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Progressing to a more sustainable aquaculture: Alternative diet for european lobster *(Homarus gammarus)* juveniles

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INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR

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Dissertation for the master's degree in Marine Science – Marine Biology submitted to the Abel Salazar Institute of Biomedical Sciences from the University of Porto.

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Dionísio Sousa

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Abstract

With the increase in aquaculture production around the globe, there is a need to make aquaculture processes as sustainable as possible. One strategy is to develop more sustainable diets that promote optimal growth while reducing the use of the natural resources. European lobster *Homarus gammarus*, a species of high economic and ecological importance, is a promising candidate for aquaculture production. However, this requires the development of diets that meet its nutritional requirements. The use of new ingredients in diets for European lobster is still understudied and more knowledge is required in order to promote the development of viable and sustainable diets. This study investigates the potential use of shrimp waste meal (SWM), a by-product of shrimp processing *Pandalus borealis*, in the formulation of diets for juvenile lobsters (stage VII – VIII, ~ 164 mg). During an 8-week trial the juveniles were fed with diets with crescent levels for SWM replacement (0 %, 7 %, 14 %, 21 %, 28 %), maintaining the same protein (~ 45 %) and lipid (~ 11 %) content. To analyse the influence of the different diets on growth performance, it was analysed survival, body mass gain, carapace length increment and the duration of the moulting cycles. The pigmentation of the exoskeleton was analysed by digital colour analysis at the end of the trial. The results suggest that SWM can be introduced as an ingredient in lobster diets up to at least 28 % inclusion without compromising growth enhancement. Despite showing no influence on growth enhancement, the inclusion of 28% SWM was found to significantly improve survival rates among juveniles. As there is a decrease in mortality without affecting growth or pigmentation, the use of SWM shows itself to be a promising alternative to other ingredients commonly used for European lobster diets. The use of ingredients such as SWM may lead to the development of a more sustainable aquaculture industry with less impact on the natural environment.

Keywords: Lobster; *Homarus gammarus*; Sustainable aquaculture; Alternative protein sources; growth performance

Resumo

Com o aumento da produção em aquacultura por todo o globo acresce uma necessidade em tornar os seus processos o mais sustentáveis possíveis. Uma das possíveis estratégias passa pelo desenvolvimento de dietas mais sustentáveis que promovam um crescimento otimizado, e que diminuam simultaneamente o impacto sobre os recursos naturais. O lavagante europeu *Homarus gammarus*, espécie ecológica e economicamente importante, é uma espécie que se revela promissora para a produção em aquacultura. No entanto, para esse fim é necessária a formulação de dietas que satisfaçam os seus requisitos nutricionais. A utilização de novos ingredientes para lavagante europeu é ainda pouco estudada pelo que o aumento de investigação com esse objetivo potencia o desenvolvimento de dietas viáveis e sustentáveis. Este estudo, investiga a utilização da farinha de resíduos de camarão (SWM), derivada de subprodutos de processamento do camarão Pandalus borealis, na formulação de dietas para juvenis de lavagante (fase VII – VIII, ~ 164 mg). Durante um ensaio de crescimento de oito semanas, os juvenis foram alimentados com dietas com inclusão crescente de SWM (0 %, 7 %, 14 %, 21 %, 28 %), mantendo o mesmo conteúdo proteico (~ 45 %) e lipídico (~ 11 %). De modo a analisar a influencia das diferentes dietas no desempenho de crescimento, foi analisada a sobrevivência, ganho de massa corporal, comprimento da carapaça e a duração dos ciclos de muda. A pigmentação do exosqueleto foi avaliada por analise de cor digital. Os resultados sugerem que SWM pode ser incluído como ingrediente em dietas de lavagante até 28 % sem comprometer o aumento de crescimento. Apesar de não ter potenciado aumentos de crescimento, verificou-se que a inclusão de 28 % SWM aumentou significativamente a taxa de sobrevivência entre os juvenis. Como se observa uma diminuição de mortalidade sem afetar o crescimento ou a pigmentação, a utilização de SWM mostra-se promissora em detrimento de outros ingredientes normalmente usados em dietas de lavagante. A inclusão de ingredientes como SWM pode desempenhar um papel fundamental no desenvolvimento de uma indústria aquícola mais sustentável com o menor impacto no meio ambiente.

Palavras-chave: Lavagante; *Homarus gammarus*; aquacultura sustentável; fontes alternativas de proteina; performance de crescimento

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Introductory Note

This MSc project was conducted in ERAMSUS at the Technical University of Denmark, Section for Aquaculture, Hirtshals, Denmark. It was carried out as part of the doctoral thesis: Goncalves, R., 2021. Ontogenetic development and nutritional requirements in early life stages of the European lobster *(Homarus gammarus*, L.). Ph.D. thesis, Section for Aquaculture, National Institute of Aquatic Resources, Technical University of Denmark. This study was financially supported by NordForsk as part of the SureAqua project (grant no. 82342), the Ph.D. School at DTU Aqua, the FiskeriLAG NORD, Denmark, and the ENV Foundation, Nord Energi, Denmark.

The results of this study were used to prepare a manuscript for publication in a scientific journal:

GONCALVES, R., LUND, I., SOUSA, D., SKOV, P.V., 2021. Shrimp waste meal (*Pandalus borealis*) as an alternative ingredient in diets for juvenile European lobster (*Homarus gammarus, L*.). **Under review, Animal Feed Science and Technology**.

1. Introduction

1.1 Species Biology

1.1.1 General Biology

The European lobster (*Homarus gammarus* Linnaeus, 1758), is a marine invertebrate belonging to the Class *Crustacea*, Order of *Decapoda* and Family *Nephropidae* (Figure 1) Together with the American lobster (*Homarus americanus* Milne-Edwards, 1873), they form the only two species of the genus Homarus. The literature on the American lobster is more extensive, as opposed to the European lobster, since the last one is a more commercially exploited species. However, the two species, even though genetically different, are quite similar (Factor, 1995). Because it is a species of high market value, the European lobster has suffered in recent decades a high fishing exploitation, with consequent reduction of its natural stocks (Smith, Collins and Jensen, 1999).

Figure 1 - European lobster Homarus Gammarus.

The segmented body of lobsters is organized into three regions: head, thorax, and abdomen, with the first two fused together to form the cephalothorax. Each segment of their body holds pairs of appendages anatomically modified to perform various functions. Appendages with sensory and with feeding function are found in the anterior area of the cephalothorax. In the posterior area appear the pereiopods, appendages with locomotor function. In the abdomen appear the pleopods,

reproductive appendages (1st pair – Figure 2) and appendages with swimming function (Factor, 1995).

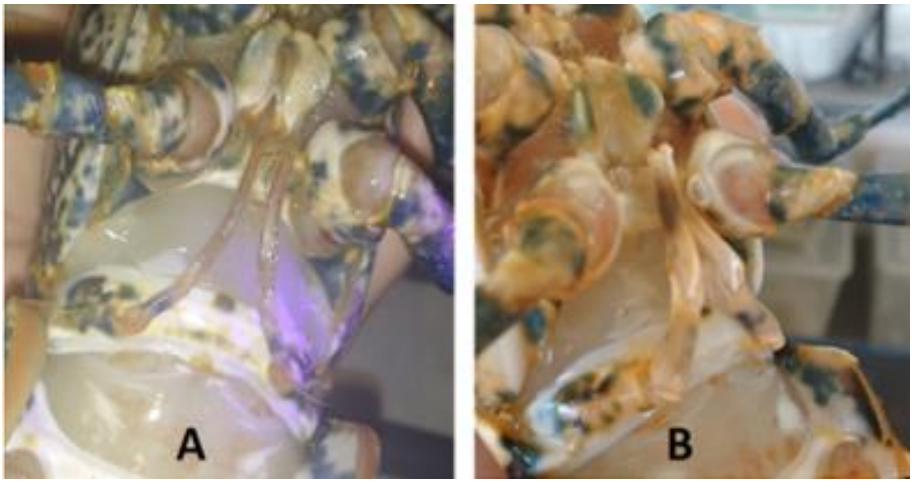

Figure 2 - Sexual dimorphism of European lobster H. gammarus. A- female, B – male.

The European lobster is geographically distributed from the Arctic Circle to the coast of Morocco. In the north it is most present in Norway, southeastern Sweden and Denmark, and is absent from the entire Baltic Sea coast. This is probably due to the different water conditions in this place, where salinity and temperature reach significantly lower values. It is also present all along the European coast, including the islands of Ireland and Britain, and north of the Mediterranean Sea. After Morocco its presence begins to decrease reaching its lower distribution limit at latitude 30º north (Phillips, 2006; Prodöhl, Jørstad and Triantafyllidis, 2006).

This species predominating habitats are coastal areas, usually with rocky or mud bottoms, and its activity is concentrated mainly at night (Moland *et al.*, 2011). They are not usually found at depths greater than 50 meters but can be occasionally observed up to 150 meters (Kristiansen, Drengstig and Bergheim, 2004). During the daylight, the lobster is predominantly restricted in its shelter, whereas during the night its activity increases, and it is during this period, that it feeds. The low daytime activity as it likely decreases the risk of predation (Smith, Collins and Jensen, 1999).

