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Replacement of Antarctic krill (*Euphausia superba*) by extruded feeds with different proximate composition: effects on growth, nutritional condition, and digestive capacity of juvenile European lobsters (*Homarus gammarus*, L.)

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1 Abstract

Extruded feeds are widely used for major aquatic animal production, particularly for finfish. 2 However, the transition from fresh/frozen to extruded/pelleted feeds remains a major obstacle to 3 progressing sustainable farming of European lobster (Homarus gammarus). The aim of this study 4 was to investigate the effects of using extruded feeds with different protein levels and 5 lipid/carbohydrate ratios on growth, feed utilization, nucleic acid derived indices (sRD), and 6 7 digestive enzymatic activity of *H. gammarus* juveniles. Six extruded feeds were formulated to contain two protein levels (400 and 500 g kg⁻¹), with three lipid/carbohydrate ratios (LOW - 1:3, 8 MEDium – 1:2, HIGH – 1:1). The extruded feeds were tested against Antarctic krill (Euphausia 9 superba) used as control (CTRL). Overall, the CTRL and 500MED feed supported the highest 10 growth and nutritional condition estimated by means of sRD, while the poorest results were 11 observed for the 400HIGH and 400MED groups. The FCR was significantly lower in the CTRL 12 than all extruded feeds, among which the most efficient, i.e., lower FCR, was the 500MED. The 13 14 highest activity of trypsin and amylase in lobsters fed the 400MED and 400HIGH feeds points to the activation of a mechanism to maximize nutrients assimilation. The highest lipase activity 15 observed for the 500LOW and 500MED groups indicates a higher capacity to metabolize and store 16 lipids. Overall, the results suggest that the 500MED feed (500 g kg⁻¹ protein, 237 g kg⁻¹ 17 carbohydrates, and 119 g kg⁻¹ lipids) is a suitable extruded feed candidate to replace Antarctic 18 krill, commonly used to grow lobster juveniles. 19

20 Keywords: Digestive Enzymatic Activity; Feed Efficiency; Macronutrients; Nucleic Acid indices.

21 Introduction

The European lobster (Homarus gammarus, L.) is an economically important decapod crustacean 22 geographically ranging from Northern Norway to Morocco and the Eastern Mediterranean⁽¹⁾. The 23 increased fishing pressure from 1930 to 1970 led to the collapse of several European lobster populations 24 25 throughout Europe⁽²⁾. To counteract the decline, multiple experimental stock enhancement programs have 26 been launched to release large numbers of juveniles to lobster grounds in France ⁽³⁾, the United Kingdom ⁽⁴⁾, Ireland ⁽⁵⁾, Norway ⁽⁶⁾, and Germany ⁽⁷⁾. Commercial aquaculture is gaining interest as an additional 27 approach. However, in comparison with other aquaculture sectors, lobster farming still faces several 28 29 constraints for their commercial production to be economically sustainable. Among them are the high mortality rates, energy and feeding costs ⁽⁸⁾. Significant advances in automated rearing systems and water 30 31 quality have been made in recent years that improve European lobster hatchery production and costeffectiveness (9-13). Nevertheless, the lack of a balanced artificial feed that is affordable, with better 32 nutritional value, and easier to handle and store compared to live, fresh, or frozen foods remains a major 33 drawback in the sustainable farming of *H. gammarus* ^(14,15). 34

Fresh, live mussels ^(16,17) and frozen krill ^(18,19) are commonly used as preferred reference diets, as they 35 36 appear to perform consistently well as food for several lobster species. However, the future success of lobster aquaculture will depend on the ability to replace live, fresh, or frozen diets by more practical and 37 38 cost-effective artificial feeds. The transition to specifically formulated, dry pelleted feeds has been applied with relative good success to several crustacean species within the Penaeidae ⁽²⁰⁾. While considerable 39 40 research efforts in the last three decades have been devoted to spiny lobster species (Palinuridae), the transition to extruded feeds has proven to be challenging ^(21,22). Results indicate that protein is the most 41 important macronutrient for optimal growth. Carbohydrates are readily digested and absorbed but their 42 43 subsequent fate is poorly understood, while lipids appear to be poorly digested, absorbed and used. These findings are in agreement with the chemical composition of the preferred prey of several spiny lobster 44 species which presents high protein, low lipid, and moderate to high carbohydrate content ⁽²³⁾. 45

The feasibility of using artificial feeds for the production of juvenile American lobster (*Homarus americanus*) was extensively investigated ^(16,24-27) but growth obtained was not as high as animals that are fed live/fresh food. The efforts on the development of artificial feeds for the European lobster have been less and only a few published studies refer to the potential application of formulated feeds for juvenile *H. gammarus* farming ^(18,28). Reported results are similar to what has been observed for the *H. americanus*,

51 i.e., lower growth performance in lobsters fed the experimental artificial diets. The relatively limited 52 success of formulated feeds for lobster juveniles can be partially explained by the lack of information on 53 how lobsters digest and assimilate formulated feeds ⁽²²⁾. Additionally, the higher growth and feeding rates 54 of lobsters fed natural diets has been generally related to: (a) their superior water stability and 55 attractiveness and (b) a nutrient profile approximating closely to their body composition ⁽²⁹⁾.

