Marine amphipods (*Paryhale hawaiensis*) as an alternative feed for the lined seahorse (*Hippocampus erectus*, Perri 1810): nutritional value and feeding trial

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Finding new alternatives to traditional live preys such as *Artemia* and rotifers, which do not always promote optimal fish growth and survival, is required for the successful aquaculture of highly specialized predatory species, including seahorses. The present study assessed the nutritional value of an interesting marine amphipod (*Paryhale hawaiensis*), and evaluates through a feeding trial its potential use as a natural prey for 10-months lined seahorse, *Hippocampus erectus*. *P. hawaiensis* showed high levels of valuable lipids (20.4-26.7% on dry matter basis) and polyunsaturated fatty acids (PUFAs) (26.4-41% of total FAs), including the long-chain PUFAs (LC-PUFAs) arachidonic acid (ARA) (2.9-7.7%), eicosapentaenoic acid (EPA) (4.3-6.5%) and docosahexaenoic acid (DHA) (2.1-6.2%). A comparison between wild-captured and cultured amphipods revealed a significant improvement of the amphipod FA profile in terms of DHA%, total omega-3 (n3) FAs and n3/n6 ratio when employing both a conventional amphipod culture based on a commercial shrimp diet, and, to a lesser extent, a large (3500 L) biofloc system. Seahorses fed with frozen/wild amphipods, either singly or in combination with *Artemia* enriched with Super Selco® (INVE Aquaculture, Belgium) for 57 days, substantially improved seahorse growth and FA profiles in terms of ARA, EPA and DHA%, including indiex associated to marine sources, such as Σn3 and n3/n6, compared to a diet based solely on enriched *Artemia*. These results support the use of marine amphipods as an alternative food organism for juvenile *H. erectus* and suggest a potential use for general marine aquaculture.

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Abstract

Finding new alternatives to traditional live preys such as *Artemia* and rotifers, which do not always promote optimal fish growth and survival, is required for the successful aquaculture of highly specialized predatory species, including seahorses. The present study assessed the nutritional value of an interesting marine amphipod (*Paryhale hawaiensis*), and evaluates through a feeding trial its potential use as a natural prey for 10-months lined seahorse, *Hippocampus erectus*. *P. hawaiensis* showed high levels of valuable lipids (20.4-26.7% on dry matter basis) and polyunsaturated fatty acids (PUFAs) (26.4-41% of total FAs), including the long-chain PUFAs (LC-PUFAs) arachidonic acid (ARA) (2.9-7.7%), eicosapentaenoic acid (EPA) (4.3-6.5%) and docosahexaenoic acid (DHA) (2.1-6.2%). A comparison between wild-captured and cultured amphipods revealed a significant improvement of the amphipod FA profile in terms of DHA%, total omega-3 (n3) FAs

36 and n3/n6 ratio when employing both a conventional amphipod culture based on a commercial
37 shrimp diet, and, to a lesser extent, a large (3500 L) biofloc system. Seahorses fed with frozen/wild
38 amphipods, either singly or in combination with *Artemia* enriched with Super Selco® (INVE
39 Aquaculture, Belgium) for 57 days, substantially improved seahorse growth and FA profiles in
40 terms of ARA, EPA and DHA%, including index associated to marine sources, such as Σn3 and
41 n3/n6, compared to a diet based solely on enriched *Artemia*. These results support the use of marine
42 amphipods as an alternative food organism for juvenile *H. erectus* and suggest a potential use for
43 general marine aquaculture.

Introduction

45 Live food organisms are indispensable for the early culture of many marine species of commercial
46 interest, including marine ornamentals (Olivotto et al., 2011, 2017a; Southgate, 2019). Due to the
47 predatory nature of some species, live food stimulates a better feeding response compared to inert
48 feeds, in addition to being more easily digested and assimilated (Conceição et al., 2010). The ideal
49 live foods are fundamentally the preys a particular species encounter in nature and these often
50 include small crustaceans such as copepods, amphipods and decapods (Olivotto et al., 2017b). The
51 production of natural food organisms is, unfortunately, laborious and expensive, at best, and
52 unsuccessful for most species (Southgate, 2019). The capture of live food organisms from the wild
53 provides a viable alternative to captive propagation. Notwithstanding its cost-effectiveness, this
54 practice is subject to seasonal availability and susceptible to the undesired introduction of
55 pathogens and pests (Cohen and Valenti, 2019).

56 Because of their relatively easy production and cost, *Artemia* sp. and rotifers are the most
57 commonly used live food organisms (Southgate, 2019). These preys are not the natural prey of
58 marine fish, but they are widely used and with relative success (Bengtson, 2003). One of their
59 main limitations is that they do not always satisfy the nutritional requirements of all organisms
60 (Sorgeloos et al., 2001). "Enriching" these preys with formulations such as oil emulsions rich in
61 essential FAs (EFAs) or microalgae, may overcome nutritional deficiencies; but even
62 implementing this costly practice, they are often inadequate for the culture of many species,
63 including seahorses (Segade et al., 2016; Randazzo et al., 2018; Planas et al., 2020).

64 In the last years, marine amphipods have received increasing attention as an alternative natural
65 food. These benthic crustaceans can form large colonies (> 100 000 individuals m^{-2}) in natural or
66 artificial aquatic habitats, such as coral reefs, seagrasses, seaweeds and biofoulings (Lourido et al.,
67 2008; Vázquez-Luis et al., 2013; Navarro-Mayoral et al., 2020), where they constitute a major
68 natural prey of small fish (< 30 cm standard length) and invertebrates (Woods, 2009). They are
69 promising candidates both for intensive and extensive culture, as they can feed on a variety of
70 foodstuffs, including decaying organic material and detritus (Guerra-García et al., 2016), and
71 tolerate wide ranges of environmental parameters, such as temperature (> 20 °C range) and salinity
72 (> 20 psu range) (Takeuchi et al., 2003; Campbell et al., 2020). Although diet and environmental

73   parameters can influence their nutritional value, they are generally rich in proteins (up to 60% of
74   dw) and lipids (up to 20% on dry weight [dw] basis), including the PUFAs (over 50% of total FAs
75   in some species) EPA (over 20% of total FAs) and DHA (up to 20% of total FAs) (Wang and Jeffs,
76   2014; Fernandez-Gonzalez et al., 2018; Jiménez-Prada et al., 2018). The few studies to assess and
77   develop potential culturing or harvesting techniques for aquaculture conducted so far are
78   promising (Guerra-García et al., 2016; Fernandez-Gonzalez et al., 2018; Xue et al., 2018; Vargas-
79   Abúndez et al., 2021). These include a recently published culture trial in biofloc systems
80   (Promthale et al., 2021).

81   Feeding trials to assess the potential use of amphipods as alternative feed conducted so far are
82   encouraging. As a partial fishmeal replacement, the Arctic amphipod (*Themsto libellula*) was
83   successfully incorporated into the diets of Atlantic salmon (*Salmo salar*) and Atlantic halibut
84   (*Hippoglossus hippoglossus*), substituting 40% of fishmeal in diet (Suontama et al., 2007).
85   *Gammarus* species have also been of interest as amphipod meal (Harlıoğlu and Farhadi, 2018).
86   Both as live and frozen feed, several amphipod species have been successfully used to replace
87   traditional live foods in the culture of seahorses (Murugan et al., 2009; Vargas-Abúndez et al.,
88   2018), octopus (Baeza-Rojano et al., 2013b) and cuttlefish (Baeza-Rojano et al., 2010).

89   According to traditional classification (Martin and David, 2001)*, Parhyale hawaiensis* (Dana,
90   1853) is a gammarid amphipod with a worldwide, circumtropical distribution (WoRMS Editorial
91   Board, 2020). It inhabits marine coastal habitats such as rocky beaches, estuaries and mangroves,
92   where it readily forms dense aggregations of up to 7 000 individuals m^{-2} (Poovachiranon et al.,
93   1986; Paz-Ríos et al., 2013). Since the early 2000's, it has been attracting interest to the scientific
94   community as a compelling crustacean model for biological research (Sun and Patel, 2019), due
95   to their small size (6.12-11.83 mm, total length), fast growth (0.15 mm day^{-1}), short life cycle
96   (from newborn to adult in 50.9 ± 5.8 days), high fecundity (up to 35 embryos per female),
97   translucent embryos and year-round reproduction (Vargas-Abúndez et al., 2021). It is amenable to
98   experimental investigation and plenty of information and experimental tools, such as the complete
99   genome and gene editing tools, are already available for this species (Kao et al., 2016; Sun and
100   Patel, 2019). Propagation of *P. hawaiensis* under laboratory conditions is straightforward and well
101   documented, with prospects for mass-scale aquaculture (Vargas-Abúndez et al., 2021). Taking
102   advantage of its environmental tolerance, opportunistic behavior and detritivorous habits, *P.*
103   *hawaiensis* could be an interesting candidate for mass production in biofloc systems. Biofloc
104   technology represents an innovative approach for environmental-friendly, cost-effective intensive
105   farming (Avnimelech, 2015; Ahmad et al., 2017).

106   The natural diet of most seahorse species is dominated by small crustaceans, primarily copepods,
107   mysid shrimps, decapods and amphipods (Manning et al., 2019). However, amphipods stand out
108   as the main food item in terms of frequency of occurrence, number or biomass ingestion for species
109   such as *H. breviceps*, *H. coronatus*, *H. erectus, H. guttulatus, H. hippocampus, H. patagonicus, H.*
110   *subelongatus* and *H. whitei* (Teixeira et al., 2001; Kendrick and Hyndes, 2005; Kitsos et al., 2008;

111 Storero and González, 2008). Adult *H. erectus*, the lined seahorse, feed almost exclusively on
112 amphipods (mainly *Gammarus muconathus*), whereas juveniles on both amphipods (mainly
113 *Ampithoe longimana*) and copepods (Teixeira et al., 2001). Feeding trials conducted by the authors
114 demonstrated that this heavily traded species showed an increased feeding response when fed with
115 frozen amphipods (*Elasmopus pectenicrus*) compared to live *Artemia* (Vargas-Abúndez et al.,
116 2018)*, confirming the potential of amphipods to overcome one of the main bottlenecks of seahorse
117 aquaculture, adequate feeding (Koldewey and Martin-Smith, 2010). Whether seahorse aquaculture
118 can meet the goal of providing a sustainable alternative to supply the traditional medicine,
119 aquarium and curio industries, highly depends on developing adequate live prey foods (Koldewey
120 and Martin-Smith, 2010).

