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TOWARDS AN EARLY WEANING IN SENEGALESE SOLE (*SOLEA SENEGALENSIS KAUP, 1858)*

Universidade do Algarve

Master in Aquaculture and Fisheries

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Abstract

This Thesis aimed to contribute towards an earlier weaning in Senegalese sole which currently starts at larvae 25 days after hatching (DAH) with good results. Two trials were conducted using different weaning strategies and microdiet formulations, targeting full weaning after larval settling (15 DAH). On the first trial, seven different treatments were tested: the control treatment was suddenly weaned at 25 DAH to a commercial diet, while the remaining six treatments were co-fed each a different microdiet and *Artemia* until full weaning at 23 DAH. Larvae achieved RGR values of around 12.4%.day^{-1}, FCR values around 1.0 and survival between 56 to 79%, which are great improvements to results obtained by other authors in Senegalese sole weaning studies. WinFlat and NCAP (WinFlat variant with mixture of encapsulated and non-encapsulated taurine instead of the microencapsulated version alone used in WinFlat) seem to be the most appropriate and cost effective microdiets used in this trial. The lowest growth performance was exhibited by the PH20 larvae that fed on a microdiet with increased inclusion levels of protein hydrolisates in relation to the commercial diet WinFlat. The control exhibited significantly inferior growth performances than all co-fed treatments, excluding PH20, and exhibited the lowest survival of all treatments. The results suggest that the use of a co-feeding regime is more appropriate when weaning larvae at such early stages of development. In the second trial, a total of seven different treatments were tested. All larvae were subject to a co-feeding regime, each with a different inert microdiet, until full weaning was performed at 19 DAH. Larvae achieved RGR values of around 11.1%.day^{-1}, FCR values averaging 4.5 and survival between 45 to 61%. The overall growth results are superior to those obtained in the first trial at an approximate larval age and such good results had never been reported before. Nevertheless, the lowest survivals observed may be related to the more aggressive weaning strategy employed. The use of a mixture of encapsulated and non-encapsulated taurine instead of a microencapsulated version alone was beneficial to the larvae growth performance. The inclusion of the microalga *Phaeodactylum tricornutum* broken cells in the microdiets at an inclusion level of 10% produced superior growth performances than the commercial diet WinFlat. The inclusion of dietary tryptophan in WinFlat may enhance larvae growth performances. The use of a moist WinFlat variant, diluted in a 1:5 ratio, caused the larvae to exhibit inferior growth performances than all remaining treatments, probably due to insufficient nutrient intake. In conclusion, results from both

trials conducted during this Thesis support that the age at which Senegalese sole currently starts to be weaned can be reduced from 25/30 DAH to 15 DAH. Nevertheless, results also suggest that improvements can still be made in weaning microdiets formulations and weaning strategies.

Keywords: Senegalese sole, *Solea senegalensis*, early weaning, inert microdiets, co-feeding

Resumo

À medida que a população mundial continua a aumentar, a necessidade de fontes de alimento aumenta também. As pescas são a principal fonte de produtos aquáticos mas a produção tem estagnado. A aquacultura é vista como uma potencial alternativa e tem sofrido uma grande expansão nos últimos anos. O sector europeu de aquacultura também se expandiu mas produz poucas espécies de peixe. O salmão do Atlântico (*Salmo salar* Linnaeus, 1758), o robalo (*Dicentrarchus labrax* Linnaeus, 1758) e a dourada (*Sparus aurata* Linnaeus, 1758) são as espécies mais produzidas e enfrentam uma tendência para saturação de mercado. Tal como a maioria dos países europeus, Portugal também produz um número de espécies reduzido. A necessidade de diversificar a produção levou a que esforços estejam a ser feitos para encontrar novas espécies que possam aumentar a diversidade das espécies produzidas. Entre estas espécies, o linguado (*Solea senegalensis* Kaup, 1858) é visto como um dos principais candidatos para a diversificação da indústria de aquacultura. Esta espécie apresenta um valor comercial elevado e as suas capturas em mar estão a diminuir. Esforços de investigação nos últimos anos levaram a que a produção atingisse sucesso comercial nos países do Sul da Europa. Muitos avanços foram feitos em elementos chave da produção, tais como regime de temperatura, fotoperíodos e densidades nos tanques. No entanto, muitos esforços de investigação ainda se focam nas primeiras fases de desenvolvimento das larvas, já que a condição nutricional destas pode ter efeitos na qualidade dos juvenis. Alimentos vivos são usualmente usados nas primeiras fases de desenvolvimento mas estes podem trazer desvantagens, tais como, actuarem como vectores de bactérias patogénicas, terem valores nutricionais variáveis e a sua produção ser complicada e dispendiosa. Consequentemente, esforços têm sido feitos visando o desenvolvimento de microdietas inertes que permitam a total substituição dos alimentos vivos. O desmame, a transição dos alimentos vivos para as dietas inertes, é uma fase muito importante na produção. O período do desmame no linguado era considerado problemático até ao final dos anos 1990 devido a mortalidades elevadas e taxas de crescimento baixas. Apesar de os resultados ainda serem variáveis, os desenvolvimentos que ocorreram nas condições zootécnicas na fase pelágica e na qualidade das microdietas inertes permitem às larvas chegar ao desmame num estado nutricional bastante mais elevado, o que tem permitido reduzir progressivamente a importância dos alimentos vivos nos protocolos de desmame no Linguado. Esta Tese teve como

objectivo contribuir para um desmame mais precoce no Linguado, que neste momento se inicia aos 25 dias após eclosão (DAE) das larvas com bons resultados. Duas experiências foram realizadas usando diferentes estratégias de desmame e formulações de microdietas, focadas no desmame total das larvas após estas se tornarem organismos bentónicos (cerca dos 15 DAE). Na primeira experiência, sete tratamentos diferentes foram testados: no tratamento controlo, a transição para alimento inerte foi feita abruptamente aos 25 DAE usando uma dieta comercial, enquanto os restantes seis tratamentos foram co-alimentados com uma microdieta (diferente para cada tratamento) e *Artemia* até desmame total aos 23 DAE. As larvas atingiram valores de RGR (taxa de crescimento relativo) à volta dos 12.4%.dia^{-1}, valores de FCR (taxa de conversão alimentar) à volta de 1.0 e sobrevivências entre 56 e 79%, resultados que são grandes melhorias em relação aos obtidos por outros autores em estudos de desmame do linguado. As dietas usadas nesta experiência mais apropriadas e com um melhor custo-beneficio foram a dieta comercial WinFlat e a NCAP (variante de WinFlat com mistura de taurina encapsulada e não encapsulada, ao invés de apenas taurina microencapsulada usada no WinFlat). A performance de crescimento mais baixa foi exibida pelo tratamento PH20 que foi alimentado com uma microdieta com níveis de hidrolisados proteicos mais elevados dos que os que são encontrados na dieta comercial WinFlat. O controlo exibiu performances de crescimento significativamente inferiores aos tratamentos que foram co-alimentados, excluindo o PH20, e exibiram a sobrevivência mais baixa de todos os tratamentos. Os resultados obtidos sugerem que o uso de um regime de co-alimentação é mais apropriado quando se efectuam desmames em etapas de desenvolvimento das larvas tão precoces. Na segunda experiência, um total de sete tratamentos diferentes foram testados. Todas as larvas foram co-alimentadas, cada com uma microdieta diferente, até ao desmame total efectuado aos 19 DAE. As larvas atingiram valores de RGR à volta dos 11.1%.dia^{-1}; valores de FCR à volta de 4.5 e sobrevivências entre 45 to 61%. Os resultados de crescimento foram superiores aos obtidos na primeira experiência em idades de desenvolvimento larval aproximadas. Nunca foram reportados resultados de crescimento similares. Os valores de sobrevivência foram, no entanto, mais baixos, o que pode estar relacionado com o uso de uma estratégia de desmame mais agressiva. O uso de uma mistura de taurina encapsulada e não encapsulada, ao invés da versão apenas com taurina microencapsulada foi benéfico para a performance de crescimento das larvas. A inclusão de células da microalga *Phaeodactylum tricornutum* com a parede celular

destruída em níveis de inclusão de 10% na microdieta produziu performances de crescimento superiores que a dieta comercial WinFlat. A inclusão de triptofano na microdieta WinFlat pode ter tido efeitos positivos na performance de crescimento das larvas. O uso de uma variante húmida de WinFlat, diluída numa proporção de 1:5, produziu performances de crescimento das larvas inferiores às observadas nos restantes tratamentos, provavelmente devido a absorções de nutrientes insuficientes. Em conclusão, os resultados das duas experiências realizadas durante esta Tese suportam a possibilidade de reduzir a idade de início de transição para alimentos inertes no linguado de 25/30 DAE para 15 DAE. No entanto, os resultados também sugerem que ainda podem ser realizadas melhorias nas formulações das microdietas de desmame e nas estratégias de desmame.

Palavras-chave: Linguado, *Solea senegalensis*, desmame precoce, microdietas inertes, co-alimentação

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AA: Amino acid

DAH: Days after hatching

DGRM: Direcção Geral de Recursos Naturais, Segurança e Serviços Marítimos

DW: Dry weight

FAA: Free amino acid

FAO: Food and Agriculture Organization of the United Nations

FCR: Feed conversion ratio

FEAP: Federation of European Aquaculture Producers

FIFO: Fish in fish out ratio

HUFA: Highly unsaturated fatty acids

INE: Instituto Nacional de Estatística

RGR: Relative growth rate

Tau: Taurine

TL: Total length

Trp: Tryptophan

1. Introduction

1.1. Aquaculture industry overview

As the world population continues to expand the demand for food sources is increasing as well. Regarding aquatic food products, fisheries captures played a big role in meeting these demands for a long time but the production has stagnated. This stagnation is occurring both due to the depletion of wild stocks and an increasing ecological awareness by governments all around the world, which are forced to legally restrict catches. Given this fact, the emergence of an alternative that can complement fisheries in the production of aquatic food urges. Aquaculture is perceived as having the potential to fulfill this role, currently being the fastest growing animal food production sector (FAO, 2016).

According to FAO (2016), in 2014, the world aquaculture animal production continued to grow and reached a total of 73.8 million tonnes, which accounted for 44.1 percent of the world total fish production. Almost all fish produced in aquaculture is destined for human consumption, although by-products may be used for non-food purposes, such as the production of fish oil and fish meal. Nevertheless, the development in the aquaculture industry has been extremely important in the supply of fish for human consumption, and for the first time in 2014, it supplied more fish than capture fisheries.

Aquaculture production still has a highly uneven distribution and unbalanced development status. Asia countries are still the major producers, responsible for 88.9 percent of the world's total production (FAO, 2016). Despite the extensive expansion over the last decades, the European aquaculture production shares have been gradually declining over the last few years and accounted for 3.9 percent of the global production in 2014 (FAO, 2016). European producers have to compete with producers from countries in which the production costs are lower due to greater production scales, lower labor costs, but also lower quality standards (DGRM, 2014). Nevertheless, Norway still is one of the world's biggest producers, having produced 1.37 million tonnes in 2014, which represents 60.9 percent of the total European aquaculture production (FEAP, 2015; FAO, 2016). Despite having a small global representation, the European aquaculture industry is characterized by state of the art technology and feed production knowledge (DGRM, 2014). The European aquaculture sector has grown but it is still producing few species. The Atlantic salmon (*Salmo salar* Linnaeus, 1758), the European seabass (*Dicentrarchus labrax* Linnaeus, 1758) and gilthead seabream (*Sparus aurata* Linnaeus, 1758) are the most produced ones and are facing market

saturation. Therefore efforts are being made to find new species that can improve diversity and ensure sustainable industry development (DIVERSIFY, 2016). Some emerging species in European aquaculture are the Senegalese sole (*Solea senegalensis* Kaup, 1858) (Morais et al., 2016), meagre (*Argyrosomus regius* Asso, 1801), the greater amberjack (*Seriola dumerili* A. Risso, 1810), the wreckfish (*Polyprion americanus* Bloch & Schneider, 1801), the atlantic halibut (*Hippoglossus hippoglossus* Linnaeus, 1758) the grey mullet (*Mugil cephalus* Linnaeus, 1758) and the pikeperch (*Sander lucioperca* Linnaeus, 1758) (DIVERSIFY, 2016).

1.2. Aquaculture in Portugal

Portugal has always been a country deeply connected to the sea. According to FAOSTAT, Portugal is the first country in the European Union and fourth country in the world with highest fish consumption per capita, reaching 57 kg per year. Due to its geographic location and its extensive coastline, Portugal has a wide range of habitats, being considered a rich area in biological terms, which gives fisheries and aquaculture activities great potential (DGRM, 2014). However, fisheries production is facing growing difficulties to meet the market demands, while aquaculture production is still small and undiversified to have a significant supplier role (DGRM, 2014).

Marine aquaculture in Portugal started as an added value to the traditional salt industry, producing fish on small scale extensive systems. After joining the European Union in 1986, aquaculture industry suffered great developments as a result of the funding received. Most of those production systems evolved to semi-intensive and intensive systems. It was not only until the 1990's that aquaculture had its biggest growth. Still, and although it is possible to see nowadays a few intensive fish farms in the coastline, the Portuguese aquaculture sector is still mainly composed by small and family-based businesses (DGRM, 2014).

