being the lowest noted as 23ºC (3.02 days).No significant differences were observed for the pairwise 21º and 23ºC as well for 19º and 21ºC (p-value>0.05; Fig. 14).

Concerning to Zoea II, the highest and the lowest IP were verified at the same temperatures of Zoea I: 5.14 days and 3.67 days, respectively at 16ºC and 23ºC (Fig. 14).However only the IP from 16ºC presented significant differences (p-value<0.05) from the remaining temperatures. No statistical differences (p-value>0.05) were observed between 19ºC, 21ºC and 23ºC.

In the last larval stage evaluated, Zoea III, no significant differences (p-value>0.05) were observed among all the tested temperatures.

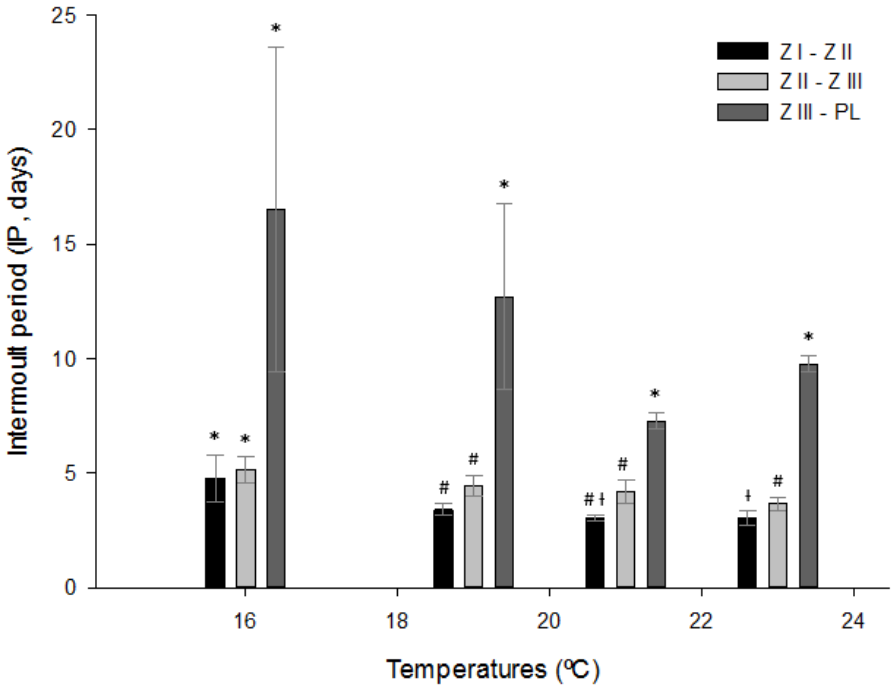


Figure 14 – Intermoult period (IP, days) for the temperatures tested of the different larval stages, the vertical lines corresponds to the SD. The symbols *, # and □ represent statistical differences between temperatures for a given larval stage.

### 4.4.4. Belehradek's equation

The equations relating intermoult period and temperature for a given larval stage are described in table 8. The results clearly show that the IP decreases with the increase of temperature for all larval stages (Fig. 15).

The relationship observed between IP and temperature for Zoea I and Zoea II were very similar (Fig. 15a and 15b), contrasting with the results obtained in Zoea III (Fig. 15c), with higher IP values comparing with previous stages. It is also observed that the development time increases with the progression of larval stages.

Belehradek's equation showed good fit for stages Zoea I and II (Table 8), however Zoea III stage duration was much more variable than other stages.

Table 8 - Equations relating stage duration and temperature for *Homarus gammarus* larvae; IP = intermoult period and T = temperature.

| Stage | Equation | $R^2$ |
|---|---|---|
| Zoea I | $IP_{ZI} = 1423.84 \times (T + 3,57)^{-1.91}$ | 0.91 |
| Zoea II | $IP_{ZII} = 586.83 \times (T + 8.40)^{-1.47}$ | 0.99 |
| Zoea III | $IP_{ZIII} = 552,11 \times (T - 4,76)^{-1.45}$ | 0.80 |