121

122 Considering the aforementioned, the aim of the present study was (a) to assess the nutritional value
123 of *P. hawaiensis* in relation to lipids and fatty acids (FA), and (b) to test their effects as a full or
124 supplemental diet on growth, survival and FA profiles of juvenile *H. erectus*. Wild-captured
125 amphipods were used in seahorse feeding trial. Additionally, and for the first time, amphipods
126 were produced in a large-scale biofloc system and their nutritional value explored. The present
127 study represents the first one evaluating the possible use of amphipods for seahorse feeding, testing
128 the actual value of a new and promising amphipod species.

Materials & Methods

130 *2.1. Ethics*

131 The present study was carried out under a permit by SEMARNAT No. SGPA/DGVS/12741/13
132 and strictly followed institutional protocols for the maintenance, manipulation, and sacrifice of the
133 experimental animals according to certified criteria established by the Guide for the Care and Use
134 of Experimental Animals in Research and Teaching of the Faculty of Superior Studies-Cuautitlán
135 (http://www.cuautitlan.unam.mx/) at Universidad Nacional Autónoma de México. During the
136 experiment, seahorse mortality was kept at zero and no apparent signs of stress were detected (*i.e.*
137 changes in color, disease, lack of feeding or mobility).

138 *2.2. Foods of different source*

139 Amphipods (*P. hawaiensis*) were collected as previously described in Vargas-Abúndez et al.
140 (2021). Specifically, they were collected both from outdoor flow-through systems in which
141 amphipods grow freely at aquaculture facilities of the National Autonomous University of Mexico
142 (UNAM) located in Sisal, Yucatán, México, and from green intertidal algae attached to rocks in
143 Sisal beach. They are abundant in Sisal beach, from where they likely infiltrate the aquaculture
144 systems. The animals were rinsed with fresh water and then some were immediately frozen at -80
145 ºC for further lipid content and FA analysis, and at -18 ˚C (commercial freezer) for use in the
146 feeding trial. Hereafter, these amphipods are referred to as captured amphipods. The rest of the
147 amphipods were acclimatized to laboratory conditions and held in an in-door 250 L tank with

148 gentle aeration. Water in the tank was partially changed twice a week and maintained at 29.1 ± 2.7
149 °C, salinity 35.9 ± 5.5 ppt, pH 8.1 ± 0.4, NO_2^- 1.3 ± 3.4 mg L^{-1}, NO_3^- 24.8 ± 26.1 mg L^{-1},
150 NH_3/NH_4^+ 0.6 ± 1.4 mg L^{-1}. Plastic mesh was introduced in the tank as artificial substratum for
151 the animals (Baeza-Rojano et al., 2013a; Vargas-Abúndez et al., 2021). Amphipods were fed daily
152 with a commercial shrimp feed (Camaronina 35® Purina, Sonora, Mexico) (crude protein 350 g
153 kg^{-1}, lipids 80 g kg^{-1}, ash < 100 g kg^{-1}, fiber < 50 g kg^{-1}, energy 21.6 kJ g^{-1}, FA profile not available).
154 Amphipods maintained this way were also used in the feeding trial and were labelled pellet-fed
155 amphipods.

156 Simultaneously, amphipods were cultured in a biofloc system, following a modified protocol for
157 shrimp biofloc systems described in Magaña-Gallegos et al. (2018), with some modifications. The
158 culture took place in a large, out-door tank (3500 L) exposed to coastal climate conditions from
159 May to December. Water in the tank was maintained at 27.2 ± 2.1 °C, salinity 28.6 ± 7.3 ppt, pH
160 8.4 ± 0.1, NO_2^- 0.7 ± 0.9 mg L^{-1}, NO_3^- 18.8 ± 19.4 mg L^{-1}, NH_3/NH_4^+ 0.6 ± 1.1 mg L^{-1}. To
161 stimulate the growth of nitrite-oxidizing bacteria, sodium nitrite ($NaNO_2$) was added at the
162 beginning of the culture (Lara et al., 2016). To promote the generation of bioflocs, sugarcane
163 molasses and wheat bran were added as carbon sources (Avnimelech, 2015). These were added at
164 the beginning of the culture trial and every two weeks thereafter, until the biofloc volume reached
165 5 ml L^{-1} (biofloc volume was measured with Imhoff cones). When the biofloc volume decreased
166 to less than 5 ml L^{-1} or total ammonia nitrogen (TAN) reached 1 mg L^{-1}, carbon addition was
167 resumed (Emerenciano et al., 2013). A high C/ N ratio of 20/1 was maintained, and water exchange
168 was limited to compensate for evaporation. Sludge was removed occasionally from the tank by a
169 central drain. The water was continuously aerated and pieces of plastic mesh were introduced as
170 substrate for the amphipods. *P. hawaiensis* amphipods were introduced into the tank three weeks
171 after the beginning of the culture. A commercial shrimp feed (Camaronina 35® Purina, Sonora,
172 Mexico) was administered three times a week in excess, as an additional source of nitrogen and
173 supplemental feed for the amphipods. This experimental group was labeled biofloc amphipods.

174 *Artemia (ProAqua ®, Sinaloa, Mexico)* was raised with wheat bran during the first 16 days and
175 then, the last 6-8 days, with *Spirulina* sp. Prior to its use, it was enriched with Super Selco® (INVE
176 Aquaculture, Dendermonde, Belgium) in 1 L tanks for six hours at a concentration of 6 mL L^{-1}.
177 Enrichment period was chosen to avoid both FA autoxidation and FA retroconversion by *Artemia*
178 (McEvoy et al., 1995; Nieves-Soto et al., 2021). Supporting this choice, preliminary observations
179 indicated a significant increase in EPA and DHA percentage within six hours, as it was further
180 confirmed by results herein (see section 3.1).

181 *2.3. Seahorses*

182 Wild pregnant *H. erectus* Perry, 1810 were captured at Laguna de Chelem, Yucatán, Mexico under
183 a scientific license (SGPA/DGVS/12741/13) from the Mexican Ministry of the Environment and
184 Natural Resources (SEMARNAT). Fish maintenance followed previously published methods by

185   Vargas-Abúndez et al., 2018. After birth, juveniles were maintained in re-circulating holding tanks
186   (30H × 28L × 18W cm, 14 L). Seawater was treated with mechanical (25, 10 and 5 μm), biological
187   and UV filtration. Water in the aquaria was maintained at 26. ± 0.5 °C (mean ± standard deviation),
188   salinity 36.4 ± 2.5 psu, pH 8.0–8.3, $NO_2^- < 0.3$ mg L^{-1}, $NO_3^- < 5$ mg L^{-1}, $NH_3/NH_4^+ < 0.1$ mg L^{-1}
189   with a gentle aeration. A 12:12 photoperiod was kept throughout experiments. Polypropylene
190   structures were placed in the aquaria to be used as holdfasts by the fish. Juvenile fish were fed
191   three times a day (09:00 h, 14:00 h, 18:00 h) with live and frozen *Artemia* enriched with Super
192   Selco® (INVE Aquaculture, Dendermonde, Belgium). At 50 mm standard length, fish were
193   weaned from *Artemia* to frozen amphipods and then fed with a mix of the two foods, according to
194   previous findings and culture recommendations (Lin et al., 2009; Vargas-Abúndez et al., 2018;
195   Del Vecchio et al., 2019). Feces and uneaten food were siphoned out after feeding.

196   *2.4. Seahorse feeding trial*

197   Forty-eight *H. erectus* juveniles (21 males and 27 females) of ca. ten months old (1.3 ± 0.4 g wet
198   weight, ranging from 0.72-2.48 g) were randomly selected and individually tagged with a collar
199   tag (Morgan and Bull, 2005). Fish were divided into 12 tanks of 15 L (30x20x30 cm) with 4 fish
200   in each tank, which were in turn evenly and randomly assigned to one of the three following dietary
201   treatments (4 tanks per dietary group) (all diets frozen): i) amphipod diet: 100% captured
202   amphipods; ii) *Artemia* diet: 100% enriched *Artemia*; iii) mixed diet: a 1:1 mix of the wild-captured
203   amphipods and the *Artemia* diets.

204   Fish were fed in excess (25% of wet body weight per day) three times a day for 57 days. Water
205   characteristics were maintained as previously described for seahorses. Fish growth was assessed
206   through individual wet weight, which was repeatedly measured in each individual at the beginning
207   of the experiment (day 0) and at days 15, 30, 45 and 57 with an OHAUS Adventurer analytical
208   balance. Fish growth among experimental groups was not influenced by animal gender, as shown
209   by data exploration (for raw data see supplementary materials). For comparative purposes, the
210   specific growth rate (SGR) for individuals of each experimental group was calculated as follows:

211    $$SGR\% = ((\ln Wf - \ln Wi)/t) \times 100$$

212   where Wf is the final wet weight, Wi, the initial wet weight, and t, the number of days. Survival
213   was recorded daily. At the end of the experiment (day 57) fish were euthanized by quick
214   submersion in a mix of ice and water (hypothermia) and stored at -80 °C for further FA acid
215   analysis.

216   *2.5. Lipid content and fatty acid analysis*

217   To assess the nutritional value of amphipods, lipid content and FA analyses were conducted on
218   samples of captured, biofloc (which treatment included shrimp food) and exclusively pellet-fed
219   amphipods. As a control group to the FA analyses, enriched *Artemia* was included. Once harvested

220 from the different sources, amphipod samples were rinsed with freshwater and sieved through a
221 710 μm mesh; retained juveniles and adults were used for the analyses.

222 Samples were minced, freeze-dried and homogenized in liquid nitrogen with a commercial
223 blender. Lipid extraction was carried out based on Folch extraction procedure with
224 dichloromethane/methanol (2:1 v/v) (Folch et al., 1987). Extracts were saponified with 20%
225 KOH:Methanol (w/v) and FAs were obtained from the saponifiable fraction (pH = 1-2) using
226 hexane as solvent. FAs were esterified with 10% BF3 in methanol (Fluka 15716) for 60 min at 80
227 °C and FA methyl esters (FAME) were obtained. FAME were separated and quantified by gas
228 chromatography using a Perkin Elmer Clarus 500 gas chromatograph (GC) equipped with a flame
229 ionization detector (FID), and a Phenomenex Zebron ZB-WAX capillary column (20 m length,
230 0.18 mm i.d., 0.18 μm film thickness). Hydrogen was used as the carrier gas at a flow rate of 40
231 mL min^{-1}. The column temperature was programmed to increase from 40 to 200 °C at a rate of 20
232 °C min^{-1} and from 200 to 250 °C at a rate of 2.5 °C min^{-1}, whereas injector and detector
233 temperatures were set at 280 and 250 °C, respectively. Individual components were identified
234 according to their retention times using analytical standards (Supelco® 37 Component FAME
235 Mix, catalog no. 47885-U) as reference. Individual FA concentrations were expressed as
236 percentages of the total FA composition.

