Effect of different live feed lipid enrichments on the growth performance of the Short-snouted seahorse, *Hippocampus hippocampus* (Linnaeus, 1758)

Neuza Alves Fernandes

Dissertation for the degree of Master in Aquaculture

Work carried out under the coordination of Dr Jorge Palma and Dr Miguel Correia

(Center of Marine Sciences, University of Algarve)

2020

Declaração de autoria de trabalho

Declaro ser autora deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

Acknowledgements

The realization of this dissertation became possible due to the support and collaboration of certain entities and people. In this way, I want to express my gratefulness and appreciation, in particular to:

Dr. Jorge Palma, for agreeing to be my advisor. I am grateful for all the availability and support demonstrated during the development of the thesis.

Dr. Miguel Correia, for agreeing to be my co-advisor, and for all the availability and support given during the thesis development.

I also want to thank the technicians Ramalhete team, for all the support and availability during the development of the practical work, in particular Helena Teixeira, João Reis, Miguel Patusco and Samuel Correia.

I am also very grateful to Dr. Margarida Castro, for all the guidance and support given during the data analysis and writing process of the thesis.

Last but not least, I want to thank all my friends and family that helped in any way possible, for all the love and support.

RESUMO

Devido às suas características únicas e peculiaridades morfológicas, os cavalos-marinhos (*Hippocampus sp*.), são procurados mundialmente para aquariofilia e medicina tradicional Chinesa, o que suscita o interesse para a sua produção em cativeiro e a um aumento do seu estudo. A alimentação e nutrição dos cavalos-marinhos continua a ser um dos fatores que mais condiciona a sua produção, pelo que continua a ser de extrema relevância estabelecer regimes alimentares adequados que atendam às necessidades nutricionais dos juvenis. A utilização de *Artemia sp*. como dieta viva é tradicionalmente mais viável, podendo ser utilizada como vetor de alimentação para atender às necessidades nutricionais, por meio do enriquecimento. Por outro lado, a utilização de copépodes, alimento base destas espécies em condições naturais, é comprovadamente um alimento adequado, no entanto, a sua cultura em cativeiro pode ser muito cara, trabalhosa ou então condicionada pelas características ambientais quando recolhidos na natureza. Neste estudo, a utilização de enriquecimentos com óleos animais de origem aquática, e a quantidade mínima de copépodes, viável para garantir o crescimento e sobrevivência ideal de juvenis de cavalos-marinhos de focinho curto (*Hippocampus hippocampus*) foram avaliados em dois ensaios diferentes. Provenientes de casais de *H. hippocampus* criados em cativeiro, foram utilizados em cada um dos ensaios, juvenis de uma única desova, e onde 20 indivíduos foram aleatoriamente distribuídos em cada dos tanques de cultivo (utilizados em triplicado) para cada um dos tratamentos alimentares testados. Num primeiro ensaio, foram utilizados quatro tratamentos alimentares; copépodes não enriquecidos (dieta controlo), *Artemia* nauplii enriquecida com emulsão de óleo de krill (*Euphasia superba*) (2, 5, 7.5 e 10 mg/L), *Artemia* nauplii enriquecida com emulsão de óleo de fígado de bacalhau (*Gadus morhua*) (2, 5, 7.5 e 10 mg/L) e *Artemia* nauplii enriquecida com DHA Selco® (10 mg/L). No segundo ensaio, foram testados quatro tratamentos alimentares: copépodes não enriquecidos (dieta controlo), mistura de copépodes com *Artemia* enriquecida com óleo de Krill (10mg/L) e DHA Selco® (10mg/L), e copépodes não enriquecidos na proporção de 2.5, 5, 7.5, 10, 20, 30 e 40% do total de presas fornecidas. Cada um dos ensaios decorreu durante um período de 28 dias (0-28 dias após o nascimento). Os dados obtidos foram usados para calcular o ganho médio de peso; ganho de comprimento médio; coeficiente de crescimento térmico; fator de condição e sobrevivência. Observou-se que nenhum dos enriquecimentos testados para a *Artemia* constituiu por si só uma alternativa adequada á utilização de copépodes na alimentação de juvenis de *H. hipocampus.* Quando incluídos na dieta,

verificou-se que os melhores resultados nos parâmetros de crescimento analisados foram obtidos com a inclusão de 40% copépodes e *Artemia* enriquecida em 10mg/L de emulsão de óleo de krill e/ou DHA Selco®, no entanto verificou-se que mesmo uma pequena percentagem de copépodes garante um crescimento assinalável e uma melhoria na taxa de sobrevivência da espécie.

Palavras-chave: Aquacultura de cavalos-marinhos; *Hippocampus hippocampus*; enriquecimento de alimento vivo; lípidos; ácidos gordos essenciais.

ABSTRACT

Due to their unique characteristics and morphological peculiarities, seahorses (*Hippocampus sp.*) are demanded worldwide for aquarium and traditional Chinese medicine, which raises interest for their production in captivity and an increase on seahorses' studies. The feeding and nutrition of seahorses continues to be the one of the major bottlenecks conditioning their production, so it remains extremely important to establish adequate diets that meet the nutritional requirements of juveniles. The use of *Artemia sp*. as a living diet it is traditionally more viable and can be used as a food vector to meet nutritional needs, through enrichment. Conversely, the use of copepods, a key feed in natural conditions, is proven to be an adequate food, but in captivity its culture can be very expensive, laborious and conditioned by environmental characteristics when harvested from the wild. In this study, the use of animal oils of aquatic origin-based enrichments, and the minimum amount of copepods, feasible to ensure the growth and optimal survival of short-snouted seahorse juveniles (*Hippocampus hippocampus*) were evaluated in two different trials. From couples of *H. hippocampus* bred in captivity, juveniles from a single batch were used in each of the trials, and where 20 individuals were randomly distributed in each of the cultivation tanks (used in triplicate) for each of the tested food treatments. In a first trial, four food treatments were used; non-enriched copepods (control diet), *Artemia nauplii* enriched with krill oil emulsion (*Euphasia superba*) (2.5, 5, 7.5 and 10 mg/L), *Artemia nauplii* enriched with cod liver oil emulsion (*Gadus morhua*) (2.5, 5, 7.5 and 10 mg/L) and *Artemia nauplii* enriched with DHA Selco® (10 mg / L). In the second trial, four dietary treatments were tested: non-enriched copepods (control diet), mixture of copepods with *Artemia* enriched with Krill oil (10 mg/L) and DHA Selco® (10 mg/L), and non-enriched copepods in the proportion of 2.5, 5, 7.5, 10, 20, 30 and 40% of the total prey supplied. Each trial ran for a period of 28 days immediately after birth. The data obtained were used to calculate the average weight gain; average length gain; thermal growth coefficient; condition and survival factor. It was observed that none of the enrichments tested for *Artemia nauplii* is an

adequate alternative to the use of copepods in the feeding of juveniles of *H. hipocampus*. When included in the diet it was found that the best results in the analyzed factors were observed with the inclusion of 40% copepods and *Artemia* enriched in 10 mg/L of krill oil emulsion and/or DHA Selco, however it was found that even a small percentage of copepods ensures remarkable growth and an improvement in the species' survival rate.

Key words: Seahorse Aquaculture; *Hippocampus hippocampus*; live feed enrichments; lipids; essential fatty acids.

Index

1 INTRODUCTION

1.1 State of the art

There are indications that the growth rate of global aquaculture may have peaked, although high growth rates may continue for some regions and species (FAO, 2018). With commercial fishing being relatively static since the late 1980s, aquaculture has been responsible for the continuing impressive growth in the supply of fish, for human consumption and others. In 2006, countries in the Asia and the Pacific region accounted for 89% of the production in weight and 77% of the value (Woods, 2007). World aquaculture (food fish and aquatic plants) has grown significantly during the past half-century. From a production under 1 million tonnes in the early 1950's, global fish production peaked at about 171 million tonnes in 2016, with aquaculture representing 47 percent of the total and a total sale value of 232 billion USD from aquaculture production alone (FAO, 2018).

Similarly, fish production for the aquarium hobbyist trade, is also a rapidly growing sector of the fishing industry and there is a directed effort and an increasing pressure within the ornamental trade to develop reliable and sustainable hatchery procedures for the captive breeding of many reef fish species (Koldewey and Martin-Smith, 2010). Aquaculture of ornamental species is considered a potential alternative for the ornamental market, which mainly relies on the collection of wild specimens to supply the growing demand, alleviating pressure from the wild stocks at the same time it allows the collection of data about species life history (Olivotto *et al*., 2011). As for seahorses in general, sustainable seahorse aquaculture can play an important role in conservation of these species, as well as in the development of reliable traceability tools to fight the illegal trade of these highly priced organisms (Cohen *et al.*, 2017).

Seahorses have a unique life history characterized by a sparse distribution, low mobility, site fidelity, small home ranges, low fecundity, mate fidelity and lengthy parental care to small broods, which might render them vulnerable to overfishing and environmental disruptions, including habitat damage and degradation (Foster and Vincent, 2004). Due to its unique characteristics and morphological peculiarities, seahorses (*Hippocampus sp.*), are in demand worldwide and are heavily traded, first and foremost as dried (wild) specimens for traditional Chinese medicine, with smaller trades in live (wild and captive bred) seahorses for aquarium display and dried (wild) seahorses for curios (Foster *et al*., 2019; Koldewey and Martin-Smith, 2010; Kuo and Vincent, 2018). The global trade of seahorse and related species is estimated to be at least 20 million

individuals a year, but recent studies indicate values over 37 million individuals traded yearly (Kitsos *et al*., 2008; Kuo and Vincent, 2018; Woods, 2007). It was estimated that at least 32 countries had traded seahorses and their relatives by 1995, but this increased to nearly 80 countries during 1996–2001, with the expansion of Africa and Latin America markets (Foster and Vincent, 2004). Trade in Asia alone was inferred to be more than 45 tons of dried seahorses in 1995, but official data, trade surveys, and qualitative evidence suggested that the Asian trade in dried seahorses in 2000 may have been considerably greater (Foster and Vincent, 2004; Koldewey and Martin-Smith, 2010). Dried specimens made up 98% of the reported trade from 2004 to 2011, and 93% of the trade was reportedly imported by Hong Kong SAR, Taiwan, Province of China and mainland China (Foster *et al.*, 2019).

Seahorses and pipefish can be valuable commodities, with prices varying among trade routes. Although data regarding import values is scarce, it was estimated that dried seahorses traded in 2000 reached a value of €1.48 million (U.S. $1.7 million), with HKSAR and Taiwan providing the only reliable data (Vincent *et al*., 2011). The trade of seahorses (dead and dried), accounts for 97% of all seahorses reported in trade, with China being the main recipient country (Koldewey and Martin-Smith, 2010; Kuo and Vincent, 2018). In addition, seahorses inhabit shallow coastal areas worldwide, where anthropogenic impacts tend to be most frequent and severe (Bell *et al*., 2003). Along with this, habitat destruction and by-catch are the main threats for seahorse conservation and led to significant declines in the wild populations over the last decades (Foster and Vincent, 2004; Kitsos *et al*., 2008; Kuo and Vincent, 2018; Woods, 2007).

Being valuable species to the international trade, there is an increasing concern about the seahorse conservation status, and measures were undertaken to protect the entire *Hippocampus* genus and to regulate seahorse exploitation and trade (Kitsos *et al*., 2008). For the first time, in 1996, two seahorse species were included in the IUCN Red List of Threatened Species, and the remaining species of the genus were included in the following years (IUCN, 2019). Presently, all 46 currently identified seahorse species are included in the IUCN Red List and in the CITES Appendix II, meaning international trade is permitted but controlled, as these species might become threatened with extinction if trade continues unregulated (Foster, 2016; IUCN, 2019; Kuo and Vincent, 2018). There are two seahorse species in coastal water habitats throughout the north-eastern Atlantic Ocean and Mediterranean Sea, the long-snouted seahorse (*Hippocampus guttulatus*) and the short-snouted seahorse (*Hippocampus hippocampus*) (Pollom, 2017; Woodall, 2017). *H. hippocampus* extinction risk cannot be reliably evaluated on a global level due to lack of data, being listed as

Data Deficient and as Near Threatened in the Mediterranean (Woodall, 2017).

The implementation of CITES trade regulations, intended to contribute to the seahorse conservation in the wild, was assessed by a recent study of Kuo and Vincent (2018). The effect of these measures contributed to an apparent decline in recorded trade volume, as well as an increase in seahorse prices (Foster *et al*., 2019). These two factors suggest a growing demand, with high prices of endangered species providing greater incentives for the community of human extraction from the wild (Kuo and Vincent, 2018).

All these factors led to an increasing concern for management and protection of the *Hippocampus* genre, as well as the species *H. hippocampus*, and an interest as a marine fish species for aquaculture in the last decades, hoping to avoid the pressure on wild populations. In the late 90's, and over the last two decades, as the continuous overexploitation of wild stocks has increased steadily, significant improvements were achieved in production, with 13 seahorse species being commercially cultured worldwide (*Hippocampus abdominalis*, *Hippocampus barbouri*, *Hippocampus breviceps*, *Hippocampus capensis*, *Hippocampus comes*, *Hippocampus erectus*, *Hippocampus ingens*, *Hippocampus kuda*, *Hippocampus reidi*, *Hippocampus spinosissimus*, *Hippocampus trimaculatus*, *Hippocampus whitei* and *Hippocampus zosterae*) (Koldewey and Martin-Smith, 2010; Wilson and Vincent, 2000; Palma *et al.,* 2012; Payne and Rippingale, 2000). Seahorse species are of interest for economic, cultural, scientific and educational ends, being used as a flagship species for biological conservation, and with rising demand in multiple sectors, these species and the overall genre are potential candidates for commercial production and the ornamental fish trade (Cohen *et al.,* 2016; Koldewey and Martin-Smith, 2010; Olivotto *et al*., 2011).