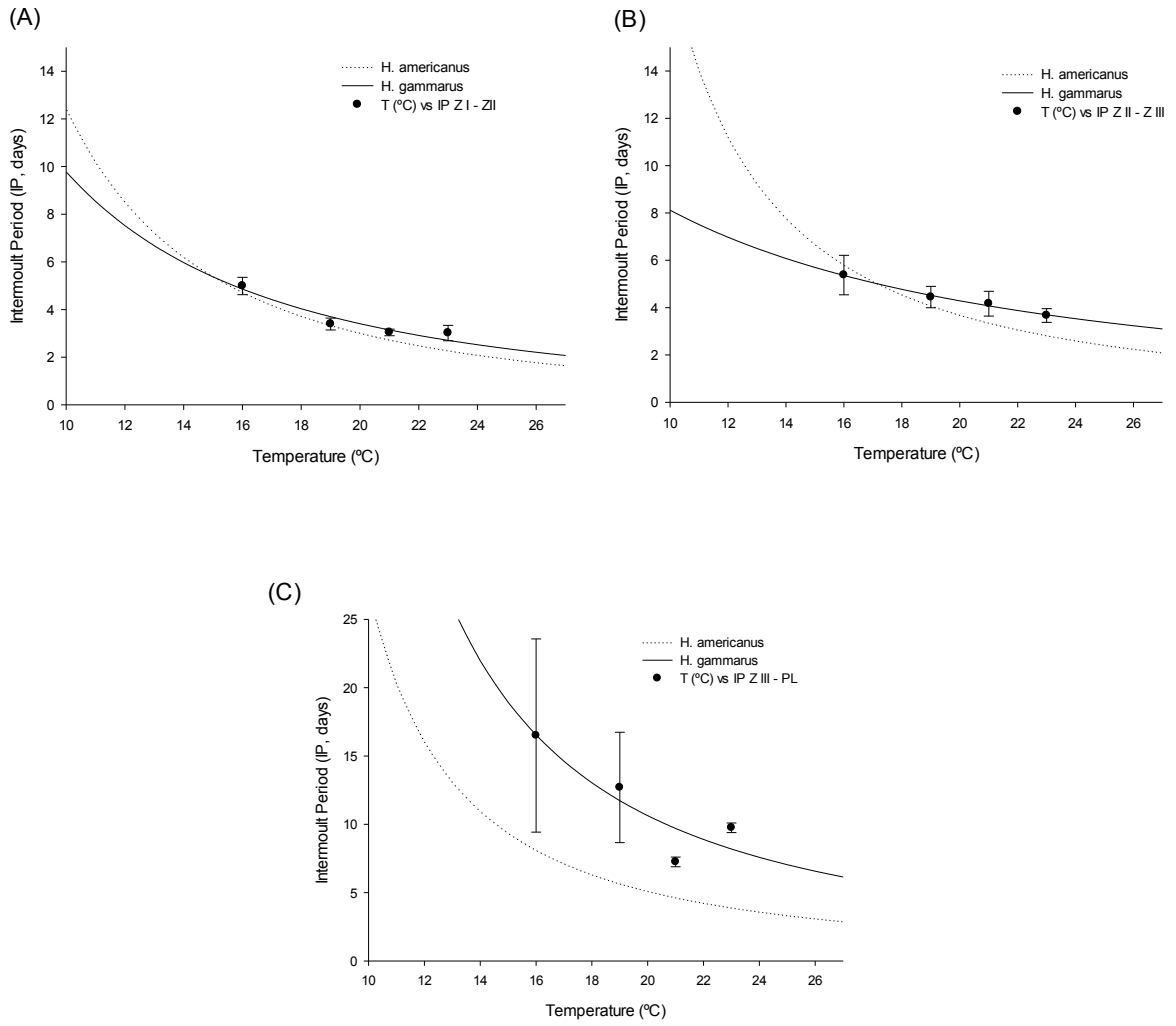


Figure 15 – Representation of relation between temperature and IP, with curve fit applying Belehradek's equation to the data. (A) Graphic of Belehradek's equation applied to data from Zoea I. (B) Graphic of Belehradek's equation applied to data from Zoea II. (C) Graphic of Belehradek's equation applied to data from Zoea III. Spotted curve was obtained from equations obtained by Mackenzie (1988) in *H. americanus*, the full curve is from the equations obtained in this study.

## 5. Discussion

Nutrition is one of the most important aspect in aquaculture, especially in the early development stages (Shields et al. 1999; Rajkumar & Kumaraguru-vasagam 2006). Lipids are particularly important in larval development, not only for supplying energy, but also for providing essential poly- and highly unsaturated fatty acids (PUFA's and HUFA's), such as ARA, EPA and DHA (Shields et al. 1999).

The unsaturated fatty acids previously mentioned are fundamental to normal cell membrane function, membrane fluidity and production of eicosanoids, biochemical compounds associated with several physiological processes (Brett & Müller-Navarra 1997; Shields et al. 1999; Phillips 2006; Arts et al. 2009).

These physiological processes include reproduction, egg production, egg laying, spawning and hatching, development and normal physiology activity of the nervous system and eye tissues, growth, survival and immunologic enhancement, provide good pigmentation and successful metamorphosis. (Brett & Müller-Navarra 1997; Evjemo et al. 2003; Arts et al. 2009; Gendron et al. 2013).

Several studies suggested that, triglycerides (TAG) content, influences settlement behaviour or habitat selection in bivalves and crustaceans, however, no studies have investigated the effect of essential fatty acids (EFAs) on settlement behaviour in marine invertebrates, therefore is likely that EFA levels in the diet influence the settlement behaviour of invertebrate larvae (Thériault & Pernet 2007).

Several studies showed that the FA composition of crustaceans and bivalves closely matches that of their diets (Brett & Müller-Navarra 1997; Arts et al. 2009). The fatty acid analysis showed that the DC, COP and DWZ samples were the most similar to the Zoea I larval stage, however these three samples were deficient in oleic acid and ARA, but very rich in EPA and DHA, two of the most important HUFA's (Arts et al. 2009).

Many studies have been made to several copepod species found in wild zooplankton (such as *T. longicornis*, *C. finmarchicus*, *Eurytemora sp.*, *C. simillimus*, *C. propinquus*, *C. finmarchicus*, *C. helgolandicus* and *P. elongates*), which evaluate their lipid

content, found that the predominant FAs are the palmitic acid, EPA and DHA (Kattner & Krause 1989; Saito & Kotani 2000; Evjemo et al. 2003; Kattner et al. 2012).

On the other hand, the diets LAN and DAN exhibited deficiencies in EFA's, ARA, EPA and DHA, compensating with high percentages of oleic acid. Brine shrimp nauplii normally have very low EFA's content, particularly DHA, and the amount of HUFA's is variable between cyst stocks, making the enrichment required to obtain adequate HUFA levels (Sorgeloos et al. 2001; Castañé et al. 2006; Rajkumar & Kumaraguru-vasagam 2006).

Although the obtained results in this experiment were very similar to previous findings, it is important to notice that the fatty acid analysis was made only in one sample of each diet, therefore, statements made must be interpreted carefully.

The results of the first trial tested for food effect on survival showed that the best diet was LAN for this type of cultivation in closed recipients without water agitation. This indicates that the best option to this methodology of *Homarus gammarus* larval rearing is the inclusion of live food. It is possible to say that, despite of the inert food being inside the rearing container, it was not available to larvae.

The inability of defined swimming, incapacity to search for food, the lack of water agitation and the natural settle tendency of larvae (Fiore & Tlusty 2005) strongly reduce the encounter probabilities between prey and predator, explaining the results.

Turbulence was reported to play an important role in the feeding of a number of zooplanktonic species by raising the encounter rate between predators and preys (Dower et al. 1997; Bermudes & Ritar 2008). In the experimentations developed by Kristiansen et al. (2004), 40 L upstream incubators were used, with water recirculating at 18-20 ºC, with 38 larvae $L^{-1}$, feed with frozen brine shrimp and frozen mysis, larvae reached PL development stage in 12 days (survival data not available).

Gendron et al. (2013) reported that the diet of natural zooplankton in the hatchery of *H. americanus* larvae from the Zoea I to PL can significantly increase the lipid content and modify the fatty acid composition, higher energy reserves (TAG and neutral lipids), better condition index, higher levels of essential fatty acids (ARA, EPA, DHA) in cell membranes of the larvae, compared to larvae fed with usual diet of live artemia. Their

study also showed that for a similar survival, better growth performance was obtained in hatchery and *in situ* with a natural zooplankton diet.

The best option is the use of live feeds, using a mixture of copepods and brine shrimp nauplii, fulfilling the nutritional requirements of larvae and making food more available to larvae, combining the results obtained, from fatty acid analysis and the food effect trial, and using the same methodology, individualized larvae in closed containers without water agitation. To this extent, the development of individual containers with water agitation and/or circulation, reducing the maintenance and maintaining water quality, is the next step, maximizing survival and growth rates. However, the development of such equipment's is a complex and laborious task.