237 Fatty acid determinations were performed on seahorses once at the end of the trial. Six whole fish
238 samples per experimental group were randomly selected from previously sacrificed fish, and
239 analyzed in duplicate. Samples were minced, freeze-dried and homogenized in liquid nitrogen with
240 a commercial blender prior to analysis. In order to compare the FA profile of samples from
241 different foods and seahorses, the following indices were calculated: total saturated FAs (ΣSFA),
242 total monounsaturated FAs (ΣMUFA), total polyunsaturated FAs (ΣPUFA), n3 highly-unsaturated
243 FAs (n3 HUFA; = C20:3n3 + C20:5n3 + C22:6n3), total n3 FAs (Σn3), total n6 FAs (Σn6), n3/n6,
244 DHA/EPA and EPA/ARA.

245 *2.6. Statistical analysis*

246 Statistical differences in total lipid content among amphipods of different source (captured
247 amphipods [n = 2], biofloc amphipods [n = 3] and pellet-fed amphipods [n = 3]) were analyzed by
248 a one-way ANOVA. The statistical software package Prism5 (GraphPad Software) was used for
249 this analysis.

250 Variations in the FA composition between sources of food and seahorses fed with three different
251 diets were assessed by means of Principal Coordinate Analyses (PCoA). Whitaker´s association
252 index (D$_9$; Legendre and Legendre, 1998; Borcard et al., 2011) was applied to the data, expressed
253 as proportions of the total FA content in each sample in order to obtain a resemblance matrix with
254 dissimilarity measures between every pair of samples. Non-metric Dimensional Scaling (nMDS)
255 was used to compare the index values calculated to characterize the FA profiles of both foods and
256 seahorses. In this case, the Gower coefficient (S$_{15}$; Legendre and Legendre, 1998) was used to

257 calculate multivariate distances between samples. Both the 2D and 3D configurations were
258 obtained together with Kruskall´s stress coefficient (Clarke et al., 2014) and the best was selected
259 on the basis of stress criteria described in Legendre and Legendre (1998).

260 Multiple ANOVAs with permutations (Anderson, 2001) were used to distinguish differences in
261 FA composition and indices related to food source and seahorse diet from random noise. In the
262 first case, the underlying model was a one-way ANOVA with food source as a fixed factor with 4
263 levels: captured amphipods (n = 4), biofloc amphipods (n = 3), pellet-fed amphipods (n = 3) and
264 enriched *Artemia* (n = 2). The underlying model in the second case had seahorse diet as a fixed
265 factor with 3 levels (amphipod, *Artemia* and a mixed diet), individual seahorses as a random factor
266 nested within each level diet (b = 6), and n = 2 replicate subsamples of every individual.
267 Permutations of residuals under the reduced model (9999) were used to generate empirical
268 distributions of *pseudo-F* values under the null hypotheses (Anderson et al., 2017). Post hoc
269 comparisons were applied following a similar procedure after the main test indicated significant
270 differences ($p < 0.05$) between at least two centroids. Multivariate procedures were carried out
271 using PRIMER 7 and PERMANOVA + for PRIMER.

272 Changes in seahorse wet weight through time was evaluated through regression analysis adjusting
273 a mixed linear model (GLMM) with diet as a fixed factor (3 levels: amphipod diet, *Artemia* diet
274 and mixed diet) and time (days) as a continuous variable. Preliminary data exploration showed
275 that data did not comply to homoscedasticity (i.e. dispersion in seahorse weight increased as mean
276 weight increased) or independence (i.e. seahorse weight was repeatedly measured on individuals
277 through time). To ensure the reliability on the estimated coefficients and standard errors and p-
278 values obtained (Zuur et al., 2007), the model was adjusted with a generalized least-square
279 procedure through restricted maximum likelihood and incorporated correlation and variance
280 structures. The intercepts and slopes of linear equations corresponding to the three diets were
281 compared with t-tests using the residual standard error estimated by the model. Different slopes
282 would indicate different seahorse growth rates (mg day^{-1}), irrespective of seahorse initial weights.
283 The goodness of fit of the model was validated by visual inspection of residuals (Montgomery and
284 Peck, 1992; Zuur et al., 2007). The R libraries nlme (Pinheiro et al., 2020) and ggplot2 (Wickham,
285 2016) were used to adjust the GLMM and generate the graphic visualization.

286 **Results**

287 *3.1. Foods of different source*

288 Biofloc and pellet-fed amphipods showed higher total lipid contents (% dw) (26.7 ± 1.3% and
289 25.5 ± 3.5% lipids, respectively) compared to captured amphipods (20.4 ± 0.8%), yet these were
290 not statistically significant ($F = 4.56$; $p = 0.07$). Table 1 shows the FA composition of all food
291 sources. Multivariate analysis on the FA composition of food sources showed an effective
292 reduction of dimensionality with the first and second principal coordinates containing 80% of the
293 total variation in the data (Fig. 1; Supplemental Table 1). Samples from captured amphipods were

294   located to the right-hand side of the ordination map and were associated with high contents of oleic
295   (C18:1n9c/t), arachidonic (C20:4n6), dihomo-gamma-linoleic (C20:3n6), pentadecylic (C15:0),
296   lauric (C12:0) and margaric (C17:0) acids. By contrast, samples from *Artemia*, pellet-fed and
297   biofloc amphipods were high in docosahexaenoic (C22:6n3), stearic (C18:0) and docosadienoic
298   (C22:2) acids and were located to the left-hand side of the map (Fig. 1). *Artemia* and biofloc
299   amphipods had the highest contents of linoleic (C18:2n6c), palmitoleic (C16:1) and alpha-
300   linolenic (C18:3n3) acids, followed by captured and pellet-fed amphipods (see Supplemental
301   Table 1 for details on the contribution of each descriptor to the linear combinations of the first
302   three principal coordinates).

303   Results of the MANOVA revealed significant differences in FA composition of foods related to
304   its source (*pseudo-F* = 7.51; $p < 0.001$; 9626 unique permutations; Table 2), and clearly separated
305   wild-captured amphipods from biofloc and exclusively pellet-fed amphipods (*pseudo-F* = 2.54 and
306   3.17; $p < 0.05$; 45 unique permutations, respectively). However, samples from *Artemia* could not
307   be statistically distinguished from any of the other groups (*pseudo-F* from 2.53 to 3.5; p from 0.07
308   to 0.1; 10 to 15 unique permutations), probably due to its low number of replicates (n = 2; Table
309   2).

310   The 3D nMDS configuration map of several indices describing the FA profiles of food sources
311   had a stress coefficient of 0.02. A 2D projection of this configuration showed that captured
312   amphipods had high values of ΣSFA and ΣMUFA, followed by biofloc and pellet-fed amphipods
313   (Fig. 2). *Artemia* samples had the highest values of ΣPUFA and Σn6, whereas pellet-fed amphipods
314   had the highest values of EPA/ARA and Σn3 (Fig. 2; see Supplemental Table 2 for details on the
315   multiple correlation coefficients of descriptors with the first three ordinal axes). Here again, the
316   MANOVA procedures statistically distinguished captured amphipods from biofloc and pellet-fed
317   amphipods (*pseudo-F* = 1.78 and 3.56; $p < 0.05$; 35 unique permutations, respectively), but was
318   unable to find significant differences between the latter (*pseudo-F* = 4.19; $p = 0.09$; 10 unique
319   permutations, Table 3). *Artemia* samples were again statistically similar to all the other groups
320   (*pseudo-F* from 2.49 to 3.64; p from 0.07 to 0.1; 10 to 15 unique permutations; Table 3).

321   *3.2. Seahorses*

322   All the animals used for the present study survived until the end of the experiment. The GLMM
323   showed a significant interaction term indicating that linear equations describing fish growth
324   differed depending on the diet ($F = 46.2$; $p < 0.0001$). Further comparisons of the regression
325   coefficients showed statistically similar intercepts but different slopes in the corresponding linear
326   equations (Table 4). Results demonstrated that captured amphipods in the diet, both solely
327   (amphipod diet: 13 ± 2 mg day^{-1}; 0.8 ± 0.3% SGR) and in combination with *Artemia* (mixed diet:
328   11 ± 1 mg day^{-1}; 0.67 ± 0.43% SGR), significantly increased the growth rate of juvenile seahorses
329   compared to *Artemia* alone (0.057 ± 0.002 mg day^{-1}; 0.04 ± 0.18% SGR; Fig.3). Moreover, results
330   showed that seahorses fed the *Artemia* diet had growth rates statistically indistinguishable from

331   zero. The GLMM significantly improved ($L.ratio$ = 497.5; p < 0.001) by adding an exponential
332   variance structure of the form:

$$\sigma^2 = RSE^2 \times exp^{\left(2\,\delta_i\,Day\right)}$$

334   where RSE is the residual standard error, δ is the parameter for each i level of diet. This allowed
335   for the variance associated to each treatment at each experimental day to be estimated (Table 5).
336   These results indicate that inclusion of amphipods in the diet made seahorse weight more variable
337   and dispersion increased with experimental days.

338   Table 6 shows the FA composition of seahorses at the end of the dietary treatment. PCoA on FA
339   composition of seahorse tissue showed that 80.5% of total variation in the data was contained in
340   the first and second principal coordinates. Ordination clearly separated samples from seahorses
341   fed diets that included amphipods to the right-hand side, from those fed *Artemia* alone to the left-
342   hand side of the map (Fig. 4). Eigenanalysis showed that the former were samples with high
343   content of eicosatrienoic (C20:3n3), lauric (C12:0) and myristic (C14:0) and to a lesser extent of
344   eicosenoic (C20:1n9), alpha-linolenic (C18:3n3), eicosapentaenoic (C20:5n3), tridecylic (C13:0),
345   palmitoleic (C16:1) and pentadecylic (C15:0) acids. These samples, however, were low in linoleic
346   (C18:2n6c), gamma-linoleic (C18:3n6), stearic (C18:0), lignoceric (C24:0) and behenic (C22:0)
347   acids. The opposite was true for seahorses fed with *Artemia*. The second coordinate separated
348   samples on the top of the map (mostly from the amphipod diet) with higher contents of oleic acid
349   (C18:1n9c/t), whereas those at the bottom (mostly from the mixed diet) were high in dihomo-
350   gamma-linoleic (C20:3n6), arachidonic (C20:4n6) and docosahexaenoic (C22:6n3) acids (Fig. 4;
351   see Supplemental Table 3 for details on the contribution of each descriptor to the linear
352   combinations of the first four principal coordinates).

