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Optimization of Live Prey Enrichment Media for Rearing Juvenile Short-Snouted Seahorse, *Hippocampus hippocampus*

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Abstract: This study aimed to determine a nutritionally adequate feeding protocol for *Hippocampus hippocampus* juveniles. In the experimental trial, seahorses were fed copepods from 0–7 days post-parturition (DPP) and, from 8–28 DPP, four different dietary treatments: (copepods (control diet) (*Cop*); microalgae-enriched *Artemia* with a docosahexaenoic acid (DHA)/eicosapentaenoic acid (EPA) ratio of 2:1 ($Art_{DHA/EPA}$); microalgae-enriched *Artemia* with a DHA/EPA ratio of 2:1 along with 5% copepods ($Art_{DHA/EPA5\%}$); and with 10% copepods ($Art_{DHA/EPA10\%}$)). At the end of the trial, juvenile seahorses fed *Cop* grew significantly more ($p < 0.05$) (5.1 mg d^{-1}) than those on fish-fed diets $Art_{DHA/EPA5\%}$ or $_{Art}DHA/EPA10\%$ (3.09 and 3.07 mg d^{-1}, respectively), or those on the fish-fed $Art_{DHA/EPA}$ (1.83 mg d^{-1}) diet, all of which performed poorly. Data suggest that feeding copepods during the first 7 DPP promotes maturation of the digestive tract of juvenile seahorses, and the addition of a limited amount of copepods to the diet improves *H. hippocampus* juvenile growth performance when compared with the use of *Artemia* as a single diet due to the improvement of the essential fatty acid profile in the diets.

Keywords: nutrition; *Hippocampus hippocampus*; live feed enrichment; microalgae; essential fatty acids; copepods

Key Contribution: The growth and survival of juvenile *H. hippocampus* is significantly enhanced when the initial diet consists exclusively of copepods; allowing for the digestive system maturation. Later, even the small inclusion of 5% to 10% copepods in the dietary protocol contributes to an improved fatty acid profile due to copepods' high EFA content and translates into substantial benefits in terms of growth and survival.

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1. Introduction

Due to their unique characteristics and morphological peculiarities, seahorses (*Hippocampus* sp.) have captured the interest of the community, thereby enhancing their cultural, scientific, educational, and economic value. As a result of this growing interest, seahorse exploitation, both for traditional medicines and the ornamental fish trade, has increased. This has led to a decline in natural populations [1] and an amplified focus on their cultivation worldwide [2].

Presently, seahorse species encounter conservation challenges, with habitat degradation and overfishing being the two primary causes of significant declines in most wild populations over the past decades. These issues have resulted in these species becoming an integral part of the IUCN Red List and Appendix II of CITES. Therefore, seahorse aquaculture holds significant potential as a conservation tool for these species, but also as a growing business, owing to the high demand for these species and their substantial

market values. Presently, it is known that at least 13 species of seahorses are commercially produced [2] at different commercial scales.

Currently, there are 42 identified seahorse species, including *Hippocampus hippocampus*, which is found in the northeastern Atlantic and Mediterranean Sea [3]. Although there is insufficient information to assess the conservation status of this species, natural populations in Portugal have exhibited a declining trend. Additionally, within the Mediterranean Sea, the species is already considered near threatened [4]. This situation has promoted research into various wild populations, often with a focus on their long-term conservation, as well as increased investigation into their captive production. Therefore, this remains a relevant topic, and numerous gaps in knowledge regarding seahorse aquaculture, including *H. hippocampus*, persist [2]. To prevent, or at the very least, mitigate the problems stemming from the illegal capture of seahorses, aquaculture can provide animals for international trade, thereby promoting the recovery of wild stocks and ensuring the conservation of natural populations [5].

It is widely acknowledged that captive breeding contributes significantly to preventing species extinction [6], and in this regard, seahorse captive breeding also assumes a preventive role in terms of species extinction [7]. Given the limited current understanding of the dietary requirements of *H. hippocampus*, the utilization and development of diets that align with the nutritional needs of the juveniles hold paramount importance. *Artemia* and rotifers have historically been utilized as live prey for feeding seahorses [7]. However, both *Artemia* nauplii and rotifers have consistently demonstrated nutritional deficiencies as food sources for larval and juvenile marine fishes, largely due to their inadequate fatty acid profiles and low levels of free amino acids [8]. Through enrichment, these live prey organisms can supply essential nutrients to the target species, with *Artemia* often serving as a carrier of essential fatty acids (EFA) [9–13]. Feeding on *Artemia* or copepods has resulted in high rates of survival and growth during the rearing of multiple seahorse species [5,14–22].

Numerous studies have also confirmed the significance of fatty acids in seahorse growth by enhancing *Artemia* with various emulsions e.g., [23–25]. This fact is related to the basis of trophic chains because marine and freshwater trophic chains are distinct in terms of fatty acid profile. Producers of marine food webs are unicellular algae rich in polyunsaturated fatty acids (PUFAs), mainly from the ω3 family, while freshwater food chain producers have a higher content of PUFAs from the ω6 family [26].

Because marine fish acquire substantial PUFA levels directly from their diet, their capacity to elongate and desaturate PUFAs has diminished, owing to a reduction in the associated enzymatic processes [27]. Considering this aspect, it can be asserted that the majority of marine species exhibit limited, inadequate, or in some instances, even non-existent abilities to elongate and desaturate linoleic acid (LA; C18:2ω6), while α-linolenic acid (ALA; C18:3ω3) is low, insufficient or, in some cases, even non-existent as they receive high amounts of these fatty acids directly from the diet [28,29]. In contrast, freshwater fish have higher levels of PUFAs with 18-carbon chains (i.e., AL, LA, and ALA) and possess substantial quantities of AA (C20:4 ω6), EPA (C20:5ω3), and DHA (C22:6ω3) resulting from metabolism [27,29,30].

When comparing the lipid composition of eggs between the vast majority of marine fish species and seahorses, it becomes evident that the former is characterized by having eggs with high concentrations of palmitic acid (PA, C16:0) (20.0%), docosahexaenoic acid (DHA: 17.4%), and eicosapentaenoic acid (EPA: 13.2%). Additionally, they contain, to a lesser extent, oleic acid (C18:1ω9), stearic acid (C18:0), palmitoleic acid (C16:1ω7), and vaccenic acid (C18:1ω7) [29].

However, as mentioned earlier, seahorse juveniles have a low content of essential fatty acids (EFAs), namely, EPA and DHA [31], which are typically associated with their survival and growth performance [32,33]. As marine fish, seahorses have high requirements for fatty acids from the ω3 family, which must be supplied through their diets [23,34–36]. Furthermore, the prey sizes may also restrict the ingesting capacity of seahorses [37]. In their natural habitat, copepods are the preferred prey for most marine fish larvae [38],

including seahorses. Multiple studies have indicated that the preference for copepods among marine fish larvae is attributed to their nutritional composition [39,40] and the provision of numerous enzymes that aid in the digestive process [41]. Consequently, in the realm of fish larval culturing, copepods are generally considered superior to rotifers and *Artemia* [42]. However, the utilization of copepods as larval fish feed is hindered by their limited availability and the challenges associated with intensive production [43].