1.1.2 Reproduction

The reproductive success of the European lobster depends on its ability to find a safe and suitable shelter for mating, spawning and hatching. Generally, the search

for shelter is the responsibility of the females. Before mating with a male, the female has to look for a sheltered place to perform the moulting. If shelter places are scarce, males may compete with each other for these, in order to attract the attention of females (Debuse, Addison and Reynolds, 2003). It is thought that mating in the post-moult period is regulated by sex pheromones released by the female and detected by the male's antennae (Skog, 2009). After mating, the female will proceed with the moult and copulation will take place in the following 30 minutes, where the male will introduce his spermatophores into the ventral area of the female. The lobster couple cohabits in the shelter for one to two weeks where the male can protect the female from potential males that may approach (Atema, 1986; Debuse, Addison and Reynolds, 2003). The male spermatophores are stored by the female until they are used in external fertilization in her abdomen about 10 months after copulation (Agnalt, Kristiansen and Jørstad, 2007). The male gametes can be stored in the ventral cavity of the female without losing their characteristics for a period of two years (Waddy, S. L., 1990).

Fecundity rate is dependently related to the female size (Agnalt, Kristiansen and Jørstad, 2007; Dove Ellis, 2016; Tully, Roantree and Robinson, 2001). The number of eggs produced varies significantly between young females (that have a smaller size) spawning for the first time and large females (between 5,000 eggs and 40,000 respectively) (Agnalt, 2008).

1.1.3 Growth

The life cycle of the European lobster is long and of high complexity, involving several stages and metamorphosis. After passing through the larval stages the lobster will start its slow and physiologically demanding growth process through moulting (Factor, 1995; Phillips, 2006).

Compared to other crustaceans of the same family, the European lobster has a very short larval development (Rötzer and Haug, 2015). When the hatching of the egg occurs, the so-called pre-larvae, remain in the maternal pleopods, followed by three larval stages (stages I, II, III). In these stages the larvae are omnivorous and pelagic, and they stay floating in the water column (Figure 3) (Mehrtens, 2008; Williamson, 1969). Reaching the fourth larval stage (stage IV), the individual, already called post-larvae, begins the process known as settlement. In this process the lobster leaves the water column and becomes benthic with swimming capacity (Factor, 1995; Rötzer and Haug, 2015).

Figure 3 - European lobster H. gammarus larvae on the water column.

The larval development is completed with the transition to stage V, the first stage of juvenile development. The entire larval process is completed in a relatively short period of time, about three weeks. However, this is influenced by external factors, the most important of them being temperature. At optimum water temperature (20º-22ºC) the rate of the development is enhanced to a period of about 12 days (Prodöhl, Jørstad and Triantafyllidis, 2006).

Growth is usually defined as the accumulation of new tissue. It is a continuous process that only becomes evident in crustaceans when the animal proceeds to moult. The individual releases its exoskeleton in a process called ecdysis (Figure 4) (Phillips, 2006). The new, soft exoskeleton stretches and expands as the animal absorbs water from the outside. In this way, there is space for growth when the new shell hardens (Burton, 2003).

Figure 4 - European lobster H. gammarus preforming a moult - ecdysis.

The lobster spends most of its lifetime preparing for the next moult or recovering from the previous one. Immediately after ecdysis, the carapace is soft, and the lobster is in the most vulnerable phase (Burton, 2003). This phase is identified as Phase A of the moult. Phase B begins right after the beginning of the hardening of the carapace, these two phases being relative to the post-moult period. Phase C, inter-moult, is the period when new tissue growth takes place, ending with Phase D, pre-moult, where the individual prepares to perform a new moult. This whole process is controlled by endocrine processes not completely known (Phillips, 2006).

With aging, the moulting frequency gradually decreases. In the first year of life each individual performs this process about 10 times. At 2 and 3 years of age, the lobster changes its exoskeleton about 3 to 4 times annually. From the fourth year onwards, the changes usually occurs once a year. When they reach a large size, lobsters may change less than once a year (Burton, 2003; Phillips, 2006).The moulting process is initiated by endocrine mechanisms but is influenced by several factors such as temperature, season, photoperiod, density, habitat, nutrition or social interactions (Tremblay and Eagles, 1997; Waddy, S. L., 1990). Of these, temperature plays a preponderant influence on lobster growth. High temperatures accelerate the metabolic processes and the growth rate. The growth rate is proportional to an increase in temperature from 8º to 25º C (Aiken, 1978; Waddy, S. L., 1990).

Age determination of crustaceans represents a challenge to the scientific community because during the moulting process these individuals release their exoskeleton, making it impossible to use conventional methods to determine this factor (Wahle and , Kathleen M. Castro, 2001). The inexistence of morphological markers of age encouraged the investigation of other indicators existing in the organism of these species, as is the case of lobster. One of the most used alternatives is based on the quantification of lipofuscin, a pigment that is deposited in the tissues during growth over time (Vogt, 2012).

In addition to the evident role of increasing tissue, the moulting process has also the function of tissue renovation, as in recovery from injuries, after losing body appendages or with the parasite elimination. In each moulting process, the entire masticatory system is renewed as well as some sensory organs (Vogt, 2012). This and other strategies allow the European lobster to avoid the usual effects of aging and reach advanced ages, being one of the marine crustacean species with the greatest longevity (Sheehy *et al.*, 1999) (Table 1).

Table 1 - Life time of different marine crustaceans (Vogt, 2012).

LifeTime (years)	Specie	Infraorder, Family	Method	Reference
1,3 (month)	Lucifer faxoni Borradaile, 1915	Dendrobranchiata, Luciferidae	LR	Lee et al. (1992)
6 (month)	Thor manningi Chace, 1972	Caridea, Hippolytidae	R	Bauer (2004)
7,1 (month)	Emerita brasiliensis Schmitt, 1935	Anomura, Hippidae	RP	Veloso and Cardoso (1999)
1	Acetes chinensis Hansen, 1919	Dendrobranchiata, Sergestidae	GM-RP	Oh and Jeong (2003)
1	Charybdis bimaculata (Miers, 1886)	Brachyura, Portunidae	GM-RP	Doi et al. (2008)
1,3	Alpheus armillatus H. Milne Edwards, 1837	Caridea, Alpheidae	GM-RP	Mossolin et al. (2006)
1,7	Litopenaeus stylirostris (Stimpson, 1871)	Dendrobranchiata, Penaeidae	GM-RP	López-Martínez et al. (2005)
2	Pagurus brevidactylus (Stimpson, 1859)	Anomura, Paguridae	GM-RP	Mantelatto et al. (2005)
2	Pinnotheres tsingtaoensis Shen, 1932	Brachyura, Pinnotheridae	RP	Soong (1997)
3,3	Crangon crangon (Linnaeus, 1758)	Caridea, Crangonidae	GM-RP	Oh et al. (1999)
4	Clibanarius antillensis Stimpson, 1859	Anomura, Diogenidae	GM	Turra and Leite (2000)
4,5	Uca pugnax (Smith, 1870)	Brachyura, Ocypodidae	GM-RP	Montague (1980)
6	Carcinus maenas (Linnaeus, 1758)	Brachyura, Portunidae	R	Yamada and Kosro (2010)
8	Callinectes sapidus Rathbun, 1896	Brachyura, Portunidae	GM-MR	Rugolo et al. (1998)
9	Aristeus antennuatus (Risso, 1816)	Dendrobranchiata, Aristeidae	GM-RP	Orsi Relini and Relini (1998)
9	Cancer pagurus Linnaeus, 1758	Brachyura, Cancridae	GM-RP, LM	Sheehy and Prior (2008)
10	Notocrangon antarcticus (Pfeffer, 1887)	Caridea, Crangonidae	GM-RP, LM	Bluhm and Brey (2001)
11	Pandalus borealis Krøyer, 1838	Caridea, Pandalidae	GM-RP	Nilssen and Aschan (2009)
15	Nephrops norvegicus (Linnaeus, 1758)	Astacidea, Nephropidae	R	Bell et al. (2006)
25	Chaceon maritae Manning and Holthuis, 1981	Brachyura, Geryonidae	GM-MR	Melville-Smith (1989)
30	Panulirus argus (Latreille, 1804)	Achelata, Palinuridae	GM-MR	Ehrhardt (2008)
72	**Homarus gammarus (Linnaeus, 1758)**	**Astacidea, Nephropidae**	**GM-MR, LM**	**Sheehy et al. (1999)**

1.1.4 Feeding Habits

Most decapod crustaceans are identified as omnivorous species, including the European lobster (Schoo *et al.*, 2014). In the wild, this species feeds on slow moving benthic invertebrates such as bivalves, polychaetes, echinoderms and gastropods. Also, part of its natural diet are algae and decomposing material like fish and other dead organisms. Being a nocturnal animal, it is during this period that the lobster performs its feeding task (Factor, 1995; Nelson *et al.*, 2006; Prodöhl, Jørstad and Triantafyllidis, 2006). In captivity, juveniles and adults, can be successfully maintained and fed using molluscs such as mussels (*Mytilus sp*.) and squid (*Loligo sp*.) (Burton, 2003).

The main food components of lobster diets in the wild are, as discussed above, well described with several concrete examples of the feeding habits of this crustacean. However, dietary and nutritional knowledge is only well known in adult individuals. There are still large gaps in the knowledge of feeding habits and dietary components of larval and juvenile crustaceans in general (Nelson *et al.*, 2006).

