The Copepod/*Artemia* Trade-Off in the Culture of Long Snouted Seahorse *Hippocampus guttulatus*

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Article

The Copepod/*Artemia* Trade-Off in the Culture of Long Snouted Seahorse *Hippocampus guttulatus*

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Abstract

This study investigated the effects of copepods and copepod conditioning strategies on the growth and survivorship of long-snouted seahorse (*Hippocampus guttulatus*) juveniles from 1 to 60 days post-parturition (DPP). Four dietary treatments were tested: 24h *Isochrysis galbana* enriched *Artemia* (control), freshly collected copepods, 48h unfed copepods, and 24h *I. galbana* enriched copepods. Juveniles fed any copepod-based diet exhibited significantly higher growth ($P < 0.05$) and survival than those fed enriched *Artemia*. Mean standard length increased from 1.3 ± 0.1 cm at release to 5.9 ± 0.2, 7.5 ± 1.4, 7.1 ± 1.2, and 7.3 ± 1.1 cm at 60 DPP for the respective treatments. Wet weight rose from 0.002 ± 1 g to 0.44 ± 0.07, 0.81 ± 0.4, 0.68 ± 0.3, and 0.76 ± 0.4 mg, while final survival reached 20%, 60%, 33.3%, and 56%, respectively. Compared with *Artemia*, copepods markedly enhanced juvenile performance, supporting faster growth and promoting beneficial behavioral traits which contributed to improved survival. These results demonstrate that copepods as a superior live feed for early juvenile *H. guttulatus*, but copepod conditioning strategies directly influence their nutritional quality and, consequently, seahorse growth and survivorship. The use of copepods for the first 60 DPP is therefore not only feasible but strongly recommended.

Keywords: seahorses; copepods; fatty acids; growth; survival

Key Contribution: This study offers a comprehensive assessment of how copepod conditioning strategies shape early growth and survival in *Hippocampus guttulatus* juveniles. It shows that copepods, regardless of conditioning, consistently outperform Artemia as a first feed, markedly improving both growth and survivorship during the first 60 days post-parturition. However, the extent of these benefits depends on conditioning, with freshly collected and short-term enriched copepods producing the most favorable outcomes. This provides evidence-based guidance for optimizing early feeding protocols in seahorse aquaculture and contributes novel insight into how live-feed nutritional quality and conditioning directly shape early seahorse performance.

1. Introduction

Seahorses (*Hippocampus* spp.) are globally traded marine fishes with considerable commercial value. Dried seahorses are predominantly used in traditional Chinese medicine (TCM) and, to a lesser extent, as curios, whereas live individuals are sold in the ornamental aquarium trade [1]. While the trade in dried seahorses constitutes 98% of the total seahorse trade [2], the aquarium trade can exert the greatest pressure on specific populations [3]. The global annual trade in seahorses and related

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syngnathid species is estimated at approximately 37 million individuals [4–6]. China, including Hong Kong, consumes about 250 tons of seahorses annually for TCM purposes [7], and nearly 80 countries were involved in the trade of seahorses and related taxa between 1996 and 2001 [8,9]. The number of wild-caught seahorse species traded internationally remained relatively stable between 1997 and 2008, while the number of captive-bred species increased between 2002 and 2004, indicating a growing trend in seahorse aquaculture [10]. Despite this, the trade in wild-caught specimens continues to exert significant pressure on native populations and is considered one of the main drivers of population declines in many species [11]. Seahorses possess unique life-history traits, including low fecundity, monogamous mating systems, and prolonged parental care for small broods [12]. These biological characteristics make seahorses particularly vulnerable to overexploitation and environmental disturbances. Consequently, according to the 2025 IUCN Red List of Threatened Species, one species is classified as *Critically Endangered*, two as *Endangered*, thirteen as *Vulnerable*, and two as *Near Threatened*, while the remaining species are listed as *Least Concern* or *Data Deficient*. Notably, 23 species are reported to have declining populations, while the remaining rest unknown [11].