Despite the increasing number of seahorse studies, there are still substantial gaps in knowledge that need to be overcome. Among those, the lack of a full understanding about seahorse nutrition is one of the major bottlenecks that affects growth and survival (especially in juveniles) of these species in captivity and prevent to date, the establishment of well-developed and improved protocols. There are records of live feed enrichment studies for some seahorse species. A few examples are *H. abdominalis* (Shapawi and Purser, 2003; Woods, 2003), *Hippocampus subelongatus* (Payne and Rippingale, 2000) and *H. guttulatus* (Palma et al., 2008; Palma *et al*., 2011), where the effect of certain specific nutrients and/or commercially available products were tested on parameters such as growth and survival. The current knowledge of *H. hippocampus* diet effect is scarce, with one study testing live prey feeding regimes (Otero-Ferrer *et al*., 2010) and a

study by Segade *et al*., (2015) testing the effect of enriched *Artemia* and mysids on parameters like growth, body colour and biochemical composition.

1.2 Seahorses

1.2.1 Biology, ecology, and distribution of species

Pipefishes, pipe-horses, seahorses and sea-dragons are all part of the Sygnathidae family, with seahorses included in the genus *Hippocampus* (Koldewey and Martin-Smith, 2010). The entire family Syngnathidae falls within the order Gasterosteiforms (Lourie *et al*., 2004). However, the taxonomy of seahorses still requires clarification, with a constant rearrangement on the number of species, forty-six species are currently known, however further studies regarding morphological, genetic and behavioural taxonomic characters, might still lead to new changes on the number of seahorse species (Lourie *et al*., 2016; Short *et al*., 2018; Short *et al*., 2020).

All seahorse species share the same basic body morphology and function, which confers them the title of unusual marine fishes. They are characterized by a scaleless skin which covers a series of bony plates that are visible as rings around the trunk and tail, a prehensile tail able to grasp and hold objects, curvaceous trunk, a bent head horse-like shaped, large eyes with independent movement, elongated tubular toothless snout ending in a small terminal mouth, as well as a digestive tract without a differentiated stomach (Lourie, 2017; Foster and Vincent, 2004). Some species also have bony bumps or skin filaments protruding from the bony rings; they can vary in colouration and have been described with and without skin filaments (Lourie, 2017; Lourie *et al*., 2004). Adult seahorses have no pelvic and caudal fins, having only one propulsive dorsal fin, two small pectoral fins which are used for stabilization and steering, and a reduced anal fin (Foster and Vincent, 2004).

Seahorses range in size from the diminutive *H. denise* (<3 cm length) to the large *H. abdominalis* (up to 35 cm length). The maximum reported size for *Hippocampus hippocampus* (Linneus, 1758) is 15cm. Males usually possess a longer tail, while females exhibit a longer body trunk. Morphological traits such as snout size, abdomen, skin colour and adult body size, allow the differentiation of the species (Blanco, 2014; Lourie *et al*., 2004; Woods, 2007).

Two seahorse species inhabit coastal water habitats throughout the north-eastern Atlantic Ocean and Mediterranean Sea, the long-snouted seahorse (*H. guttulatus*) and the short-snouted seahorse (*H. hippocampus*) (Pollom, 2017; Woodall, 2017). Despite overlapping distribution areas, these

two sympatric species show distinct habitat preferences, with a common association with seagrass assemblages; *H. guttulatus* is generally associated with shallow waters and enclosures, preferring higher habitat complexity, while *H. hippocampus* is able to exploit habitats with lower complexity, showing preference for deeper areas, with lower holdfast availability (Correia, 2014; Foster and Vincent, 2004; Kitsos *et al*., 2008).

The life span, mortality rate and growth rate of the different seahorse species are largely unknown in their natural environment. However, inferred life spans range from 1 year in smaller species to 3 to 5 years for larger species (Foster and Vincent, 2004; Woods, 2007).

The duration of "pregnancy" in seahorses varies between approximately 9–45 days, depending on species and water temperature. For *H. hippocampus*, gestation period varies from 3 to 4 weeks, with breeding season occurring from April to October (Lourie *et al.*, 2004). Male parturition occurs during the night and the juveniles are actively ejected from the pouch. Individuals hatch from their eggs inside the parent male's pouch and develop into their juvenile form, passing the transition phase from endogenous feeding to fully prepared exogenous feeding inside the parent male's pouch, with no free larval stage (Woods, 2007). Egg, new-born and brood size vary according to species, from 0.9 to 2.0 mm in egg diameter, 2.0 to 16.2 mm in new-born length and 34 to 2000 in maximum brood size (Faleiro and Narciso, 2010; Foster and Vincent, 2004). The maximum reported brood size of *H. Hippocampus* is 865 juveniles, with egg diameter and length at birth averaging 1.6mm and 9.3mm respectively (Lourie *et al.,* 2004). After pouch release, seahorse juveniles are fully formed and independent, with an open and well differentiated digestive tract, physically capable of active swimming and immediate exogenous feeding. They show pelagic behaviour for several weeks and feed on selected planktonic species (Foster and Vincent, 2004; Woods, 2007).

Seahorses are visual predators which predominantly rely on stealth and camouflage, ambushing and striking their prey while keeping visual focus on the selected prey-item and swallowing them through the tubular snout to a stomach less digestive tract. Feeding typically occurs during diurnal or crepuscular hours but can also occur at night in some nocturnally active species (Foster and Vincent, 2004; Woods, 2007). New-born juveniles bear a relative short and broad snout, which becomes significantly longer and slenderer as they grow (Roos *et al*., 2011). On parturition, seahorse juveniles possess a fully functional feeding system and begging pivot feeding, a specialized suction feeding. Capture occurs with a high rotational velocity of the snout towards the prey and simultaneous buccal cavity expansion, which creates a strong inhalant current (Roos

et al., 2011; Woods, 2007). Prey are then consumed whole without mastication or, depending on the species, broken into smaller pieces by repeated feeding strikes before ingestion (Blanco, 2014; Woods, 2007).

1.2.2 Diet and Nutritional Requirements

Seahorses are typically carnivorous, feeding upon a wide range of epifaunal and planktonic prey. The diet of *H. hippocampus* was studied and found to be mainly dominated by Amphipoda, Anomura Decapoda and Mysidacea, with an incidence in crustaceans such as copepods, amphipods, isopods, and caridean, euphausid and mysid shrimps (Kitsos *et al*., 2008; Otero-Ferrer *et al*., 2010). Seahorses may change their diet ontogenetically with smaller individuals consuming smaller prey, yet it is known that prey selection by marine fish larvae and juveniles strongly depend on the size of both fish and prey (Blanco, 2014; Woods, 2007).

Conversely to most fish larvae, in some species juvenile seahorses are capable of actively forage and predate on available prey, immediately after birth. Therefore, feeding needs to start soon after the release from the male pouch due to the lack of endogenous reserves (Faleiro and Narciso, 2010; Sheng *et al*., 2006). It is at this stage in life that juveniles are most vulnerable and where large-scale mortalities commonly occur in the captive environment (Chang and Southgate, 2001; Shapawi and Purser, 2003). The lack of knowledge on specific nutritional requirements has raised major difficulties in culturing marine organisms, with nutrition constituting one major challenge in marine larviculture. Appropriate diets that fulfil all the nutritional requirements must be determined for juveniles, to ensure high survival and maximal growth (Faleiro and Narciso, 2010; Woods, 2007).

It is well established that larvae and juveniles of most marine fish species require live prey with a high nutritional content, especially at the first feeding stage. In addition to proteins, lipids are the main energy source to seahorse new-borns. Fish larvae have the capacity to digest and assimilate lipids from the start of the exogenous feeding and are essential for fish larvae growth as the most important fuel source. The action of lipases is directed towards the hydrolysis of dietary essential fatty acids (EFA's) from fats, which are very important for development, growth and survival of larvae/juveniles (Izquierdo, 1996; Sargent *et al*., 1999a; Sargent *et al*., 1999b; Sorgeloos *et al*., 2001).

Fish larvae require food organisms with relatively high concentrations of long-chain, n-3

polyunsaturated or essential fatty acids (EFA) such as 20:5n-3 (EPA, eicosapentaenoic acid) and 22:6n-3 (DHA, docosahexaenoic acid) (Ajiboye *et al*., 2011Ca). The HUFA requirements of young seahorses are still understudied, however, some studies indicate that seahorse's EFA's requirements might be similar, or maybe even be higher, compared to other marine fish larvae individuals (Faleiro and Narciso, 2010; Payne and Rippingale, 2000; Woods, 2003). In other commercially cultured species, dietary DHA content showed a greater influence on growth (when compared to EPA). Fatty acid enrichments of *Artemia* improved their nutritional value, confirming the beneficial effects of improved dietary n-3 HUFA on fish larvae. In a different study, Faleiro and Narciso (2010) found that the optimal diet for *H. guttulatus* reproduction should reflect the PUFA-rich profile of eggs. Extremely high levels of n−3 HUFA must be provided, while low AA concentrations should be enough to fulfil the n−6 HUFA requirements.

1.2.3 Feeding Regime

In captivity, mysids and amphipods are currently used to fed adult fish (Vargas-Abúndez *et al*., 2018; Woods, 2003), sometimes combined with *Artemia* metanauplii. Juvenile seahorses feeding relies mainly on copepods and enriched *Artemia* nauplii (Instar I) or metanauplii (Instar II) (Palma *et al*., 2008; Segade *et al*., 2015; Sheng *et al*., 2006; Woods, 2007). However, a successful feeding schedule has been barely achieved for first feeding and juvenile stages in seahorses (Otero-Ferrer *et al*., 2010).

Artemia is commonly used as live prey in aquaculture due to easiness of access and culture, but it poorly fulfils the nutritional requirements of marine fish larvae, especially regarding dietary essential fatty acids and amino acids at first feeding (Gardner, 2004; Sorgeloos *et al*., 2001). Most attempts to rear new-born seahorses with diets consisting entirely of *Artemia* nauplii, have resulted in very poor juvenile survival; an example is Payne and Rippingale (2000), with the species *H. subelongatus*.

Feeding preferences and rearing performance (growth and survival) have been already assayed in some seahorse species (e.g. Gardner, 2004; Olivotto et al., 2008; Payne and Rippingale, 2000; Pham and Lin, 2013; Sheng et al., 2006) and the importance of copepods as supplement or exclusive prey have been recognized, as well as enriched *Artemia* (Palma *et al*., 2011; Willadino *et al*., 2012; Zhang *et al.*, 2010).

Copepods are considered the best live food to be used mainly due to their biochemical profile.

Calanoid copepods in particular, have been recognized as an ideal prey for first feeding of many fish species, including seahorses, since growth and survival rates result significantly increased (Drillet *et al*., 2006; Gardner, 2004; Sorgeloos *et al*., 2001). Contrary to *Artemia*, copepods do not need to be enriched as they are a natural source of Highly Unsaturated Fatty Acids n-3 HUFA, especially Eicosapentaenoic Acid (EPA) and Docosahexaenoic Acid (DHA), as well as Arachidonic Acid (ARA), vitamins and antioxidants (Ajiboye *et al*., 2011; Lindley *et al*., 2011; Rajkumar and Kumaraguru, 2006). However, their use in aquaculture can be very expensive. Copepods do not reach nearly as high a density in culture conditions as other live foods and require larger volumes of water and larger culture vessels (Gardner, 2004).

The nutritional quality of commercially available *Artemia* strains is relatively poor in EPA and DHA, so it is essential and common practice to enrich this live prey with emulsions of marine oils. Enriched *Artemia* provides the fish culturist with a convenient ‘‘carrier’’ vehicle for desirable dietary components such as essential fatty acids (EFAs), as well as being a cheap and easily cultured prey. The most commonly applied boosting technique is a 24-h enrichment period after hatching (Job *et al*., 2002; Martínez-Cárdenas and Purser, 2007; Olivotto *et al*., 2008; Otero-Ferrer *et al*., 2010; Sheng *et al*., 2006; Sorgeloos *et al*., 2001).

1.3 Live Feed Enrichment

As nutrition requirements are still largely unknown, commercial seahorse culturists often prefer existing, well- known commercial products, that have been shown to increase growth and survival in other marine finfish, to enrich live foods. However, such commercial products may, or may not, be suitable for optimising seahorse growth and survival (Palma *et al*., 2011; Woods, 2003; Woods, 2007).

High levels of linolenic (18:3n-3) acid is found in *Artemia* and related to the cyst origin. High EPA (20:5n-3) is more likely to succeed in the enrichment process (Otero-Ferrer *et al*., 2010). Taking advantage of the primitive feeding characteristics of *Artemia* nauplii, it is possible to manipulate the nutritional value of HUFA-deficient *Artemia*. Since brine shrimp nauplii that moult into the second instar stage (about 8 h following hatching), are non-selective particle feeders, simple methods have been developed to incorporate different kinds of products into the *Artemia* prior to feeding to predator larvae. Bioencapsulation, also called *Artemia* enrichment or boosting, is widely applied in marine fish and crustacean hatcheries for enhancing the nutritional value of *Artemia*

with essential fatty acids (Sorgeloos *et al*., 2001).