Hence, as copepods are a more nutritionally suitable prey, *Artemia* cultivation is a more feasible option and, through enrichment, can serve as a "vehicle" to fulfill the nutritional requirements of seahorses through a process known as "bio-encapsulation". Conversely, advancements have been made in marine fish incubation techniques over recent years. Nonetheless, these improvements have not fully addressed the mortality issues observed during the early developmental stages of certain marine juvenile fish species [7,44,45], which are often linked to feeding, feeding behavior, and periods of food scarcity [46–48].

Considering the above, the objective of this study was to address some of the existing gaps in understanding the dietary requirements of the short-snout seahorse, *H. hippocampus*, during its early life stages, through the utilization of different live feed (*Artemia*) dietary enrichments and the implementation of an appropriate copepod co-feeding protocol.

2. Materials and Methods

A total of 420 *H. hippocampus* juveniles obtained from a captive-bred broodstock kept at the Ramalhete research station, CCMAR—Universidade do Algarve, Portugal were used to conduct two different rearing trials. The broodstock fish were kept in one 250 L plastic tank assembled in a semi-open flow system with a water turnover of approximately 125 L/h per tank. Holdfast units were added to provide support for seahorses [49]. Individuals were fed a mix of live mysid shrimp (*Mesopodopsis slabberi* and *Leptomysis* sp.) daily in different proportions, depending on availability. After natural spawning, juvenile seahorses from a single brood were randomly collected and used in the trials just after release from the male's pouch. The juvenile seahorses were gently transferred to the rearing tanks using a 250 mL beaker with the utmost caution to avoid exposure to air.

In this study, two rearing trials were conducted. The first one, referred to as the preliminary trial, was designed to test an enrichment protocol for the seahorses' feeding regime. The second, the experimental trial, was developed using the information obtained from the preliminary trial to create an optimized feeding protocol that will be described later. In both trials, the rearing tanks (10 L glass rectangular tanks) were supplied with water at a stable flow rate within a semi-closed recirculation system. Prior to being introduced into the rearing tanks, water was circulated from the reservoir tank through a UV sterilization unit. To ensure that dissolved oxygen was always kept close to saturation, water was vigorously aerated in a filtration reservoir (sump). To prevent bubbling, the rearing tanks' water inflow was supplied through a transparent plastic tube ending below the water surface. The water outflow structure was assembled in the corner of the tanks. It consisted of a black polystyrene tube covered at the water surface with a 150 µm diameter mesh to prevent live feed from being flushed from the tanks. To improve prey detection, lateral and back tank walls were covered with a black adhesive similarly to [50], while the front wall remained uncovered for observation. Each of the rearing tanks had two artificial holdfasts adjusted to juvenile size. For illumination, 2 × 36 W fluorescent tubes were placed above the tanks. The light intensity at the water surface was around 900 ± 40 lux, with a photoperiod of 16L: 8D (06:00–22:00 h), controlled by a timer. Water temperature and chemical parameters pH, salinity, and dissolved oxygen were recorded daily and maintained at 20.4 ± 0.3 °C, 7.8, 37.5 ± 0.1, and 7.6 ± 0.1 mg/L, respectively. Seawater quality parameters were recorded biweekly, ammonia values were always below detectable levels, nitrate values < 0.3 mg/L, and nitrite values < 1.25 mg/L. The tanks were cleaned daily by siphoning to remove feces, uneaten feed, and dead juvenile seahorses (accounted for mortality).

2.1. Live Feed Culture and Enrichment

AF *Artemia* cysts (Sanders®, Ogden, UT, USA) were hatched according to the procedures described by [51]. Phytobloom Prof *Isochrysis galbana* and Phytobloom Prof *Nannochloropsis* spp. pure powders from Necton's Phytobloom Green Formula (Necton, S.A., Olhão, PT) were used as enrichment according to the manufacturer specifications. In brief, each microalgae quantity was weighted individually, added to 250 mL of seawater, mixed, and left to hydrate for 15 min. The mix was then poured into a blender and homogenized for 2 min. The *Artemia* (approx. 48,000 nauplii) were added into the enrichment cup at a volume not higher than 750 mL and the enrichment media were added. This procedure was repeated for each dietary treatment. *Artemia* nauplii were left to enrich for 24 h at room temperature (20–22 °C), under continuous moderate aeration and illumination.

2.2. Copepod Harvesting

Copepods (*Acartia clausi*) were naturally produced in the outflow pond of the Ramalhete research station and collected daily using a 60 µm hand net. After acclimation to room temperature, the copepods were strained through a 150 µm sieve to remove large plankters and debris, and were counted to the adequate feed ratio.

2.3. Feeding trials

In the preliminary trial, the single use of enriched *Artemia* was tested. For this, three different enrichment media with different DHA/EPA ratios were tested using two microalgae in different proportions: *I. galbana*, rich in DHA, and *Nannochloropsis* sp., rich in EPA. The calculations of the DHA and EPA ratios were derived from the fatty acid (FA) profile provided by the manufacturers. A total of 180 juvenile *H. hippocampus* from the same brood were distributed across nine rearing tanks using a completely randomized design, with three replicate tanks allocated to each dietary treatment. Twenty juvenile *H. hippocampus* were stocked in each replicate tank. The first dietary treatment (*Art2:1*), contained *Artemia* metanauplii, enriched with 2:1 DHA/EPA ratio; the second (*Art1:1*) contained *Artemia* metanauplii enriched with 1:1 DHA/EPA ratio; and the third (*Art1:2*) contained *Artemia* metanauplii enriched with 1:2 DHA/EPA ratio. The experiment was designed to last 28 days.

In the experimental trial, based on the results of the preliminary trial, the minimum copepod (*A. clausi*) addition to sustain the optimal growth and survival of juvenile *H. hippocampus* was evaluated and compared with copepod and *Artemia* diets. A total of 240 juvenile *H. hippocampus* from the same brood were assembled into twelve rearing tanks according to a completely randomized design, with three replicate tanks assigned to each dietary treatment. Twenty juvenile *H. hippocampus* were stocked in each replicate tank at a density of 2 fish l^{-1}. All groups were first fed natural copepods from 0–7 days post-parturition (DPP), and only on the 8th day the transition to each of the following dietary treatments was made. The first dietary treatment (*Cop*) consisted of daily captured copepods (control diet); the second dietary treatment ($Art_{DHA/EPA}$) consisted of 2:1 DHA/EPA ratio enriched *Artemia* metanauplii used in the previous trial (dietary treatment *Art2:1*); the third ($Art_{DHA/EPA5\%}$) dietary treatment consisted of a mixture of 95% 2:1 DHA/EPA ratio enriched *Artemia* metanauplii and 5% natural copepods; and the fourth dietary treatment ($Art_{DHA/EPA10\%}$) consisted of a mixture of 90% 2:1 DHA/EPA ratio enriched *Artemia* metanauplii and 10% natural copepods. The experiment lasted for 28 days.

