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Nutritional bases of microdiet development for the early stages of two crustacean species: whiteleg shrimp (*Penaeus vannamei*) and European lobster (*Homarus gammarus*)

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Master in Aquaculture and Fisheries

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Masters in Aquaculture and Fisheries

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Universidade do Algarve, 1 de Outubro de 2020

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Acknowledgements

I would like to start by thanking my family, specially my parents for the constant support throughout this adventure. I also would like to thank my closest friends who always motivated me and pushed me further.

I would like to thank Luís Conceição for giving me the opportunity and privilege to work with Sparos. I also would like to thank Wilson Pinto for his constant support and share of knowledge with me and for always being available when I needed.

I also would like to thank ELA and Riasearch for allowing me to conduct my thesis in their facilities. Thank you to the people which have helped and taught me so much, Bruna Silva, Adriana Laranjeira and André Barreto at Riasearch; and José Pedro Oliveira, Carlos Teixeira, Mike Webber and Dionísio Teixeira de Sousa at ELA.

This work was supported by project FA_05_2017_005 SHELLWIN, financed by the Blue Fund program of the Ministry of the Sea, Portuguese Republic.

Abstract

Crustaceans are an important source of aquatic food protein with a growing global demand leading to one of the fastest growing aquaculture sectors. Aquaculture of crustaceans is highly dependent on meeting their nutritional requirements and producing high quality stocks. However, knowledge on the nutrition, feeding ecology, body composition and nutrient requirements in crustacean larval culture is still limited. This thesis aimed at creating nutritional bases for the development of diets for two crustaceans species, whiteleg shrimp (*Penaeus vannamei*) and European lobster (*Homarus gammarus*), at their early development stages.

A first feeding trial was conducted to study the dietary protein requirements of whiteleg shrimp postlarvae. For this trial, six experimental diets were formulated to include 34, 44, 49, 54, 58 and 63% protein levels, designated as treatments P34, P44, P49, P54, P58 and P63, respectively. In this trial, 3.18 mg initial mean body weight postlarvae were reared in triplicate groups for 21 days, being fed one of the experimental diets. Growth performance and survival of shrimp from different treatments were affected by the dietary protein levels, with shrimp fed diet P34 registering significantly lower results than the remaining treatments. Final weight, weight gain, RGR and survival of shrimp only increased until diet P44 and FCR decreased. Broken-line analysis of weight gain indicated that the optimal dietary level of crude protein is 47.1% for whiteleg shrimp postlarvae. This requirement level of protein is slightly higher than previous research on posterior stages of development. This can be due to higher growth rates in earlier stages of development.

A second feeding trial was conducted to study the growth performance of European lobster juveniles fed on three different experimental diets. These diets were rich in protein, protein and calcium or in carbohydrates and calcium, designated as PROT, PROTCA and STASH, respectively. For this trial, 23 lobster juveniles were reared for 90 days fed one of the experimental diets with 8 replicates per diet, except diet PROT which had 7 replicates. Survival in this trial was 100%. Weight gain, FCR and RGR were not affected by the diet treatments. At the end of the trial, body weight and total body length showed to be significantly higher in juveniles fed PROTCA than in the remaining treatments. Cephalothorax length, colouration changes and molting were not affected by the diet treatments.

This thesis concludes that a diet with a medium-high level of protein has shown good performance in shrimp postlarvae, with an optimal dietary level of crude protein estimated at 47.1%. Moreover, from the diets tested on European lobster juveniles, the diet rich in protein and calcium was considered the most suitable for early stages of development. This can be a result of this diet being the most related to the body composition of the lobster at the life stage studied.

Key words: Whiteleg shrimp, *Penaeus vannamei*, European lobster, *Homarus gammarus*, dry feeds, protein requirement

Resumo

Os crustáceos são uma importante fonte de proteína aquática com uma alta procura a nível global, o que tem levado a carcinicultura a ser um dos sectores da área de aquacultura com expansão e crescimento mais rápidos. A aquacultura de crustáceos é muito dependente de cumprir os requisitos nutricionais destas espécies para produção de lotes de alta qualidade. No entanto, os conhecimentos sobre nutrição, ecologia alimentar, composição corporal e requisitos de nutrientes em fases iniciais de desenvolvimento de crustáceos ainda é deveras limitado. Esta tese tem como objetivo otimizar dietas para duas espécies de crustáceos em fases iniciais de crescimento, o camarão-de-patas-brancas (*Peneaus vannamei*) e o lavagante europeu (*Homarus gammarus*).

Na primeira experiência, pós-larvas de camarão-de-patas-brancas foram utilizadas para estudar os seus requisitos de proteína. Nesta experiência, seis dietas experimentais foram formuladas para incluir 34, 44, 49, 54, 58 e 63% nível de proteína, designados como P34, P44, P49, P54, P58 e P63, respetivamente. Nesta experiência, pós-larvas com peso médio corporal de 3,18 mg foram utilizadas durante 21 dias em triplicados, sendo alimentadas uma das dietas experimentais. A performance de crescimento e a sobrevivência das pós-larvas demonstraram ser influenciadas pelo nível de proteína nos camarões alimentados com a dieta P34, registando resultados significativamente menores. O peso final, ganho de peso, RGR e sobrevivência registaram aumento até a dieta P44, enquanto que o FCR decresceu até P44. A análise de "broken-line model" dos dados de crescimentos mostrou que o nível ideal de proteína bruta para pós-larvas de Camarão-de-patas-brancas é 47.1%. Este nível de proteína é um pouco mais alto que o reportado em estudos anteriores, para fases de desenvolvimento posteriores, o que pode ser devido à alta taxa de crescimento em fases iniciais de desenvolvimento.

Na segunda experiência, juvenis de lavagante europeu foram utilizados para estudar a performance de crescimento quando alimentados com três dietas experimentais, ricas em proteína, proteína e cálcio ou em hidratos de carbono e cálcio, designados como PROT, PROTCA e STASH, respetivamente. Nesta experiência, 23 lavagantes foram criados durante 90 dias sendo alimentados uma das dietas experimentais, com 8 replicados por dieta, exceto a dieta PROT com 7 replicados. A sobrevivência nesta experiência foi de 100% e o ganho de peso, FCR e RGR demonstraram não ser influenciado pelas diferentes dietas. O peso e o comprimento total dos lavagantes mostrou ser significantemente maior no final da experiência em juvenis alimentados com a dieta PROTCA. No entanto, o comprimento do cefalotórax, as mudanças de coloração e as mudas por lavagante não foram influenciados pelas diferentes dietas.

Em conclusão, uma dieta com um nível médio-alto de proteína demonstrou uma melhor performance em pós-larvas de camarões, sendo requisito estimado em 47.1% de proteína bruta. Conclui-se também, que entre as diferentes dietas testadas em juvenis de lavagante, a dieta rica em proteína e cálcio demonstrou ser a mais adequada para a fase inicial de crescimento dos lavagantes. Isto pode ser devido a esta dieta ser a mais correlacionada com a composição corporal dos lavagantes nesta fase de desenvolvimento.

Palavras-chave: Camarão-de-patas-brancas, *Penaeus vannamei*, Lavagante europeu, *Homarus gammarus*, microdietas, requisitos de proteína

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1. Introduction

1.1. Aquaculture overview

As world's population continues to expand so does the demand for food sources. The demand for seafood supply has been widespread and increasing rapidly and to meet these demands, captures fisheries have played an important role. Alongside, global aquaculture production has contributed to the impressive growth of world's seafood supply for human consumption. The contribution of aquaculture in world fish production has been constantly increasing whilst dominating the aquatic food production sector globally (FAO, 2020). According to FAO (2020), in 2018, as world aquaculture fish production continues to increase, its productions has reached 82.1 million tonnes accounting for 46% of global fish production. The rapid increase of aquaculture industry has allowed for it to become the main supplier of fish and seaweed for human consumption, exceeding global capture fisheries. This industry is also highly diversified not only in species but in processes and product forms which are not only for the purpose of human consumption but increasingly generating by-products for non-food uses, such as production of fishmeal and fish oil.

Aquaculture production still has an uneven distribution, even though being globally spread, Asian countries are the major producers, contributing to 91% of global aquaculture production (FAO, 2020). Despite aquaculture being the fastest growing production sector, in Asia particularly, the expansion of European Union aquaculture production has not followed this pattern, even dropping slightly in the last few years. Europe, only accounting for 3.8% of the global production in 2018 has not represented an alternative to capture fisheries, which have also been declining in this region (DGRM, 2014; FAO, 2020). European aquaculture production has had a slow and weak growth compared to global aquaculture production, however it still is renowned by its technology, feed production quality, sustainability, consumer protection standards and suitable climate for specific species. However, European producers have to compete with other countries productions which have lower labor costs respective of their higher production scales (DGRM, 2014). Still, Norway is one of the major global fish producers, known as the top producer of Atlantic Salmon, producing 1.35 million tonnes in 2018 (FAO, 2020). European aquaculture sector is still making efforts to produce more species, being the most produced species the Atlantic salmon (*Salmo* *salar*), and the rainbow trout (*Oncorhynchus mykiss*). Moreover the Mediterranean area where market is becoming saturated for gilthead seabream (*Sparus aurata*) and seabass (*Dicentrarchus labrax*) production, which still are between the most valuable species in the European market (DGRM, 2014). More recently, Member states of European Union have promoted various actions for a sustainable development of the aquaculture industry in the EU. Therefore, due to their biological and economical potential, emerging finfish species have been selected for the expansion and diversification of European aquaculture such as meagre (*Argyrosomus regius*), the wreckfish (*Polyprion americanus*), the Atlantic halibut (*Hippoglossus hipoglossus)*, the grey mullet (*Mugil cephalus)* and the pikeperch (*Sander lucioperca*) (DIVERSIFY, 2017). The progress in technological developments has become increasingly important to the seafood production sector.

The need for further diversification in aquaculture has created opportunities for expansion of crustaceans aquaculture leading to its rapid increase for decades, specially shrimp aquaculture. Of all the sectors of aquaculture, shrimp aquaculture has expanded rapidly across the world becoming one of the most important sources of protein available for human consumption (Ayisi et al., 2017).