In this study, three different products, krill oil, cod liver oil and DHA Selco® were tested through incorporation in *Artemia* enrichments in order to evaluate their potential usefulness in juvenile *H. hippocampus* early diets. Krill oil has a uniquely high content of phospholipid-bound n-3 LC-PUFA, which are thought to be more bioavailable, as well as being involved in regulating more metabolic pathways than the triacylglycerol-bound EPA and DHA found in most fish oils (Sprague *et al.*, 2017). Krill oil has many attributes, like phospholipids, triglyceride, and non-esterified fatty acid forms of EPA and DHA. It is also a rich source of astaxanthin, a carotenoid pigment and powerful antioxidant (Linder *et al.*, 2010; Salem and Kuratko, 2014). Cod liver oil is rich in omega 3 and 6, essential fatty acids for the better utilization of the vitamins A, D, G and K; composed of 75% cod liver oils and 25 % cardamom oil, with a fat percentage of 99.9% and a 0.1% of protein. Marine lecithin was used to prepare the emulsions of cod liver and krill oil, and is a HUFA-rich and polar lipids (PL)-based product with great potential as enrichment diet, as phospholipids, a predominant fraction of PL's, have emulsifying properties, which facilitate lipid absorption and increases tolerance to stress conditions (Guinot *et al*., 2013).

1.4 Objectives

Feeding trials with *H. hippocampus* juveniles took place with the objective of facilitating and improving the captive culture of this species, with a focus on diet. The two main objectives are:

- To test the effect of different lipid emulsions and inclusions ratios on the growth and survival of juvenile *H. hippocampus*, in order to determine the best supplement and inclusion level that could serve as an alternative for the control feed;
- To evaluate the effect of partial *Artemia* replacement by copepods and to determine the minimum inclusion level to sustain optimal growth.

2 METHODOLOGY

2.1 Experimental units

The Master Thesis was performed at the Resources, Restoration, Connectivity and Climate (R2C2)

research group from the Centre of Marine Sciences (CCMAR), University of Algarve (UAlg). This experimental work was conducted at the Ramalhete Aquaculture field station and University laboratories.

A total of 2220 *H. hippocampus* juveniles were used to conduct 6 different rearing trials, obtained from a captive-bred broodstock kept at the CCMAR facilities. The broodstock was maintained in two 250 litre plastic tanks assembled in a semi-closed system. Seahorses were daily fed a mix of mysid shrimp, *Mesopodopsis slabberi* and *Paramysis sp.* in different proportions, depending on availability. Males were regularly observed to determine the pregnancy stage and when close to parturition were separated along with one female and placed into glass tanks (8L capacity) with adequate aeration, and artificial holdfasts in order to minimize stress. Spawning occurred naturally, and after release from the male's pouch, juvenile seahorses from a single brood were randomly collected and used in the experimental trials.

2.2 Experimental design

In this study, two different trials [1)*Artemia* enrichments and 2) enriched *Artemia* AF with copepod percentages] were sequentially performed using the same experimental conditions. Each trial was conducted using individuals from one single batch, to prevent uneven growth performances motivated by their condition index on parturition. Juveniles were assembled into the rearing thanks according to a completely randomised design.

Twenty juvenile *H. hippocampus* were stocked in each of the three 8L glass replicate tanks per dietary treatment at a density of 2 fish L^{-1}. In each tank, lateral and back walls were covered with a black adhesive to improve prey detection, leaving the front wall uncovered for observation. Artificial holdfasts were placed in each of the rearing tanks. Holdfast structures were changed as the individuals grew to provide adequate grip for the seahorses. Floating structures were used from 0 days after parturition (DAP) until 14 DAP, since the juveniles would prefer to attach near the tank surface. Through the ontogenic process, as individuals would start to swim and feed near mid/bottom of the tank (around 14 DAP), the floating structures were replaced by bottom structures that remained until the end of the trial. Each trial lasted for 28 days, from parturition until 28 DAP.

A semi-close recirculation system was already set up in the laboratory was used to perform the trials. Water flowed into the tanks at a constant rate, pumped from the reservoir tank and passing

through a UV sterilization unit before distribution into the rearing tanks. The water was vigorously aerated in the reservoir (sump) to ensure that dissolved oxygen was always kept close to saturation. A sump protein skimmer was added to collect the excess from the enriched feed, ensuring high water quality standards. Seawater would enter the rearing tanks through a transparent plastic tube placed below the water surface, to prevent bubbling and water turbulence. The water outflow structures were assembled in the corner of the tanks and were composed of a black polystyrene tube covered at the water surface with a mesh of 15 µm diameter to prevent prey to be flushed from the thanks. Outflow water would then be drained into a settling tank equipped with a bio-filter. Tanks were illuminated from above with fluorescent tubes, light intensity at water surface around 900 lx, and a photoperiod of 16 L:8 D (06:00 – 22:00 h), controlled by a timer. Seawater temperature and dissolved oxygen were recorded daily.

Daily maintenance routines consisted of tank cleaning through siphoning to remove faeces and uneaten feed, feed preparation and feeding, including removal of dead individuals.

2.3 Feeding regimes

In the first trial, enriched *Artemia* with krill oil and cod liver oil at four different lipid inclusion levels (2.5, 5, 7.5, 10 mg/L) and enriched *Artemia* with DHA Selco® from INVE Aquaculture were tested and compared to a natural copepod control diet (*Oithona nana* and *Tigriopus brevicornis*). In the second trial, different inclusion rates of copepods (same species as mentioned above) and enriched *Artemia* with krill oil (10mg/L) and DHA Selco® (10mg/L) were tested and compared to a control diet (100% copepods).

DHA Selco® enrichment was used according to the manufacturer instructions. The two different lipid emulsions (krill oil and cod liver oil) were prepared according to an adapted protocol from Watanabe *et al.* (1982). Each lipid emulsion batch was prepared by mixing 100 ml of sea water, 1.5g of marine lecithin granulate along with the desired concentration of the enrichment media, either krill or cod liver oil. Emulsions were homogenized in a mixer for 3 minutes, divided in units for each replicate and stored below 4ºC for around 3-4 days, until supplied to the *Artemia.*

In all trials, seahorses were fed daily, one ration given on the same schedule in late morning. Feeding was supplied *ad libitum*, with the initial ratio adapted to approximately 2 prey per mL (Woods, 2007; Palma *et al.*, 2008; Segade *et al*., 2015) during the first part of the trial, and subsequently increased to approximately 2.25 preys/tank day^{-1}, to maintain the desired prey

availability from that point on. This was equivalent to a respective total of 16,000 and 18,000 *Artemia*/tank day^{-1}. The same protocol was used in later trials when different copepod inclusion rates along with enriched *Artemia* were tested.

Copepods were daily harvested in the University of the Algarve Ramalhete Aquaculture field station natural ponds. *Artemia* AF cysts (Sanders, Ogden, UT, USA) was hatched according to protocol defined by Sorgeloos *et al*. (2001). In brief, 1-2g of *Artemia* cysts were daily hatched in 20-L acrylic cylindrical-conical tanks, under strong aeration, temperature 25–28 °C, salinity15–35, minimum pH of 8.0, near saturated oxygen levels, and continuous illumination of 2000 lux. The *Artemia* had an approximate composition of 60% protein, 24% fat, 4.4% ash and 8.5% moisture. The amount using during the experiments (1-2g) was adjusted according to hatching rate and desired amount of prey.

After being counted, newly hatched *Artemia* nauplii were separated into each desired concentration (to the respective trial) and placed into each of 4 individual 1.5L plastic cylindrical-conic thanks held at room temperature (20–22 °C) under moderate aeration. In each enrichment thank, 100 ml of the enrichment emulsion level was mixed with 900 ml of seawater to attain the desired lipid concentration (mg/L). The enrichment period lasted for approximately 16h.

In the trials with DHA Selco®, the necessary *Artemia* nauplii for the daily feeding was enriched according to the manufacturer instructions in a 20-L acrylic cylindrical-conical tank, under the same conditions as described above.

2.3.1 *Artemia* enrichments

In a preliminary trial, non-enriched *Artemia* feed (0mg/L lipid inclusion), was used as a potential negative control, and daily harvested copepods as a potential positive control. Juvenile seahorses feed unenriched *Artemia* nauplii recorded 100% mortality at 10 DAP, which confirmed the inadequacy of this diet. Juvenile *H. hippocampus* fed copepods had what was considered a normal growth and survival, were sampled for growth performance at 14 DAP, and in the absence of further comparison the trial was ended. From this point on, only the copepod control diet was selected for further use.

In the first trial, five dietary treatments were tested: daily harvested copepods (control diet), cod liver oil enriched AF *Artemia* at 2.5, 5, 7.5 and 10 mg/L inclusion rate, krill oil enriched AF

Artemia at 2.5, 5, 7.5 and 10 mg/L inclusion rate, krill oil enriched EG *Artemia* at 2.5, 5, 7.5 and 10 mg/L inclusion rate and DHA Selco® enriched AF *Artemia*. AF and EG refer, respectively, to small and large sized *Artemia* nauplii, thus the two different sized nauplii were used to test the prey size adequacy to the juvenile *H. hippocampus*. DHA Selco® enrichment was prepared according to the supplier recommendations. In brief, 10mg DHA Selco® (10 mg/L) was diluted in seawater, homogenized, and supplied for enrichment.

2.3.2 Enriched *Artemia* AF and copepod percentages

As *Artemia* may induce potential nutritional imbalances when used as a sole diet, different inclusion levels of copepods were tested to evaluate its likely beneficial effect on the growth and survival of juvenile *H. hippocampus* as a compensatory nutritional provision.

Different percentages of copepods were tested following a co-feeding protocol, along with either *Artemia* AF enriched with krill oil (10 mg/L) or DHA Selco® (10 mg/L). In the first trial, copepod inclusion rates at 10, 20, 30 and 40% on the expanses of with *Artemia* AF enriched with krill oil were tested. In a second trial, and due to the fact that DHA Selco® enriched *Artemia* showed positive results, copepods were included at lower inclusion rates of 2.5, 5, 7.5, 10, 20, 30 and 40% on the expanses of DHA Selco® enriched *Artemia*.

Feeding protocol was the same as in the previous trials. Fed was initially provided at 2 prey ml^{-1} (16000 prey/tank/day^{-1}) and raised to 2.25 prey ml^{-1} (18000 prey/tank/day^{-1}) at 14 DAP, to maintain prey availability.

2.4 Sampling and laboratory analysis

Whole body mass and total length were measured for each individual seahorse. Data was collected on the start of the experiment (0 DAP), 14 DAP, and 28 DAP at the end of the experiment. For the initial sample, 10 individuals were randomly selected and euthanized (according to the CCMAR guidelines for animal experimentation), placed in sea water and preserved at -18 ºC for posterior analysis. For the initial sampling of the new-born seahorses, total length was accessed using digital image processing with DinoCapture 2.0 and weight was obtained using a high precision scale (Sartorius).

In the two subsequent sampling events (at 14 and 28 DAP), fish were measured using a digital

Vernier calliper. To minimize stress during sampling, instead of the three measurements proposed by Lourie *et al*. (2004) (the sum of head, trunk and tail lengths), seahorses were measured by the sum of the head length (distance from the tip of the snout (upper jaw) to the midpoint of the tip of the coronet) and fish height (from that same point to the tip of the outstretched tail).

Fish were gently blot dried to remove excess water and swiftly weighted using a high precision Kern microgram scale. Daily observations and data collection took place for mortality analysis, temperature and dissolved oxygen; collecting dead individuals (if existing) and siphoning of faeces and leftover feed.

2.5 Statistical analysis

Data was used to calculate:

1) Mean Weight Gain (WG)

$$Mean\ Weight\ Gain\ WG\ (mg/fish) = \frac{(Wf - Wi)}{Wi}$$

Where Wf is the final seahorse wet weight (g) and Wi is the individuals' initial wet weight (g);

2) Mean Length Gain (LG) (according to Palma, *et al*., 2011)

$$Mean\ Lenght\ Gain\ LG\ (cm/fish) = \frac{Lf - Li}{Li},$$

Where Lf is the final seahorse length (cm3) and Li is the initial length (cm3);

3) Growth rate, using the Thermal-unit Growth Coefficient method (TGC) (modified from Iwama and Tautz, 1981; Cho, 1990)

$$Thermal - unit\ Growth\ Coefficient\ (TGC) = \left[\frac{\left(Wf^{1/3} - Wi^{1/3}\right)}{\sum(T \times D)}\right] \times 100$$

Where Wf is the final seahorse wet weight (g) and Wi is the initial wet weight (g), T is the water temperature (ºC) and D is the number of days in each trial;

4) Condition Factor (CF) (according to Ighwela *et al*., 2011; Kachari *et al*., 2017)

$$Condition\ Factor\ (CF) = \left(\frac{WW}{L^b}\right) \times 100$$

Where WW is the total wet weight (g) and L the total length (cm^3) and b the regression

coefficient of the relationship Log W = Log a + b Log L (W is weight in grams and L total length in mm).

5) Survival (S) (according to Otero-Ferrer *et al*., 2010)

$$Survival\ (S\ \%) = \left(\frac{(Ni - Mt)}{Ni}\right)\text{x}100$$

Where Ni is the initial number of seahorses placed on each thank on day 1 and Mt was the total number of individuals that died in each trial.

All data was tested for normality and homogeneity of variances. The treatment levels (with three replicates each) were compared for each trial (1 to 8) and day (14 and 28). To study the effect of the diets on growth and weight gain as well as Thermal-unit Coefficient, Condition Factor and Survival differences between groups, a One-Way ANOVA, using Turkey's test for multiple comparisons was used. Exploratory data analysis and preparation of the databases was done using EXCEL and the statistical analysis was performed using GraphPad Prism software version 8.0.0 for Window statistical package. Significance was considered at α=0.05 level.

3 RESULTS

3.1.1 *Artemia* enrichments

In the first three diets tested, 100% mortality was observed in all dietary treatments (non-enriched *Artemia* (control), AF enriched *Artemia* with cod liver oil at 2.5, 5, 7.5 and 10 mg/L, and both AF and EG enriched *Artemia* with krill oil at 2.5, 5, 7.5 and 10 mg/L) within 7 days after the start of the experiment (7 DAP), except for the copepod control diet. Fish fed the copepod control diet attained a weight gain (WG) of respectively, 9.475±0.031, 10.420±2.367 and 10.580±1.100 mg/fish at 14 days, and a survival of 56.7, 58.3 and 61.7% at 14 days (14 DAP), when the trial was terminated, as no further comparisons could be obtained (Table 1). As a result of these trials, it was possible to observe that the use of a *Artemia* control diet was unnecessary, and from that point on, only copepods were used as control diet in the following trials.

