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Establishment of a feeding protocol to improve survival and growth of whiteleg shrimp (*Penaeus vannamei*) at RiaSearch

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Todas as correções determinadas pelo júri, e só essas, foram efetuadas.

O Presidente do Júri,

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Abstract

Shrimp is one of the most valuable globally traded seafood products and shrimp industry has been particularly relevant to food sector. Nevertheless, with the overexploitation of natural resources, aquaculture has been an important alternative to meet the demand of quality shrimp, contributing to the world total shrimp production.

Whiteleg shrimp *Penaeus vannamei* is currently the most farmed penaeid shrimp species in the world, representing about eighty percent of the global total shrimp aquaculture production. However, their larval and early juvenile production phases are the most critical in aquaculture. Relatively low survival rates and sub-optimal quality are associated to inadequate feed and feeding practices and incidence of cannibalism, which seriously affects the productivity and profitability of several farming enterprises. So, optimizing feed and feeding management is of paramount importance to reduce whiteleg shrimp mortality during early developmental stages.

This is the case of RiaSearch, Lda., a research and development company that performs experimental feed trials with several marine fish species for aquafeed industries and companies, being currently dedicated to whiteleg shrimp. This company has been confronted with a high mortality of whiteleg shrimp, especially during the postlarvae and juvenile stages, which represents a significant economic loss for the company. Reducing the rate of mortality and increasing the growth performance of whiteleg shrimp, while ensuring its correct nutritional status, becomes essential. The supply of proper diets meeting the nutritional requirements of shrimp, along with the use of optimal feeding regimes may constitute useful measures to achieve this goal.

In this context, the present internship aimed to develop feeding strategies to reduce mortality and increase growth performance during the early developmental stages of whiteleg shrimp, at RiaSearch. Accordingly, two experimental growth trials were conducted with whiteleg shrimp postlarvae and juveniles, respectively, in a recirculation aquaculture system, to compare the survival and growth of individuals fed different feeding protocols, including different types of feed and feeding frequencies.

In the first experimental trial, two feeding protocols were tested. One consisted of a co-feeding regime combining a commercial inert microdiet and *Artemia* nauplii during the first eight days, followed by the inert microdiet alone until the end of the trial; the other regime consisted in feeding the commercial inert microdiet alone during the entire trial. Higher survival and growth performance were observed for co-fed shrimp postlarvae. With this feeding protocol, shrimp survival, final body weight and daily growth index were, respectively, 9%, 96% and 67% higher than that of postlarvae fed only with the inert microdiet. This represents a considerable improvement of *Penaeus vannamei* postlarvae growth and survival, at RiaSearch, by using a co-feeding regime, besides suggesting that the nutritional requirements of the species were not satisfied when only the commercial inert microdiet was provided, thus reducing the postlarvae nutritional status and increasing their susceptibility to diseases and to cannibalistic behaviors.

In the second growth trial, shrimp juveniles were fed using three different feeding frequencies – 4, 6 and 8 times per day. Survival increased with increasing feeding frequency, and juveniles given 8 meals per day showed the highest growth performance. Final body weight of shrimp fed 6 times per day was 38% higher than those fed 4 times a day, while shrimp fed 8 times per day exhibited a final body weight 53% and 11% higher than those fed 4 and 6 times a day, respectively. In comparison with shrimp fed 4 times per day, increasing the feeding frequency to 6 or 8 times per day increased the daily growth index in about 18% and 25%, respectively. Additionally, feed efficiency in shrimp fed 6 and 8 times per day was, respectively, 1.4 and 1.6-fold higher than that of shrimp fed 4 times a day. This indicates that feeding 8 times per day remarkably improves growth performance and feed utilization efficiency of *Penaeus vannamei* juveniles, at RiaSearch, also suggesting that providing 4 or 6 meals per day did not ensure the maintenance of shrimp nutritional status, leading to increased mortality.

Results of the present studies demonstrate that using co-feeding regimes with *Artemia* nauplii and inert diets, during early developmental stages, and providing 8 meals per day are the best feed and feeding management to improve survival and growth of *Penaeus vannamei* at RiaSearch, thus enhancing profitability.

Keywords: Feeding protocol; Whiteleg shrimp; Cannibalism; Growth; Co-feeding; Feeding frequency; *Penaeus vannamei*

Resumo

A indústria do camarão tem assumido particular relevância no âmbito do setor alimentar, dado que este crustáceo é, atualmente, um dos produtos aquáticos mais comercializados e consumidos em todo o mundo. Todavia, com a sobreexploração dos recursos naturais, a aquacultura tem-se demonstrado uma importante alternativa no que diz respeito ao fornecimento de camarão, contribuindo de forma cada vez mais significativa para a produção total de camarão, à escala global.

Com mais de oitenta por cento de toda a produção global de camarão em aquacultura, o camarão de patas brancas (*Penaeus vannamei*) é, de facto, a espécie de camarão mais cultivada a nível mundial. Contudo, as fases de produção larvar e juvenil desta espécie são as mais críticas em aquacultura. Taxas de sobrevivência relativamente baixas e uma qualidade sub-ótima do camarão de patas brancas estão associadas a práticas inadequadas de alimentação e à incidência de canibalismo, o que afeta consideravelmente a produtividade e rentabilidade das empresas aquícolas. Deste modo, otimizar a alimentação em aquacultura constitui uma importante estratégia para reduzir a mortalidade durante as fases iniciais de desenvolvimento desta espécie.

A RiaSearch, Lda., uma empresa de investigação e desenvolvimento que se dedica fundamentalmente à realização de ensaios nutricionais para validação de dietas destinadas a organismos aquáticos, trabalhando atualmente com camarão de patas brancas, tem-se deparado com uma elevada mortalidade em pós-larvas e juvenis da espécie, o que tem causado significativas perdas económicas para a empresa. Neste sentido, torna-se essencial reduzir a taxa de mortalidade e aumentar o crescimento do camarão de patas brancas, garantindo a manutenção do seu estado nutricional. O fornecimento de dietas adequadas que satisfaçam as necessidades nutricionais desta espécie, bem como a otimização de protocolos alimentares constituem possíveis medidas para alcançar tal objetivo.

Assim sendo, o presente trabalho teve como objetivo desenvolver estratégias alimentares para reduzir a mortalidade e melhorar o crescimento do camarão de patas brancas durante as suas fases iniciais de desenvolvimento, na empresa RiaSearch.

Para tal, dois ensaios de crescimento foram realizados com pós-larvas e juvenis de *Penaeus vannamei*, respetivamente, em sistema de recirculação de água, a fim de se comparar a sobrevivência e crescimento de indivíduos alimentados de acordo com diferentes protocolos alimentares, incluindo diferentes tipos de alimento e frequências alimentares.

Em relação ao primeiro ensaio experimental, foram testados dois protocolos alimentares. O primeiro consistiu num regime de co-alimentação em que se forneceu alimento inerte suplementado com náuplios de artémia até ao oitavo dia do ensaio e só alimento inerte nos restantes dias; o segundo protocolo consistiu em fornecer apenas alimento inerte durante todo o ensaio experimental. Maior sobrevivência e crescimento foram observados nas pós-larvas co-alimentadas com dieta inerte e náuplios de artémia. Comparativamente às pós-larvas alimentadas apenas com a dieta inerte, a sobrevivência, o peso médio final e o índice de crescimento diário das pós-larvas co-alimentadas foram respetivamente superiores em 9%, 96% e 67%. Estes resultados demonstram que a utilização de um regime de co-alimentação beneficiou consideravelmente o crescimento e sobrevivência das pós-larvas de *Penaeus vannamei*, na RiaSearch, além de sugerir que o fornecimento apenas de alimento inerte não terá sido suficiente para atender às exigências nutricionais da espécie em estudo, o que por sua vez terá afetado o estado nutricional das pós-larvas, tornando-as mais suscetíveis a doenças e a comportamentos canibais.

No segundo ensaio de crescimento, três frequências alimentares diferentes foram testadas, nomeadamente 4, 6 e 8 vezes por dia. A sobrevivência dos juvenis aumentou com o aumento da frequência alimentar, e os juvenis alimentados 8 vezes por dia apresentaram o maior crescimento. Os juvenis alimentados 6 vezes por dia apresentaram um peso médio final superior ao dos indivíduos alimentados 4 vezes por dia em cerca de 38%, enquanto o peso médio final dos juvenis alimentados 8 vezes por dia foi 53% e 11% superior ao dos indivíduos alimentados 4 e 6 vezes por dia, respetivamente. Comparado com os juvenis alimentados 4 vezes por dia, o aumento da frequência alimentar para 6 e 8 vezes por dia aumentou o índice de crescimento diário em cerca de 18% e 25%, respetivamente. Também a eficiência alimentar nos juvenis alimentados 6 e 8 vezes por dia foi, respetivamente, 1,4 e 1,6 vezes superior à dos juvenis alimentados 4 vezes por dia. Estes resultados indicam que fornecer alimento 8 vezes por dia melhora o crescimento e a eficiência alimentar em juvenis da espécie *Penaeus vannamei*, na RiaSearch, sugerindo, ainda, que fornecer 4 ou 6

refeições por dia não terá garantido a manutenção do estado nutricional dos juvenis, aumentando a mortalidade.

Os presentes estudos demonstram, então, que a utilização de regimes de co-alimentação com náuplios de artémia e alimento inerte, durante os estados iniciais de desenvolvimento, e o fornecimento de 8 refeições por dia representam a melhor estratégia alimentar para melhorar a sobrevivência e o crescimento do camarão de patas brancas na RiaSearch.

Palavras-chave: Protocolo alimentar; Camarão de patas brancas; Canibalismo; Crescimento; Co-alimentação; Frequência alimentar; *Penaeus vannamei*

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Abbreviations

ABS – Adsorptive bubble separation

ABW – Average body weight

AFI – *Artemia* feed intake

CO_2 – Carbon Dioxide

D – Dark

DFI – Dietary feed intake

DGI – Daily growth index

DO – Dissolved oxygen

FBW – Final body weight

FE – Feed efficiency

GIH – Gonad-inhibiting hormone

IBW – Initial body weight

IMTA – Integrated multi-trophic aquaculture

L – Light

NH_3 – Ammonia

NH_4^+ – Ammonium ion

NO_2^- – Nitrite ion

NO_3^- – Nitrate ion

ORP – Oxidation reduction potential

O_2 – Oxygen

O_3 – Ozone

PL – Postlarvae

RAS – Recirculating aquaculture system

R&D – Research and development

SD – Standard deviation

SS – Suspended solids

USD – United States Dollar

UV – Ultraviolet

WG – Weight gain

Chapter 1: Introduction

The accelerated growth of world's human population poses one of the world's major challenges – how to feed more than 9 billion people by 2050, in a context of climate change, economic and financial uncertainty, and growing competition for natural resources (FAO, 2016). Meeting the demand for food and energy for this population will require millions of tons of new biomass resources and the oceans' potential as a natural resource will be essential to ensure adequate nutrition and food security for human beings (FAO, 2016; Skjermo *et al*., 2014).

Marine resources contribute, since an early stage, to provide diverse and quality food, including fish, mollusks, crustaceans, seaweeds and other aquatic plants. The capture of these organisms has been an important way for humans to obtain essential nutrients and satisfy their food requirements. The improvement of fishing methods/techniques over the time, along with the progress of technology and industrial development not only allowed easier and more effective capture fisheries, but also produced negative effects on world marine ecosystems, such as the drastic reduction of natural fishing stocks. Considering the depletion of marine resources and the current stagnation state of the world fishing activity, it becomes necessary to develop feasible alternatives capable of minimizing potential environmental risks (Olmos *et al*., 2011).

Aquaculture industry represents an important alternative to meet future demand of high quality food and to help accommodate expansion of human population (Hatje *et al*., 2016; Tantipanatip *et al*., 2014; Van, 2016). Its contribution to the global seafood consumption reached 49% in 2012, which is expected to increase up to 62% in 2030 (Figure 1; FAO FIPS, 2014). It is, currently, the fastest growing food-producing sector, being of major relevance the implementation of a sustainable aquaculture for the supply of seafood to human population and for the recovery, stabilization and proper preservation of aquatic ecosystems and their biodiversity (Díaz *et al*., 2012; FAO, 2016, Olmos *et al*., 2011, SEAFISH, 2018).

In general, aquaculture includes production of freshwater or saltwater species under controlled conditions, being this activity carried out onshore or offshore.

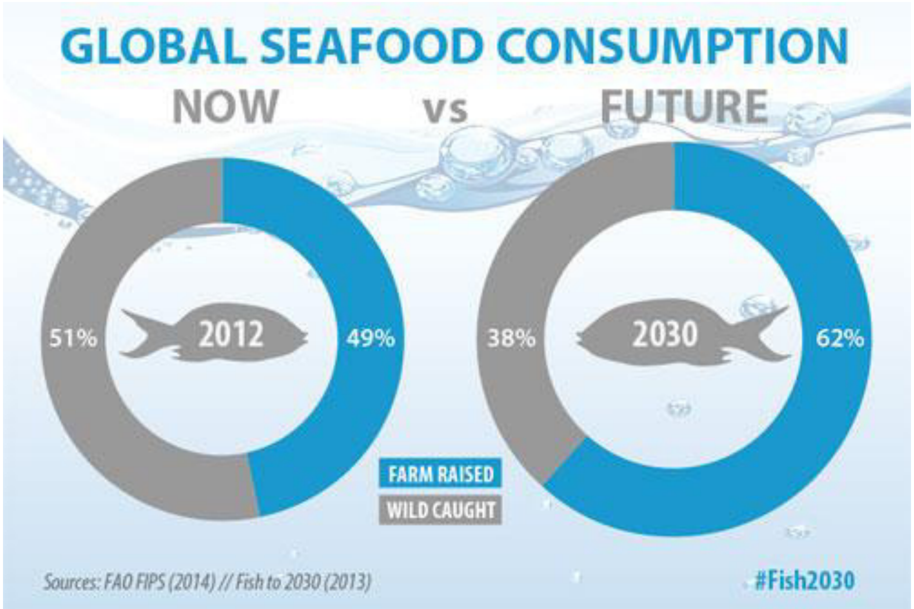

Fig. 1 – Global seafood consumption (fisheries and aquaculture). Source: FAO FIPS, 2014.

1.1 Shrimp industry

Shrimp is the expression commonly used to refer to a group of crustaceans from the order Decapoda, with five pairs of legs (*pereopods*). It comprises the suborder Dendrobranchiata, with more than 500 species that release thousands of embryos into the water column, and also the suborder Pleocyemata, with nearly 3 000 species that typically incubate the embryos in their abdomen (Ackefors, 2009; Carvalho and Calado, 2018; Hickman *et al.*, 2011; Thorp and Rogers, 2016).