1.2. Crustacean aquaculture industry overview

Crustacean aquaculture production is one of the most important sectors of aquaculture industry. Crustaceans are an important source of aquatic food protein and also an essential revenue for people engaged in this sector. In 2018, crustacean production reached 9.4 million tonnes, contributing at around 11% to global aquaculture production. Since the expansion of aquaculture globally, crustaceans have showed an average annual growth of 2.9% (FAO, 2020). The production of crustaceans is a valuable component of the current growth of aquaculture industry and it is expected that it carries on in the future, contributing to a higher relative contribution to the overall aquatic protein production.

Crustacean farming has had a very strong growth particularly due to the aquaculture of marine shrimp which registers the highest in terms of quantity and value, followed by lobster, marine crabs and freshwater crustaceans (Bondad-Reantaso et al., 2012; FAO, 2020). Crustacean production grows at an average annual rate of 9.92% per year since 2000, with marine shrimp, specially *P. vannamei* and *P. monodon,* currently dominating the production of crustaceans reaching 5.5 million tonnes, accounting for 65.3% of total crustacean production. They are followed by the valuable freshwater crustaceans which account for 29.9% of crustaceans production reaching 2.5 million tonnes (Tacon, 2019). Shrimp are one of the most heavily traded fish products where its production is a valuable source of foreign exchange earnings to some developing countries in Asia and Latin America and also where the bulk of production takes place (FAO, 2020). World shrimp production is led by the Asian continent, reflecting on the dramatic increase of white leg shrimp (*Penaeus vannamei*) farming in China, Thailand and Indonesia which are also the major shrimp exporting countries. However, USA is the world's largest shrimp market, having a major effect on these species exporting countries throughout the world (Bondad-Reantaso et al., 2012; FAO, 2020).

Crustaceans are not only important for human consumption, they are also part of an important and valuable global trade of ornamental aquatic species which is of extreme importance for developing countries (Bondad-Reantaso et al., 2012). Therefore, governments must take in consideration sustainability issues concerning shrimp capture fisheries, sustainable shrimp trade and aquaculture. These may affect the contribution of crustaceans, particularly shrimp, to global security, therefore should be given attention whilst implementing practical solutions (Bondad-Reantaso et al., 2012; FAO, 2020). Consequently, for this study two different crustacean species were used, *Penaeus vannamei* which has well established production both commercially and research wise, however lacks further knowledge on the optimal protein levels required for an adequate postlarvae growth and development. On the other hand, *Homarus gammarus* is a crustacean species where aquaculture production is less established and further research and knowledge is necessary on the nutrient requirements of this species in order to produce a suitable dry feed for appropriate development and survival of early life stages.

1.3. *Penaeus vannamei*

1.3.1. Biology

The whiteleg shrimp, *Penaeus vannamei* (Boone, 1931), is a decapod crustacean native to the Eastern Pacific coast of Mexico and as far south as Northern Peru, in areas where water temperatures are usually over 25ºC throughout the year (Dugassa and Gaetan, 2018). They live in tropical marine habitats and are omnivorous diggers feeding on a wide variety of benthic organisms and detritus. The adults live and spawn in the open ocean while postlarvae and juveniles migrate inshore to areas such as coastal estuaries, lagoons and mangrove areas until they reach sub-adult stages. The life cycle of the whiteleg shrimp is quite complex, with males and females becoming mature from 20g and 28g onwards, respectively, at the age of 6 to 7 months. A matured whiteleg shrimp female can weigh between 30 to 45g and spawn between 100 to 250 thousand eggs in the offshore waters where fertilization occurs in the external environment. Approximately 16 hours after spawning and fertilization, hatching of the first larval stages occurs where nauplii are released from the hatching eggs and live of their egg yolk sack reserve. Through metamorphosis, nauplii develop into the next larval stages (protozoa, mysis and early stage of postlarvae) which remain planktonic. In farming conditions, during these stages, protozoa feeds on phytoplankton while mysis and early stage of postlarvae feed on zooplankton (rotifer, copepods and *Artemia*). Once they molt into their postlarvae (PL) stage, their morphology is similar to their juvenile and adult stages, and they start feeding on benthic detritus, worms, bivalves and crustaceans (Dugassa and Gaetan, 2018; Zhang et al., 2019).

The whiteleg shrimp has been in the last decade one of the most important farming shrimp species, occupying a vital position in aquaculture industry. By the early 1980s, commercial culture of this species and intensive breeding had begun in United States of America, Central and South America and rapidly growing. The rapidly expanding of the production of the whiteleg shrimp is due to its various competitive advantages such as faster growth rate, good survival in high density stocking, tolerance to a wide range of temperatures and salinities, higher resistance to diseases in intensive grow-out productions and lower protein requirements (Bondad-Reantaso et al., 2012; Kannan et al., 2015; Lee and Lee, 2018). Furthermore, as shrimp farming overcame other sectors and advancements on the species management evolved, key techniques were developed in hatcheries for culture improvement. The best technique for a better performance in maturation and spawning of the whiteleg shrimp relies on the use of the eye stalk ablation method for the induction of ovarian maturation in the shrimps (Kannan et al., 2015). By using such techniques, it has led to more spawning and egg production but also higher fertilization or hatching rates. Therefore, further research on the whiteleg shrimp production and management has led to vital developments which have improved and intensified their production, facilitating the increased demand.

1.3.2. *Penaeus vannamei* aquaculture production techniques

Shrimp farming, which currently supplies most of the volume of the global market is dominated by the farming of Penaeidae shrimp such as the whiteleg shrimp (*P. vannamei*), the top reported crustacean aquaculture species and of great economical value. In 2017, shrimp farming had reached 35.1% in China, the major producer, followed by Indonesia with a significant lower percentage, 13.3%. Total shrimp commercial feed usage is estimated to increase from 7.6 million tonnes in 2017 to 10.5 million tonnes by 2025, while shrimp production is estimated to reach 7.8 million tonnes in 2025 (Tacon, 2019). The whiteleg shrimp is the most important farming shrimp species in the last decade, representing 52.9% of all crustaceans production, reaching 4.9 million tonnes in 2018 (FAO, 2020). The whiteleg shrimp has well established aquaculture production techniques including proper feeding strategies for production at all life stages. With the expansion of the whiteleg shrimp aquaculture, various hatchery production systems have been developed in last decades, from small, unsophisticated hatcheries to larger sophisticated intensive hatcheries installations. Aquaculture production of the whiteleg shrimp is based on the capture of wild broodstock and then purchase of tank-reared SPF (specific pathogen free) and/or SPR (specific pathogen resistant) broodstock which have been vital for avoiding diseases outbreaks, still an ongoing problem in productions. Shrimp health concerns have been one of the main reasons that revived the interest in hatchery productions of post larvae shrimp. This has led to the development of genetic selection programs and using pond-reared broodstock with maturation facilities (FAO, 2003). Some farms still use nurseries, where postlarvae are kept until they reach larger sizes, before the grow-out stage instead of purchasing and transporting postlarvae. The choice of ongrowing techniques to be used is not only based on stocking densities but also on the production yield that is required as the different techniques result in different survival percentages, FCR and number of batches per year (Briggs, 2006).

Successful culture of the whiteleg shrimp and growth enhancement depend mainly on important factors such as water quality and feed management. Low water quality has shown to cause stress which eventually leads to loss of appetite, slow growth and a greater susceptibility to diseases. In order to maintain water quality and prevent diseases, efforts have been employed in the development of innovative practices while maintaining and increasing sustainability such as producing shrimp under biofloc technology (BFT) and closed recirculating aquaculture systems (RAS) which have gained a lot of attention in the last years (Ray and Lotz, 2017; Yun et al., 2015). By using clear water RAS which have more filtration components, system control is higher which can accommodate better higher animal biomass while controlling and stabilizing the system, specially the nitrogen cycle. Both these systems contribute to reduce water exchange, wastewater while enhancing biosecurity and intensive nurseries with larger animals (Ray and Lotz, 2015). Although commercial production of the whiteleg shrimp has grown enormously in the past decades, it has been severed impacted due to diseases outbreaks which has limited production and caused great economic losses. Diseases such as White spot and Taura Syndrome have led to development of biosecurity measures and use of disease free SPF (specific pathogen free) and resistant SPR (specific pathogen resistant) broodstock (Briggs et al., 2004; Gunalan et al., 2014). Moreover, during nursery and early juvenile stage it is essential that shrimp have an appropriate feeding strategy and do not suffer from nutritional deficiencies while maintaining water quality as it may trigger their cannibalistic nature (Romano and Zeng, 2017).

1.3.3. Larval nutrition

The lack of knowledge concerning nutrition requirements of the whiteleg shrimp larvae may lead to sub-optimal rearing condition (Xia et al., 2010). At early life stages a wide range of feeds and nutritional supplements are used in feeding protocols of commercial hatcheries. During the early life stages, nauplii, mysis and protozoa stages, microalgae are added to the tanks for availability as direct food where it is maintained or reduced slightly until post-larval stages are reached. A*rtemia* spp. increasingly replace algae until the postlarvae stage when microdiets become the dominant feed. Microdiets have been used as alternative or alongside live feeds on the whiteleg shrimp since their early life stages, from mysis and early post larvae stages until adults (Gamboa-Delgado and Le Vay, 2009).

Protein is an essential and the most expensive nutrient in compound feed for shrimp, reducing its level may lead to drastic reduction in growth, therefore it should be carefully formulated in order to meet the needs of the cultured shrimp at a certain life stage (Shahkar et al., 2014; Yun et al., 2015). Studies on protein requirement at the various stages of life of the whiteleg shrimp have allowed to formulate various feeds for a better nutrient digestibility whilst using optimal protein quantities (Yun et al., 2015; Zhou et al., 2014). As nutritional requirements differ at different life stages of the whiteleg shrimp, studies have been conducted to estimate these requirements to apply formulated microdiets in feeding protocols, as addition or replacement of live feeds. However, shrimp are known to shred their food particles prior to ingestion, this leads to rapid discharge making their food consumption measurements and nutritional studies rather difficult. Though, a minimum dietary protein level is required in order to supply the adequate amino acids for maintenance of physiology and metabolism (Lee and Lee, 2018). As growth performance depends highly on dietary protein and the quality of feed, it is important that this is well established as protein in excess relative to energy may lead to higher excretion of ammonia nitrogen affecting also water quality and consequently diseases outbreaks and cannibalism (Lee and lee, 2018; Hu et al., 2008). Increase demand of dietary protein has led to the production of commercial feeds with various formulations, being the most widely used ingredients fishmeal and soybean meal which are known to be mostly used in feeding trials in juveniles and subadults (Xie et al., 2016).