1.1.5 Wild Population

As a highly valued species, the European lobster has been suffering enormous pressure from its natural population over the last few decades. Although the main type of fishing used in the capture of this crustacean is small-scale fishing (Figure 5), in the last decade it is estimated that the capture values are range between 4500 and 5600 tons (FAO, 2020). These values are mainly due to the fishing carried out in the northern European countries and less in the Mediterranean (Pavičić *et al.*, 2020; Prodöhl, Jørstad and Triantafyllidis, 2006).

These facts explain the low population levels that are observed from the Scandinavian to the Mediterranean coast, where a low or non-existent population recovery rate is observed (Ellis *et al.*, 2017). The first evidence for this lack of recovery appeared between 1930 and 1970 where a collapse in lobster catches was observed due to overexploitation of stocks and inappropriate fishing practices. In Denmark, Norway and Sweden stocks had declines of 99 %, 92 % and 90 %, respectively, representing high and worrying values when it comes to the conservation and ecological importance of this species (Ellis *et al.*, 2017; Phillips, 2006).

Figure 5 - Small scale fishery boat used for ecological e scientific purposes.

1.2 Lobster Aquaculture

1.2.1 Aquaculture Through Time

The cultivation of nature through the production of useful organisms has been part of human culture since its origins (Meeren, Van Der, 2005). Aquaculture is the production of aquatic organisms, such as fishes, molluscs, crustaceans, amphibians, invertebrates and algae. The production of this sector, that is currently growing, already exceeds the total global fish catch by 18.32 million tonnes (FAO, 2020).

Like the fisheries sector, fish represent the most significant species in aquaculture production, standing at 47.7 %, followed by aquatic plant production - 28.4 %. Molluscs, an equally important group in the aquaculture business, represent 15.4 % of the overall total production. In the group in which lobsters are included, crustaceans, the percentage of the total global production is around 7.5 %, with the remaining percentage relating to amphibians and invertebrate animals (FAO, 2020). Although 70 % of the entire planet is covered by seawater or brackish water, most of the aquaculture production is of freshwater species, around 83.6 %, compared with the fish catches of freshwater species which only represents 13.4 % of total catches (FAO, 2020; Tacon, 2020).

The aquaculture production of crustaceans has been growing significantly in recent years. Since 2000, the annual aquaculture production of crustaceans has grown

by 9.92 %, with more than 30 different species, one of the most representative the tiger shrimp *Penaeus monodon*. Compared with the other aquaculture species, crustaceans represent the product with the highest economic value per unit. Even though it is the 4th group of species to be most produced it is the 2nd in relation to the most economically productive (FAO, 2020).

Scientific advances made over the last 50 years have produced a major positive impact on the knowledge and functioning of aquatic ecosystems and resources, in addition to increased environmental awareness and sustainable use of resources. The importance of fish stocks and aquaculture products is now widely recognised and valued. However, these efforts have only been more significantly realised following the implementation of the Sustainable Development Goals (SDGs), which include the sustainable use of oceans and marine resources and other objectives relevant to aquaculture and fisheries (FAO, 2020).

Global fish consumption has been increasing at a rate of 3.1 % per year from 1961 to 2017, a rate twice the population growth rate (1.6 %). Compared to other food products such as meat or dairy products, among others, fish consumption has also seen a higher increase. The state of fish resources, based on permanent monitoring show continuous declines, a worrying fact, given the increase in consumption of these products. In 2018 the European Union was the largest importer of fish products (34 %) followed by the United States of America (14 %) (FAO, 2020). Observing these facts, there is a need to reduce the stress caused on natural stocks as well as to sustainably increase aquaculture production.

1.2.2 Lobster Culture

Regarding aquaculture, it can be conducted in various ways and using different techniques, depending on the final objective. The production of crustaceans, particularly lobsters, is no exception. Lobster aquaculture can be conducted in different ways: if the objective is to enhance the environment and support and increase the natural stocks, is considered re-stocking aquaculture. If the objective is production for consumption, this can be carried out through two different ways, product enhancement or production of the species in an integral way (Kristiansen, Drengstig and Bergheim, 2004). The interest in re-stocking aquaculture, as a result of declining natural stocks, started several decades ago when many hatcheries were formed in Europe and North America with the aim of hatching lobster eggs and releasing them into the wild during

or immediately after their larval stages (Nicosia and Lavalli, 1999). The enhancement of the product happens when the objective is to capture individuals in the wild and keep them in captivity proceeding to feeding routines to be later commercialized with a higher weight and value (Factor, 1995). The full production of the species consists of a closed cycle independent of the natural stocks where lobsters are cultivated from eggs until they reach commercial weight (Kristiansen, Drengstig and Bergheim, 2004).

The interest in lobster production started in the 1970's following several research programmes on lobster production in the United States and Canada. Through this research, and other previous studies, together with a well-established knowledge of this crustacean (Factor, 1995) lobster production was well specified from larval rearing until the individual reaches commercial size (Kristiansen, Drengstig and Bergheim, 2004). Several companies have started production of American lobster, however, none of the projects proved to be commercially viable. This was due to an increase in the lobster catch at that time coupled with an interruption of research programmes that ended before all production strategies were well established (Nicosia and Lavalli, 1999).

In order to establish production strategies for a particular species, it is initially necessary to understand the viability and advantages that this species has for a profitable production. Compared with other decapod crustacean species, European and American lobsters are more robust and have a simple and rapid larval development. They are easily fed either on live food or formulated diets, as long as they meet their nutritional requirements, they are resistant to pathologies and with a growth rate considered fast when exposed to higher temperature waters (Kristiansen, Drengstig and Bergheim, 2004; Phillips, 2006). Thus, the viability of these species compared with other decapod crustaceans is promising for their production and commercialisation.

In Europe, a research and development project was carried out by Norwegian Lobster Farm LTD. in southwest Norway between 2000 and 2007. As a result, this company is now the only one in the world producing European lobster (Drengstig and Bergheim, 2013). The production uses an advanced water recirculation system and patented individual production systems. The research project included the experimentation with six different formulated diets to meet the nutritional requirements of the individuals, various automatic and robotic systems for feeding, collection, cleaning and image processing, as well as the development of biological protocols and several market studies. In this way, Norwegian Lobster Farm managed to close the

production cycle of European lobster, from egg to reach commercial size ensuring the quality and valorisation of the final product (Drengstig and Bergheim, 2013).

1.3 Lobster Nutrition

Over the last decades, several studies have been conducted in order to fill the knowledge gaps in the nutritional requirements of crustaceans and the development of formulated diets. Mussels have been used as one of the standard diets for several crustaceans, as with lobster, as they show positive growth performances (Nelson *et al.*, 2006). However, the use of artificial diets can positively influence the growth of individuals if these diets are formulated to nutritionally supply the needs of the species. Physical properties should also be considered when formulating diets such as texture and resistance to degradation (stability) (Nelson *et al.*, 2006), as well as palatability in order to promote ingestion (D'Abramo *et al.*, 1980). By gradually replacing the use of fresh or frozen feed, by formulated diets, the production process becomes simpler and more efficient, and the quality of the final product is improved (Silva, 2020).

Vital nutritional aspects have been identified for formulated diets for lobsters and other crustaceans. These include the determination of appropriate ratios of macromolecules and the essential requirements of nutritional elements (Nelson *et al.*, 2006). Several studies indicate a dietary protein inclusion of 30 to 60 % as essential (Boghen and Castell, 1981; D'Abramo *et al.*, 1980). The inclusion values of lipids in diets are directly related to digestion, reproduction and growth processes and therefore their introduction in diet formulation can have positive effects (D'Abramo *et al.*, 1980). A diet with carbohydrate levels twice that of lipids is associated with greater energy availability (Nelson *et al.*, 2006).

Within the protein content in the diet, 10 essential amino acids have been shown to be indispensable in crustacean diets, these being arginine, methionine, valine, threonine, isoleucine, lysine, histidine, phenylalanine and tryptophan. Arginine and lysine, together with leucine, have been known as the main amino acids in whole-body tissue of *H. gammarus* juveniles, traducing in a high requirement of these three amino acids for the species (Mente, Houlihan and Smith, 2001).

As for the dietary lipid content, five polyunsaturated fatty acids are considered essential: linolenic acid, eicosapentanoic acid, linoleic acid, docosahexanoic acid and arachidonic acid (Nelson *et al.*, 2006). The inclusion of minerals is, in addition, a very

relevant factor with the presence of five minerals considered essential to the healthy growth of crustaceans such as calcium, copper, phosphorus, potassium, magnesium, selenium and zinc (Davis and Gatlin, 1996).

In addition to the nutritional requirements for optimal physiological functioning of the animal, nutritional supplements that promote normal pigmentation can also be beneficial. European lobster is known to have a natural blue/navy colouration. This pigmentation is usually due to astaxanthin, a predominant carotenoid present in aquatic animals (Yu and Liu, 2020). In decapod crustaceans it is described that the pigmentation of the exoskeleton is determined by astaxanthin incorporated into a macromolecular protein complex known as crustacyanin (Chayen *et al.,* 2003). Astaxanthin also exhibits high antioxidant properties and is considered to be beneficial for the health of aquatic species (Lim *et al.,* 2018).