Economically, shrimp has shown an increasing importance, being currently one of the most valuable globally traded seafood products. However, only a few species are commercially important, counting a total of 83 species with commercial value (FAO-FIGIS, 2016; Hatje *et al.*, 2016; Sakas, 2016). On the world market, the family Penaeidae stands out with large and valuable tropical shrimp species caught in tropical or subtropical areas and largely cultivated in Southeast Asia and Latin America essentially. In many countries, penaeid shrimp aquaculture is an important economic activity (Ackefors, 2009; Kannan *et al.*, 2015; Varadharajan and Pushparajan, 2013).

Over the years, shrimp industry has developed remarkably. In 1950, the vast majority of the global total shrimp production derived from fisheries (412 165 tonnes), with almost no aquaculture production (1 325 tonnes), and in 1970 capture fishery production was already exceeding 1 million tonnes, while aquaculture remained below 10 000 tonnes (Figure 2). But as time went on, aquaculture became increasingly important for global shrimp production, exceeding, in 2007, the capture production. In 2016, shrimp farming was higher than 5 million tonnes, currently contributing with more than 50% of the global total shrimp production and consumption (Ackefors, 2009; FAO-FIGIS, 2016; Hatje *et al.*, 2016; Portley, 2016; Schock *et al.*, 2013).

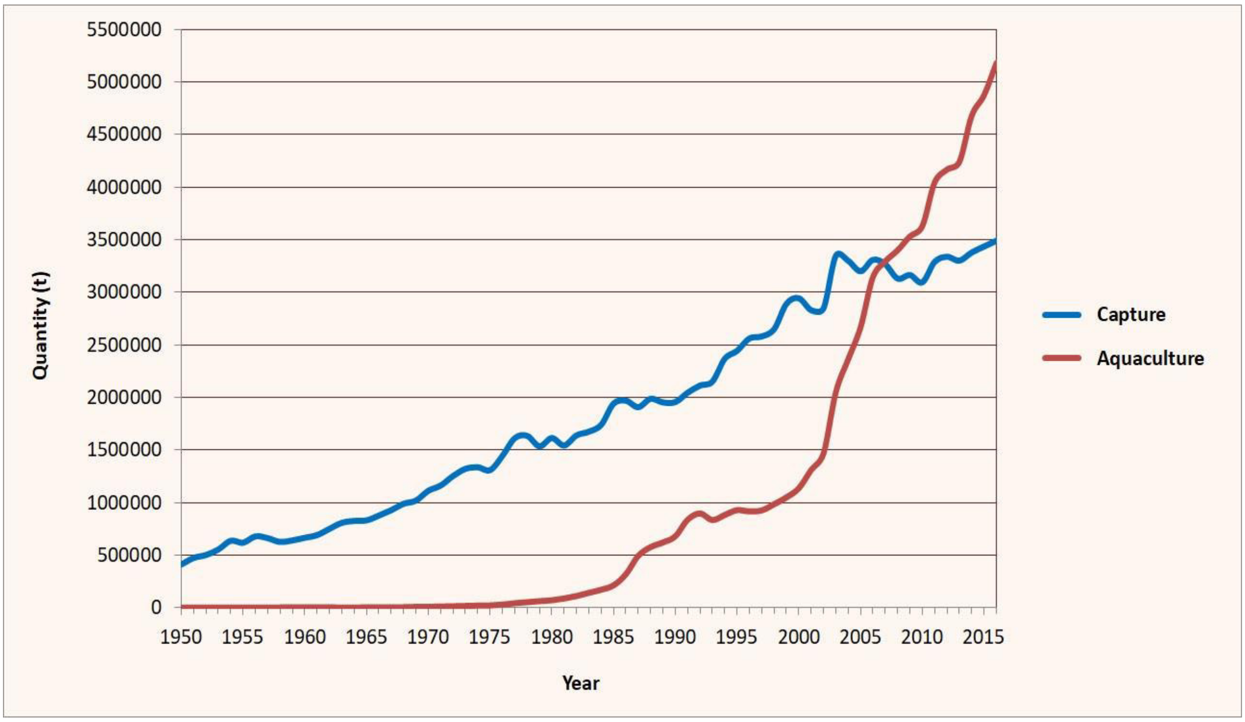

Fig. 2 – Annual global total shrimp production, in tonnes. Source: FAO-FIGIS, 2016.

The Asian continent takes the lead in the world shrimp production. It accounted for approximately 2.5 million tonnes in capture fisheries (72% of the world fisheries production) and more than 4 million tonnes in aquaculture (86% of the world aquaculture production), in 2016, followed by Americas with more than 700 000 tonnes both in capture fisheries and in aquaculture. Europe has the lowest shrimp aquaculture production, with only 262 tonnes (FAO-FIGIS, 2016; OCTOFROST, 2017). In the same year, China, Indonesia, Viet Nam, India, Ecuador, Thailand, Mexico, Bangladesh, Philippines and Myanmar were the world ten largest shrimp producer countries in aquaculture (FAO-FIGIS, 2016).

Shrimps and prawns are the most produced and commercialized crustacean around the world, having reached, in 2016, a global total production of 8 671 358 tonnes, besides generating a total value of 32 012 410 USD in aquaculture (FAO-FIGIS, 2016). A social advantage of shrimp sector is its employability level, since shrimp capture fisheries employ more than one million fishers and shrimp farms largely contribute to the creation of new jobs (Portley, 2016).

However, both shrimp capture fisheries and aquaculture have a high environmental risk, on a global scale (Tovar *et al.*, 1999). In industrial shrimp fishery, two situations require special attention: (1) the alteration/destruction of seabed caused by the use of highly destructive fishing techniques, such as the deep-sea trawling, and (2) the bycatch of non-targeted species, which leads to future unbalances on aquatic ecosystems (Ackefors, 2009; FAO, 2016; Lapointe, 2013; Portley, 2016; Sakas, 2016). Environmental impacts of shrimp aquaculture are related to four different factors – (1) the siting locations where shrimp ponds are constructed, (2) the type of management and technology applied during the operation of shrimp ponds, (3) the size/scale of the aquaculture production, as well as the surface area dedicated for it, and (4) the characteristics and capacity of the receiving waters where aquaculture effluents are discharged (Páez-Osuna, 2001b).

During establishment of shrimp aquaculture facilities, the coastal lowlands converted into areas for shrimp pond construction include salt flats, salt marshes, mangrove areas and agricultural lands. Therefore, the most alarming environmental impact of shrimp aquaculture is the direct and extensive destruction of mangroves and wetlands in order to accommodate shrimp ponds, which results in loss of habitats, breeding/nursery grounds or even shelter areas for many important species that use mangrove ecosystems during early development; decrease of ecosystem biodiversity and occurrence of coastal erosion. Salt marshes are important routes of discharge of waters during floods and storms and its conversion, as well as that of agricultural lands and salt flats, changes water drainage patterns (Páez-Osuna, 2001a; Páez-Osuna, 2001b; Páez-Osuna *et al.*, 1998; Ronnback, 2002; Sohel and Ullah, 2012). Maintaining an acceptable balance between mangroves/wetlands and shrimp pond areas is crucial to minimize the impact of shrimp aquaculture (Páez-Osuna, 2001b).

During the operation of shrimp aquaculture facilities, different types of environmental problems may occur, including bycatch associated with wild-caught postlarvae, if the aquaculture production is dependent on the capture of individuals in the environment; environmental effluents discharge from shrimp ponds; escape of individuals from aquaculture facilities; release and spread of diseases in natural ecosystems and salt water intrusion into groundwater aquifers or other adjacent freshwater wetlands (Páez-Osuna, 2001a; Páez-Osuna, 2001b).

Bycatch is one of the most critical issues of aquaculture that still depends on the capture of wild postlarvae/juveniles to replenish the culture stocks. Although the development of hatcheries for shrimp species has reduced the dependency on wild-caught postlarvae, these environmental captures are still a frequent reality in many places. Reducing and regulating the capture of wild postlarvae and juveniles, together with the establishment of definite specific areas for such activity could mitigate this problem (Páez-Osuna, 2001b; Páez-Osuna *et al.*, 2003; Ronnback, 2002).

Another problematic issue is the discharge of pond effluents into natural environment, which may affect water quality of adjacent coastal waters (estuaries and/or lagoons), depending on different factors such as the total magnitude of discharge, chemical and microbiological composition of pond effluents (suspended solids, nutrients, organic matter, microorganisms) and the characteristics of the receiving water bodies where effluents are discharged (dilution rate, residence time, receiving water quality). Shrimp pond effluents are typically enriched in suspended solids (SS) and nutrients, and its environmental discharge can cause water deterioration and changes in the structure of benthic communities. The higher is the aquaculture's degree of intensification, i.e. higher stocking density and increased use of water and artificial feeds, the higher will be the final waste load on pond effluents (Páez-Osuna, 2001a; Páez-Osuna, 2001b; Sohel and Ullah, 2012).

To minimize the negative effects of environmental discharge of shrimp pond effluents, different options may be considered. Development of integrated multi-trophic aquaculture (IMTA), where shrimp pond water is used to feed oysters, mussels and seaweed or even to irrigate halophytes crops, has been shown as an important way of reducing effluents waste load (Cruz-Suárez *et al.*, 2010; Páez-Osuna, 2001a). Additionally, the use of biofloc systems has been reported to have beneficial effects on shrimp culture, as this technology includes the formation of floccules – made up of bacteria, algae, protozoa, nematodes and detritus (bioflocs) – that are capable of assimilating the organic wastes in the water (Samocha, 2016; Thong, 2014). Reduction of water exchange rates and improvement of feed composition are other possible mitigation measures for this problem (Páez-Osuna, 2001b).

In countries where the aquaculture production of non-indigenous species is allowed, the escape of individuals from farming facilities is a major environmental concern due to the possible introduction of non-indigenous species on local ecosystems and "biological pollution" of wild populations. So, it is important to optimize the management and control of aquaculture facilities, as well as to include equipment effective enough to prevent these occurrences (Páez-Osuna, 2001b).

Diseases represent the biggest obstacle for shrimp aquaculture development and its release and spread into natural environment are one of the most alarming impacts of this industry. Rapid expansion of aquaculture and lack of environmental control for transfer of diseases are commonly associated with disease occurrence. Effluents discharged from shrimp ponds are the responsible for the introduction of pathogens into environment, affecting wild populations and other aquaculture facilities, as the outlet receiving waters often serve as inlet water for neighboring farms. Ensuring good feed supplemented with the use of prophylactic agents (e.g. probiotics), along with the maintenance of good water quality, low stocking densities and high level of disease environmental control contribute to mitigate the occurrence of disease outbreaks (Páez-Osuna, 2001a; Páez-Osuna, 2001b; Sohel and Ullah, 2012).

In addition, the pumping of large volumes of groundwater to shrimp ponds leads to the lowering of groundwater levels, emptying of aquifers and salinization of adjacent waters and land. Water salinization reduces the water supply for domestic and agricultural uses. This environmental impact can be minimized by avoiding pumping groundwater into shrimp ponds and, in critical cases, using pond liners to prevent the seepage of brackish water (Páez-Osuna, 2001b; Sohel and Ullah, 2012).

1.2 *Penaeus vannamei*

Commonly known as whiteleg shrimp or Pacific white shrimp, *Penaeus vannamei* is currently one of the most important commercial penaeid species and the most farmed worldwide, with about 80% of the global total shrimp aquaculture production (Figure 3; Cobo *et al.*, 2012; FAO-FIGIS, 2016; Sanudin *et al.*, 2014).

This crustacean species is native to the eastern Pacific coast, having a distribution that extends from Sonora, in Mexico, to as far south as Tumbes, in Peru, and it lives in tropical marine habitats with water temperatures normally above 20 °C throughout the year (FAO, 2018; Galil *et al.*, 2011; Medina-Reyna, 2001; Wakida-Kusunoki *et al.*, 2011; Zhou, 2014).

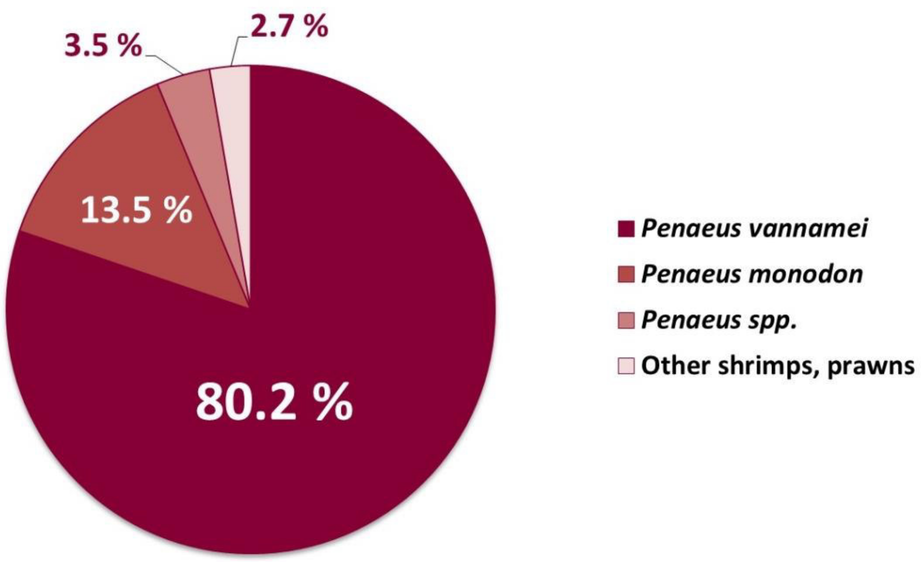

Fig. 3 – Global total shrimp aquaculture production, in 2016. Source: FAO-FIGIS, 2016.

It has a typically translucent-white color, sometimes displaying a bluish hue due to the presence of blue chromatophores near the margins of telson and uropods, at the end of the body (Figure 4), and is considered omnivorous (FOFONOFF *et al.*, 2018; FOX and BRIGGS, 2018).

Adult individuals of this species live and breed in the ocean, with males and females becoming mature from 20 g and 28 g onwards, respectively (at the age of 6-7 months). After mating, spawning occurs and females with more than 40 g can release into the water 100 000 to 250 000 eggs. Approximately 16 hours after spawning, eggs hatch into non-feeding nauplii larvae that live on their yolk reserves and are positively phototactic. Nauplii develop into the next larval stages of the species, namely protozoea, mysis and early postlarvae, which already eat phytoplankton and zooplankton. In the phase of postlarvae (PL), individuals migrate to inshore brackish nursery grounds (coastal estuaries, lagoons or mangrove areas) and begin feeding on benthic detritus, worms, bivalves and other crustaceans. In the coastal area, postlarvae become juveniles, sub-adults and, then, adults, being in this last phase that the species returns to the open sea for subsequent maturation and reproduction, completing its life cycle (Brito *et al.*, 2001; FAO, 2018; FOFONOFF *et al.*, 2018; Kannan *et al.*, 2015; Medina-Reyna, 2001; Rothlisberg, 1998).