Probiotics can protect their host against the surrounding environment or invading pathogens, by interfering with their cellular functions through production of metabolites that inhibit the colonization or growth of other microorganisms or by competing for resources, such as nutrients or space (Vine et al. 2006).

The appropriate selection and colonization methods are two of the most challenging tasks in developing probiotic bacteria (Gullian et al. 2004). Several selection steps have been defined, however many failures in probiotic research are attributed to inadequate selection of microorganisms and they need to be adjusted for different host species and environments (Gomez-Gil et al. 2000).

Probiotics can be provided by several ways, such as addition via life food (brine shrimp and rotifers) or artificial diet (pellets), bathing, addition to culture water and injection (Irianto & Austin 2002; Balcázar et al. 2006). Probiotics should be administrated at an early age of the host, being easier to manipulate and improve the indigenous gut micro flora (Rengpipat et al. 2000; Balcázar et al. 2006).

It was clear in this study that the addition of *Lactobacillus plantarum* in rearing water did not have any benefit in *Homarus gammarus* cultivation. The ineffectiveness of these bacteria can be explained by several factors. One factor that can explain such results, is that the specie used in the experiment wasn't capable of provide greater survival and growth rates, and faster development in *Homarus gammarus* larvae.

Lactic acid bacteria, such as *L. plantarum*, are rarely isolated from larvae of aquatic organisms, because, incubation temperature, incubation time and lack of glucose in the medium are limiting factors for its growth, being the last two the most important for some strains, since their growth rate is slow and require some specific nutrients, such as sugar and vitamins (Farzanfar 2006).

Despite these limitations, Gatesoupe (1991) reported the benefit of using *Lactobacillus plantarum* and *Lactobacillus helveticus* in turbot. *Scopthalmus maximus*. Nikoskelainen et al. (2003) showed that the administration of *Lactobacillus rhamnosus*, at a level of $10^4$ CFU $g^{-1}$ to $10^8$ CFU $g^{-1}$ of feed, improved immune parameters in rainbow trout. Although, Gildberg et al. (1995) did not find any improvement in using lactic acid bacteria in *Salmo salar* fry, challenged with *A. salmonicida*.

Another explanation for the results can be related with the administration method used in the trials, where probiotic was directly added to rearing water and the bacteria concentration used in the water was $10^5$ CFU $mL^{-1}$. The methodology applied wasn't capable to fulfil the objectives, since the administration method applied wasn't efficient in colonizing both water and digestive tract of larvae and the concentration used was too low and incapable to overcome harmful threats.

Rengpipat et al. (1998) used *Bacillus* S11 as a probiotic, via feed with a concentration of ~$10^{10}$ CFU $g^{-1}$, in *P. monodon* and showed that the bacteria was able to colonize the culture water and the shrimp digestive tract, increasing their survival. However, Shariff et al. (2001) found that using a commercial *Bacillus* probiotic in cultivation water, $10^3$-$10^4$ CFU $mL^{-1}$, of *P. monodon* larvae did not increase significantly the survival of shrimp.

To directly evaluate and compare studies using probiotics is not easy, because the efficacy of their application depends on many factors, such as environmental conditions, species composition and interactions, probiotic application level and frequency of application (Gatesoupe 1999; Gomez-Gil et al. 2000; Verschuere et al. 2000; Bomba et al. 2002). This means that the application method and the concentration administrated are only two factors promoting larval growth and survival (Zhou et al. 2009). A reduction of *in vivo* mortality in one system does not necessarily indicate a universal effect (Li et al. 2006).

In the probiotic trial was used just one bacteria specie as probiotic but no improvement in growth, survival or reduction in larval development was observed. Studies indicate that the use of a mixture of bacteria can be more effective (Farzanfar 2006; Kesarcodi-Watson et al. 2008), resulting in greater growth rates, survival, feed conversion ratio (FCR) and protection against diseases (Balcázar 2003; Balcázar et al. 2006).

Balcázar (2003) showed that the administration of *Bacillus* and *Vibrios sp*. had positive effects in growth and survival rates of juveniles of *Litopenaeus vannamei* and conferred protective characteristics against *Vibrio harveyi* and white spot syndrome virus (WSSV). This protection was due to a stimulation of the immune system, by increasing phagocytosis and antibacterial activity (Balcázar et al. 2006).

Prebiotics, jointly with probiotics, have been used to maximize aquaculture production, and these feed components, indigestible by the host, are added to feed providing beneficial consequences to the host, because it selectively stimulates metabolism, growth and/or activity of bacteria present in gastrointestinal tract, thus improving host health. (Yousefian & Amiri 2009).

Daniels et al. (2010) tested the effect of brine shrimp enriched with *Bacillus sp*. and mannan oligosaccharides (MOS) together, separated and un-enriched brine shrimp, in *H. gammarus* larval development. The study not only showed survival improvement but also greater growth parameters, such as FCR, SGR, live weight gain, weight/carapace length ratio and moulting condition, when combined both enrichments.

The aquatic candidate probiotics for larviculture have been isolated from adults and healthy larvae, and it's been suggested that their efficiency is likely to be highest in the host species from which they were isolated (Vine et al. 2006). However, in this study, the bacteria used as probiotic, wasn't isolated from *H. gammarus* adults or larvae, indicating that *L. plantarum* may not be the best option.

Considering the results obtained in the probiotic experiment, it is necessary to adjust the procedures used in the study. It is important to evaluate the effect of *L. plantarum* applying a different methodology. It would be interesting to test different concentrations using these bacteria and compare the method used in the study with a different one, as well as the use of live feeds enriched with probiotic.

Another approach that can be tested, is to experiment different bacteria, such as *Bacillus sp*., and/or combine more than one probiotic. The isolation, identification, modes of action and effect in host of bacteria collected from digestive tract of *H. gammarus* is another methodology that can result in interesting findings.

The temperature is one of the principal factors affecting hatching success, growth rates, larval development rates, metamorphosis and survival, and the responses among crustacean species is very similar (Mene et al. 1991; Fiore & Tlusty 2005; Bermudes & Ritar 2008; Johnston et al. 2008; Drengstig & Bergheim 2013).

The survival of decapod larvae commonly declines at the lower and upper extremes of a species specific temperature tolerance range (Bermudes & Ritar 2008). Temperature manipulation, combined with photoperiod, are particularly important for maximizing growth rate, achieving market size in the shortest period possible (Bermudes & Ritar 2008; Johnston et al. 2008).

Survival obtained in the IBM experiment revealed an overall decreased in survival rates with larval development, the same results were reported by Schmalenbach & Franke (2010). However, at a temperature of 23ºC, this tendency was not observed, survival rate had an accentuate decrease between Zoea II and Zoea III, followed by an increase between Zoea III and PL.