Yet, there is a lack of research conducted on the protein requirements of this species postlarvae as most studies have been conducted on juveniles and sub-adults. Such studies have estimated the varying optimum dietary protein requirements under different conditions which can improve growth performance of the whiteleg shrimp. Protein requirements varying between 30% and 56% have been reported to be optimal for better growth and performance. However, these estimated values can be influenced by various factors such as shrimp size, body weight, type of culture system, stocking density and biological value of protein sources (Brown et al., 2013; Shahkar et al., 2014; Yun et al., 2015). For instance, Velasco et al. (2000) reported optimum protein level of 21.5% and 22% to achieve better growth of whiteleg shrimp postlarvae in a feeding trial using between 5 and 25% protein levels. In various feeding trials with juveniles, for maximum growth performance, Hu et al. (2008) and Shahkar et al. (2014) recorded similar results in feeding trials of juveniles where optimal dietary protein level for better growth could be of 34% and 33.4%, respectively. In another feeding trial where both juvenile and sub-adult shrimp were fed with 16% and 32% level of protein diets, both stages had a greater weight gain when fed with 32% protein diet, complying with relatable information obtained in the latter studies (Kureshy and Davis, 2002). Despite the little information on the protein requirements during the adult stage of the whiteleg shrimp, Lee and Lee (2018) used larger shrimp between 10 and 20 g size in a feeding trial with six experimental diets between 25 and 50% level of protein. Here it was estimated a 32.2% optimal dietary protein level based on weight gain. Moreover, in the same trial juveniles and sub-adults were also studied where the optimal dietary protein levels would be 34.5 and 35.6%, respectively. It is extremely important to understand these nutritional requirements in all life stages of the whiteleg shrimp, but specially in larval stages. This will support the development of appropriate formulated diets and feeding protocols, not only to aid in the transition of live feeds to artificial diets but also to improve growth and feed intake, and ultimately increase the efficiency of production of this species.

1.4. *Homarus gammarus*

The European lobster, *Homarus gammarus* (Linneaus, 1758) is a large decapod crustacean of highly ecological and economic importance. By 2013, world lobster production had reached 231 thousand tonnes while the European lobster landings reached 4 571 tonnes (FAO, 2017). Despite its landings not reaching the ones of the American lobster (*Homarus americanus), H. gammarus* is of high value in the market and of great importance to local fishing communities contributing to the regional economic growth. Moreover, this species has a key role in maintaining a healthy and diverse ecosystem (Ellis et al., 2015; Moland et al., 2011). They are primarily nocturnal animal with a broad geographical distribution inhabiting sub-tidal eastern North Atlantic waters from the north of Norway to the Azores and Morocco (Moland et al., 2011; Wahle et al., 2013). They are primarily found from the low intertidal to depths exceeding 50 m and they have preference for rocky substrates with holes and cracks where they can find shelter and hide (Burton, 2003; Prodöhl et al., 2007; Wahle et al., 2013). European lobsters are omnivorous and hunt mainly nocturnally, they forage during dark hours as light hours are spent inside shelters to reduce predation risk. Their diet is mainly composed of protein-rich animal diet as they feed on a great range of benthic organisms such as crabs, molluscs, sea-urchins, starfish and polychaeta worms (Factor, 1995; Moland et al., 2011).

European lobsters have a relatively long life cycle with a slow growth rate and as other crustaceans their growth occurs through molting (Rötzer and Haug, 2015). The main factor influencing growth rate in lobster has been considered to be an optimum water temperature (around 20 to 22ºC) and water quality (Prodöhl et al., 2007). Molting occurs continuously throughout their life cycle decreasing in frequency during juvenile stages where adults lobsters molt only once every one or two years as it becomes an annual part of the mating, spawning and egg hatching cycle (Prodöhl et al., 2007). The molting cycle and sexual maturation of these species are vital processes which are influenced by a complex of glands in crustacean eyestalks (Wickins and Lee, 2002). Both female and male lobsters reach sexual maturation at around the same age where the females mature between 5 to 7 years of age with a carapace length of 75 to 80 mm (Prodöhl et al., 2007). Premolt females search for shelters where they will be able to mate, here they remain until the eggs are fertilized which are held underneath their abdomen for approximately one year until hatching occurs during their hatching season, between June and September (Prodöhl et al., 2007). After hatching, prelarva are released and begin their planktonic stage floating on the surface layers (Rötzer and Haug, 2015; Burton, 2003). These prelarva will go through three larvae developmental stages (zoea stages) until metamorphosis into the fourth larval stage, called postlarvae stage where they go into their benthonic stage and begin to appear anatomically like a miniature adult lobster (Prodöhl, et al., 2007; Rötzer and Haug, 2015).

1.4.1. *Homarus gammarus* aquaculture production techniques

The European lobster is considered to be one of the most exclusive seafood products in the world and identified as one of the most promising aquaculture species due to its high prospected margins. Over the past decades, there has been an increase concerning fisheries pressures on lobster species, mainly *Homarus spp.* which has led to lower lobster stocks not complying with an increasing demand in these species market (Carere et al., 2015; Kristiansen et al., 2004). Throughout the 1980s-1990s, various efforts were put into renewing and enhancing European stocks as response to the fisheries collapse throughout Scandinavia from 1930 to 1970 as result of overexploitation and inadequate management (Ellis et al., 2015). Due to the large fluctuations in lobster landings worldwide and the importance of these highly prized species throughout the world, several breakthroughs have been achieved and are ongoing in lobster aquaculture production. This has included the development of hatchery techniques to ensure that stocks of valuable species such as the European lobster remain sustainable for future generations (Pereira and Josupeit, 2017; Kitada, 2018). Aquaculture production of the European lobster has been increasing in the last decades, despite less literature available comparing to other homarid species, research efforts have been put towards improving water quality conditions and development of novel rearing systems while improving cost-effectiveness, although less towards the development of a species-specific formulated diet (Goncalves et al., 2020). Research attention on the farming of this species has been focused on restocking programs and stock enhancement by releasing juvenile lobsters into the wild and on the emerging sector of commercial lobster farming (Ellis et al., 2015; Hinchcliffe et al., 2019).

Farming of lobsters beyond postlarvae size requires further innovation to maintain feeding, cleaning and separation of juvenile lobsters as they grow (Powell and

Elce, 2016). Although there are well established rearing techniques for lobster larviculture and juveniles, long term ongrowing of juveniles has proved to be a challenge due to high mortality rates and aggressive behavior when communally cultured, due to their natural cannibalistic behaviour (Powell and Elce, 2016; Romano and Zeng, 2017). To prevent mortality, juveniles are reared in separate containers which can affect molting frequency, size increase at molt and survival of lobsters if each holding container is not the of proper size (Aiken and Waddy, 1995). Moreover, optimized water conditions have proven to be essential to reach maximum productivity and improve growth of lobsters since larval stage, specially temperature which has been shown to be the primary controller of growth (Drengstig and Bergheim, 2013; Schmalenbach and Buchhol, 2013). With major progresses made in recirculation technology, it has improved land-based aquaculture of this species and allowed the use of heated water contributing for an accelerated growth in lobsters (Drengstig and Bergheim, 2013). Despite novel rearing techniques for the European lobster being studied for the past decades, bottlenecks remain for the commercial culture of lobsters due to the lack of high quality dry feed (Drengstig and Bergheim, 2013).

1.4.2. Juvenile nutrition

There is a lack of knowledge on the feeding ecology and nutritional requirements of the early stages of the European lobster. Therefore improvements on the rearing of this species relies on the development and use of suitable dry feed for different lobster life stages (Carere et al., 2015; Powell et al., 2017).). However, some studies have been conducted with European lobster where live/frozen feed has been replaced by various commercial dry diets. By embracing the use of formulated feeds, a simpler and more reliable production is possible, as storage will be more stable and nutritional quality more consistent whilst influencing growth, development and survival of lobsters. The study conducted by Powel et al. (2017) has shown that it is possible to successfully rear European lobster larvae whilst using a dry commercial food diet under the similar conditions of a commercial hatchery. Furthermore, a study conducted by Haché et al. (2015) on American lobster showed that by feeding larvae with Artemia and a mixture of dry commercial feed had optimized growth and survival as the typical feeding regime used was suboptimal. In order to improve *H. gammarus* larval performance, it is crucial that efforts are put into development of a species-specific diet, formulated taking into consideration its proximate composition as shown by Powell et al. (2017). A promising research by Hinchcliffe et al. (2019) has shown how the development of formulated feeds is essential and how these can be tailored to meet the different nutritional requirements of the various European lobster life stages. Here they suggest how different protein concentrations in diets should be investigated as their conclusion show that a diet containing high protein content from shrimp could be the best source for a sustainable lobster feed in this emerging aquaculture sector. Moreover, a nutritional trial has previously been conducted on European lobster juveniles with a diet produced by SPAROS, Lda. where a weaning microdiet rich in protein led to enhanced growth (Sousa, 2018). This trial has shown that by using this diet not only the growth of the lobsters was enhanced when compared with a control diet (fresh blue mussels) but it also promoted a higher voluntary feed ingestion (Sousa, 2018). Development of experimental diets for European and American lobster has been frequently using findings on the dry matter biochemical composition content, in order to reach an appropriate diet (Goncalves et al., 2020). Despite various studies showing that formulated dry feeds contribute to enhancement of growth and survival, knowledge on the feeding behavior and nutrition of this species is still needed in order to develop a balanced diet according to species-specific information of the European lobster.

1.5. Objectives

This thesis aims at establishing nutritional bases to formulate appropriate diets suited for the early development stages of crustaceans. Therefore, the performance of two crustacean species (*P. vannamei* and *H. gammarus*) was evaluated, reared on customized experimental diets during the early developmental stages. For this purpose, two trials were conducted: the first trial aimed at evaluating the dietary protein requirement of whiteleg shrimp postlarvae. The effect of six experimental microdiets differing in protein composition (34 to 63%) was evaluated in shrimp growth performance, survival and feeding efficiency. The second trial, aimed at determining the performance of European lobster juveniles fed on three different diets (rich in protein, protein and calcium or in carbohydrates and calcium). Thus, the effect of these diets was evaluated on lobster growth, molting, survival and coloration changes.