Portuguese aquaculture produced a total of 10.8 thousand tonnes of fish in 2014, which is only around 8% of the total production, while wild fish caught represent around 92% (INE, 2016). Only five fish species are currently being produced in a significant manner: turbot (*Psetta maxima* Linnaeus, 1758; 2.7 thousand tonnes), gilthead seabream (1.5 thousand tonnes), rainbow trout (*Oncorhynchus mykiss* Walbaum, 1792; 1 thousand tonnes), European seabass (500 tonnes), and Senegalese sole (60 tonnes) (FEAP, 2015). The remaining production is extensive production of clams and oysters.

1.3. Senegalese sole

Senegalese sole (*Solea senegalensis* Kaup, 1858) is a flatfish from the Soleidae family that inhabits sandy or muddy bottoms throughout the Atlantic and Mediterranean, from the Golf of Biscay to Senegal coasts, which feeds mainly on benthonic invertebrates, such as polychaets and bivalves (Whitehead, 1986). It is a gonochoric species that matures after 3 years of age, at around a total length of 32 cm (Dinis et al., 1999). The spawning season occurs naturally during Spring, from March to April/May and in September, a second shorter period may occur. In farming conditions, eggs may be obtained from natural spawns of wild broodstocks kept in captivity (Dinis et al., 1999) although induced spawning by manipulation of photoperiod and water temperature has been successfully achieved, leading farms to control Senegalese sole spawning according to production requirements (Wilson Pinto, personal communication). Temperature plays a big role in the spawning season because egg emission stops below 16ºC (Dinis et al., 1999). The eggs start to hatch after 36 to 48 hours, depending on the water temperature, which can vary between 16 and 18ºC (Dinis et al., 1999; Engrola, 2008). The newly hatched larvae are pelagic and have bilateral symmetry, measuring around 2.4 mm. Exogenous feeding starts at 2 DAH (days after hatching) when the larvae measure around 3.4 mm (Dinis et al., 1999). Although Senegalese sole larvae can feed upon *Artemia* nauplii at first feeding, enriched rotifers are usually the first prey for a few days (Dinis et al., 1999; Imsland et al., 2003; Conceição et al., 2007). The larvae undergo an accentuated metamorphosis climax which usually starts at 11 DAH and is completed by 19 DAH (Dinis et al., 1999). This metamorphosis is characterized by a 90º rotation of the body, a migration of the left eye and a reorganization of the internal organs, resulting in a benthonic postlarva (Ribeiro et al., 1999a; Fernández-Díaz et al., 2001).

Senegalese sole has been receiving a lot of attention lately and is a prime candidate to have a role in the diversification of the European aquaculture industry, particularly in Spain and Portugal. It has a high market value and demand due to its flesh quality and decreasing number of wild catches (Imsland et al., 2003; Makridis et al., 2009; Dâmaso-Rodrigues et al., 2010; Morais et al., 2016). Research efforts over the last years led Senegalese sole production to reach commercial success in southern Europe. The expansion signs in the species culture have been so important that it attracted substantial investment to intensify production. Many advances were made in key elements of production, such as temperature regime, photoperiod and stocking

densities. It was not long ago that Senegalese sole production was made in salt marshes as an added value product to semi-intensive polycultures of seabream and seabass. These fish would rely on the natural productivity of the ponds and, consequently, production was very variable. Nowadays, culture is made in more intensive systems with much more controlled environments and using commercial feeds. In 2015, Spain was the main producer of Senegalese sole, with 664 tonnes, followed by France, Portugal and Iceland which produced around 330 tonnes, 180 and 100 tonnes, respectively (APROMAR, 2016). Some new farms are now using a recirculation aquaculture system which has been an important technological development to the production of the species. This type of system enables a better control of the environmental conditions which often reflects in increased growths and survival (Morais et al., 2016).

1.4. Senegalese sole weaning

With culture protocols for Senegalese sole juveniles established and relatively standardized, research is mainly focused in the first life stages, since nutritional conditions of larvae may have strong effects on the quality of juveniles (Morais et al., 2016). Live feeds are usually given in the first live stages but they can have disadvantages such as acting as vectors of pathogenic bacteria, having variable nutritional values, as well as, being expensive, labour-intensive and complicated to culture (Ribeiro et al., 2005; Hamre et al., 2013). Consequently, a lot of effort has been employed in the development of microdiets (inert diets with small granulometry) that can fully replace live feeds in fish first developmental stages (Cañavate and Fernández-Díaz, 1999; Ribeiro et al., 2005; Engrola et al., 2008; Pinto et al., 2010).

Weaning, the transition from live feeds to an inert diet, is an extremely important phase in production. The early introduction of inert diets without live feeds may have negative effects on larvae survival and growth (Robin and Vincent, 2003; Fletcher et al., 2007; Parma et al., 2013), which may be caused by low ingestion rates, poor nutrient digestion and absorption (Fletcher et al., 2007). Although diets that can replace live feeds are currently widely used in fish larviculture and promising results have been obtained in weaning experiments in many species (Hamre et al., 2013), little is known about the nutritional requirements of marine fish larvae (Holt, 2011) (see section 1.5). Lately, research efforts led to the development of weaning protocols and to improvements on the formulated microdiets quality. Microdiets are designed focusing

on several objectives depending on the weaning strategy, which normally is to partially or completely replace live feeds from first feeding or at a given young age. These microdiets need to have several structural and biochemical characteristics to create a compromise between stability in the water and nutrient digestibility. First, these microdiets need to meet the energetic and nutritional requirements for larval growth and development. Secondly, the feed particles must be stable enough to avoid disintegration after immersion in water and feed formulation must ensure the retention of water soluble micronutrients. Leaching of these micronutrients is common in microdiets due to a high surface/volume ratio. Lastly, the particles need to have the right diameter so the larvae can identify them as feed and ingest them. Also, they have to be digestible by the larval digestive system since most marine fish hatch with an immature digestive system (Hamre et al., 2013). Senegalese sole lacks of a functional stomach at first feeding which implies a high dependence from the pancreatic enzymes, like trypsin, lipase and amylase, for digestion (Ribeiro et al., 1999a). Acid digestion is only achieved when the gastric glands become functional several weeks after first feeding (Ribeiro et al., 1999b). Nevertheless, pancreatic digestive enzyme activity may not be enough for a complete feed hydrolisys. Since dietary protein have a primary relevance, inclusions of low to medium levels of protein hydrolisates, which are pre-digested proteins mostly containing low molecular weight peptides, in fish larvae weaning diets have been shown to improve survival and growth. In European seabasss and cod (*Gadus morhua*), the inclusion of protein hydrolisates has been shown to improve survival (Cahu et al., 1999; Kvåle et al., 2009) and both survival and growth in common carp (*Cyprinus carpio*) (Carvalho et al., 2004). It has been proposed that inert diets rich in protein hydrolisates may be used in earlier stages of larvae development, and can have a positive effect on the maturation of the digestive tract of Senegalese sole larvae when used in a co-feeding regime (Engrola et al., 2009a). According to Canada (2016), optimal protein quality seems to change during Senegalese sole larval development: Inclusion of moderately hydrolyzed protein improves growth in early larval stages, while larger peptides and intact protein seem to be more suitable to sole post-larvae and young juveniles. Although not built into protein, taurine is part of the free amino acid (FAA) pool and is used for cell volume regulation and bile salt synthesis, among other functions (Hamre et al., 2013). In Senegalese sole, higher taurine levels lead to increased retention of protein in the larval body and accelerates metamorphosis completion (Pinto et al., 2010).

The weaning period in Senegalese sole larvae was historically problematic due to high mortalities and low growth rates and was considered one of the major bottlenecks in the species farming until the late 1990's (Dinis et al., 1999; Conceição et al., 2007). Although the weaning results are still variable, the developments made in zootechnical conditions and inert microdiets quality allow the larvae to reach the weaning phase in a much better nutritional state and have allowed to progressively reduce the importance of live feeds in Senegalese sole weaning protocols (Pinto et al., 2015).

Currently, two different weaning strategies are used: sudden weaning and co-feeding with *Artemia* until full weaning. The choice of feeding strategy to adopt should be based on post-larvae weight since it is a better indicator of larval nutritional status than age (Engrola et al., 2007). In pelagic or small post-larvae below 1 mg of dry weight, a co-feeding regime with low *Artemia* replacement seems to present better results and improve post-larval quality (Engrola et al., 2009a; Engrola et al., 2009b). Additionally, co-feeding with live feeds and inert diets may condition the larvae to more readily accept the manufactured diets when live feed is removed (Cañavate and Fernández-Díaz, 1999). However, in larvae with a dry weight between 5 and 10 mg sudden weaning should be performed (Engrola et al., 2007). Nevertheless, it is paramount the larvae are in very good nutritional condition prior to sudden weaning (Pinto et al., 2015). It is the strategy that further progressed during the last decades, with the age of sudden weaning advancing over the years. In the 1990's, weaning was done at 60 DAH, while in 2010, weaning at 40 DAH was achieved. Nowadays, it is possible to achieve survivals close to 100% and growth around 10% per day by weaning between 25 and 30 DAH. While until 2010, Senegalese sole reached mean wet weights of 1g at 90 DAH in best case scenarios, nowadays, it is possible to obtain that at 70 DAH (Pinto et al., 2015).

1.5. Larval nutritional requirements and feed intake

The larvae nutritional requirements may differ from those of juveniles or adult fish, since fish undergo extreme morphological and physiological changes during ontogenesis (Hamre et al., 2013). Current knowledge of nutrition in early stages is based on experimental laboratory studies carried out using artificial conditions based on limited prey types and relatively constant abiotic and biotic conditions (Hamre et al., 2013). Nutritional requirement studies are few, mainly due to the lack of appropriate

diets that can be used for the purpose. Nutrient concentrations in live feeds may be difficult to control and formulated feeds have technical limitations, such as high leaching rates and low digestibility. Additionally, marine fish larvae are extremely vulnerable in the first stages of development and require specific conditions to survive, develop and grow properly (Hamre et al., 2013). This lack of knowledge in fish larval nutritional requirements is one of the main causes for high mortalities and quality problems commonly observed in marine larviculture (Conceição et al., 2010). Research efforts focused on overcoming this knowledge gap contributed to the appearance of several methods for measuring larval nutrient requirements and have been reviewed in detail by Hamre et al. (2013). Alongside understanding those requirements, knowledge on feeding behavior and the factors that modulate feed detection, acquisition and processing is also extremely important for the development of diets and feeding protocols in larviculture (Rønnestad et al., 2013). Feed intake greatly impacts larval growth and development, as it determines the amount of nutrients the larvae can use for the high structural and energy demands of rapid growth and organogenesis (Hamre et al., 2013). Methods that determine feed intake in fish can be used to infer important aspects regarding diet adequacy, namely, palatability and nutritional value. Nevertheless, most developed methods are essentially used in juvenile fish studies and their application to fish larvae may not be appropriate or even possible due to biological and technical constrains (Conceição et al., 2007; Bonacic et al., 2016). Therefore, the development of a method that would reliably determine feed intake in fish larvae would be an extremely important tool to evaluate the adequacy of ingredient formulations on fish larval diets, which would ultimately aid in the transition from live feeds to inert diets.

1.6. Objectives

This Thesis aims to contribute towards an earlier weaning in Senegalese sole, which may currently be started at larvae 25 DAH with good results. For this purpose, two trials were conducted using different weaning strategies and microdiet formulations, targeting a partial or, if possible, full weaning immediately after larval settling (15 DAH). Commercial weaning diets for Senegalese sole and other species were tested, as well as new prototypes focusing on the improvement of fish performance. Several parameters were analyzed, focusing on larval growth, survival and feeding efficiency. The adequacy of the microdiets for Senegalese sole larvae during

settling was assessed using a feed intake method that has been developed by Sparos Lda.

2. Trial 1 – Effects of different weaning microdiets on the performance of newly settled Senegalese sole larvae

2.1. Materials and methods

2.1.1. Dietary treatments

In trial 1 a total of seven different dietary treatments were tested: Control, NCAP, Flat, Plus, Fast, nPea and PH20. These were randomly distributed by the rearing tanks used in triplicates (Control, NCAP, nPea and PH20) and quadruplicates (Flat, Plus and Fast). The inert microdiets used were Sparos Lda commercial diets WinFlat, a cost-effective microdiet for flatfish; WinFlat^{plus}, a premium microdiet for flatfish; WinFast, formulated for fast growing species such as meagre, cobia (*Rachycentron canadum* Linnaeus, 1766) and the greater amberjack; and three WinFlat experimental variants. The WinFlat variants used differed in the ingredient formulation but had similar proximal compositions to WinFlat (Table 1). In NCAP, a mixture of encapsulated and non-encapsulated taurine was used instead of the microencapsulated version alone used in WinFlat; in nPea, the pea protein concentrate was substituted by other sources of protein of marine and vegetable origin; and in PH20, the amount of protein hydrolisates used was 20%, higher than what is found in WinFlat (10 to 15%).

Table 1 – Proximal nutritional composition of the diets used in Trial 1.