By including the essential nutritional requirements in the diet, commercialisation success and increased growth performance become possible. By improving the formulation of diets and making them more sustainable can it be ensured that lobster production is established in more countries and more markets.

1.4 Sustainability in Aquaculture Feeds

The main objective of sustainable aquaculture production, where food production is carried out through the sustainable use of natural resources, is achieved by using production systems with minimal ecological impact. Aquaculture has been the subject of many concerns about sustainability as well as the diets used, production errors and the discharge of waste into the natural environment (Martins *et al.*, 2010). Aquaculture production currently represents the fastest growing agricultural sector with the greatest potential, as it provides a food source rich in easily digestible proteins, healthy fats and a content of several micronutrients essential to many societies around the globe (Sarkar *et al.*, 2021). Over the years, it is observed a rapidly increase on the production of farmed fish and shellfish due to an enhanced intensive aquaculture practices (FAO, 2020).

With the increase of intensive production systems, the reliance on natural stocks for human consumption consequently decrease (Sarkar *et al.*, 2021). However, one of the greatest demands in aquaculture is the use of diets that provide an optimal growth rate. Fishmeal is widely used as a source of protein in aquaculture diets as it

has a high digestible protein content, a balanced amino acid profile, is rich in unsaturated fatty acids and promotes intake due to its high palatability (Wang *et al.*, 2021). Fishmeal production is not able to meet the needs of aquaculture production, which has expanded rapidly since 1970. These combined factors have led to an excessive increase in the price of fishmeal resources and there is currently a bottleneck between the demand for this resource and its supply (FAO, 2020). The rising price of fishmeal also results in increased demand for carnivorous fish that feed preferentially on diets rich in high levels of fishmeal resources (Wang *et al.*, 2021).

A sustainable increase in the aquaculture sector implies a sustainable use of the available resources as well as substitutes for less bioavailable ingredients such as fishmeal or fish oil. Therefore, research into the use of sustainable functional diets is the way forward as a strategy to reduce the effects on growth performance and health derived from diets with low concentrations of fishmeal (Torrecillas *et al.*, 2021). This work has already been developed over the last decade in which we can observe a decrease in the use of fish meal and fish oil in formulated diets with the aim of being replaced by other more sustainable sources such as plants, insects, animal by-products, among others (Tacon, 2020). A well-studied example is the case of salmonid species that, being carnivorous, require high quantities of fishmeal in their diets in order to have optimum growth. Several studies have therefore tested the replacement of fishmeal with other protein sources like plant-based alternatives in order to decrease the reliance on fishmeal in formulated diets (Zaretabar *et al.*, 2021). For example, soybean meal (Voorhees *et al.*, 2019), rapeseed meal (Carter and Hauler, 2000), potato protein (Tusche *et al.*, 2013), safflower (Ustaoglu *et al.*, 2015), corn gluten meal (Stone *et al.*, 2005) and rice (Palmegiano *et al.*, 2006).

Fishmeal can then be replaced by other sources of more sustainable origin but, in most cases, high or total substitution results in a decline in the health of the individual as well as conditioning of optimal growth. This is due to an imbalanced amino acid and micronutrient profile, as well as the presence of anti-nutritional factors or reduced digestion due to the presence of plant proteins, normally associated with low palatability (Torrecillas *et al.*, 2021). Despite these studies for replacing non sustainable ingredient on fish production, fish meal remains the main ingredient in formulated diets for marine crustaceans' species (Turchini, Trushenski and Glencross, 2019). There are still barriers and gaps in the knowledge of new, more sustainable ingredients for fish production despite all the research carried out over the last few

years. In the case of crustaceans, it is an even bigger challenge due to the lack of knowledge about their nutritional requirements.

1.5 Case Study

In place of the use of plant protein sources, which have nutritional values vastly different from the animal protein sources, the use of by-products from fishing or fish processing and by-products from aquaculture show great promise as a sustainable option to replace fishmeal in crustacean diets. In addition to the economic advantages, as these resources are less valued, by-products of animal origin lead to more sustainable aquaculture production (Hua *et al.*, 2019; Stevens *et al.*, 2018).

Northern shrimp, *Pandalus borealis*, are the commercially most important Caridean species in the North Atlantic and represent 10% of the global shrimp production (Paterson, 2003). The total global landings of P. borealis amounted to ~295.000 tonnes annually between 2010 and 2016 (FAO, 2020). Although being an important species in marine industry, Northern shrimp has an enormous amount of unprofitable waste. During Shrimp process, large amounts of shrimp by-products are generated, these ones including shrimp heads, shells, appendages and tails, which traduce in 65% of the initial shrimp weight (Dave *et al.*, 2020). These by-products of shrimp processing are also of great concern as they are very slow to biodegrade (Shahidi and Synowiecki, 1991). Being a source of animal protein with the possibility of positive effects on the growth of crustaceans, as opposed to vegetable protein sources, these by-products of shrimp processing show a promising potential not only in terms of reducing the cost of formulating diets, but also in terms of greater sustainability for aquaculture and for the production of shrimp or other crustaceans.

The use of shrimp waste meal (SWM) represents an enormous potential as a protein source for crustacean diets such as lobster, as it translates into a high protein content and a well-balanced amino acid profile (Cruz-Suárez *et al*., 1993). Processing waste of P. borealis has previously been identified as a high protein source content (44.5%, dry weight basis) with high levels of arginine and lysine (Heu, Kim and Shahidi, 2003) which were already been indicated with a strong presence on lobster tissue. The SWM is a natural source of astaxanthin which is known as a good addictive to formulated diets in aquatic species (Yu and Liu, 2020).

In contrast to the many positive factors of shrimp waste meal, it also contains high levels of chitin. Although chitin is a known inhibitor of nutrient digestion in fish (Karlsen *et al*., 2017), its action on the digestibility of crustaceans is more controversial. Chitinase, the digestive enzyme that breaks down chitin, has been documented in most crustaceans to facilitate the digestion of prey exoskeletons as well as their own exoskeleton after *ecdysis* (Ceccaldi, 1989). Even though the metabolic cost of moulting cannot be accurately quantified, *ecdysis* is related with large losses of nitrogen and carbon, which must be recovered from the diet (Zoutendyk, 1988). In addition, high levels of chitin have been described as having no negative effect on the growth or survival of crustaceans such as black tiger shrimp (Fox *et al.*, 1994). Thus, it may be advantageous to use chitin-rich diets to obtain a suitable growth rate for crustaceans such as lobsters, as the results of chitin degradation will be essential for the formation of a new exoskeleton after moulting and the synthesis of new tissue (Niu *et al.*, 2013; Powell and Rowley, 2007).

With these indications *P. borealis* processing waste can be used as a more sustainable alternative for the formulation of diets for *H. gammarus*. In this study, an experiment was conducted in order to discover the potentiality of shrimp waste meal produced from *P. borealis* processing waste (heads, appendages and exoskeletons) as a diet with positive results for *H. gammarus* juveniles. The experiment examined growth performance, survival, feed efficiency, proximate composition and exosqueleton colouation of early juvenile lobsters beginning at stages VII to VIII fed one of the five experimental diets formulated to include crescent levels of shrimp waste meal (SWM) inclusion (0% to 28% of total dietary protein).

2. Materials and Methods

2.1 Experimental Animals

The animals used in this study were hatched from eggs at the aquaculture facilities at the Technical University of Denmark, Section of Aquaculture, Hirtshals, where the experiment was conducted. The eggs were obtained from ovigerous wild European lobster females (body weight 500-700 g) caught in the Limfjord (North Jutland, Denmark). The larval stages (I-IV) were communally reared in 500 L rectangular tanks equipped with a top seawater inflow (50 L/h) and a vertical outflow filter (1 mm mesh of diameter). Cleaning routines were carried out daily (filter cleaning, checking of water flow, etc.) as well as the maintaining the water quality parameters (temperature of 18 ºC and salinity of 34 PSU). The larvae were daily fed with Antartic Krill, *Euphorbia superba* (Akudim A/S, Denmark). When reached to stage IV, the post-larvae were transferred to individual compartments in a raceway system. The individual compartments used was a 3D printed polylactic acid (PLA) bioplastic cassette system (Figure 6).

Figure 6 - 3D compartments used for rearing lobster juveniles.

Each compartment, with 200 mL, has perforated grids, one side the grid was in the top and the opposite near the bottom to induce a “up and down “enhanced water flow. The cassettes were placed in raceways (Figure 7) supplied by a saltwater recirculation system with a constant flow rate of 330 L/h. The water quality was

maintained with temperature, salinity, dissolved oxygen and ammonia-N values of 19 ºC, 34 PSU, >90% and <0.1 mg/L, respectively. The individuals were subjected to a photoperiod time of 8h light and 16h dark by fluorescent lamps with a light intensity at the water surface of 7-8 Lux. The post-larvae were daily fed with the same diet (Antartic Krill, *Euphorbia superba*) and were kept with these conditions for approximately 8 weeks. At this time the lobsters were reaching the stage VI of their development (Goncalves *et al.*, 2021b).

Figure 7 - Raceway system used to perform the growth essay.