2. *Penaeus vannamei* trial

2.1. Material and methods

2.1.1. Dietary treatments

Experimental diets were produced at Sparos Lda (Olhão, Portugal) and tested on *P. vannamei* postlarvae. The shrimp were fed on six different experimental diets (P34, P44, P49, P54, P58 and P63) which were formulated to contain 34%, 44%, 49%, 54%, 58% and 63% crude protein, respectively. Ingredients with similar percentages of inclusion in experimental microdiets were as follows: fish meal, fish protein hydrolysate, wheat gluten and cholesterol. Variations on ingredients, including squid meal, wheat meal and fish oil were conducted to have isolipidic diets.

All diets were produced following these procedures: the powder ingredients of the diets were initially mixed according to each target formulation in a double-helix mixer and later ground twice in a micropulverizer hammer mill. Subsequently, the oil fraction of the formulation was added and the diets were humidified and agglomerated through low-shear extrusion. After extrusion, each diet was dried in a convection oven at 60ºC for 4 hours and later crumbled and sieved to the desired size ranges. For the feeding trial, diets were sieved as 400-600 µm pellets.

2.1.2. Rearing conditions

The trial was conducted at Riasearch facilities in Murtosa (Aveiro, Portugal) and had a duration of 21 days. Whiteleg shrimp postlarvae were produced in the United States of America (Sea Product Development – Division of Global Blue Technologies, Texas) and transported in six plastic bags to RiaSearch facilities. Upon arrival, before the start of the trial, an acclimatization process was carried out where the plastic bags containing the shrimp postlarvae were distributed equally between 3 tanks of 580L. Water parameters were continuously checked during the acclimation process until the required conditions were reached, pH at 7.8, dissolved oxygen at 8mg/L, salinity at 22 ppt and temperature at around 21ºC. These tanks were part of a RAS (Recirculation Aquaculture System) system where water is renewed every hour.

For three days, postlarvae were co-fed with *Artemia EG* and WinFlat (Sparos Lda, Portugal). *Artemia* cysts were incubated in a 80L conical-cylindrical tank under constant aeration, maintaining a salinity between 25 and 30 ppt, pH between 8.0-8.5 and temperature between 28 to 30ºC. After 24h of hatching, artemia was harvested using a SEP-art GSL Margnetic Artemia cysts (INVE Aquaculture) and collected and retained in an 80µm filter. The artemia was then distributed by hand between the three tanks in four meals (10:00, 13:00, 15:00 and 17:00 hours) and the microdiet was provided using an automatic feeder (Fish Farmer Feeders, Spain) every hour during a 24 hour period.

After three days, at PL18 stage, 4 500 postlarvae were counted and distributed by 18 tanks of 47L each and 892 m^2 shrimp density (250 per tank) (Figure 1). These tanks were part of a RAS system where each individual tank contained aeration, a screen outlet filter (125µm) to eliminate any remaining feed, a net covering the tank to prevent any escape through jumping and an automatic feeder Fish Mate. Both during acclimatization and the trial, water parameters were recorded daily using a commercial probe whilst nitrites and ammonia twice a week. Temperature and salinity were maintained at 28.3 ± 0.6ºC and at 21.5 ± 0.8 ppt, respectively, while dissolved oxygen was maintained at 7.23 ± 0.2 mg/L and pH at 8.05 ± 0.1. Water renewal in each tank was 100% $hour^{-1}$. This system included a mechanical and biological filtration through two 440L sumps where Kaldnes bio media are used as biofilters with aeration for constant movement. Mechanical filtration was obtained through a housing unit with a 50 µm polypropylene filter bag inside which retains any suspended particles such as fecal matter and remaining feed. Connected to the sumps was a chiller to maintain water temperature in all system between 27 and 29 ºC. Water renewal was performed everyday where 10% of the sump water was replaced by water supplied from the borehole. Both the acclimation and trial system were kept in a greenhouse facility and under a natural daily photoperiod.

During the trial, daily feeding was provided by an automatic feeder FishMate providing feed every 3h, on a 24h period, where for 2 hours provides feed and stops for one hour. Every morning, feed remnants from the day before were evaluated qualitatively, from -2 to 2 to adjust amount of feed to be provided to each tank, where -2 represents 20% less feed and 2 stands for 20% more. Automatic feeders were also cleaned everyday to weight feed remnants and later calculate the correct feed provided each day and they were also checked numerous times daily to ensure proper functioning. After remnants evaluation and before providing daily feed, the outlet filter mesh was cleaned and feed remainings and feces were siphoned.

Figure 1. *P. vannamei* postlarvae experimental system, sumps and tanks.

2.1.3. Sampling

At the start of the experiment, at PL18, before distributing the postlarvae through the tanks, 60 Whiteleg shrimp postlarvae were sampled for body weight (BW) to calculate feed to be given on the first day of the experiment. At the end, after 21 days of experiment, larvae from each tank were collected by reducing the water in each tank and collecting them with a net. These were then counted to calculate survival. Afterwards, 90 larvae were weighted in randomly selected groups of 10 to 20 larvae, for analysis of feed conversion ratio (FCR) and relative growth rate (RGR).

2.1.4. Data analysis

Feed conversion ratio (FCR) was calculated as: FCR= (Fi / Ww) where Fi is feed intake (g) and Ww is the wet weight gain (g). Relative growth rate (RGR) was calculated as: RGR(% day^{-1})= (e^g – 1) x 100, where g = (lnWt – ln$W0$) x t^{-1}, Wt is the final weight (g) and $W0$ the initial weight (g) at a chosen time t. Survival was calculated as: S(%)= (Lf / Li) x 100, where Li and Lf are the initial and final number of shrimp postlarvae in the tanks, respectively. Shapiro-Wilk and Levene's tests were performed to analyze normal distribution and homogeneity of variances, when necessary in all parameters evaluated. Before statistical analysis, data expressed as percentage were arcsin transformed: T= *ASIN* (SQRT(value/100)). To evaluate the differences between growth performance, feed intake and survival between the dietary treatments, a one-way ANOVA was performed followed by a Tukey multiple comparison test whenever significant differences were identified. Non-linear regression was performed on the data regarding the final postlarvae weight whereby using the broken-line model it was possible to estimate an optimal dietary protein level. All statistical analysis were performed using the statistical software Rstudio (Version 1.2.5001 - © 2009-2019 Rstudio, Inc.). Differences between the different diet treatments were considered significant when $p<0.05$ in all tests performed.

2.2. Results

2.2.1. Dietary treatments

The proximal composition of the six experimental diets used in the whiteleg shrimp postlarvae trial are shown in Table 1. Crude protein level was registered higher in diet P63 while crude fat was higher in diet P34.

Table 1. Proximal composition of the six experimental diets used in the whiteleg shrimp feeding trial.

Diets	**P34**	**P44**	**P49**	**P54**	**P58**	**P63**
Dry Matter (DM, %)	93.5 ± 0.0	94.1 ± 0.0	93.7 ± 0.1	93.4 ± 0.0	93.2 ± 0.0	93.7 ± 0.0
Crude protein (% DM)	33.8 ± 0.1	44.2 ± 0.0	48.7 ± 0.0	53.6 ± 0.1	58.3 ± 0.2	63.4 ± 0.1
Crude fat (% DM)	14.0 ± 0.0	14.0 ± 0.1	13.7 ± 0.0	13.5 ± 0.2	13.0 ± 0.1	13.1 ± 0.0
Ashes (% DM)	9.6 ± 0.0	9.7 ± 0.0	9.9 ± 0.0	9.8 ± 0.0	9.9 ± 0.0	10.0 ± 0.1
Phosphorous (% DM)	1.5 ± 0.0	1.5 ± 0.0	1.5 ± 0.0	1.5 ± 0.0	1.5 ± 0.0	1.6 ± 0.0
Energy (KJ g^{-1} DM)	18.3 ± 0.1	19.0 ± 0.0	19.3 ± 0.0	19.5 ± 0.0	19.7 ± 0.0	20.1 ± 0.0

Values are expressed as means ± SD. (n=2).

2.2.2. Growth, survival and feed conversion efficiency

The shrimp postlarvae mean initial body weight was 3.18mg. The results obtained concerning growth performance and survival of the Whiteleg shrimp postlarvae are shown in Table 2. After 21 days fed under different diet treatments, significant differences were found between dietary treatments in the shrimp final weight, weight gain, FCR, RGR and survival (Table 2). However, no significant differences were found between dietary treatments above 44% protein level. Shrimp fed diets above 44% protein level showed significantly higher final weight, weight gain, RGR and survival, whereas P34 showed significantly lower values. FCR was significantly lower in shrimp fed above 44% protein level.

Table 2. Whiteleg shrimp postlarvae growth performance and survival reared under different diet treatments for 21 days.

Diets	**P34**	**P44**	**P49**	**P54**	**P58**	**P63**
Final weight (mg)	27.5 ± 0.7 [a]	45.2 ± 2.1 [b]	52.3 ± 5.1 [b]	60.0 ± 2.4 [b]	56.5 ± 8.8 [b]	67.5 ± 16.4 [b]
Weight gain (mg)	24.3 ± 0.7 [a]	42.1 ± 2.1 [b]	49.1 ± 5.1 [b]	56.9 ± 2.4 [b]	53.3 ± 8.8 [b]	64.3 ± 16.4 [b]
FCR	2.1 ± 0.1[a]	1.4 ± 0.2[b]	1.2 ± 0.2 [b]	1.3 ± 0.1 [b]	1.1 ± 0.1 [b]	1.0 ± 0.3 [b]
RGR	10.8 ± 0.1[a]	13.5 ± 0.3[b]	14.2 ± 0.5 [b]	15.0 ± 0.2 [b]	14.6 ± 0.9 [b]	15.5 ± 1.3 [b]
Survival (%)	75.3 ± 4.4 [a]	83.1 ± 4.9 [b]	81.1 ± 2.7 [b]	84.1 ± 1.9 [b]	86.8 ± 3.1 [b]	86.7 ± 0.8 [b]

Values are expressed as means ± SD. Means with different superscript letters within the same row are significantly different ($p<0.05$). ($n=3$).