353   Results of the MANOVA confirmed significant differences in FA content associated to seahorse
354   diet (*pseudo-F* = 15.0; p < 0.001; 9920 unique permutations, Table 7). No significant differences
355   were found between subsamples of seahorse tissue (*pseudo-F* = 1.19; p = 0.28; 9907 unique
356   permutations), indicating that the method for the determination of FA content was highly
357   consistent. Paired comparisons of centroids showed that the diet based on *Artemia* resulted in
358   seahorses with a significantly different FA content compared to those fed with either the amphipod
359   or mixed diets (*pseudo-F* = 6.32 and 3.67; both p < 0.01; 461 and 462 unique permutations,
360   respectively). Statistical differences were also found between the two diets containing amphipods
361   (*pseudo-F* = 1.94; p < 0.05; 461 and 462 unique permutations, Table 7).

362   The nMDS applied on indices describing the FA profiles also successfully separated tissue samples
363   from seahorses fed with different diets (3D Stress = 0.04; Fig. 5). The permutational MANOVA
364   showed that FA profiles differed significantly depending on diet (*pseudo-F* = 7.63; p < 0.001; 9913
365   unique permutations; Table 8), whereas variation amongst individual seahorses subjected to the
366   same diet were not larger than those expected by chance (*pseudo-F* = 1.26; p = 0.24; 9914 unique

367 permutations). Here again, the FA profile of seahorses fed with *Artemia* was statistically different
368 from the amphipod and mixed diets (*pseudo-F* = 4.23 and 2.26; both *p* < 0.01; both 462 unique
369 permutations, respectively), but these two were not statistically distinguishable (*pseudo-F* = 1.81;
370 *p* = 0.06; 462 unique permutations). The 3D configuration projected on two dimensions showed
371 that samples from the amphipod and mixed diets had high Σn3, n3HUFA, EPA/ARA and ΣMUFA
372 values, whereas those from *Artemia* had high DHA/EPA and Σn6 (Fig. 5). The n3/n6 ratio was
373 higher in the former than in the latter. The amphipod diet resulted in slightly higher ΣMUFA,
374 whereas the mixed diet had higher ΣPUFA (Fig.5); but these differences can only be considered
375 marginally significant (see Supplemental Table 4 for details on the multiple correlation
376 coefficients of descriptors with the first three ordinal axes).

Discussion

378 *4.1. Nutritional value of P. hawaiensis from different production sources*

379 Results in the present study revealed that captured *P. hawaiensis* contains high levels of lipids
380 (20.4 ± 0.8% of dw), with a FA profile suitable for feeding *H. erectus* juveniles, namely rich in
381 the EFAs ARA, EPA and DHA. Interestingly, amphipod production based both on the use of a
382 large biofloc system and a small in-door tank with commercial pellets tended to increase the lipid
383 content (26.7 ± 1.3% and 25.5 ± 3.5%, respectively). This was true despite the relatively low lipid
384 content of the commercial feed used (80 g lipid kg^{-1}) and the typically low content of bioflocs (1.6-
385 8.3% lipids) (Ahmad et al., 2017; Magaña-Gallegos et al., 2018; Sgnaulin et al., 2018). There is a
386 limited number of studies analyzing lipid content in the context of aquaculture to compare with,
387 but these lipid levels are slightly higher than those found in amphipods harvested from an off-
388 shore aquaculture farm (13%) (Fernandez-Gonzalez et al., 2018), shrimp biofloc ponds (4.7-6.3%),
389 rivers (7.5-13%) (Kolanowski et al., 2007), different marine areas (5.1-19.15%) (Baeza-Rojano et
390 al., 2014; Jiménez-Prada et al., 2018), as well as in other commonly used live food organisms,
391 such as mysids (6.7-8.0%) (Planas et al., 2020) and copepods (11.3–12.4%) (Wang et al., 2014).
392 The optimal dietary lipid levels for seahorses are still unknown, but researchers agree that beyond
393 lipid content, a well-balance FA fraction plays a pivotal role in seahorse nutrition (Faleiro and
394 Narciso, 2010; Segade et al., 2016; Planas et al., 2020).

395 Amphipods from all three sources (captured, biofloc and pellet-fed amphipods) showed valuable
396 levels of PUFAs (26.38-41%), almost as high as SFAs (39.6-41.7%). These levels are similar to
397 those reported for the marine gammarid *Hyalella media* collected from the same coastal area
398 (Baeza-Rojano et al., 2013b) and others from the strait of Gibraltar (Baeza-Rojano et al., 2014);
399 but lower than highly nutritious organisms such as copepods (Zhang et al., 2015), mysids
400 (Schlechtriem et al., 2008; Herrera et al., 2010) and long-time enriched *Artemia* (Planas et al.,
401 2017), particularly regarding DHA and n3/n6 ratio. In contrast to enriched *Artemia,* that showed a
402 FA profile rich in Σn6 and linoleic acid typical of freshwater organisms (Sargent et al., 1999),
403 amphipods were characterized by high contents in SFAs and MUFAs. Amphipods did not

404 dramatically differ in terms of the presence of nutritionally relevant FAs, such as ARA, EPA and
405 DHA, compared to *Artemia*. However, both PCoA and nMDS ordinations clearly separated
406 amphipods from *Artemia*, suggesting that amphipods used herein could present a more "marine
407 profile", similar to that in studies of amphipods from littoral areas (Woods, 2009; Jiménez-Prada
408 et al., 2018; Alberts-Hubatsch et al., 2019). This was especially true for pellet-fed amphipods,
409 which showed the highest values of Σn3, n3 HUFAs, n3/n6, DHA, DHA/EPA and EPA/ARA.
410 DHA, along with ARA and EPA, are important components of cellular membranes and precursors
411 of bioactive molecules involved in essential metabolic and physiological processes (Tocher, 2003).
412 These FAs are considered essential for marine fish and shrimp nutrition, as they have limited
413 enzymatic capacity to synthesize them *de novo* (Tocher et al., 2003). Required absolute values for
414 these nutrients are species-specific, but they are generally needed at levels around 1% dry weight
415 of diet (National Research Council (NRC), 2011). Besides providing sufficient amounts of these
416 FAs to meet requirements, it is also important to optimize their relative proportions, as their
417 essential functions can be influenced by the presence and relative amounts of other FAs (Izquierdo
418 and Koven, 2011). For example, in Atlantic salmon, addition of EPA to a DHA rich diet enhanced
419 DHA tissue retention and fish growth, whereas addition of ARA did not enhance growth but
420 reduced DHA retention (Glencross et al., 2014). Although captured amphipods in the present study
421 showed higher ARA contents (Fig. 1 and 2), DHA was highest in both biofloc and pellet-fed
422 amphipods, suggesting a better FA balance in these cultured amphipods. Absolute FA amounts
423 were not quantified in the present study but, with ARA, EPA and DHA contents ranging from 2.9
424 to 9.1% of total FAs, *P. hawaiensis* could fulfill such dietary requirements (NRC, 2011; Zhang et
425 al., 2015).

426 The improved FA profile of pellet-fed amphipods, and to a lesser extent of biofloc amphipods, was
427 not surprising, given that the commercial shrimp feed used is expected to meet the nutritional
428 requirements of penaeid shrimps (Martinez-Cordova et al., 2003). The main ingredient of the feed
429 is fishmeal (Chávez-Sánchez, 1993), which may contain ideal sources of PUFAs and amino acids
430 for aquafeeds (Vargas-Abúndez et al., 2019). Whilst both biofloc and pellet-fed amphipods were
431 fed the commercial shrimp diet, it is likely that the biofloc culture provided an additional source
432 of food for the amphipods, hence explaining the slight differences in FA composition between
433 biofloc and pellet-fed amphipods. *P. hawaiensis* is a detritivorous species and, as an opportunistic
434 grazer, it is expected to feed efficiently on different floc particles. Amphipod gut content was not
435 analyzed but amphipods introduced into the biofloc system changed markedly in color compared
436 to pellet-fed amphipods, turning from almost translucence to a dark brown body with a green belly
437 after a few days (personal observations), possibly as a result of microalgae and biofloc ingestion.

438 As previously reported for a similar biofloc setup (Magaña-Gallegos et al., 2018), the FA
439 composition of the biofloc particles in the present study was probably low in EPA and DHA.
440 However, the actual nutritional value of bioflocs can vary according to a number of factors,
441 including biofloc particle size, carbon source, biofloc maturation, floc density, food preference by
442 cultured animals and their ability to ingest and digest the different biofloc particles (Ahmad et al.,

443 2017; Magaña-Gallegos et al., 2018; Promthale et al., 2021). Results herein are insufficient to
444 ascertain specific causes, but indicate that resulting biofloc amphipods present a FA profile highly
445 suitable for applications in marine aquaculture, *i.e.* with significant amounts of ARA, EPA and
446 DHA and better n3/n6, DHA/EPA, EPA/ARA ratios compared to enriched *Artemia*. Further
447 research should assess other nutrients such as proteins and their amino acid constituents, as they
448 also play foremost important roles in fish growth and development (D'Abramo, 2019).

449 *4.2. Effect of amphipods on the seahorse growth and fatty acid profile*

450 The use of captured amphipods substantially enhanced the growth of juvenile seahorses when used
451 both solely (amphipod diet) or in combination with enriched *Artemia* (mixed diet) (Fig. 3). Whilst
452 all individuals in all dietary treatments survived throughout the 57-day trial, the use of enriched
453 *Artemia* as the only food source did not promote seahorse growth, since no significant change in
454 the wet weight in animals in this treatment could be demonstrated. Results similar to these have
455 been previously reported in other *H. guttulatus* adults (Palma et al., 2008). It is well documented
456 that *Artemia* is not an adequate prey for many seahorse species due to nutritional deficiencies, poor
457 digestibility and absorption (Payne and Rippingale, 2000; Blanco et al., 2015; Randazzo et al.,
458 2018). Despite its limitations, *Artemia* is largely used given its extensive availability (Sorgeloos
459 et al., 2001; Bengtson, 2003; Olivotto et al., 2008; Del Vecchio et al., 2019; Southgate, 2019;
460 Planas et al., 2020).