As for the DHA Selco® enriched *Artemia* diet, fish fed the control diet showed a much higher growth performance and survival in both sampling periods, with differences getting more obvious as the trial continued (Table 1, and in Figure 1 and 2). At 14 DAP fish feed the DHA Selco®

enriched diet had a survival of 40%, which decreased to 5% at 28 DAP. At the same period (28 DAP) fish fed the copepod control diet had a survival of 81.7%. As for WG, control had values of 9.644±0.954 at 14 DAP and 31.784±6.171 mg/fish at 28 DAP, while the DHA Selco® treatment showed values of 1.923±2.013 and 4.230±0.412 mg/fish, respectively.

Table 1 - Descriptive table of diets 1 through 4. S (%) – Survival, WG - Weight Gain (fish/mg), TLG - Total Length Gain (cm/fish), and TGC - Thermal Growth Coefficient were calculated for the periods between 0-14 DAP (days after parturition) and from 0 to 28 DAP. CF is the condition factor of the juveniles measured at parturition, 14 DAP and 28DAP. Mean and standard deviation (±) are showed for the calculated formulas and for each level tested [0 (Artemia control), 2.5, 5, 7.5, 10 mg/L Artemia enrichments and control of copepods]. Within the same row, different subscripts refer to significant differences.

		Cod liver oil enriched *Artemia* AF					**Krill oil enriched *Artemia* AF**					**Krill oil enriched *Artemia* EG**					**DHA Selco enriched *Artemia* AF**	
	Control copepods	**0 mg/L**	**2.5 mg/L**	**5 mg/L**	**7.5 mg/L**	**10 mg/L**	**Control copepods**	**2.5 mg/L**	**5 mg/L**	**7.5 mg/L**	**10 mg/L**	**Control copepods**	**2.5 mg/L**	**5 mg/L**	**7.5 mg/L**	**10 mg/L**	**Control copepods**	**10 mg/L**
Juvenille standard weight on release (mg)	0.003±0	0.003±0	0.003±0	0.003±0	0.003±0	0.003±0	0.003±0	0.003±0	0.003±0	0.003±0	0.003±0	0.003±0.001	0.003±0.001	0.003±0.001	0.003±0.001	0.003±0.001	0.003±0.004	0.003±0.004
Juvenille standard weight at 14 DAP (mg)	0.033±0.013	n.a.	n.a.	n.a.	n.a.	n.a.	0.035±0.016	n.a.	n.a.	n.a.	n.a.	0.037±0.014	n.a.	n.a.	n.a.	n.a.	0.037±0.011	0.008±0.001
Juvenille standard weight at 28 DAP (mg)	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	0.111±0.028	0.018±0.007
Juvenille standard lenght on release (mm)	12.918±0.312	13.066±0.534	13.066±0.534	13.066±0.534	13.066±0.534	13.066±0.534	13.800±0.405	13.800±0.405	13.800±0.405	13.800±0.405	13.800±0.405	13.845±0.764	13.845±0.764	13.845±0.764	13.845±0.764	13.845±0.764	13.036±0.720	13.036±0.720
Juvenille standard lenght at 14 DAP (mm)	26.488±4.170	n.a.	n.a.	n.a.	n.a.	n.a.	24.359±3.693	n.a.	n.a.	n.a.	n.a.	25.985±3.046	n.a.	n.a.	n.a.	n.a.	26.027±2.944	17.253±2.062
Juvenille standard lenght at 28 DAP (mm)	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	39.894±3.159	23.803±2.495
S (%) 0-14 DAP	56.7	0	0	0	0	0	58.3	0	0	0	0	61.7	0	0	0	0	90^{a}	40^{b}
S (%) 0-28 DAP	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	81.7^{a}	5^{b}
WG (mg d-1) 0-14 DAP	9.475±0.031	n.a.	n.a.	n.a.	n.a.	n.a.	10.420±2.367	n.a.	n.a.	n.a.	n.a.	10.580±1.100	n.a.	n.a.	n.a.	n.a.	$9.644±0.954^{a}$	$1.923±2.013^{b}$
WG (mg d-1) 0-28 DAP	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	$31.784±6.171^{a}$	$4.230±0.412^{b}$
TLG (mm) 0-14 DAP	1.003±0.165	n.a.	n.a.	n.a.	n.a.	n.a.	0.746±0.102	n.a.	n.a.	n.a.	n.a.	0.847±0.085	n.a.	n.a.	n.a.	n.a.	$0.989±0.144^{a}$	$0.380±0.169^{b}$
TLG (mm) 0-28 DAP	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	$2.070±0.210^{a}$	$0.826±0.001^{b}$
TGC 0-14 DAP	0.589±0.009	n.a.	n.a.	n.a.	n.a.	n.a.	0.596±0.080	n.a.	n.a.	n.a.	n.a.	0.605±0.042	n.a.	n.a.	n.a.	n.a.	$0.609±0.036^{a}$	$0.195±0.157^{b}$
TGC 0-28 DAP	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	$0.559±0.052^{a}$	$0.186±0.013^{b}$
CF 0 DAP	$0.963±0.000^{a}$	$0.963±0.000^{a}$	$0.963±0.000^{a}$	$0.963±0.000^{a}$	$0.963±0.000^{a}$	$0.963±0.000^{a}$	$0.759±0.000^{a}$	$0.759±0.000^{a}$	$0.759±0.000^{a}$	$0.759±0.000^{a}$	$0.759±0.000^{a}$	$0.792±0.000^{a}$	$0.792±0.000^{a}$	$0.792±0.000^{a}$	$0.792±0.000^{a}$	$0.792±0.000^{a}$	$1.073±0.000^{a}$	$1.073±0.000^{b}$
CF 14 DAP	1.3±0.2	n.a.	n.a.	n.a.	n.a.	n.a.	1.5±0.1	n.a.	n.a.	n.a.	n.a.	1.3±0.1	n.a.	n.a.	n.a.	n.a.	$1.043±0.000^{a}$	$0.845±0.064^{b}$
CF 28 DAP	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	$1.001±0.100^{a}$	$0.802±0.000^{b}$

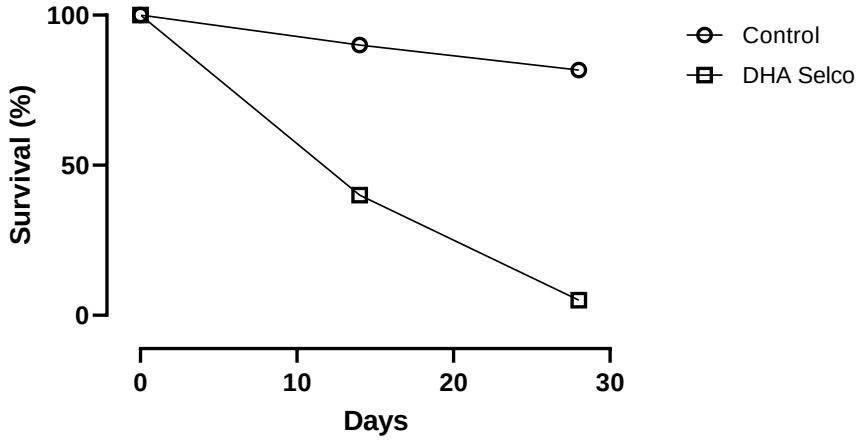

Figure 1 - Survival (t28-t0) of juveniles fed on DHA Selco® *Artemia* enrichment *and copepods as control.*

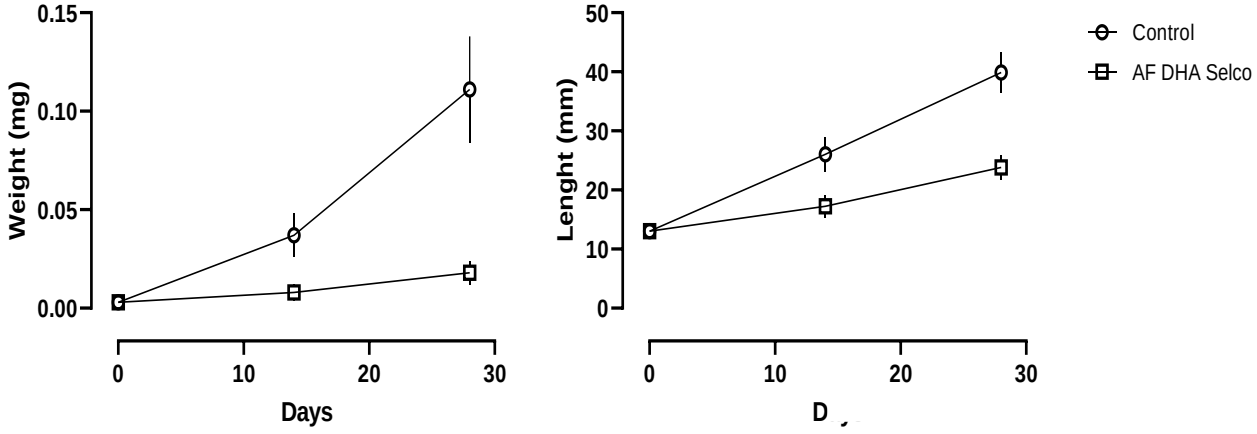

Figure 2 - Weight Gain and Length Gain (t28-t0) of juveniles fed DHA Selco® *Artemia* enrichment and copepods as control. *The vertical line represents the standard deviation.*

Significant statistical differences ($p<0.0001$) were found relative to weight, both at 14DAP and 28DAP, with the DHA Selco® treatment having a much lower overall weight gain 4.230±0.412 than control 31.784±6.171, as it can be seen in the descriptive Table 1, and Figure 2, where differences between the DHA Selco® treatment and control can be seen at 14DAP, becoming even more accentuated at 28DAP.

The same was observed regarding length gain (Figure 2), the DHA Selco® treatment had an overall growth of 0.826±0.001 and control 2.070±0.210 (Table 1). However, as it can be seen on Figure

2, the differences between these two treatments are less than those seen relative to weight gain and the juveniles fed DHA Selco® seem to follow the same growth pattern as those in control.
The overall of the Thermal Growth Coefficient and Condition Factor were also significantly higher in the copepod control, as it can be seen in the descriptive Table 1, juveniles fed the DHA Selco® treatment showed lower growth and condition at both sample periods (14DAP and 28DAP).

3.1.2 Copepod inclusion rates

An inclusion rate of 10-40% copepods and enriched krill oil *Artemia* diet was also tested. Regarding survival, both at 14DAP and 28DAP there were no significant statistical differences between the control and the 20% copepod and enriched krill oil *Artemia*, with similarities between the 20 and 30% mixture levels and control, at 28DAP (Figure 3). In this trial, at 28 DAP, it was observed that fish fed the control diet attained the highest survival (81.7%), followed by fish fed the 20% copepod inclusion diet (76.7%), whereas fish fed the 40% copepod inclusion diet attained the lowest value of 58.3% survival, as it can be seen in Table 2 and Figure 3.

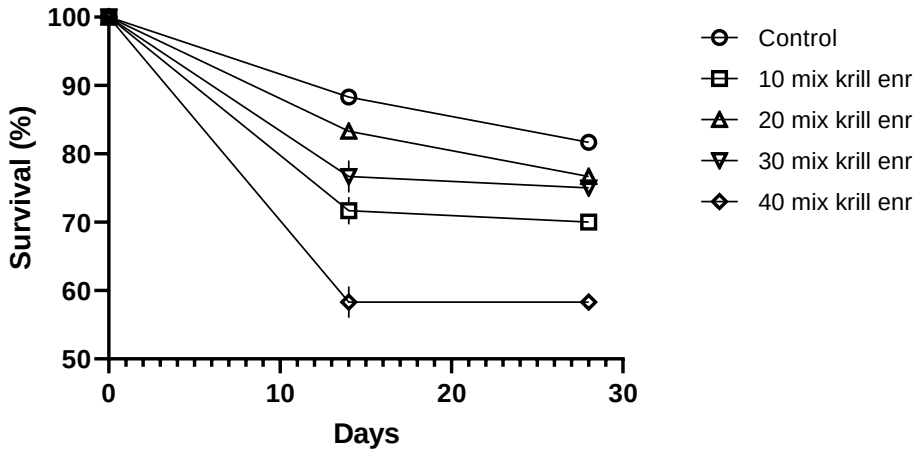

Figure 3 - Survival % (t28-t0), 10-40% copepods and enriched krill oil Artemia diet with copepod control. The vertical lines indicate the standard deviation.

Table 2 - Descriptive table of 10-40% copepods and enriched krill oil Artemia diet. S (%) – Survival, WG - Weight Gain (fish/mg), TLG - Total Length Gain (cm/fish), and TGC - Thermal Growth Coefficient were calculated for the periods between 0-14 DAP (days after parturition) and from 0 to 28 DAP. CF is the condition factor of the juveniles measured at parturition, 14 DAP and 28DAP. Mean and standard deviation (±) are showed for the calculated formulas and for each copepod proportion tested (10, 20, 30, 40%) with krill oil Artemia enrichment and control of copepods. Within the same row, different subscripts refer to significant differences.