2.4. Sampling, Data Collection, and Statistical Analysis

To obtain the accurate weight and length information at 0 DPP, due to their small size and to avoid morphological distortions, ten juveniles were immediately and rapidly sacrificed with an excess of anesthetic (2-phenoxyethanol solution, 0.40 mg L^{-1}). The individuals were placed in the anesthetic solution for at least 20 min and removed no less than 10 min after ventilation stopped. The fish were than weighted using a high-precision scale and measurements (total length as the sum of the head, trunk, and tail

length) were conducted using a binocular microscope associated with image analysis software (DinoCapture 2.0 (AnMo Electronics Corporation, Sanchong, New Taipei City, Taiwan)). Later, experimental fish were sampled at 14 and 28 DPP for growth performance analysis. To determine the total length, the sum of the head length and the height of the fish were considered. Head length was measured as the distance from the rostral zone of the snout (maxillary) to the midpoint of the cleithral ring. The height of the seahorse was measured based on the length between the cleithral ring to the end of the tail, considering it in extension (Annex I), as suggested by [52]. This procedure was related to the adoption of a methodology that would inflict the least possible stress on seahorses, which is more advantageous than the traditional three-measurement method proposed by [53]. Mortality was monitored daily.

The seahorses' wet weight and total length as well as mortality were used to calculate: Survival (S) = $((N_i - M_{ij})/N_i) \times 100$, where Ni is the initial number of seahorses placed in each tank on day 1 and M_{ij} the total number of surviving individuals at the end of the trial; Mean Length Gain (LG) (cm/fish) = $(L_f/L_i)/t$, where L_f is the final length (cm), L_i is the initial length (cm), and t is the number of experimental days; Mean Weight Gain (WG) (mg/fish) = $(W_f - W_i)/t$, where W_f is the final wet weight (mg), W_i is the initial wet weight (mg), t is the number of experimental days; and the Thermal-unit Growth Coefficient (TGC) (modified from [54] by [55]) = $[({W_f}^{1/3} - {W_i}^{1/3})/\Sigma(T \times D)] \times 100$, where W_f is the final seahorse wet weight (g), W_i is the initial wet weight (g), T is the water temperature (°C), and D is the number of days in each trial; and Condition Factor (CF) = $(WW/L^3) \times 100$, where WW is the wet weight (g) and L is the total length (cm). Data processing and statistical analysis were conducted using the statistical analysis program package, GraphPad Prism (version 8.4.2 for Windows, GraphPad Software, San Diego, CA, USA). All data were tested for normality and homogeneity of variances. To study the effect of the diets on growth performance (Weight gain, Length gain, Thermal-unit Coefficient, Condition Factor, and Accumulative survival) between groups, a One-Way ANOVA, using Tukey's test for multiple comparisons, was used.

2.5. Dietary Treatments Proximate Analysis and Fatty Acid Composition

To analyze the nutritional quality of prey and the dietary treatments used in the experimental trial, samples of natural copepods (*Cop*), unenriched *Artemia* (Art_{Ref}), enriched *Artemia* ($Art_{DHA/EPA}$), 24 h enriched *Artemia* plus 5% copepods ($Art_{DHA/EPA5\%}$), and enriched *Artemia* plus 10% copepods ($Art_{DHA/EPA10\%}$) were collected. Even though unenriched *Artemia* (Art_{Ref}) was not a dietary treatment tested in any of the groups, it was analyzed along with all the regimes for comparison purposes and to determine the efficiency of the enrichment used and copepod addition.

The four dietary treatments and the unenriched *Artemia* were analyzed following the procedures outlined in [56]. In brief, the dry matter was analyzed by drying samples at 105 °C to constant weight; ash by incineration of samples at 550 °C for 5 h; crude protein (N × 6.25) using a Leco nitrogen analyzer (Leco Corporation, St. Joseph, MI, USA); total lipid by extraction according to the method of [57], using accelerated solvent extraction (ASE) and lyophilized samples; crude fiber by acid and basic digestion (Fibertec System M., 1020 Hot Extractor, Tecator); and gross energy content using an automatic oxygen bomb calorimeter (Parr 6400, U.S.A.) according to ISO 9831.

For the fatty acid analysis, to achieve the extract derivatization, total lipids were transferred to methylation tubes using 1.5 mL of $CHCl_3$/MeOH (3:2) solution and placed under a nitrogen stream to evaporate the transfer solvent and dry the extract for saponification and methylation. For the methylation phase, the samples were cooled for approx. 1 min, and 1 mL of BF_3-methanol was added; the tubes were then heated at 70 °C for 10 min. Once methylation was complete, the solution was cooled to room temperature, 2 mL of distilled water and 2 mL of petroleum ether were added and stirred vigorously. The tubes were then placed in the fridge for 12 h at 2 °C. After separation of the phases was verified, each sample's supernatant (1.5 mL) was collected into 2 mL vials, which were hermetically

sealed, labeled, and placed in the fridge until further use. Chromatographic analysis of methyl esters was performed as described by [58]. In brief, the analysis of the peaks and their respective MS was obtained by electronic impact at 70 eV, with a sweep of m/z = 40 to 450 and analyzed using MSWS 8.2 software. The Fame standards used were 37-component FAME MIX and BAME MIX 26 (Supelco). Fatty acids were designated according to the nomenclature of the International Union of Pure and Applied Chemistry (IUPAC) for carbon chain length: number of double bonds and position of the double bond closest to the omega carbon.

2.6. Ethical Statement

CCMAR facilities and the research are certified to house and conduct experiments with live animals (Group-C licenses from the Direção Geral de Alimentação e Veterinária, Ministério da Agricultura, Florestas e Desenvolvimento Rural, Portugal). The experimental design of the present study was part of the Projects HIPPONUTRE (reference 1602-01-FMP-54) and HIPPOSAVE (reference MAR-01.04.02-FEAMP-0029), which obtained approval from the ethics committee of the Veterinary Medicines Directorate, Ministry of Agriculture, Rural Development and Fisheries, Portugal (protocol code 0421/000/000, 10/11/2015). The study adhered to the guidelines outlined by the European Union Council (86/609/EU) and the relevant Portuguese legislation concerning the use of laboratory animals.

3. Results

The proximate composition of each dietary treatment is presented in Table 1. The dry matter of *Cop* dietary treatment was considerably lower (13.1%) compared with the remaining diets (ranging between 29.9 and 33.2%). As for the gross protein, the $Art_{DHA/EPA10\%}$ had the highest content (20 g/100 g DM), followed by $Art_{DHA/EPA5\%}$ (19.1 g/100 g DM), $Art_{DHA/EPA}$ (17.9 g/100 g DM), Art_{Ref} (16.5 g/100 g DM), and *Cop* (10.9 g/100 g DM). Concerning the total lipid content, the *Cop* diet had the lowest value of total lipids, (4.9 g/100 g DM), a value two times lower than all other dietary treatments. Conversely, the *Cop* diet had the highest gross energy content (503.9 Kcal/100 g DM), followed by $Art_{DHA/EPA5\%}$ (495.62 Kcal/100 g DM), $Art_{DHA/EPA10\%}$ (492.18 Kcal/100 g DM), and $Art_{DHA/EPA}$ (489.84 Kcal/100 g DM) (Table 1).