2.2.3. Estimation of dietary protein requirement

Accordingly, with broken-line model, a dietary protein level of 47.1% was estimated for maximum weight gain (Figure 2).

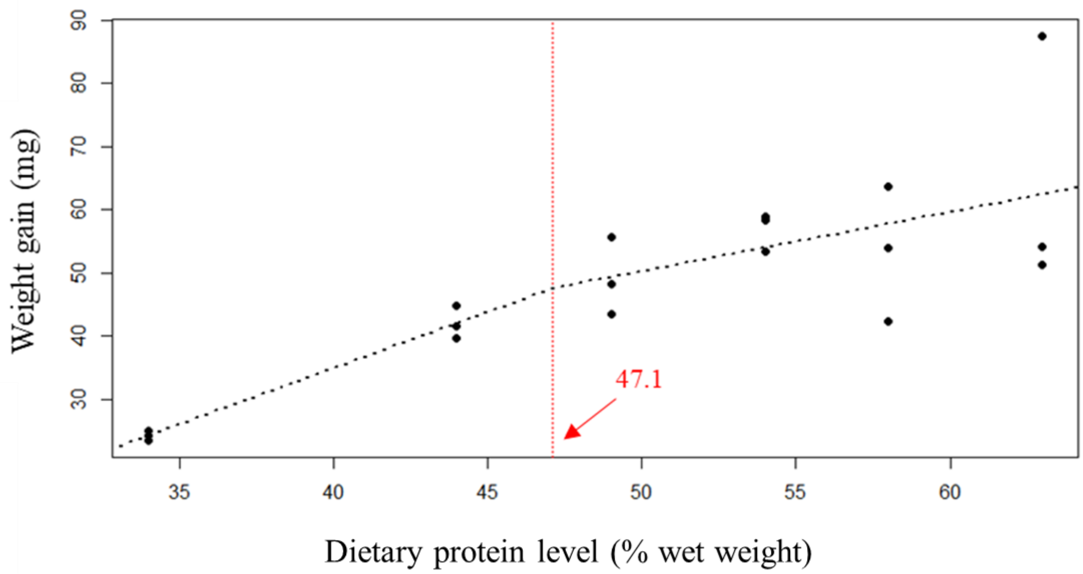

Figure 2. Relationship between dietary protein levels (%) and mean weight gain (mg) of the Whiteleg shrimp postlarvae. It is estimated the protein requirement according to the broken-line model which is given by the abscissa value at the breakpoint.

2.3. Discussion

In this trial, six different experimental diets were tested to study the dietary protein requirements, survival and growth performance in the whiteleg shrimp postlarvae. Six different dietary protein levels were used in this trial, 34, 44, 49, 54, 58 and 63%. In the current trial, postlarvae survival varied between 75 to 87% and averaged 83%, which is similar than those obtained in another study by Velasco et al. (2000) which had a survival between 67 to 93% in postlarvae fed diets between 10 and 25% dietary protein levels. Despite reared under different conditions and fed under protein levels between 12 and 35%, Wang et al. (2016) obtained a mean postlarvae survival of 93.7%. Further studies conducted on juveniles and sub-adults have also registered higher percentage of survivals, with values reaching above 90%, such as Hu et al. (2008) where juveniles survival ranged between 86 to 93%, and Lee and Lee (2018) registered from 83 to 93% juvenile survival. Kureshy and Davis (2002) registered in both trials of juveniles and sub-adults survival above 90%, similar results were obtained in the juveniles trial by Shahkar et al. (2014). In general terms, these results were considered positive, showing that shrimp postlarvae from the current experiment had a good survival, which is a clear indication of good zootechnical conditions and quality of microdiets used. The experimental diets used in this study were formulated with fish meal as one of the main protein sources, considered adequate for protein requirement studies (Lee and Lee, 2018; Yun et al., 2015). Fish meal has shown to be a good ingredient for protein requirement studies due to its nutritional content and many advantages such as its bioavailability, balanced amino acids composition, an rich in vitamins and minerals, making it an attractive protein source (Bowyer et al., 2013).

Final weight of shrimp from different treatments varied from 28 to 68 mg, weight gain varied from 24 to 64 mg, FCR between 1 and 2.1 and RGR values varied between 11 and 16% day^{-1}. Significant differences were found in growth performance between P34 and the remaining diets. Previous studies have recorded different growth performance results than the present study, however it is difficult to make comparisons with the present study as initial body weight of shrimp are different. Weight gain results observed were lower than the studies performed on postlarvae by Velasco et al. (2000) which recorded weight gain between 47 and 88 mg in both trials despite using lower protein levels and postlarvae with lower initial weight, 0.9 and 1.0 mg. Concerning FCR and RGR, Velasco et al. (2000) which used diets between 5 and 25% of protein level observed for both trials, a FCR between 1 and 3, whereas in the present study FCR was closer to 1, and RGR values between 21 and 25% day^{-1}, considerable higher than the present study. These higher RGR values showed by Velasco et al. (2000), demonstrated a higher growth than expected with the level of protein tested. However, this may be due the use of larvae at an earlier stage than the current study, PL7, and also different rearing conditions and different components in feeds which makes it difficult to compare results with Velasco et al. (2000) being the only similar previous study in postlarvae.

Good weight gain was recorded in shrimp juveniles by both Shahkar et al. (2014) and Kureshy and Davis (2002), where higher weight gain was observed when fed with diets above 35% protein level and with 32% protein level diet, respectively. However, these studies used higher initial body weight than the present study. Despite using posterior life stages of development which make comparisons with the current study difficult, these results show that higher weight gain is obtained when fed lower protein levels. Lee and Lee (2018), recorded higher FCR values in juveniles, sub-adults and adult shrimp fed with lower protein levels (25%) which can also be observed in the present study where higher FCR was observed in shrimp fed under P34 diet. The same pattern can be observed in the studies by Shahkar et al. (2014) and Xia et al. (2010). These results show that when lower protein levels are used, higher FCR values are obtained.

Results from the current trial showed that all diets with a crude protein level above 44% resulted in post-larvae with a higher survival, weight gain, RGR and lower FCR values than the diet with 34% crude protein level. Based on the broken-line regression analysis on weight gain of postlarvae of whiteleg shrimp, the optimal dietary protein requirement is 47.1%, under these trials' conditions. Previous studies have reported varying optimum protein requirements under different rearing conditions and depending on the shrimp size. Also using broken-line analysis, Velasco et al. (2000) suggested that optimal dietary crude protein level would be 20.2 and 21.5% in their postlarvae trials. Despite Velasco et al. (2000) obtaining lower estimates of optimum protein level than the current study, they tested lower protein levels and different diet formulations. However, they still obtained a high optimal dietary crude protein level considering the levels tested. Kureshy and Davis (2002) suggested that protein requirement for maximum growth of juveniles and subadults shrimp was found when fed under 32% protein diet and that diets containing low protein did not show maximum weight gain. Lee and Lee (2018) suggested that for juveniles, sub-adults and adults the optimal dietary level of crude protein to be 34.5, 35.6 and 32.2%, respectively and Xia et al. (2010) suggested that a dietary protein level of around 43% could be optimum for sub-adults. Xia et al. (2010) showed that weight gain in sub-adults increased linearly up to 43% protein level, slowly decreasing thereafter but registering an increase at 47% (Xia et al., 2010). The results of the protein requirement obtained in this study is higher than the mentioned previous studies, suggesting that under these trials conditions, postlarvae of whiteleg shrimp will reach maximum growth when fed a 47% protein level diet. The higher level of protein requirement for postlarvae in the present study might be explained by the current stage of development of shrimp where they grow exponentially and have higher growth rates. This means postlarvae may require a higher percentage of protein level for maximum growth.

3. *Homarus gammarus* trial

3.1. Material and methods

3.1.1. Dietary treatments

Test diets were produced at Sparos Lda (Olhão, Portugal) and tested on European lobster juveniles, *Homarus gammarus*. Three different diets were developed following the known and anticipated dietary requirements of the European lobster early life stages studied. These diets differ in their composition where Diet PROT was formulated to contain higher protein levels, PROTCA high in protein and calcium and STASH was high in carbohydrates and calcium. Ingredients with similar percentages of inclusion in experimental microdiets were as follows: fish meal, fish protein hydrolysate, wheat gluten and cholesterol. Variations on ingredients, including squid meal, wheat meal, marine zooplankton meal, shrimp meal and calcium carbonate were conducted to attain the targeted nutritional concepts. These diets were produced using the same procedure as the one described in section 2.1.1. However, they were directly extruded at low temperatures as 2 mm pellets due to the lobster's larger size.

3.1.2. Rearing conditions

This trial was conducted at Estação Litoral da Aguda (Vila Nova de Gaia, Portugal) and had a duration of 90 days. All the twenty-three European lobster juveniles used in this trial were hatched in the same facilities in the summer of 2018 and kept there for a year until the start of the trial. The lobsters were reared individually in 12L aquariums in a closed water recirculating system (Figure 3). Here they were randomly attributed one of the diets (PROT with n=7, PROTCA and STASH with n=8). This system consisted of three sumps with the same characteristics, each 120 L sump containing mechanical and biological filters. These filtration systems were divided in the sumps by a glass division to ensure that the water flowed through each section. As the water came into the sump it first went through the coarse-pored filter sponge which filtered larger waste such as feed and residues. Thereafter, there was the biological filter including sand and filter medium rings which aid in the nitrification cycle. There was also in each sump airstones and universal pumps to achieve water distribution with an output of 3400 l/h, also aiding in supplying oxygen throughout the sumps and aquariums. A protein skimmer was also connected to all sumps to remove organic matter and dissolved/particulated protein from the system. A water chiller was also connected to the water distribution pump in the first sump to aid in maintaining the temperature throughout all system. However, due to colder environmental temperatures it was necessary to place in each sump submersible heaters to control and maintain the required temperature alongside the chiller. Water level was regulated throughout the sumps by using plastic pipes which connected sump 1 to sump 2 and sump 2 to sump 3. Water parameters were checked daily and ammonia, nitrites and nitrates were checked weekly. Temperature was maintained at 20.8 ± 1.1 ºC, salinity = 35.4 ± 1.3 ppt, pH = 7.74 ± 0.1, dissolved oxygen in the sump was 84.9 ± 2.5 % whilst in the tanks was 80 ± 3.3 %, subjected to a natural photoperiod during autumn time.