	Control copepods	**10%**	**20%**	**30%**	**40%**
Juvenille standard weight on release (mg)	0.003±0.004	0.003±0.004	0.003±0.004	0.003±0.004	0.003±0.004
Juvenille standard weight at 14 DAP (mg)	0.037±0.011	0.021±0.009	0.028±0.009	0.034±0.009	0.037±0.012
Juvenille standard weight at 28 DAP (mg)	0.112±0.028	0.069±0.022	0.079±0.021	0.088±0.025	0.104±0.032
Juvenille standard lenght on release (mm)	13.036±0.746	13.036±0.746	13.036±0.746	13.036±0.746	13.036±0.746
Juvenille standard lenght at 14 DAP (mm)	25.986±2.988	23.490±3.759	24.011±2.712	25.948±3.506	27.311±3.352
Juvenille standard lenght at 28 DAP (mm)	39.558±3.761	35.578±4.580	36.474±3.142	37.660±4.109	39.397±3.810
S (%) 0-14 DAP	88.3^{a}	71.7^{b}	83.3^{a}	76.6^{b}	58.3^{c}
S (%) 0-28 DAP	81.7^{a}	70^{b}	76.7^{a}	75^{b}	58.3^{c}
WG (mg d-1) 0-14 DAP	$9.699±1.005^{a}$	$5.360±1.820^{b}$	$7.019±0.199^{b}$	$9.025±0.709^{a}$	$9.699±0.960^{a}$
WG (mg d-1) 0-28 DAP	$31.896±6.108^{a}$	$19.886±5.331^{b}$	$22.055±1.157^{bc}$	$25.240±4.280^{c}$	$30.251±4.349^{a}$
TLG (mm) 0-14 DAP	$0.991±0.147^{a}$	$0.840±0.280^{b}$	$0.840±0.104^{b}$	$0.994±0.133^{a}$	$1.088±0.042^{a}$
TLG (mm) 0-28 DAP	$2.042±0.195^{a}$	$1.767±0.228^{b}$	$1.798±0.018^{bc}$	$1.918±0.174^{ac}$	$2.049±0.111^{a}$
TGC 0-14 DAP	$0.610±0.039^{a}$	$0.429±0.087^{b}$	$0.508±0.008^{b}$	$0.588±0.027^{b}$	$0.612±0.033^{a}$
TGC 0-28 DAP	$0.560±0.052^{a}$	$0.459±0.031^{b}$	$0.469±0.011^{b}$	$0.501±0.042^{b}$	$0.542±0.037^{a}$
CF 0 DAP	$1.073±0.000^{a}$	$1.073±0.000^{a}$	$1.073±0.000^{a}$	$1.073±0.000^{a}$	$1.073±0.000^{a}$
CF 14 DAP	$1.326±0.193^{a}$	$0.994±0.077^{b}$	$1.279±0.191^{a}$	$1.248±0.246^{a}$	$1.141±0.126^{a}$
CF 28 DAP	$1.067±0.131^{a}$	$0.902±0.016^{b}$	$0.974±0.049^{b}$	$0.966±0.024^{b}$	$1.003±0.034^{a}$

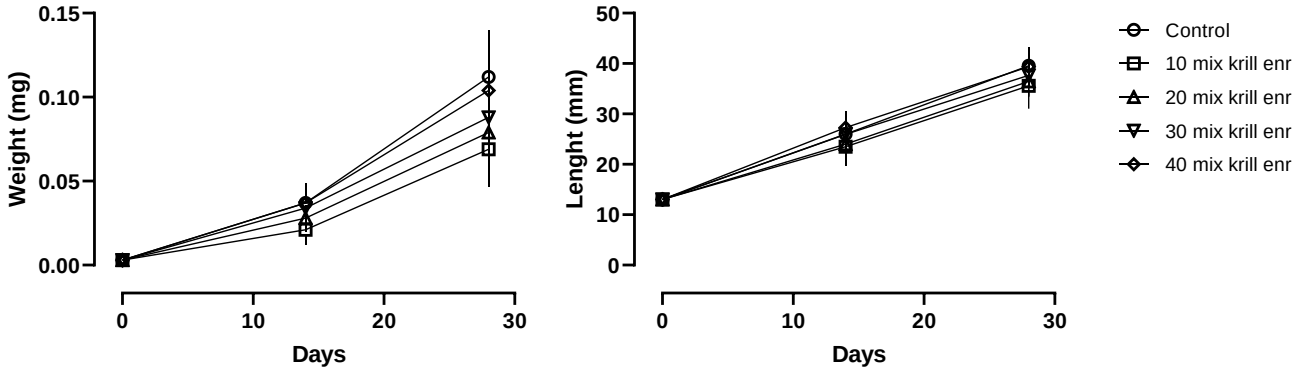

Figure 4 - Weight mg and Length mm (t28-t0), 10-40% copepods and enriched krill oil Artemia diet with copepod control. The vertical lines indicate the standard deviation.

Data on the growth performance of fish fed the different inclusion rates is reported in Table 2. At the end of the trial, no significant differences ($p>0.05$) were found between fish fed the control and the 40 % copepod inclusion rate diets. Fish fed 10, 20 and 30% copepod inclusion rates grew significantly less ($p<0.05$) (Figure 4) compared to a 40% inclusion rate. Seahorse fed the control showed the highest weight gain (WG) in the trial (31.896±6.108 mg/fish), followed by the 40 % copepod inclusion rate with a WG of 30.251±4.349 mg/fish.

Both at 14 DAP and at 28 DAP there were no significant statistical differences between control and the 40% treatment ($p>0.05$), regarding Total Length Gain (TLG), with the other treatments showing significant differences between control and the 40% treatment ($p<0.05$). This can be seen in Figure 4 and the descriptive Table 2. Generally, the best length gain results were observed in the 40% inclusion group 2.049±0.111 cm/fish, followed by control 2.042±0.195 cm/fish and 30% inclusion rate 1.918±0.174 cm/fish, with the three treatments having very similar results at 28 DAP.

Regarding the Thermal Growth Coefficient, control had the best results 0.560±0.052, closely followed by the 40% group 0.542±0.037, during this period the 10% group also had good results with a growth of 0.459±0.031 and the 30 % group with 0.501±0.042. As for the Condition Factor, in the overall of the trial control showed the best condition, with a value of 1.067±0.131, followed by 40%, with 1.003±0.034. This can be seen in the descriptive Table 2.

After this trial, a second one was conducted, where inclusion rates of 2.5-40% copepods and enriched DHA Selco® *Artemia* diet were tested. As it can be seen on Figure 5, the 20% and 30% treatments had a survival higher, but similar, to control. In the totality of the trial, the 30% group (81.7%) and 20% group (80%) showed the highest survivals, followed by the control (73.3%). As the percentage of copepods in the feed decreased so did survival, with the lowest levels of copepod inclusion having the lowest survivals. However, the 7.5% group showed a survival of 58.3%, higher than the 10% treatment (Table 3).

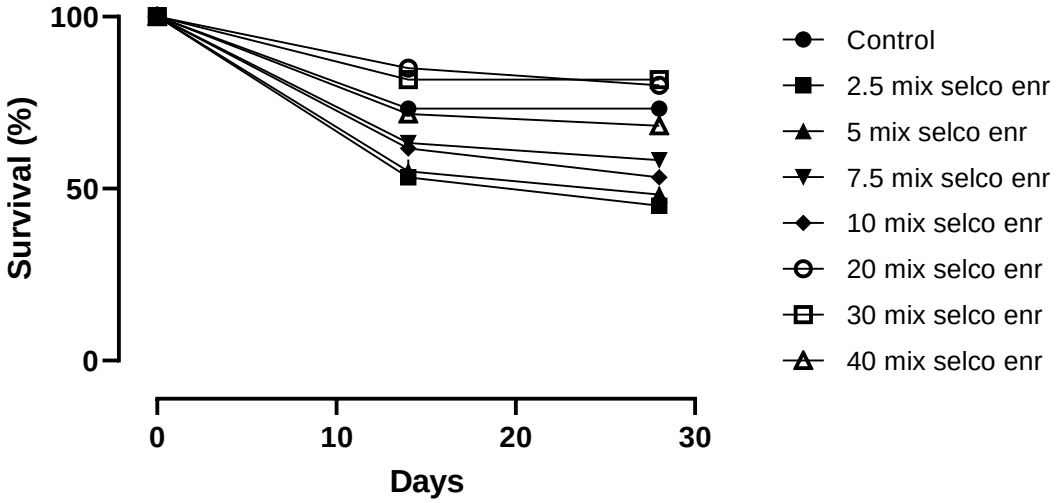

Figure 5 - Survival % (t28-t0), 2.5-40% copepods and enriched DHA Selco® Artemia diet with copepod control. The vertical lines indicate the standard deviation.

Table 3 - Descriptive table of 2.5-40% copepods and enriched DHA Selco® Artemia diet. S (%) – Survival, WG - Weight Gain (fish/mg), TLG - Total Length Gain (cm/fish), and TGC - Thermal Growth Coefficient were calculated for the periods between 0-14 DAP (days after parturition) and from 0 to 28 DAP. CF is the condition factor of the juveniles measured at parturition, 14 DAP and 28DAP. Mean and standard deviation (±) are showed for the calculated formulas and for each copepod proportion tested (2.5, 5, 7.5, 10, 20, 30, 40%) with DHA Selco® *Artemia* enrichment and control of copepods. Within the same row, different subscripts refer to significant differences.

	Control copepods	**2.5%**	**5%**	**7.5%**	**10%**	**20%**	**30%**	**40%**
Juvenille standard weight on release (mg)	0.003±0.002	0.004±0.004	0.004±0.004	0.004±0.004	0.003±0.001	0.003±0.001	0.003±0.001	0.003±0.001
Juvenille standard weight at 14 DAP (mg)	0.039±0.014	0.014±0.006	0.018±0.008	0.022±0.006	0.024±0.010	0.023±0.007	0.043±0.013	0.038±0.013
Juvenille standard weight at 28 DAP (mg)	0.103±0.041	0.052±0.026	0.055±0.024	0.059±0.018	0.079±0.029	0.071±0.023	0.094±0.025	0.115±0.041
Juvenille standard lenght on release (mm)	13.438±0.693	13.181±0.753	13.181±0.753	13.181±0.753	13.696±0.666	13.696±0.666	13.696±0.666	13.696±0.666
Juvenille standard lenght at 14 DAP (mm)	33.980±3.864	23.772±3.314	25.429±3.453	27.128±2.815	30.177±3.802	29.751±3.400	33.252±2.955	33.486±3.525
Juvenille standard lenght at 28 DAP (mm)	48.433±8.701	32.662±9.899	32.967±6.052	33.077±3.448	43.198±4.521	42.660±4.818	45.293±4.733	47.688±5.463
S (%) 0-14 DAP	73.3^{a}	53.3^{b}	55^{b}	63.3^{c}	61.7^{c}	85^{ad}	81.7^{ad}	71.7^{a}
S (%) 0-28 DAP	73.3^{a}	45^{b}	48.3^{b}	58.3^{c}	53.3^{bc}	80^{ad}	81.7^{ad}	68.3^{a}
WG (mg d-1) 0-14 DAP	$14.197±4.810^{a}$	$2.809±0.092^{bc}$	$4.484±2.104^{bcd}$	$5.003±0.894^{bd}$	$8.667±1.073^{bd}$	$7.922±1.419^{bd}$	$16.165±3.208^{ae}$	$14.757±3.838^{be}$
WG (mg d-1) 0-28 DAP	$50.344±13.011^{a}$	$13.845±2.408^{bc}$	$15.371±3.973^{bc}$	$15.431±1.034^{bcd}$	$31.389±9.578^{bde}$	$27.451±5.884^{bcde}$	$36.438±6.103^{be}$	$47.214±16.296^{b}$
TLG (mm) 0-14 DAP	$1.545±0.081^{a}$	$0.799±0.136^{b}$	$0.981±0.225^{bc}$	$1.057±0.053^{c}$	$1.214±0.067^{d}$	$1.187±0.126^{d}$	$1.433±0.065^{a}$	$1.489±0.199^{a}$
TLG (mm) 0-28 DAP	$2.645±0.411^{a}$	$1.570±0.709^{b}$	$1.554±0.337^{b}$	$1.511±0.017^{b}$	$2.196±0.294^{cd}$	$2.152±0.225^{c}$	$2.317±0.100^{acd}$	$2.540±0.333^{ad}$
TGC 0-14 DAP	$0.693±0.082^{a}$	$0.292±0.006^{b}$	$0.390±0.112^{b}$	$0.426±0.048^{bc}$	$0.502±0.038^{c}$	$0.475±0.049^{c}$	$0.700±0.074^{a}$	$0.667±0.088^{a}$
TGC 0-28 DAP	$0.664±0.041^{a}$	$0.380±0.033^{b}$	$0.401±0.055^{b}$	$0.405±0.014^{b}$	$0.494±0.073^{c}$	$0.462±0.046^{b}$	$0.532±0.043^{c}$	$0.596±0.091^{a}$
CF 0 DAP	$0.930±0.208^{a}$	$0.930±0.208^{a}$	$0.930±0.208^{a}$	$0.930±0.208^{a}$	$0.930±0.208^{a}$	$0.930±0.208^{a}$	$0.930±0.208^{a}$	$0.930±0.208^{a}$
CF 14 DAP	$0.677±0.014^{a}$	$0.665±0.150^{b}$	$0.670±0.022^{b}$	$0.670±0.047^{b}$	$0.542±0.009^{c}$	$0.523±0.030^{c}$	$0.714±0.101^{a}$	$0.606±0.030^{b}$
CF 28 DAP	$0.782±0.020^{a}$	$1.001±0.517^{ac}$	$0.938±0.172^{ac}$	$0.982±0.042^{ac}$	$0.565±0.054^{b}$	$0.523±0.033^{b}$	$0.587±0.063^{b}$	$0.605±0.062^{b}$