	WinFlat / WinFlat variants	**WinFlat^{plus}**	**WinFast**
Crude protein (%)	62	62	63
Crude fat (%)	18	15	17
Crude ash (%)	9	11	12
Fiber (%)	0.5	0.5	0.3
Tau (%)	1	1	1
Phosphorus (%)	1.9	2.5	2.1
Calcium (%)	1.3	1.3	1.5
Sodium (%)	0.5	0.5	0.5

For each diet, a fluorescent labeled version was also produced to be used for feed intake analysis. In these variants, an hydrophobic fluorescent dye was added at a 1% concentration to the diet composition, following procedures by Pereira (2016). This feed intake method developed by Sparos Lda allowed to evaluate inert microdiet acceptance by calculating the ingestion of each inert microdiet at various developmental stages. All diets were produced at Sparos Lda facilities (Olhão, Portugal) using extrusion at low temperatures as main production process, as follows: powder ingredient mixing according to target formulation using a double helix mixer; grinding

in a micropulverizer hammer mill (SH1, Hosokawa-Alpine, Germany); addition of the oil fraction; humidification and agglomeration through low temperature extrusion (Dominioni Group, Italy); drying of resultant pellets in a convection oven (OP 750-UF, LTE Scientifics, United Kingdom) for 4 h at 60ºC, crumbling (Neuero Farm, Germany) and sieving to desired size ranges.

Two different weaning strategies were used: sudden weaning for the control and co-feeding for the remaining treatments. Larvae from the control were fed exclusively frozen enriched *Artemia* until they were suddenly weaned to inert microdiets at 25 DAH, following the procedures used by Pereira (2016). Larvae from the remaining treatments were initially co-fed frozen enriched *Artemia* and inert microdiets in a 20% to 80% respective proportion and were fully weaned at 23 DAH. Full weaning was performed as soon as visual inspection of the larval guts confirmed an adaption to the inert microdiet. The inert diets used for each treatment and the period in which they were used are shown in Figure 1. The inert diet granulometry used changed according to fish age as follows: 16 to 24 DAH, 200-400 µm; 25 to 54 DAH, 400-600 µm; 55 to 60 DAH, 600-800 µm.

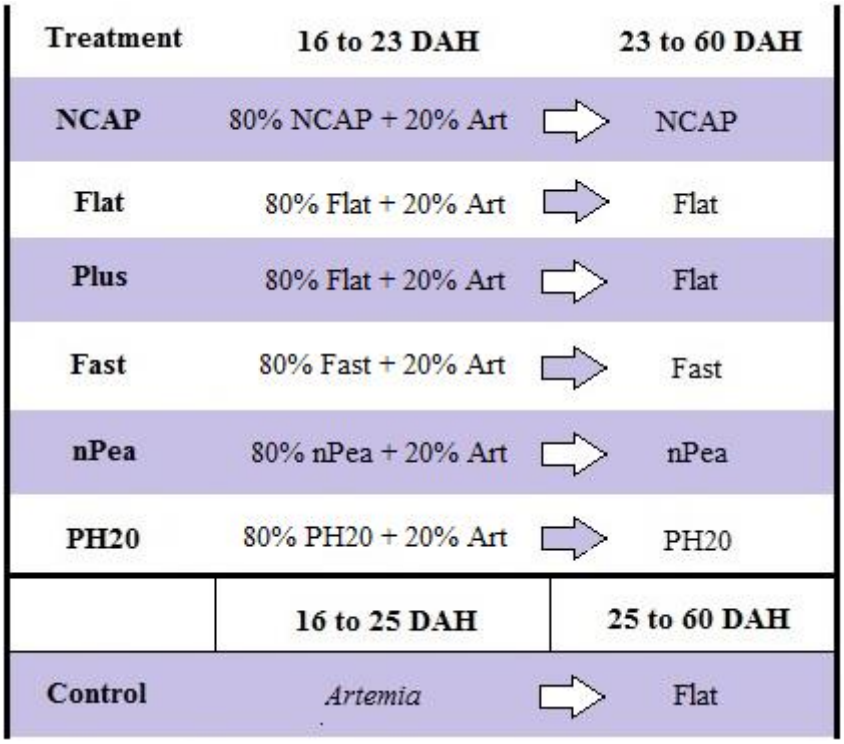

Figure 1 – Experimental design used in Trial 1. During the co-feeding period (16 to 23 DAH) Art (frozen enriched *Artemia*) and inert microdiets were supplied in a 20% to 80% proportion. Flat (WinFlat microdiet), Plus (WinFlat^{plus} microdiet) and Fast (WinFast microdiet) – commercial diets by Sparos Lda; NCAP, nPea and PH20 – experimental variants of WinFlat.

2.1.2. Fish rearing

Trial 1 was conducted at Sparos Lda research facilities (Olhão, Portugal) and had a duration of 44 days, where Senegalese sole larvae, originated from SEA8's hatchery Safiestela (Póvoa de Varzim, Portugal), were reared from 16 DAH to 60 DAH. Upon arrival, the larvae were randomly distributed in 24 tanks with 8L each previously prepared with clean seawater and aeration. Larval density was kept in each tank at 2650 larvae/m^2. These were kept under a natural daily photoperiod, but light intensity was kept to a minimum to promote feed ingestion (Navarro et al., 2009). The tanks were part of a semi-open recirculating system. The amount of external seawater input in the system was kept at a total of 3.2 system water volume renewals per day. Water renewal in each tank was kept at 4 renewals per hour. The system and tanks water renewals were adjusted when needed according to the amount of nitrogen compounds found in the water, in order to keep them to a minimum. The water parameters were measured and recorded daily using commercial probes. Temperature was maintained at 20.1 ± 0.4ºC, dissolved oxygen concentration at 94.4 ± 3%, salinity at 35g/L and nitrogen compounds ideally below 0.1 mg/L. Tanks were cleaned every day using a sponge to clean the walls and filters and a water siphon to remove waste.

During the co-feeding period (16 to 23 DAH), the daily routines were as presented in Table 2. The distribution of inert microdiets by the tanks was done by hand in this period since the quantities were too small to be able to be efficiently performed by an automatic feeder. The inert microdiets were offered in 4 meals, each consisting of 25% of the total daily amount (Annex 1). The *Artemia* was also offered in 4 meals but in different proportions in each meal (Table 2). For the control group the routines were the same but the first meal of *Artemia* was offered at 9.30h and no inert diet was offered. The estimated total daily amounts of *Artemia* offered to the control in this period can be seen in Annex 1. After weaning, the daily routines were as presented in Table 3. At this point, the amount of microdiets provided (Annex 2) allowed the use of the automatic feeders, which were set to supply 8 meals in a 24 hour period. Meals had a duration of 3 hours, in which the feeder continuously supplied feed for 2 hours and stopped for 1 hour before the next meal. Feeders were checked and cleaned every day to ensure proper functioning. Undischarged feed was quantified and registered. Larvae from all groups were fed *ad libitum* and it was made sure that feed was always present in the tanks but in amounts that would not deteriorate the water quality. The amounts of

microdiet given daily were adjusted according to the amount of feed remnants from the day before.

Table 2 – Daily routines used during the co-feeding period (16 to 23 DAH) in Trial 1.

Hours	Daily Routine
9:00h	Behavior observation, mortality check and removal of dead fish, registry of feed remnants from day before
9:15h	Cleaning of major feed waste
9:30h	Feeding – Diet (25 % of total amount)
10:00h	Measure of environmental parameters
10:30h	Feeding – *Artemia* (30 % of total amount)
11:00h	Filter cleaning
12:00h	Feeding – *Artemia* (10 % of total amount)
14:00h	Feeding – Diet (25 % of total amount)
15:00h	Feeding – *Artemia* (10 % of total amount)
15:30h	Sump cleaning
16:00h	Tank cleaning
17:00h	Feeding – Diet (25 % of total amount)
17:15h	Weighing of diet for the following day
18:00h	Feeding – *Artemia* (50 % of total amount)
23:00h	Feeding – Diet (25 % of total amount)

Table 3 - Daily routines used after weaning was performed (23 to 60 DAH) in Trial 1.

Hours	Daily Routine
9:00h	Behavior observation, mortality check and removal of dead fish, registry of feed remnants from day before
9:15h	Measure of environmental parameters
10:00h	Thorough tank cleaning
11:00h	Weighing of diet
11:30h	Feeder cleaning/quantification of remnants in feeder
12:00h	Feeder charging for 24 hours
14:00h	Filter cleaning
15:00h	Sump cleaning

2.1.3. Sampling

2.1.3.1. Growth performance, survival and feeding efficiency

At the start of the experiment (16 DAH) 125 Senegalese sole larvae were sampled from the initial pool of larvae that was distributed to the tanks and at 23, 50 and 60 DAH, 50 larvae were sampled from each tank for dry weight (DW) and total length (TL) determination. To determine TL all larvae were photographed and measured using AxioVision Microscopy software. These were then freeze dried and weighted on a digital scale. Only larvae from the two upper quartiles in each tank were considered for DW, TL and RGR analysis to simulate the sorting process that the larvae typically go through in commercial hatcheries, which eliminates the smallest individuals. Feed conversion ratio (FCR) and relative growth rate (RGR) for each treatment were assessed from 16 DAH to 60 DAH. For the control treatment, FCR was calculated for the period in which inert diets were offered (25 to 60 DAH). Condition factor (K) and survival were determined at the end of the experiment. In all samplings, larvae were harvested randomly to a mesh sieve and then euthanized with Phenoxyethanol. Larvae were then washed in distilled water to remove residual feed and salt, collected into white sheets and stored at -20ºC until analysis. Prior to harvesting, it was made sure that larvae were not feeding for at least 8 hours so at the time of sampling the amount of feed in their digestive tract would be minimal and would not affect weight analysis.

2.1.3.2. Feed intake

Larvae were sampled for feed intake analysis at 19, 25 and 40 DAH. All automatic feeders were removed and all tanks were cleaned, making sure to remove all feed remnants. The equivalent to one meal of unlabeled feed on the automatic feeder was supplied and the larvae were allowed to feed for one hour. After this period, the tank was cleaned and the uneaten feed was removed. Another meal of the respective labeled diet was then supplied and the larvae were allowed to feed for one hour. Afterwards, 20 larvae from each tank were then harvested, euthanized, thoroughly washed and stored at -20ºC as described in section 2.1.3.1. The larvae were photographed and TL measurements were made using AxioVision Microscopy software. These were then freeze dried, weighed and stored in individual eppendorfs.

To determine feed intake, 1 ml of solvent (Ethyl Acetate) was added to each eppendorf. The larvae were homogenized at 20000 rpm using an ultraturrax with a S10N-5G dispersion tool (Ultra Turrax T10, IKA, Staufen, Germany). The homogenate

was then centrifuged at 10000 x g for 1 minute at room temperature (ScanSpeed 1236R, Labogene, Denmark). The resultant supernatants were applied to 96 well polypropylene microplates. Stock solutions using the feed used in each treatment were made so calibration curves could be established for each microdiet. These stock solutions were homogenized, centrifuged and their supernatants applied to the plates using the same procedure used for the larvae. The fluorescence of each well was then measured at a specific emission and excitation wavelength, on a wavelength reader Synergy ™ HT (BioTek Instruments, USA). The fluorescence of each larva was then compared to the calibration curve of their respective diet in order to determine the amount of feed ingested.

2.1.4. Data analysis

Feed conversion ratio (FCR) was calculated as: FCR = (Fi / Ww), where Fi corresponds to feed intake (g) and Ww to the mean wet weight gain (g). Relative growth rate (RGR, % day^{-1}) was calculated as: RGR = $(e^{g} - 1)$ x 100, where $g = (\ln Wt - \ln W0)$ x t^{-1}. Wt and $W0$ correspond to the final and initial dry weights, respectively, at a chosen period t. Condition factor (K) was calculated as: $K = 100 \times (W / L^3)$, where W is the wet weight and L is the total length. Survival was expressed as percentage and calculated as: $S = (Lf / Li) \times 100$, where Li and Lf correspond to the initial and final number of larvae in the tanks, respectively. The amount of sampled larvae was taken in consideration. Differences in growth performance and survival between dietary treatments were evaluated using a One-way ANOVA, followed by a Tukey multiple comparison test. When the one-way ANOVA assumptions were not complied, Krukall-Wallis tests were used, followed by Mann-Whitney tests. Differences in the percentage of larvae that fed on the offered labeled diets between treatments were assessed using chi-square tests. Results were expressed as means ± standard deviation (SD). In results expressed as percentage, an arcsine transformation was performed prior to any statistical test: $T = ASIN(SQRT(value / 100))$. The significance level considered was $p<0.05$ for all tests performed. All statistical analyses were performed in IBM SPSS Statistics 24 software. The amount of labeled feed ingested by the larvae in relation to their bodyweight was represented in notched boxplots and differences between treatments were evaluated by visually comparing the notches which display confidence intervals around the medians. Non-overlapping intervals imply a median difference with a significance level of 95% (Chambers et al., 1983).