The oscillations in survival rates between larval stages at 23ºC are explained by an abrupt reduction of larvae number, 32 larvae reached Zoea II but only 3 reached Zoea III, and just 2 have made metamorphosis to PL stage. However, it is important to notice that the stage specific survival rate was quantified but considering survival rate during all larval development, all temperatures had the same behaviour, decreasing with larval development.

The survival rate results also showed that a temperature of 19ºC had the highest survival rate, leading to 5 larvae reaching PL stage, contrasting with 2 larvae metamorphosing in the remaining temperatures. These survival rates indicate that 19ºC was the best temperature tested, being between the ideal temperature described in literature, 18-22ºC (Kristiansen et al. 2004).

However, Schmalenbach & Franke (2010) reported increasing of survival rates with increasing temperature up to 22ºC, and suggested that the upper limit of thermal tolerance must be well above of 22ºC. The same authors reported that no moults occurred at 10ºC or below, and at 11, 12 and 13ºC an increasing percentage of larvae moulted to Zoea II or even to Zoea III, but none was able to proceed to PL.

The temperature effect on larval survival was also reported to its American relative, *Homarus americanus*, but, unlike European lobster larvae, they can develop successfully at temperatures as low as 8ºC, furthermore, optimal larval survival, 11ºC, of *H. americanus* is much lower than *H. gammarus* (Mackenzie 1988; Schmalenbach & Franke 2010).

Considering all the experiments of this study, temperature, food and probiotic effect, the survival until PL stage obtained in each experiment was very low, approximately 5%. The low survival rates obtain here are not atypical for *H. gammarus* and for *H. americanus*, ranging from less than 5% to more than 90%, using different rearing methodologies (Nicosia & Lavalli 1999; Uglem et al. 2006; Contarini et al. 2008; Browne et al. 2009).

One of the reasons for these results, other than those previously mentioned, may be related to the provenience of larvae coming from one female. Uglem et al. (2006) reported the variation of survival rates in larvae from different females, and showed that survival ranged from 2.8% to 93%, being related with egg quality between different females and Vibrio sp. outbreaks, with typical abrupt mortality immediately before moulting to PL stage.

In all temperatures, the specific growth rates (SGR) decreased with the larval development, but the increase in growth rate did not follow the temperature increase. Between Zoea I and II, 19ºC provided the highest SGR, however, for the following larval stages, 19ºC provided lower SGR comparing with remaining temperatures. At 23ºC between Zoea II and III, the SGR was much higher than 16, 19 and 21ºC, however this value represents just three larvae and should be interpreted carefully. Higher SGR was expected with increased temperature, however, that was not observed in the experiment. Temperature is the primary controller of growth (Drengstig & Bergheim 2013).

Little is known about larval growth rates for clawed lobster, and even less is known about stage specific growth rates (Table 1). The variations in SGR obtained were more dependent of intermoult period, longer with larval development, than the size of larvae, which was very similar between temperatures for a given larval stage.

However, larval size can be affected by temperature, colder water, typical from offshore waters, appear to produce larger larvae than inshore waters, although this temperature-dependent size difference was not observed beyond the PL stage (Mercaldo-Allen & Kuropat 1994).

Kurmaly et al. (1990) evaluated different diets in larval growth rate of American lobster, reared in individual containers at 25ºC, ranging from 0.032 mm $d^{-1}$ to 0.114 mm $d^{-1}$, from surviving larvae after 16 days feed with six different diets. Daniels et al. (2010) evaluated the probiotic effect, added to live feeds, in larval development of *H. gammarus,* at 18.5±1 in 80 L upwelling cylindrical conical tanks, and obtained SGR ranging 4.25±0.2 % $d^{-1}$ and 6.59±0.1 % $d^{-1}$, from Zoea I to PL stage.

The intermoult period (IP) results showed, for all temperatures tested, that IP increases with the larval development, as Beard et al. (1985) described. Additionally, the IP decreased with temperature increasing, but this was not true between Zoea III and PL at 23ºC, showing higher IP than 21ºC, however no significant differences were observed between all temperature between Zoea III and PL, due to great SD in each temperature and the few individuals reaching PL stage. As previously referred, higher temperatures result in faster growth and larval development, translating in lower IP's.

Nicosia & Lavalli (1999) presented the typical stage specific development time for *H. gammarus*, showing for Zoea I IP of 3, 3 and 2 days, for Zoea II 4, 4 and 3 days and for Zoea III 5, 4, 4 days for 16, 20 and 22ºC, respectively, corresponding to 12, 11 and 9 days of total development time for 16, 20 and 22ºC, respectively.

Schmalenbach & Franke (2010) tested the temperature effect on larval development showing for Zoea I IP of 4.4±0.3, 3.3±0.7 and 3.0±0 days, for Zoea II 6.2±1.0, 4.7±1.4 and 3.7±1.0 days and for Zoea III 5.2±1.8, 6.4±1.1, 7.1±2.6 days for 16.0±0.3, 19.9±0.6 and 22.0±0.7 ºC, respectively, corresponding to 19.7±2.0, 14.4±1.5 and 13.5±2.8 days of total development time for the same temperatures, respectively.

The Belehradek's equation described the curvilinear relationship between temperature (ºC) and stage specific IP (days), showing that intermoult period is closely related with temperature. The equation produced a good fit to results and good growth model correlating the temperature effect in larval development time, however, the model formulated need further and deeper testing.

For a better larval development model it is fundamental to test lower and higher temperatures and smaller gaps between temperatures, formulating a more accurate growth model. However, using this model it is possible to predict the larval responses to temperature changes.

Little was developed in larval growth models in decapods, and just a few studies were made to define temperature-dependent larval growth models for clawed lobster. Mackenzie (1988) evaluated the temperature effect on larval development of *H. americanus*, using the Belehradek's equation, formulating a model of larval development, with similar findings; however, the ideal growth temperatures obtained for *H. americanus* were lower.

The equation comparison between the study of Mackenzie (1988) and this study, showed that the intermoult period for the same temperatures of *H. gammarus* is higher than *H. americanus*. Just in Zoea II at 16ºC, the IP of European lobster is slightly lower than American lobster, however, the difference is really low and can be considered negligible.

For *Homarus gammarus*, Schmalenbach & Franke (2010), using a different equation, found similar responses of larvae to temperature, but tested lower temperatures, and concluded that larvae grew better and had higher survival rates at 22ºC, not the same results obtained here, but with similar intermoult period behaviour, decreasing with temperature increase.