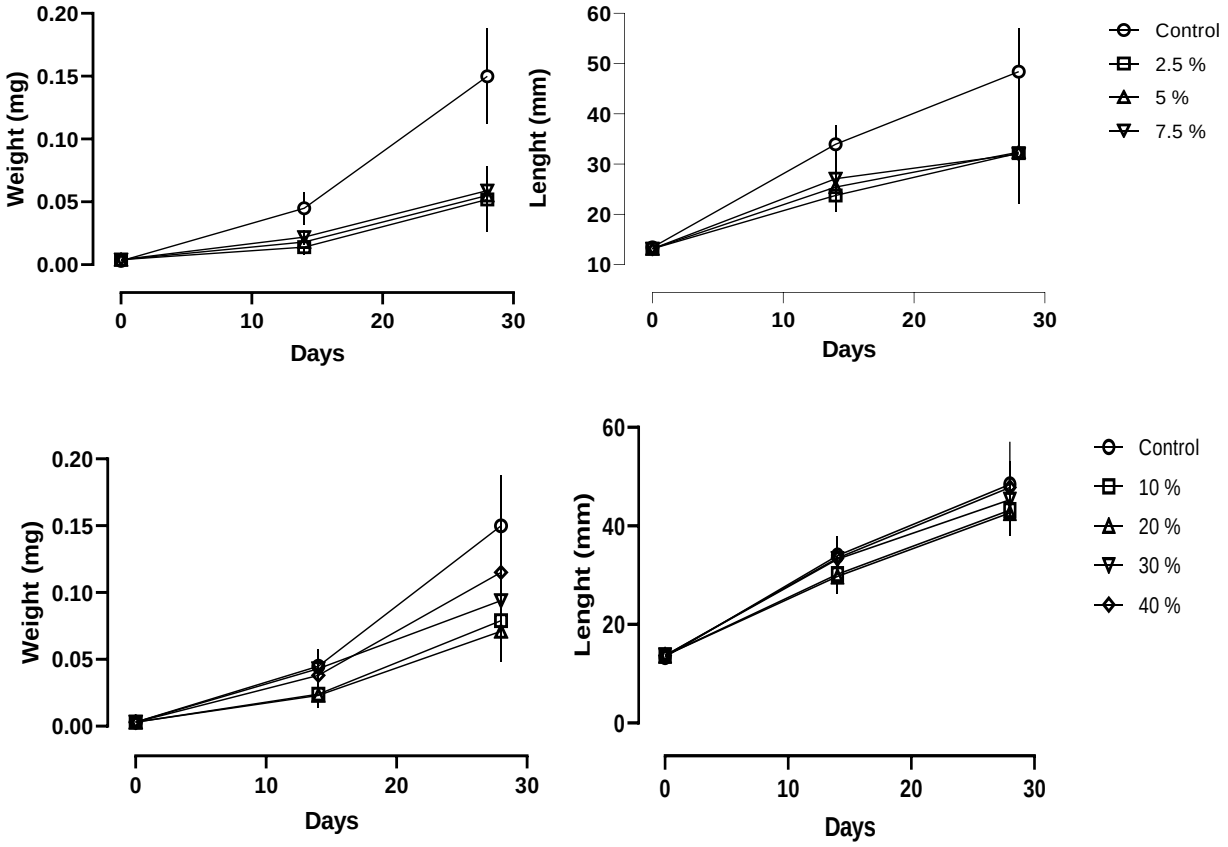

Figure 6 - Weight mg and Length mm (t28-t0), 2.5-40% copepods and enriched DHA Selco® Artemia diet with copepod control. The vertical lines indicate the standard deviation.

As for Weight gain (WG), at 14DAP there were no significant statistical differences ($p>0.05$) between control and the 30%, but as the trial continued the differences became more accentuated, and at 28DAP significant differences can be seen between the treatments and the control ($p<0.05$), with treatments 2.5-7.5% showing similar results, as did the 10% and 20%, and the 30% and 40% treatments (Figure 6). Overall, the control diet provided the best results 50.344±13.011 mg/fish followed by the group 40% 47.214±16.296 mg/fish, as the proportion of copepods in the feed decreased so did weight (e.g. 7.5% treatment 15.431±1.034 mg/fish), as can be seen in the descriptive Table 3 and Figure 6.

In the descriptive Table 3 and Figure 6, it can also be seen that regarding Length gain (TLG), the trial had the best results in control 2.645±0.411 cm/fish, followed by 40% 2.540±0.333 cm/fish and the 30% group 2.317±0.100 cm/fish, without significant statistical differences between control

and the 30% and 40% ($p>0.05$) treatments. Treatments with a percentage of copepods from 2.5% to 20% showed significant differences ($p<0.05$) between the control at both sampling times (14 and 28 DAP), with more accentuated differences in the feeds with the lowest percentages of copepods, becoming even more heightened with the continuity of the trial (Figure 6). However, low percentages of copepods still obtained a relatively good length gain (TLG), like the 2.5% group (1.570±0.709 cm/fish) and 5% (1.554±0.337 cm/fish) (Table 3).

As for the Thermal-Growth coefficient, the highest values were observed in control 0.664±0.041 and in the 40% group 0.596±0.091, followed by the 30% group 0.532±0.043. In the Condition factor the inverse was observed, with lower levels of copepods in the feed resulting in individuals with a better condition; individuals with the best condition were obtained in the 7.5% inclusion rate 0.982±0.042, followed by the 2.5% group 1.001±0.517 and 5% groups 0.982±0.042. This can be seen in the descriptive Table 3.

In order to identify the correct value of b to be used in the Condition factor (Equation 4) calculations, a Weight-Length relationship was performed (Figure 7) in all the juveniles from the control groups of the experiments, a total of 755 fish were measured and taken into account for the calculations, resulting in a b value of 3.19.

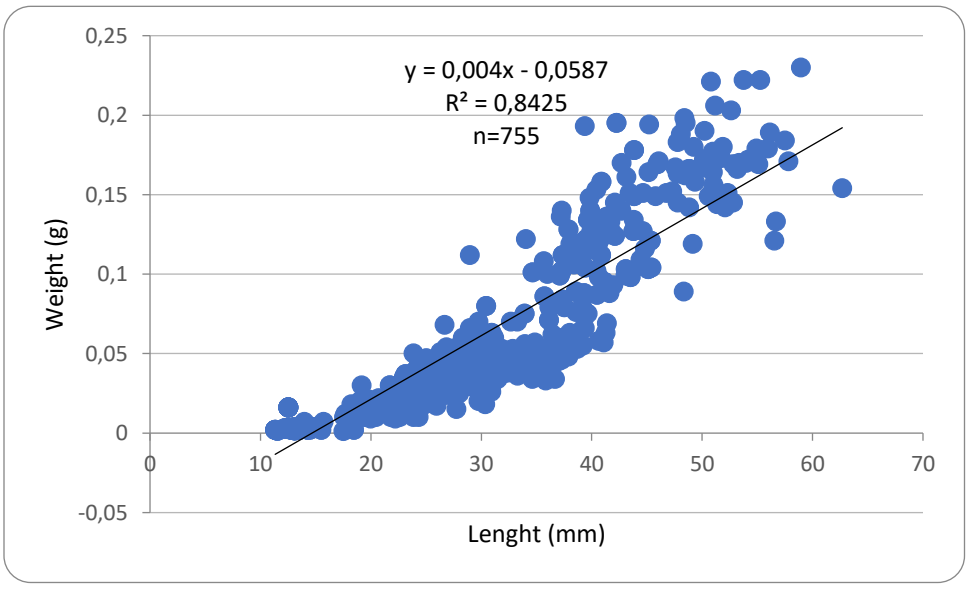

Figure 7 – Weight-Length relationship of H. hippocampus juveniles in the control groups of the experiments.

4 DISCUSSION

In the first experiment, both *Artemia* enrichments tested (cod liver oil and krill oil) failed to provide sustainable growth and survival for juvenile *H. hippocampus*, as only the seahorses fed the copepod control diet survived after the first week of the trial. This implied a significant difference between the tested dietary treatments, thus concluding that these feeds are unfit to fulfil *H. hippocampus* nutritional requirements. In this experiment, two sizes of *Artemia* were used. *Artemia* AF of smaller size on hatch (used in an attempt to provide prey of adjusted size to juvenile *H. hippocampus*), and *Artemia* EG with a larger size on hatch. In the first, as it hatches with a smaller size, this implies that the granted 24-hours enrichment period would be shorter, while the second, for having a larger size on hatch, could complete a full 24-hours enrichment period. Considering the results, it was found that neither the different size of the strains used, nor the enrichment period had a significant effect on the growth and survival of juvenile seahorse, continuing the trials with *Artemia* AF.

Some commercial products have shown good results in larvae and juvenile feeding of fish species (e.g. Pham and Lin, 2013; Watanabe *et al*., 2016; Woods, 2003). Considering the poor performance of the previously used emulsions, a trial using DHA Selco® was performed. When differences were tested between a copepod control diet and a feed of 10mg/L DHA Selco® enriched *Artemia* AF, regarding survival, the DHA Selco® enrichment worked to a certain point, but significant differences were found ($p<0.05$) in all periods sampled and indexes calculated, having a much lower performance than control. Palma *et al*. (2011), found that *H. guttulatus* juveniles fed *Artemia* enriched with DHA Selco had 100% mortality at 10DAP and a much higher incidence of gas bladder disorders than juveniles fed copepods. The fact that in the present trial there were juveniles alive at 28DAP in the DHA Selco treatment shows that there might be some differences between *H. hippocampus* and *H. guttulatus* relative to prey acceptance and nutritional requirements, with *H. hippocampus* being more tolerant of this commercial product.

The diets with 10-40% copepod inclusion rates and krill oil enriched *Artemia* were the ones with the best allometric growth and condition factor results and has similar results as the same percentages of copepods when used with enriched DHA Selco® *Artemia*. Out of all the treatments tested, the most feasible and with the best condition is a feed of 30 to 40% copepods and DHA Selco® *Artemia* nauplii, with similar results to control. It was still possible to obtain reasonable levels of growth and survival with proportions of 2.5-7.5% copepods and enriched DHA Selco®

Artemia. Low percentages of copepods with enriched DHA Selco®, like the 7.5% and 10% still showed good survival, having almost half the juveniles alive at 28DAP; the most significant differences can be seen in weight gain, when comparing lower percentages of copepods to higher levels. Therefore, in order to increase survival and obtain healthy juveniles, a feed of 30% or 40% copepods supplemented with enriched Krill oil *Artemia* or 40% copepods supplemented with enriched DHA Selco® *Artemia* are the more suitable feeds to use as an alternative to 100% copepods. This decreases significantly decreases the daily concentration of copepods needed, as well as less contaminants from these, since they are harvested from natural ponds and can induce pathogens in the system. However, different *Artemia* enrichments still need to be tested on the survival and growth performance of *H. hippocampus*, since a more suitable option could decrease or completely eliminate the need to incorporate copepods in the diet.

In previous studies, relatively high survival and growth rates for seahorse juveniles fed with copepod diets have been observed (Payne and Rippingale, 2000; Olivotto *et al*., 2008; Celino *et al*., 2012; Schubert *et al*., 2016).

Economically, culturing calanoid copepods at high densities has yet to be achieved (Olivotto *et al*., 2008) and collecting wild plankton can be labor intensive and introduce harmful pathogens and/or unwanted organisms into aquaculture systems. Wild caught plankton also varies temporally and spatially, making it difficult to manage and replicate, and copepod-based rearing is expensive due to fluctuating in survival and reproduction rates within copepod cultures (Pham and Lin, 2013), as well as labor intensive when collected from the wild (Schubert *et al*., 2016). Therefore, seahorse larval feeding protocols are commonly based on a mixed diet of rotifers, copepods, and/or strains of the brine shrimp *Artemia spp*. However, as *Artemia* nauplii display a deficiency in HUFAs (Sorgeloos *et al*., 2001; Otero-Ferrer *et al*., 2010), they are usually enriched by incubation in special HUFA solutions (Job *et al*., 2002; Woods, 2003; Wong and Benzie, 2003; Palma *et al*., 2014; Schubert *et al*., 2016). It is therefore of the outmost importance to become copepod independent or to decrease the density of copepods necessary to obtain high survival and growth while rearing *H. hippocampus.*

The importance of highly unsaturated fatty acids (HUFA) in *Hippocampus* spp. has been demonstrated. For example, it was found that juvenile *H. abdominalis* growth increased when fed-enriched *Artemia* with higher levels of arachidonic acid (AA), eicosapentanoic acid (EPA) and somewhat smaller amounts of docosahexanoic acid (DHA) (Woods, 2003). As DHA, EPA, and AA play important roles as constituents in bio membranes and in immune response (Sargent *et al*., 1999b; Watanabe, 1993) this rely as the probable explanation for these findings. In fish nutrition,

DHA is selectively retained in the polar lipids of developing larvae during starvation, and when it is fed it is selectively incorporated into the larval glycerophospholipids, essential components of biological membranes (Izquierdo, 1996). Chang and Southgate (2001) also found a significant correlation between mean survival of *Hippocampus* spp. juveniles and dietary n3-HUFA, EPA, and DHA contents. Seahorse embryos have a high metabolic turnover of DHA, AA, EPA, and C16:0 (Palmitic-acid) resulting in a low amount of lipids and largely depleted HUFA contents in new-born juveniles. They are completely dependent on exogenous food resources, as marine fishes are neither able to synthesize DHA *de novo* or to convert it from precursors in sufficient amount (Sargent *et al*., 1999b). Furthermore, an undersupply of DHA can cause malformation, slower growth, and enhanced mortality in fishes; high mortalities of *Hippocampus* spp., generally observed during the first week of life, might be related to the exhaustion of the juvenile's fatty acid (Schubert *et al.,* 2016; Sheng *et al*., 2007; Shields *et al*., 1999). Faleiro and Narciso (2010) showed that PUFA constitutes the major source of metabolic energy and the fatty acids 16:0, EPA and DHA are the main fatty acids to fulfil the energetic demands of seahorse embryos.