56 Most nutritional studies performed with crustacean species address weight and length gains as growth performance indicators. However, maximum size *per se* does not necessarily imply the best condition. 57 58 This is particularly relevant in decapod crustacean species since their body weight is highly influenced by the water content and moulting stage (30). The use of biochemical indicators can aid in a more 59 60 comprehensive evaluation of the nutritional status of the reared animals. The ratio of RNA:DNA concentrations has been previously used as an indicator of recent growth and nutritional condition in a 61 variety of crustacean species including the *H. gammarus* ⁽³¹⁻³⁴⁾. The main premises are that the amount of 62 RNA in a cell varies in proportion to protein synthesis, whereas the amount of DNA remains fairly 63 constant ⁽³⁵⁾. Thus, theoretically, poor nutritional condition contributes to low protein synthesis and slow 64 65 growth, resulting in a low RNA:DNA ratio ⁽³⁶⁾.

Increasing the knowledge on the activity of digestive enzymes of European lobsters is essential to establish 66 a better understanding of their digestive ability. Analysing the digestive response might help revealing 67 68 why some diets show better performance than others might. There are few published studies referring to the digestive capacity of *H. gammarus*. A range of carbohydrases ⁽³⁷⁾ and proteases ⁽³⁸⁾ have been detected 69 70 in the hepatopancreas and gastric juice of wild caught adults. However, changes in the digestive enzyme 71 activity with type of food or nutritional composition have not yet been determined in European lobster. 72 To the best of our knowledge, only one such study on lobsters has been published ⁽³⁹⁾ but on a different 73 species. In this cited work, the rearing of juvenile spiny lobsters (Jasus edwardsii) on a formulated diet 74 for six months resulted in a pronounced decrease in the enzymatic activity (protease, trypsin, α -amylase, and α -glucosidase) of the foregut and digestive gland compared to juveniles reared on fresh mussels. 75

In the present study, we aimed to evaluate the effect of protein levels and the ratios between lipid and carbohydrates in formulated extruded feeds in terms of survival, growth, feed utilization, nutritional condition, and enzymatic activity to explore the potential nutritional requirements of European lobster juveniles. The experimental extruded feeds were tested against a conventional control diet (Antarctic krill, *Euphausia superba*).

81 Material and Methods

82 *Experimental animals*

Juveniles of *H. gammarus* were obtained from a wild European lobster female caught in the 83 Skagerrak coast of North Jutland, Denmark. Animals used in this experiment were procured from 84 the same female to reduce phenotypic variation. The fertility/fecundity of the chosen female, as 85 well as the survival and apparent quality of her progeny was observed to be as usual in our 86 facilities. From hatching and until settling, larvae were reared in 500-1 rectangular tanks installed 87 88 at the North Sea Oceanarium, Hirtshals. Each tank was equipped with a top seawater inflow (501 h⁻¹) and a vertical outflow filter (1mm mesh diameter). Temperature and salinity were kept at 18 89 \pm 1 °C and 34 \pm 1 PSU, respectively. Strong aeration was provided from the bottom of the tank 90 91 using airstones to maintain larvae in the water column. Larvae were stocked sequentially into tanks over three consecutive days after hatching at an initial density of 5-7 larvae l⁻¹ and daily fed thawed 92 Antarctic Krill, Euphausia superba (Akudim A/S, Denmark). The bottom of the tanks was 93 syphoned every week to remove debris. Upon metamorphosis to stage IV ⁽⁴⁰⁾, post-larvae were 94 transferred to the aquaculture facilities at the Technical University of Denmark, Section for 95 96 Aquaculture, Hirtshals. Animals were individually reared in 3D printed PolyLacticAcid (PLA) bioplastic cassette systems of 200 ml compartments with perforated grids for water in- and outlet. 97 Cassettes were distributed and immersed in raceway tanks of 80-1 capacity supplied by 98 recirculation seawater system at a constant flow rate of 330 l h⁻¹ (18 ± 0.5 °C temperature, 34 ± 1 99 PSU salinity, > 90% dissolved oxygen, $< 0.1 \text{ mg } l^{-1}$ ammonia-N) subjected to a photoperiod cycle 100 of 8h light:16 h dark. Lobsters were adapted to the trial conditions for one month, during which 101 they were daily fed thawed Antarctic Krill. 102