Fig. 4 – Individual from *Penaeus vannamei* species. Source: ANONYMOUS, 2007.

Historically, the first spawning of *Penaeus vannamei* in captivity was achieved in 1973, in Florida, from a wild mated female caught in Panama. Commercial production began in South and Central America, following the discovery of unilateral eyestalk ablation to induce female penaeids maturation, in 1976, in Panama (FAO, 2018; Kannan *et al.*, 2015). The eyestalk ablation allows the removal of the X-organ sinus gland that produces the gonad-inhibiting hormone (GIH), a potent inhibitor of the vitellogenin synthesis, thus accelerating the maturation of female gonads (Chen *et al.*, 2014; Li *et al.*, 2016; Pervaiz *et al.*, 2011; Reis, 2017; Sainz-Hernández *et al.*, 2008; Uawisetwathana *et al.*, 2011). By the early 1980s, the continuous development of intensive breeding and rearing techniques of this crustacean spread its production in Hawaii, United States of America and Latin America. In 2000, Asia began to produce *Penaeus vannamei* with a total of 2 310 tonnes, rapidly increasing to 252 919 tonnes in 2002, and exceeding the 235 001 tonnes produced by Americas in the same year (FAO, 2018; FAO-FIGIS, 2016).

Recently, whiteleg shrimp aquaculture has been growing steadily in both American and Asian continents, having also started in Oceania, Europe and Africa, although its contribution to the global total aquaculture production is very low. In 2016, the world aquaculture production of *Penaeus vannamei* reached a total value of 4 155

827 tonnes, of which Asia accounted 3 428 506 tonnes, representing more than 80% of the global total aquaculture production of this crustacean, while Americas had a total production of 727 166 tonnes, less than 20% of the global production. China, Indonesia and India were the three main aquaculture producer countries in the same year (Figure 5; FAO-FIGIS, 2016).

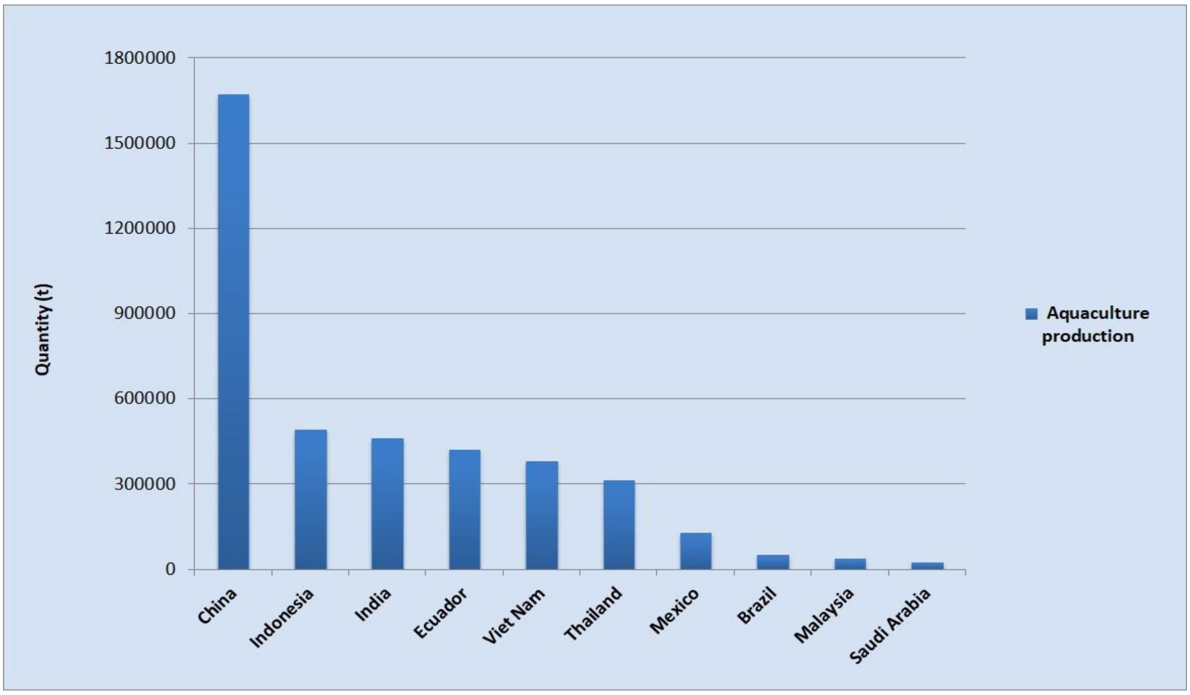

Fig. 5 – Main aquaculture producer countries of *Penaeus vannamei*, in 2016. Source: FAO-FIGIS, 2016.

Among some of the main reasons for the growing aquaculture production of *Penaeus vannamei*, many authors stand out its capacity for rapid growth, good survival in high stocking densities, tolerance to a wide range of salinities and temperatures, high resistance against certain diseases in intensive grow-out productions and its great economic value. Whiteleg shrimp is a highly profitable species in this industry, being one of the most consumed farmed shrimp in the world (An, 2011; Chong-Robles *et al.*, 2014; Funge-Smith *et al.*, 2003; Hu *et al.*, 2008; Lu *et al.*, 2018; Pakravan *et al.*, 2017; Panini *et al.*, 2017; Zeng *et al.*, 2017; Zhou, 2014).

In general, aquaculture production cycle of *Penaeus vannamei* (Figure 6) involves the hatchery production (including separate facilities for broodstock quarantine, acclimatization, maturation and reproduction, for eggs hatching and for larval and nursery rearing) and, also, the grow-out production phase (FAO, 2003; FAO, 2018; Quagrainie, 2015).

Hatchery systems may range from small and simple hatcheries to large, sophisticated and environmentally controlled installations, associated with maturation units. In the breeding phase, the broodstock, whether cultivated or wild-caught, are kept in maturation tanks, in dark rooms, with access to clean filtered seawater, and usually fed with a mixture of fresh and formulated broodstock feeds. It is at this phase that one eyestalk from each female is ablated, thus inducing repeated maturations and spawnings. Females reproduce effectively at 8-10 months of age, while males reach the peak of reproduction with over 10 months (FAO, 2003; FAO, 2018; Reis, 2017).

Spawning usually occurs during nighttime and the fertilized eggs should be transferred to hatching tanks. In the afternoon of the following day, the healthy nauplii larvae are harvested using a light to attract them to the water surface, collected, washed with seawater, disinfected with iodine and/or formalin, rinsed again and, finally, counted and transferred to holding tanks, under optimal conditions, until they are stocked or sent directly to the larval rearing facilities (FAO, 2003; FAO, 2018; Kannan *et al*., 2015; Vu, no date).

Nauplii are cultured until they reach the stage of PL_{10-12} (postlarvae with 10 to 12 days after metamorphosis) in a single larval rearing tank or harvested at the stage of $PL_{4–5}$, transferred to larger tanks and then reared to $PL_{10–30}$. During this phase, water is regularly exchanged and shrimp are fed with live food (*Artemia* and microalgae) and formulated diets. Precautions are taken to prevent contamination of the larval facilities, including periodic dry-out and disinfection of the facilities, water filtration and chlorination, nauplii disinfection and the use of antibiotics or probiotics (FAO, 2003; FAO, 2018).

Before being stocked in grow-out ponds, $PL_{10–20}$ may be transferred to nurseries where they are maintained until they reach larger sizes (0.2–0.5 g), which is especially useful in colder areas with limited growing seasons. During this phase, shrimp quality is evaluated, ensuring the quality of postlarvae to be transferred to the grow-out phase (FAO, 2018; Quagrainie, 2015).

In the grow-out production, shrimp is raised to commercial size (until harvest), under extensive, semi-intensive, intensive or super-intensive productions systems, depending on the shrimp stocking densities used, i.e. low, medium, high and extremely high stocking densities, respectively (FAO, 2018; Reis 2017).

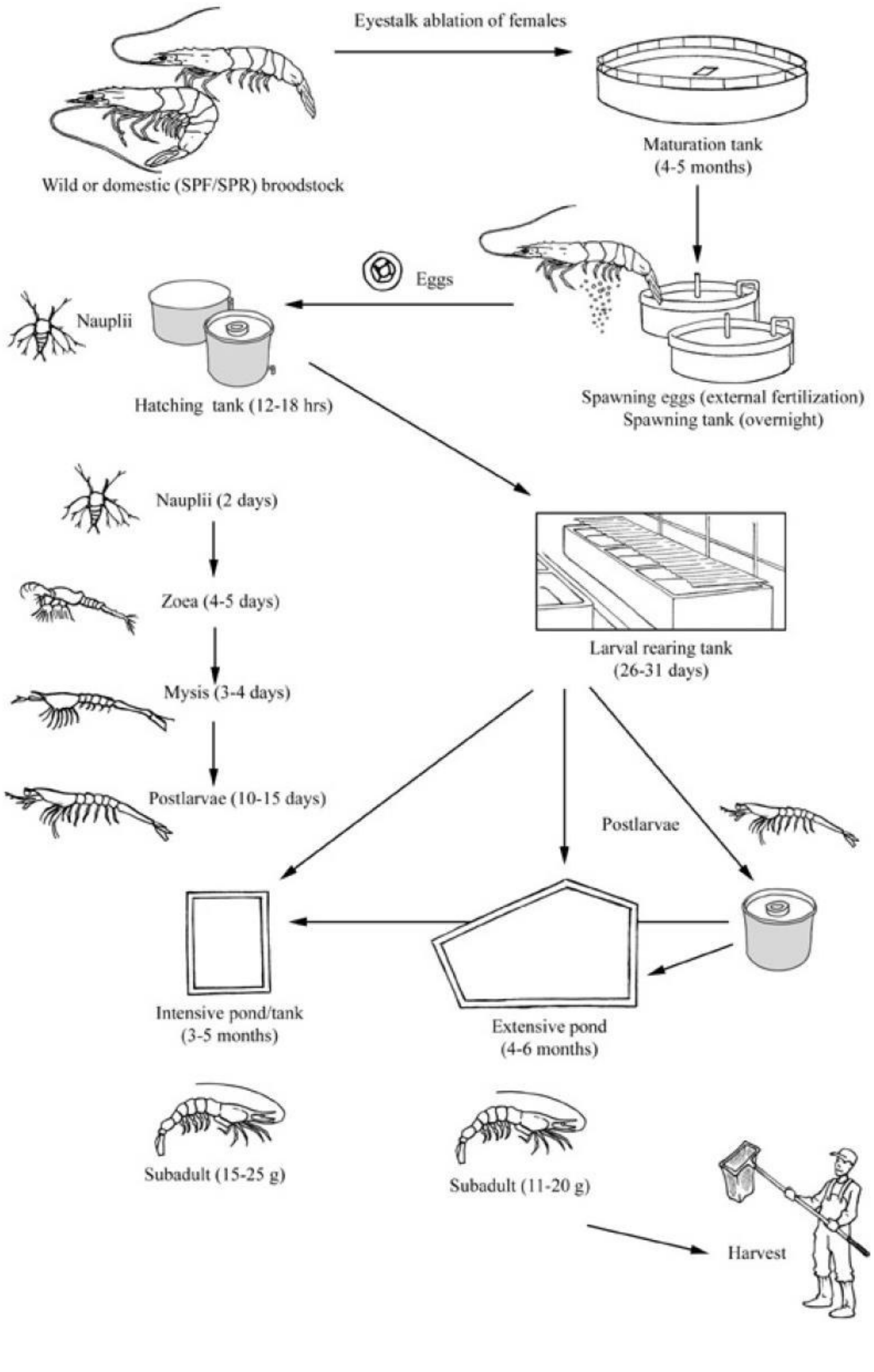

Fig. 6 – Production cycle of *Penaeus vannamei*. Source: FAO, 2018.

Extensive grow-out of whiteleg shrimp is usually conducted in tidal areas with minimal or no water pumping or aeration and with low stocking densities. Shrimp are mainly fed with natural foods enhanced by pond fertilization, and supplemented with low protein formulated diets once a day. Within 4 to 6 months, shrimp weighing 11–20 g are harvested. Semi-intensive ongrowing systems use medium stocking densities, water is regularly exchanged by pumping and aeration is minimal. Shrimp are fed with natural foods enhanced by pond fertilization and also with formulated diets 2 to 3 times per day. Intensive shrimp farms are typically located in non-tidal areas, with ponds being completely drained and prepared before each stocking, high stocking densities are applied and heavy aeration ensures the oxygenation of water. Shrimp are fed 4 to 5 times daily with artificial diets that satisfy their nutrient requirements. Shrimp weighing 15–25 g are harvested after 3 to 5 months. Super-intensive grow-out systems are recently developed in the United States of America and are conducted in raceway systems in greenhouses with no water exchange or discharges. Besides using extremely high stocking densities, these systems are biosafe and eco-friendly, having a small ecological footprint and producing cost-efficient, high quality shrimp (FAO, 2018).

1.3 Cannibalism during shrimp early development

Cannibalism is the concept commonly used to define a relationship of predation between conspecific individuals. It includes the agonistic behavior of attacking, injuring, killing and eating individuals of the own species, as well as the consumption of already dead animals (Fox, 1975; Polis, 1981; Romano and Zeng, 2017).

Being a usual trait in the biology of several species, cannibalistic behaviors may have influence on the structure, size and dynamics of many natural populations, but their occurrence and intensity are predominantly related to environmental and ecological factors, including the availability (and quality) of food, the density of individuals in the habitat, the existence of shelter areas, the physiological or psychological stress induced in individuals and the presence of vulnerable individuals (victims/preys) as well as their interaction with conspecifics (Fox, 1975; Lovrich and Sainte-Marie, 1997; Polis, 1981).

In decapod crustaceans, cannibalism is quite common especially during early developmental stages. Unlike other animals, this group of aquatic organisms has its body surface covered by a rigid exoskeleton and undergoes a step-wise growth which implicates the periodic shedding of their exoskeleton in order to grow in size – a cyclic physiological process known as molting. Cannibalistic timing and frequency mainly occurs during this molting process, when crustaceans are more fragile, flexible and unable to defend themselves, being hence highly vulnerable to cannibalism until their new shell is fully calcified (Corteel *et al.*, 2012; Romano and Zeng, 2017). As molting more frequently happens during crustacean's larval growth, the incidence of cannibalistic behaviors is also higher in those early developmental stages.