Within each aquarium, it was placed sand ranging coarse to fine as substrate and small PVC tubes to stimulate their natural burrowing behavior providing shelter and replicating their natural habitat as much as possible. There was also the water inlet and outlet to regulate the water level containing a grid to avoid any clogging caused by the pellets. Here water is renewed in an average of 9 times per hour in each aquarium.

Lobsters were fed twice daily (10:00 and 17:00 h) and every morning feed remnants were evaluated qualitatively, using the same method described in 2.1.2., and registered to adjust the amount of feed for the next day. Feed remnants were cleaned through siphoning alongside feces in the aquariums, this led to an average of 10% water loss in the system therefore each sump was refilled at the end of cleaning leading to water renewal within the system.

Figure 3. System for the rearing of European lobster juveniles.

3.1.3. Sampling

To evaluate growth performance, at the start of the experiment, in the middle (45 days) and at the end of the experiment (90 days) each juvenile was measured for total body length (TL), cephalothorax length (CL) and weighted (BW), carefully dried in paper to remove water excess. Total length was measured from the start of the rostrum until the end of the telson whilst cephalothorax length was measured from the rostrum to the rear of the carapace, both in the center line of the body. In each sampling, photographs were also taken of each lobster to further analyze coloration changes between molts.

3.1.4. Data analysis

Feed conversion ratio (FCR) and relative growth rate (RGR, % day^{-1}) were calculated using the same formulas mentioned in the *P. vannamei* trial. Molt occurrences were recorded daily. The number of molts on lobsters of each treatment was evaluated using a repeated measures ANOVA. Before statistical analysis, data expressed as percentage were arcsin transformed: T= *ASIN* (SQRT(value/100)). Shapiro-Wilk and Levene's tests were performed to analyse normal distribution and homogeneity of variances, when necessary. To evaluate differences in growth performance and feed intake between the different treatments a one-way ANOVA was performed followed by a Tukey multiple comparison test whenever significant differences were identified. To evaluate differences in colouration a chi-square test of independence was performed. When data did not comply with normal distribution, data was transformed using a normalization transformation. All statistical analysis were performed using the statistical software RStudio (Version 1.2.5001 - © 2009-2019 RStudio, Inc.). Differences between the different diet treatments were considered significant when $p<0.05$.

3.2. Results

3.2.1. Dietary treatments

The proximal composition of the experimental diets used in the European lobster juveniles trial is shown in Table 3. Essentially, between all diets the higher level of protein was registered in diet PROT while the highest percentage of fat was registered in diets PROT and PROTCA.

Table 3. Proximal composition of experimental diets used in the European lobster juveniles feeding trial.

Diets	PROT	PROTCA	STASH
Dry Matter (DM, %)	93.4 ± 0.0	94.4 ± 0.0	93.5 ± 0.4
Crude protein (% DM)	74.7 ± 0.1	68.5 ± 0.1	61.7 ± 0.1
Fat (% DM)	10.7 ± 0.1	10.1 ± 0.2	8.9 ± 0.1
Ash (% DM)	11.3 ± 0.3	17.5 ± 0.0	19.7 ± 0.2
Phosphorous (% DM)	1.3 ± 0.0	1.2 ± 0.0	1.1 ± 0.0
Energy (KJ g $^{-1}$ DM)	22.3 ± 0.0	20.4 ± 0.0	19.2 ± 0.1

Results are expressed as means ± SD (n=2). DM- dry matter.

3.2.2. Growth, survival and feed conversion efficiency

The results obtained showed that are no significant differences between weight gain, FCR and RGR between the different diet treatments (Table 4). Weight gain was higher in juveniles fed with PROTCA whilst STASH showed the lowest weight gain. Survival was not affected by the different diets and was 100% in all diet treatments.

Table 4. European lobster juveniles growth performance and survival reared under different diet treatments.

Diets	PROT	PROTCA	STASH
Weight gain (g)	9.2 ± 3.6	9.4 ± 3.4	8.7 ± 3.5
FCR	2.8 ± 0.6	2.7 ± 1.0	3.1 ± 1.3
RGR	0.8 ± 0.1	0.8 ± 0.1	0.7 ± 0.2
Survival (%)	100	100	100

In PROT, n=7 and PROTCA and STASH, n=8 replicates juveniles per treatment. Values are expressed as means ± SD.

Body weight analysis showed that after 45 and 90 days there were significant differences between the diet treatments (Figure 4). PROTCA showed to be the diet with significantly higher body weight values in all samplings whereas PROT and STASH showed similar body weight values. Total body length also showed at 45 and 90 days significant differences between the diet treatments (Figure 5). At 45 and 90 days of highest total body length was recorded in juveniles fed under diet PROTCA. Concerning cephalothorax length, after 45 days significant differences were found between the diet treatments (Figure 6). PROTCA showed to be the diet with highest values of cephalothorax length throughout the rest of the samplings, but with no significant differences between diets in the last sampling at 90 days.

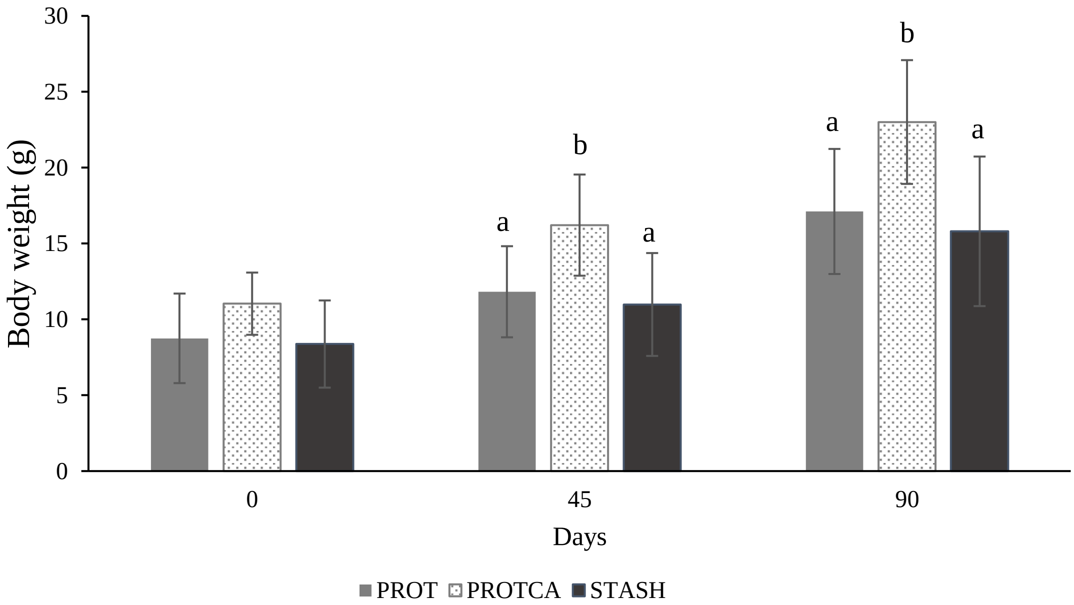

Figure 4. Final body weight of European lobster juveniles reared under different diet treatments. In PROT, n=7 and PROTCA and STASH, n=8 replicates juveniles per treatment. Values are expressed as means ± SD. Means with different superscript letters are significantly different ($p<0.05$) at the same age.

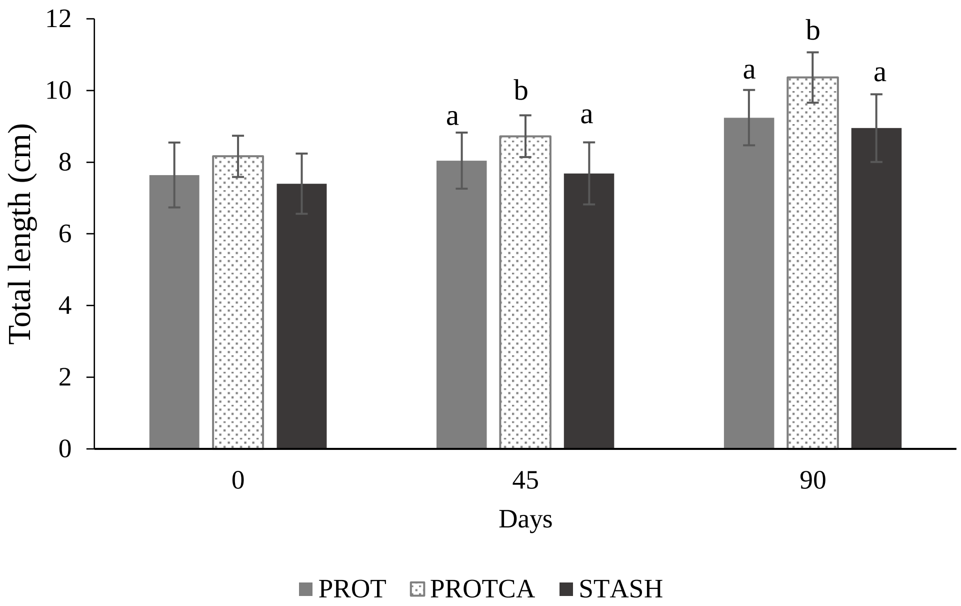

Figure 5. Total body length of European lobster juveniles reared under different diet treatments. In PROT, n=7 and PROTCA and STASH, n=8 replicates juveniles per treatment. Values are expressed as means ± SD. Means with different superscript letters are significantly different (p<0.05) at the same age.