In this context, seahorse aquaculture represents a promising alternative to meet the growing demand for ornamental marine species while potentially reducing fishing pressure on wild populations (Martin-Smith and Vincent, 2006). The global market for aquaculture-sourced seahorses is dominated by live specimens [10]. Of the 45 recognized *Hippocampus* species [25], seven, *H. abdominalis*, *H. barbouri*, *H. breviceps*, *H. comes*, *H. ingens*, *H. kuda*, and *H. reidi*, account for over 99% of internationally traded captive-bred individuals [13]. The long-snouted seahorse, *Hippocampus guttulatus* (Cuvier, 1829), is among the wild-caught species traded in relatively large numbers [10] and may thus represent a promising candidate for diversification within the aquaculture industry. Due to its geographical distribution, the optimal temperature range for *H. guttulatus* is lower than that of several other species commonly traded in the ornamental market, such as *H. kuda* [14,15], *H. comes* [16], *H. barbouri* [17], and *H. reidi* [18,19]. However, its temperature tolerance is comparable to other widely traded species such as *H. ingens* [20], *H. whitei* [21], *H. erectus* [22], and *H. abdominalis* [23,24], supporting its potential for production as an ornamental species. Nevertheless, seahorses remain challenging candidates for aquaculture development due to persistent knowledge gaps concerning their nutritional requirements. Feed formulation and delivery continue to represent a major bottleneck in the successful rearing of seahorses, particularly during the early juvenile stages. The low survival in the early juvenile stages [25] is still one of the bottlenecks affecting commercial economic return of seahorse culture. Although high survival rates in some juvenile seahorse species have been reported (e.g., *H. abdominalis* [26], *H. erectus* [27], *H. comes* [16] or *H. guttulatus* [28]), a consensual husbandry protocol including feeding preventive of substantial mortalities in the first day's post parturition (DPP) haven't been achieved. Water quality, light intensity and rearing density do play a role in seahorse juvenile survival [e.g. 21;29], but inadequate feeds have been identified as the major cause for juvenile mortality in the first day's post parturition (DPP) [30,31]. As juvenile seahorses feed only on live prey, to date most rearing attempts have relied on the use of *Artemia* nauplii [e.g. 14,18,32-43]. Yet, *Artemia* nauplii and metanauplii have repeatedly proven to be nutritionally deficient as a food source for larvae and juvenile marine fishes compared to natural zooplankton, mainly copepods, which consistently produce much better results in terms of growth, survival and overall health condition regardless of the copepod species [6,18,29,33,44–48]. Copepods are known as the preferred natural prey for most marine fish larvae and often form the dominant component of their diets [49]. Copepods not only possess nutritional profiles that meet larval fish requirements [50–52]; but also contain high levels of digestive enzymes [53] and capable of producing appetite stimulatory effects on larvae [54,55]. Delbare et al. (1996) [56] summarized the advantages of using copepods, to their wide range of body size between nauplii and adults, typical movement, and high content of HUFAs.

Fish, like other vertebrates, require three long-chain HUFAs, docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), and arachidonic acid (AA) for growth, development and reproduction.

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Conversely, a deficit in these fatty acids may cause a general decrease in larval health, poor growth, low feed efficiency, anemia, and high mortality [57–62]. These compounds play an important role in cell membrane structure and function and act as precursors to a group of highly biologically active hormones known as eicosanoids [57,58]. Eicosanoids are involved in a wide range of physiologic functions and are produced in response to stressful situations. Unlike terrestrial vertebrates and most freshwater and marine fishes are unable to synthesize these HUFAs from other molecules. DHA, EPA, and AA are therefore considered essential fatty acids in marine-fish nutrition [44]. Recent research has demonstrated that because of competitive interactions between these fatty acids and between their chemical precursors and products, dietary ratios of DHA, EPA, and AA may be more important than actual levels. Therefore, HUFA levels in aquaculture feeds and enrichment products must be considered in relative terms rather than as absolute amounts. No matter the considerable variation in HUFA requirements among marine fish species many of those species seem to benefit from a high DHA-to-EPA ratio [57,58].

Copepods are considered an optimal diet for marine fish larvae most due to their balanced fatty acids profile, but their practical use in aquaculture can be very expensive. Copepods do not reach nearly as high density in culture conditions as other live food thus requiring larger volumes of water and larger culture vessels [44]. The use of wild-caught copepods can eliminate this problem, but wild populations may be subjected to a high degree of fluctuations. When collected from the wild and later kept until use, or even captive breed, it is necessary to maintain the adequate nutritional profile, so their conditioning is fundamental.

This study aimed to evaluate not only the usefulness of using copepods as a live diet, but also, to evaluate if different copepod conditioning prior to use, either by limited time food deprivation or through enrichment, would have a significant effect on growth and survival of juvenile long snout seahorse, *H. guttulatus*.

2. Materials and Methods

2.1. Broodstock Maintenance and Feeding

A broodstock of twenty F_3 generation adult *H. guttulatus* (10 females, 10 males), were stocked in each of 2 units of 250L white plastic flat bottom rectangular tanks at 10 animals (5 males, 5 females) per tank. Tanks were assembled in a flow-through system with a constant water flow (100 l h^{-1}) and moderate aeration. Temperature and salinity were not controlled and followed the natural seasonal pattern. Tanks were illuminated from above with 2×36W fluorescent tubes, with an intensity of 600±25 lux at the water surface and a photoperiod controlled by a timer. Photoperiod was adjusted every two weeks to match the natural photoperiod. Water quality parameters (ammonia, nitrates and nitrites) were recorded twice a week and kept stable throughout the experiment; ammonia values were always below detectable levels, nitrate <0.3 mg l^{-1} and nitrite <1.25 mg l^{-1}. Seahorses were fed a mixed diet composed of mysid shrimp (*Mesopodopsis slabberi and Diamysis lagunaris*) and frozen shrimp (Atlantic ditch shrimp, *Palaemonetes varians*).