103 Experimental extruded feeds and control diet

Details of the experimental feeds and control diet are reported in ⁽¹⁸⁾. Briefly, experimental extruded feeds were formulated to contain 400 or 500 g kg⁻¹ protein, with lipid contents ranging from 86 to 233 g kg⁻¹ and carbohydrates from 210 to 347 g kg⁻¹ resulting in three ratios of lipid to carbohydrate: low (1:3), medium (1:2), and high (approx. 1:1). This resulted in six experimental feeds referred as 400LOW, 400MED, 400HIGH, 500LOW, 500MED, and 500HIGH. The different protein, carbohydrate, and lipid contents were achieved by altering squid meal, wheat gluten, wheat starch, and fish oil inclusion levels. Experimental feeds were extruded as 4 mm 111 pellets and were manufactured by SPAROS, Lda. (Olhão, Portugal). Thawed Antarctic krill was used as control feed (CTRL). Proximate analysis of the Antarctic krill and extruded feeds were 112 performed in duplicate. Feeds and krill were finely ground using a Krups Speedy Pro homogenizer 113 and analysed for crude protein (i.e. Kjeldahl N \times 6.25 ⁽⁴¹⁾), total lipid ⁽⁴²⁾, dry matter, and ash ⁽⁴³⁾. 114 Likewise, amino acid analyses was performed in duplicate by use of hydrolysed feed samples ⁽⁴⁴⁾. 115 The amino acid content was determined by HPLC⁽⁴⁵⁾. The ingredients and proximate chemical 116 composition of the experimental extruded feeds and control diet are shown in Table 1. The amino 117 118 acid profiles of the extruded feeds and control diet are listed in Table 2.

119 *Growth trial*

Prior to the start of the experiment, 24h unfed lobster juveniles (stage V to VI) were individually weighed and measured (carapace length). Homogeneous groups of 30 individuals (initial weight of 90.0 ± 2.8 mg per lobster; carapace length of 6.5 ± 0.6 mm, mean \pm SD) were randomly allocated to each dietary treatment. As each lobster was held separately, the experimental unit in this study was the individual.

125 Individual lobsters were hand-fed in excess a pre-weighed amount of thawed krill or extruded pellet each morning, and allowed to feed for 4h. Thus, all lobsters had equal access to feed for a 126 127 limited amount of time per day. Additionally, lobsters were permitted to consume their shedded 128 exoskeletons. After each meal, the uneaten feed was siphoned off and stored in 50 ml tubes at -20 °C. To estimate feed intake (FI), each treatment was divided into subgroups of 10 individuals. The 129 uneaten feed fraction of each subgroup (minus sampled or dead animals, when applicable) was 130 131 collected daily, accumulated over 6 weeks, and stored in a single tube, allowing triplicate values to be obtained for FI estimation. At the end of the trial each tube content was filtered, dried (24h 132 at 60 °C), and weighed for FI estimation using the following formula ⁽⁴⁶⁾: 133

134
$$FI (\% BW_i^{-1} day^{-1}) = (dF - uF - L) \times BW_i^{-1} \times \Delta t^{-1} \times 100$$

where: dF = distributed feed, uF = unconsumed feed, L = leaching after 4 h, $BW_i = initial$ body weight, $\Delta t = number$ of days during which uneaten feed was collected (42 days). Leaching was estimated by placing a pre-weighed quantity of each diet in the cassette compartments under the same conditions as in the trial but in this case, without animals. FI was expressed in percent of dry matter ingested per initial body weight per day. Energy intake was calculated by multiplying thedaily individual intake (mg) by the gross energy content of each diet.

The presence of shedded exoskeletons and mortality were recorded daily. The experiment was 141 conducted for 8 weeks and lobsters were individually weighed and measured every second week. 142 143 Significant mortality was gradually observed in the group of animals fed the 400HIGH diet. Consequently, at week 6, samples for enzyme activity (9 individual lobsters per treatment) were 144 collected and the growth trial ended for the 400HIGH group. The growth trial continued for two 145 more weeks for the other groups. Body wet weight was recorded to the nearest 0.001 g after gently 146 147 blotted dry each individual lobster with a paper towel. Carapace length was recorded to the nearest 0.1 mm with a vernier calliper from the base of the eye socket to the posterior edge of the 148 cephalothorax. The following formulas were used to determine growth performance: 149

150
$$FCR = FI \times BG^{-1}$$

where: FCR = feed conversion ratio; FI = feed intake (dry weight, mg); BG = biomass gain (wet
weight, mg).

153
$$SGR (\% day^{-1}) = [\ln(BW_f) - \ln(BW_i)] \times \Delta t^{-1} \times 100$$

where: SGR = specific growth rate; BW_f = final wet body weight (at week 6); BW_i = initial wet body weight, Δt = number of growth trial days (42) considered for SGR estimation.

156
$$iCL(\%) = (CL_f - CL_i) \times CL_i^{-1} \times 100$$

where: iCL = increment in carapace length; CL_f = final carapace length (at 42nd day); CL_i = initial carapace length.

159 Juvenile lobsters proximate chemical composition

At the end of the trial lobsters were lethally anesthetized in ice-cold seawater, weighed, measured, rinsed in distilled water, and stored at -80°C for proximate chemical composition analysis. Proximate analysis of the juveniles was performed in analytical triplicates per dietary treatment. Briefly, the pool of six to twelve individuals per treatment were finely ground using a Krups Speedy Pro homogenizer and analysed for dry matter and ash ⁽⁴³⁾. Protein was determined spectrophotometrically at 750 nm using a commercial Lowry-based, micro-protein determination kit (BIO-RAD 500-0112). Lipids were extracted with chloroform - methanol (2:1 by volume)
according to the Folch method ⁽⁴⁷⁾ and lipid content determined gravimetrically.