The same kind of study was made to spiny lobster, *Jasus edwardsii*, and similar findings were obtained. However, the study was applied just to one larval stage of spiny lobster due to greater larval development time, comparing with European lobster (Bermudes & Ritar 2008).

## 6. Conclusions and further perspectives

Fatty acid analysis made to the different diets tested in the food experiment showed that the copepods and the wild zooplankton are very similar to Zoea I larvae, being the ideal diets for larval rearing of *Homarus gammarus*. Despite of showing shortage of oleic acid, these diets fulfil all the larval need of essential fatty acids, EPA and DHA. However it will be interesting in further studies to test the effect of different microalgae in copepods fatty acid content, evaluating their effect in larval development of decapods, such as European lobster. The inclusion of alternative species of copepods would also be interesting, such as *Acartia clausi* and species belonging to *Calanus* genus, like *C. helgolandicus*.

Food experiment clearly showed that live feeds are the best option for larval development of *Homarus gammarus* reared in individual containers without water circulation and agitation. Despite copepods and wild zooplankton having the best fatty acid content, the lack of water agitation and/or circulation and the biological characteristics of European lobster larvae made these food items unavailable, resulting in total mortality of larvae fed with copepods and zooplankton. Using the fatty acid analysis and the results from food experiment, the ideal feed is live copepods and brine shrimp combined, fulfilling all fatty acid requirements, however further investigation and experiments are necessary.

The use of probiotics have been very promising in decapod larval rearing, maximizing survival and growth rates, however, such result were not observed in this study, without any differences between treatments, making the methodology used ineffective. Nevertheless, it is fundamental to test different methodologies, adjusting the procedures, testing different probiotics and applying different administration techniques.

The temperature experiment showed that *Homarus gammarus* grows best at 19ºC, with greater survival among tested temperatures, however all temperatures showed low survival rates. This experiment also showed a decrease in growth rates with larval development at all temperatures, and no increase at higher temperatures.

Larval development analysis showed high relationship between intermoult period and temperature, and faster larval development with temperature growth for all larval stages. The relation also showed that intermoult period increase with more advanced larval stages. The intermoult period comparison between *H. gammarus* and *H.*

*americanus*, using Belehradek's equation, revealed similar larval development in both species of clawed lobster.

The evaluation of temperature effect on larval development of *H. gammarus* made in this study revealed good data for formulation of growth model for this species; nevertheless, further validation is needed to obtain more accurate models.

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# 8. Appendix

## 1. Eye index

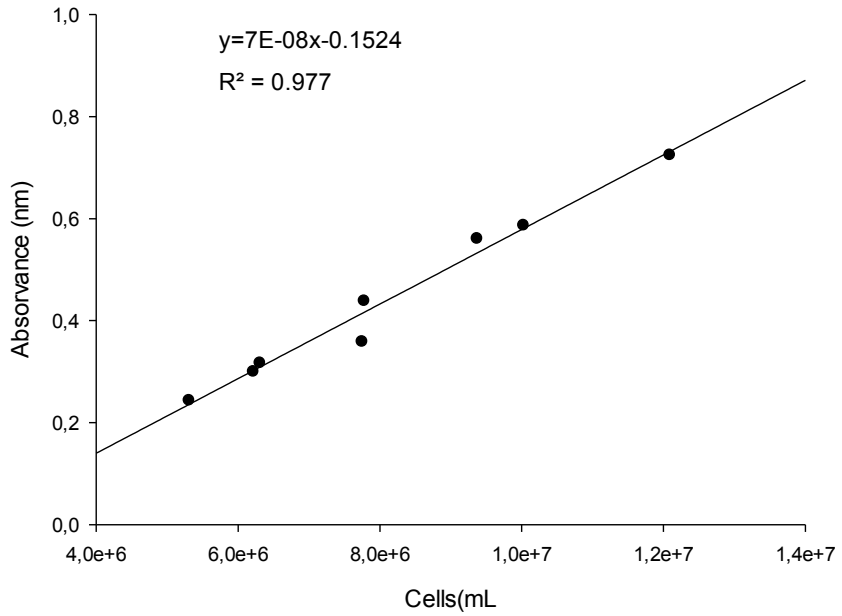
Embryonic development is temperature dependent. The approximate time to hatch may be predicted at known temperatures by observing the Embryonic Eye Index (Browne et al. 2009; Beard et al. 1985). This is done by periodically measuring the increase in size of the oval embryonic eye in a small sample of eggs.

$$\text{Embryonic Eye Index (µm)} = \frac{\text{(length + width of eye pigment)}}{2}$$

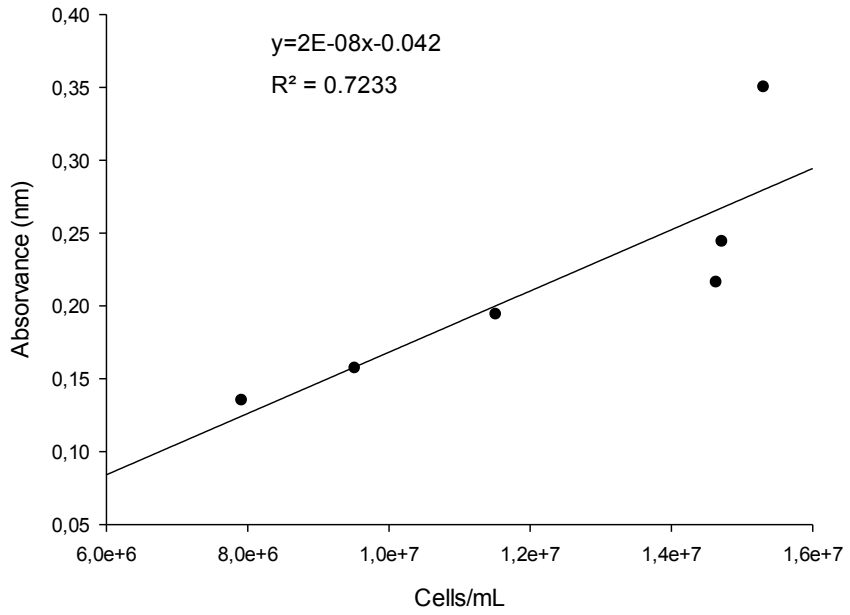
An eye condition index of 600 to 620 µm represents complete embryonic development for *H. gammarus* (Beard et al. 1985).