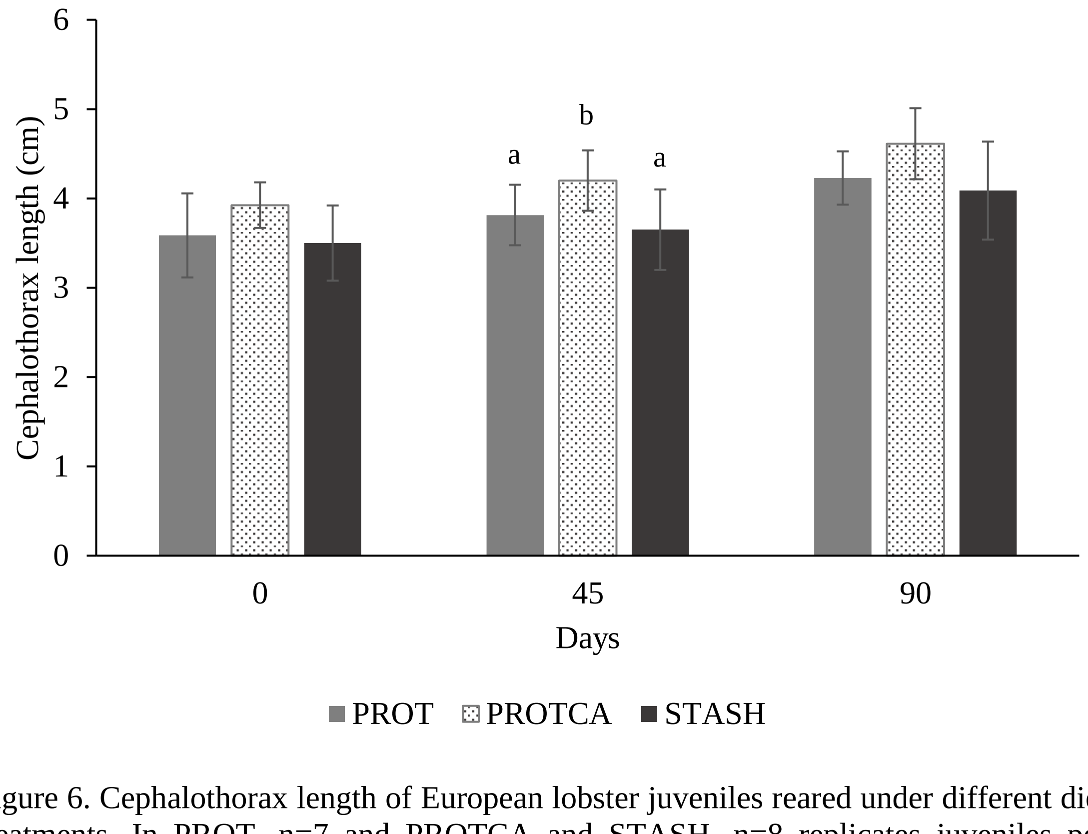

Figure 6. Cephalothorax length of European lobster juveniles reared under different diet treatments. In PROT, n=7 and PROTCA and STASH, n=8 replicates juveniles per treatment. Values are expressed as means ± SD. Means with different superscript letters are significantly different (p<0.05) at the same age.

3.2.3. Colouration

The different colouration in the lobsters were not significantly affected by the different diets (Figure 7 and 8). Before the start of the trial, lobsters showed a lighter shell pigmentation, light blue (Figure 7a), followed by a similar occurrence of dark blue (Figure 7b and 8a). Here, it was also recorded some albino lobsters (Figure 7c). After 45 days, there was no occurrence of lighter blue pigmentation, however in juveniles fed under diets PROT and STASH juveniles showed a pink pigmentation (Figure 7d and 8b). Moreover, more juveniles were found to have blue with purple pigmentation (Figure 7e) and juveniles fed under the diet PROTCA showed a high occurrence of dark blue. After 90 days, at the end of the trial, no significant differences were found between the die

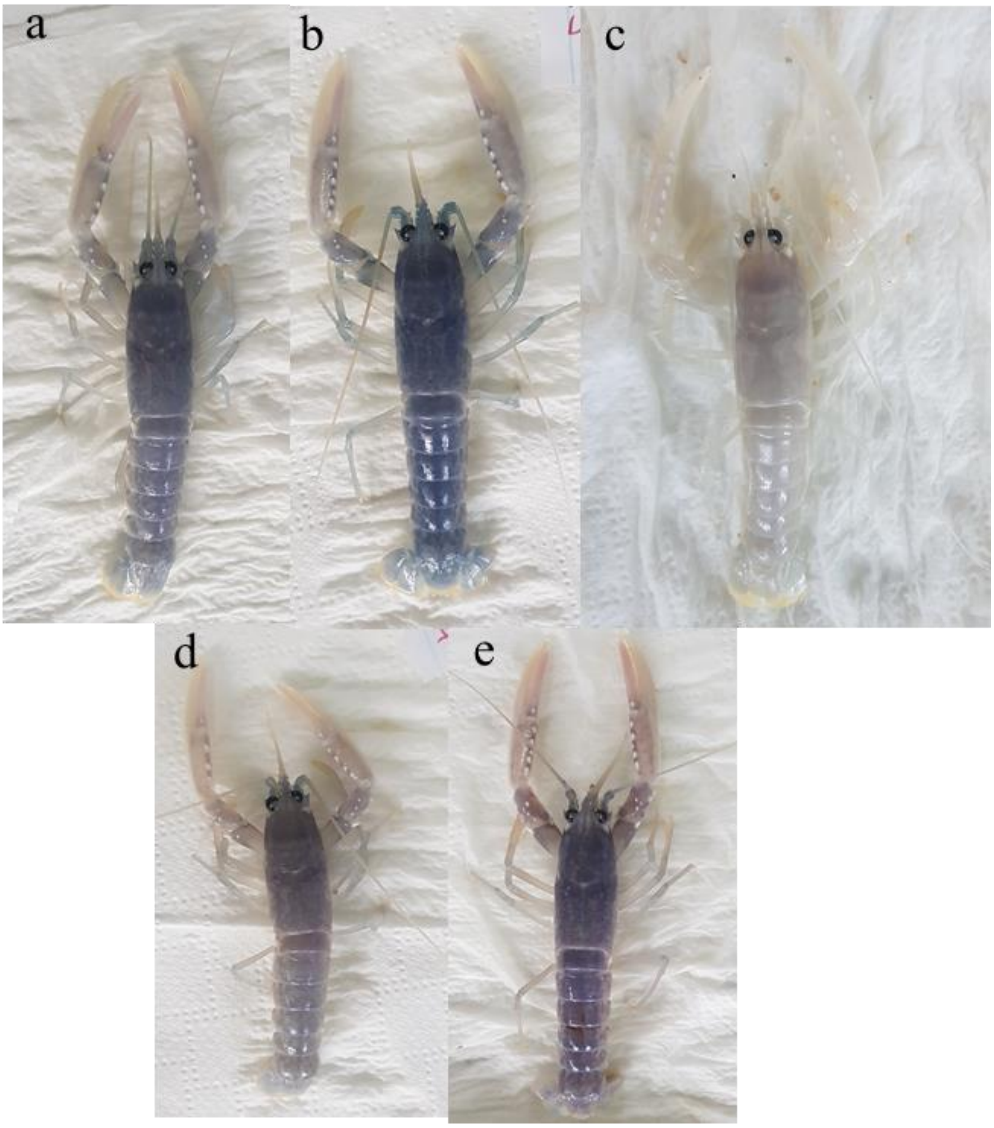

Figure 7. Different shell colouration recorded in the European lobster juveniles in the last sampling at 90 days; a) light blue, b) dark blue, c) albino, d) pink and e) blue with purple.

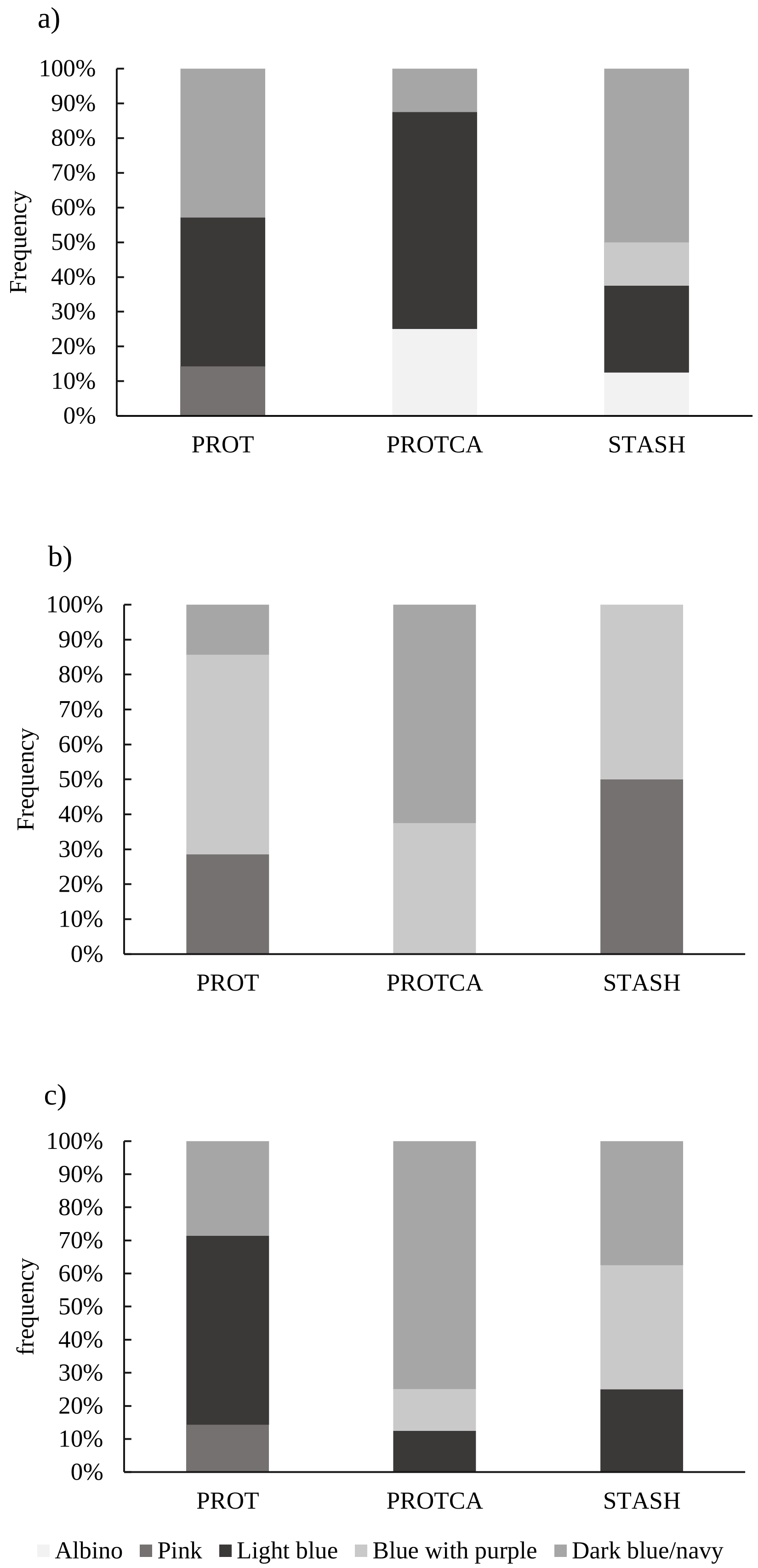

Figure 8. Shell colouration recorded in the samplings a) 0, b) 45 and c) 90 days. In PROT, n=7 and PROTCA and STASH, n=8 replicates juveniles per treatment.