Table 1. Proximate composition of the four tested dietary treatments (per 100 g of Dry Matter (g/100 g DM)). *ArtRef* *—Although *ArtRef* was not directly tested, it served as the basis for the enriched *Artemia* dietary treatments; therefore, it is presented for comparison purposes. *Cop*—Natural copepods; *ArtRef*—Unenriched *Artemia* nauplii; *ArtDHA/EPA*—2:1 DHA/EPA ratio enriched *Artemia* metanauplii; $Art_{DHA/EPA5\%}$—95% 2:1 DHA/EPA ratio enriched *Artemia* metanauplii and 5% natural copepods; $Art_{DHA/EPA10\%}$—90% 2:1 DHA/EPA ratio enriched *Artemia* metanauplii and 10% natural copepods. Row values with different superscripts are significantly different ($p < 0.05$).

Proximate Composition	*Cop*	Art_{Ref} *	$Art_{DHA/EPA}$	$Art_{DHA/EPA5\%}$	$Art_{DHA/EPA10\%}$
Moisture (%)	86.9 ± 1.1^{a}	70.1 ± 0.7^{b}	70.1 ± 0.6^{b}	68.5 ± 0.8^{b}	66.9 ± 0.8^{b}
Dry Matter (%)	13.1 ± 0.9^{b}	29.9 ± 1.1^{a}	29.9 ± 1^{a}	31.4 ± 1.3^{a}	33.2 ± 1.1^{a}
Gross Protein (g/100 g DM)	10.9 ± 0.7^{c}	16.5 ± 0.6^{b}	17.9 ± 0.8^{ab}	19.1 ± 0.9^{ab}	20 ± 1.1^{a}
Total Lipids (g/100 g DM)	4.9 ± 0.9^{b}	11.8 ± 0.7^{a}	10.2 ± 0.7^{a}	10 ± 0.8^{a}	10.5 ± 0.9^{a}
Gross Energy (Kcal/100 DM)	503.85 ± 1.1^{a}	497.8 ± 0.6^{ab}	489.8 ± 0.7^{c}	495.6 ± 0.7^{ab}	492.2 ± 1.2^{b}

Regarding the presence of EFAs in the two microalgae used, *I. galbana* had higher percentages of LA (C18:2ω6) (7.5%), ALA (C18:3ω3) (15.5%), and DHA (C22:6ω3) (16.5%) than *Nannochloropsis* sp. Conversely, although *Nannochloropsis* sp. did not contain any DHA in its profile, it had a higher concentration of ARA (C20:4ω6) (5.3%) and substantially more EPA (C20:5ω3) (36.1%) (Table 2). Concerning fatty acid classes (SFA, MUFA, PUFA, and HUFA) and families (ω3, ω6 and ω9), the biggest differences were in the percentage of HUFAs, EFAs, and ω9 fatty acids. *Nannochloropsis* sp. presented higher amounts of HUFAs

(42.3%) and EFAs (45,6%), while *I. galbana* showed a greater percentage of ω9 fatty acids, with a value of 15.2%. As for the ratios, *I. galbana* showed higher values for all the ratios presented, especially the very high DHA/EPA ratio (165), whereas *Nannochloropsis* sp. has a value of 0 for the same ratio (Table 2).

Table 2. Fatty acid profile (% total FA) of the two microalgae used as *Artemia* nauplii enrichment in the diets, $Art_{DHA/EPA}$, $Art_{DHA/EPA5\%}$, and $Art_{DHA/EPA10\%}$.

Fatty Acid	*Isochrysis galbana*	*Nannochloropsis* sp.
C18:2ω6 (LA)	7.5	4
C18:3ω3 (ALA)	15.5	0.2
C20:4ω6 (ARA)	0.2	5.3
C20:5ω3 (EPA)	0.1	36.1
C22:6ω3 (DHA)	16.5	-
ΣSFA	25.5	26.2
ΣMUFA	20.2	22.9
ΣPUFA	50.1	48.1
ΣHUFA	27.3	42.3
ΣEFA	39.8	45.6
Σω3	39.8	37.2
Σω6	10.6	10.9
Σω9	15.2	3.2
DHA/EPA	165	0
ω3/ω6	3.75	3.41
PUFA/SFA	1.96	1.84

ALA—Alpha-linolenic acid; ARA—Arachidonic acid; DHA—Docosahexaenoic acid; EFA—Essential fatty acid; EPA—Eicosapentaenoic acid; HUFA—Highly unsaturated fatty acid; LA—Linoleic acid; MUFA—Monounsaturated fatty acid; PUFA—Polyunsaturated fatty acid; SFA—Saturated fatty acid.

Table 3 presents the lipid profile of the control diet (Cop) and tested dietary treatments ($Art_{DHA/EPA}$, $Art_{DHA/EPA5\%}$, and $Art_{DHA/EPA10\%}$), as well as the additional Art_{Ref}, for the most expressive fatty acids and respective sums of SFA, MUFA, PUFA, HUFA, and EFA. The *Cop* diet contained high values of all fatty acid classes except for MUFA. The *Cop* and Art_{Ref} diets also revealed the highest percentage values for PUFA and HUFA. Regarding PUFA, the *Cop* diet showed a value of 31%, followed by Art_{Ref}, with 23.6%, and by the three diets including enriched *Artemia*. Dietary treatments with enriched *Artemia* had much higher values of MUFA (≈34%) and, consequently, lower PUFA and HUFA values (≈20% and 10%, respectively).

The most relevant SFAs were C12:0, C14:0, C16:0, C18:0, and C20:0. C16:0 was the SFA with the highest value in all tested diets, with the *Cop* diet having 31.2%, followed by $Art_{DHA/EPA10\%}$ (17.1%), $Art_{DHA/EPA5\%}$ (16.9%), and $Art_{DHA/EPA}$ (16%) (Table 3). The second most expressive SFA was C14:0, whereas for C18:0, there were no major differences between dietary treatments. Amongst the PUFAs, all EFAs (LA, ARA, ALA, EPA, and DHA) were present in the tested dietary treatments (Table 3). Regarding LA and ARA, all diets containing *Artemia* ($Art_{DHA/EPA}$, $Art_{DHA/EPA5\%}$, and $Art_{DHA/EPA10\%}$) had about twice the amount of those fatty acids compared with the *Cop* control diet (Table 3). No relevant differences in the ALA amount (≈3%) were found among dietary treatments. Regarding EPA, the *Cop* control diet had the highest value (10.48%), followed by Art_{Ref}, and 8.26%, while the remaining samples had similar amounts (≈6%). The *Cop* dietary treatment contained a high percentage of DHA (11.7%) compared with the remaining diets, which had concentrations ranging from 0.2% (due to the $Art_{DHA/EPA}$ enrichment process) to 0.6% (due to the $Art_{DHA/EPA}$ enrichment process and the additional copepod inclusion levels) (Table 3).