Under aquaculture facilities, animal mortality associated with cannibalism represents a major concern, since it seriously affects the productivity and profitability of aquaculture (Fox, 1975; Romano and Zeng, 2017). To reduce the incidence of cannibalism, aquaculture producers have been applying different strategies, such as reducing the stock densities and heterogeneity of lots, creating appropriate shelter areas to reduce physical confrontations between individuals and improving feed quality and quantity to minimize possible nutritional deficiencies (Romano and Zeng, 2017). These strategies are species- and size-specific.

In aquaculture of marine shrimp and freshwater prawns, larval and early juvenile production phases are the most critical, since it is in these early life stages that shrimp metabolic rates are higher and molting is more frequent, so animals are much more susceptible to cannibalism and mortality increases (Abdussamad and Thampy, 1994; Romano and Zeng, 2017; Wasielesky *et al.*, 2013). During larval rearing, reducing the size heterogeneity is not an effective method to control cannibalism, because shrimp larvae have irregular body shapes and are extremely fragile, with the risk of being damaged during that process. So, it is essential to supply live feed in appropriate quantity and quality to ensure the correct nutritional status of shrimp larvae (Romano and Zeng, 2017; Ventura *et al.*, 2008). During nursery phase, the quality and frequency of feeding are also important to reduce starvation periods and nutritional deficiencies, which are triggers for cannibalistic behaviors in shrimp juveniles (Abdussamad and Thampy, 1994; Romano and Zeng, 2017).

This indicates that establishing proper feeding strategies, in order to ensure an efficient feed utilization and satisfy shrimp nutritional requirements, is of paramount importance to reduce shrimp mortality associated with cannibalism during larval and juvenile growth. In other words, optimizing larval feeding management contributes to maximize shrimp growth and survival in aquaculture (Hasan and New, 2013).

Cannibalism may also be aggravated during disease outbreaks or changes in environmental parameters (e.g. low dissolved oxygen, high ozone levels in water), when individuals become more vulnerable, which then results in a higher shrimp mortality associated with cannibalism. In addition, in species with a cannibalistic nature, the risk of exposure to pathogens, such as White Spot Syndrome Virus and Yellow Head Virus, is increased when compared to less cannibalistic species, since there is horizontal transmission of some pathogens through cannibalism and the infected, weak shrimps are more easily cannibalized by their conspecifics (Romano and Zeng, 2017; Wu *et al*., 2001). This emphasizes the relevance of establishing adequate methods and procedures in order to avoid the occurrence of cannibalism in aquaculture units.

Chapter 2: RiaSearch, Lda.

RiaSearch is a research and development (R&D) company, focused in physical and natural sciences and located in Cais da Ribeira de Pardelhas, Murtosa Municipality, in Aveiro (Portugal). Founded in 2016, this company works essentially in two business areas, performing feed trials with marine species for aquafeed industries and companies, as well as offering scientific and technical consulting services in the aquaculture sector and recirculating aquaculture system (RAS) technology.

RiaSearch facilities are divided into three main sections – a feeding trials room, a water treatment area and a laboratory. The feeding trials room comprises a total of fifty-five 200-350 L tanks and twelve 160 L tanks, in a recirculating aquaculture system. In the water treatment area all the equipment utilized in the system's water collection, treatment and monitoring are localized, including the pumps used to circulate the water in the system. At the laboratory, water quality tests are performed, as well as weighing the inert diet amount that is necessary to provide to the animals in the tanks.

Currently, RiaSearch company has performed experimental feed trials with several marine fish species, like European sea bass (*Dicentrarchus labrax*), gilthead sea bream (*Sparus aurata*) and Senegalese sole (*Solea senegalensis*), but is mainly dedicated to whiteleg shrimp (*Penaeus vannamei*), in marine recirculating aquaculture system. The shrimp are received in the postlarvae phase (PL) from a disease free commercial hatchery located in Florida, United States of America.

2.1 Transport of shrimp postlarvae

Postlarvae are produced in the United States of America and transported, by plane to Portugal and by car to RiaSearch company, in plastic bags (polyethylene bags resistant to puncturing) filled with one-third of filtered seawater and two-thirds with pure oxygen. Two plastic bags are typically used, one inside the other, as a precautionary measure, and some granules of activated carbon are also added to each bag, in order to help maintaining low ammonia levels during the transportation time (FAO, 2003, FAO, 2018; Kungvankij and Chua, 1986; Lekang, 2013).

After being sealed, the bags are placed into polystyrene boxes for further transport and water temperature is reduced to about 17-18 °C (by using ice on the bottom, side and top of the polystyrene box), with the major purpose of lowering shrimp metabolic rate and, thus, minimizing their oxygen consumption and waste excretion, as well as keeping them calm during transportation (FAO, 2003, Kungvankij and Chua, 1986; Lekang, 2013).

2.2 Reception and acclimation of shrimp postlarvae

At RiaSearch, and according to the acclimation guidelines of the commercial hatchery, the plastic bags are opened and poured into the 160 L tanks (reception tanks), where they will be maintained during the acclimation period. Initially, the temperature, salinity, pH and oxygen level of the bags' water are measured with a probe. Then, the filtered seawater from RiaSearch is slowly mixed with the bag water, while the same water parameters are continuously checked. It is highly important that, throughout the acclimation process, the RiaSearch seawater flow rate is correctly adjusted to ensure that, every 15 min, water temperature rises a maximum of 1 °C, salinity decreases 1, and the pH increase does not exceed 0.1. Dissolved oxygen is initially above saturation, being then monitored with a probe during acclimation. After the acclimation to RiaSearch experimental conditions, postlarvae (PL_{10-12}) begin to be fed with inert diet.

2.3 Performance of feeding trials

Postlarvae generally remain in the 160 L tanks for about two weeks (nursery phase), before being manually separated and distributed, at lower stocking densities, by the 200-350 L tanks (Figure 7), according to the specifications of the requested feeding trials. However, depending on the requirements and experimental conditions of the feeding trials requested to RiaSearch, it may be necessary to use the postlarvae earlier, right after the acclimatization period.

During the feeding trials, water quality parameters (oxygen, pH, salinity, temperature, redox potential) are daily monitored by probes and ammonia, nitrite and nitrate concentration values are checked twice a week.

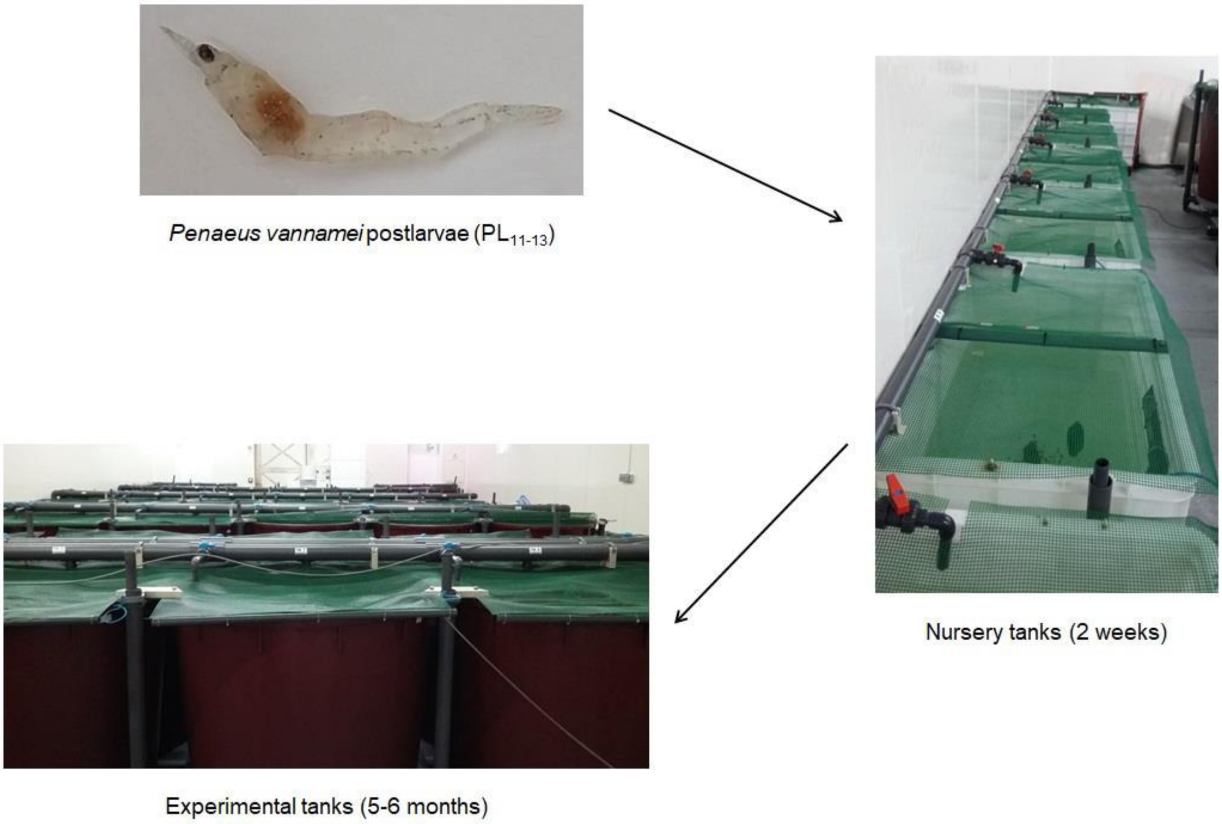

Fig. 7 – Growth cycle of *Penaeus vannamei* at RiaSearch.

Daily, stock tanks are manually cleaned before the first feeding time, in order to remove exoskeletons, fecal matter and dead individuals. During the day, postlarvae are fed by hand several meals per day and, at nighttime, feed is provided through the use of automatic feeders. The amount of feed per meal is weighed at the laboratory, using a precision scale, in accordance with a shrimp daily growth table.

2.4 Recirculating Aquaculture System (RAS)

Although aquaculture has been a feasible solution to provide sufficient aquatic/sea food for a growing world population, it has also raised some public and governmental concerns regarding its sustainability, since the several culture methods that are practiced in this industry are commonly associated with the occurrence of harmful effects on the natural environment (Martins *et al*., 2010; Mirzoyan *et al*., 2010; Rijn, 2013; Schumann *et al*., 2017).

Aquaculture production in earthen ponds, for example, requires an extensive use of land, being difficult to remove the solids that typically accumulate on the bottom of the ponds, while in flow-through systems (raceway aquaculture) water moves through the production facilities (inland aquaculture) and the effluents may be discharged into a receiving stream with little or no wastewater treatment. With regard to net-pen aquaculture, an offshore operation that uses movable buoyant enclosures to culture aquatic organisms, the main problem is the direct discharge of waste (uneaten feed, fecal matter and excreted metabolic products) into the surrounding water body, which represents a potential way of polluting natural ecosystems (Interdonato, 2007; Mirzoyan *et al.*, 2010; Piedrahita, 2003).

Faced with economic and environmental constraints, such as the lack of space for expansion, limitations of existing water resources and the discharge of waste into natural environment, a sustainable and efficient production method of seafood needs to be adopted (Díaz *et al.*, 2012; Ferreira, 2012; Martins *et al.*, 2010; Mirzoyan *et al.*, 2010; Piedrahita, 2003; Rijn, 2013; Zhang *et al.*, 2011). In this context, recirculating aquaculture systems (RAS) are emerging as one of the possible opportunities to further develop aquaculture, while an adequate culture water quality is provided and some of the above mentioned issues are mitigated in order to decrease aquaculture's ecological impact (Badiola *et al.*, 2012; Díaz *et al.*, 2012; Rijn, 2013; Zhang *et al.*, 2011).

In recirculating aquaculture systems, also known as water re-use systems, the outlet water from the aquaculture tanks, instead of being released into a recipient water body, is (partially) re-used after undergoing a series of treatment processes (mechanical, chemical and biological treatments), thus remaining to flow between the culture tanks and the water treatment equipment (recirculation), by the use of a water transport pump (Ferreira, 2012; Interdonato, 2007; Lekang, 2013; Martins *et al.*, 2010; Mirzoyan *et al.*, 2010; Rijn, 2013; Zhang *et al.*, 2011). Developed as a technology for intensive farming, these land-based aquatic systems are considered the most economical and environmental friendly aquaculture systems. Since they were introduced in the late 80's, their aquaculture production in Europe has increased significantly in volume and species diversity, although it is still small when compared to that of sea cages, flow-through systems or ponds (Badiola *et al.*, 2012; Ferreira, 2012; Martins *et al.*, 2010; Zhang *et al.*, 2011; Zhang *et al.*, 2018).

Several advantages of RAS can be mentioned. One great example is the reduction of water consumption in aquaculture farms, since the re-use of water allows to minimize its replacement in the culture tanks and to compensate for an insufficient water supply. Besides that, these systems also enables a high level of control over water quality parameters to ensure optimal culture conditions, a higher hygienic and disease management, reduced waste production and discharge into natural environment, and a lower visual impact of the farm (Badiola *et al*., 2012; Carvalho, 2017; Ferreira, 2012; Gutierrez-Wing and Malone, 2006; Lekang, 2013; Martins *et al*., 2009; Martins *et al*., 2010; Mirzoyan *et al*., 2010; Rijn, 2013; Zhang *et al*., 2011).

Conversely, investment and operating costs of RAS represent two main disadvantages. The initial capital investments, the operational and energetic costs required by RAS are higher than that of flow-through systems, which operates without recirculation of water. Moreover, due to its mechanical complexity, RAS are more exposed to technical faults. So, it is required a careful management in terms of water treatment as well as equipment maintenance (Badiola *et al*., 2012; Interdonato, 2007; Lekang, 2013; Martins *et al*., 2010; Ray and Lotz, 2017; Schneider *et al*., 2012).

Under RAS conditions, water treatment typically includes the collection and removal of solid waste in a smaller effluent stream that can be handled more easily, the ammonia removal/conversion generally through a biological process, the addition of oxygen into the water by aeration or oxygenation as well as the removal of carbon dioxide by degassing, and water disinfection usually using ozone and/or ultraviolet light (Carvalho, 2017; Díaz *et al*., 2012; Ferreira, 2012; Interdonato, 2007; Mirzoyan *et al*., 2010; Piedrahita, 2003; Ray and Lotz, 2017; Rijn, 2013; Suhr *et al*., 2015).

At RiaSearch, feeding trials with whiteleg shrimp are performed in a marine RAS, as aforementioned. In this system, from the outlet of shrimp tanks water flows to the mechanical filtration equipment, composed by waste water reservoirs, from which is pumped into a set of two mechanical polypropylene bag filters (filtration size of 50 and 25 µm), each one of them placed inside a housing unit (Figure 8-A), for the collection of suspended solids (fecal matter and uneaten feed). Further, water passes through a protein skimmer or foam fractionator (Figure 8-B), where dissolved organic substances, colloids and fine suspended particles are trapped. In this protein skimming process, a mix of air and ozone (O_3) is introduced, via a venturi injector, at the bottom of the protein skimmer's reaction chamber, to create gas bubbles in the water column. As bubbles rise to the water surface, they adsorb the particles, colloids and dissolved solids – adsorptive bubble separation process (ABS) – and when they reach the surface, they are pushed out of the water by its buoyancy, thus creating foam. This foam passes into an upper riser tube, being ejected over the top of it and, then, collected in a waste drain cup.