2.2. Breeding Protocol

For the porpoise of the experiment, adult seahorses were allowed to mate and reproduce freely. After parturition, juveniles from a single brood were gently collected, counted and randomly stocked in each of 16 (10 l glass rectangular) tanks at a density of 1.5 fish l^{-1} (15 juveniles per tank) (n=240). Density was intentionally kept low to prevent detrimental effects on juvenile seahorse growth. Husbandry conditions and experimental design used were the same as described by [40]. The rearing trial was conducted according to a completely randomised design, with four replicate tanks assigned to each tested diet. In each replicate tank, the front wall was left uncovered for observation, but lateral and back walls were covered with a black adhesive to improve prey detection [26]. Seawater temperature, salinity and dissolved oxygen were kept, respectively, at 20.5 ± 0.3ºC, 37.5 ± 0.1‰ and 7.4 ± 0.1mg l^{-1}. Tanks were illuminated from above with 2 × 36 W fluorescent tubes, with a light

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intensity of 900 ± 40 lux at the water surface and a photoperiod controlled by a timer adjusted as described above. Seawater quality data (ammonia, nitrates, and nitrites) were recorded biweekly. Values remained stable throughout the experiment; ammonia values were always below detectable levels, nitrate <0.3 mg l^{-1} and nitrite <1.25 mg l^{-1}. The rearing trial was run for 60 days (0-60 DPP), period after which, copepods became size inadequate to fed juvenile *H. guttulatus*.

Juvenile *Hippocampus guttulatus* were assigned to one of four dietary treatments: (1) 24 h–enriched *Artemia* (control diet), (2) freshly captured copepods, (3) 24 h–enriched copepods, and (4) 48 h–unfed copepods. For the control diet, AF *Artemia* cysts (Inve®, Dendermonde, Belgium) were hatched following the protocol described by [63]. The nauplii were subsequently enriched for 24 h in a 20 L acrylic cylindrical–conical tank at a maximum density of 50,000 *Artemia* L^{-1}, maintained at room temperature (20–22 °C) under constant moderate aeration. The enrichment medium consisted of cultured *Isochrysis galbana* provided at a concentration of 6×10^6 cells mL^{-1}.

Copepods (*Oithona nana*) were collected daily from the outflow ponds of the research station, where they occurred naturally. Following collection, copepods were counted and divided equally into three portions corresponding to the respective dietary treatments. The first portion was supplied immediately after collection (fresh copepod diet). The second portion was enriched for 24 h in a 20 L acrylic cylindrical–conical tank with *I. galbana*, following the same enrichment procedure applied to *Artemia* (enriched copepod diet). The third portion was maintained unfed for 48 h in a 20 L tank before use (unfed copepod diet). In all dietary treatments, juvenile seahorses were fed *ad libitum* once daily at an approximate prey density of 3,000 prey L^{-1}. Each morning, two hours prior to feeding, uneaten prey were removed by siphoning, and faeces and other debris were eliminated using the same procedure.

During the experimental period, seahorses were sampled every two weeks, using a simplified protocol as an alternative to the measuring protocol proposed by [64] in order to minimize stress during sampling. Instead of the three measurements proposed by this author (the sum of head, trunk and tail lengths) juvenile seahorses were measured by the sum of the head length and fish height. Both, length and weight were collected using [35] protocol. Juveniles were individually collected from the broodstock tank with a small container and transferred to a shallow tray where were measured with Vernier digital calliper. Seahorse wet weight was measured on a Kern microgram balance following quick blot-drying. This data was used to calculate: 1) Mean Weight Gain WG(mg/fish) = $(W_f\text{-}W_i)/W_i$, where W_f is the final seahorse wet weight and W_i is the initial wet weight, 2) Mean Length Gain LG(cm/fish) = $(L_f\text{-}L_i)/L_i$, where L_f is the final seahorse length and L_i is the initial length, 3) Growth rate was calculated using the thermal-unit growth coefficient (TGC) method (modified from [65] by Cho [66]), using the equation $TGC=[(W_f^{1/3} – Wi^{1/3})/\Sigma(T\times D)]\times 100$, where W_f is the final seahorse wet weight and W_i is the initial wet weight; T=water temperature, ºC; D=number of days, 4) Condition Factor (CF)=(wet weight (g)/length (cm^{-3}))×100.

2.4. Proximate Analysis

Artemia and copepod analyses were performed on triplicate 50g (wet weight) of each of the three dietary treatments. Samples were freeze-dried, grinded and stored at -18 ºC until analysis. Samples were analysed for dry matter and ash contents according to the methods of [67], crude protein (N × 6.25) by Dumas method using a Leco Nitrogen Analyser, and total lipid by petroleum ether extraction using a XT20 ANKOM analyser (Ankom Technology, Macedon, NY, USA).

2.5. Lipid Analysis

Triplicate samples of each of the four dietary treatments were frozen and stored at -80ºC. The samples were then freeze-dried for 2 days. Total dry weights were recorded and then samples were taken for lipid extraction. Total lipid samples were separated into classes by one-dimensional double-development high-performance thin-layer chromatography (HPTLC) using methyl acetate/ isopropanol/ chloroform/ methanol/ 0.25% (w/v) KCl (25:25:25:10:9 by vol.), as the polar solvent system and hexane/diethyl ether/glacial acetic acid (80:20:2 by vol.), as the neutral solvent system.