2.2 Growth Trial

Before start of the 8 weeks trial, 75 stage VII lobsters were individually weighted using a PG503 Delta Range balance (Mettler Toledo, Ohio, USA) and the Body weight (BW) was determined to the nearest 0.001 g with an average weight of 0.164 ± 0.057 g. The Carapace length (CL) was measured with a vernier calliper, from the base of the eye socket to the posterior edge of the cephalothorax, to the nearest 0.1 mm. Lobster had an average length of 8.2 ± 1.0 mm. Individuals were randomly distributed into five dietary treatments (N=15, per diet). Each dietary group was divided into 3 replicates (N=5, per replicate) which were intercalated with the other dietary replicates to ensure that the location inside the raceway was not a variable. Between each replicate was left a line gap to avoid that one diet passed to another dietary group. Every day the individuals in each dietary group were hand-fed with one pellet (55 mg average weight) of the respective diet and allowed to feed for a 4-hour period. After that period the

uneated food was removed. Moulting and mortalities were daily recorded, and the weight and length of dead lobsters were measured. When moulting occurred, the exoskeletons were left in the compartments to allow the individual to consume them. The water temperature was recorded daily, and dissolved oxygen, pH, salinity, ammonia, nitrite and nitrate were recorded weekly in order to ensure the water quality. Every second week all the individuals were measured, and the body weight (BW) and carapace length (CL) were recorded until the end of the trial, 8-week later (Figure 8). To estimate the growth performance the following formulas were used:

Specific Growth Rate (SGR, % day $^{-1}$) = {ln (BW_f) – ln (BW_i)} x Δt^{-1} x 100%

Where: SGR = specific growth rate; BW_f = final body weight; BW_i = initial body weight; Δt = duration of the growth trial (56 days).

Carapace Length Increment (CLI, %) = (CL_f – CL_i) x CL_i^{-1} x 100%

Where: CLI = increment in carapace length; CL_f = final carapace length; CL_i = initial carapace length.

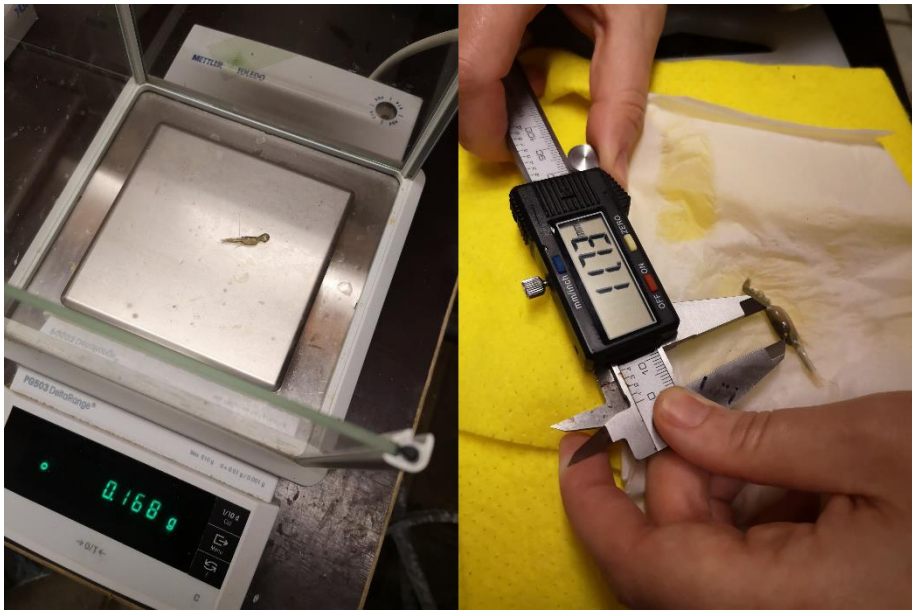

Figure 8 - Sampling of body weight and carapace length of a juvenile.

To determine the intake, the individuals were transferred to a closed chamber supplied with aeration with the same water conditions as of the raceways (Figure 9). After a 24 hour starvation period one pre-weighed pellet was offered to each lobster for a period of 4 hours. At the end of the meal, juveniles were transferred back into the

raceway compartments. Feed intake was estimated from the uneaten feed fraction after collection, filtration and drying, employing the formula:

$FI\ (\%\ BW^{-1}\ day^{-1}) = (P_{weigh} - P_{unconsumed} - L) \times BW^{-1} \times 100\%$

Where: FI = feed intake, P_{weigh} = distributed pellet, $P_{unconsumed}$ = unconsumed pellet, L = leaching after 4h, BW = body weight. Leaching was estimated by placing a pre-weighed pellet in the chamber under the same conditions as in the feeding period but without animals. Leaching was estimated for 6 pellets of each diet.

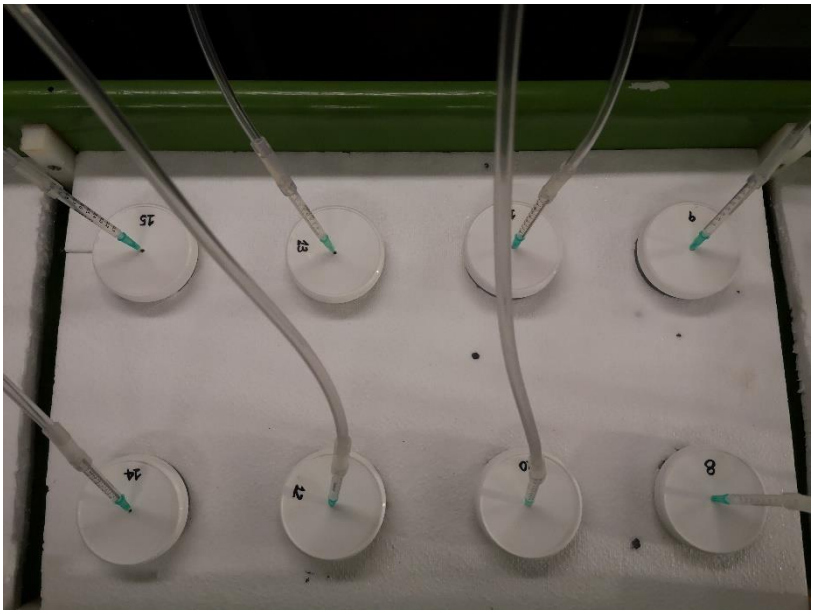

Figure 9 - Aerated chamber used to measure the food intake.

At the end of the growth trial all individuals were lethally anesthetised in ice-cold seawater, weighed, measured, individually photographed, rinsed in distilled water and stored as -80 ºC until further analysis (Goncalves *et al.*, 2021b).

2.3 Experimental Diets

The growth trial consisted of five different dietary treatments, each one with different inclusion levels of Fish Meal (FM) and Shrimp Waste Meal (SWM). The five diets were formulated to incorporate crescent levels of SWM (0 %, 7 %, 14 %, 21 % and 28 % of total dietary protein), while preserving the same lipid and protein content. It was not possible to include more than 28 % SWM in order to maintain a total protein content of 54% which is known to be the optimal protein percentage for European lobster juveniles (Gonçalves et al., 2021a). The five experimental diets (~ 40 % DM)

were formulated to contain 22 % of total crude protein (CP – 54 % of DM) and 4.3 % of total crude fat (CF – 11 % of DM). The diets were made by combining different proportions of fish meal, FM (South America, CP 68.1 %, CF 8.9 %, Ash 15 %) and shrimp waste meal, SWM (SWM - heads, appendages, and exoskeletons from Northern shrimp *Pandalus borealis*, CP 47.2 %; CF 98 6.1 %, Ash 33.5 %). Squid meal (CP 77.2 %, CF 4.4 %, Ash 8 %) was as well included in all diets. The Shrimp waste meal (SWM) and the formulated diets were prepared at DTU Aqua facilities (Hirtshals, Denmark) (Figure 10). The proximate composition, carotenoid and chitin content as well as the amino acid composition of the SWM is shown in Table 2. Ingredient inclusion, proximate composition and the amino acid content of all five experimental diets are presented in Tables 3, 4 and 5 respectively.

Figure 10 -Mixture of the ingredients in order to prepare de formulated diet.

The SWM ingredient was purchased in form of a mixture of heads, appendages and exoskeletons of Northern shrimp *Pandalus borealis* from a shrimp factory (Launis A/S, Skagen, Denmark). These waste parts of the shrimp were oven-dried at 60ºC until constant weight. Dried SWM was pre-grounded to a particle size < 5mm (Krups Speedy Pro homogenizer) and then milled to a final particle size of 120 µm using an ultra-centrifugal mill (ZM 200, Retsch GmbH, Dusseldorf, Germany). The dry ingredients of each diet, except the carrageenan, were thoroughly mixed and heated to 85 ºC. Carrageenan, krill oil and water were mixed and heated to 85 ºC to form a carrageenan emulsion. The dry ingredients were mixed with the carrageenan emulsion

until obtained a homogeneous paste. Water loss by evaporation, estimated by weight difference, was compensated by adding heated water (85 ºC). The fluid viscous mixture was poured into a steel tray, evenly distributed in a thin layer, and cooled down until the mixture solidified. At last, moist pellets with a size of 4 x 4 x 4 mm were obtained by pushing a 3D printed plastic grid (i3 MK2, Prusa, Prague, Czech Republic) into the solidified mixture. The pellets were distributed in labelled containers and stored at – 20 ºC until further usage (Goncalves *et al.*, 2021b).