Table 3. Fatty acid composition of each dietary treatment (mean ± standard deviation) expressed as a percentage (%) of total identified fatty acids. * Although *ArtRef* was not directly tested, it served as the basis for the enriched *Artemia* dietary treatments; therefore, it is presented for comparison purposes. Row values with different superscripts are significantly different ($p < 0.05$).

Fatty Acid	*Cop*	Art_{Ref} *	$Art_{DHA/EPA}$	$Art_{DHA/EPA5\%}$	$Art_{DHA/EPA10\%}$
C12:0	0.19 ± 0.01 ^{b}	0.16 ± 0.02 ^{c}	0.15 ± 0.02 ^{c}	0.18 ± 0.01 ^{b}	0.23 ± 0.07 ^{a}
C14:0	10.25 ± 0.19 ^{a}	3.06 ± 0.19 ^{c}	4.26 ± 0.16 ^{b}	4.25 ± 0.2 ^{b}	4.24 ± 0.15 ^{b}
C16:0	31.23 ± 0.4 ^{a}	15.69 ± 0.41 ^{b}	15.94 ± 0.88 ^{b}	16.91 ± 1.15 ^{b}	17.07 ± 0.47 ^{b}
C18:0	6.13 ± 0.28 ^{a}	4.84 ± 0.3 ^{b}	5.35 ± 0.2 ^{b}	5.19 ± 0.14 ^{b}	5.07 ± 0.29 ^{b}
C20:0	0.11 ± 0 ^{a}	0.11 ± 0.03 ^{b}	0.14 ± 0 ^{b}	0.14 ± 0.02 ^{a}	0.09 ± 0.03 ^{b}
ΣSFA	47.9 ^{a}	23.87 ^{c}	25.84 ^{c}	26.65 ^{c}	36.7 ^{b}
C14:1ω5	-	0.33 ± 0.02 ^{a}	0.23 ± 0.01 ^{b}	0.21 ± 0 ^{b}	0.23 ± 0.02 ^{b}
C16:1ω7c	8.88 ± 0.2 ^{b}	13.79 ± 0.67 ^{a}	12.74 ± 0.46 ^{a}	13.06 ± 0.61 ^{a}	13.58 ± 0.47 ^{a}
C16:1ω7t	0.27 ± 0.07 ^{b}	0.47 ± 0.09 ^{a}	0.40 ± 0.04 ^{a}	0.36 ± 0.02 ^{a}	0.39 ± 0.02 ^{a}
C18:1ω9c	2.91 ± 0.04 ^{c}	6.07 ± 6.64 ^{b}	12.36 ± 0.92 ^{a}	12.21 ± 1.12 ^{a}	11.82 ± 1.64 ^{a}
C18:1ω9t	1.9 ± 0.09 ^{b}	8.08 ± 1.8 ^{a}	8.3 ± 1.3 ^{a}	7.73 ± 1.44 ^{a}	7.43 ± 1.29 ^{a}
C18:1ω7	0.29 ± 0.42 ^{a}	0.12 ± 0.06 ^{b}	0.10 ± 0.01 ^{b}	0.09 ± 0.07 ^{bc}	0.07 ± 0.02 ^{c}
C20:1ω9c	-	0.34 ± 0.08 ^{a}	0.33 ± 0.03 ^{a}	0.33 ± 0.01 ^{a}	0.32 ± 0.02 ^{a}
ΣMUFA	14.26 ^{c}	29.2 ^{b}	34.46 ^{a}	33.99 ^{a}	33.82 ^{a}
C16:2ω4	0.27 ± 0.1^{d}	1.04 ± 0.05 ^{a}	0.73 ± 0.45 ^{b}	0.71 ± 0.38 ^{b}	0.38 ± 0.22 ^{c}
C18:2ω6 (LA)	2.09 ± 0.04 ^{b}	5.05 ± 0.27 ^{a}	4.56 ± 0.02 ^{a}	4.49 ± 0.06 ^{a}	4.52 ± 0.13 ^{a}
C18:2ω4	-	0.72 ± 0.02 ^{a}	0.48 ± 0.03 ^{b}	0.46 ± 0.02 ^{b}	0.39 ± 0.08 ^{b}
C18:3ω6	0.34 ± 0.02^{d}	0.83 ± 0.13 ^{b}	0.63 ± 0.09 ^{c}	0.60 ± 0.06 ^{c}	1.56 ± 1.6 ^{a}
C18:3ω3 (ALA)	2.9 ± 0.02 ^{a}	3.23 ± 0.22 ^{a}	3.18 ± 0.1 ^{a}	3.07 ± 0.08 ^{a}	2.93 ± 0.18 ^{a}
C18:4ω3	1.5 ± 0.09 ^{a}	0.56 ± 0.04 ^{c}	1.14 ± 0.04 ^{a}	1.07 ± 0.03 ^{a}	0.85 ± 0.06 ^{b}
C20:3ω6	0.23 ± 0.02 ^{a}	0.24 ± 0.08 ^{a}	0.16 ± 0.05 ^{a}	0.17 ± 0.04 ^{a}	0.17 ± 0.08 ^{a}
C20:4ω6 (ARA)	1.28 ± 0.1 ^{c}	3.44 ± 0.17 ^{a}	2.55 ± 0.02 ^{b}	2.62 ± 0.01 ^{b}	3.14 ± 0.57 ^{a}
C20:4ω3	0.25 ± 0.08 ^{a}	0.24 ± 0.06 ^{a}	0.11 ± 0.05 ^{b}	0.14 ± 0.05 ^{b}	0.08 ± 0.04 ^{c}
C20:5ω3 (EPA)	10.48 ± 1.46 ^{a}	8.26 ± 0.3 ^{b}	5.58 ± 0.3 ^{c}	5.86 ± 0.46 ^{c}	5.94 ± 0.89 ^{c}
C22:6ω3 (DHA)	11.67 ± 0.79 ^{a}	-	0.23 ± 0.04 ^{c}	0.47 ± 0.09 ^{b}	0.58 ± 0.05 ^{b}
ΣPUFA	31.01 ^{a}	23.6 ^{b}	19.35 ^{c}	19.63 ^{c}	20.78 ^{c}
ΣHUFA	25.18 ^{a}	12.51 ^{b}	9.61 ^{c}	10.13 ^{c}	10.58 ^{c}
ΣEFA	28.42 ^{a}	19.98 ^{b}	16.1 ^{c}	16.51 ^{c}	17.11 ^{c}
Σ Others	6.83^{d}	23.33 ^{a}	20.35 ^{b}	19.73 ^{b}	17.7 ^{c}

Figure 1 shows the distribution of fatty acid families by dietary treatment. The *Cop* control diet had the highest value of ω3 fatty acids (27%), followed by Art_{Ref} (12.3%), $Art_{DHA/EPA5\%}$ (10.5%), $Art_{DHA/EPA10\%}$ (10.4%), and $Art_{DHA/EPA}$ (10.2%). Concerning the ω6, ω7, and ω9 fatty acid families, all diets but the *Cop* revealed higher percentages. The diets with the highest ω6 fatty acids were $Art_{DHA/EPA10\%}$ (9.4%), $Art_{DHA/EPA}$ (7.9%), and $Art_{DHA/EPA5\%}$ (7.9%). Regarding the ω7 fatty acids, all regimes with enriched *Artemia* were revealed to have high concentrations with similar values (approximately 22%), while the *Cop* diet had half the concentration of the remaining diets (9.4%). Finally, for ω9 fatty acids, it was observed that the $Art_{DHA/EPA}$ regime presented the highest value (20.7%), followed by $Art_{DHA/EPA5\%}$ (19.9%), and $Art_{DHA/EPA10\%}$ (19.2%). The *Cop* diet presented the lowest value of ω9 fatty acids, with 4.8%.