Fig. 8 – Equipment used at RiaSearch for solid waste removal, including a housing unit with a bag filter inside (A) and the protein skimmer (B). Photography courtesy of Adriana Laranjeira, RiaSearch.

Ozone is a powerful oxidizing agent as well as an effective bactericide, parasiticide and virucide. So, its use in the protein skimming process aim at reducing potential pathogens and disease outbreaks, oxidizing organic wastes, reducing water turbidity, improving the effectiveness of other water treatment units and thus increasing the general water quality. To prevent the production of toxic by-products associated with ozone disinfection, such as hypobromous acid and hypobromite ion, the ozone added is always at the recommended levels. An Oxidation Reduction Potential (ORP) probe (along with the establishment of a safety limit of redox potential) is used in the protein skimmer to monitor and indicate the availability of oxidation agents in the water.

From the protein skimmer, water is pumped to the biological trickling filter (Figure 9) for the removal of ammonia, an excreted toxic compound, and for carbon dioxide degassing/stripping. This biofilter has a cylindrical shape filled up with a plastic filter medium over which a bacterial biofilm is established. Water is pumped to the top of the trickling filter and, then, trickles down through the fixed plastic medium by gravity, flowing over the biofilm. It is in this biofilm that a nitrification process takes place and is performed by nitrifying bacteria that oxidize ammonia, using oxygen (O_2) as oxidizing agent and carbon dioxide (CO_2) as a carbon source for growth. The nitrification process is carried out in two steps, where ammonium (NH_4^+) is converted to nitrite (NO_2^-) by *Nitrosomonas* bacteria and nitrite is converted to nitrate (NO_3^-) by *Nitrobacter* bacteria, and since there is an equilibrium between the concentrations of ammonia (NH_3) and ammonium ion (NH_4^+) in water, reducing one of them also reduces the other.

Fig. 9 – Trickling filter used at RiaSearch for ammonia removal and carbon dioxide degassing. Photography courtesy of Adriana Laranjeira, RiaSearch.

As the water trickles down in the biofilter, gas exchange occurs – oxygen is absorbed from the air inside the filter, while carbon dioxide is removed by degassing. This makes the trickling filter independent of an oxygen supply for nitrification to occur, besides ensuring the maintenance of adequate oxygen levels in shrimp tanks' water. However, whenever low oxygen levels in the tanks water are detected by an oxygen probe, a blower is automatically activated, ensuring the water aeration through the air stones that are placed in each shrimp tank.

After the biological filtration, water is directed to clean water reservoirs, from which is pumped to a filter of ultraviolet (UV) irradiation for disinfection. This disinfection process works by applying light in ultraviolet wavelengths to damage/destroy the genetic material (DNA and/or RNA) of microorganisms, which results in their inactivation and dead, thus allowing the control of potential bacterial, viral, fungal and parasitic infections. Ultraviolet irradiation is a technology widely used in aquaculture, as it does not generate toxic residuals (as does ozone) and it is also effective not only to inactivate microorganisms but also to remove ozone disinfection by-products from the water and to destroy dissolved ozone residuals (Lekang, 2013; Powell *et al*., 2015; Sharrer and Summerfelt, 2007; Summerfelt, 2003).

Finally, water passes through a plate-type heat exchanger prior to return to shrimp tanks. In this heat exchanger, energy is transferred from a heated freshwater medium to the seawater medium of the recirculating aquaculture system. The freshwater medium is heated by the action of a heat pump, and this heat exchanger is used only when there is a need to heat the system's seawater.

When it is necessary to renew/replace the water in the recirculating aquaculture system, new seawater is introduced from a water supply. In this case, saltwater is supplied from a borehole (6 m of depth) and pumped to a reservoir located in the outside area of the company's facilities. This water has a high content of dissolved iron and to remove it water passes through an iron oxidation equipment, where air is injected, oxidizing the dissolved iron. Then, water passes through a sand filter where particles and precipitated iron are filtered. Finally, water is pumped to the system reservoirs, from where it is directed to the set of bag filters, passing through all the above mentioned water treatment steps before reaching the shrimp tanks.

Chapter 3: Objectives

Feed and feeding management of marine shrimp represents a major challenge to maximize productivity and profitability in aquaculture. Strategies to increase shrimp survival and enhance its growth performance, particularly during the first phases of production, are required. The application of adequate feeding protocols as well as the supply of proper diets meeting the nutritional requirements of shrimp may be of particular importance to reduce mortality associated with cannibalism in aquaculture, although knowledge of shrimp nutrition and feeding is more limited than that of fish.

RiaSearch has been confronted with a high mortality of whiteleg shrimp, especially during the postlarvae and juvenile stages. This represents a significant economic loss for the company. In this context, the present study aimed to define feeding strategies to improve whiteleg shrimp larval growth and survival at RiaSearch. For that purpose, the effect of different feeding protocols, including different types of feed and feeding frequencies, on the survival and growth of postlarvae and juveniles from *Penaeus vannamei* species was evaluated, with the following null hypotheses being tested:

- ✓ There is no effect of co-feeding with *Artemia* nauplii on the survival and growth of whiteleg shrimp postlarvae;
- ✓ There is no effect of feeding frequency on the survival and growth of whiteleg shrimp juveniles.

In addition, this internship at RiaSearch was benefic, since it allowed to acquire valuable knowledge on good management practices in shrimp and live feed production, as well as on operation and maintenance of recirculating aquaculture systems; and to develop professional skills within a real work context.

Chapter 4: Material and Methods

4.1 Experimental animals

Whiteleg shrimp (*Penaeus vannamei*) postlarvae (PL) were obtained from a commercial hatchery located in Florida, United States of America, transported to Portugal by plane and then to RiaSearch by car.

After transportation in plastic bags filled with one-third of filtered seawater and two-thirds of pure oxygen, placed into polystyrene boxes, postlarvae were acclimatized to the experimental conditions in tanks containing shelters, following the acclimation guidelines for shrimp larvae of the commercial hatchery.

4.2 Growth trials

Two growth trials with whiteleg shrimp postlarvae and juveniles, respectively, were carried out in experimental systems at RiaSearch facilities, in Murtosa municipality, Aveiro (Portugal).

4.2.1 Effect of co-feeding with *Artemia* nauplii on the survival and growth of whiteleg shrimp postlarvae

A first trial aimed to evaluate the effect of two different feeding protocols – a co-feeding combining live feed and inert diet or an inert diet alone – on the survival and growth of whiteleg shrimp postlarvae.

- **Experimental system:**

The feeding trial lasted 13 days and was performed in a recirculating aquaculture system equipped with a battery of four tanks of 160 L (Figure 10), two bag filters (filtration size of 50 and 25 µm), a protein skimmer, a biological filter and a UV sterilizer, and the water temperature in tanks was controlled by using a heat pump. Tanks were supplied by a continuous filtered seawater flow (1.0-2.5 L min^{-1} in each tank) and a photoperiod regime of 14 h light (L): 10 h dark (D) was made by artificial illumination.

At the beginning of the trial, four groups of 5 000 individuals were constituted from a homogeneous batch of PL_{12}, i.e. postlarvae with twelve days after metamorphosis, and randomly distributed by the experimental tanks. To determine the average initial body weight of postlarvae (4.3 ± 0.0 mg, mean ± SD), a representative sample of the initial batch was group weighed. Samples of twenty PL from each experimental tank were, also, weighed every other day throughout the feeding period, for PL growth monitoring. At the end of the trial, the total number of PL from each experimental tank was determined and a representative sample weighed, for both survival and growth performance evaluation.

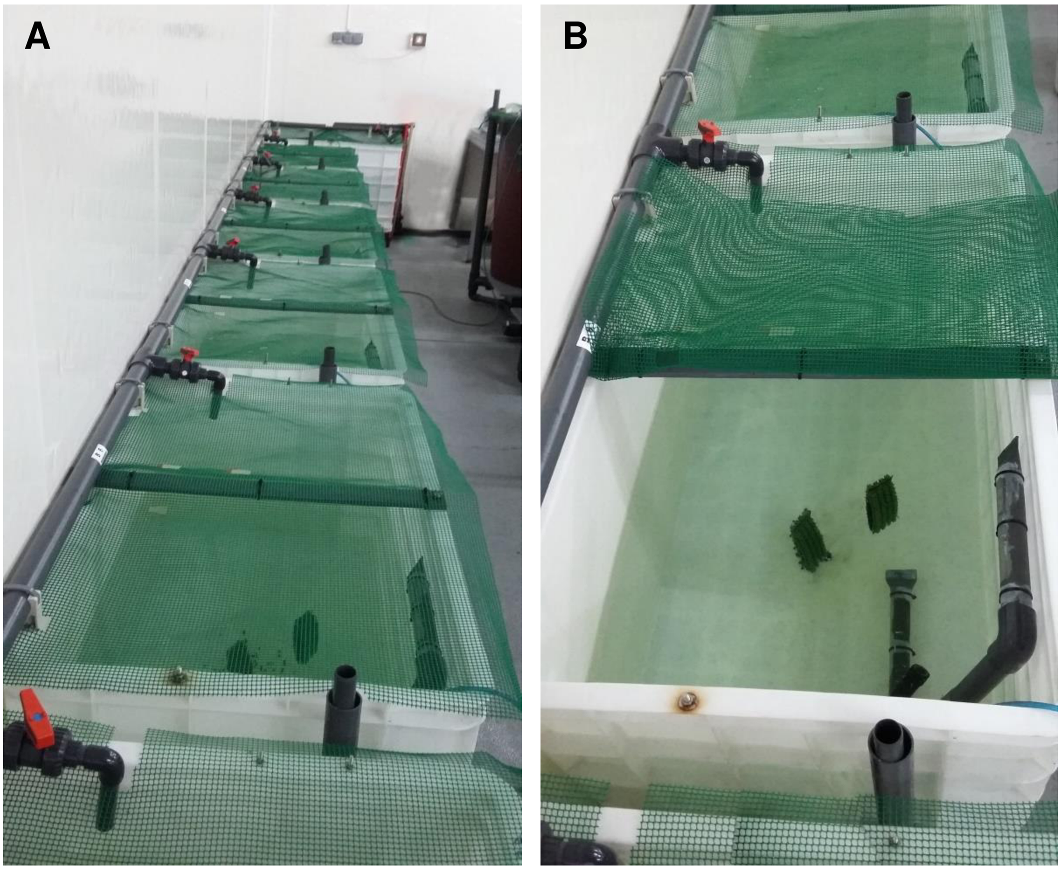

Fig. 10 – Experimental system of the first growth trial at RiaSearch (A), showing the tanks with shelters (B).

- **Feeding protocols:**

Two feeding protocols were tested in duplicate groups of shrimp postlarvae and consisted of:

With Artemia – co-feeding regime combining *Artemia* nauplii and a commercial inert microdiet, during the first eight days, and then just inert microdiet until the end of the trial (Figure 11);

Without Artemia – providing only the commercial inert microdiet during the entire trial.

Fig. 11 – Scheme of the feeding protocols used in the first growth trial.

Eight meals per day were established for both treatments, four during the day (at 09.00 h, 12.00 h, 15.00 h and 18.00 h) and the other four at night (at 21.00 h, 24.00 h, 03.00 h and 06.00 h).

In the treatment "With Artemia", live feed was provided by hand, only during daytime (at 09.00 h, 12.00 h, 15.00 h and 18.00 h). The quantity of *Artemia* nauplii was determined in order to ensure at least two *Artemia* nauplii per postlarva at 09.00 h, 12.00 h and 15.00 h meals, and at least ten *Artemia* nauplii per postlarva at 18.00 h meal. The inert diet was provided by hand during daytime (at 09.00 h, 12.00 h, 15.00 h and 18.00 h) and using automatic feeders at nighttime (at 21.00 h, 24.00 h, 03.00 h and 06.00 h).

For the treatment "Without Artemia", the inert diet was provided following the same schedule of protocol "With Artemia", i.e. by hand during the day (at 09.00 h, 12.00 h, 15.00 h and 18.00 h) and using automatic feeders during the night (at 21.00 h, 24.00 h, 03.00 h and 06.00 h).

Automatic feeders consisted of feed dispensers with rotary system, each one regulated to provide just two meals per night, at previously scheduled times, so two automatic feeders were used per experimental tank.

The amount of inert microdiet provided per meal was similar among the feeding protocols, and was determined considering the shrimp initial biomass in each experimental tank as well as their daily weight gain (as percentage), in accordance with the shrimp daily growth table. In addition, the amount of feed not dispensed at nighttime was quantified for the corrected dietary feed intake calculation.

During the day, the bottom of the experimental tanks was siphoned before each meal, in order to remove faecal matter, feed wastes, exoskeleton and dead individuals. The water outlet filter tube of each experimental tank was, also, cleaned and changed every day, just before the first and last diurnal feeding times (at 09.00 h and 18.00 h).

- **Inert diet:**

The commercial inert microdiet used was WIN Fast (pellet size 150-500 µm) from SPAROS Lda., Faro, Portugal. It had a proximate composition of 63% crude protein, 17% crude fat, 0.3% crude fiber, 12% crude ash, 2.1% total phosphorus, 1.5% calcium and 0.5% sodium, and contained squid meal, fishmeal, fish hydrolysate, shrimp meal, wheat gluten, fish gelatin and fish oil.

- ***Artemia* preparation:**

Artemia was locally prepared using two cylindrical tanks of 9 L water volume (Figure 12) and following the hatching protocol of RiaSearch. *Artemia* sp. cysts (INVE Aquaculture) were daily incubated in filtered seawater, at a density of 2.5 g/L, with a photoperiod regime of 24 h light (L). Water temperature and dissolved oxygen were maintained at 25.1 ± 0.6 °C (mean ± SD) and 6.8 ± 0.2 mg/L, respectively, using a heater and air stones, while salinity was around 18.7 ± 0.4 and pH averaged 7.9 ± 0.1. After a 24 h incubation time, the amount of nauplii obtained in the cylindrical tanks was estimated by performing five samplings (and subsamplings) of the incubation medium (seawater with *Artemia*), in order to calculate the volume that was necessary to provide to postlarvae, in each experimental tank.

Fig. 12 – *Artemia* sp. production in cylindrical tanks, at RiaSearch.