Payne and Rippingale (2000) showed that their copepod and *Artemia f*eeds contained overall similar HUFA amounts, but that copepods had five times the DHA content than the *Artemia* feeds. This would indicate that the superior effect of the copepod feed might be largely due to its high DHA amount. It could also be related to copepods having HUFAs mainly in the polar lipid fraction, while the HUFAs in *Artemia* nauplii these are mainly in the neutral lipid faction, and HUFAs in the polar lipid fraction are better available and utilizable than esterified HUFAs within triacylglycerols (Schubert *et al*., 2016; Sorgeloos *et al*., 2001). As krill oil is also rich in DHA, containing phospholipid, di- and tri-glycerides as well as non-esterified fatty acid forms and primarily triglycerides (Salem and Kuratko, 2014) that was the underlying reason it was used in this study.

However, other key components like protein, vitamins and antioxidants might also have a key role in successful juvenile rearing, helping with optimal growth. Schubert *et al*. (2016) fed *H. reidi* with *Artemia* enriched containing higher values in HUFA and DHA than the copepods used in this study. However, the copepod treatment showed the best results in growth and survival, showing that feed species might also be a determining factor. Hence, these factors should be considered for further studies.

Since this study was performed with a protected species and aiming towards conservation, the sacrifice of individuals throughout the experiment in order to perform histology and carcass

analysis was avoided, although the biochemical analysis would be an interesting factor to consider in order to clarify the prey/predator nutrient shift and assimilation. This investigation dealt only with the effects of varying *Artemia* enrichment on growth and survival of *H. hippocampus*. In the present study, a biochemical analysis of the live prey would have been beneficial and should be performed in further experiments. The nutritional analysis of the copepods and *Artemia* could have provided a better idea if the enrichments were done properly (Otero-Ferrer *et al.*, 2010), especially considering that the enrichments prepared in the laboratory could have been degrading over time, or not being incorporated properly in the *Artemia*, unlike DHA Selco®, which is a manufactured and commercially distributed emulsion and has been seen to show better results with other species. This could provide information regarding the nutritional profile of the harvested copepods and their potential properties that favour juvenile seahorse's growth, when compared to other crustacea and most life feed enrichments. The *Artemia (*both *AF* and EG) enriched with the different media should have also been analysed, to assess its effectiveness and if the necessary nutrients, especially the essential fatty acids were properly incorporated into the prey and if there were significant differences in the nutritional profile. However, the obtained data on the growth and survival of juvenile *H. hippocampus* serve as very good indicator of the nutritional quality of the different tested diets.

Larvae have different specificities in digestion and nutritional requirements when compared to juveniles. Compound diets can slightly delay the onset secretion mechanisms and therefore enzymatic function, as well as maturation of enterocytes, which are related to normal peptidase activity; despite the fact that the use of natural products and/or manufactured enrichments can affect and delay the digestive process, certain enrichments provide acceptable growth and survival results, even if they are not the most suitable option (Cahu and Zambonino Infante, 2001).

Natural microalgae enrichments such as *Chlorella sp*., have been used to rear *H. guttulatus* (Palma *et al*., 2011) and *H. erectus* (Zhang et al. 2010), although these still have major setbacks due to variability in n-3 HUFA content under different conditions and increase in labor. Thus, additional studies using various commercially available enrichments should be performed because they are generally more convenient and cheaper to use (Pham and Lin, 2013; Shapawi and Purser, 2003). Pham and Lin (2013) tested the effects of live feed enrichment on newly released *H. reidi* fed with rotifers and *Artemia* enriched with Dan's Feed®, a commercially available product, which outperformed those that were fed with rotifers and *Artemia* enriched with the algae *Isochrysis galbana*. Otero-Ferrer *et al*. (2010) also tried to replace copepods by testing *Artemia* and rotifers diets, obtaining good survival (60%) in the first. In a similar study with *H. reidi*, Schubert *et al.*

(2016) started the feeding trials at 8 DAP, after the brood fluctuations stabilize, with enriched Selco S.presso® *Artemia* feed and a feed composed of a mixture of copepods and *Artemia* nauplii. At this point of ontogeny perhaps the juveniles are more fit to sustain *Artemia* as a prey, since mouth size could be a factor that affect the ingestion of prey. Payne and Rippingale (2000), for example, reported for *H. subelongatus* survival rates after day 14 of >80% for specimens fed with copepods compared to >40% for those fed with *Artemia* nauplii, and Job *et al.* (2002) noted survival rates for *H. kuda* ranging between 40 and 75%, depending on type of enrichment (Schubert *et al.*, 2016).

High size variations within reared groups of fish are frequently associated with stocking density (Irwin *et al.*, 1999) and seahorses seem to be no exception. In fact, higher survival rates have been reported when culturing seahorses at densities as low as ≤1 individual L^{-1} (Job *et al.*, 2006; Lin *et al.*, 2008). This might be also related to post hatching behaviour, since seahorses use their tails for anchoring. At higher densities this behaviour can cause interference amongst co-specifics. Thus, low density culture conditions were used in the present study (~2 individuals L^{-1}), but despite that, high variations both in length and weight were registered at the end of the experiment, fact that have also been reported for other seahorse species like *H. erectus* and *H. guttulatus* (Lin *et al.*, 2008; Palma *et al*., 2014). In commercial seahorse culture, low survival, particularly in the early juvenile stages, is still one of the bottlenecks affecting commercial economic return. Although high survival rates of several seahorse species in the early juvenile stages have been reported (e.g., *H. abdominalis* (Woods, 2003), *H. erectus* (*Lin et al.*, 2008), and *H. comes* (Job *et al*., 2006), these have been obtained at stocking densities around or lower than 1 individual L^{-1}, which may be too low for economically viable commercial mass production (Palma *et al*., 2014).

In addition to the effect of prey nutritional value on animal growth, prey densities can also affect growth rate by affecting assimilation efficiencies. *Artemia* can be harder for fish to digest compared to other live prey such as copepods, and when prey densities are high may actually pass through the digestive tract intact (Payne and Rippingale, 2000) and sometimes even still alive (Woods, 2003). *H. hippocampus* was fed *ad libitum* in the trials conducted, at a density near 2 prey/mL, and an optimal prey digestion and absorption might not have occurred, leaving the full beneficial contents of the enrichments used might have not been realised, hence the digestive capacity and activity through ontogeny for new-born *H. hippocampus* should be studied. In the wild, new-born seahorses may be selecting certain prey items over others; therefore, conducting gut content analysis to evaluate new-born prey preference through ontogeny will also be advantageous (Pham and Lin, 2013).

The gut and faeces analyses of species like *H. subelongatus* and *H. guttulatus* have shown a better digestibility of copepods compared to enriched *Artemia* nauplii, with *Artemia* sometimes still alive and intact within the faeces (Payne and Rippingale, 2000; Schubert *et al*., 2016). The superior digestibility of copepods may be ascribed to the evolutionary adaptation of the digestive tract and enzymatic activity of seahorses to copepods, as they constitute a major part of the natural diet of most seahorse juveniles (Tipton and Bell, 1988; Castro *et al*., 2008; Schubert *et al*., 2016). It is possible that a nutritional deficiency related to HUFA levels in prey can cause alteration in juvenile seahorses' behaviour, like reduction in swimming and feeding activity, as well as a delay in the settlement process, since in *Artemia* nauplii feeding, the prey is eaten but their nutrients are not completely assimilated (Koldewey and Martin-Smith, 2010; Otero-Ferrer *et al.*, 2010). Additionally, Palma *et al.* (2014) found that *H. guttulatus* juveniles fed on enriched *Artemia* nauplii had a higher percentage of gas bubble infections compared to those fed on copepods, suggesting that seahorse feed should be as similar as possible as that found in the natural habitat in order to obtain healthy individuals.

Evaluating the micro-components, such as HUFAs and polyunsaturated fatty acids, vitamins, and essential amino acids of feed items enriched with DHA Selco® as well as the emulsion of krill oil and contrasting these to wild caught plankton may reveal the key dietary components that promote growth and survivorship in *H. hippocampus*. Identifying these components is crucial to developing highly targeted feeds that will promote high survivorship and fast growth, which are vital to the success of aquaculture enterprises (Pham and Lin, 2013).

5 CONCLUSION

Unenriched *Artemia* nauplii on its own it is not a suitable alternative to copepods for *H. hippocampus* juveniles and need to be supplemented with copepods, with higher levels of the latest providing the best results in growth and condition of fish. *Artemia* EG is also a live feed that is not suitable, producing even worse results that *Artemia* AF, which could be due to size of prey being unfit for the juvenile's mouth at parturition or the enrichment being expended by the *Artemia* metabolism.

Despite it not have been possible to analyse the lipidic profile of the live feed prey, it is possible to conclude that none of the used enrichments would work on its own and the feeding of *H. hippocampus* juveniles needs to be supplemented with copepods, with a 40% mixture of copepods

with enriched krill oil *Artemia* AF providing results similar to a diet of 100% copepods, only being possible to determine what is the component they require with further analysis of their HUFA profile. Knowing which levels of HUFAs and other components provide an optimal growth and survival in seahorses is the next step, allowing the production of emulsions with the required nutritional profile, facilitating the rearing and production of this species on a commercial scale, lowering the impact on wild populations by providing another product source and a tool that can be of great use for conservation, by restocking populations.

6 REFERENCES

Ajiboye, O., Yakubu, A., Adams, T., Olaji, E. and Nwogu, N. (2011). A review of the use of copepods in marine fish larviculture. *Reviews in Fish Biology and Fisheries*, 21(2), pp.225-246.

Blanco, A. (2014). Rearing of the seahorse *Hippocampus guttulatus:* Key factors involved in growth and survival. *Ph.D, pp.293. Universitat de les Illes Balears*.

Bell, E., Lockyear, J., McPherson, J., Dale Marsden, A. and Vincent, A. (2003). First field studies of an endangered South African seahorse, *Hippocampus capensis*. *Environmental Biology of Fishes*, 67(1), pp.35-46.

Cahu, C. and Zambonino Infante, J. (2001). Substitution of live food by formulated diets in marine fish larvae. *Aquaculture*, 200(1-2), pp.161-180.

Castro, A., Diniz, A., Martins, I., Vendel, A., Oliveira, T. and Rosa, I. (2008). Assessing diet composition of seahorses in the wild using a non-destructive method: *Hippocampus reidi* (Teleostei: *Syngnathidae*) as a study-case. *Neotropical Ichthyology*, 6(4), pp.637-644.

Celino, F., Hilomen-Garcia, G. and del Norte-Campos, A. (2012). Feeding selectivity of the seahorse, *Hippocampus kuda*, (Bleeker), juveniles under laboratory conditions. *Aquaculture Research*, 43(12), pp.1804-1815.

Chang, M. and Southgate, P. (2001). Effects of varying dietary fatty acid composition on growth and survival of seahorse, *Hippocampus sp*., juveniles. *Aquarium Sciences and*

Conservation, 3, pp.205-214.

Cho, C.Y. (1990). Fish nutrition, feeds and feeding: with special emphasis on salmonid aquaculture. *Food Rev*iews *International*, 6, pp.333–357.

Cohen, F., Valenti, W., Planas, M. and Calado, R. (2017). Seahorse aquaculture, biology and conservation: Knowledge gaps and research opportunities. *Reviews in Fisheries Science & Aquaculture*, 25(1), pp.100-111.

Correia, M. (2014). Trends in seahorse abundance in the Ria Formosa, South Portugal: recent scenario and future prospects. *Ph.D, pp148. Universidade of Algarve*.

Curtis, J. (2006). A case of mistaken identity: skin filaments are unreliable for identifying *Hippocampus guttulatus* and *Hippocampus hippocampus*. *Journal of Fish Biology*, 69(6), pp.1855-1859.

Curtis, J. and Vincent, A. (2005). Distribution of sympatric seahorse species along a gradient of habitat complexity in a seagrass-dominated community. *Marine Ecology Progress Series*, 291, pp.81-91.

Dhert, P. and Sorgeloos, P. (1995). Live feeds in aquaculture. *Infofish International*, 2, pp.31-39.

Drillet, G., Jorgensen, N., Sorensen, T., Ramlov, H. and Hansen, B. (2006). Biochemical and technical observations supporting the use of copepods as live feed organisms in marine larviculture. *Aquaculture Research*, 37(8), pp.756-772.

Dytham, C. (2003). Choosing and using statistics. *Oxford: Blackwell*, pp.98-109.

Faleiro, F. and Narciso, L. (2010). Lipid dynamics during early development of *Hippocampus guttulatus* seahorses: Searching for clues on fatty acid requirements. *Aquaculture*, 307 (1-2), pp.56-64.

FAO (2018). The State of World Fisheries and Aquaculture 2018 - Meeting the sustainable development goals. Rome. Licence: CC BY-NC-SA 3.0 IGO.

Foster, S.J. (2016). Seahorses *(Hippocampus spp.)* and the CITES review of significant trade. *Fisheries Centre Research Reports* 24(4): 48 pp.

Foster, S. and Vincent, A. (2004). Life history and ecology of seahorses: implications for conservation and management. *Journal of Fish Biology*, 65(1), pp.1-61.

Foster, S., Kuo, T., Wan, A. and Vincent, A. (2019). Global seahorse trade defies export bans under CITES action and national legislation. *Marine Policy*, 103, pp.33-41.

Fowler, J. and Cohen, L. (1990). Practical statistics for field biology. *Milton Keynes (Pa.): Open UP*, pp.87-89.

Gardner, T. (2004). The copepod/*Artemia* trade-off in the captive culture of *Hippocampus erectus*, a vulnerable species in New York State. *Master of Science*, pp.42. *Hofstra University*.

Guinot, D., Monroig, Ó., Hontoria, F., Amat, F., Varó, I. and Navarro, J. (2013). Enriched on-grown *Artemia* metanauplii actively metabolise highly unsaturated fatty acid-rich phospholipids. *Aquaculture*, 412-413, pp.173-178.