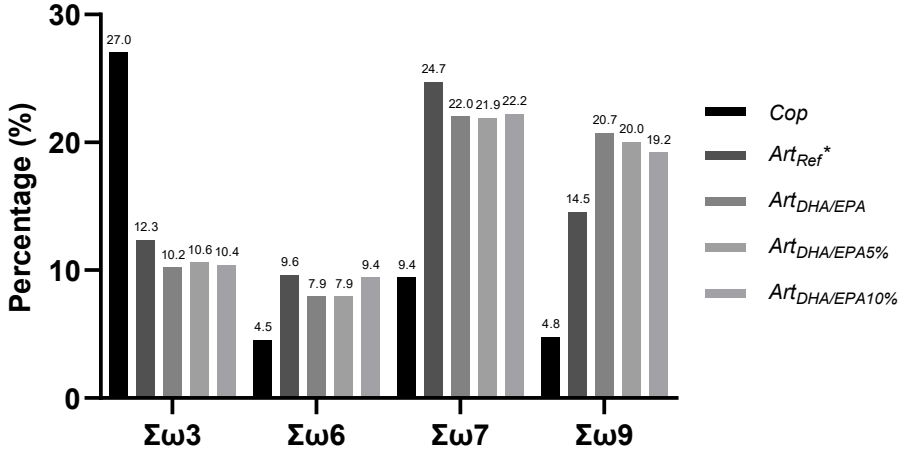

Figure 1. Characterization of the fatty acid families ω3, ω6, ω7, and ω9 in each of the four tested dietary treatments. *Cop*—Natural copepods; Art_{Ref}—Unenriched *Artemia* nauplii; $Art_{DHA/EPA5\%}$—95% 2:1 DHA/EPA ratio enriched *Artemia* metanauplii and 5% natural copepods; and $Art_{DHA/EPA10\%}$—90% 2:1 DHA/EPA ratio enriched *Artemia* metanauplii and 10% natural copepods. * Although *ArtRef* was not directly tested, it served as the basis for the enriched *Artemia* dietary treatments; therefore, it is presented for comparison purposes.

Some relevant ratios in terms of nutritional assessment of the tested diets are presented in Table 4. DHA/EPA and ω3/ω6 ratios in the *Cop* diet revealed the highest values (1.11 and 6, respectively), while the DHA/EPA ratio in the Art_{Ref} was 0. Regarding the PUFA/SFA ratio, *ArtRef* presented the highest value (0.99), followed by $Art_{DHA/EPA10\%}$ (0.78), $Art_{DHA/EPA}$ (0.75), $Art_{DHA/EPA5\%}$ (0.74), and *Cop* (0.65) dietary treatments.

Table 4. Main fatty acid ratios in each of the four tested dietary treatments. * Although *ArtRef* was not directly tested, it served as the basis for the enriched *Artemia* dietary treatments; therefore, it is presented for comparison purposes.

Fatty Acid Ratios	*Cop*	Art_{Ref} *	$Art_{DHA/EPA}$	$Art_{DHA/EPA5\%}$	$Art_{DHA/EPA10\%}$
DHA/EPA	1.11	0	0.04	0.08	0.1
ω3/ω6	8	1.29	1.29	1.34	1.1
PUFA/EFA	0.65	0.99	0.75	0.74	0.78

In the preliminary trial, all dietary treatments (*Art2/1*, *Art1/1*, and *Art1/2*) resulted in 100% mortality by the 9th DPP, leading to the termination of the trial at that point. Regarding the subsequent experimental trial, comprehensive data regarding the survival and growth performance of juvenile *H. hippocampus* fed each of the four dietary treatments (*Cop*, $Art_{DHA/EPA}$, $Art_{DHA/EPA5\%}$, and $Art_{DHA/EPA10\%}$) are presented in Table 5. It was observed that both the $Art_{DHA/EPA}$ and $Art_{DHA/EPA5\%}$ groups exhibited similar survival rates, 88% and 87%, respectively, while the $Art_{DHA/EPA10\%}$ and *Cop* groups had lower survival rates, with 77% and 72%, respectively (Table 5).

At the end of the trial, juvenile *H. hippocampus* fed the *Cop* diet grew significantly more ($p < 0.05$) than all the remaining groups. $Art_{DHA/EPA5\%}$ and $Art_{DHA/EPA10\%}$ attained intermediate growth performance, non-significantly different ($p < 0.05$) between the two. The group fed $Art_{DHA/EPA}$ grew significantly less ($p < 0.05$) than all the remaining groups (Table 5). This is corroborated by a *Cop* group WG of 5.1 mg d^{-1} and a final MW of 146.3 ± 29.6 mg at 28 DPP, the highest observed. The $Art_{DHA/EPA5\%}$ and $Art_{DHA/EPA10\%}$ groups showed virtually identical WG (3.09 and 3.07 mg d^{-1}, correspondingly) and a MW of 89 ± 22.7 mg and 88.5 ± 28.4 mg, respectively. The group fed with the $Art_{DHA/EPA}$ attained a WG of 1.8 mg d^{-1} and a final MW of 53.6 ± 19.3 mg, showing the lowest growth performance.

Table 5. Survival and growth parameters (mean ± st. dev.) of juvenile *H. hippocampus* fed each of the four tested dietary treatments. (ML—Mean Length, MW—Mean Weight, LG—Length Gain, WG—Weight Gain, TGC—Thermal-unit Growth Coefficient, CF—Condition Factor). Row values with different superscripts are significantly different ($p < 0.05$).