2.2. Results

2.2.1. Growth performance, survival and feeding efficiency

The Senegalese sole larvae DW values obtained throughout Trial 1 are shown in Table 4 and represented in Figure 2. At 23 DAH, with the exception of treatment NCAP and Flat, all other co-fed groups showed significantly higher DW values than the control. At this point, Flat had the lowest values of all co-fed treatments. In contrast, at 40 DAH, it had significantly superior DW values than the remaining treatments, while there were no observed differences between these. At 50 DAH, larvae from all treatments, excluding treatment PH20, showed significantly higher DW values than the control. Larvae from NCAP had significantly higher DW values than all treatments. At the end of the trial (60 DAH), it was possible to verify that NCAP larvae achieved the highest DW values which were significantly higher than the remaining treatments excluding Flat. Larvae from the co-fed treatments weighed significantly more than the control with the exception of PH20 which reached the lowest DW values observed.

Table 4 – Senegalese sole larvae DW (mg) values observed throughout the course of Trial 1.

Treatment	Control	NCAP	Flat	Plus	Fast	nPea	PH20
16 DAH				0.88 ± 0.1			
23 DAH	1.25 ± 0.2 ^{ab}	1.33 ± 0.2 ^{bc}	1.23 ± 0.3 ^{a}	1.38 ± 0.2 ^{d}	1.33 ± 0.2 ^{cd}	1.37 ± 0.2 ^{cd}	1.37 ± 0.2 ^{cd}
40 DAH	4.7 ± 3.2 ^{a}	6.5 ± 6.5 ^{a}	10.3 ± 6.4 ^{b}	7.2 ± 6.7 ^{a}	6.6 ± 6.4 ^{a}	6.7 ± 5.9 ^{a}	6.42 ± 5.3 ^{a}
50 DAH	36.8 ± 9.4 ^{a}	67.6 ± 16.8 ^{d}	50.1 ± 17.9 ^{b}	55.7 ± 11.5 ^{c}	49.2 ± 15.4 ^{b}	45.7 ± 15 ^{b}	41.9 ± 15.4 ^{a}
60 DAH	137.1 ± 36.5 ^{b}	184.5 ± 52 ^{d}	168.7 ± 36 ^{cd}	166.1 ± 27.2 ^{c}	167.4 ± 50 ^{c}	141.8 ± 41.2 ^{c}	117.5 ± 42.2 ^{a}

Values presented as mean ± standard deviation. At 40 DAH, n = 20 and at 23, 50 and 60 DAH, n = 50 observational units. Different superscript letters indicate statistical differences ($p<0.05$) between larvae from different treatments at the same age. Values refer to the two upper quartiles of weight distribution (excluding 40 DAH values due to the lower number of observational units).

The Senegalese sole larvae TL (mm) values obtained throughout Trial 1 are shown in Table 5. Results obtained followed similar trends to those observed in DW analysis. At the end of the trial (60 DAH), NCAP larvae achieved significantly higher TL values than all other treatments excluding Fast, whereas PH20 larvae achieved significantly lower values than all other treatments.

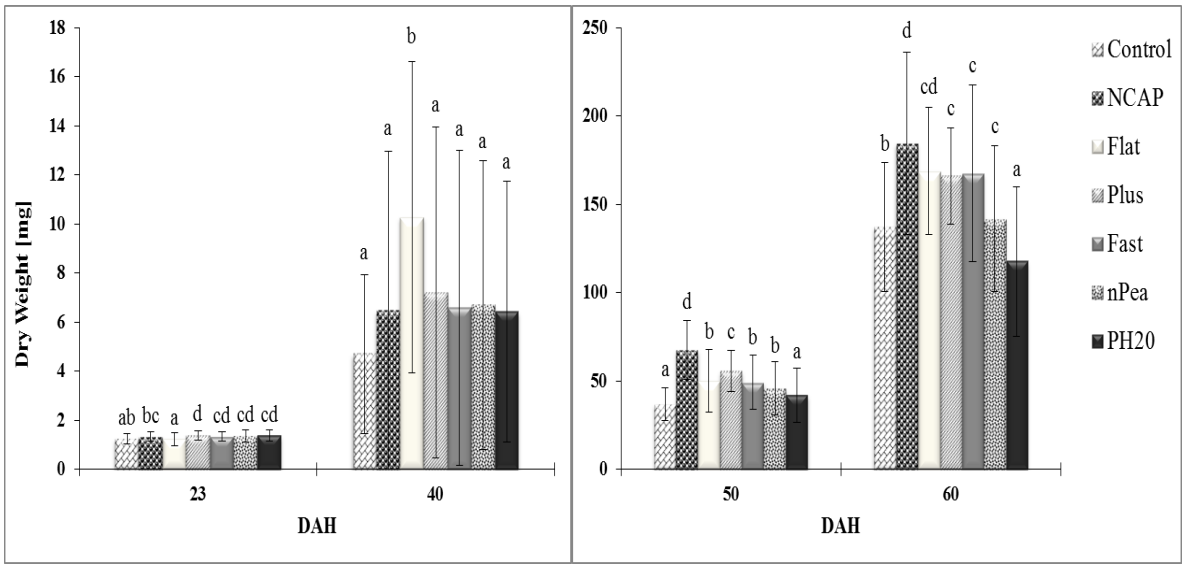

Figure 2 – Dry weight of Senegalese sole larvae reared under different dietary treatments. Values presented as mean ± standard deviation. At 40 DAH, n = 20 and at 23, 50 and 60 DAH, n = 50 observational units. Different superscript letters indicate statistical differences ($p<0.05$) between larvae from different treatments at the same age. Values refer to the two upper quartiles of weight distribution (excluding 40 DAH values due to the lower number of observational units).

Table 5 – Senegalese sole larvae TL (mm) values observed throughout the course of Trial 1.

Treatment	Control	NCAP	Flat	Plus	Fast	nPea	PH20
16 DAH				6.7 ± 0.4			
23 DAH	8.5 ± 0.4 ^{a}	8.7 ± 0.3 ^{bc}	8.6 ± 0.5 ^{ab}	8.9 ± 0.4 ^{e}	8.8 ± 0.4 ^{cd}	8.7 ± 0.5 ^{ab}	8.9 ± 0.5 ^{de}
40 DAH	13.3 ± 2.7 ^{ab}	13.9 ± 4.5 ^{ab}	16.6 ± 4.1 ^{c}	14.4 ± 4.5 ^{b}	13.1 ± 4.4 ^{a}	14.7 ± 4.8 ^{b}	13.7 ± 4.1 ^{ab}
50 DAH	27.3 ± 3.5 ^{a}	33.4 ± 2.5 ^{e}	29.7 ± 3.4 ^{bc}	31.1 ± 2.7 ^{d}	30.5 ± 3.6 ^{cd}	28.8 ± 3.2 ^{b}	27.4 ± 3.2 ^{a}
60 DAH	39.2 ± 3.1 ^{bc}	43.3 ± 3.6 ^{e}	40.7 ± 3 ^{cd}	41.1 ± 2.2 ^{d}	42.5 ± 3.7 ^{e}	39 ± 3.7 ^{b}	36.2 ± 4.2 ^{a}

Values presented as mean ± standard deviation. At 40 DAH, n = 20 and at 23, 50 and 60 DAH, n = 50 observational units. Different superscript letters indicate statistical differences ($p<0.05$) between larvae from different treatments at the same age. Values refer to the two upper quartiles of weight distribution (excluding 40 DAH values due to the lower number of observational units).

There were no significant differences observed between dietary treatments in FCR, RGR and K at larvae 40 DAH (Table 6). Table 7 shows the values obtained at the end of Trial 1 for FCR, RGR, K and survival. There were no significant differences observed between dietary treatments in FCR, RGR and K. The control exhibited a significantly lower survival than the remaining treatments, excluding treatments Plus and Fast.

Table 6 – Senegalese sole larvae FCR, RGR and K values obtained at larvae 40 DAH in Trial 1.

Treatment	Control	NCAP	Flat	Plus	Fast	nPea	PH20
FCR	10.6 ± 1.4	7.5 ± 1.3	5.6 ± 1.1	7.1 ± 0.7	9.8 ± 4	7.5 ± 2.3	8.9 ± 4.1
RGR (%DW day^{-1})	3.88 ± 0.3	4.61 ± 0.5	5.34 ± 0.3	4.90 ± 0.1	4.47 ± 1.1	4.69 ± 0.5	4.51 ± 1.0
K	0.75 ± 0.04	0.89 ± 0.10	0.83 ± 0.05	0.92 ± 0.10	1.03 ± 0.25	0.81 ± 0.32	0.90 ± 0.04

Results expressed as mean ± standard deviation (n = 20 observational units).

Table 7 – Senegalese sole larvae FCR, RGR, K and survival values obtained at the end of Trial 1.

Treatment	Control	NCAP	Flat	Plus	Fast	nPea	PH20
FCR	1.3 ± 0.5	0.94 ± 0.03	0.95 ± 0.2	1.05 ± 0.2	1.09 ± 0.2	1.07 ± 0.3	1.5 ± 0.5
RGR (%DW day^{-1})	12.1 ± 0.9	12.9 ± 0.3	12.7 ± 0.5	12.7 ± 0.3	12.6 ± 0.6	12.2 ± 0.5	11.7 ± 0.9
K	0.92 ± 0.07	0.92 ± 0.05	1 ± 0.03	1.01 ± 0.03	0.88 ± 0.07	0.98 ± 0.04	0.98 ± 0.04
Survival (%)	55.7 ± 4 ^{a}	73.9 ± 7.6 ^{b}	71.7 ± 2.5 ^{b}	61.1 ± 12 ^{ab}	66.1 ± 6.6 ^{ab}	78.7 ± 7.2 ^{b}	71.1 ± 4 ^{b}

Results expressed as mean ± standard deviation (n = 50 observational units). Different superscript letters indicate statistical differences ($p<0.05$) between larvae from different treatments. RGR values refer to the two upper quartiles of weight distribution.

2.2.2. Feed intake

Feed intake analysis results showed that at 19 DAH there were significant differences between treatments in the number of larvae that ingested the offered labeled diets (Figure 3). Flat treatment had close to 60% of larvae feeding on that particular meal which was significantly higher than the remaining treatments, excluding NCAP. In contrast, nPea had only close to 20% of ingestion which was significantly lower than the remaining treatments, excluding PH20. Figure 4 shows that there were no significant differences between treatments on the amounts of labeled microdiet ingested by the larvae that fed on that particular meal.

At 25 DAH, Control larvae did not feed on the offered labeled diet. The percentage of larvae that fed from treatments NCAP, Flat, Plus and PH20 varied between 79 ± 1% to 90 ± 17%, which was significantly higher than Fast and nPea, both close to 60% (Figure 5). Larvae from treatments Flat, Plus and PH20 ingested significantly higher amounts of labeled diet than treatments NCAP, Fast and nPea (Figure 6).

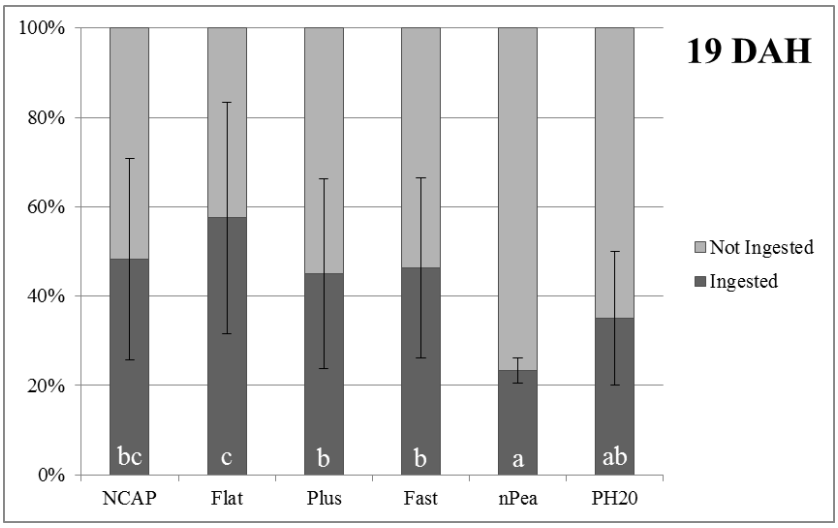

Figure 3 – Ingestion or no ingestion. Senegalese sole larvae ingestion pattern of different experimental labeled feeds offered at 19 DAH. Results expressed as treatment mean ± standard deviation (n = 20 observational units). Different superscript letters indicate statistical differences ($p<0.05$) between treatments.