- **Water quality parameters measurement:**

During the growth trial, water quality parameters including salinity, temperature, dissolved oxygen and pH were recorded daily with a probe. Water salinity and temperature were maintained at 23.3 ± 0.5 (mean ± SD) and 27.3 ± 0.5 °C, respectively, dissolved oxygen (DO) averaged 5.2 ± 0.6 mg/L and pH ranged between 6.5 and 7.4. Ammonium, nitrite and nitrate values (0.0-0.5 mg/L, 0.15-0.25 mg/L and 50.0 ± 0.0 mg/L (mean ± SD), respectively) were determined using water quality chemical tests twice a week.

4.2.2 Effect of feeding frequency on the survival and growth of whiteleg shrimp juveniles

A second growth trial was performed with shrimp individuals previously fed according to protocol "With Artemia" of the first trial, and it aimed to determine the effect of feeding frequency on the survival and growth of whiteleg shrimp juveniles.

- **Experimental system:**

The feeding trial lasted 22 days and was carried out in a recirculating aquaculture system comprising a battery of six tanks of 200 L (Figure 13), two mechanical bag filters (filtration size of 50 and 25 µm), a protein skimmer, a biological filter, a UV sterilizer and a heat pump. Experimental tanks were supplied by a continuous flow (5 L min^{-1} in each tank) of filtered seawater and a photoperiod regime of 14 h light (L): 10 h dark (D) was made by artificial illumination.

Initially, six groups of 300 individuals with an average initial body weight of 35.9 ± 0.0 mg (mean ± SD) were established from a homogeneous batch of PL_{25} and randomly distributed by the experimental tanks. At the end of the trial, the total number of shrimp juveniles from each experimental tank was determined and individually weighed, for both survival and growth performance evaluation.

Fig. 13 – Experimental system of the second growth trial at RiaSearch (A and B).

- **Feeding protocols:**

Three feeding frequency protocols were tested in duplicate groups of shrimp and consisted of:

Feed 4t – supply of a commercial inert microdiet four times per day (Figure 14);

Feed 6t – supply of a commercial inert microdiet six times per day;

Feed 8t – supply of a commercial inert microdiet eight times per day.

Fig. 14 – Scheme of the feeding protocols used in the second growth trial.

The total amount of inert microdiet provided every day was similar among the feeding protocols, and was determined taking into account the shrimp initial biomass in each experimental tank and their daily weight gain, as percentage, in accordance with the shrimp daily growth table.

For protocol "Feed 4t", the inert diet was provided by hand during daytime (at 09.00 h, 12.00 h, 15.00 h and 18.00 h). No inert diet was provided during nighttime. For protocol "Feed 6t", the inert diet was provided by hand during the day (at 09.00 h, 12.00 h, 15.00 h and 18.00 h) and using automatic feeders at nighttime (at 23.00 h and 04.00 h). For protocol "Feed 8t", the inert diet was provided by hand four times during the day (at 09.00 h, 12.00 h, 15.00 h and 18.00 h) and other four at night (at 21.00 h, 24.00 h, 03.00 h and 06.00 h), using automatic feeders.

Automatic feeders, similar to those used in the first growth trial, were used to distribute the meals during the night. The amount of feed not dispensed during the night was quantified for the corrected dietary feed intake calculation.

Every day, the bottom of the experimental tanks was siphoned before the first and last diurnal meals (at 09.00 h and 18.00 h) and the water outlet filter tube of the experimental tanks was cleaned.

- **Inert diet:**

The inert diet used was the commercial microdiet WIN Flat for flatfish (pellet size 500-800 µm) from SPAROS Lda., Faro, Portugal. It had a proximate composition of 62% crude protein, 18% crude fat, 0.5% crude fiber, 9% crude ash, 1.9% total phosphorus, 1.3% calcium and 0.5% sodium, containing the following ingredients: krill meal, squid meal, wheat gluten, fish meal, shrimp meal, fish hydrolysate, pea protein concentrate, fish gelatin, fish oil and lecithin.

- **Water quality parameters measurement:**

Throughout the growth trial, water temperature, salinity, dissolved oxygen and pH were recorded daily with a probe. Water temperature averaged 27.6 ± 0.2 °C (mean ± SD), salinity was around 22.2 ± 0.5, dissolved oxygen was kept at 6.8 ± 0.4 mg/L and pH remained about 7.2 ± 0.1. Ammonium, nitrite and nitrate values (0.0-0.5 mg/L, 0.25-0.50 mg/L and 50.0 ± 0.0 mg/L (mean ± SD), respectively) were determined using water quality chemical tests twice a week.

4.3 Data analysis

Data from both growth trials were presented as mean and standard deviation (SD). Average body weight (ABW), weight gain (WG), dietary feed intake (DFI), *Artemia* feed intake (AFI, in the first growth trial), feed efficiency (FE), daily growth index (DGI) and survival were determined, as followed:

Average body weight

ABW = (FBW + IBW) / 2

(where ABW is the average body weight, FBW is the average final body weight and IBW is the average initial body weight)

Weight gain

WG = ((FBW – IBW) x 1000) / (ABW x time in days)

(where WG is the weight gain in g kg ABW^{-1} day^{-1} and FBW, IBW and ABW are in g)

Dietary feed intake

DFI = (total dry feed intake × 1000) / (ABW × time in days)

(where DFI is the dietary feed intake in g kg ABW^{-1} day^{-1}, total dry feed intake is in g and ABW is in g)

***Artemia* feed intake**

AFI = total *Artemia* nauplii intake / (ABW × time in days)

(where AFI is the *Artemia* feed intake in nauplii g ABW^{-1} day^{-1} and ABW is in g)

Feed efficiency

FE = (FBW – IBW) / total dry feed intake

(where FE is the feed efficiency, FBW and IBW are in g and total dry feed intake is in g)

Daily growth index

DGI = (($FBW^{1/3}$ – $IBW^{1/3}$) / time in days) x 100

(where DGI is the daily growth index as percentage and both FBW and IBW are in g)

Survival

Survival = 100 – ((Initial number – Final number) x 100 / Initial number)

(with survival as percentage)

- **Statistical analysis:**

Data regarding shrimp final body weight in the second growth trial were subjected to statistical analysis using the software IBM SPSS Statistics 25.0 for Windows. Data of both trials were subjected to descriptive statistics.

Final weight data of the second trial were subjected to a one-way analysis of variance (ANOVA) after testing the normality and homogeneity of variances by the Shapiro-Wilk and Levene tests, respectively. Differences were considered significant at $P < 0.05$ and the Tukey's test was performed in order to ascertain which specific feeding protocols were significantly different ($P < 0.05$).

Chapter 5: Results

5.1 Effect of co-feeding with *Artemia* nauplii on the survival and growth of whiteleg shrimp postlarvae

Results on shrimp survival and growth performance, as well as on feed efficiency, which was determined according to the inert feed intake only, are presented in Table 1, as mean ± SD, Figures 15, 16 and 17.

Survival of shrimp postlarvae that were co-fed with *Artemia* nauplii and inert diet ("With Artemia"; 71.2%) was 9.4% higher than that of postlarvae fed with the inert diet alone ("Without Artemia"; 65.1%) (Table 1).

Average final body weight (FBW) of co-fed shrimp ("With Artemia") was almost twice that of non-co-fed shrimp ("Without Artemia"), and daily growth index (DGI) was 67.4% higher in co-fed shrimp ("With Artemia") when compared to those fed only the inert diet ("Without Artemia") (Table 1; Figure 15). Figure 16 shows that the postlarvae fed with the protocol "With Artemia" exhibited higher body weight values than the postlarvae that were fed with the protocol "Without Artemia" from the day 18 after metamorphosis onwards (body weight of PL_{18}, PL_{20}, PL_{22} and PL_{24} fed protocol "With Artemia" vs "Without Artemia": 15.2 vs 8.8; 23.1 vs 9.9; 27.5 vs 10.8 and 34.0 vs 17.4 mg, respectively).

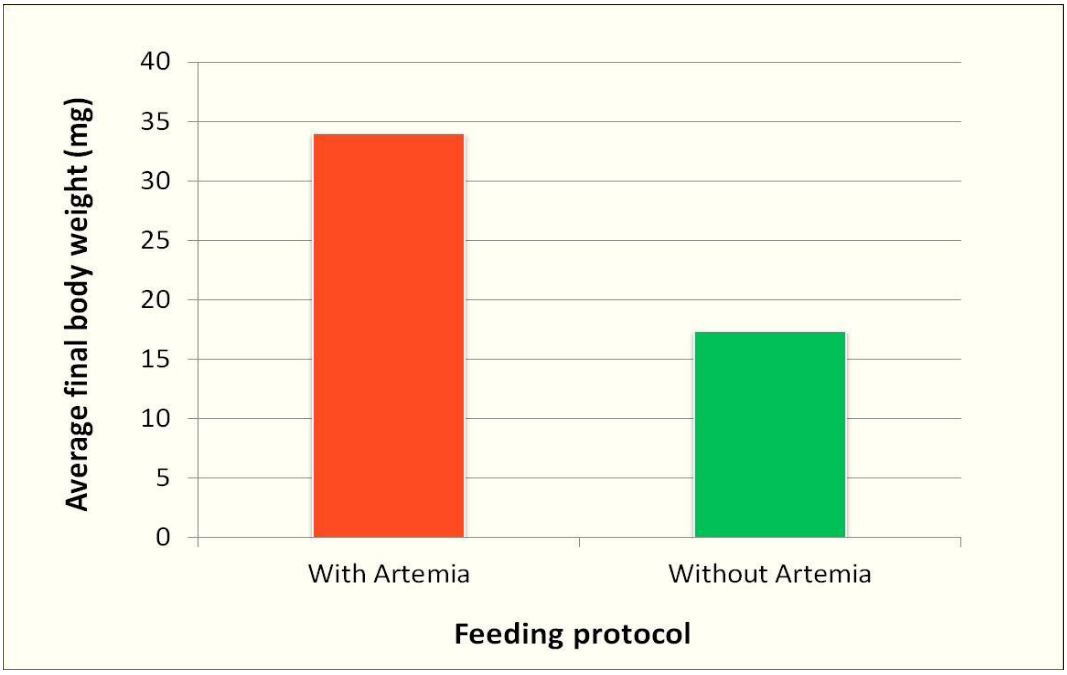

Fig. 15 – Shrimp average final body weight (mg) at the end of the trial (24 days after metamorphosis). Values are mean of duplicate from each feeding protocol.

Table 1 – Growth response of shrimp postlarvae fed according to the different feeding protocols for 13 days[1].

	With Artemia	Without Artemia
Initial number	5000.0 ± 0.0	5000.0 ± 0.0
Final number	3559.0 ± 185.3	3253.5 ± 169.0
Survival (%)	71.2 ± 3.7	65.1 ± 3.4
IBW (mg)	4.3 ± 0.0	4.3 ± 0.0
FBW (mg)	34.0 ± 0.003	17.4 ± 0.001
DGI (%)	1.24 ± 0.06	0.74 ± 0.05
AFI (nauplii shrimp^{-1} day^{-1})	16.0 ± 0.0	-
AFI (nauplii g ABW^{-1} day^{-1})	1124.9 ± 23.8	-
DFI (g kg ABW^{-1} day^{-1})	135.2 ± 4.36	232.9 ± 11.69
FE	0.79 ± 0.03	0.30 ± 0.05

[1] Values (mean ± SD) are mean of duplicate.

Survival = 100 – ((Initial number – Final number) x 100 / Initial number);

IBW, Average initial body weight;

FBW, Average final body weight;

DGI, Daily growth index = (($FBW^{1/3}$ – $IBW^{1/3}$) / time in days) x 100;

AFI, *Artemia* feed intake = total *Artemia* nauplii intake / (ABW × time in days);

DFI, Dietary feed intake = (total dry feed intake × 1000) / (ABW × time in days);

FE, Feed efficiency = (FBW – IBW) / total dry feed intake.

The *Artemia* feed intake of co-fed shrimp postlarvae ("With Artemia") was about 1 125 nauplii g ABW^{-1} day^{-1} (Table 1).

Daily dietary feed intake (g as is per feeding protocol) during the growth trial was relatively similar among protocols (Figure 17). However, dietary feed intake (DFI) evaluated as g kg ABW^{-1} day^{-1} was higher in shrimp postlarvae fed only with the inert diet ("Without Artemia"; Table 1), and the opposite was true for feed efficiency (FE), whose value was 2.7-fold higher in the co-fed shrimp postlarvae (Table 1).

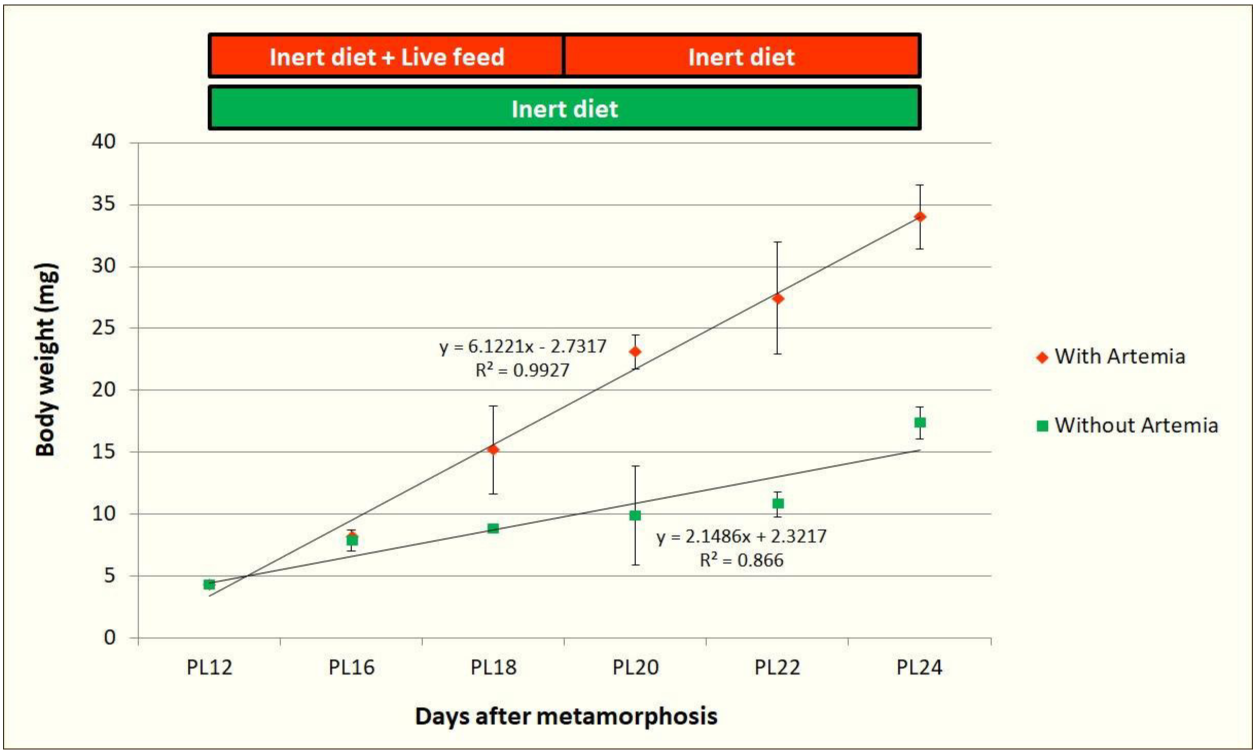

Fig. 16 – Shrimp postlarvae body weight (mg) during the growth trial. Daily values are mean ± SD of duplicate from each feeding protocol.