	Cop	$Art_{DHA/EPA}$	$Art_{DHA/EPA5\%}$	$Art_{DHA/EPA10\%}$
Survival	72	88	87	77
ML (cm) 0 DPP	1.1 ± 0.1	1.1 ± 0.1	1.1 ± 0.1	1.1 ± 0.1
ML (cm) 28 DPP	4.4 ± 0.3 ^{a}	3.2 ± 0.4 ^{c}	3.8 ± 0.3 ^{b}	3.7 ± 0.3 ^{b}
MW (mg) 0 DPP	2.4 ± 0.4	2.4 ± 0.4	2.4 ± 0.4	2.4 ± 0.4
MW (mg) 28 DPP	146 ± 39.6 ^{a}	53.1 ± 19.3 ^{c}	89 ± 22.7 ^{b}	88.5 ± 28.4 ^{b}
LG (cm day^{-1})	0.12 ± 0.003 ^{a}	0.07 ± 0.004 ^{c}	0.09 ± 0.004 ^{b}	0.09 ± 0.004 ^{b}
WG (mg day^{-1})	5.14 ± 0.33 ^{a}	1.83 ± 0.33 ^{c}	3.09 ± 0.82 ^{b}	3.07 ± 0.23 ^{b}
TGC	0.008 ^{a}	0.003 ^{c}	0.005 ^{b}	0.005 ^{b}
CF	0.17 ^{a}	0.17 ^{a}	0.17 ^{a}	0.17 ^{a}

Concordantly, the *Cop* group had the highest LG (0.12 cm d^{-1}) averaging 4.4 ± 0.3 cm at the end of the experiment. $Art_{DHA/EPA5\%}$ and $Art_{DHA/EPA10\%}$ groups were not significantly different ($p > 0.05$), and the LG was 0.09 cm d^{-1}, resulting in 0.03 cm d^{-1} less than the *Cop* group. The lowest LG and ML values were observed for the $Art_{DHA/EPA}$ regime group, with 0.07 cm d^{-1} and a ML of 3.2 ± 0.4 cm (Table 5).

Concerning the TGC, the highest value was observed in the *Cop* group (0.008), followed by both the $Art_{DHA/EPA5\%}$ and $Art_{DHA/EPA10\%}$ groups (0.005) and the $Art_{DHA/EPA}$ group (0.003) the lowest TGC value was observed. All groups attained similar and non-significantly different ($p > 0.05$) CF values at the end of the experiment (Table 5).

4. Discussion

Juvenile seahorses require large amounts of good quality live feed [2] in which the fatty acids are a prevalent nutritional requirement. However, ensuring its appropriate provision remains one of the most significant challenges in successful seahorse mass farming. *Artemia* is a commonly used feed for seahorses due to its easy cultivation and commercial availability [14,16] but available *Artemia* enrichments are still inadequate for most seahorse species. *Artemia* nauplii has been identified as deficient in highly unsaturated fatty acids (HUFAs) [5,11], requiring HUFA enrichment media [14,25,59,60]. Conversely, copepods are regarded as an ideal diet for marine fish larvae, seahorses included, primarily due to their well-balanced fatty acid composition. However, copepods often cannot be cultured at high densities under controlled conditions as other live food options because they need larger volumes of water and larger culture vessels [61].

Thus, enriched *Artemia*-based diets cannot be ignored and can be used as an alternative or complement to an exclusive copepod-based diet if its enrichment is adequately tailored to the target species. Additionally, its utilization can also offer advantages by potentially reducing associated contaminants, as copepods frequently originate from wild populations and can introduce pathogens [62].

In the current study, all dietary treatments containing *Artemia* ($Art_{DHA/EPA}$, $Art_{DHA/EPA5\%}$, and $Art_{DHA/EPA10\%}$) had higher levels of total protein and total lipids compared with the *Cop* control diet. However, it was the *Cop* control diet that yielded a higher gross energy content. This same trend in gross energy content was observed when $Art_{DHA/EPA}$ was supplemented with 5% and 10% copepod inclusion, resulting in an increase in gross energy content in these dietary treatments. From a nutritional perspective, dietary lipids have been shown to play a critically important role in the early development of marine finfish larvae [29]. They serve as the primary energy source for larvae and are a vital source of highly unsaturated fatty acids (HUFA) and essential fatty acids (EFA), which are necessary for the development of new cellular structures. Additionally, they contribute to normal larval growth, morphogenesis, and bone formation [63]. Therefore, a deficiency in HUFA levels in prey can lead to detrimental effects on juvenile seahorse performance, including reduced feeding and swimming activity,

as well as a delay in the settlement process, as observed in previous studies where *Artemia* was used, due to its incomplete assimilation [2,5]. Concordantly, a correlation between the type of prey enrichment and the occurrence of gas bubble disorder has also been observed. Ref. [17] reported that juveniles of *H. guttulatus* fed *Artemia* enriched with microalgae exhibited a significantly lower incidence of this disorder compared with those fed on *Artemia* enriched with commercial products. Similarly, it was noted that *H. guttulatus* juveniles fed DHA-Selco®-enriched *Artemia* metanauplii displayed symptoms of gas bladder overinflation and intestinal disorders, unlike individuals fed solely on natural copepods [59].

In the present study, the selection of the two microalgae for *Artemia* enrichment was based on their DHA and EPA profiles. The analysis revealed that *I. galbana* had a higher proportion of C22:6ω3 (DHA) in its profile, while *Nannochloropsis* sp. lacked any significant concentration of this essential fatty acid. In contrast, *Nannochloropsis* sp. exhibited a substantial percentage of C20:5ω3 (EPA), while *I. galbana* displayed only a trace amount of this fatty acid. The presence of EPA in *Nannochloropsis* sp. notably influenced the HUFA content, leading to an increase compared with that of *I. galbana*. Moreover, *I. galbana* was rich in ω9 HUFAs, largely attributed to its high content of oleic acid (C18:1ω9), which represented most of the ω9 HUFAs in this species. The *Cop* diet had a higher PUFA percentage than $Art_{Ref,}$ base diet. Notably, out of the PUFA content in the *Cop* diet, most of it comprised HUFA, indicating a significant presence of EPA and DHA compared with the Art_{Ref} base diet. In contrast, only a smaller part of PUFA in the Art_{Ref} diet constituted HUFA. Additionally, the analysis of both the Art_{Ref} and $Art_{DHA/EPA}$ diets revealed that the enrichment process contributed to elevating the SFA and MUFA content. Conversely, the incorporation of 5% or 10% copepods in combination with the enriched *Artemia* increased the PUFA and HUFA content.

Regarding the fatty acid families, the *Cop* diet showed high concentrations of ω3 PUFA. Its fatty acid profile showed that EPA and DHA were the predominant polyunsaturated fatty acids. Furthermore, the substantial presence of C16:1ω7c (palmitoleic acid) in the *Artemia* profile contributed to the heightened ω7 content in *Artemia*-based dietary treatments ($Art_{DHA/EPA}$, $Art_{DHA/EPA\ 5\%}$, and $Art_{DHA/EPA\ 10\%}$). Moreover, the enriched *Artemia* diets exhibited notably high levels of ω9 fatty acids, which declined with the incorporation of copepods. This outcome is attributed to the elevated concentration of C18:1ω9 (olieic acid) in the *I. galbana*, as mentioned earlier. The analysis of essential fatty acid profiles in the tested dietary treatments revealed the presence of all five essential fatty acids (LA, ALA, ARA, EPA, and DHA), highlighting the content of EFA in the *Cop* diet (∑EFA = 28.4%) (Table 3). The higher content of these fatty acids in the *Cop* diet could potentially justify a better lipid profile of this diet when compared with that of *Artemia* for juvenile seahorses, since high levels of these fatty acids are typically found in marine fish eggs [29]. In comparison, the EFA content in the Art_{Ref} base diet was significantly lower than that of the *Cop* diet, which can be attributed to the instability of highly unsaturated fatty acids (HUFAs) and their metabolism in *Artemia* [31].