168 Nucleic acid determinations

169 At week 4, six juveniles per dietary treatment were collected for nucleic acid analysis 24h after being fed. Lobsters were lethally anesthetized in ice-cold seawater, weighed, measured, and rinsed 170 171 in distilled water before being frozen at -80°C. Lobsters were freeze-dried and weighed ($\pm 1 \mu g$ 172 dry weight [DW]) on an electronic microbalance (Sartorius M5P). The concentration of nucleic acids (RNA, DNA) was quantified in approx. 1 mg of dry abdominal muscle tissue from each 173 individual lobster following procedures described previously ⁽⁴⁸⁾. Briefly, the muscle samples were 174 chemically (cold sarcosyl Tris-EDTA extraction buffer) and mechanically homogenized in an 175 ultrasonic homogenizer unit (4710 Series, Cole Parmer Instruments Co.). The homogenate was 176 centrifuged for 15 min at 1,200 g, 4 °C, and the supernatant extract was used for the analysis of 177 RNA and DNA. The supernatants (30 µl) were placed into fluorescent microplate wells with Tris 178 179 buffer (140 μ l). Finally, a specific nucleic acid fluorochrome dye GelRED (30 μ l) was added into each well for the fluorescent reading of nucleic acids. Fluorescence was measured on a microplate 180 181 reader (Biotek Synergy HT model SIAFRTD - BioTek Instruments, Inc., Vermont, USA) with an 182 excitation wavelength of 365 nm and an emission wavelength of 590 nm. Following the first scan to determine total fluorescence of RNA and DNA, a ribonuclease A (Type-II A) solution (30 µl) 183 was added to each well and activated by incubating the fluorescent plates at 37°C for 30 minutes. 184 The plates were read again, and the concentration of DNA was calculated directly from the 185 186 standard curve described below. The RNA fluorescence was calculated subtracting the DNA 187 fluorescence (second scan) from total fluorescence (first scan). Concentrations were determined by running standard curves of DNA-GelRED and RNA-GelRED with known concentrations of λ -188 phagus DNA (0.25 µg µl⁻¹) and 16S-23S E. coli RNA (4 µg µl⁻¹) (Roche). The average ratio of 189 190 DNA and RNA slopes (average \pm SD) was 5.85 \pm 0.01. The RNA/DNA ratios were standardized (sRD) using DNA and RNA slope ratios and the reference slope ratio of 2.4⁽⁴⁹⁾. The physiological 191 192 condition of the juveniles was estimated with nucleic acid derived indices: RNA (µg mg⁻¹ DW), DNA (µg mg⁻¹ DW), and sRD (standardized RNA/DNA ratios). 193

194 *Digestive enzyme activities*

195 At week 6 nine lobsters per treatment were collected 4h to 5h after being fed for the analysis of 196 digestive enzyme activities. Individuals were rinsed in distilled water and stored at -80 °C. To 197 prepare the enzyme extracts, juveniles were previously freeze-dried. Rostrum, chelipeds, legs, pleopods, uropods, and telson were removed and the abdominal section of each individual was 198 mechanically homogenized in 1 ml distilled water. The homogenate was centrifuged for 10 min at 199 15,800 g, 4 °C, and the supernatant extract was used for the analysis of trypsin, amylase, and lipase 200 201 activities. All samples were kept in ice all times to avoid enzymes denaturation or damage. Enzyme extract aliquots were stored at -80 °C until analysis. 202

For trypsin analysis, the fluorogenic substrate Boc-Gln-Ala-Arg-7-methylcoumarin hydrochloride (BOC-SIGMA B4153) was diluted in dimethyl sulfoxide (DMSO), to a final concentration of 20

 μ M. For analysis, 5 μ l of this substrate, 190 μ l of 50mM Tris + 10 mM CaCl₂ buffer (pH 8.5) and

 15μ l of the enzyme extract were added to the microplate ⁽⁵⁰⁾. Fluorescence was measured at 355

207 nm (excitation) and 460 nm (emission).

Ultra Amylase Assay Kit (E33651) from Thermo Scientific was used for amylase analysis. The kit contains a starch derivate labelled with a fluorophore dye as a substrate. This substrate was diluted in 3-(N-morpholino) propane sulfonic acid (MOPS; pH 6.9) and substrate solvent (sodium acetate; pH 4.0), to a final concentration of 200 μ g/ml. For analysis, 50 μ l of the substrate solution and 15 μ l of the enzyme extract were added to the microplate. Fluorescence was measured at 485 nm (excitation) and 538 nm (emission).