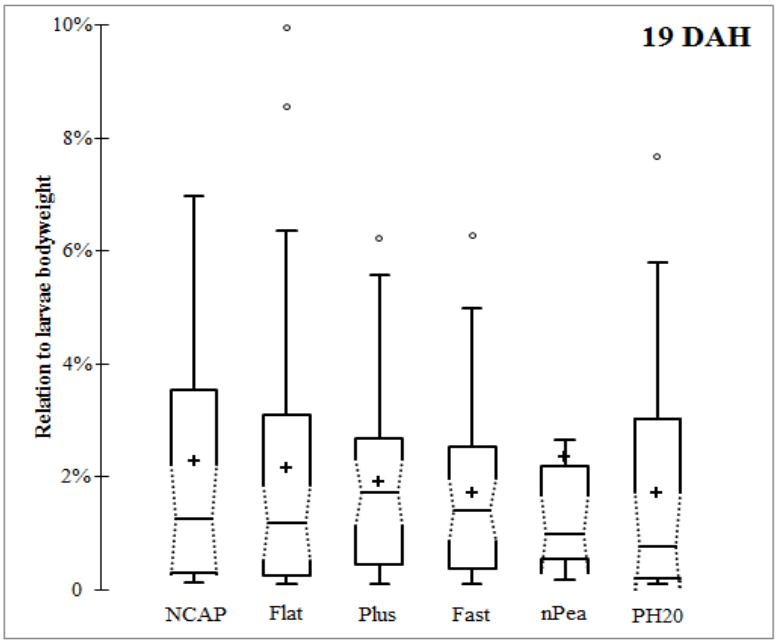

Figure 4 – Amounts of labeled feed ingested in relation to bodyweight by Senegalese sole larvae fed different experimental microdiets at 19 DAH (n = 20 observational units). Box shows the interquartile range of distribution (IQR) (25 to 75%). Horizontal line crossing the box represents the median. Notches in the box represent 95% confidence intervals around the median. Whiskers represent values outside the IQR, excluding outliers. Cross and open circle symbols represent the mean and outliers, respectively. Different superscript letters indicate non-overlapping intervals which imply a median difference with a 95% significance level.

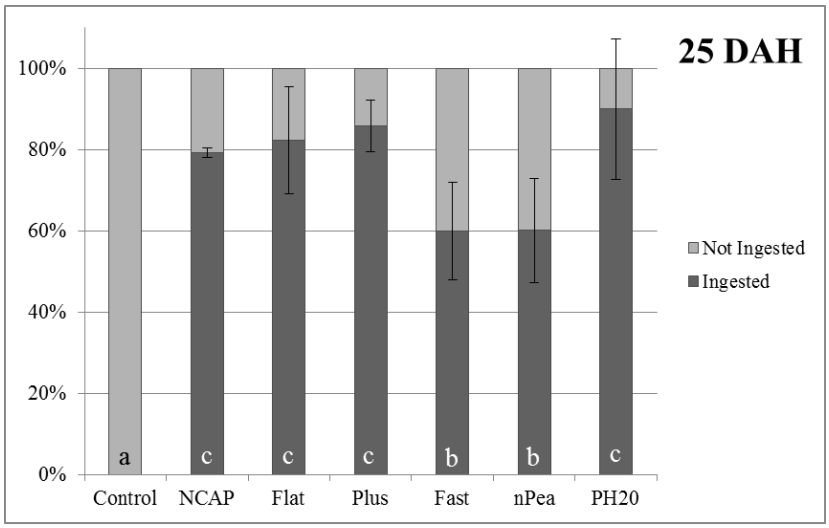

Figure 5 – Ingestion or no ingestion. Senegalese sole larvae ingestion pattern of different experimental labeled feeds offered at 25 DAH. Results expressed as treatment mean ± standard deviation (n = 20 observational units). Different superscript letters indicate statistical differences ($p<0.05$) between treatments.

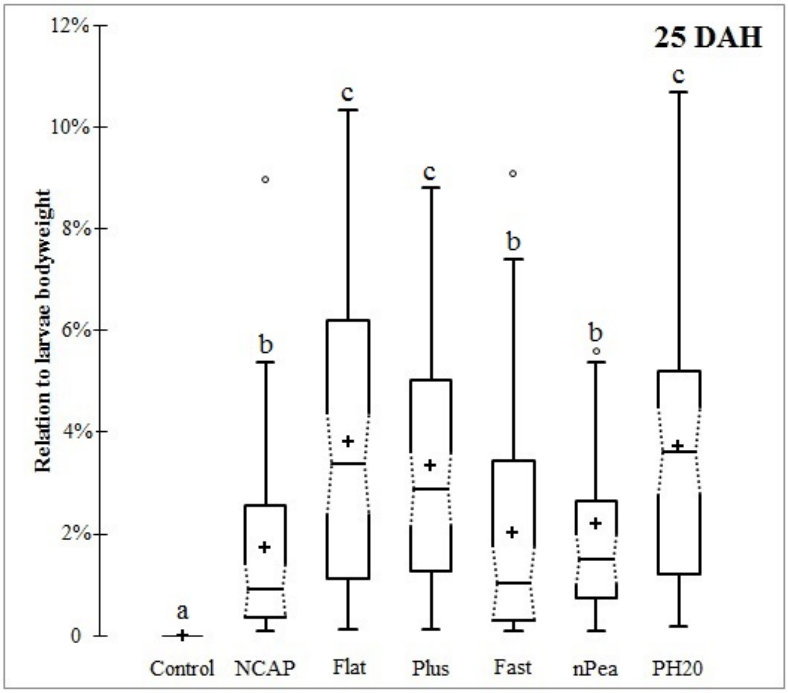

Figure 6 – Amounts of labeled feed ingested in relation to bodyweight by Senegalese sole larvae fed different experimental microdiets at 25 DAH (n = 20 observational units). Box shows the interquartile range of distribution (IQR) (25 to 75%). Horizontal line crossing the box represents the median. Notches in the box represent 95% confidence intervals around the median. Whiskers represent values outside the IQR, excluding outliers. Cross and open circle symbols represent the mean and outliers, respectively. Different superscript letters indicate non-overlapping intervals which imply a median difference with a 95% significance level.

At 40 DAH, Flat treatment had over 90% of larvae feeding on the offered labeled diets, which was significantly higher than treatments Control, NCAP, Fast and nPea but similar to Plus and PH20. Control had the highest increase in the percentage of feeding larvae from 0% at 25 DAH to around 74% at 40DAH, value only significantly lower than Flat (Figure 7). Larvae from Flat also ingested significantly higher amounts of labeled diet than the remaining treatments, whereas no other significant differences were found (Figure 8).

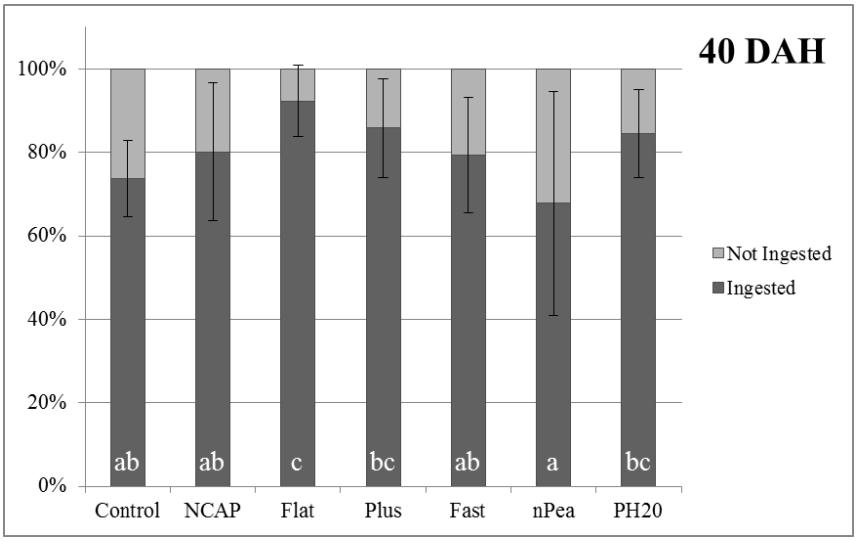

Figure 7 - Ingestion or no ingestion. Senegalese sole larvae ingestion pattern of different experimental labeled feeds offered at 40 DAH. Results expressed as treatment mean ± standard deviation (n = 20 observational units). Different superscript letters indicate statistical differences ($p<0.05$) between treatments.

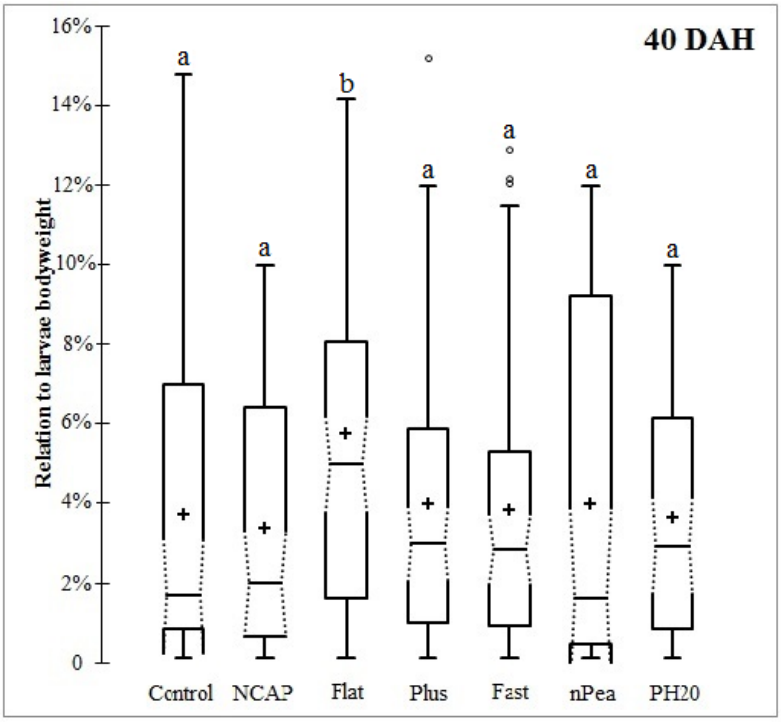

Figure 8 – Amounts of labeled feed ingested in relation to bodyweight by Senegalese sole larvae fed different experimental microdiets at 40 DAH (n = 20 observational units). Box shows the interquartile range of distribution (IQR) (25 to 75%). Horizontal line crossing the box represents the median. Notches in the box represent 95% confidence intervals around the median. Whiskers represent values outside the IQR, excluding outliers. Cross and open circle symbols represent the mean and outliers, respectively. Different superscript letters indicate non-overlapping intervals which imply a median difference with a 95% significance level.

2.3. Discussion

In this trial, several inert microdiets were tested targeting an early weaning right after settlement in Senegalese sole larvae. The use of an early co-feeding strategy allowed the performance of full weaning at 23 DAH, with good larvae growth performance and survival results. Larvae achieved DW values of 185 ± 52 mg at 60 DAH; RGR values around 12.4%.day^{-1}; FCR values around 1, K values averaging 0.95 and survival varied between 56 to 79%. The growth results obtained are a great improvement to those obtained by other authors on Senegalese sole larvae co-feeding studies such as Cañavate and Fernández-Díaz (1999), that obtained DW values of 50.9 mg at 60 DAH; Ribeiro et al. (2005), that obtained DW values of 4.2 mg at 39 DAH; Engrola et al. (2007), that obtained DW values of 50.9 mg at 60 DAH and RGR values of 8%.day^{-1}; Engrola et al. (2009a), that obtained DW values of around 100mg at 68DAH and RGR values of 5.5%.day^{-1}; Lobo et al. (2014), that obtained DW values of around 50 mg at 56 DAH; and Pereira (2016), that obtained DW values 25 mg at 51 DAH and RGR values averaging 11.4%.day^{-1}. These results are also an improvement to

those obtained on studies using a sudden weaning strategy such as Engrola et al. (2005), that obtained RGR values of 6.3%.day^{-1}; Dâmaso-Rodrigues et al. (2010), that obtained DW values of around 25 mg at 60 DAH; and Pinto et al. (2016), that obtained RGR values around 4.5%.day^{-1}. Despite performing full weaning at an earlier larval age, the RGR and DW values obtained are amongst the highest values ever reported in Senegalese sole experimental trials. The fact that only larvae from the two upper quartiles of weight distribution were considered for growth analysis had little influence on these conclusions, since RGR values of around 11.7%.day^{-1} would be achieved if all larvae had been considered, which still are good values. The survivals observed were also higher than those obtained in other studies, such as Cañavate and Fernández-Díaz (1999) (39%), Engrola et al. (2005) (44% and 70%), Engrola et al. (2007) (39%), Engrola et al. (2009a) (24%) and Pereira (2016) (between 48% and 73%). Engrola et al. (2007), Lobo et al. (2014) and Pinto et al. (2016) registered higher survivals, but the weaning strategy adopted by such authors was also more conservative. The overall good results obtained in the current study may most likely be explained by the improvement that has occurred on the zootechnical conditions during the pelagic phase, the quality of weaning microdiets and the feeding protocols during weaning. These allow the larvae to grow faster in the first developmental stages achieving a faster digestive maturation and a shorter period of adaption to inert microdiets.

The type of microdiet used in the current trial had an effect on the larvae growth performance but not on survival. The overall good growth performance results obtained in this trial confirm the quality of the microdiets used. Nevertheless, improvements can still be made in order to increase its suitability for younger larvae and reduce the cost-benefit relation. NCAP larvae exhibited the best growth performance of all treatments and only Flat larvae achieved similar final weights. The microdiet used for this treatment differed from WinFlat on the inclusion of a mixture of encapsulated and non-encapsulated taurine as opposed to the microencapsulated taurine alone. Since taurine is an hydrosoluble AA (Amino acid), microencapsulation can be performed to decrease leaching (Hamre et al., 2013). However, encapsulation involves the formation of a particle where a complete enclosure of a given nutrient by a binder occurs. This may make it more difficult for the larvae to digest the encapsulated particles where taurine is incorporated, especially at early stages of larval development, when the digestive system is still not completely developed. Still, NCAP larvae did not exhibited superior growth performances during the first stages of development, which does not provide

clear information on the digestibility of these particles. The results of this trial did not allow assessing the benefit of using microencapsulated taurine in the WinFlat diet, since similar results were obtained with non-encapsulated taurine. This adds an extra step to the production process, increasing its overall costs. Nevertheless, this WinFlat variant shall be further investigated in Trial 2.