461 In contrast to *Artemia*, amphipods are a natural prey for seahorses and other marine organisms
462 (Manning et al., 2019). Several authors have found food preferences in seahorses over natural
463 preys, such as mysids and copepods (Buen-Ursua et al., 2015, Blanco and Planas, 2015), although
464 depending on the stage of development. Regarding amphipods, a previous study by the authors
465 (Vargas-Abúndez et al., 2018), found very different ingestion rates in *H. erectus* juveniles (7.7-96
466 mm standard length) when comparing amphipods and *Artemia* diets. With frozen amphipods (*E.
467 pectenicrus*), seahorses ingested $4.1 \pm 1.7\%$ of its wet body weight within 12 minutes, whereas
468 with live *Artemia* seahorses ingested equivalent biomass only after 90 minutes. In the present
469 study, ingestion rates were not measured, but based on the previous one, it is likely that fish
470 ingested a higher amphipod biomass compared to that of *Artemia,* thereby partially explaining the
471 increased growth in the two diets that contained amphipods. These results are encouraging and
472 consistent with previous observations on other marine organisms kept under controlled conditions.
473 In a trial with *Octopus maya*, live marine gammarids induced a higher growth rate ($6.9 \pm 0.2\%$ day^{-1})
474 and survival ($92.2 \pm 6.8\%$) compared to adult *Artemia* ($4.8 \pm 0.2\%$ day^{-1} growth and $74.5 \pm$
475 23.8% survival) and freshwater gammarids ($5.0 \pm 0.3\%$ day^{-1} growth and $41.2 \pm 21.2\%$ survival)
476 (Baeza-Rojano et al., 2013b). Both gammarids and caprellids have been recognized as nutritionally
477 adequate prey for cuttlefish hatchlings (Baeza-Rojano et al., 2010). However, gammarids induce
478 a better feeding response and consequently growth and survival in cuttlefish, compared to
479 caprellids, due to differences in behavior (Baeza-Rojano et al., 2010). In Atlantic salmon and
480 Atlantic halibut, amphipod meal successfully replaced up to 40 % of fish meal in compound diets,

481   with no negative effects on feed conversion ratio, dry matter digestibility, protein digestibility and
482   muscle composition (Suontama et al., 2007).

483   The improved growth of *H. erectus* fed either the amphipod or the mixed diet could also be related
484   to the nutritional value of the diets, particularly with regard to FAs. The nutritional requirements
485   of seahorses are still unknown, but high levels of PUFAs, particularly of the LC-PUFAs ARA,
486   EPA and DHA, seems to be determinant for seahorse growth and survival (Faleiro and Narciso,
487   2010; NRC, 2011). In the wild, seahorses mostly consume small crustaceans such as copepods,
488   mysid shrimps and amphipods (Teixeira et al., 2001; Manning et al., 2019), known to be rich
489   sources of LC-PUFAs (Woods, 2009; Guerra-García et al., 2014; Alberts-Hubatsch et al., 2019).

490   In the present study, both PCoA and nMDS ordination methods applied on their respective FA
491   profiles (Fig. 4 and 5) clearly separated samples of seahorses fed either amphipod diets from those
492   fed *Artemia*, and this separation was statistically distinguished from random noise. *Artemia* was
493   enriched with PUFAs (Super Selco®*)* and thus resulted in higher PUFA percentages with respect
494   to both amphipod diets. However, the higher PUFA content was mainly the result of increased
495   linoleic acid (C18:2n6c), whereas nutritionally relevant markers such as, ARA, EPA and DHA
496   were found in similar percentages in all three diets. Linoleic acid is characteristic of terrestrial
497   plants and consequently of non-marine organisms such as *Artemia*, especially when fed ingredients
498   of terrestrial origin (Balachandar and Rajaram, 2019). Linoleic acid is considered an EFA for fresh-
499   water fish, but not for marine fish (NRC, 2011). In the present study, *Artemia* was raised with
500   wheat bran, which could have increased the abundance of this FA. Interestingly, the higher PUFA
501   content in *Artemia* did not translate into increased levels of PUFAs in the seahorses. On the
502   contrary, despite similar ARA, EPA and DHA compositions amongst all three diets, seahorses fed
503   enriched *Artemia* showed lower percentages of these FAs. This trend became more clearly
504   evidenced by the nMDS projection applied to indices describing the FA profile, where samples of
505   seahorses fed either amphipod diets showed closer association to vectors representing Σn3, n3
506   HUFAs, EPA/ARA and ΣMUFAs, whereas those from *Artemia* were closer to Σn6 and DHA/EPA
507   (Fig. 5). In fact, the higher n6 percentage detected in the *Artemia* diet was reflected in the seahorse
508   n6 percentage and, consequently, in a substantial reduction in the n3/n6 ratio amongst seahorses
509   fed with this diet. Marine fish have higher requirements of n3 FAs than of n6, and, as stated earlier,
510   optimal ratios tend to be high due to competitive interactions in FA biosynthesis (Faleiro and
511   Narciso, 2010; NRC, 2011). n3/n6 ratios of about 2.5 - 3.5 are common in natural prey ingested
512   by seahorses (Zhang et al., 2015; Segade et al., 2016; Planas et al., 2020). In egg, newborn and
513   juvenile seahorses, this ratio ranges from 1 to 16, being generally higher in earlier developmental
514   stages (Saavedra et al., 2014; Segade et al., 2016; Planas et al., 2020). In newborn *H. erectus*,
515   improved growth with an n3/n6 ratio of 2.5 was achieved with the use of calanoid copepods
516   collected from fish ponds (Zhang et al., 2015). Thus, the n3/n6 ratio observed in the present study
517   when using the amphipod diet (0.97 ± 0.17) was comparatively low but highly satisfactory for
518   feeding late juveniles of *H. erectus*.

519 A third element that could explain the better performance of the amphipod diets is digestibility.
520 Poor digestibility due to limited enzymatic capacity is a common issue in newborn and early stage
521 seahorses (Blanco et al., 2015; Novelli et al., 2016; Ofelio et al., 2018). However, it was suggested
522 that adult seahorses can display differences in the digestibility of zooplanktonic organisms (Corse
523 et al., 2015). The digestibility of amphipods has only been evaluated in Atlantic salmon and
524 Atlantic halibut compound diets, and it was comparable to the excellent digestibility of fish meal
525 and krill meal (Suontama et al., 2007). That brings the possibility that amphipods, as natural prey
526 of *H. erectus*, could be more efficiently digested and absorbed compared to *Artemia*. It may explain
527 why diets containing amphipods outperformed the *Artemia* diet despite small differences in key
528 nutrients (ARA, EPA and DHA). It remains unclear why seahorses fed exclusively *Artemia* did
529 not display any growth after two months. More information on other nutrients such as energy and
530 protein content, as well as amino acid profiles, which are essential for fish growth, is required to
531 properly address this issue.

532 In the present study, neither pellet nor biofloc amphipods were used in the seahorse feeding trials,
533 but given their FA profile, it can be expected similar or even enhanced fish performances compared
534 to captured amphipods. Further research is required to confirm the suitability of using cultured
535 amphipods. The use of biofloc for amphipod production represents a more sustainable and cost-
536 effective new technology (Ahmad et al., 2017), which could be easily scalable for the commercial
537 production of valuable feed for marine fish, well beyond seahorses.
538

Conclusions

540 Both captured and cultured *P. hawaiensis* showed adequate levels of lipids and n3 fatty acids. *H.*
541 *erectus* fed with captured *P. hawaiensis*, either alone or in combination with enriched *Artemia*,
542 improved seahorse growth and fatty acid profiles in terms of ARA, EPA, DHA, Σn3 percentages,
543 as well as n3/n6 ratio. The present research supports the potential use of amphipods as an
544 alternative prey for feeding seahorses. Further research addressing the nutritional value of other
545 important nutrients, such as amino acids and microelements is required for a comprehensive
546 understanding of the amphipod nutritional value.

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Figure 1

Principal coordinate analysis of fatty acid composition of four diets elaborated with amphipod *P. hawaiensis* of different source and enriched *Artemia.*