Lipase activity was assayed using 4-methylumbelliferyl heptanoate (M2514, Sigma-Aldrich). The substrate was dissolved in phosphate buffer pH 7.0 to a final concentration of 0.4 mM, modified method from ⁽⁵⁰⁾. Fifteen μ l of the lobster extract was added to the microplate and mixed with 250 μ l of 0.4 mM substrate for the analysis. Fluorescence was measured at 355 nm (excitation) and 460 nm (emission).

All enzyme activities were expressed as RFU (Relative Fluorescence Units) per mg lobster dryweight before dissection.

221 Statistical analyses

The amino acid profiles of control diet and experimental feeds were analysed by principal component analysis performed using R version 3.5.1 software ⁽⁵¹⁾ and the factoextra version 1.0.7

package ⁽⁵²⁾. Survival and moult occurrence data were analysed by a Kaplan-Meier procedure. Log-224 rank (Mantel-Cox) test was used to determine significance (p<0.05). Whenever significance was 225 detected, a Chi-square table with multiple comparisons was generated to identify differences 226 among dietary treatments. All other parameters shown are expressed as means \pm SEM unless 227 otherwise specified. Before analyses, the ANOVA assumptions of normality of residuals and 228 229 homogeneity of variances were tested using the Shapiro-Wilk and Levene's test, respectively. In instances where assumptions were not met, data were square root or log-transformed. A second 230 231 order polynomial regression with time was used as an estimate of the average growth, both in terms of carapace length and body weight. The models were validated via residual plot analysis and 232 generated using R version 3.5.1 software ⁽⁵¹⁾. Curve coefficients were compared in a one-way 233 ANOVA and whenever significant differences were detected, the coefficients from experimental 234 235 feed curves were tested against the CTRL using the Dunnett *t*-test. For the remaining estimated growth parameters, RNA and DNA concentrations, sRD, and digestive enzyme activities, the 236 237 dietary treatments were subjected to a one-way ANOVA to test the experimental formulated dry feeds against the CTRL diet. Whenever significant differences were identified, comparisons 238 239 against the CTRL diet were conducted using the Dunnett *t*-test. Data from experimental extruded feed treatments were subsequently subjected to a two-way ANOVA, considering protein level and 240 241 L:CHO ratio as explanatory variables. Following the two-way ANOVA and, whenever significant differences were identified, means were compared by the Holm-Sidak post hoc test. Standardized 242 RNA/DNA ratio was expected to reflect changes in growth performance parameters ⁽⁵³⁾. Thus, a 243 244 simple linear regression was calculated to predict sRD based on SGR. Apart from the principal component analysis and the second order polynomial regression for carapace length and body 245 weight, all statistical tests were performed using the IBM SPSS Statistics 25.0 and graphics were 246 generated by GraphPad Prism version 5.0 software package. 247

248 **Results**

249 Diets amino acid composition

The CTRL and 500MED feed contained higher levels of both essential (EAA) and non-essential 250 251 (NEAA) amino acids while the 400MED and 400HIGH contained the lowest levels for EAA and NEAA (Table 2). A principal component analysis (Fig. 1) revealed that the 500LOW and 500MED 252 253 were more similar to the CTRL than any other experimental feeds. The majority of the variance 254 was explained by PC1 (84.1%) which mostly identified differences related to the concentration of 255 the total amino acids that was lower in all the three 400 and in the 500HIGH compared to the other groups. PC2 explained 14.3% of the variability and showed that methionine and taurine were the 256 257 amino acids with higher contribution for differences between the CTRL and the extruded 500MED 258 and 500LOW feeds.

259 Survival, growth, and feed utilization

Survival was not significantly affected by dietary treatment ($\chi 2 = 8.2$, df = 6, p = 0.22) varying between 65% and 90% (Fig. 2). Although no statistical significant difference was detected in the survival rate of the 400HIGH group, the projected curve indicates a marked divergence from the other dietary groups. In order to secure enough sample size for subsequent analysis we decided to end the growth trial for this treatment two weeks earlier. Therefore, despite the survival curves were calculated for an 8-week period in all treatments other than the 400HIGH, their comparison was performed only for the first 6 weeks period (Fig. 2).

As for survival, moulting curves comparison was restricted to the first 6 weeks. Moulting 267 occurrence was significantly affected by dietary treatment (Fig. 3) and the rate of a first moult 268 varied between 62% and 100% among diets. Mean cumulative first moult was significantly higher 269 270 for lobsters offered the 500MED feed compared to juveniles fed the 400HIGH, 400MED, and 500LOW feeds ($\chi^2 = 14.4$, df = 6, p = 0.03). Juvenile lobsters offered the 400HIGH diet did not 271 succeed a second moult. In the remaining groups, the occurrence rate of the second moult varied 272 between 21% and 60%. Mean cumulative successful second moult was significantly higher for the 273 500MED than the other groups, except for CTRL and 400LOW ($\chi^2 = 32,3$, df = 6, p < 0.001). 274

Changes in weight and carapace length throughout the trial were evaluated by second orderpolynomial regression with time. Regression curves are represented for all dietary treatments in