Table 2 - Proximate and amino acid composition of the shrimp waste meal (SWM).

Proximate composition (% content)		**Amino acid composition (g 100g^{-1} SWM)**	
Dry matter	97.2	***Essential***	
Crude protein	47.8	Arginine	2.7
Crude fat	6.1	Histidine	1.0
Ash	33.5	Isoleucine	1.8
Chitin	16.0	Leucine	2.7
		Lysine	2.6
		Methionine	1.0
		Phenylalanine	1.9
		Threonine	1.8
		Valine	2.1
		ΣEAA	18.1
Astaxanthin content (mg kg^{-1})		***Nonessential***	
Esterified astaxanthin	69.0	Alanine	2.3
Free astaxanthin	3.6	Aspartic acid	4.0
		Cysteine + Cystine	0.5
		Glutamic acid	5.4
		Glycine	2.1
		Proline	3.6
		Serine	2.0
		Tyrosine	1.6
		Tryptophan	0.5
		ΣNEAA	21.5
		EAA/NEAA	0.84
		ΣAA	39.6

Table 3 - Ingredients used (g 100 g-1 diet) on the experimental diets.

Ingredients (as used 100g^{-1} diet)	**Level of SWM as % of dietary protein**				
	0	**7**	**14**	**21**	**28**
Fish meal	19.1	17.2	15.5	13.6	11.8
Shrimp waste meal	0	3.5	6.4	9.8	13.2
Squid meal	7.1	7.3	7.3	7.4	7.4
Krill oil	2.6	2.6	2.6	2.6	2.5
Maize starch	1.7	1.6	1.4	1.2	1.0
Potato starch	1.7	1.6	1.4	1.2	1.0
Wheat starch	3.5	3.1	2.8	2.4	2.1
Vitamin premix	1.2	1.2	1.2	1.2	1.2
Carrageenan	1.6	1.6	1.6	1.6	1.6
Copper supplement	0.005	0.005	0.005	0.005	0.005

Table 4- Proximate composition of the experimental diets.

Proximate composition (as fed g 100g^{-1})	**Level of SWM as % of dietary protein**				
	0	**7**	**14**	**21**	**28**
Crude protein	23.5	22.2	21.4	20.7	22.5
Crude fat	4.6	4.4	4.2	4.0	4.4
Moisture	59.5	59.7	58.9	60.6	56.5
Ash	5.4	6.0	6.7	7.2	8.8
NFE[1]	7.0	7.7	8.8	7.5	7.8
Gross energy[2] (KJ g^{-1})	8.1	7.8	7.8	7.3	7.9
Chitin[3] (% of DM)	0	1.4	2.5	4.0	4.9

[1] Nitrogen-free extract (NFE), calculated as: 100% - crude protein% - crude fat% - ash% - moisture%.
[2] Coefficient for energy concentration: 21.3 kJ, 39.5 kJ, and 17.6 kJ for protein, lipid, and carbohydrate (NFE), respectively (Cuzon and Guillaume, 1997).
[3] Estimated from chitin content on SWM (16%) and assuming SWM is the only ingredient containing chitin.

Table 5 - Amino acid profile of the experimental diets (g 100 g-1 diet).

Amino acid composition (g 100g^{-1} diet)	**Level of SWM as % of dietary protein**				
	0	**7**	**14**	**21**	**28**
Essential					
Arginine	1.2	1.1	1.1	1.1	1.2
Histidine	0.7	0.5	0.5	0.5	0.6
Isoleucine	0.9	0.8	0.8	0.8	0.9
Leucine	1.6	1.4	1.4	1.4	1.5
Lysine	1.5	1.4	1.4	1.4	1.4
Methionine	0.6	0.5	0.5	0.5	0.5
Phenylalanine	0.8	0.7	0.8	0.8	0.8
Threonine	0.9	0.8	0.8	0.9	0.9
Valine	1.0	0.9	0.9	0.9	1.0
ΣEAA	9.1	8.0	8.4	8.4	8.9
Nonessential					
Alanine	1.3	1.1	1.2	1.2	1.3
Aspartic acid	1.8	1.7	1.8	1.9	2.0
Cysteine + Cystine	0.2	0.2	0.2	0.2	0.2
Glutamic acid	2.7	2.5	2.6	2.6	2.8
Glycine	1.4	0.9	1.0	0.9	1.1
Proline	1.0	0.9	1.0	0.9	1.1
Serine	0.8	0.7	0.8	0.8	0.9
Tyrosine	0.8	0.6	0.7	0.7	0.7
Tryptophan	0.2	0.2	0.2	0.2	0.3
Hidroxyproline	0.3	0.3	0.0	0.0	0.0
ΣNEAA	10.5	9.4	9.7	9.7	10.6
EAA/NEAA	0.86	0.85	0.87	0.86	0.84
ΣAA	19.6	17.4	18.1	18.1	19.5

2.4 Analytical Methods

The analytical composition of the SWM ingredient and the five experimental diets (proximate composition, amino acid profile and carotenoid content) was analysed by Eurofins Steins Laboratory (Vejen, Denmark). The SWM chitin content was determined spectrophotometrically following the method described in (Tsuji, Kinoshita and Hoshino, 1969). The proximate composition analysis of lobster juveniles was performed for three individuals per dietary treatment. Whole body juvenile lobsters were freeze-dried and then combusted for 16h at 500ºC (NMKL 23, 1991) for dry matter and ash content determination, respectively. Protein was determined spectrophotometrically at 750 nm using a commercial Lowry-dried, microprotein determination kit (BIO-RAD 500-0112, Bio-155 Rad Laboratories, California, USA). Lipids were extracted with chloroform-methanol (2:1 by volume) using the Folch method (Christie and Han, 2010) and the content determined gravimetrically (Goncalves *et al.*, 2021b).

2.5 Colouration Analysis

The analysis of exoskeleton pigmentation was carried out according to a method similar to the one used in (Tlusty, 2005). Each lobster was photographed with a digital camera. The camera was fixed at a focal length of 6.1 mm and pictures were taken at an exposure time of 1/30 s with an aperture of F2.8. Individuals were photographed against a white background after removal of excess water. The pictures were stored in a 4000 × 2664 pixels format. Each image was analysed for RGB scores using ImageJ 1.52n software (Abramoff, 2004). RGB scores were measured for each individual at 3 different locations on the body, including the claw (CLW), the last abdominal segment (LAS) and the telson (TEL) (Figure 11). The white background was used to correct the deviation to pure white (100, 100, 100) by standardizing the exoskeleton RGB measurements by the values obtained for the white surface (Goncalves *et al.*, 2021b).

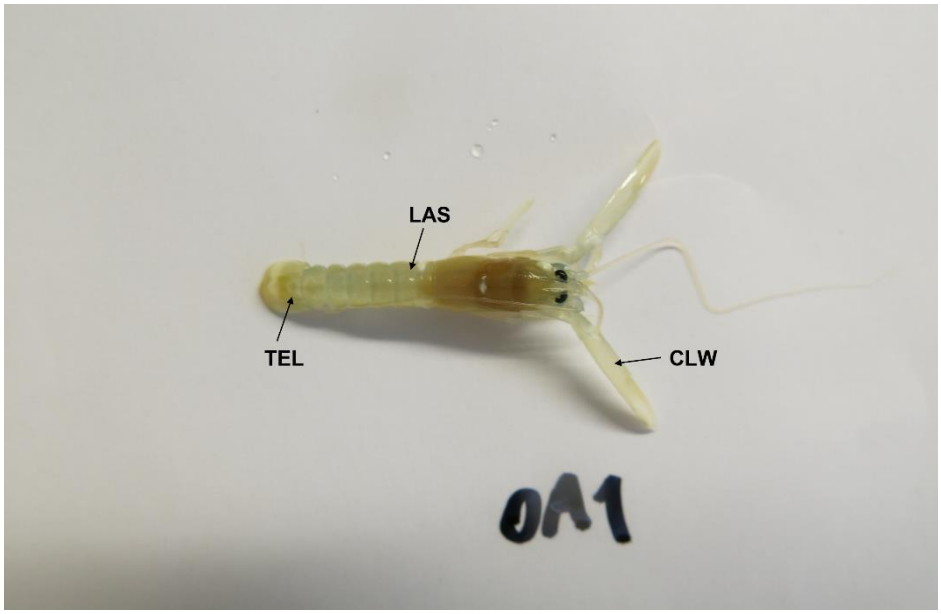

Figure 11 - Body locations of H. gammarus juveniles examined for digital colour analysis. CLW = claw, LAS = last abdominal segment, TEL = telson.

2.6 Data Analysis and Statistics

Data were expressed as mean ± SEM unless otherwise specified. All dietary treatments were subjected to a one-way ANOVA to test the comparisons of the different diets. Whenever significant differences were observed the Tuckey post hoc test was used. Survival data was analysed by a Kaplan-Meier survival test and Log-Rank (Mantel-Cox) test was used to determine the significance. Before analysis, parametric assumptions of normality of residuals and homogeneity of variances were tested. A significance value of 0,05 was used. All statistical tests were performed using IBM SPSS Statistics 26.0.