## 2. Relation between cells count and optical density

A – *Isochrysis galbana*



B – *Rhodomonas lens*




**3. Protocol of decapsulation of Artemia**

a. Artemia cysts were placed in a container with fresh water, with strong aeration, for 60 minutes.

b. The hydrated cysts were screened through a sieve with a mesh of 150 µm.

c. Was prepared a solution with saltwater and commercial bleach, 1:1.

d. Cysts were placed in the previous solution, with strong aeration. When the cysts change the color from brown to orange color, indicating the beginning of decapsulation. When aeration is removed and the cysts settle the decapsulation is finished.

e. The solution was sieve with a mesh of 150 µm. The cysts were washed with fresh water until the odor of bleach was gone.

f. Cysts were placed in fresh water with 0.1 mL of sodium thiosulfate and with strong aeration, to neutralize sodium hypochlorite residues.

g. The cysts were again riddled with 150 µm sieve and washed.

h. The cysts were placed in a bottle to hatch, 1.5 L, at 28-30 °C and salinity 35 for 24 hours.

**4. MRS broth preparation**

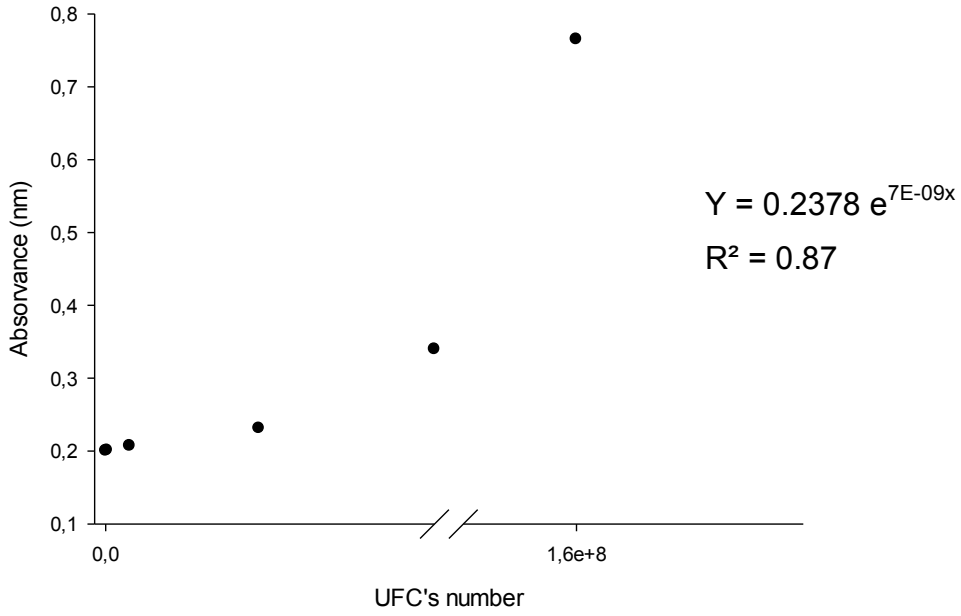
a. MRS broth was weight, 13.025 g (corresponding to a concentration of 52.1 g/L) and was placed in 250 mL Erlenmeyer.

b. It was added distilled water, performing 250 mL of total volume, and mixed.

c. The Erlenmeyer with MRS broth was autoclaved (Trade Raypa), during 15 minutes at 121ºC.

d. After cooling, the Erlenmeyer was inoculated with *L. plantarum*.

**5. Growth equation from *L. plantarum***



**6. Fatty acid analysis and GC equipment**

After weighing the samples, these were placed in glass test tubes, with lid, and accomplished the modified method of Bligh and Dyer. Was added 2.5 ml of methanol (Carlo Ebra Reagents) and acetyl chloride (Scharlau) (20:1) to each tube, followed by a 30 second vortex cycle to homogeneity and 10 minutes in ultrasound (VWR, Ultrasonic Cleaner). The test tubes were placed in a water bath (Memmert), 80°C for 60 minutes. After incubation, the tubes were cooled on ice, then added 1 mL of MiliQ water (Fisher Scientific) and 2 ml of n-heptane (Scharlau) in each tub test, followed by vortex cycle for 1 minute. Then the test tubes were centrifuge (Centrifuge 5804 R, Eppendorf®), for 1500 G's for 10 minutes at 4°C. Then the organic phase, upper phase, was removed from each sample and filtered through a column of anhydrous sodium sulfate (Panreac), to remove residual water, and placed in vials of gas chromatography (GC).

The samples were injected into the (GC) (Finnigan TRACE GC Ultra, Thermo Electron Corporation®) equipped with a column TR-FAME (30mx0.25mm IDx0.25m), an auto sampler AS 3000 Thermo Electron Corporation, and a flame ionization detector. The injector operating in split less mode, and the detector were programmed to 250ºC and 260ºC, respectively. Helium was used as carrier gas at a flow rate of 1,5 $mL.min^{-1}$. Air and hydrogen were supplied to the detector, at a flow rate of 350 $mL.min^{-1}$ and 35 $mL.min^{-1}$, respectively. The oven temperature was set to have a slow growth, increasing to 60ºC for 1 min, increased to 150ºC at 15ºC $min^{-1}$ and held in level for 1min. Further increase to 180ºC, 5ºC $min^{-1}$, where it remained 3 min, a further increase in temperature up to 220ºC, 10ºC $min^{-1}$ and a landing late this temperature for 1min.

The retention time of each fatty acid was identified by comparison with the retention time of standard commercial [PUFA-1 Marine Source and PUFA-3 Menhaden oil (Supelco Analytical)].

## 7. Chromatograms from fat acid profile

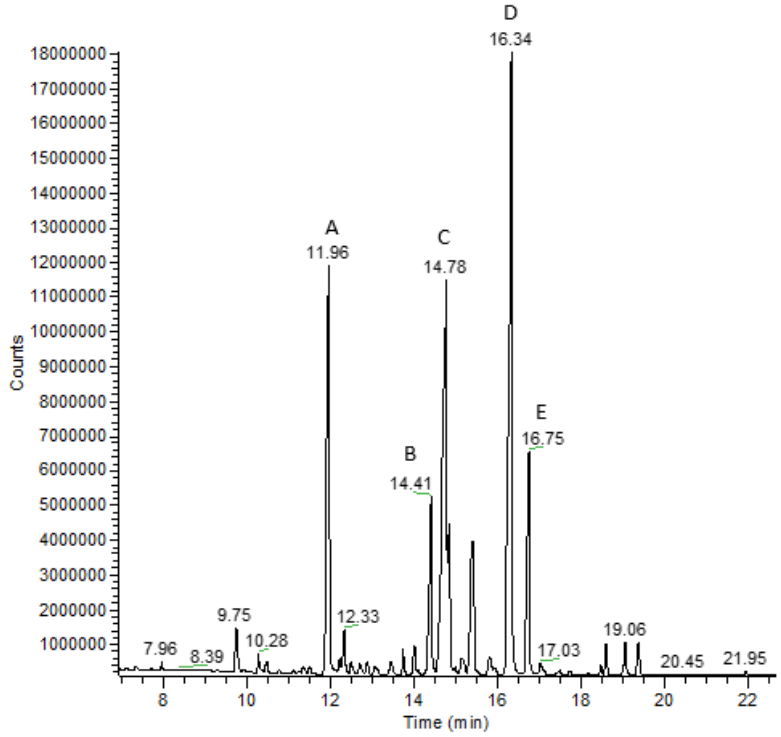



Figure 16 – Chromatogram with spectrum of GC reading from LAN diet. The letters indicate the highest peaks. A: palmitic acid; B: stearic acid; C: oleic acid; D: parinaric acid; E: eicoseic acid.