277 Fig. 4. However, because the trial ended at week 6 for the 400HIGH treatment, the curve 278 coefficients from this group were not statistically compared with the CTRL. During the 8-week 279 experimental period, lobsters grew from an initial mean weight of 90 mg (6.5 mm carapace length) to mean weights ranging from 134 to 254 mg (7.9 - 9.5 mm carapace length). The juveniles fed 280 281 the 500MED and CTRL diets sustained an increase in carapace length and body weight over the 8-week growth trial. The remaining groups reached a growth plateau, both in terms of length and 282 283 weight, after 6 weeks (Fig. 4). The 400MED and 500LOW experimental feeds had a significant lower carapace length increase over time compared to the CTRL (Fig 4, panels a and b). In terms 284 of body weight gain over the trial period, all the experimental dietary groups except the 500MED 285 and 400LOW showed a significantly slower growth compared to the reference group (CTRL) (Fig 286 4, panels c and d). 287

288 The growth performance indices - SGR and iCL- were determined for a 6 weeks period in all treatments (Table 3). A significantly higher SGR was observed for krill-fed lobsters in comparison 289 290 to lobsters fed the extruded feeds, except for the 500MED diet (Table 3). Within the extruded feeds, SGR was significantly affected by protein level, L:CHO ratio and the interaction of protein 291 292 \times L:CHO ratio. Results showed that for the 400-protein group, juveniles fed the 400LOW diet grew faster than the ones fed the 400MED and 400HIGH feeds. Within the 500-protein level, a 293 294 significantly faster growth was observed in the 500MED treatment. The protein content did not affect the SGR in the LOW L:CHO ratio category while in the MED and HIGH groups juveniles 295 296 fed the 500 g kg⁻¹ protein feeds grew significantly faster (Table 3). Juveniles fed the 400MED, 400HIGH, and 500HIGH feeds had a significantly lower iCL than the CTRL group. The protein 297 298 content, L:CHO ratio, and protein × L:CHO all had a significant effect on the iCL. Among the 400-protein level, the highest iCL was achieved for the 400LOW and the lowest for the 400HIGH 299 300 group. Within the 500 level, the highest iCL was observed for the 500MED and the lowest for the 500HIGH treatment. The protein content only had a significant effect on iCL at the intermediate 301 level of L:CHO for which the 500MED were larger than the 400MED group (Table 3). 302

As for SGR and iCL, the feed efficiency indices - energy intake, FI, and FCR - were calculated for the first 6 weeks period in all treatments (Table 3). Feed intake was significantly higher for all experimental feeds compared to the CTRL except for the 400HIGH. Within the experimental feeds L:CHO ratio and the interaction of protein × L:CHO had a significant effect on the FI. A higher

feed intake in extruded feeds with higher carbohydrate content was observed for both protein levels 307 examined (400 and 500 mg kg⁻¹) with most pronounced differences in feeds with a lower protein 308 309 content. As such, lobsters fed LOW feeds presented higher FI than HIGH with intermediate levels 310 observed in the MED treatments (Table 3). Intake expressed in terms of energy followed the same 311 trend as FI. The FCR was significantly lower for the CTRL diet compared to the experimental extruded feeds except for the 500MED group. There was a significant effect on FCR from the 312 313 L:CHO ratio among the experimental dry feeds, for which FCR in juveniles reared on MED feeds was significant lower compared to LOW feeds (Table 3). 314

315 *Proximate composition*

316 No statistical analysis was performed on the proximate composition data set since only analytical 317 replicates were used in the analysis of a single pooled sample. The dry matter content varied from 20% to 29%, with a minimum observed for the 400HIGH and maximum for the CTRL group. The 318 inverse trend was detected for the ash content, with a minimum of 32% observed for the CTRL 319 320 and maximum of 43% for the 400HIGH treatment. The protein fluctuated between 41% and 50%, lipids between 4% and 7%, and carbohydrates between 9% and 18%. Juveniles reared on the 321 500LOW experimental diet had the highest lipid and carbohydrate, and the lowest protein content, 322 while the lobsters fed the 400LOW diet had the lowest protein and highest carbohydrate contents. 323 324 Minimum lipid content was observed for the 400HIGH group (Table 3).

325 Nucleic acid derived indices

- The changes in the weight-specific RNA and DNA concentrations and sRD are shown in figure 5.
- 327 The RNA content was significantly lower ($F_{6,41} = 5.61$, p < 0.001) in animals fed the 400MED,
- 400HIGH and 500HIGH feeds when compared to the krill fed lobsters. Within the experimental
- feeds, the RNA content was affected by L:CHO ratio ($F_{2,35} = 7.61$, p < 0.002). Feeds in the HIGH
- category triggered a significant reduction in the RNA content of the abdominal muscle (Fig. 5 a).

331 In comparison to the CTRL group the 400HIGH treatment presented a significant increase ($F_{6,41} =$

- 332 5.08, p = 0.001) in DNA content. Among the experimental extruded feeds both protein ($F_{1,35}$ =
- 333 10.41, p = 0.003) and L:CHO ratio ($F_{2,35} = 3.75$, p = 0.035) significantly affected the DNA
- concentration in the muscle. Feeds within the 400-protein group supported a significant increase
- in DNA content compared to the 500-protein group. The DNA content of the abdominal muscle

of juveniles reared on the L:CHO MED category feeds was significantly lower compared to theHIGH groups (Fig. 5 b).