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Lipid classes were quantified by charring with a copper acetate reagent followed by calibrated scanning densitometry using a SHIMADZU CS-9001PC (Kyoto, Japan) dual wavelength flying spot scanner [68]. Total lipid extracts were subjected to acid-catalysed transmethylation for 16 h at 50ºC, using 1mL of toluene and 2 mL of 1% sulphuric acid (v/v) in methanol. The resulting fatty-acid methyl esters (FAME) were purified by thin-layer chromatography (TLC) and visualized with iodine in chloroform:methanol (2:1 v/v) 98% (v/v) containing 0.01% BHT [69]. Prior to transmethylation, heneicosanoic acid (21:0) was added to the TL as an internal standard. FAME were separated and quantified using a SHIMADZU GC 2010 (Kyoto, Japan) gas chromatograph equipped with a flame-ionisation detector (250ºC) and a fused silica capillary column RTX - WAXTM (10 m x 0.1 mm I.D.). Helium was used as a carrier gas and the initial oven temperature was 150ºC, followed by an increase at a rate of 90ºC min^{-1} to a final temperature of 250ºC for 3 min. Individual FAME were identified by reference to authentic standards and to a well-characterized fish oil. BHT, potassium chloride, potassium bicarbonate, and iodine were supplied by Sigma Chemical Co (St. Louis, USA). TLC (20x20 cm x 0.25 mm) and HPTLC (10x10 cm x 0.15 mm) plates, pre-coated with silica gel (without fluorescent indicator) were purchased from Macheren-Nagel (Düren, Germany). All organic solvents used for GC were of reagent grade and were purchased from PANREAC (Barcelona, Spain).

2.5. Statistical Analysis

Statistical analyses were conducted using the package GraphPad Prism (version 9.0, GraphPad Software, San Diego, CA, USA). Differences in seahorse length, wet weight, CF, TGC and FCR tested using nested ANOVA with post hoc Neuman-Keul's (NK) multiple comparison test (P=0.05). One-way ANOVA followed by a pair-wise multiple comparisons of means using Tukey's test following testing for normality and homogeneity of variance were used to determine differences in fatty acid composition ($P < 0.05$).

3. Results

The proximate composition of each dietary treatment is presented in Table 1. The moisture content differed significantly among diets, with enriched *Artemia* showing the lowest moisture (70.3 ± 0.4%), while all copepod-based diets exhibited significantly higher values (84.6–86.6%), with no differences among the copepod groups. Consequently, enriched *Artemia* had a significantly higher dry matter content (29.7 ± 1%) compared with daily-collected, 48 h unfed, and 24 h enriched copepods (13.4–15.4%). Enriched *Artemia* also displayed the highest gross protein (17.7 ± 0.8 g/100 g DM) and total lipid levels (9.8 ± 0.7 g/100 g DM), both significantly higher than any of the copepod treatments. Among copepods, protein and lipid contents were lower and similar across daily-collected, 48 h unfed, and 24 h enriched groups. Gross energy content was significantly higher in daily-collected copepods (504.28 ± 1.2 kcal/100 g DM) and in the 24 h enriched copepods (502.53 ± 1.4 kcal/100 g DM) compared with enriched *Artemia* and 48 h unfed copepods, which showed lower but comparable energy values.

Table 1. Proximate composition of the four tested dietary treatments (per 100 g of Dry Matter (g/100 g DM)) (24h *I. galbana* enriched *Artemia*, daily captured copepods, 48h unfed copepods, and 24h *I. galbana* enriched copepods).

Proximate Composition	Enriched *Artemia*	Daily copepods	48h unfed copepods	24h enriched copepods
Moisture (%)	70.3±0.4^{b}	86.6±1.1^{a}	84.6±1.0^{a}	85.5±1.0^{a}
Dry Matter (%)	29.7±1^{a}	13.4±1.1^{b}	15.4±1.3^{b}	14.5±1.3^{b}
Gross Protein (g/100g DM)	17.7±0.8^{a}	10.4±0.6^{b}	8.9±1.1^{a}	9.8±1.1^{b}
Total Lipids (g/100g DM)	9.8±0.7^{a}	4.9±0.8^{b}	4.3±0.7^{b}	4.8±0.9^{b}
Gross Energy (Kcal/100 DM)	488.9±0.7^{b}	504.28±1.2^{a}	491.53±1.4^{b}	502.53±1.4^{a}

The fatty acid profiles of the four dietary treatments (enriched *Artemia*, daily copepods, starved copepods, and enriched copepods) revealed marked differences in the proportions of saturated (SFA), monounsaturated (MUFA), and polyunsaturated fatty acids (PUFA) (Table 2). SFA were