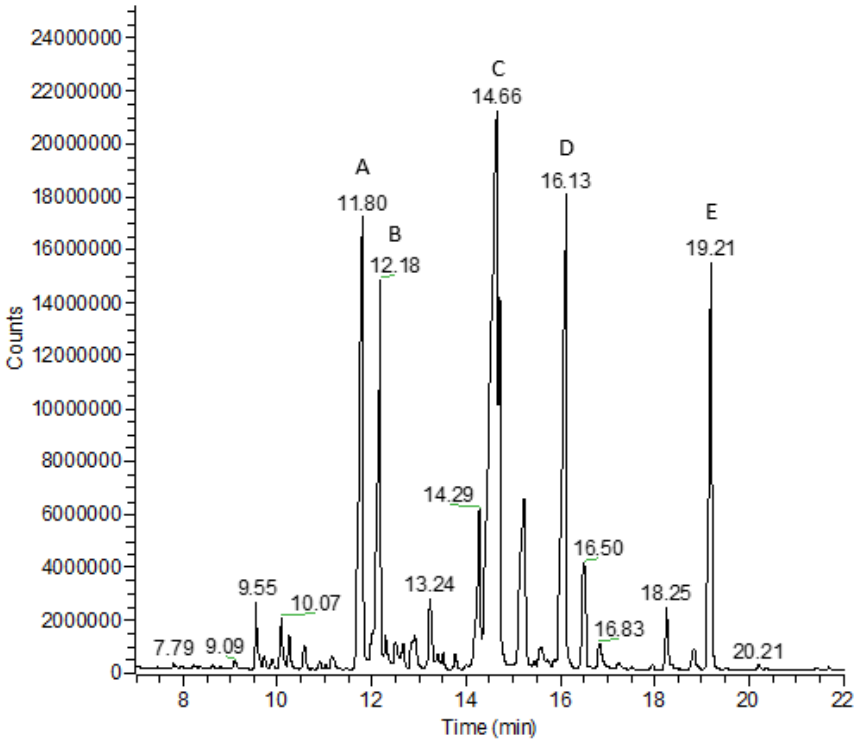



Figure 17 - Chromatogram with spectrum of GC reading from DAN diet. The letters indicate the highest peaks. A: palmitic acid; B: palmitoleic acid; C: oleic acid; D: parinaric acid; E: eicoseic acid.

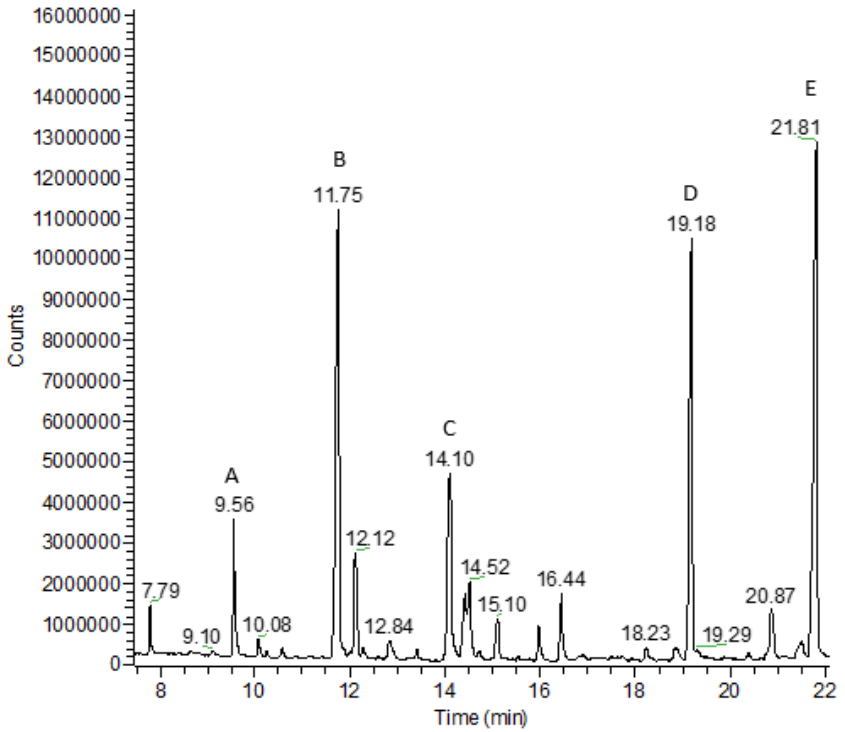



Figure 18 - Chromatogram with spectrum of GC reading from DC diet. The letters indicate the highest peaks. A: myristic acid; B: palmitic acid; C: stearic acid; D: EPA; E: DHA.