Ighwela, K., Ahmed, A. and Abol-Munafi, A. (2011). Condition factor as an indicator of growth and feeding intensity of Nile tilapia fingerlings (*Oreochromis niloticus*) feed on different levels of maltose. *American-Eurasian Journal of Agriculture and Environmental Sciences*, 11(4), pp.559-563.

Irwin, S., O'Halloran, J. and FitzGerald, R. (1999). Stocking density, growth and growth variation in juvenile turbot, *Scophthalmus maximus* (Rafinesque). *Aquaculture*, 178(1-2), pp.77-88.

IUCN (2019). The IUCN Red List of Threatened Species. Version 2018-2. [online] Available at: http://www.iucnredlist.org [Accessed Jan. 2019].

Iwama, G.K. and Tautz, A.F. (1981). A simple growth model for salmonids in hatcheries. *Canadian Journal of Fisheries and Aquatic Sciences,* 38, pp.649–656.

Izquierdo, M. (1996). Essential fatty acid requirements of cultured marine fish larvae. *Aquaculture Nutrition*, 2(4), pp.183-191.

Job, S., Buu, D. and Vincent, A. (2006). Growth and survival of the tiger tail seahorse, *Hippocampus comes*. *Journal of the World Aquaculture Society*, 37(3), pp.322-327.

Job, S., Do, H., Meeuwig, J. and Hall, H. (2002). Culturing the oceanic seahorse, *Hippocampus kuda*. *Aquaculture*, 214(1-4), pp.333-341.

Kachari, A., Abujam, S. and Das, D. (2017). Length- weight relationship (LWR) and condition factor of *Amblyceps apangi*. *Journal of Aquaculture Engineering and Fisheries Research*, pp.97-107.

Kitsos, M., Tzomos, T., Anagnostopoulou, L. and Koukouras, A. (2008). Diet composition of the seahorses, *Hippocampus guttulatus (*Cuvier, 1829) and *Hippocampus hippocampus* (L., 1758) (Teleostei, Syngnathidae) in the Aegean Sea. *Journal of Fish Biology*, 72(6), pp.1259-1267.

Koldewey, H. and Martin-Smith, K. (2010). A global review of seahorse aquaculture. *Aquaculture*, 302(3-4), pp.131-152.

Kuo, T. and Vincent, A. (2018). Assessing the changes in international trade of marine fishes under CITES regulations – A case study of seahorses. *Marine Policy*, 88, pp.48-57.

Lin, Q., Lin, J. and Zhang, D. (2008). Breeding and juvenile culture of the lined seahorse, *Hippocampus erectus* (Perry, 1810). *Aquaculture*, 277(3-4), pp.287-292.

Linder, M., Belhaj, N., Sautot, P. and Arab Tehrany, E. (2010). From Krill to Whale: an overview of marine fatty acids and lipid compositions. *Oléagineux, Corps gras, Lipides*, 17(4), pp.194-204.

Lindley, L., Phelps, R., Davis, D. and Cummins, K. (2011). Salinity acclimation and free amino acid enrichment of copepod *nauplii* for first-feeding of larval marine fish. *Aquaculture*, 318(3-4), pp.402-406.

Lourie, S. (2017). Seahorses: A life-size guide to every species. *Chicago (Illinois): University of Chicago Press*. ISBN: 978-0-226-33841-5 (hc); 978-0-226-33855-2 (eb). 2017. *The Quarterly Review of Biology*, 92(2), pp.211-211.

Lourie, S., Foster, S., Cooper, E. and Vincent, A. (2004). A Guide to the identification of seahorses. *Project Seahorse and TRAFFIC North America.* Washington D.C.: University of British Columbia and World Wildlife Fund.

Lourie, S., Pollom, R. and Foster, S. (2016). A global revision of the seahorses *Hippocampus*

Rafinesque 1810 (Actinopterygii: Syngnathiformes): Taxonomy and biogeography with recommendations for further research. *Zootaxa*, 4146(1), p.1.

Martinez-Cardenas, L. and Purser, G. (2007). Effect of tank colour on *Artemia* ingestion, growth and survival in cultured early juvenile pot-bellied seahorses (*Hippocampus abdominalis*). *Aquaculture*, 264(1-4), pp.92-100.

Olivotto, I., Avella, M., Sampaolesi, G., Piccinetti, C., Navarro Ruiz, P. and Carnevali, O. (2008). Breeding and rearing the long snout seahorse *Hippocampus reidi*: Rearing and feeding studies. *Aquaculture*, 283(1-4), pp.92-96.

Olivotto, I., Planas, M., Simões, N., Holt, G., Avella, M. and Calado, R. (2011). Advances in breeding and rearing marine ornamentals. *Journal of the World Aquaculture Society*, 42(2), pp.135-166.

Otero-Ferrer, F., Molina, L., Socorro, J., Herrera, R., Fernández-Palacios, H. and Soledad Izquierdo, M. (2010). Live prey first feeding regimes for short-snouted seahorse *Hippocampus hippocampus* (Linnaeus, 1758) juveniles. *Aquaculture Research*, 41(9), pp.8-19.

Palma, J., Andrade, J. and Bureau, D. (2012). Growth, reproductive performances, and brood quality of long snout seahorse, *Hippocampus guttulatus*, fed enriched shrimp diets. *Journal of the World Aquaculture Society*, 43(6), pp.802-813.

Palma, J., Bureau, D. and Andrade, J., (2011). Effect of different *Artemia* enrichments and feeding protocol for rearing juvenile long snout seahorse, *Hippocampus guttulatus*. *Aquaculture*, 318(3-4), pp.439-443.

Palma, J., Bureau, D. and Andrade, J. (2014). The effect of diet on ontogenic development of the digestive tract in juvenile reared long snout seahorse *Hippocampus guttulatus*. *Fish Physiology and Biochemistry*, 40(3), pp.739-750.

Palma, J., Stockdale, J., Correia, M. and Andrade, J. (2008). Growth and survival of adult long snout seahorse (*Hippocampus guttulatus*) using frozen diets. *Aquaculture*, 278(1-4), pp.55-59.

Payne, M. and Rippingale, R. (2000). Rearing West Australian seahorse, *Hippocampus subelongatus*, juveniles on copepod nauplii and enriched *Artemia*. *Aquaculture*, 188(3-4),

pp.353-361.

Pham, N. and Lin, J. (2013). The effects of different feed enrichments on survivorship and growth of early juvenile long-snout seahorse, *Hippocampus reidi*. *Journal of the World Aquaculture Society*, 44(3), pp.435-446.

Pollom, R. (2017). *Hippocampus guttulatus. The IUCN Red List of Threatened Species 2017*: e.T41006A67617766. [online] Available at: https://dx.doi.org/10.2305/IUCN.UK.2017-3.RLTS.T41006A67617766.en. [Accessed Apr. 2021].

Roos, G., Van Wassenbergh, S., Aerts, P., Herrel, A. and Adriaens, D. (2011). Effects of snout dimensions on the hydrodynamics of suction feeding in juvenile and adult seahorses. *Journal of Theoretical Biology*, 269(1), pp.307-317.

Sargent, J., Bell, G., McEvoy, L., Tocher, D. and Estevez, A. (1999a). Recent developments in the essential fatty acid nutrition of fish. *Aquaculture*, 177(1-4), pp.191-199.

Sargent, J., McEvoy, L., Estevez, A., Bell, G., Bell, M., Henderson, J. and Tocher, D. (1999b). Lipid nutrition of marine fish during early development: current status and future directions. *Aquaculture*, 179(1-4), pp.217-229.

Salem, N. and Kuratko, C. (2014). A reexamination of krill oil bioavailability studies. *Lipids in Health and Disease*, 13(1).

Schubert, P., Vogt, L., Eder, K., Hauffe, T. and Wilke, T. (2016). Effects of feed species and HUFA composition on survival and growth of the long-snout seahorse (*Hippocampus reidi*). *Frontiers in Marine Science*, 3.

Segade, Á., Robaina, L., Otero-Ferrer, F., García Romero, J. and Molina Domínguez, L. (2015). Effects of the diet on seahorse (*Hippocampus hippocampus*) growth, body colour and biochemical composition. *Aquaculture Nutrition*, 21(6), pp.807-813.

Shapawi, R. and Purser, G. (2003). The value of enriched *Artemia* in supporting growth and survival of juvenile pot-bellied seahorses *Hippocampus abdominalis*. *Journal of the World Aquaculture Society*, 34(4), pp.533-541.

Sheng, J., Lin, Q., Chen, Q., Gao, Y., Shen, L. and Lu, J. (2006). Effects of food, temperature and light intensity on the feeding behaviour of three-spot juvenile seahorses, *Hippocampus trimaculatus* Leach. *Aquaculture*, 256(1-4), pp.596-607.

Sheng, J., Lin, Q., Chen, Q., Shen, L. and Lu, J. (2007). Effect of starvation on the initiation of feeding, growth and survival rate of juvenile seahorses, *Hippocampus trimaculatus* Leach and *Hippocampus kuda* Bleeker. *Aquaculture*, 271(1-4), pp.469-478.

Shields, R., Bell, J., Luizi, F., Gara, B., Bromage, N. and Sargent, J. (1999). Natural copepods are superior to enriched *Artemia* nauplii as feed for halibut larvae (*Hippoglossus hippoglossus*) in terms of survival, pigmentation and retinal morphology: relation to dietary essential fatty acids. *The Journal of Nutrition*, 129(6), pp.1186-1194.

Short, G., Claassens, L., Smith, R., De Brauwer, M., Hamilton, H., Stat, M. and Harasti, D. (2020). *Hippocampus nalu*, a new species of pygmy seahorse from South Africa, and the first record of a pygmy seahorse from the Indian Ocean (Teleostei, Syngnathidae). *ZooKeys*, 934, pp.141-156.

Short, G., Smith, R., Motomura, H., Harasti, D. and Hamilton, H. (2018). *Hippocampus japapigu*, a new species of pygmy seahorse from Japan, with a redescription of *H. pontohi* (Teleostei, Syngnathidae). *ZooKeys*, 779, pp.27-49.

Sorgeloos, P., Dhert, P. and Candreva, P. (2001). Use of the brine shrimp, *Artemia spp*., in marine fish larviculture. *Aquaculture*, 200(1-2), pp.147-159.

Sprague, M., Betancor, M. and Tocher, D. (2017). Microbial and genetically engineered oils as replacements for fish oil in aquaculture feeds. *Biotechnology Letters*, 39(11), pp.1599-1609.

Tipton, K. and Bell, S. (1988). Foraging patterns of two syngnathid fishes: importance of harpacticoid copepods. *Marine Ecology Progress Series*, 47, pp.31-13.

Vargas-Abúndez, J.A., Simões, N., Mascaró, M. (2018). Feeding the lined seahorse *Hippocampus erectus* with frozen amphipods. *Aquaculture*. 491, 82-85.

Vincent, A., Foster, S. and Koldewey, H. (2011). Conservation and management of seahorses and other Syngnathidae. *Journal of Fish Biology*, 78(6), pp.1681-1724.

Watanabe, T. (1993). Importance of docosahexaenoic acid in marine larval fish. *Journal of the World Aquaculture Society*, 24(2), pp.152-161.

Watanabe, W., Alam, M., Ostrowski, A., Montgomery, F., Gabel, J., Morris, J. and Seaton, P.

(2016). Live prey enrichment and artificial microdiets for larviculture of Atlantic red porgy *Pagrus pagrus. Aquaculture Reports*, 3, pp.93-107.

Watanabe, T., Ohta, M., Kitajima, C., Fujita, S. (1982). Improvement of dietary value of brine shrimp *Artemia salina* for fish larvae by feeding them on 3 highly unsaturated fatty acids. *Bulletin of the Japanese Society of Scientific Fisherie*s, 48(12).

Whittington, R. and Chong, R. (2007). Global trade in ornamental fish from an Australian perspective: The case for revised import risk analysis and management strategies. *Preventive Veterinary Medicine,* 81(1-3), pp.92-116.

Willadino, L., Souza-Santos, L., Mélo, R., Brito, A., Barros, N., Araújo-Castro, C., Galvão, D., Gouveia, A., Regis, C. and Cavalli, R. (2012). Ingestion rate, survival and growth of newly released seahorse *Hippocampus reidi* fed exclusively on cultured live food items. *Aquaculture*, 360-361, pp.10-16.

Wilson, M. and Vincent, A. (2000). Preliminary success in closing the life cycle of exploited seahorse species, *Hippocampus spp*., in captivity. *Aquarium Sciences and Conservation,* 2, pp.179–196.

Wong, J. and Benzie, J. (2003). The effects of temperature, *Artemia* enrichment, stocking density and light on the growth of juvenile seahorses, *Hippocampus whitei* (Bleeker, 1855), from Australia. *Aquaculture,* 228(1-4), pp.107-121.

Woodall, L. (2017). *Hippocampus hippocampus*. *The IUCN Red List of Threatened Species 2017*. [online] Available at: http://dx.doi.org/10.2305/IUCN.UK.2017-3.RLTS.T10069A67618259.en [Accessed Nov. 2018].

Woods, C. (2003). Effects of varying *Artemia* enrichment on growth and survival of juvenile seahorses, *Hippocampus abdominalis*. *Aquaculture*, 220(1-4), pp.537-548.

Woods, C. (2007). Aquaculture of the big-bellied seahorse *Hippocampus Adominalis* Lesson 1827 (Teleostei*: Sygnathidae). Ph.D, pp.251. Victoria University*.

Zhang, D., Zhang, Y., Lin, J. and Lin, Q. (2010). Growth and survival of juvenile lined seahorse, *Hippocampus erectus* (Perry), at different stocking densities. *Aquaculture Research*, 42(1), pp.9-13.