The standardized ratio (sRD) was significantly affected by dietary treatment. The sRD was 338 significantly lower ($F_{6,41} = 7.87$, p < 0.001) in animals fed the 400MED, 400HIGH and 500HIGH 339 340 feeds when compared to the CTRL. Within the experimental feeds, sRD was significantly affected by protein ($F_{1,35} = 7.63$, p = 0.010), L:CHO ratio ($F_{2,35} = 7.09$, p = 0.003) and the interaction protein 341 \times L:CHO (F_{2,35} = 5.83, p = 0.007). For the 400-protein level, juveniles fed the 400MED and 342 400HIGH feeds showed a significantly lower ratio compared to the lobsters fed the 400LOW diet. 343 For the 500 level the 500MED showed higher sRD than the 500HIGH group. Protein level had a 344 345 significant effect within the MED L:CHO category, for which the 500 resulted in a higher sRD than the 400 group (Fig. 5 c). A significant regression equation was found between sRD and SGR 346 $(F_{1,40} = 49.05, p < 0.001)$, with an R^2 of 0.55. The predicted sRD for juvenile lobsters was equal to 347 -0.143 + 0.644 (SGR) when SGR is estimated in % d⁻¹ (Fig. 6). 348

349 *Digestive enzyme activities*

350 Trypsin activity was significantly higher for the animals fed the experimental feeds than for the CTRL group, except for the 400LOW treatment ($F_{6.62} = 8.37$, p < 0.001). Within the experimental 351 feeds trypsin activity was affected by the L:CHO ratio ($F_{2.53} = 9,86$, p < 0.001) and the interaction 352 protein x L:CHO ($F_{2,53} = 6.49$, p = 0.003). No differences were observed among the three feeds in 353 354 the 500-protein level but in the 400 group the 400HIGH treatment promoted a significant increase in trypsin activity compared to the 400LOW. The protein content significantly affected the trypsin 355 activity for the LOW (higher for the 500 level) and HIGH (higher for the 400 level) categories 356 357 (Fig. 7 a).

358 As for trypsin, a similar trend was observed for the activity of amylase. When compared to the 359 CTRL, amylase activity was significantly higher in the 400MED and 400HIGH treatments ($F_{6,62}$ = 5.00, p < 0.001). Protein (F_{1.53} = 28.91, p < 0.001), L:CHO ratio (F_{2.53} = 12.79, p < 0.001), and 360 the interaction of both factors ($F_{2,53} = 4.97$, p = 0.011) had a significant effect on amylase activity. 361 No significant differences were observed within the 500-protein level. For the 400-protein group 362 amylase activity was significantly higher in juveniles fed the 400MED and 400HIGH feeds. 363 Within the MED and HIGH categories, amylase activity increased for the 400-protein level (Fig. 364 7 b). 365

- 366 Juveniles fed the 500LOW and 500MED feeds had a higher lipase activity in comparison to
- 367 juveniles fed the CTRL diet ($F_{6,62} = 8.33$, p < 0.001). Within the experimental dietary groups, both
- 368 the protein level ($F_{1,53} = 23.36$, p < 0.001) and L:CHO ratio ($F_{2,53} = 8.39$, p = 0.001) significantly
- affected the lipase activity. The activity of this enzyme was significantly higher for the 500-protein
- 370 content feeds. Within the L:CHO ratio, juveniles fed the LOW feeds had a significantly higher
- lipase activity than the ones fed the MED and HIGH feeds (Fig 7 c).

372 Discussion

373 *Growth performance*

Results obtained indicate that the overall performance of European lobster juveniles reared on the 374 375 500MED extruded feed for an 8-week period was comparable to the performance of juveniles fed thawed Antarctic krill (CTRL). Previous nutritional studies carried out on H. gammarus ⁽²⁸⁾, H. 376 americanus⁽²⁷⁾, and several Palinuridae species⁽²²⁾ showed that the growth rate was generally 377 poorer in lobsters fed formulated compared to fresh diets. A recent study ⁽¹⁸⁾, investigated the effect 378 379 of the same feeds that are used here on the performance, metabolic rate, and nitrogen retention in European lobster juveniles. The results indicated that *H. gammarus* grew faster when fed the 500 380 g kg⁻¹ compared to the 400 g kg⁻¹ protein feeds. The results also suggested a potential protein 381 sparring capacity in the formulated feeds, when compensated by the inclusion of carbohydrates, 382 383 given that the growth obtained for the 400LOW was comparable to that of the three 500 feeds. In 384 the present study, we found that the growth rate of juveniles fed the 400LOW, 500LOW, and 385 500HIGH feeds was lower than previously projected. This is most likely due to the too short growth trial period in the previous study. We thus confirm what had already been hypothesized in 386 the previous work, i.e., a period of 4 weeks is not long enough to assess the effects of experimental 387 388 feeds on the growth of juvenile European lobsters. Even the present study is arguably a little too short. As growth in crustaceans, as opposed to fish, rely on sequential moulting ⁽⁵⁴⁾, nutritional 389 390 trials performed in crustacean species aiming growth performance as the primary outcome must consider a minimum duration that allows individuals to complete two moults. 391