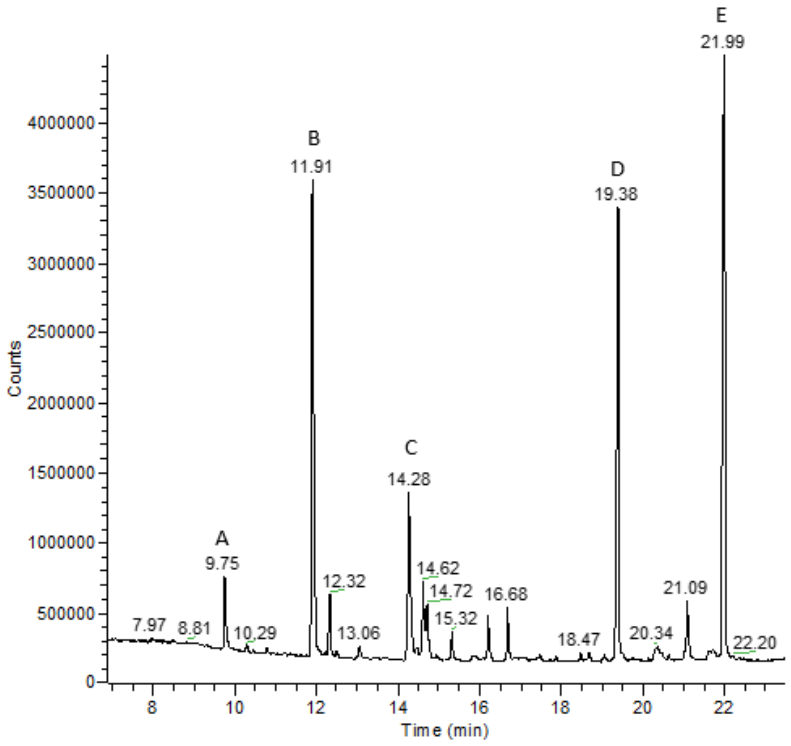



Figure 19 - Chromatogram with spectrum of GC reading from copepods. The letters indicate the highest peaks. A: myristic acid; B: palmitic acid; C: stearic acid; D: EPA; E: DHA.

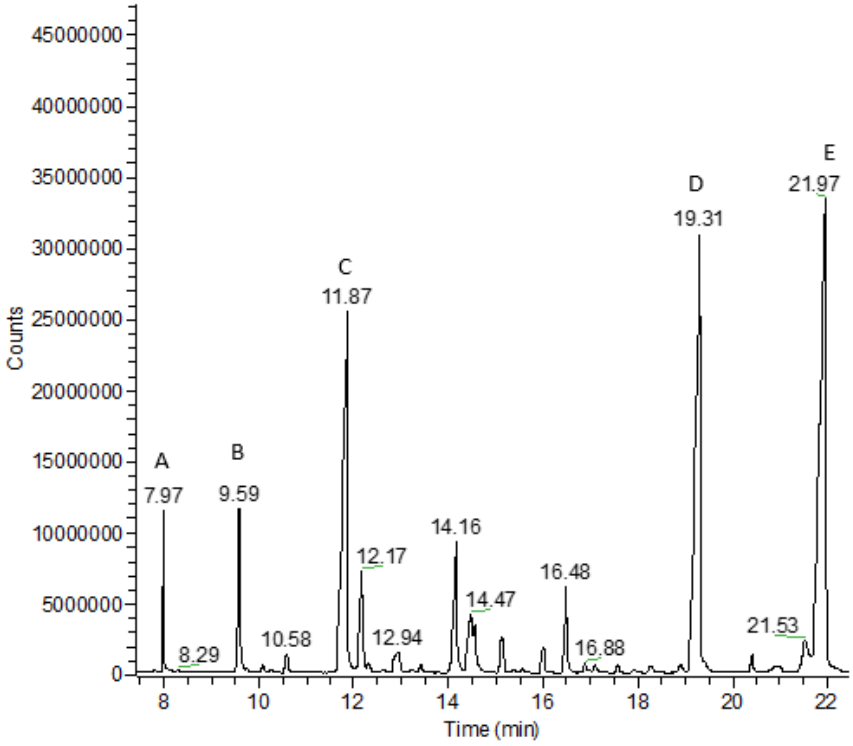



Figure 20 - Chromatogram with spectrum of GC reading from DWZ diet. The letters indicate the highest peaks. A: lauric acid; B: myristic acid; C: palmitic acid; D: EPA; E: DHA.

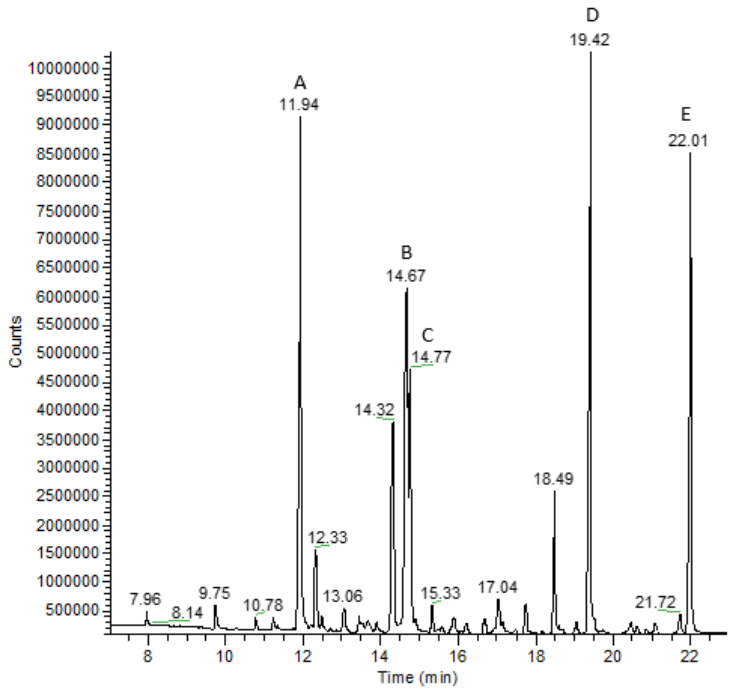



Figure 21 - Chromatogram with spectrum of GC reading from Zoea I larval stage. The letters indicate the highest peaks. A: palmitic acid; B: oleic acid; C: Vaccenic acid; D: EPA; E: DHA.

## 7. Fat acid identification

| Number of carbons and double bonds | Systematic name (common name) |
|---|---|
| C 12:0 | Dodecanoic acid (lauric) |
| C 14:0 | Tetradecanoic acid (myristic) |
| C 14:1 | Tetradecenoic acid (myristoleic) |
| C 16:0 | Hexadecanoic acid (palmitic) |
| C 16:1 | Hexadecenoic acid (palmitoleic) |
| C 18:0 | Octadecanoic acid (stearic) |
| C 18:1 n9 | Octadecenoic acid (oleic) |
| C 18:1 n7 | Vaccenic acid |
| C 18:2 n6 | Linoleic acid |
| C 18:3 n3 | Octadecatrienoic acid (linolenic, LA) |
| C 18:4 n3 | Octadecatetraenoic acid (parinaric) |
| C 20:1 n9 | Eicosenoic acid |
| C 20:4 n6 | Eicosatetraenoic acid (arachidonic, ARA) |
| C 20:5 n3 | Eicosapentaenoic acid (timnodonic, EPA) |
| C 22:6 n3 | Docosahexaenoic acid (DHA) |
| C 24:1 n9 | cis-Tetracosenoic acid (nervonic) |