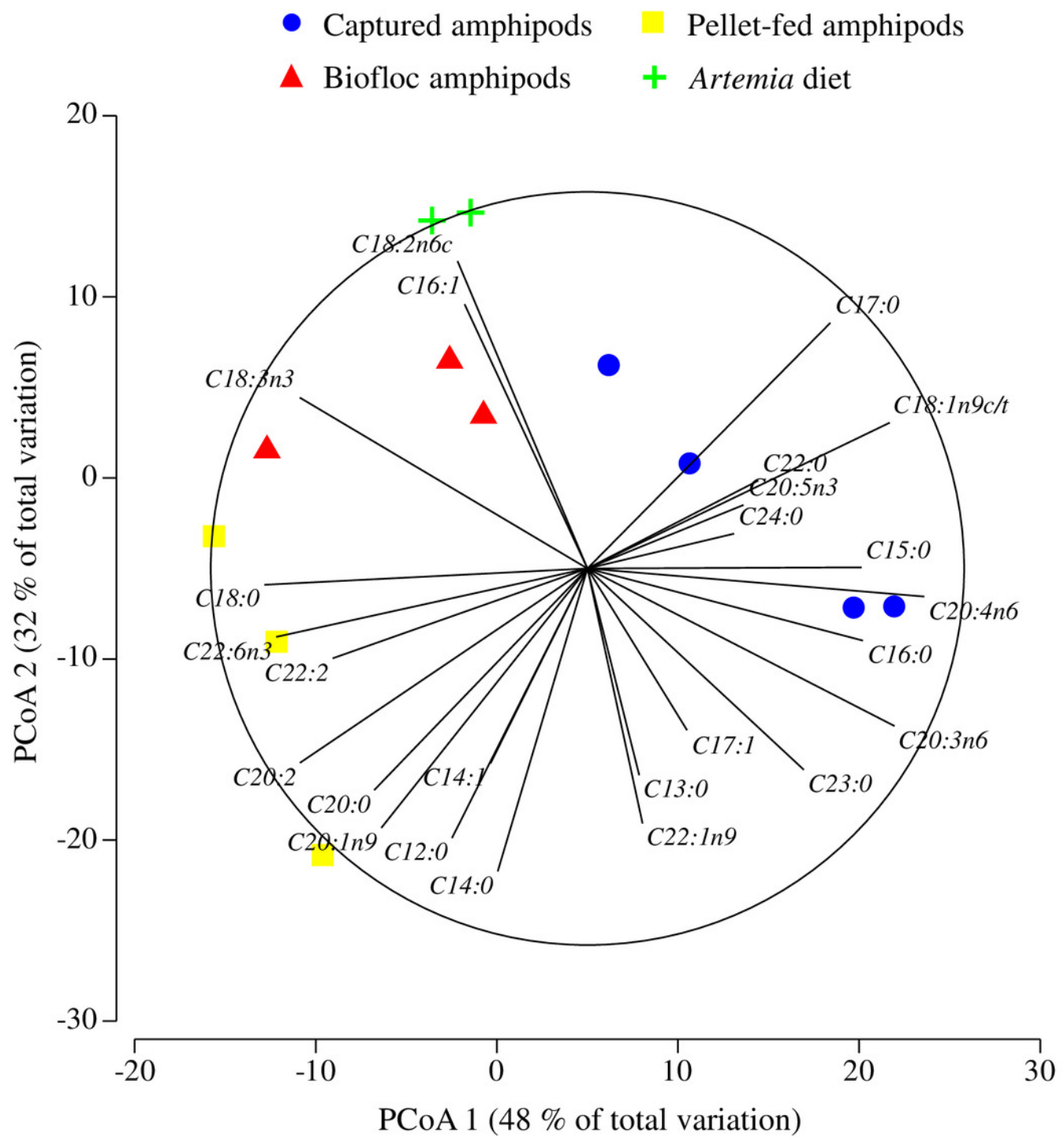



Figure 2

Non-metric Multidimensional Scaling (2D projection) of indices describing the fatty acid composition of amphipods *P. hawaiensis* of different source and enriched *Artemia.*

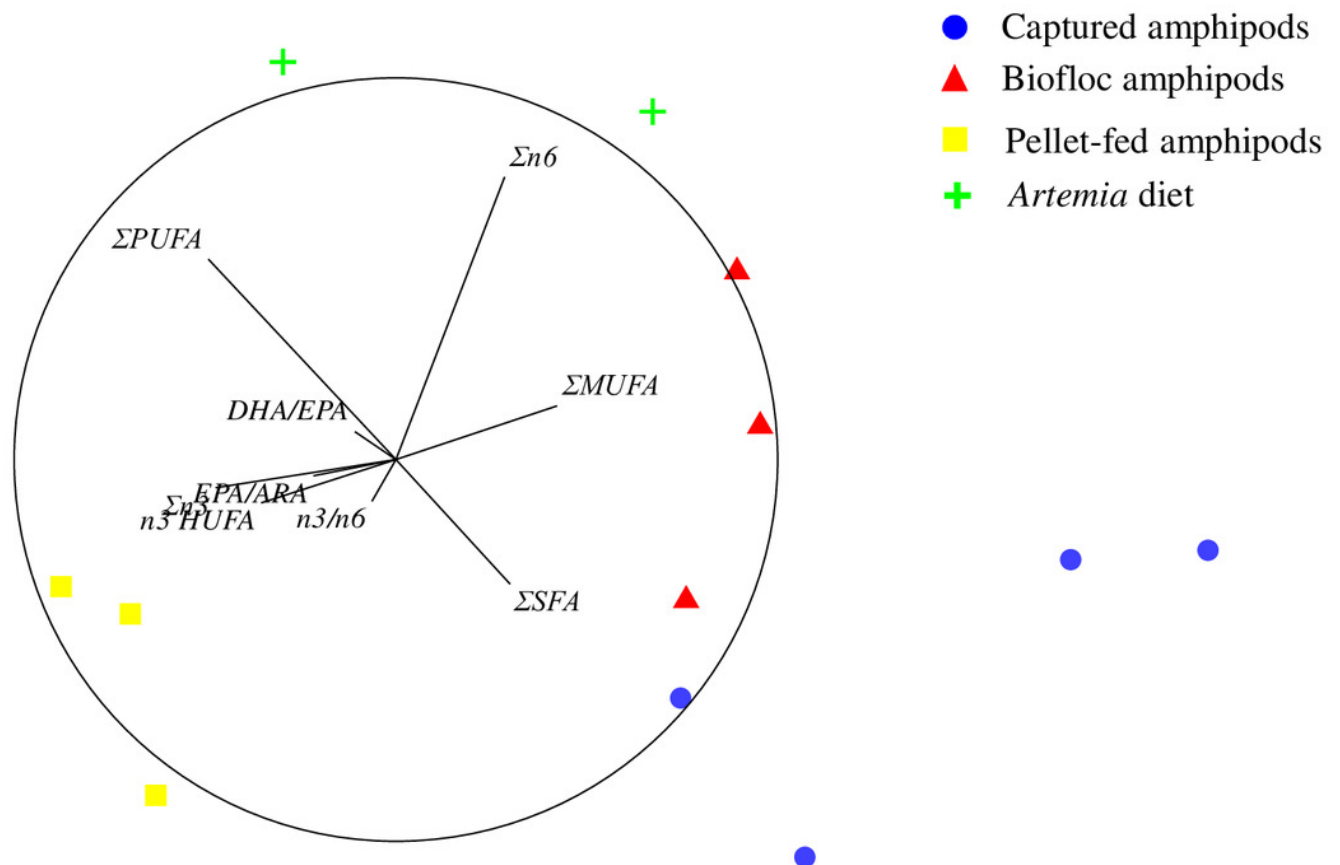



Figure 3

Wet weight (g) of *H. erectus* fed captured amphipods (amphipod diet), enriched *Artemia* (*Artemia* diet), and a mixture (1:1) of the amphipod and the *Artemia* diets (mixed diet).

Error bars show ± one standard deviation of the mean.

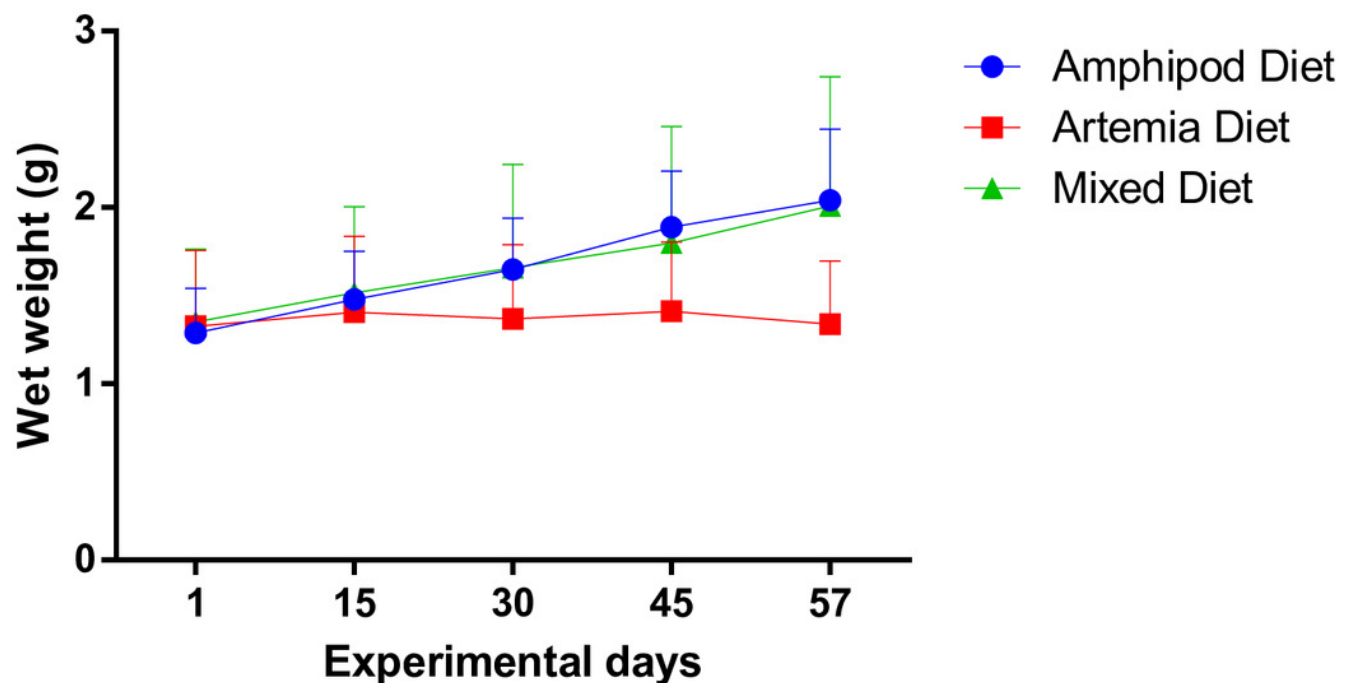



Figure 4

Principal coordinate analysis of fatty acid composition in tissue samples of *H. erectus* fed captured amphipods (amphipod diet), enriched *Artemia* (*Artemia* diet), and mixed (1:1) diet.