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significantly higher in all copepod-based diets compared to enriched *Artemia* ($p < 0.05$). The highest SFA levels were found in daily and starved copepods (47.77 ± 0.75% and 47.90 ± 1.01%, respectively), while enriched *Artemia* showed the lowest (32.46 ± 1.43%). The predominant SFA, 16:00, was significantly higher in copepod diets (24.6–27.3%) than in *Artemia* (20.9%). MUFA were most abundant in enriched *Artemia* (45.15 ± 2.31%), significantly exceeding those in copepod treatments (14.13–18.05%) ($p < 0.05$). This was mainly due to higher proportions of 16:1n7 and 18:1n9 in *Artemia* (12.42% and 16.37%, respectively), compared to lower values in copepods (2.06–6.93% and 2.22–5.99%). PUFA, particularly highly unsaturated fatty acids (HUFA, ≥C20), were markedly higher in copepod diets. ΣHUFA reached 27.14 ± 0.14% in daily copepods and 28.73 ± 2.17% in starved copepods, significantly greater than in enriched *Artemia* (8.37 ± 0.58%) ($p < 0.05$). Enriched copepods also contained elevated HUFA (22.35 ± 0.60%), though slightly lower than other copepod treatments. The high HUFA content was mainly due to 22:6n3 (DHA) and 20:5n3 (EPA), which were especially abundant in copepods (DHA: 16.28–18.66%; EPA: 5.31–9.67%) relative to *Artemia* (0.23% and 5.77%, respectively). Regarding PUFA families, the n-3 fatty acids were significantly higher in copepod diets (27.48–30.15%) compared to enriched *Artemia* (10.61%) ($p < 0.05$), while n-6 fatty acids showed the opposite trend, being higher in *Artemia* (7.49%) and enriched copepods (6.88%) than in daily and starved copepods (2.86–3.52%). Consequently, the n-3/n-6 ratio was significantly lower in copepod diets (0.33–0.52) than in *Artemia* (1.42). Ratios among key fatty acids further highlighted compositional shifts between diets. Copepods presented significantly higher DHA/EPA, EPA/AA, and DHA/AA ratios (1.92–3.06, 9.14–9.39, and 17.9–28.07, respectively) than *Artemia* (0.04, 3.43, and 0.14, respectively), indicating a stronger enrichment in long-chain n-3 HUFA. Overall, copepod-based diets, particularly daily and starved copepods, were characterized by high levels of SFA and HUFA, especially DHA and EPA, while enriched *Artemia* contained substantially higher MUFA proportions but lower HUFA levels.

Table 2. Fatty acid composition of each dietary treatment, 24h *I. galbana* enriched *Artemia*, daily captured copepods, 48h unfed copepods and 24h *I. galbana* enriched copepods (mean ± standard deviation, (n=3)) expressed as a percentage (%) of total identified fatty acids. SMA (saturated fatty acids), MUFA (monounsaturated fatty acids), PUFA (polyunsaturated fatty acids), HUFA (highly unsaturated fatty acids). Row values with different superscripts are significantly different ($p < 0.05$).