Larvae from treatments Flat, Plus, Fast and nPea showed similar weights at the end of the experiment which were higher than those achieved by the Control larvae. Nevertheless, FCR, RGR and K values were similar to the Control larvae. The premium WinFlat version, WinFlat^{plus}, seems, at least during good husbandry conditions, to be less cost-effective than the normal version since its use did not improve larvae growth or survival. The use of WinFast, a high energy diet for fast growing fish species, did not improve larvae survival and did not increase larvae growth which suggests that Senegalese sole larvae may not have such energy requirements. As for the nPea treatment, the pea protein concentrate used in WinFlat was substituted by other sources of protein of marine and vegetable source and no effects on the survival and on final weight achieved by the larvae were observed, which does not support this replacement. These results suggest that all these microdiets can be used for an early weaning in Senegalese sole but amongst these, WinFlat seems to be the most cost-effective one.

PH20 larvae exhibited the lowest growth performance of all treatments. During the co-feeding these were fed a microdiet with 20% of protein hydrolisates, which is a higher amount than what is used in WinFlat, and fed WinFlat after weaning. In Senegalese sole, the inclusion of moderate levels of protein hydrolisates seems to result in improved growth in early larval stages (Canada, 2016). Inclusions of protein hydrolisates in weaning diets have been shown to improve survival and growth, but the inclusion of high levels has been proven to be detrimental to several species such as the gilthead seabream (Kolkovski & Tandler, 2000), common carp (Carvalho et al., 1997) and European seabass (Cahu et al., 1999). High levels of protein hydrolisates increase the amount of leached compounds which can make the diets less nutritive (Hamre et al., 2013). Additionally, it has been suggested by Cahu et al. (1999) and Carvalho et al. (2004) and discussed in De Vareilles et al. (2012) that the inclusion of high levels of protein hydrolisates can result in an excess of FAA, di and tri-peptides which saturate the peptide and AA intestinal transport mechanisms, which could be responsible for the detrimental effects observed. Furthermore, FAA's are absorbed faster than protein-bound AA which may lead to AA imbalances and consequent decrease on protein

utilization and larval performance (Rønnestad et al., 2000). Despite showing good survival, PH20 larvae grew significantly less than the remaining treatments, which does not support increasing the level of protein hydrolisates in WinFlat.

The type of weaning strategy used had an effect on larvae growth performance and survival. All co-fed treatments, excluding PH20, exhibited superior performances than the control, which was suddenly weaned. This effect was also visible on the larvae survival, with the control exhibiting the lowest survival values of all treatments. Studies made on Senegalese sole weaning over the years confirm the viability of both weaning strategies. Results obtained by authors such as Cañavate and Fernández-Díaz (1999) and Engrola et al. (2009a) support the use of a co-feeding strategy while results obtained by Engrola et al. (2005), Ribeiro et al. (2005), Engrola et al. (2007) and Pereira (2016) support the use of a sudden weaning strategy. The feeding strategy to adopt should be based on larvae weight: in post larvae below 1 mg of DW a co-feeding strategy seems to present better results (Engrola et al., 2009a) while sudden weaning should be performed if larvae are already around 5 to 10 mg of DW (Engrola et al., 2007). In this trial, larvae starting DW was 0.88 mg at 16 DAH, therefore the use of a co-feeding regime is in compliance with what is suggested. The Control treatment was suddenly weaned at 25 DAH when larvae were around 1.5 mg which is considerably lower than what is recommended. The feed intake analysis results have also shown that these larvae also did not feed on the inert microdiet at the moment of weaning, and at 40 DAH, larvae that fed were significantly less than treatment Flat, which was fed the same microdiet but was co-fed before full weaning. These facts confirm the need of an adaption period to the inert microdiets and may explain the lower DW values and survival achieved for the Control treatment at the end of the experiment. Additionally, it is worth to mention that these larvae were subject to transportation and introduced to a new environment before the start of the experiment. Choosing to suddenly wean larvae after these stressful conditions may not have been ideal since it has been shown that larvae require an adaption period to the inert microdiets. The inclusion of a small fraction of *Artemia* in their diets may be crucial so larvae do not fast for long periods of time when introduced to inert diets and don't reach full weaning in suboptimal nutritional conditions. Nevertheless, Pereira (2016) performed sudden weaning at 27 DAH when larvae were around 2.7 mg and still observed comparable results to those achieved by the co-feeding treatments in this trial. Pereira (2016) obtained RGR values of around 12.7%.day^{-1}, while values of around 12.4% in the present trial. However,

these values refer to an experiment where Pereira (2016) opted for a more conservative weaning strategy, performing sudden weaning at a later larvae age with no previous co-feeding. On a second experiment, when co-feeding was used from 19 to 35 DAH, Pereira (2016) obtained values of around 11.4%.day^{-1}. Considering that Pereira (2016) also used Sparos Lda commercial diet WinFlat variants, the differences in these results suggest that the shorter co-feeding period used in this trial was more suitable since the inert microdiets have and added nutritional value in comparison with *Artemia*, which may have had a beneficial effect on the growth performance of Senegalese sole larvae. Furthermore, the survival values observed in this trial for the co-fed treatments are a great improvement to previous studies, which reflect the improvement of the co-feeding strategies and the microdiets used and how suitable these have become to earlier stages of larval development.

The feed intake method used was extremely important in relating the growth performances with the inert microdiets and the weaning strategies used. This method also allowed assessing how attractive the different microdiets were to the larvae at different stages of development and how the ingestion amounts related to weight increases. The use of such tool is of critical importance on the development of the weaning strategies and on the formulation of inert microdiets. Feed intake analysis results have shown that the amounts of feed ingested by NCAP larvae were significantly less than what larvae fed WinFlat ingested after weaning. These facts suggest that the larvae were more successful in dealing with the nutritional composition of this microdiet since they were able to eat less and grow more. Nevertheless, FCR values do not confirm this assumption, which makes additional studies necessary. The percentage of larvae that fed on WinFlat throughout the trial and the amounts of feed ingested were always significantly higher than those that fed on WinFast which suggests that it may not be as attractive for Senegalese sole larvae. WinFlat^{plus} was only offered during the co-feeding and during that period significantly more larvae fed on WinFlat which does not support the need for a premium diet, at least during good husbandry conditions. The substitution of the pea protein concentrate made the microdiet less attractive to the larvae since, throughout the trial, significantly higher percentages of ingestion were observed in larvae fed WinFlat. Protein hydrolisates are more soluble in the water which can make the microdiets more attractive in initial stages. The feed intake analysis results have shown that increasing the amount of protein hydrolisates did not increase the microdiet attractability during the co-feeding stages since the percentage of larvae

that fed on this microdiet were lower than larvae fed WinFlat. These results are in compliance with the growth results: amongst the microdiets tested, WinFlat seems to be the most appropriate one to use in a Senegalese sole early weaning.

In conclusion, this trial represents a significant progress on the age of full weaning in Senegalese sole, since full weaning was performed at 23 DAH with good growth and survival results. WinFlat seems to be the most appropriate inert microdiet for Senegalese sole larvae weaning. Nevertheless, the use of non-encapsulated taurine in WinFlat produced similar results to using microencapsulated taurine, which will be further investigated in Trial 2. Results from this trial also support the use of a small co-feeding period during an early weaning in Senegalese sole, particularly if it is initiated immediately after larval settling.

3. Trial 2 – Targeting full weaning after settlement on Senegalese sole larvae

3.1. Materials and methods

3.1.1. Dietary treatments

In trial 2 a total of eight different dietary treatments were tested in triplicates: Flat, Plus, NCAP, Tryp, WP5, BP5, BP10 and GW. The inert microdiets used for Flat and Plus were Sparos commercial diets for flatfish WinFlat and WinFlat^{plus}, respectively. For the remaining treatments, five experimental WinFlat variants were used, which differed in the ingredient formulation but had similar proximal compositions (Table 1, section 2.1.1.). The NCAP formulation was the same as the one used in Trial 1 (section 2.1.1.); in Tryp, a supplement of encapsulated tryptophan was used; in WP5, whole cells of diatom *Phaeodactylum tricornutum* (Bohlin, 1897) were used in a concentration of 5%; in BP5 and BP10, broken cells of *Phaeodactylum tricornutum* were used in concentrations of 5% and 10%, respectively. GW was a moist variant of WinFlat which jellified in contact with water. This microdiet had the same proximal composition as WinFlat but was diluted in a 1:5 ratio. All diets were produced at Sparos Lda facilities (Olhão, Portugal) using the same manufacturing process as the one described in section 2.1.1.

The same weaning strategy as in Trial 1 was used for all treatments: initially larvae were co-fed frozen enriched *Artemia* and inert diet in a 20% to 80% respective proportion and were fully weaned at 19 DAH. Weaning was performed as soon as visual inspection of the larval guts confirmed an adaption to the inert diets. The inert diet granulometry used changed according to fish age as described in section 2.1.1.

3.1.2. Fish rearing

Trial 2 was conducted at Sparos Lda research facilities (Olhão, Portugal) and had a duration of 29 days. Senegalese sole larvae, originated from SEA8's hatchery Safiestela (Póvoa de Varzim, Portugal), were reared from 15 DAH to 43 DAH. Upon arrival, the larvae were randomly distributed in 24 tanks with 8L each previously prepared with clean seawater and aeration. Larval density was kept in each tank at 3000 larvae/m^2. These were kept under the same zootechnical conditions described in Trial 1 (section 2.1.2). The amount of external seawater input in the system was kept at a total of 4 system water volume renewals per day. Water renewal in each tank was kept at 4 renewals per hour. The system and tanks water renewals were increased when needed according to the amount of nitrogen compounds found in the water, in order to keep

them to a minimum. Temperature was maintained at 20.1±0.9ºC, dissolved oxygen concentration at 96.2±1.6%, salinity at 35g/L and nitrogen compounds ideally below 0.1 mg/L. During the co-feeding period (15 to 19 DAH), the daily routines were as presented in Table 2 (section 2.1.2). The *Artemia* was offered in 4 meals, each consisting of 25% of the total daily amount. The inert microdiets distribution was done using automatic feeders as described in section 2.1.2. As for the GW treatment, no automatic feeder was used since a moist diet was used. Administration was done using a syringe and in a way the feed was distributed in strings across the bottom surface of the tanks. The moist diet was offered in 4 meals: at 10:15, 14:00, 18:00 and 00:00 hours. After full weaning, the daily routines were as presented in Table 3 (section 2.1.2). For the GW treatment, feed administration remained in the same schedule. The estimated total daily amounts of feed administrated during Trial 2 can be seen in Annex 3. Larvae from all groups were fed *ad libitum* and it was made sure that feed was always present in the tanks but in amounts that would not deteriorate the water quality. The amounts of microdiet administrated were adjusted according to the amount of feed remnants from the day before.

3.1.3. Sampling

3.1.3.1. Growth performance, survival and feeding efficiency

At the start of the experiment (15 DAH) 125 Senegalese sole larvae were sampled from the initial pool of larvae that was distributed to the tanks and at 25, 32 and 43 DAH, 50 larvae were sampled from each tank for dry weight (DW) and total length (TL) determination. To determine TL all larvae were photographed and measured using AxioVision Microscopy software. These were then freeze dried and weighted on a digital scale. Only larvae from the two upper quartiles in each tank were considered for DW, TL and RGR analysis to simulate the sorting process that the larvae typically go through in commercial hatcheries, which eliminates the smallest individuals. Feed conversion ratio (FCR) and relative growth rate (RGR) for each treatment were assessed from 15 DAH to 43 DAH. Condition factor (K) and survival were determined at the end of the experiment. In all samplings, the larvae were harvested randomly following the procedures described in section 2.1.3.1. of Trial 1.

3.1.3.2. Feed intake

One sampling was made at 25 DAH in which 20 larvae from each tank were harvested. All procedures used prior to and post harvesting were the same as the ones described in section 2.1.3.2. In this feed intake analysis, the same labeled inert diet (WinFlat) was used for all dietary treatments.

3.1.4. Data analysis

As in Trial 1, feed conversion ratio (FCR), relative growth rate (RGR, % day^{-1}), condition factor (*K*) and survival were calculated using the same formulas as presented in section 2.1.4. Differences in growth performance and survival between dietary treatments were evaluated using a One-way ANOVA, followed by a Tukey multiple comparison test. When the one-way ANOVA assumptions were not complied, Krukall-Wallis tests were used, followed by Mann-Whitney tests. Differences in the percentage of larvae that fed on the offered labeled diets between treatments were assessed using chi-square tests. Results were expressed as means ± standard deviation (SD). In results expressed as percentage, an arcsine transformation was performed prior to any statistical test: $T = ASIN\ (SQRT\ (value\ /\ 100))$. The significance level considered was $p<0.05$ for all tests performed. All statistical analyses were performed in IBM SPSS Statistics 24 software. The amount of labeled feed ingested by the larvae in relation to their bodyweight was represented in notched boxplots and differences between treatments were evaluated by visually comparing the notches which display confidence intervals around the medians. Non-overlapping intervals imply a median difference with a significance level of 95% (Chambers et al., 1983).