Fig. 17 – Dietary feed intake (g as is) by shrimp postlarvae during the growth trial. Daily values are mean ± SD of duplicate from each feeding protocol.

Data from this trial shows that co-feeding with *Artemia* nauplii increased survival and growth of whiteleg shrimp postlarvae, therefore rejecting the null hypothesis.

5.2 Effect of feeding frequency on the survival and growth of whiteleg shrimp juveniles

Zootechnical performance of shrimp juveniles is presented in Table 2, as mean ± SD, Figures 18 and 19.

Survival ranged from 93.5% in shrimp fed 4 times per day ("Feed 4t") to 97.3% in those fed 8 times daily ("Feed 8t"), and it trended to increase with increasing feeding frequency (Table 2).

At the end of the growth trial, the average final body weight of shrimp juveniles significantly increased ($P < 0.05$) with the increase of the number of meals per day from 4 ("Feed 4t"; 608.0 mg) to 6 times a day ("Feed 6t"; 836.6 mg) and from this to 8 times daily ("Feed 8t"; 928.4 mg) (Table 2; Figure 18).

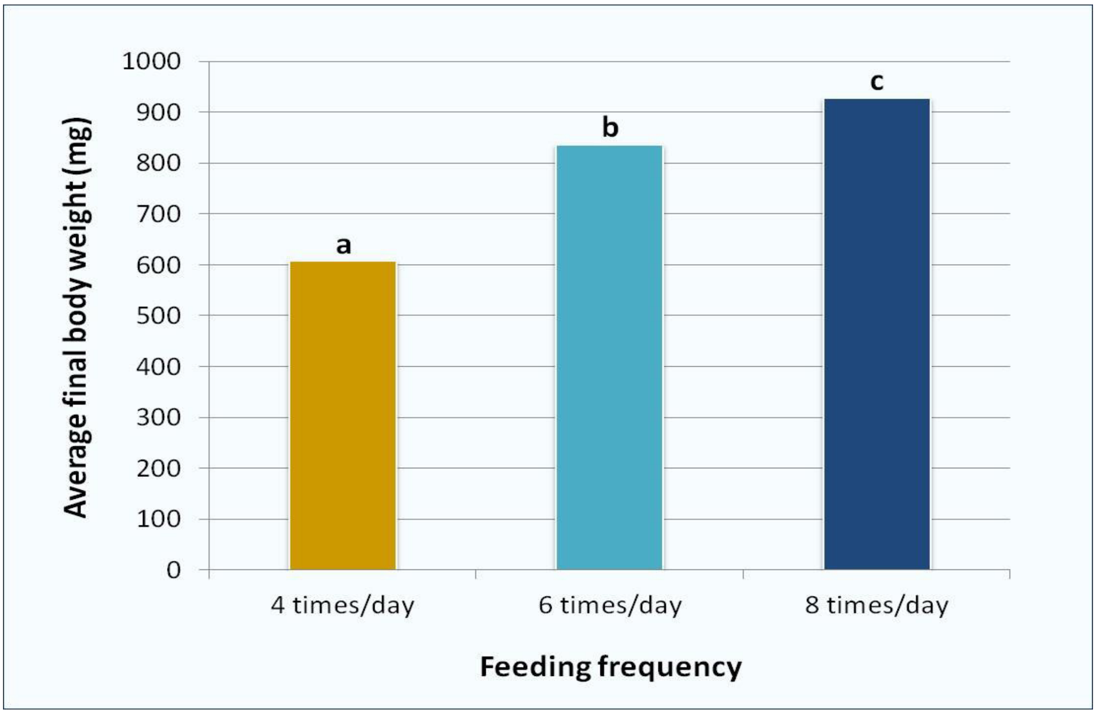

Fig. 18 – Shrimp average final body weight (mg) at the end of the trial (46 days after metamorphosis). Values are mean of duplicate from each feeding protocol; variables were compared by one-way ANOVA ($P < 0.05$); different letters indicate significant differences among the feeding protocols ($P < 0.05$).

Daily growth index (DGI) of shrimp juveniles increased with the increase of feeding frequency (Table 2).

Table 2 – Growth response of shrimp juveniles fed four, six or eight times per day for 22 days[1].

	Feed 4t (4 times/day)	Feed 6t (6 times/day)	Feed 8t (8 times/day)	*p*-value
Initial number	300.0 ± 0.0	300.0 ± 0.0	300.0 ± 0.0	
Final number	280.5 ± 4.9	286.0 ± 1.4	292.0 ± 0.0	
Survival (%)	93.5 ± 1.6	95.3 ± 0.5	97.3 ± 0.0	
IBW (mg)	35.9 ± 0.0	35.9 ± 0.0	35.9 ± 0.0	
FBW (mg)	608.0 ± 0.02[a]	836.6 ± 0.01[b]	928.4 ± 0.01[c]	0.000
DGI (%)	2.35 ± 0.04	2.78 ± 0.02	2.93 ± 0.16	
DFI (g kg ABW^{-1} day^{-1})	72.9 ± 1.12	54.1 ± 0.75	49.5 ± 4.69	
FE	1.10 ± 0.02	1.54 ± 0.02	1.70 ± 0.18	

[1] Values (mean ± SD) are mean of duplicate.

Survival = 100 – ((Initial number – Final number) x 100 / Initial number);

IBW, Average initial body weight;

FBW, Average final body weight (Means in the same row with different superscript letters are significantly different (P < 0.05));

DGI, Daily growth index = $((FBW^{1/3} - IBW^{1/3})$ / time in days) x 100;

DFI, Dietary feed intake = (total dry feed intake × 1000) / (ABW × time in days);

FE, Feed efficiency = (FBW – IBW) / total dry feed intake.

Irrespectively of the number of meals per day, daily voluntary feed intake (g as is per feeding protocol) was similar (Figure 19). However, dietary feed intake (DFI) expressed as g kg ABW^{-1} day^{-1} decreased with the increase of feeding frequency. In contrast, feed efficiency (FE) increased with increasing number of meals per day (Table 2).

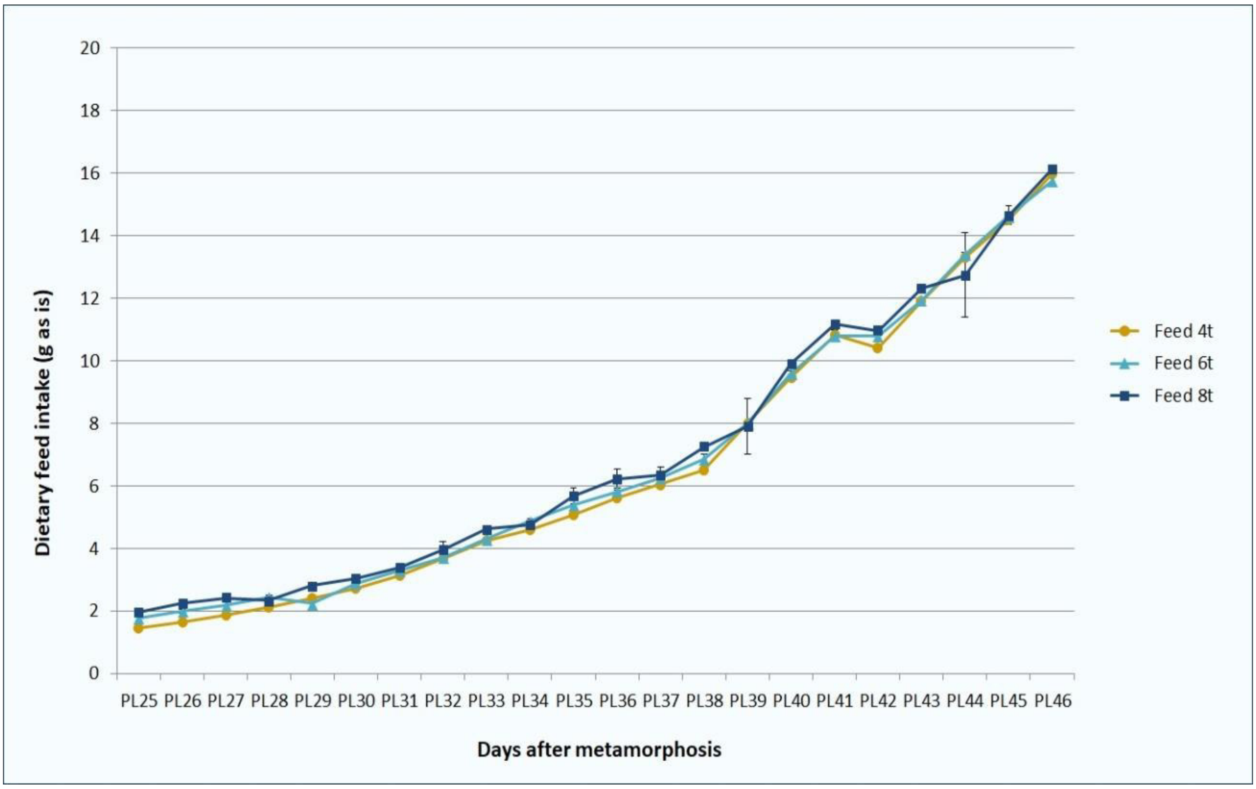

Fig. 19 – Dietary feed intake (g as is) by shrimp juveniles during the growth trial. Daily values are mean ± SD of duplicate from each feeding protocol.

Results of this trial showed that a higher feeding frequency increased survival and growth of whiteleg shrimp juveniles, thus rejecting the null hypothesis.

Chapter 6: Discussion

Effect of co-feeding with Artemia nauplii on the survival and growth of whiteleg shrimp postlarvae

To ensure a good growth performance and feed conversion efficiency, as well as a high survival rate of farmed aquatic species, a reliable and balanced diet capable of satisfying the nutritional requirements of the species, both qualitatively and quantitatively, along with the utilization/application of optimal feeding regimes is essential (Conceição *et al*., 1998; Engrola *et al*., 2009a; Engrola *et al*., 2009b; Pousão-Ferreira *et al*., 2003; Russo *et al*., 2017).

Intensive production of commercially important fish and shrimp species in hatcheries is still based on the use of live feeds (algae, rotifers, *Artemia*) for the initial feeding of larval stages, followed by a combination of live and dry inert feed until larvae are completely weaned (Cahu and Infante, 2001; Gamboa-Delgado and Vay, 2009; Naegel and Rodríguez-Astudillo, 2004; Pousão-Ferreira *et al*., 2003; Rosenlund *et al*., 1997). The use of live feed, during the early production phases, requires not only specialized culture infrastructures but also an extra effort in terms of management, labor, time and energy, which inevitably leads to increased operating costs (Naegel and Rodríguez-Astudillo, 2004; Pousão-Ferreira *et al*., 2003; Pradhan *et al*., 2014; Robinson *et al*., 2005; Russo *et al*., 2017; Sambhu *et al*., 2014; Sangha *et al*., 2000; Watanabe *et al*., 2016). In addition, the availability and nutritional value of live feeds may also be variable, which may represent a sub-optimal source of nutrients, not supporting acceptable growth rates of farmed species if fed exclusively with live feed (Calderon *et al*., 2004; Conceição *et al*., 2007; Gallardo *et al*., 2002; Gamboa-Delgado and Vay, 2009; Pousão-Ferreira *et al*., 2003; Robinson *et al*., 2005; Rosenlund *et al*., 1997; Watanabe *et al*., 2016). To attenuate this situation, one common practice is the enrichment of live prey with essential nutrients (fatty acids, vitamins, proteins, etc.) before feeding them to larvae, but even so, any alternative diet or feeding protocol that may reduce or eliminate the dependence on live feeds' utilization is of technical and economic interest for aquaculture (Abatzopoulos *et al*., 2002; Cañavate and Fernández-Díaz, 1999; McVey, 1993; Pradhan *et al*., 2014; Sorgeloos *et al*., 2001; Watanabe *et al*., 2016).

The development of cost-effective, nutritionally complete and stable inert feeds to substitute live feed would be of tremendous benefit to commercial hatchery operations (Gamboa-Delgado and Vay, 2009; Robinson *et al*., 2005; Watanabe *et al*., 2016), since artificial formulated inert microdiets can be adjusted to specific farmed species and developmental stages, supplying all the essential nutrients that are not found in unenriched live prey and promoting higher larval survival rates (Gamboa-Delgado and Vay, 2009; Pousão-Ferreira *et al.*, 2003). Apart from offering lower production costs, these manufactured inert diets are also easily stored in the aquaculture facilities and, thus, immediately available for larval feeding (Pousão-Ferreira *et al.*, 2003).

On the other hand, the acceptance of inert microdiets, provided from the onset of exogenous feeding, by larvae of several farmed species is limited, often leading to low or variable ingestion rates and to poor growth performances (Conceição *et al.*, 2007; Liu *et al.*, 2012; Pradhan *et al.*, 2014; Rosenlund *et al.*, 1997). This limited success may be related to the composition, palatability, attractiveness or physical characteristics (e.g. particle size) of the inert diet as well as to an inability of the cultured larvae to properly digest the feed, as a result of inadequate development of digestive enzymes (Liu *et al.*, 2012; Nhu *et al.*, 2010; Pradhan *et al.*, 2014; Rosenlund *et al.*, 1997). Providing an inert diet may also reduce culture water quality, due to degradation and leaching of nutrients from the inert feeds, which in turn contributes to loss of appetite by larvae, poor growth and high mortality (Calderon *et al.*, 2004; Robinson *et al.*, 2005; Rosenlund *et al.*, 1997).

A feeding protocol that combines live feed and manufactured inert diets (co-feeding) from the start of exogenous feeding or from an early developmental stage represents an alternative strategy to alleviate the problems of low acceptance and digestibility of inert diets by the cultured larvae and, thus, increase the success of early weaning to a formulated microdiet (Gamboa-Delgado and Vay, 2009; Liu *et al.*, 2012; Pradhan *et al.*, 2014; Rosenlund *et al.*, 1997).