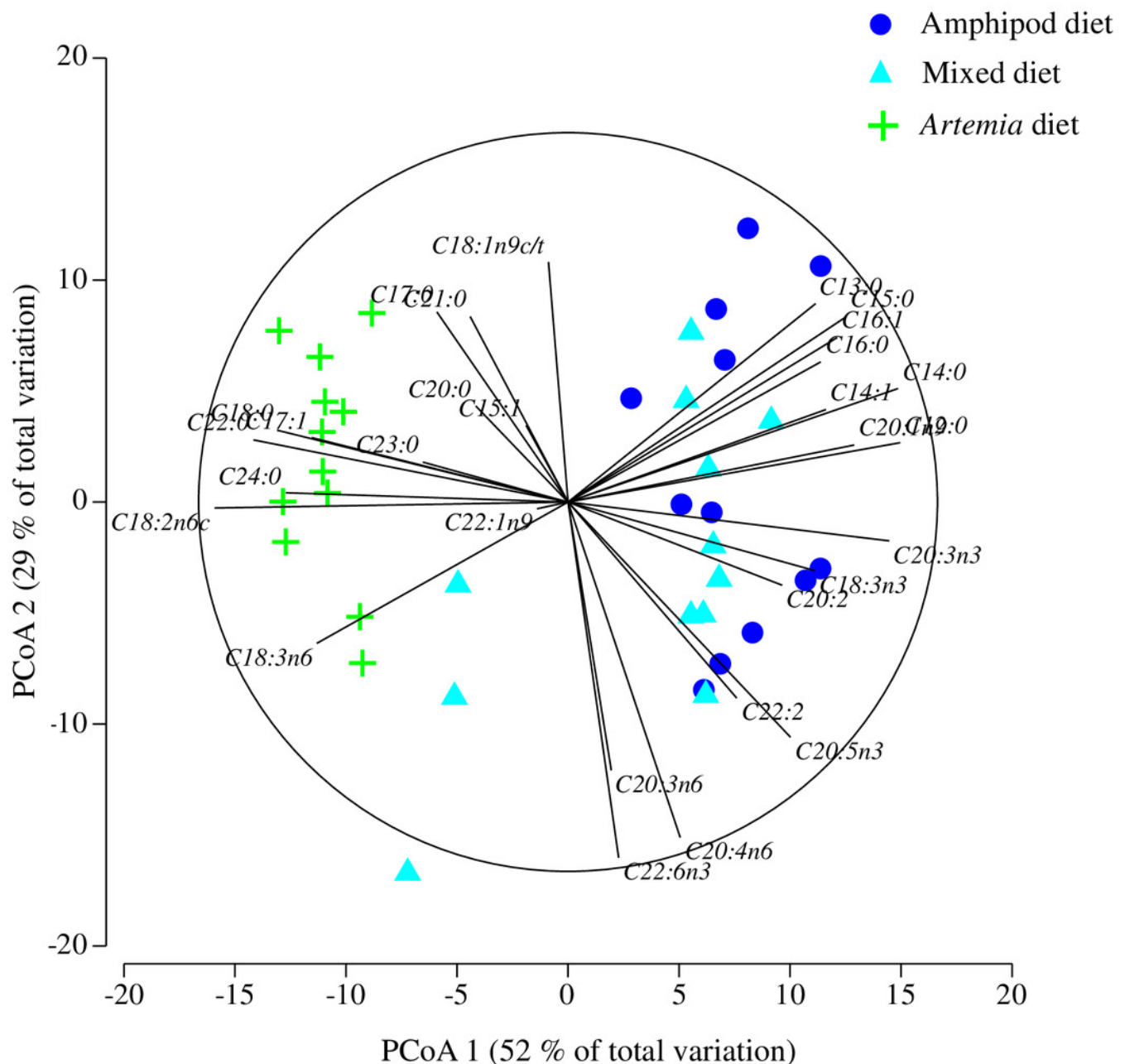
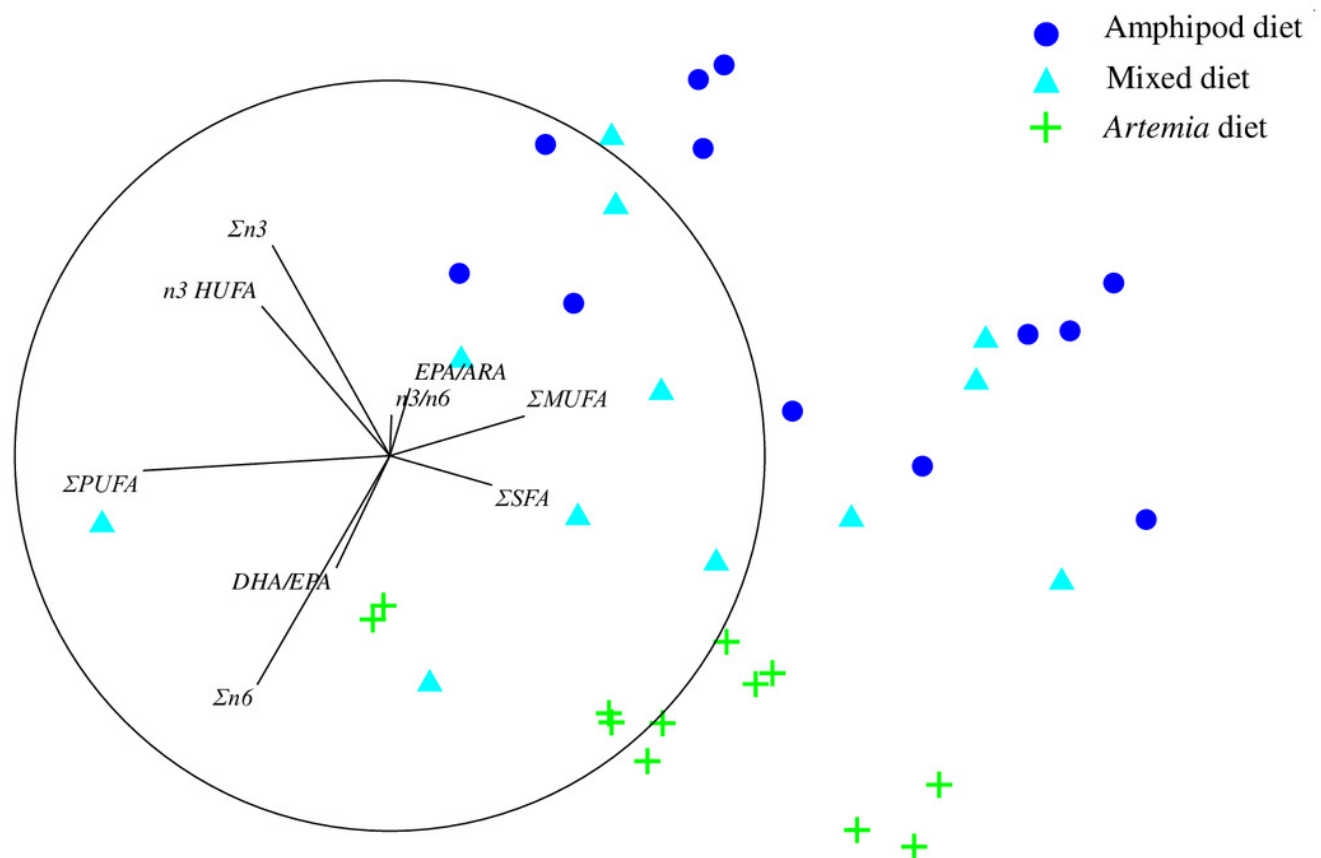



Figure 5

Non-metric Multidimensional Scaling (2D projection) of indices describing the fatty acid composition in tissue samples of seahorses *H. erectus* subjected to different dietary treatments.

Amphipod diet: captured amphipods; *Artemia* diet: enriched *Artemia*; mixed diet: a 1:1 mixture of the amphipod and Artemia diets.

Fatty acid composition (as percentage of total FAs) of *P. hawaiensis of different source and enriched Artemia.*

Data show ± one standard deviation of the mean.

FAs	Captured amphipods	Biofloc amphipods	Pellet-fed amphipods	*Artemia*
C12:0	0.4 ± 0.21	0.74 ± 0.08	0.81 ± 0.12	0.05 ± 0.01
C13:0	0.06 ± 0	0.06 ± 0.01	0.06 ± 0.01	0.04 ± 0
C14:0	5.74 ± 1.62	7.36 ± 0.68	8.59 ± 0.91	1.71 ± 0.18
C14:1	0.04 ± 0	0.05 ± 0	0.04 ± 0	0.01 ± 0
C15:0	0.97 ± 0.06	0.78 ± 0.06	0.59 ± 0.19	0.64 ± 0.07
C16:0	22.49 ± 7.96	15.75 ± 5.59	14.97 ± 0.69	17.47 ± 1.53
C16:1	5.71 ± 6.32	6.44 ± 1.67	4.86 ± 3.14	9.22 ± 1.08
C17:0	2.35 ± 0.4	1.3 ± 0.08	0.34 ± 0.13	2.43 ± 0.19
C17:1	0.54 ± 0.05	0.23 ± 0.14	0.45 ± 0.27	0.14 ± 0.02
C18:0	8.23 ± 2.64	12.66 ± 4.12	13.89 ± 2.71	9.41 ± 0.7
C18:1n9c/t	22.9 ± 1.44	18.23 ± 5.21	8.6 ± 3.34	15.71 ± 2.81
C18:2n6c	5.58 ± 0.94	14.8 ± 2.11	7.04 ± 2.99	20.78 ± 1.06
C18:3n6	1.19 ± 0.77	0.17 ± 0.04	5.51 ± 1.76	0 ± 0
C18:3n3	0.65 ± 0.7	2.12 ± 0.12	1.92 ± 1.04	2.23 ± 0.09
C20:0	0.43 ± 0.12	0.44 ± 0.06	0.68 ± 0.05	0.27 ± 0.03
C20:1n9	2.34 ± 0.18	3.22 ± 0.18	4.12 ± 1.06	1.58 ± 0.07
C20:2	0.8 ± 0.14	3.06 ± 0.14	4.19 ± 0.17	0.45 ± 0.01
C20:3n6	0.74 ± 0.33	0.19 ± 0.03	0.25 ± 0.01	0.05 ± 0.03
C21:0	0.17 ± 0.01	0.12 ± 0	0.15 ± 0.01	0.02 ± 0
C20:3n3	0.35 ± 0.04	0.65 ± 0.03	0.81 ± 0.04	0.03 ± 0.01
C20:4n6	7.68 ± 2.62	2.92 ± 0.13	2.98 ± 0.12	4.97 ± 0.35
C20:5n3	6.63 ± 0.53	4.26 ± 0.2	5.65 ± 0.23	6.94 ± 0.95
C22:0	0.45 ± 0.06	0.22 ± 0.04	0.32 ± 0.03	0.49 ± 0.12
C22:1n9	0.34 ± 0.03	0.23 ± 0.06	0.35 ± 0.05	0.19 ± 0.03
C22:2	0.09 ± 0.08	0.12 ± 0.02	0.18 ± 0.01	0.08 ± 0.09
C23:0	0.21 ± 0.07	0.11 ± 0.03	0.13 ± 0.02	0.04 ± 0.02
C24:0	0.25 ± 0.16	0.05 ± 0.01	0.09 ± 0	0.08 ± 0.02
C22:6n3	2.66 ± 0.71	3.72 ± 0.19	6.17 ± 0.25	4.72 ± 1.33
ΣSFA	41.74 ± 6.19	39.59 ± 4.79	40.61 ± 1.53	32.65 ± 2.84
ΣMUFA	31.87 ± 5.97	28.4 ± 3.42	18.41 ± 6.18	26.84 ± 1.6
ΣPUFA	26.38 ± 2.35	32.01 ± 1.43	34.7 ± 3.71	40.22 ± 1.34
n3 HUFA	9.64 ± 0.94	8.63 ± 0.42	12.63 ± 0.49	11.66 ± 2.28
Σn3	10.29 ± 1.46	10.75 ± 0.53	14.55 ± 1.49	13.89 ± 2.19
Σn6	15.2 ± 2.96	18.07 ± 1.98	15.78 ± 2.17	25.8 ± 0.74
n3/n6	0.71 ± 0.21	0.6 ± 0.1	0.93 ± 0.06	0.54 ± 0.1
DHA/EPA	0.4 ± 0.12	0.87 ± 0	1.09 ± 0.02	0.67 ± 0.1
EPA/ARA	0.95 ± 0.34	1.46 ± 0.03	1.9 ± 0.01	1.39 ± 0.09

Results of a permutational MANOVA applied on the fatty acid composition of four diets elaborated with amphipod *P. hawaiensis* of different source and enriched *Artemia.*

df: degrees of freedom; SS multivariate sums of squares; multivariate mean squares; *pseudo-F*, *pseudo-t* and *p*: *F* and *t* values obtained through permutations of the reduced model and the *p* values associated; number of unique permutations used to obtain each *pseudo-F* and *pseudo-t* value.