3.2. Results

3.2.1. Growth performance, survival and feeding efficiency

The Senegalese sole larvae DW values obtained throughout Trial 2 are shown in Table 8 and represented in Figure 9. At 25 DAH, GW larvae had significantly higher DW values than Plus and BP10, whereas no other significant differences between the remaining treatments were observed. On the following sampling, at 32 DAH, larvae from WP5 had significantly higher DW values than all other treatments, followed by the Tryp treatment, which also showed significantly higher values than the remaining treatments. Larvae from Plus weighed significantly less than all other treatments. At the

end of the experiment (43 DAH) larvae from NCAP had achieved significantly higher DW values than the remaining treatments, with the exception of Tryp and BP10. GW larvae reached significantly lower DW values than the remaining treatments, followed by Plus, which also showed significantly lower DW than the remaining treatments.

Table 8 – Senegalese sole larvae DW (mg) values observed throughout the course of Trial 2.

Treatment	Flat	Plus	NCAP	Tryp	WP5	BP5	BP10	GW
15 DAH	0.86 ± 0.2							
25 DAH	0.98 ± 0.4^{ab}	0.90 ± 0.4^{a}	0.95 ± 0.4^{ab}	0.92 ± 0.4^{ab}	1.03 ± 0.5^{ab}	0.94 ± 0.4^{ab}	0.86 ± 0.4^{a}	1.14 ± 0.3^{b}
32 DAH	2.1 ± 0.9^{bc}	1.7 ± 0.5^{a}	2.2 ± 0.6^{c}	2.9 ± 1.3^{d}	3.5 ± 1.6^{e}	2.2 ± 0.9^{bc}	2.2 ± 1.1^{bc}	1.9 ± 0.3^{b}
43 DAH	16 ± 4.4^{de}	8.1 ± 2.7^{b}	18.3 ± 5.6^{f}	17.2 ± 4.7^{ef}	15.5 ± 4.9^{cd}	14.4 ± 5.3^{c}	17.9 ± 5.6^{f}	3.1 ± 1^{a}

Values presented as mean ± standard deviation. At 25 DAH, n = 20 and at 32 and 43 DAH, n = 50 observational units. Different superscript letters indicate statistical differences ($p<0.05$) between larvae from different treatments at the same age. Values refer to the two upper quartiles of the weight distribution.

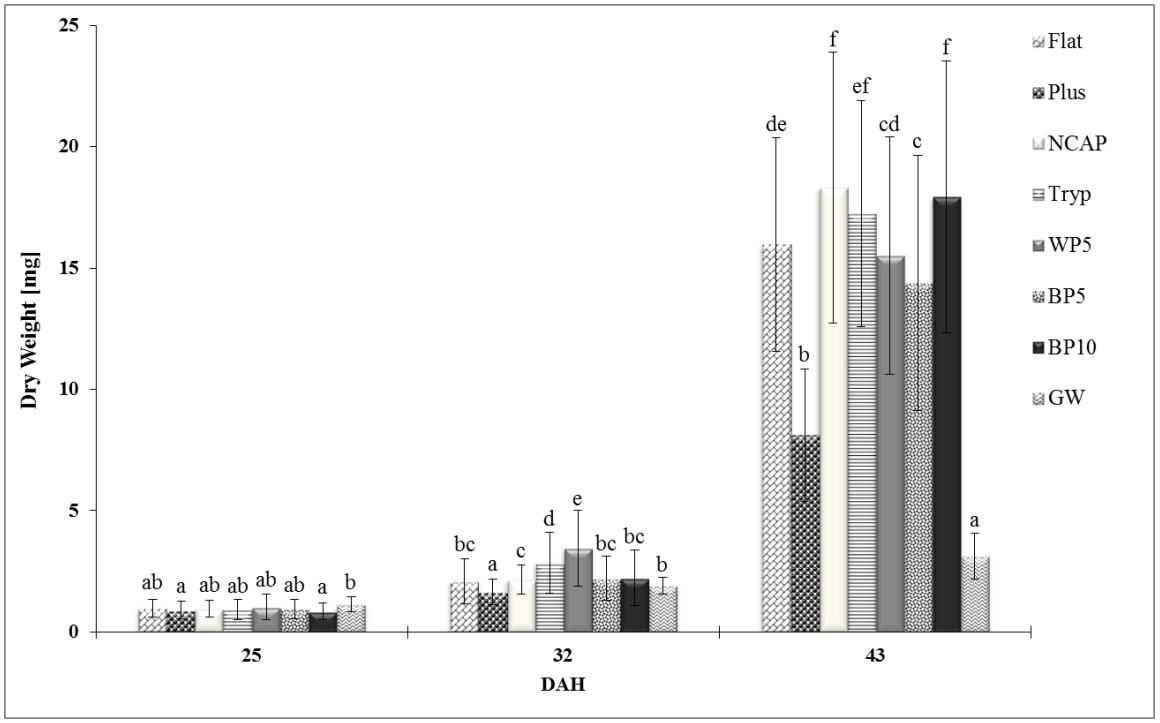

Figure 9 – DW of Senegalese sole larvae reared under different dietary treatments. Values presented as mean ± standard deviation. At 25 DAH, n = 20 and at 32 and 43 DAH, n = 50 observational units. Different superscript letters indicate statistical differences ($p<0.05$) between larvae from different treatments at the same age. Values refer to the two upper quartiles of the weight distribution.

The Senegalese sole larvae TL (mm) values obtained throughout Trial 2 are shown in Table 9. Results obtained followed similar trends to those observed in DW analysis. At the end of the experiment (43 DAH), the highest TL values observed were those of Tryp larvae, which were significantly higher than the remaining treatments, with the exception of treatments NCAP and BP10. Larvae from GW had achieved significant lower TL values than the remaining treatments, followed by Plus treatment, which also achieved significantly lower values than the remaining treatments.

Table 9 – Senegalese sole larvae TL (mm) values observed throughout the course of Trial 2.

Treatment	Flat	Plus	NCAP	Tryp	WP5	BP5	BP10	GW
15 DAH	7.47 ± 0.5							
25 DAH	8.1 ± 0.7^{ab}	7.7 ± 0.8^{a}	8 ± 0.9^{ab}	7.9 ± 0.8^{ab}	8.1 ± 1.1^{ab}	7.8 ± 0.9^{ab}	7.9 ± 0.9^{ab}	8.3 ± 0.7^{b}
32 DAH	10.2 ± 1.3^{b}	9.6 ± 0.7^{a}	10.5 ± 0.9^{c}	11.5 ± 1.4^{d}	12.2 ± 1.5^{e}	10.6 ± 1^{c}	10.5 ± 1.4^{bc}	10.2 ± 0.7^{b}
43 DAH	20.8 ± 2^{cd}	16.8 ± 1.9^{b}	21.4 ± 1.9^{de}	21.9 ± 1.9^{e}	20.8 ± 2.1^{c}	20.2 ± 2.2^{c}	21.4 ± 1.9^{de}	12.8 ± 1.2^{a}

Values presented as mean ± standard deviation. At 25 DAH, n = 20 and at 32 and 43 DAH, n = 50 observational units. Different superscript letters indicate statistical differences ($p<0.05$) between larvae from different treatments at the same age. Values refer to the two upper quartiles of the weight distribution.

Table 10 shows the values obtained at the end of Trial 2 for FCR, RGR, K and survival. GW larvae had a significantly higher FCR value than all other treatments, followed by Plus larvae, which also had a significantly higher FCR value than the remaining treatments. Regarding RGR, GW larvae had a significantly lower value than the remaining treatments, followed by Plus which also had a significantly lower RGR value than the remaining treatments. No significant differences regarding FCR and RGR were found between the remaining treatments. K values obtained were not significantly different between treatments. As for survival, treatments NCAP, Tryp, WP5 and GW achieved significantly higher survivals than Plus.

Table 10 – Senegalese sole larvae FCR, RGR, K and Survival values obtained at the end of Trial 2.

Treatment	Flat	Plus	NCAP	Tryp	WP5	BP5	BP10	GW
FCR	4.5 ± 1.6^{a}	11.3 ± 1^{b}	3.9 ± 1.1^{a}	4.3 ± 0.5^{a}	4.6 ± 0.8^{a}	6.1 ± 2.3^{a}	3.9 ± 0.4^{a}	29.8 ± 6.3^{c}
RGR (%DW day^{-1})	11 ± 0.5^{c}	8.3 ± 0.1^{b}	11.5 ± 1^{c}	11.3 ± 1^{c}	10.9 ± 0.6^{c}	10.6 ± 0.8^{c}	11.5 ± 0.5^{c}	4.7 ± 0.7^{a}
K	0.79 ± 0.07	0.76 ± 0.13	0.8 ± 0.01	0.73 ± 0.1	0.75 ± 0.03	0.75 ± 0.03	0.79 ± 0.03	0.59 ± 0.02
Survival (%)	49.5 ± 9^{abcd}	45.1 ± 3^{ab}	60.9 ± 5^{d}	59.9 ± 4^{d}	60.1 ± 7^{cd}	53.7 ± 7^{abcd}	50.2 ± 1^{abc}	56.8 ± 5^{cd}

Results expressed as mean ± standard deviation (n = 50 observational units). Different superscript letters indicate statistical differences ($p<0.05$) between larvae from different treatments. RGR values refer to the two upper quartiles of weight distribution

3.2.2. Feed intake

Feed intake analysis results shown that at 25 DAH the larvae that ingested the offered labeled diet from treatment GW were significantly less than the larvae from the remaining treatments. Close to 10% of GW larvae fed on that particular meal while it varied between around 30 to 45% on the remaining treatments (Figure 10). The amount of labeled feed ingested by the larvae on that meal can be seen in Figure 11. Larvae from Tryp ingested significantly higher amounts of labeled feed than larvae from treatments Flat and GW.

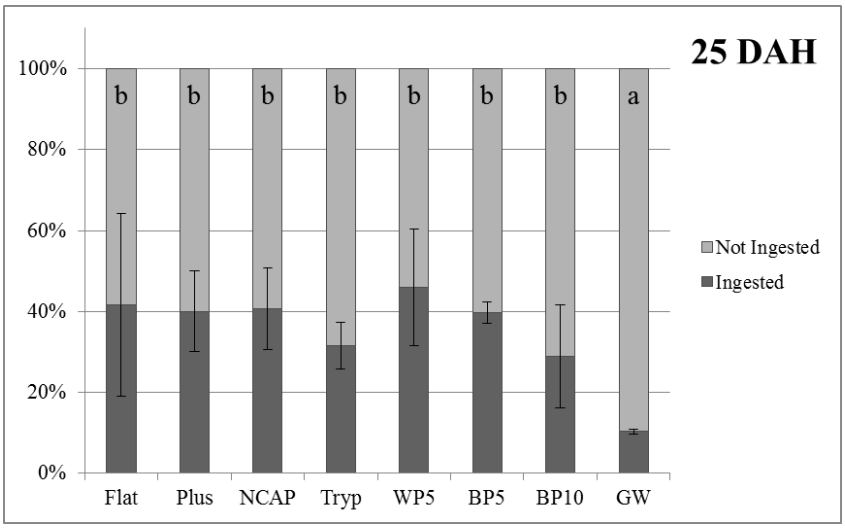

Figure 10 – Ingestion or no ingestion. Senegalese sole larvae ingestion pattern of labeled WinFlat offered at 25 DAH. Results expressed as treatment mean ± standard deviation (n = 20 observational units). Different superscript letters indicate statistical differences ($p<0.05$) between treatments.

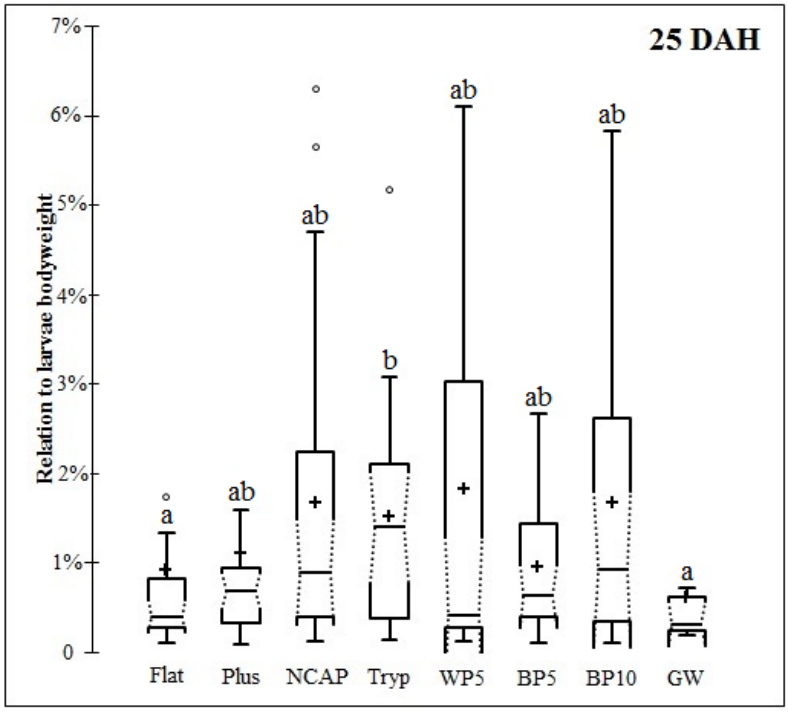

Figure 11 – Amounts of labeled WinFlat ingested in relation to bodyweight by Senegalese sole larvae at 25 DAH (n = 20 observational units). Box shows the interquartile range of distribution (IQR) (25 to 75%). Horizontal line crossing the box represents the median. Notches in the box represent 95% confidence intervals around the median. Whiskers represent values outside the IQR, excluding outliers. Cross and open circle symbols represent the mean and outliers, respectively. Different superscript letters indicate non-overlapping intervals which imply a median difference with a 95% significance level.