3.2.4. Molting

The number of molts per lobster was not significantly affected by the different diets (Figure 10). Lobsters fed under diet PROT did not record any molts from day 30 to day 60 and reached 1.5 molts per lobsters, as did STASH. Both PROTCA and STASH recorded a steady increase in the average number of molts per lobster throughout the trial. Throughout the trial, a tendency can be observed on molts between all diets where at the end of the trial molts vary between 1.5 and 1.7 molt per lobster.

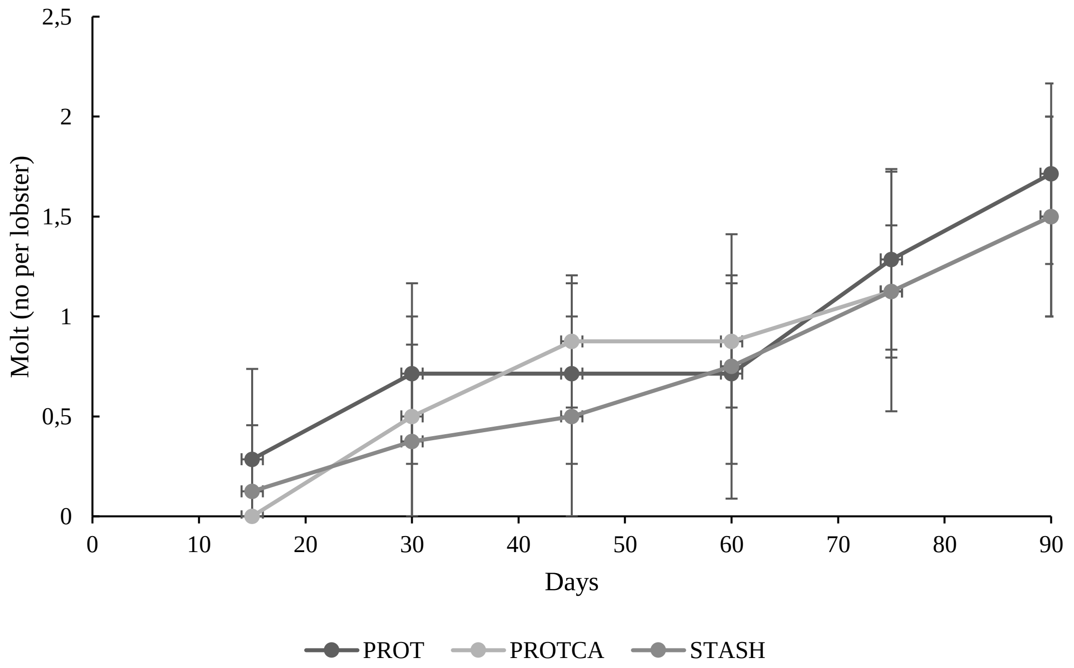

Figure 9. Number of molts of European lobster juveniles reared under different diet treatments. In PROT, n=7 and PROTCA and STASH, n=8 replicates juveniles per treatment. Values are expressed as means ± SD.

3.3. Discussion

In this trial, three different experimental diets were used to evaluate the growth performance, survival, colouration and molting of the European lobster. Juveniles weight gain varied between 8.7 and 9.4 g, FCR between 2.7 and 3.1, RGR between 0.7 and 0.8 %/day while survival was 100%. Colouration registered was varied between diets with different shell pigmentations being recorded at the end of the trial, showing to not be affected by the different diets.

Studies related with growth performance while using dry diets are very limited with little information available for the European lobster. The results from this study establish that the different diets did not affect weight gain, FCR and RGR. FCR values in this study are lower than the previous study by Sousa (2018) which registered 5.91 and 6.35 when comparing a dry feed and frozen feed on juveniles, respectively. Concerning survival, the results obtained in this trial were higher than previous studies. Hinchliffe et al. (2019) recorded in post-larva survivals between 44 and 80% when fed different experimental feeds. Powell et al. (2017) registered very low survival below 20% in early life stages European lobster larvae, however it recorded higher survival (80%) throughout the experiment in lobster fed with only dead conspecifics. Despite the previous studies being conducted on larvae stages of European lobster, the current survival results might be related to a lower sensitiility of the posterior stages of development used in the current study. Sousa (2018) registered a survival of 50% in juveniles fed with a test diet. Relating these results with American lobster, Lim et al. (1997) registered similar FCR values to the present study, with FCR between 2 and 3 in American lobster juveniles fed with formulated feeds. Moreover, they also recorded lower survival results between 79 and 86%. These results demonstrate that the trial was successful and the positive survival percentages observed are higher than the literature, which may also be related to the good rearing conditions employed.

Weight and length parameters (final body weight, total body and cephalothorax length) were all recorded highest in lobster fed with diet PROTCA, which is a diet formulated with protein and calcium. This trend can be seen in the study by Lim et al. (1997) where the formulated diet with higher percentage of crude protein registers the highest final weight. Sousa (2018) also showed that average body weight, body and cephalothorax length were higher in juvenile fed with an experimental microdiet rich in protein.

Colouration differences in shell pigmentations of lobsters fed with dry diets has not had research efforts, therefore a comparison it not possible to be made with further studies. However, it is known that the typical colouration of mature European lobster is blue/navy and also the colour of interest (Chayen et al., 2003). In the present study, at the end of the trial, after molting, some juveniles presented a darker blue/navy, being recorded when fed diet PROTCA. Light blue lobster were also recorded in higher proportion when fed with diets PROT and STASH and pink colouration until 45 days of trial. The lack of pigmentation in some of the juveniles at the beginning of the trial can be associated with deficiencies in food as it has been shown that dietary carotenoids are necessary are in diets of cultured lobsters for normal pigmentation and also growth (Chayen et al., 2003; Kristiansen et al., 2004). The good results concerning darker blue pigmentation in some of the juveniles shows that the formulation of these diets can contribute to a natural exoskeleton colour after the lobster molt.

The number of molts per lobsters in this trial showed to be smaller than a previous study by Lim et al. (1997) where American lobsters juveniles molted at a frequency of 2.2 and 2.5 when reared for 150 days. Although the frequency of molting was higher, in the present study molting frequency was almost 2 per lobster in 90 days of trial. As it is known, molting frequency in lobsters can be influenced by many factors such as diet, light, temperature, water quality which control molting and growth altogether (Aiken and Waddy, 1995).

From the results obtained in this trial, diet PROTCA showed better overall results in all growth parameters studied. This experimental diet has been formulated to be high in protein and calcium. Within the diets used, the diet which contained carbohydrates did not show such positive results in this study. These results can be related to the requirement of carbohydrates at different life stages of development, as earlier life stages may require higher levels according to their nutritional composition (Powell et al., 2017). Although research has not been conducted on the dietary demands of protein in the European lobster, research efforts have been made towards other lobster species, such as the spiny lobster. A study conducted by Williams (2007) showed a dietary demand for high protein in the development of formulated feed for this species. Haché et al. (2015) also showed that through the proximate composition content of American lobster postlarvae, a formulated diet for this species should contain 53% protein. Moreover, protein content in diets has been shown to affect growth and carapace length increment in European lobster juveniles (Goncalves et al., 2020). It is important to study not only the lobsters biochemical composition, but specially their protein content in early life stages of the European lobster so that a diet can meet appropriately the organism's biochemical composition (Goncalves et al., 2020; Powell et al., 2017). In order for lobster to grow, they need to produce a new carapace after each molt. As their exoskeleton consists of calcium carbonate and chitin, diets supplemented with calcium can provide a good source of minerals and nutrient to help solidify their new shell, as shown to happen in crustaceans when they eat their cast shells after molting (Burton, 2003; Schoo et al., 2013). Diet PROTCA showed better performance for these reasons as it provides two essential nutrients for growth: protein as their body is mainly composed of it not having much fat content, and calcium which is essential for their exoskeleton.

4. Conclusions

In this thesis different diets with different levels of protein were used to study the protein requirement of whiteleg shrimp postlarvae. It was concluded that for the conditions of this trial, a 47.1% level of protein is optimal for maximum growth of shrimp. Although further research is still needed in protein requirements in early stages of this species, the results of this trial allowed to understand that above 44% level of protein no significant differences were found in growth performance and survival.

Further studies should not only focus on protein requirement, but also further nutrient requirements which are crucial in early stages of development of this species. By increasing the knowledge in nutrient requirements of this species it will be possible to formulate a suitable diet for maximum growth and survival of this species. Moreover, improvement of water quality, less food waste and environmental impact and lower diet production cost can be achieved.

The three different experimental diets used in the European lobster trial significantly influenced final weight and total body length of juveniles at the end of the trial whilst registering a survival of 100%. The results from this trial have shown that European lobster juveniles have a better growth performance when fed diets formulated rich in protein and calcium.

Due to limited information on these species nutritional requirements, this thesis contributes to increase the knowledge about these species. However, further research on the composition analysis of this species early development stages is essential and still needed. As this has not been determined yet, calculating their general composition such protein, lipids, carbohydrates and minerals composition can be a first approach in order to formulate appropriate species-specific diets for the early stages of the whiteleg shrimp and the European lobster.

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