In the preliminary growth trial, the survival rate was 0% after 9 days of the experimental trial across all dietary treatments. This result suggests that the exclusive use of enriched *Artemia* diets since 0 DPP, even with differing DHA/EPA ratios, is not a viable alternative to copepods as a suitable diet for *H. hippocampus* juveniles. While these diets match the necessary fatty acid profile, the outcome agreed with the findings of other studies, which linked this observation with the underdeveloped nature of a seahorse's digestive tract in the initial phase of its life cycle. Consequently, the correct assimilation of nutrients is hindered [7,64,65]. As *Artemia* can be more difficult to digest by fish compared with other live prey, such as copepods, when prey densities are high, continuous feeding reduces digestion time. This lowers the assimilation efficiency of the gut, possibly leading to the intact passage through the digestive tract. In some cases, prey might even remain alive upon passage [18], occasionally maintaining vitality [25]. In their natural habitat, newly born seahorses might exhibit preferences for specific prey items, making it advantageous to perform gut content analysis to assess prey preferences as they develop [66]. Since

copepods constitute the primary prey for wild juvenile seahorses, it is expected that seahorses will possess a greater capacity for digesting copepods compared with other prey. This capability could arise from an adaptive evolution of the gastrointestinal system and its secretions tailored for copepod digestion [60,67,68]. Moreover, the composition and permeability of the exoskeleton can also account for variations in digestive efficiency [69]. The exoskeleton of a copepod comprises a thin, easily breakable, segmented cuticle constructed from a protein matrix containing lipids and chitin rods. Conversely, the cuticle forming the exoskeleton of *Artemia* is thicker and more resistant to breaking [70]. This difference constitutes a significant barrier to the digestion and assimilation of the contents of specific live prey [65]. Additionally, the fact that *Artemia* is not a natural prey item for seahorses adds complexity. For instance, [18] discovered that *H. subelongatus* encountered difficulties in nutrient assimilation from the provided diet. Ref. [71] observed *Artemia* being excreted live during the first DPP when fed to juvenile *H. guttulatus*. Similarly, [7] found that *H. guttulatus* newborns exhibited a limited ability to efficiently digest *Artemia* until around 10 DPP, with significant improvement only after 15 DPP [65]. An additional explanation for the less effective use of *Artemia* as food could be attributed not only to the development of the seahorse's digestive system [7,64,72], but also to the size of their oral cavity [64,73], which may limit its capacity to accommodate larger prey. In terms of prey size, since both prey types (*Artemia* and copepods) were measured during the trials and copepods were found to be almost twice the size of the *Artemia* used, this hypothesis is not plausible, and the current results only suggest that the maturation of the digestive system is the most probable underlying cause. This idea is reinforced by the results of [64], as their study did not establish a clear correlation between the mouth size of the juveniles and the size of the prey.

In contrast, the use of copepods as a preconditioning diet played a pivotal role in achieving the survival and growth of juvenile *H. hippocampus*. The preliminary use of a natural copepod diet appeared to facilitate the maturation of the digestive tract, subsequently enabling the ability to effectively digest other enriched crustacea diets with minimal mortality. Seahorses belong to the category of agastric teleost species, lacking a functional stomach. In these species, the primary site of digestion is the intestine [74], which undergoes developmental changes during ontogeny, transitioning from a short, direct tube to an elongated, segmented duct. Within this context, the exocrine pancreas assumes the responsibility of synthesizing and releasing digestive enzymes, including proteases, glycosidases, and lipases, into the intestine. This biochemical process aids in the breakdown of dietary nutrients, transforming them into readily absorbable molecules [75].

Given the effectiveness of employing copepods as the primary diet for most finfish species, their inclusion, often co-fed with other diets to address the challenges of their exclusive use, has the potential to significantly enhance the growth and survival of juvenile seahorses e.g., [59,64]. Therefore, considering the unsatisfactory performance of *Artemia* in the preliminary trial, the second trial was conducted to test the beneficial use of limited copepod inclusion levels. During this trial, survival rates exhibited considerable fluctuation, yet the average survival rate across all dietary treatments reached 81%, with the lowest recorded at 72%. This promising outcome is noteworthy compared with the findings of other studies [17,23,70]. Ref. [18] reported survival rates of >80% at 14 DPP for *H. subelongatus* juveniles fed copepods compared with >40% for those fed enriched *Artemia*. In the present study, considering the appropriateness of a copepod diet, one would anticipate a higher survival rate among fish fed this diet. However, contrary to this expectation, the opposite was observed, as fish fed the *Cop* diet exhibited the lowest survival rate (72%) among the four tested groups. During the initial 7 days of the trial (0–7 DPP), all groups were provided with the same copepod diet, and only on the 8th day the diet was changed to the respective dietary treatments. Therefore, the initial mortality observed within the first 7 DPP cannot be attributed to diet. Similarly, previous studies achieved reasonably high rates of survival and growth for seahorse juveniles fed copepod-based diets [16,18,76].

Regarding the growth performance of fish fed various dietary treatments, it is noteworthy that the mean LG displayed greater homogeneity than the mean WG during the analyzed time period. Furthermore, LG exhibited a more pronounced similarity among the groups. Once again, a recurring observation was made: smaller LG was associated with individuals fed $Art_{DHA/EPA}$, whereas larger LG was evident among those fed according to other dietary regimes, with the *Cop* diet resulting in the highest measurements. Consequently, juvenile seahorses fed the enriched *Artemia* diet ($Art_{DHA/EPA}$) without any inclusion of copepods demonstrated the lowest MW gain at the conclusion of the experimental trial. As previously mentioned, this outcome likely resulted from the inadequate DHA/EPA ratio (0.04) identified in this particular diet, negatively impacting juvenile growth. Additionally, it was evident that when diets included either enriched *Artemia* co-fed with copepods ($Art_{DHA/EPA5\%}$ and $Art_{DHA/EPA10\%}$) or exclusively comprised copepods (*Cop*), the MW gain increased significantly ($p < 0.05$). This consequently yielded substantially higher mean WG values, thereby contributing to elevated thermal-unit growth coefficient (TGC) values. These findings can also be attributed to the heightened HUFA content observed in these diets compared with that of $Art_{DHA/EPA}$. This observation agrees with [77], who demonstrated that optimal growth performance in *H. erectus* juveniles fed copepods was attributed to the abundant content of HUFAs.

5. Conclusions

In conclusion, the growth and survival of juvenile *H. hippocampus* are significantly enhanced when the initial diet consists exclusively of copepods, allowing for the maturation of the digestive tract. Subsequently, even the small inclusion of 5% to 10% copepods in the diet contributes to an improved fatty acid profile due to the copepods' high EFA content. This dietary adjustment aligns with the species' requirements, translating into substantial benefits in terms of growth and survival.

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Data Availability Statement: The data used during the current study are available from the corresponding author upon reasonable request.

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