Category	Fatty acid	24h enriched *Artemia*	Daily copepods	48h unfed copepods	24h enriched copepods
SMA (saturated)	14:00	4.32 ±0.14^{b}	10.02 ±0.6^{a}	3.67 ±0.21^{b}	8.48 ±0.2^{a}
	15:00	0.71 ±0.12^{a}	1.13 ±0.11^{a}	0.67 ±0.08^{a}	0.88 ±0.08^{a}
	16:00	20.92 ±0.89^{c}	27.31 ±0.4^{a}	26.58 ±0.75^{a}	24.6 ±0.05^{b}
	17:00	0.61 ±0.09^{c}	1.09 ±0.05^{b}	1.92 ±0.11^{a}	1.3 ±0.1^{a}
	18:00	5.24 ±0.15^{c}	6.91 ±0.15^{b}	13.24 ±0.21^{a}	7.57 ±0.13^{b}
	21:00	0.47 ±0.07^{a}	0.20 ±0.03^{b}	0.38 ±0.09^{b}	0.60 ±0.03^{a}
	24:00	0.19 ±0.04^{a}	0.30 ±0.06^{a}	0.17 ±0.15^{a}	0.20 ±0.17^{a}
Σ Saturated		32.46±1.43^{b}	47.77 ±0.75^{a}	47.90 ±1.01^{a}	44.46 ±0.35^{a}
MUFA (monoenes)	16:1n7	12.42 ±0.32^{a}	6.93 ±0.23^{b}	2.06 ±0.20^{d}	4.14 ±0.02^{c}
	17:01	0.19 ±0.20b	0.24 ±0.02a	0.26 ±0.24a	0.40 ±0.01a
	18:1n9	16.37 ±0.05^{a}	2.22 ±0.03^{c}	2.71 ±1.18^{c}	5.99 ±0.12^{b}
	18:1n7	12.81 ±0.04^{a}	3.24 ±0.11^{b}	3.03 ±1.20^{b}	3.22 ±0.06^{b}
	22:1n9	1.94 ±0.21^{b}	1.61 ±0.12^{b}	4.51 ±0.03^{a}	2.35 ±0.05^{b}
	24:01	1.42 ±0.59^{a}	0.60 ±0.07^{b}	0.78 ±0.68^{b}	0.99 ±0.03^{a}
Σ Monoenes		45.15±2.31^{a}	15.95 ±0.60^{b}	14.13 ±1.39^{b}	18.05 ±0.14^{b}
PUFA (non-HUFA)	16:2n4	0.42 ±0.07^{a}	0.29 ±0.01^{a}	0.04 ±0.07^{a}	0.21 ±0.07^{a}
	16:3n4	0.15 ±0.21^{a}	0.35 ±0.01^{a}	0.30 ±0.30^{a}	0.25 ±0.07^{a}
	16:4n1	0.18 ±0.09^{a}	0.20 ±0.06^{a}	0.07 ±0.13^{a}	0.14 ±0.12^{a}
	18:2n6	4.86 ±0.06^{a}	1.05 ±0.05^{b}	0.92 ±0.05^{b}	3.78 ±0.09^{a}
	18:3n6	0.63 ±0.09b	0.75 ±0.07b	0.66 ±0.21b	1.39 ±0.11a
	18:3n3	3.21 ±0.05^{a}	1.31 ±0.00^{b}	0.85 ±0.06^{b}	2.47 ±0.05^{a}
	18:4n3	1.02 ±0.04b	0.97 ±0.00b	0.57 ±0.04b	2.66 ±0.05a
Σ PUFA (non-HUFA)		10.47±2.45			
HUFA (=C20)	20:4n6 (AA)	1.68 ±0.03^{a}	0.96 ±0.02^{a}	1.03 ±0.08^{a}	0.58 ±0.05^{b}
	20:5n3 (EPA)	5.77 ±0.32^{b}	8.77 ±0.01^{a}	9.67 ±0.50^{a}	5.31 ±0.17^{b}
	22:5n3	0.37 ±0.23^{a}	0.74 ±0.02^{a}	0.40 ±0.04^{a}	0.45 ±0.05^{a}

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22:5n6	0.32 ±0.16^{b}	0.49 ±0.04^{a}	0.25 ±0.21^{b}	0.82 ±0.19^{a}
22:6n3 (DHA)	0.23±0.07^{b}	17.18 ±0.12^{a}	18.66 ±1.72^{a}	16.28 ±0.56^{a}
Σ HUFA	8.37±0.58^{c}	27.14 ±0.14^{a}	28.73 ±2.17^{a}	22.35 ±0.60^{b}
Σ PUFA (total) (non-HUFA + HUFA)	18.84±1.48^{b}	29.42 ±0.14^{a}	30.15 ±2.19^{a}	27.48 ±0.62^{a}
Total Σ (SMA + MUFA + PUFA)	96.45±1.42^{a}	97.61 ±0.36^{a}	95.90 ±3.53^{a}	97.52 ±0.42^{a}
Unknown	3.55±1.42^{a}	2.39±0.36^{a}	4.13±3.53^{a}	2.48±0.42^{a}
Saturated	32.46±1.09^{b}	47.77±0.75^{a}	47.9±1.01^{a}	44.46±0.35^{a}
Monoenes	45.15±2.14^{a}	15.95±0.6^{b}	14.13±1.39^{b}	18.05±0.14^{b}
n-3	10.6±1.45^{b}	29.42±0.14^{a}	30.15±2.19^{a}	27.48±0.62^{a}
n-6	7.49±0.5^{a}	3.52±0.2^{b}	2.86±0.15^{b}	6.88±0.24^{a}
n-9	18.31±0.53^{a}	4.06±0.12^{c}	7.65±1.09^{b}	8.78±0.14^{b}
n-3 HUFA	6.37±0.61^{c}	27.14±0.14^{a}	28.73±2.17^{a}	22.35±0.6^{b}
n-3/n-6	1.42±0.07^{a}	0.51±0.004^{b}	0.52±0.02^{b}	0.33±0.005^{b}
DHA/EPA	0.039±0.02^{a}	1.96±0.002^{b}	1.92±0.01^{b}	3.06±0.008^{b}
EPA/AA	3.43±0.2^{a}	9.14±0.15^{b}	9.39±0.21^{b}	9.16±0.22^{b}
DHA/AA	0.14±0.02^{a}	17.9±0.1^{b}	18.12±0.1^{b}	28.07±0.12^{b}

Data on standard length, body weight, mean weight gain (WG), condition factor (CF), thermal-unit growth coefficient (TGC), and survival are presented in Table 3. After 60 days, juvenile growth performance was similar between seahorses fed daily copepods and those fed 24 h enriched copepods ($p > 0.05$), but significantly lower ($p < 0.05$) in fish fed enriched *Artemia*. This difference in growth performance was already evident from the first sampling period (Figure 1a,b) and persisted until the end of the experiment. Seahorses fed 48 h unfed copepods showed intermediate growth and survival values, positioned between those of fish fed daily or 24 h enriched copepods and those receiving enriched *Artemia* (Table 1).