3. Results

3.1 Growth performance

Results for growth performance, specific growth rate (SGR), carapace length increment (CLI) and moult cycle are presented in Table 6. During the growth trial, the juveniles grew from an initial mean weight of 164 mg and 8.2 mm of carapace length, to mean weights ranging from 308 to 362 mg (10.2 to 11.2 mm of carapace length). The group of individuals that showed the highest mean for final weight and total length was the group of juveniles fed the SWM 28 % (362 ± 47 mg of BW and 10.8 ± 0.4 mm of CL). SGR and CLI were not significantly different among the dietary treatments ($p > 0.05$), SGR varied from 1.05 to 1.25 and the CLI between 24 % and 35 % (Table 6).

Juveniles completed an average of 1.7 moults for the duration of the experiment. The number of moults ranged from 0 to 3 moults. The minimum value was observed for the SWM 28 % and in opposite the maximum moult cycles were observed with the lowest level of SWM inclusion (SWM 0 %). Despite this difference the intermoult period was not significantly affected by the dietary treatment ($p > 0.05$) and varied between 6 and 31 days (Table 6).

3.2 Survival

The results of survival among the dietary treatments are presented in Table 7. Each dietary group began with 15 individuals and a total mortality rate of 35 % was observed during the eight-week trial. A significant difference between the lobsters fed with the SWM 7 % and the SWM 28 % ($x^2 = 5.44$, $p = 0.02$) was found. The juveniles fed with SWM 28 % showed a significantly higher survival (87 %) compared with the ones fed with the SWM 7 % (47 %).

3.3 Intake

The food intake ranged from 1.8 % to 2.6 % BW^{-1} day^{-1}. The lowest value corresponding to the juveniles fed with SWM 28 % and highest when fed SWM 0 %. Significative differences were not observed trough dietary treatments ($p > 0.05$).

Table 6 - Growth performance of juveniles Homarus gammarus fed with the experimental diets over an eight-week period.

Bioassay	**Level of SWM as % of dietary protein**					
	0	**7**	**14**	**21**	**28**	**One-Way ANOVA**
BW_i (mg)	168 ± 18	162 ± 12	164 ± 16	160 ± 12	167 ± 16	$F_{1.74} = 0.05$
CL_i (mm)	8.2 ± 0.3	8.1 ± 1.1	8.2 ± 0.2	8.2 ± 0.3	8.4 ± 0.3	$F_{1.74} = 0.24$
N (initial)	15	15	15	15	15	
BW_f (mg)	342 ± 40	315 ± 26	309 ± 54	308 ± 44	362 ± 47	$F_{1.48} = 0.31$
CL_f (mm)	11.2 ± 0.5	10.7 ± 0.3	10.2 ± 0.6	10.3 ± 0.5	10.8 ± 0.4	$F_{1.48} = 0.68$
SGR (% d^{-1})	1.14 ± 0.19	1.24 ± 0.19	1.25 ± 0.21	1.05 ± 0.14	1.25 ± 0.13	$F_{1.48} = 0.31$
CLI (% CL_i^{-1})	34.87 ± 4.74	33.64 ± 5.75	26.91 ± 3.72	24.08 ± 4.29	26.02 ± 2.86	$F_{1.48} = 1.31$
N (final)	9	7	9	11	13	
Moult cycle (days)	29.3 ± 1.6	26.0 ± 2.4	28.3 ± 2.0	30.7 ± 2.1	30.9 ± 1.8	$F_{1.37} = 1.03$
N (individuals with 2 moults)	7	8	7	7	9	

Table 7- Survival of Homarus gammarus juveniles (% of initial numbers, N=15 per group) fed experimental diets containing different levels of shrimp waste meal over an eight-week period.

Survival	**Level of SWM as % of dietary protein**				
	0	**7**	**11**	**21**	**28**
N_i	15	15	15	15	15
N_f	9	7	9	11	13
Survival (% of N_i)	60	47[a]	60	73	87[b]

[a,b] Different letters denote statistically significant difference ($p < 0.05$) determined by Log-rank test.

3.4 Colouration Analysis

The RGB scores of the three areas (LAS, CLW TEL) among the different dietary groups are presented on Table 8. High RGB scores translate into lighter colour while low scores translate into a darker colouration of the exoskeleton.

Table 8 - RGB colour scores at three different body locations of Homarus gammarus juveniles fed experimental diets containing different levels of shrimp waste meal over an eight-week period. Body locations are as identified in figure 11.

RGB score	**Level of SWM as % of dietary protein**				
	0	**7**	**14**	**21**	**28**
LAS	(80, 80, 57)	(79, 80, 57)	(75, 78, 59)	(77, 76, 53)	(74, 75, 54)
CLW	(95, 94, 79)	(97, 96, 79)	(91, 91, 76)	(93, 92, 75)	(87, 88, 69)
TEL	(83, 79, 50)	(78, 74, 44)	(78, 76, 51)	(78, 74, 45)	(77, 74, 49)

LAS – last abdominal segment, CLW – claw, TEL – telson.

Despite the tendencies to lower scores in all three colours (Red, Green and Blue) with increasing the SMW % of dietary content, no significantly differences were observed between dietary treatments ($p > 0.05$). Two individuals representative of the extremes in the RGB score (lightest and darkest subjects among the analysed individuals) are presented in Figure 12 for comparison.

Figure 12 – Two juveniles representing the highest (left) and lowest (right) RGB scores.

3.5 Body Composition

The results of body composition (dry matter, ash, protein and lipid content) are presented on Table 9. There was no effect of the dietary treatment on the body composition of the juveniles, not observing any significant differences ($p > 0.05$). The dry matter content ranged from 24 % to 29 % and the ash content between 31 % and 35%. The protein content ranged between 24% and 32% and lipid content between 5 % and 8 %.

Table 9 - Proximate composition of juveniles Homarus gammarus fed with the experimental diets over an eight-week period.

Proximate composition (% of DM)	**Level of SWM as % of dietary protein**					
	0	**7**	**14**	**21**	**28**	**One-Way ANOVA**
Dry matter	23.9 ± 1.2	26.4 ± 2.5	28.8 ± 1.4	26.2 ± 1.3	24.4 ± 2.7	$F_{1.14}$ =1.00
Protein	29.1 ± 2.2	24.0 ± 4.1	30.6 ± 2.2	32.2 ± 1.1	31.5 ± 1.7	$F_{1.14}$ =1.74
Lipid	5.2 ± 1.0	6.2 ± 0.8	6.7 ± 1.0	7.8 ± 0.7	6.3 ± 0.2	$F_{1.14}$ =1.41
Ash	34.4 ± 0.4	31.6 ± 2.3	35.4 ± 4.9	32.9 ± 2.9	31.2 ± 1.7	$F_{1.14}$ =0.40
N	3	3	3	3	3	

4. Discussion

In this study it was examined the influence of substituting fish meal as a protein source with a more sustainable protein source (SWM). This, in order to understand how growth performance would be influenced. Although the group of individuals that was fed with the SWM 28 % exhibited the highest mean for final weight and total length the values of SGR and CLI showed no significant differences between the dietary treatments ($p > 0.05$).

Values of SGR ranged between 1.05 and 1.25 % day^{-1} , which were slightly lower than the SGR results for european lobster (1.72 % day^{-1}) obtained in a study that had used extruded diets with similar macronutrient ratios (Goncalves *et al.*, 2021a). While in the study performed by Goncalves *et al.*, 2021, juveniles of a younger size and stage (stage V to VI, ~ 90 mg wet body weight) were used, in this trial juveniles were at a higher stage of development (stage VII - VIII, ~ 164 mg wet body weight) were used. This may be one hypothesis to explain the lower SGR rate, as with increasing stage of development the ingestion also decreases, with higher values of ingestion being associated with earlier stages (Factor, 1995; Phillips, 2006). Other possible explanation for the higher SGR observed by Goncalves *et al.*, 2021 could be associated to a higher dry mass feed intake of the juveniles fed extruded feed (5.3 % BW day^{-1}) compared to the semi-moist diets used in this study (1.8 to 2.6 % BW day^{-1}). The semi-moist diets used on this study had a lower DM content (~ 40 %) compared to the previous study (92 %). It was suggested that lobsters may thrash and tear the feed more easily when reared on diets with a softer consistency than the extruded feeds, and this may result in an increased feed intake. However, the observed lower intake results may suggest that this statement is not necessarily accurate, possibly due to satiation induced by mechanical expansion of the gut.

The present study was admittedly a little too short. As growth in crustaceans, as opposed to fish, depends on sequential moulting, nutritional trials conducted on crustacean species targeting growth performance as a primary outcome should consider a minimum duration that allows individuals to complete two moults (Goncalves *et al.*, 2021a; Simon *et al.*, 2015). In the present study, juveniles completed an average of 1.7 moults for the eight-week trial. The number of moults ranged from 0 to 3 moults, which indicates that an increase in the trial duration could translate into better or more significant results to analyse the growth performance.

The most relevant finding of this study is the survival of juvenile European lobster among the different dietary treatments. The dietary inclusion of SWM was shown to interfere positively in survival between the different test groups, with a statistically significant difference between the survival of juveniles fed with the 28 % SWM and the 7% SWM. The highest survival (87 %) was observed for the lobster group fed the SWM 28 % while the lowest survival (47 %) was reported for the SWM 7 % dietary group. These results support the view that the content of chitin and astaxanthin in the SWM is potentially nutritionally advantageous to *H. gammarus* juveniles since SWM is the only source of either chitin or astaxanthin in the diets.