Source of variation	df	SS	MS	pseudo-F	p	Unique permutations
Food source	3	2617.2	872.4	7.5	< 0.001	9626
Residual	8	929.3	116.2			
Total	11	3546.5				

Post-hoc comparisons	pseudo-t	p	Unique permutations
Wild amphipods *vs* Biofloc amphipods	2.5	< 0.05	35
Wild amphipods *vs* Pellet amphipods	3.2	< 0.05	35
Biofloc amphipods *vs* Pellet amphipods	3.5	0.10	10
Artemia *vs* Biofloc amphipods	2.3	0.11	10
Artemia *vs* Pellet amphipods	3.5	0.10	10
Artemia *vs* Wild amphipods	2.5	0.07	15

Results of a permutational MANOVA applied on several indices describing the fatty acid composition of amphipods *P. hawaiensis* of different source and enriched *Artemia.*

df: degrees of freedom; SS multivariate sums of squares; multivariate mean squares; *pseudo-F*, *pseudo-t* and *p*: *F* and *t* values obtained through permutations of the reduced model and the *p* values associated; number of unique permutations used to obtain each *pseudo-F* and *pseudo-t* value.

Source of variation	df	SS	MS	*pseudo-F*	*p*	Unique permutations
Food source	3	6777.1	2259	8.4	< 0.001	9586
Residual	8	2148.7	268.6			
Total	11	8925.8				

Post-hoc comparisons	*pseudo-t*	*p*	Unique permutations
Wild amphipods *vs* Biofloc amphipods	1.8	< 0.05	35
Wild amphipods *vs* Pellet amphipods	3.6	< 0.05	35
Biofloc amphipods *vs* Pellet amphipods	4.2	0.09	10
Artemia *vs* Biofloc amphipods	2.6	0.09	10
Artemia *vs* Pellet amphipods	3.4	0.09	10
Artemia *vs* Wild amphipods	2.5	0.07	15

Results of *t*-tests comparing the intercepts and slopes of three lineal regressions on the changes in wet weight (g) of *H. erectus* as a function of time (days) when fed the experimental diets.

Amphipod: amphipod diet; *Artemia*: *Artemia* diet; Mixed: mixed diet, ns: non-significant; *** p < 0.001 (see text for details on the GLMM adjusted to the data).

	Intercept		Slope	
	Amphipod	Mixed	Amphipod	Mixed
Mixed	0.47 ns	-	1.08 ns	-
Artemia	0.16 ns	0.62 ns	8.76 ***	6.49 ***

Variance estimates (σ^2) associated to mean biomass of *H. erectus* fed with three diets (captured amphipods, enriched *Artemia* and a mixed (1:1) diet) on days 0, 15, 30, 45 and 57 of the experiment.

Estimates were obtained with a generalized least-square procedure through restricted maximum likelihood and included a variance exponential structure.

Days	Diet		
	Amphipod	Mixed	*Artemia*
0	0.121	0.121	0.121
15	0.144	0.157	0.107
30	0.170	0.202	0.093
45	0.201	0.261	0.082
57	0.231	0.320	0.074

Fatty acid composition (% of total FAs) of *H. erectus* fed captured amphipods (amphipod diet), enriched *Artemia* (*Artemia* diet), and a mixed diet (1:1; mixed diet) throughout a 57-day experiment

Data show ± one standard deviation of the mean.

FAs	Amphipod diet	*Artemia* diet	Mixed diet
C12:0	0.25 ± 0.06	0.09 ± 0.15	0.2 ± 0.1
C13:0	0.08 ± 0.02	0.05 ± 0.01	0.1 ± 0
C14:0	4.49 ± 0.9	1.6 ± 0.22	3.6 ± 1.1
C14:1	0.03 ± 0.01	0.09 ± 0.29	0.01 ± 0.01
C15:0	0.88 ± 0.15	0.59 ± 0.06	0.78 ± 0.14
C15:1	0.03 ± 0.02	0.03 ± 0.03	0.02 ± 0.02
C16:0	24.05 ± 3.12	19.52 ± 2.24	24.84 ± 4.16
C16:1	8.31 ± 2.2	4.72 ± 0.43	6.2 ± 2.44
C17:0	1.57 ± 0.56	1.78 ± 0.27	1.5 ± 0.47
C17:1	0.53 ± 0.24	1.09 ± 0.25	0.61 ± 0.38
C18:0	12.42 ± 3.93	17.72 ± 1.81	15.23 ± 2.58
C18:1n9c/t	20.37 ± 2.96	20.28 ± 2.95	16.24 ± 4.48
C18:2n6c	3.43 ± 0.24	15 ± 1.09	6.21 ± 2.36
C18:3n6	0.34 ± 0.08	0.58 ± 0.15	0.43 ± 0.14
C18:3n3	1.01 ± 0.22	0.61 ± 0.15	0.77 ± 0.17
C20:0	0.48 ± 0.09	0.56 ± 0.09	0.41 ± 0.16
C20:1n9	1.43 ± 0.24	0.8 ± 0.08	1.22 ± 0.28
C20:2	0.51 ± 0.06	0.35 ± 0.1	0.44 ± 0.1
C20:3n6	0.35 ± 0.06	0.32 ± 0.05	0.34 ± 0.07
C21:0	0.14 ± 0.02	0.17 ± 0.09	0.09 ± 0.05
C20:4n6	7.48 ± 2.24	5.53 ± 1.39	8.39 ± 2.33
C20:3n3	0.3 ± 0.05	0.07 ± 0.04	0.22 ± 0.06
C20:5n3	4.61 ± 1.68	2.39 ± 0.85	4.08 ± 1.28
C22:0	0.45 ± 0.06	0.82 ± 0.11	0.49 ± 0.16
C22:1n9	0.31 ± 0.05	0.3 ± 0.09	0.35 ± 0.26
C22:2	0.18 ± 0.1	0.08 ± 0.06	0.15 ± 0.11
C23:0	0.09 ± 0.03	0.14 ± 0.05	0.11 ± 0.06
C24:0	0.28 ± 0.05	0.45 ± 0.08	0.3 ± 0.12
C22:6n3	5.6 ± 2.46	4.29 ± 1.92	6.65 ± 2.8
ΣSFA	45.18 ± 3.11	43.5 ± 4.22	47.66 ± 5.62
ΣMUFA	31.02 ± 4.91	27.31 ± 2.95	24.67 ± 5.02
ΣPUFA	23.81 ± 6.41	29.21 ± 5.33	27.67 ± 7.43
n3 HUFA	10.51 ± 3.9	6.75 ± 2.75	10.96 ± 3.61
Σn3	11.51 ± 4	7.35 ± 2.87	11.72 ± 3.64
Σn6	11.61 ± 2.43	21.42 ± 2.47	15.36 ± 4.19
n3/n6	0.97 ± 0.17	0.33 ± 0.1	0.76 ± 0.15
DHA/EPA	1.22 ± 0.32	1.75 ± 0.21	1.66 ± 0.59
EPA/ARA	0.62 ± 0.15	0.42 ± 0.08	0.5 ± 0.16

Results of a permutational MANOVA applied on the fatty acid composition in samples of *H. erectus* fed captured amphipods (amphipod diet), enriched *Artemia* (Artemia diet), and mixed (1:1) diet.

df: degrees of freedom; SS multivariate sums of squares; multivariate mean squares; *pseudo-F*, *pseudo-t* and *p*: *F* and *t* values obtained through permutations of the reduced model and the *p* values associated; number of unique permutations used to obtain each *pseudo-F* and *pseudo-t* value.

Source of variation	df	SS	MS	*pseudo-F*	*p*	Unique permutations
Diet	2	2607.2	1303.6	15.0	< 0.001	9920
Individual (Diet)	15	1301.9	86.8	1.2	0.28	9907
Residual	18	1310.4	72.8			
Total	35	5219.5				

Post-hoc comparisons	*pseudo-t*	*p*	Unique permutations
Amphipod *vs* Mixed diet	1.9	< 0.05	462
Amphipod *vs* *Artemia* diet	6.3	< 0.01	461
Artemia *vs* Mixed diet	3.7	< 0.01	462

Results of a permutational MANOVA applied on indices describing the fatty acid composition of *H. erectus* fed captured amphipods (amphipod diet), enriched *Artemia* (Artemia diet), and mixed (1:1) diet.

df: degrees of freedom; SS multivariate sums of squares; multivariate mean squares; *pseudo-F*, *pseudo-t* and *p*: F and *t* values obtained through permutations of the reduced model and the *p* values associated; number of unique permutations used to obtain each *pseudo-F* and *pseudo-t* value.

Source of variation	df	SS	MS	*pseudo-F*	*p*	Unique permutations
Diet	2	5799.7	2899.8	7.6	< 0.001	9913
Individual (Diet)	15	5701.6	380.1	1.3	0.24	9914
Residual	18	5419.7	301.1			
Total	35	16921				

Post-hoc comparisons	*pseudo-t*	*p*	Unique permutations
Amphipod *vs* Mixed diet	1.8	0.06	462
Amphipod *vs* *Artemia* diet	4.4	< 0.01	462
Artemia *vs* Mixed diet	2.3	< 0.01	462