Table 3. Standard length (cm), Body weight (mg), Thermal-unit growth coefficient (TGC), and Condition Factor (CF) (mean ± SD) of juvenile *H. guttulatus* fed 24h *I. galbana* enriched *Artemia*, daily captured copepods, 48h unfed copepods or 24h *I. galbana* enriched copepods at the end of the 60-day study.

	Enriched *Artemia*	**Daily copepods**	**24h unfed copepods**	**24h enriched copepods**
Standard length (cm)	5.9±0.2^{b}	7.5±1.4^{a}	7.1±1.2^{a}	7.3±1.1^{a}
Body weight (g)	0.44±0.07^{c}	0.81±0.4^{a}	0.68±0.24^{b}	0.76±0.25^{a}
WG (g.d^{-1})	0.007±0.001^{b}	0.013±0.002^{a}	0.011±0.002^{a}	0.013±0.002^{a}
TGC	0.15^{b}	0.27^{a}	0.23^{a}	0.25^{a}
CF	0.21±0.02^{a}	0.19±0.03^{a}	0.19±0.03^{a}	0.2±0.04^{a}
% survival	20^{c}	60^{a}	33.3^{b}	56^{a}
Initial weight=0.002±0.001g: Initial length=1.3±0.1cm, Initial Condition Factor=0.09±0.01.				

Among the measured parameters, CF was the only one unaffected by diet, with no significant differences ($p > 0.05$) observed among *H. guttulatus* juveniles across treatments (Table 3). Survival ranged from 20% to 60% among dietary groups (Figure 1), being markedly lower in fish fed enriched *Artemia* (20%) compared to those fed daily copepods (60%) and 24 h enriched copepods (56%) (Figure 1c).

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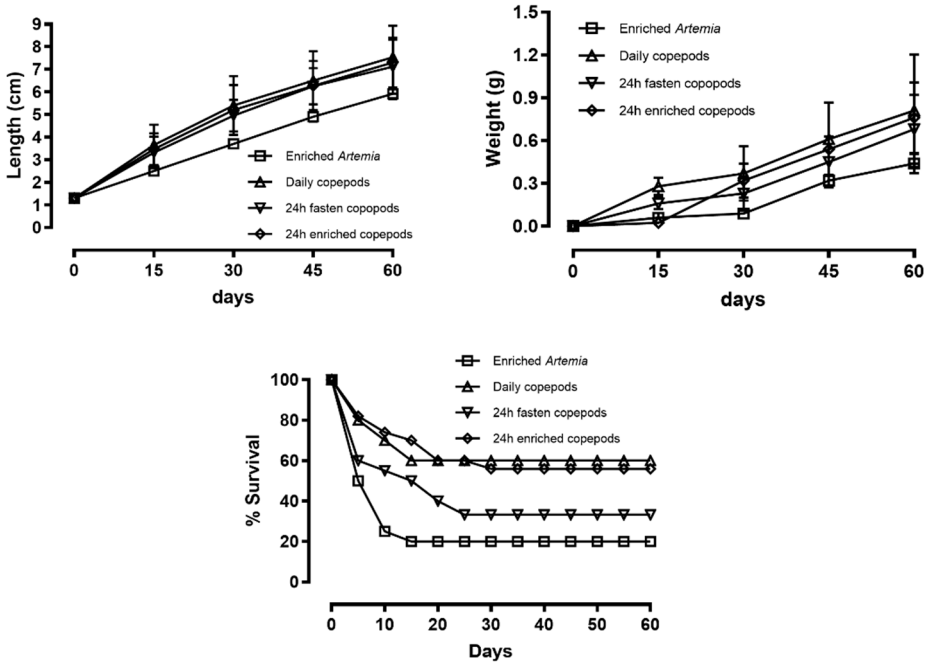

Figure 1. (a) Length increase, (b) weight increase, and (c) percent survival of juvenile *H. guttulatus* fed each of the four tested diets (24h *I. galbana* enriched *Artemia* (control diet), daily captured copepods, 48h unfed copepods, and 24h enriched copepods).