3.3. Discussion

Given the good results obtained in Trial 1, this trial aimed to further advance the weaning age of Senegalese sole larvae and full weaning was performed at 19 DAH. Larvae achieved DW values of 18.3 ± 6 mg at 43 DAH; RGR values around 11.1%.day^{-1} (excluding treatments Plus and GW, that exhibited significantly lower values); FCR values averaging 4.5 (excluding treatments Plus and GW, that exhibited significantly higher values); K values averaging 0.75 and survival between 45 to 61%. The overall growth results are an improvement to those obtained in Trial 1 at an approximate larval age and such good results had never been reported before. In trial 1, at 40 DAH, larvae had achieved DW values of 10.3 ± 6; RGR values around 4.8%.day^{-1}; FCR values averaging 7.7 and K values averaging 0.90. When comparing the estimated amounts of feed offered in Trial 1 with Trial 2 (Annexes 1, 2 and 3), it is possible to see that larvae ate considerably less in Trial 2. As for survival, the values obtained were higher than those reported by Engrola et al. (2009a), with values around 25% and Pereira (2016) with values between 44% and 59% using co-feeding regimes. Nevertheless, the values obtained are low when compared to those obtained in trial 1 and by other authors such

as Engrola et al. (2005), Ribeiro et al. (2005), Engrola et al. (2007), Lobo et al. (2014), Pinto et al. (2016) and Pereira (2016), which may have been caused by larvae poor condition or may be related to the more agressive weaning strategy employed. In this trial, larvae were fully weaned four days after arrival and were co-fed with a low *Artemia* proportion during this period, while in Trial 1, the co-feeding period lasted for seven days. These results suggest that the co-feeding period may have to be extended when dealing with such young larvae and particularly after these have been subjected to stress conditions such as transportation to ensure these are fully adapted to the inert microdiet before performing full weaning. Additionally, the overall good condition of the larvae at weaning is of critical importance and is something that may be subject to variation according to the egg batch quality.

The type of microdiet used had an effect on the larvae growth performance. As in Trial 1, larvae from NCAP treatment achieved one of the highest weight values, which were significantly higher than the remaining treatments, excluding Tryp and BP10. The inclusion of a mixture of encapsulated and non-encapsulated taurine was beneficial to the larvae growth performance which suggests that the larvae were more successful in dealing with the inclusion of this type of taurine than with the microencapsulated version used in WinFlat. The results obtained in both trials do not seem to justify the use of taurine microencapsulation since similar (Trial 1) or higher (Trial 2) growth performances were obtained using non-encapsulated taurine. Additionally, the microencapsulation technic adds an extra step to production increasing its overall costs and is only justified when it adds value to the microdiets.

The inclusion of the diatom *Phaeodactylum tricornutum* in the microdiets produced promising growth performance results. Aquaculture production is the major consumer of fish meal and fish oil on the global market (FAO, 2016). The dependence of the aquaculture feed industry on these products and its consequences for wild fish stocks are often used as arguments against the sustainability of fish farming. Therefore, the future of aquaculture must rely on an increase in the use of alternative sources of lipid and protein (Ytrestøyl et al., 2015). The gradual replacement of marine ingredients with plant ingredients on the salmon industry has allowed it to grow without consuming more fish biomass than what it produces (FIFO<1) (Sørensen et al., 2016). Microalgae are natural feed resources for zooplankton and fish, and are used in aquaculture to feed fish larvae, crustaceans and mollusks (Brown et al., 1997). Nevertheless, nutrient digestibility may vary according to the species of microalgae used (Sørensen et al.,

2016). According to Sørensen et al. (2016), *Phaeodactylum tricornutum* appears to be a promising microalga in comparison to other commercially produced ones, because there is a high correlation between inclusion levels and digestibility. In this trial, the use of *Phaeodactylum tricornutum* in the microdiets presented good results using broken cells and in inclusion levels of 10%. In fact, larvae from BP10 achieved higher weight values than larvae fed the commercial diets WinFlat and WinFlat^{plus}. The use of broken cells over whole cells at an inclusion level of 5% did not have an effect on larvae growth performance. This may be due to the fact that *Phaeodactylum tricornutum* is very poor in silica, with the cell wall being essentially composed of organic compounds (Cerezuela et al., 2012), which may allow the larvae to have a similar capacity to digest broken and whole cells, even at such early developmental stages. Studies on fish meal replacement by plant sources in diets for Senegalese sole juveniles have shown that high plant-protein diets support fish growth while also having a positive environmental impact by reducing phosphorous fecal waste and reducing the fishmeal used per kg of sole produced (Silva et al., 2010; Cabral et al., 2011), but no studies have been reported on Senegalese sole larvae. The results obtained in this trial show that inclusion of microalgae biomasses in weaning microdiets for Senegalese sole larvae can have positive effects on growth performance and provide preliminary data for further research.

For Tryp treatment, tryptophan (Trp) was added to the microdiets. Trp is an essential AA which is a precursor for serotonin (5-hydroxytryptamine), a neurotransmitter associated with several behavioral patterns including fear, stress, aggression, appetite regulation, social dominance and sex behavior in humans and animals including fishes (Kumar et al., 2014). Inclusion of dietary Trp has been shown to modulate aggressive behavior or reduce cannibalism and reduce stress associated with farming practices in different fish species such as European seabass, brown trout (*Salmo trutta*) and pikeperch (Herrero et al., 2007; Höglund et al., 2007; Król and Zakęś, 2016). It has been shown that dietary Trp supplementation can contribute to increasing levels of serotonin which tend to reduce feed intake and thereby result in depressed fish growth (Papoutsoglou et al., 2005). Furthermore, Trp is also an indispensable AA, and its supplementation may also improve protein retention. In this trial, larvae from Tryp treatment exhibited good growth performances, similar (weight) or better (length) to those achieved by the larvae fed the commercial diet WinFlat, which suggests that growth was not suppressed, and may eventually be enhanced by

trypyophan supplementation. The effects of the inclusion of different Trp dietary levels on serotonin levels in Senegalese sole inert microdiets need to be assessed in further research if the goal is to use Trp as a stress reducer.

Larvae from Plus treatment exhibited the second lowest growth performance of all treatments. The results obtained in this trial are not in compliance with the results obtained in Trial 1 and with those obtained by Pereira (2016). Such unsatisfactory results had never been obtained using WinFlat^{plus}, which suggest that some degradation might have occurred since the microdiet belonged to a batch that was not produced specifically for this trial and was stored for some time.

Larvae from GW treatment exhibited the lowest growth performance of all treatments at the end of Trial 2. The fact that these larvae ate less frequently and that this microdiet was diluted in a 1:5 ratio, may have had effects on the final weight achieved by the larvae as a result of insufficient nutrient intake. Nevertheless, until 25 DAH, GW larvae showed comparable weight values to the remaining treatments and even higher values than larvae from Plus and BP10 which suggest an increase in nutrient requirements as larvae grow. This new type of feed arises as an interesting weaning diet alternative as it is a soft textured feed with high structural stability in the water that allows the larvae to feed continuously. In fact, larvae readily accepted this variant. Nonetheless, further research should be conducted in order to adjust feeding protocols where this diet can be used successfully, namely by increasing feeding frequency or its nutritional value, since this microdiet produced promising results initially but did not seem to sustain a desirable growth after 25 DAH.

The feed intake results did not show any significant difference between treatments on the percentage of larvae feeding on the offered labeled diet, excluding treatment GW which only had around 10% of larvae feeding on that particular meal. These results were to be expected since GW larvae were being fed a soft textured inert diet and the offered labeled diet may have not been recognized as feed. The remaining treatments had similar amounts of larvae feeding on the offered labeled diet which may be due to the fact that they were all fed the same microdiet and due to the similarity of the variants with the commercial microdiet WinFlat. By comparing the feed intake results obtained in this trial with the ones obtained in Trial 1 at 25 DAH, it is possible to see that considerably less larvae were feeding at this point.

In conclusion, this trial also represents a significant progress on the age of full weaning in Senegalese sole. Despite the lower survivals observed in this trial in

comparison to Trial 1, higher growth performances were achieved by using a short co-feeding period and performing full weaning at 19 DAH. The survival results obtained in this trial suggest that the larvae need to be in excellent conditions in order to perform such an early weaning. Non encapsulated taurine seems to be a more cost effective alternative to the microencapsulated version and confirms the results obtained in Trial 1; *Phaeodactylum tricornutum* seems to have positive effects on growth performance when using broken cells in inclusion levels of at least 10%; and Trp may enhance larvae growth, but further studies need to be conducted.

4. Final conclusions

The present Thesis supports the following conclusions:

- The importance of live feeds in Senegalese sole production can be reduced by advancing the age at which full weaning is performed. Although research is still needed for further progress, the current microdiet quality and feeding protocols used allow the performance of full weaning at earlier larval ages maintaining good growth performances and survival.

- There is still room for improvement in Senegalese sole weaning microdiets formulation. Although good results have been reported in this Thesis using commercial diets, some experimental variants produced even superior larvae growth performances. Additionally, this Thesis has shown that it is also possible to make changes in formulation to accomplish goals such as cost reduction and environmental sustainability, without compromising larvae growth performances.

- Further development of weaning strategies is crucial. The use of a co-feeding regime seems to be critical when performing a full weaning at such early stages of development and when larvae, like in this Thesis, are subject to stress factors such as transportation that may inhibit them to feed right away. By supplying live feeds, even if in small proportions, it is ensured that the larvae do not fast and can be in good nutritional conditions when full weaning is performed.

- The use of feed intake methods is of extreme importance in the development of feeding protocols and microdiet formulations. The feed intake method used in this Thesis has allowed relating growth performances with the different microdiets and weaning strategies used. Additionally, important aspects such as how attracted the larvae were to the microdiets and how ingestion amounts related to weight increases were also possible to assess using this method.

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6. Annexes

6.1. Annex 1

Table 11 - Estimated total daily amount of frozen enriched *Artemia* and inert microdiets given from 16 to 24 DAH in Trial 1. The amounts are estimated for 1000 larvae.

	Control	Co-feeding	
DAH	**Artemia per day ($x10^6$)**	**Artemia per day ($x10^3$)**	**Inert microdiet per day (g)**
16	0.78	45	0.9
17	0.98	41	1.2
18	1.22	37	1.6
19	1.44	35	2.4
20	1.80	31	2.8
21	2.24	23	3.5
22	2.65	13	4.0
23	2.89	-	4.7
24	3.41	-	5.5

6.2. Annex 2

Table 12 - Estimated total daily amount of inert microdiets given after weaning in Trial 1. The amounts are estimated for 1000 larvae.

DAH	Inert microdiet per day (g)
23	4.7
24	5.5
25	6.0
26	6.1
27	6.2
28	6.4
29	6.6
30	6.7
31	6.9
32	7.3
33	7.5
34	7.8
35	8.0
36	8.5
37	9.0
38	9.3
39	9.6
40	10.1
41	10.7
42	11.2
43	11.7
44	12.3
45	12.8
46	13.3
47	13.6
48	14.0
49	14.4
50	14.8
51	15.5
52	15.5
53	16.0
54	16.5
55	17.0
56	17.5
57	18.0
58	18.5
59	19.0

6.3. Annex 3

Table 13 - Estimated total daily amount of frozen enriched *Artemia*, inert microdiets and moist microdiets given in Trial 2. The amounts are estimated for 1000 larvae.

DAH	Artemia per day ($x10^3$)	Inert microdiet per day (g)	Moit microdiet per day (g)
15	72	2.4	0.8
16	64	2.7	1
17	49	3.1	1
18	43	3.5	1.3
19	-	3.8	1.3
20	-	3.8	2.8
21	-	3.8	3.4
22	-	4.2	4.2
23	-	4.7	4.5
24	-	4.7	4.5
25	-	4.7	4.7
26	-	4.7	5
27	-	4.7	5.2
28	-	4.7	5.5
29	-	4.9	5.5
30	-	4.5	5.9
31	-	4	6.0
32	-	4	6.0
33	-	4	5
34	-	4	4.2
35	-	4	3.4
36	-	4.5	4.2
37	-	4.5	3.8
38	-	4.7	3.4
39	-	4.7	3.4
40	-	4.7	3.4
41	-	4.7	3.8
42	-	4.7	4.2