In the present study, the effect of using different feeding protocols on the survival and growth performance of postlarvae from whiteleg shrimp *Penaeus vannamei* was evaluated. Protocols consisted of a co-feeding regime combining *Artemia* nauplii and a commercial inert microdiet, during the first eight days, and then just inert microdiet until the end of the trial ("With Artemia") or providing only the commercial inert microdiet during the entire trial ("Without Artemia"). Survival was higher when postlarvae were co-fed with *Artemia* nauplii than when they were fed only a commercial inert microdiet. Compared with the supply of inert microdiet alone

("Without Artemia"), the co-feeding with *Artemia* nauplii increased the survival of whiteleg shrimp by 9.4% (65.1% "Without Artemia" against 71.2% "With Artemia"). Within a business context as in RiaSearch, this increase in survival may represent an economical advantage.

This result agrees with previous studies with shrimp larval stages showing that co-feeding regimes with *Artemia* and inert diets lead to a higher survival than feeding an inert diet alone. For example, for Northern brown shrimp larvae, *Farfantepenaeus aztecus*, it was observed that an inert diet may be used to replace 50% of *Artemia* nauplii, but higher replacement levels resulted in low survival (Robinson *et al.*, 2005). Similarly, for whiteleg shrimp larvae, *Penaeus vannamei*, it was demonstrated that co-feeding regimes in which *Artemia* nauplii were replaced by inert diets at different levels, 25, 50 or 75%, lead to higher survival than that of feeding either an inert diet alone or only *Artemia* nauplii (Gamboa-Delgado and Vay, 2009).

Growth performance of whiteleg shrimp postlarvae in this experimental trial was affected by feeding protocol. Throughout the trial, the growth of postlarvae that were co-fed with inert diet and *Artemia* nauplii was higher than that of postlarvae fed the inert diet alone, as the co-fed postlarvae exhibited higher body weight values from the day 18 after metamorphosis (PL_{18}) onwards. Daily growth index of co-fed whiteleg shrimp was 67.4% higher than that of whiteleg shrimp fed only the inert diet. Additionally, a higher feed efficiency was observed in co-fed postlarvae, which was 2.7-fold higher than that of postlarvae fed the inert diet alone. These results suggest that the commercial inert microdiet used did not fulfil the nutritional requirements of whiteleg shrimp postlarvae due, at least in part, to diet formulation issues or limited digestive and metabolic utilization of the inert diet, reducing shrimp survival, which in turn can be related to an increase of cannibalistic behaviors to compensate the nutritional deficiencies.

Comparing the whiteleg shrimp final body weight, it is worth stressing that co-fed postlarvae showed a final body weight 95.7% higher than the non-co-fed postlarvae. Together, these data demonstrate a considerable improvement of whiteleg shrimp larval growth and survival, at RiaSearch, if a co-feeding regime combining *Artemia* nauplii and a commercial inert microdiet, until 19 days after metamorphosis (PL_{19}), is used. These results are in accordance with previous studies reporting nutritional benefits and higher growth performance of penaeid shrimp larvae when inert diets are complemented with live feed (Brito *et al*., 2000; Brito *et al*., 2001; Gallardo *et al*., 2002; Robinson *et al*., 2005). Brito *et al*. (2004) observed that a significant higher proportion of dietary energy was retained in both Northern white shrimp *Penaeus setiferus* and whiteleg shrimp *Penaeus vannamei* larvae, if co-fed with *Artemia* nauplii, algae and a microparticulate commercial diet, in comparison with shrimp fed only the microparticulate diet. Some authors have referred that the success of co-feeding regimes is associated to the fact that live prey may facilitate the intake of microdiets by visual and chemical stimulation, thus improving larval growth and survival in the aquaculture production units (Liu *et al*., 2012; Pousão-Ferreira *et al*., 2003). Moreover, *Artemia* nauplii as live feed for shrimp postlarvae, in this study, was also beneficial, as far as live prey size and nutritional value are important characteristics to take into account in shrimp hatchery production (Abatzopoulos *et al*., 2002; McVey, 1993; Sambhu *et al*., 2014), and the small size of the nauplii favored its intake by postlarvae.

Several studies with fish species have also reported improved larval growth and survival during the weaning period when co-feeding regimes, combining live feed and compound inert diets, are adopted at earlier larval stages (Cañavate and Fernández-Díaz, 1999; Engrola *et al*., 2009a; Fletcher *et al*., 2007; Liu *et al*., 2012; Pousão-Ferreira *et al*., 2003; Rosenlund *et al*., 1997). These results have been associated to some particularities of live feed, including natural attractants and nutritional factors, that are not present in the inert diets and have important influence on food attractiveness, ingestion and stimulus of endocrine responses that modulate digestion and absorption processes of inert feeds (Fletcher *et al*., 2007; García-Ortega *et al*., 2010; Liu *et al*., 2012; Nhu *et al*., 2010; Pousão-Ferreira *et al*., 2003).

In this context, the use of co-feeding regimes, combining *Artemia* nauplii and inert diets, during shrimp earlier developmental stages is the best management to maximize productivity. Co-feeding protocols allow to improve and stabilize the nutritional condition of shrimp larvae, enhancing their growth performance, and pre-condition the larvae to more readily accept inert diets when live feed is withdrawn, which results in shorter weaning periods and increased profitability (Cañavate and Fernández-Díaz, 1999; Fletcher *et al*., 2007; Nhu *et al*., 2010; Rosenlund *et al*., 1997).

Effect of feeding frequency on the survival and growth of whiteleg shrimp juveniles

The utilization efficiency of an inert diet in aquatic medium depends largely on its rapid intake before nutrient leaching occurs. Many other factors as feed particle size, feeding time and frequency and the amount of feed provided per meal can also affect the survival and growth performance of farmed species at their early life stages (Aalimahmoudi *et al*., 2016; Pontes *et al*., 2015; Tian *et al*., 2015). Among these, feeding frequency is a key parameter in nutrition management strategies that has an influence on the digestion and absorption processes of live and inert feeds. Indeed, it has been observed that distributing the daily amount of feed in multiple meals, several times a day, promotes shrimp growth and improves feed conversion efficiency and water quality (Carvalho and Nunes, 2006; Ding *et al*., 2017; Pontes *et al*., 2015; Velasco *et al*., 1999). Thus, efforts should focus on optimizing the feeding strategy in order to reduce feed waste and water quality deterioration, while maximizing the productivity and profitability of aquaculture companies (Aalimahmoudi *et al*., 2016; Ding *et al*., 2017; Moorhead and Zeng, 2017; Navarro-Guillén *et al*., 2017; Smith *et al*., 2002; Velasco *et al*., 1999; Wang *et al*., 2009; Watanabe *et al*., 2016).

In the second growth trial of the present study, the effect of three different feeding frequencies – 4, 6 and 8 feedings per day – on the survival and growth performance of whiteleg shrimp juveniles (*Penaeus vannamei*) was evaluated. Survival was generally high and trended to increase with increasing feeding frequency. Survival of whiteleg shrimp fed 6 ("Feed 6t") or 8 times per day ("Feed 8t") was, respectively, 2% and 4% higher than those fed 4 times per day ("Feed 4t"), which within a business context may be relevant.

Growth performance, evaluated as final body weight and daily growth index, of whiteleg shrimp fed 6 or 8 times per day was higher than those fed 4 times daily. At the end of the experimental trial, the juveniles fed 6 times per day exhibited a final body weight 38% higher than those fed 4 times a day, whereas those fed 8 times per day had a final body weight 53% and 11% higher than those fed 4 or 6 times per day, respectively. Similarly, compared to whiteleg shrimp fed 4 times per day, the increase of the feeding frequency to 6 or 8 times per day increased the daily growth index in about 18% and 25%, respectively. In addition, feed efficiency in shrimp fed 6 and 8 times per day was, respectively, 1.4 and 1.6-fold higher than that of shrimp fed 4 times a day. These data indicate that feeding 8 times per day remarkably improves the growth performance and feed utilization efficiency of whiteleg shrimp juveniles, in comparison with feeding frequencies of 4 or 6 times per day. These results also suggest that feeding frequencies lower than 6 meals per day may not ensure the maintenance of shrimp nutritional status, due to a possible increase of starvation periods and nutritional deficiencies, which may intensify cannibalism as an alternative way of obtaining food.

Previous studies on shrimp feeding frequency have demonstrated enhanced growth response by increasing the feeding frequency. For instance, Robertson *et al*. (1993) studied the effect of three feeding frequencies (1, 2 and 4 feedings per day) on the growth and survival of whiteleg shrimp *Penaeus vannamei* juveniles and observed a significant increase of growth with the increase of feeding frequency, while survival was not significantly different among the treatments. Similarly, Tacon *et al*. (2002) obtained high growth performances in whiteleg shrimp juveniles fed 8 times per day (throughout the day and the night), in comparison with individuals fed only 4 times daily or 4 times nightly. Moradizadeh *et al*. (2011) and Ding *et al*. (2017) also found benefits of increased feeding frequency on growth response of Indian white shrimp *Penaeus indicus* and Oriental river prawn *Macrobrachium nipponense*, respectively. More recently, Aalimahmoudi *et al*. (2016) reported that increasing the feeding frequency of whiteleg shrimp juveniles from 4 to 6 times per day positively affected its growth, leading to significantly higher values of weight gain, specific growth rate, daily growth rate and survival. However, other authors reported contradictory results on the effect of feeding frequency in shrimp. Pontes *et al*. (2008) recorded a decrease of whiteleg shrimp juveniles growth performance with the increase of feeding frequency from 3 or 4 to 7 times a day. Velasco *et al*. (1999) did not observe significant differences on the survival and growth performance of whiteleg shrimp fed 3, 5, 8, 11 and 15 times per day. Similarly, Smith *et al*. (2002) and Carvalho and Nunes (2006) also reported that feeding frequency had no effect on growth or survival of tiger shrimp *Penaeus monodon* and whiteleg shrimp, respectively. Available information suggests that these conflicting results can be related not only with the species used in the experimental studies, including differences on its genetic background, age or developmental stage, body weight and size, but also with the composition and attractiveness of the inert diet, feed particle size, water quality and culture conditions (Carvalho and Nunes, 2006; Ding *et al*., 2017; Manley *et al*., 2015; Russo *et al*., 2017; Thongprajukaew *et al*., 2017; Velasco *et al*., 1999).

Research projects using fish species have also been carried out in order to understand the relation between feeding frequency and growth response (Moorhead and Zeng, 2017; Thongprajukaew *et al*., 2017; Tian *et al*., 2015; Wang *et al*., 2009; Wu *et al*., 2015). Some authors mentioned that insufficient feeding frequencies lead to poor growth and high mortality of fish, while feeding at shorter intervals than that required for gastric evacuation and return of appetite may decrease the feed efficiency utilization, indicating that growth and feed utilization usually increase with feeding frequency up to a given limit (Thongprajukaew *et al*., 2017; Tian *et al*., 2015; Wu *et al*., 2015). In addition, other studies have documented that feeding restrictions promote food competition, variability in food intake and size heterogeneity, and that aggressive behaviors and cannibalism tend to increase during periods of low food availability, as conspecifics present the only type of alternative food (Manley, 2014; Manley *et al*., 2015; Ribeiro *et al*., 2015; Russo *et al*., 2017).

Under the present conditions, the increase of the feeding frequency up to 8 times per day has increased considerably the growth performance and feed efficiency of whiteleg shrimp juveniles, at RiaSearch. The optimization of shrimp feeding frequency in aquaculture units is of paramount importance to allow better access to feed, reduce starvation periods and ensure the correct nutritional status of shrimp. Moreover, mortality trended to decrease which may suggested a reduction of cannibalistic behavior as a way of compensating nutritional deficiencies imposed by the prolonged starvation periods. Together, these results may have a considerable economic impact for RiaSearch.

Chapter 7: Conclusions and Future Perspectives

The present study aimed to optimize the feed and feeding management of whiteleg shrimp (*Penaeus vannamei*) under the conditions of RiaSearch company. For that purpose, two experimental growth trials were performed.

The first growth trial focused on the study of the effect of co-feeding a combination of a commercial inert microdiet and *Artemia* nauplii or just feeding the commercial inert microdiet on the survival and growth performance of whiteleg shrimp postlarvae. Results showed a remarkable enhancement of whiteleg shrimp growth performance by using the co-feeding regime with a commercial inert microdiet and *Artemia* nauplii until the 19th day after metamorphosis (PL_{19}). Moreover, survival increased 9.4% when postlarvae were fed the co-feeding regime. Together, these results may indicate that the nutritional requirements of postlarvae were not met when fed with the commercial inert microdiet alone, decreasing whiteleg shrimp nutritional status and well-being. Additionally, lower nutritional status may also increase cannibalism as a way of compensating nutritional deficiencies.

The second trial aimed at evaluating the effect of three feeding frequencies (4, 6 and 8 times per day) on the survival, growth response and feed utilization of whiteleg shrimp juveniles. Results of the second growth trial showed higher growth performance of whiteleg shrimp fed 8 times per day, in comparison with those fed 4 and 6 times per day, with the juveniles fed 8 times per day exhibiting a final body weight 53% and 11% higher than those fed 4 or 6 times per day, respectively. Furthermore, feed efficiency in whiteleg shrimp fed 6 and 8 times per day was, respectively, 1.4 and 1.6-fold higher than that of whiteleg shrimp fed 4 times a day. Survival of juveniles also increased with the increase of the number of meals per day. In comparison with the feeding frequency of 4 times per day, survival increased circa 2% and 4% when whiteleg shrimp were fed 6 and 8 times per day, respectively. These results indicate that using a feeding frequency of 8 times per day instead of 4 or 6 times per day improves the growth performance of whiteleg shrimp juveniles, since a higher number of daily meals may reduce starvation periods and nutritional deficiencies, which in turn attenuates the occurrence of cannibalistic behaviors as an alternative way of obtaining food.

With respect to RiaSearch, the use of co-feeding regimes, combining *Artemia* nauplii and inert diets, during early developmental stages, and the supply of 8 meals per day constitute the best feed and feeding management to improve survival and growth of whiteleg shrimp *Penaeus vannamei*, while enhancing the company's profitability. Nevertheless, additional studies on whiteleg shrimp feeding protocols may be of particular relevance to RiaSearch. Extending the experimental period time of the first growth trial beyond the 24^{th} day after metamorphosis (PL_{24}) to ascertain if the growth of co-fed and non-co-fed postlarvae remain different; testing other commercial inert microdiets, including diets specifically produced for whiteleg shrimp; studying the effect of using other feeding frequencies, namely higher than 8 times per day; studying the effect of different photoperiods; or even evaluating the influence of the daily amount of feed provided to whiteleg shrimp on their growth response are examples of possible experiments to further optimize feeding strategy at RiaSearch, in order to enhance whiteleg shrimp growth performance.

Chapter 8: References

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