Chitin is one of nature's major complex polysaccharides, found in the cuticle of arthropods and fungal cell walls. Several observations have suggested that some crustaceans eat their cuticle after ecdysis, implying a beneficial dietary effect (Niu *et al.*, 2013; Powell *et al.*, 2017). When present, chitin and its derivates have been reported to increase survival in crustacean. Chitin has also been found as a dietary component that enhances growth if well digested. (Powell and Rowley, 2007). In order to digest this molecule, it needs to be degraded by enzymes. This enzymes (Chitinase, NAGase, Lysozymes) will break the chitin molecule into glucosamine compounds (Niu *et al.*, 2013) that are known to be involved in the moulting process, by helping the synthesis of the new exoskeleton (Fox *et al.*, 1994).

In addition to its positive nutritional effects, chitin and its derivatives have also been shown to stimulate immune activity in fish and crustaceans (Powell and Rowley, 2007). In parallel to the stimulation of immune system responses, chitin also has positive antimicrobial properties against a variety of micro-organisms by binding to their surfaces and changing the permeability of their membranes (Tsai and Hwang, 2004). This could lead to elimination of potential pathogenic microorganisms from the gut of lobster juveniles (Holt *et al.*, 2019). In Powell and Rowley, 2007, the authors describe a higher survival for crabs, *Carcinus maenas*, when fed with 5 % chitin supplemented diets, by reducing the number of bacteria in the hepatopancreas. These facts could explain the higher survival of the group fed with 28 % SWM diet probably by its higher bioavailability of chitin and its derivates.

Another factor that may explain the increased survival rate of individuals fed 28 % SMW diets is the presence of astaxanthin, as it was only found in the diets including shrimp waste by-products. This carotenoid is present in the exoskeleton of numerous crustaceans, including the shrimp *P. borealis*, (Dave *et al.*, 2020), that was used in the SWM incorporated diets.

Astaxanthin (3,3′-dihydroxy-β,β″-carotene-4,4′-dione) is an orange-red carotenoid pigment that belongs to the xanthophyll family. This molecule cannot be biosynthesised by animals and is therefore only available through the food chain. It is present mostly in crustaceans (shrimps, lobsters and crabs) but also in fish (salmon and trout) and is responsible for the orange-red colouration of both the exoskeleton and the flesh (Dave *et al.*, 2020). Although astaxanthin is mostly associated with the pigmentation of the crustacean exoskeleton (Wade, Gabaudan and Glencross, 2017), it also exhibits strong antioxidant properties by scavenging a variety of reactive radicals and oxygen species (Dave *et al.*, 2020; Yu and Liu, 2020).

As discussed previously, together with chitin, the presence of larger levels of astaxanthin may have positively influenced the survival of individuals fed diets with a higher percentage of inclusion of shrimp waste by-products. Future studies may help to explain whether the inclusion of these compounds in formulated diets will effectively translate into higher survival rates by playing an active role in the elimination of bacteria and other microorganisms or by assisting the demanding moulting process. More research effort will have to be employed to understand how these compounds act physiologically and chemically in juvenile european lobster.

Astaxanthin is biosynthesised by organisms such as microalgae, plants and bacteria and is therefore only accumulated by other organisms such as zooplankton, bivalves, crustaceans and fish through the food chain. As the latter cannot synthesise carotenoids *de novo,* they must obtain them from their diets. Because of this, astaxanthin has been used as an additive on a large scale to formulate diets for marine organisms (Yu and Liu, 2020). Astaxanthin is identified as the major colour pigment of the exoskeleton of crustaceans and is consequently the most commonly used pigment in diet formulation (Crear *et al.*, 2002). As crustaceans are then completely dependent on exogenous sources of carotenoids for their pigmentation (D'Abramo *et al.*, 1980), the use of astaxanthin-source diets (as is the case of the SWM-included diets in this study) shows hypothetical promise in translating positive improvements in the development of colouration in crustaceans such as European lobster.

In this study it was hypothesised that increasing the inclusion of SWM in the diets would translate into an increase in the natural colouration of the juvenile's exoskeleton. Various studies have shown that the inclusion of carotenoids influences pigmentation in *Homarus americanus* (Lim *et al.,* 2018) and the use of diets with added astaxanthin induces increased pigmentation in exoskeletons of juvenile *H. gammarus* (Hinchcliffe *et al.*, 2020). The results obtained in this study, however, do not support these

allegations. Despite the tendencies to lower RGB scores in all three colours (Red, Green and Blue) with increasing SMW % of dietary content, no significant differences were observed between dietary treatments, which shows that the inclusion of SWM in the diet did not influence, in this study, the change in pigmentation of juveniles of European lobster.

The colour of decapod crustacean's exoskeleton is mainly determined by the carotenoid astaxanthin incorporated into a macromolecular protein complex known as crustacyanin. Within this complex, astaxanthin is found in a free or unesterified state (Chayen *et al.*, 2003). Since unesterified astaxanthin is present in the crustacyanin complex, it might be expected that the available content of unesterified astaxanthin could be a decisive factor for the development of the colour of the crustacean shell (Wade *et al.*, 2005). One possibility that might explain the lack of significant results is that the amount of astaxanthin present in the diets, that might not have been sufficient to produce positive effects on the pigmentation of the juvenile carapace. Literature says that astaxanthin extracted from cooked shells of *Pandalus borealis* was around 284 mg Kg^{-1} (Dave *et al.*, 2020), and Crear *et al.*, 2002, support that to achieve a pigmentation equivalent to wild caught juveniles of southern rock lobster, *Jasus edwardsii*, a carotenoid intake of about 115 mg Kg^{-1} is required.

Assuming that these values are required to obtain increased colouration of crustacean's shell, such as with European lobster, it can be assumed that the amount of astaxanthin in the diets with SWM inclusion (72.6 mg kg^{-1} of astaxanthin content, which only 3.6 mg kg^{-1} was unesterified) was not sufficient to translate into better gain in pigmentation.

With the objective of more sustainable aquaculture diets, fishmeal replacement is one of the most obvious options, since its production is not able to meet the needs of aquaculture production. Fishmeal can, and should, then be substituted by other sources of more sustainable origin, but in most cases high or total substitution leads to a deterioration in the health of the individual as well as conditioning of optimal growth. The use of shrimp waste meal represents an enormous potential as a protein source for crustacean diets such as lobster, as it translates into a high protein content and a well-balanced amino acid profile. Nevertheless, there was no effect of the dietary treatment on the body composition of the juveniles. As no significant differences were observed between diets with different levels of SWM inclusion, it can be assumed that the inclusion of this alternative protein source instead of fishmeal, should not hinder body composition, while achieving its objective of obtaining a more sustainable origin.

5. Conclusion

This study aimed to evaluate the growth and weight results of juvenile European lobster when fed with a diet in which the main protein source (Fishmeal) was replaced by increasing percentages of SWM (0 %, 7 %, 14 %, 21 %, 28 %). In this way, it was attempted to understand if SWM, a more sustainable source of protein, could be used without compromising the development of juveniles of this species.

Regarding this subject, the results of this thesis show that the inclusion of SWM, in increasing amounts to replace fish meal, shows promise in meeting the objective of using an alternative ingredient to produce the feed used in aquaculture, thus turning them more sustainable. Since a higher survival rate was observed only when using diets with a higher percentage of SWM, the use of these shrimp waste by-products is demonstrated than can be a possible alternative to fish meal. The highest survival rate (87%) was observed in the group of juveniles fed the diet with the highest inclusion of SWM (SWM 28%). As there was no record of lower growth rate and the differences were not significant between the dietary treatments, the inclusion of SWM showed to be a safe alternative to the inclusion of less sustainable ingredients in a diet for H. Gammarus without compromising the growth performance. The positive results regarding lower mortalities when SWM was included showed the possible positive influence of the presence of compounds only present in the shrimp by-products, such as chitin and astaxanthin.

However, future studies may be necessary to understand how these compounds promote increased survival and how they are assimilated in the organism of European lobster. On other hand, investigating further factors that may have interfered with the growth enhancement predicted for the juvenile groups fed diets with higher SWM inclusion can also be considered useful. The use of higher dry matter diets instead of high moisture diets, as in this study (~ 60 %), may translate into higher rates of intake and thus provide growth gains even when using fish meal replacement by other protein sources. Future research is also needed to better understand the nutritional requirements for pigmentation changes in juvenile European lobster. The astaxanthin content of the SWM-included diets may have promoted health gains in the individuals but did not promote the expected gains in colouration. This may have been influenced by the processing of shrimp by-products which can influence the quantity and quality of astaxanthin present to influence pigmentation.

This study shows that there is an open window for the development of new approaches to crustacean aquaculture feeds with a higher degree of sustainability. Promoting the substitution of ingredients of very low sustainability, but of high nutritional importance, as is the case of fish meal, by other protein sources, either of vegetable origin (which carry greater nutritional problems) or of animal origin, but more sustainable, as is the case with the by-products of shrimp processing, should therefore be considered a priority in aquaculture research.

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