4. Discussion

During fish embryogenesis, nutrients required for growth, cellular and organ differentiation and metabolism originate on the yolk reserves, until the onset of exogenous feeding [70]. Seahorses and *H. guttulatus* in particular are no exception, as according to [70] this species as an extremely high rate of total fatty acid (TFA) consumption (67.8%) a value higher than observed in other fish, thus reflecting the high fatty acid requirements for development. As many other fish species presently breed in captivity, it was expectable that the use of enriching products could fulfil *H. guttulatus* TFA requirements. However, as reported by [40] the use of *Artemia* metanauplii enriched with DHA-Selco® resulted in 100% mortality just 10 DPP mostly due to the occurrence of gas bladder overinflation triggered by the use of enriching products which result in nutritionally unbalanced diets [30]. Due to this, and despite the available enriching products on the market intended to manipulate the levels and ratios of HUFAs in *Artemia* and other live foods, the use of copepods continues to produce better fish yields [33,71–76]. This suggests that either the ideal levels or proportions of HUFAs are not being attained through enrichment, or that there are other factors affecting growth and survival of fishes reared on copepods versus those reared on *Artemia* [44]. The latter hypothesis is supported by the results obtained by [77] in which two groups of the pipefish *Stigmatopora argus* fed with copepods containing either high or low HUFA levels did not evidenced significant differences in growth or survival. Naess et al. (1995) [78] analysed the fatty acid composition of the live feeds used on first feeding of Atlantic halibut (*Hippoglossus hippoglossus*) found that copepod-dominated wild plankton have a higher DHA/EPA ratio ranging from 1.4 to 1.7, compared to un-enriched and SuperSelco™ enriched *Artemia* which had DHA/EPA ratios of 0.1 and 0.5, respectively. The present study demonstrated that differences in dietary fatty acid composition had a clear effect on the growth performance and survival of fish. Despite all experimental parameters being standardized, fish fed copepod-based diets (daily, fasted, or enriched copepods) exhibited significantly higher standard length, body weight, and growth indices (WG and TGC) compared to those fed enriched *Artemia*. Not surprisingly, the best performance was achieved with the daily copepod diet, which yielded the greatest body weight (0.81 ± 0.4 g), weight gain (0.013 ± 0.002 g·d^{-1}), and thermal growth coefficient (0.27), while enriched *Artemia* resulted in markedly lower

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growth (0.007 ± 0.001 g·d^{-1}; TGC = 0.15) and the poorest survival (20%). These results align with previous findings that copepods constitute a superior live prey source for marine fish larvae due to their balanced and naturally rich fatty acid profile (31, 79-81].

The enhanced growth and survival in copepod-fed groups are likely associated with their higher levels of highly unsaturated fatty acids (HUFA), particularly docosahexaenoic acid (DHA, 22:6n3) and eicosapentaenoic acid (EPA, 20:5n3). In the present study, total HUFA in copepod treatments reached 22–29%, compared to only 8% in enriched *Artemia*. DHA levels were especially elevated in copepods (16–18%), while *Artemia* contained only trace amounts (0.23%), resulting in markedly higher DHA/EPA and DHA/AA ratios in copepod diets. Such fatty acid profiles are essential for maintaining membrane integrity, neural and visual development, and optimal metabolic function in fish larvae [57,82]. The superior growth obtained with copepods therefore reflects the larvae's better access to essential long-chain n-3 PUFA, particularly DHA, which is often the limiting nutrient in *Artemia*-based rearing systems. In contrast, enriched *Artemia* was characterized by elevated monounsaturated fatty acids (45.15%) and lower HUFA proportions, leading to an unbalanced n-3/n-6 ratio (1.42) compared to copepods (0.33–0.52). Although enrichment protocols can improve the nutritional quality of *Artemia*, achieving HUFA levels equivalent to natural copepods remains challenging [53,83]. The insufficient supply of long-chain n-3 HUFA likely contributed to the lower growth and survival observed in *Artemia*-fed fish, as has been previously reported for several marine species including *Solea senegalensis*, *Gadus morhua*, and *Hippocampus spp.* [40,84,85].

Interestingly, no significant differences in growth performance were found among the three copepod-based treatments, despite slight variations in their fatty acid profiles. Although enriched copepods exhibited somewhat lower total HUFA than daily or fasted copepods, the DHA and EPA concentrations were still within ranges known to support optimal juvenile development. This suggests that once a sufficient threshold of essential fatty acids is met, further enrichment may not produce measurable improvements in growth. The relatively high survival (56–60%) in copepod-fed groups compared to *Artemia* (20%) reinforces the importance of adequate DHA and EPA levels for maintaining larval robustness and resistance to stress.

Overall, these results highlight the critical role of dietary fatty acid composition, particularly DHA and EPA availability, in determining growth and survival outcomes in early fish development. Copepods provided a superior balance of HUFA and an optimal n-3/n-6 ratio, supporting improved growth efficiency and survival compared to *Artemia*. These findings underscore the nutritional advantages of using copepods in seahorse juvenile rearing protocols and emphasize the need to refine *Artemia* enrichment strategies to more closely replicate the fatty acid profiles of natural zooplankton prey.

5. Conclusions

This study provides the first integrated evaluation of how different copepod feeding and conditioning strategies influence early growth and survival of Hippocampus guttulatus juveniles. By directly comparing freshly collected, unfed, and enriched copepods against enriched Artemia, the work demonstrates that copepods, regardless of conditioning, substantially outperform Artemia as a first feed, enhancing both growth trajectories and survivorship during the critical first 60 days post-parturition. Importantly, the study also shows that copepod conditioning modulates the magnitude of these benefits, revealing that simple daily-collected or short-term enriched copepods yield the best overall outcomes. This provides evidence-based guidance for optimizing early feeding protocols in seahorse aquaculture and contributes novel insight into how live feed nutritional quality and conditioning directly shape early seahorse performance.

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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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