The poorest overall performance observed in the group of lobsters reared on the 400HIGH diet is 392 393 most likely related to the low feed intake. The reason for such low intake in this dietary group 394 remains unclear but it might be associated with a lower palatability, different smell, or that the 395 higher lipid content caused faster satiation. Results from a feeding trial carried out with European lobster juveniles of similar size (55) showed that animals fed 5% dry feed of body mass grew 396 significantly less than juveniles fed 10%. In our study, only lobsters fed the 400LOW diet were 397 close to the 10% level recommended (55) but a direct comparison between the current and the 398 mentioned study is not possible since both test diets and feeding duration were different. In the 399 cited study ⁽⁵⁵⁾, juveniles were allowed to feed from the afternoon until the next morning, while 400

401 only 4 hours in our set up. Nonetheless, the fact that low intake compromised growth was validated 402 in both studies. In animals fed the 400HIGH diet, not only growth but also survival was 403 compromised. Results suggest that juveniles were not taking enough feed to support growth, nor even enough to sustain the minimum standard metabolic rate (SMR). In our previous study using 404 the same feeds ⁽¹⁸⁾, a significant decrease was observed in the SMR of European lobster juveniles 405 406 reared on the 400MED and 400HIGH feeds for a 32-day period. Another likely reason for such a low performance on this diet may be related to a low tolerance to high lipid diets by crustaceans 407 ⁽⁵⁶⁾. It has been previously suggested ⁽⁵⁷⁾ that high dietary lipids reduce the capacity for amino acid 408 absorption due to an increase in bile acid concentration in response to a high-fat diet. 409

410 *Feed efficiency*

Although the CTRL group grew faster with the lowest FI among all treatments, the low FI observed 411 412 for this group is masked by the fact that calculations were performed on a dry weight basis. The CTRL diet composed of frozen Antarctic krill had a much lower dry matter (DM) content (approx. 413 414 11%) than the experimental extruded feeds (DM approx. 90%) which complicates FI comparisons. Also, the energy intake was lower in lobsters fed with krill compared to the extruded feeds. 415 416 Moreover, the form in which krill is presented is so different from an extruded feed, which may 417 cause differences in the overall nutrient absorption and digestibility. The highest intake observed for the experimental feeds in the LOW L:CHO category suggests that feeds with high carbohydrate 418 content are more attractive to European lobster juveniles. This is supported by previous studies 419 420 showing that the composition of the preferred prey of spiny lobster is generally high in protein 421 content, moderate to high in carbohydrate content, and low in lipid content ⁽²³⁾. In the present study, we observed that the CTRL group was the most efficient in terms of FCR but highly influenced 422 423 by the low DM content of the thawed Antarctic krill. When FCR is estimated on a wet weight basis, the trend inverts. For example, FCR increases from 2.0 to 2.2 and from 0.7 to 6.0 in the 424 425 500MED and CTRL groups, respectively. More meaningful is the comparison of FCR values among the experimental extruded feeds. Results indicate a lower FCR in diet 500MED and points 426 427 to a better utilization of this diet. The general best performance of animals fed the 500MED when compared to the other extruded feeds could also be related to a more balanced amino acid 428 429 composition. This diet, together with the 500LOW, showed the most similarity to the Antarctic krill among the experimental extruded feeds. Still, the apparent lower level of taurine in all the 430

431 extruded feeds might have limited the growth of juveniles. This amino acid is known to elicit a feed-attractant stimulus in crustaceans (22). A potential solution to increase the performance of 432 lobsters fed on artificial feeds could be the incorporation of taurine to formulated feeds to increase 433 feed intake of lobsters ⁽¹⁶⁾. The 500 mg kg⁻¹ protein level in the experimental feeds was achieved 434 by increasing the proportion of squid meal. This ingredient is often used in crustaceans' diets to 435 increase growth and attractiveness of artificial feeds and the replacement of fish by squid meal has 436 been proven beneficial for growth in several crustacean species ⁽⁵⁸⁾. However, since the amino acid 437 profiles of fish and squid meal are similar (56), it is unlikely that the dissimilarities observed among 438 439 the experimental extruded feeds were caused by the different inclusion levels of this ingredient.

440 *Nucleic acid derived indices*

The decrease in sRD for the 400MED and 500HIGH dietary groups were mainly caused by a reduction in the RNA content, while for the 400HIGH group, results show that the low sRD was a combined effect of decreased RNA and increased DNA concentrations in the muscle tissue.

444 The quantified RNA includes ribosomal RNA (rRNA), messenger RNA (mRNA), and transfer RNA (tRNA) which respond in different ways and have different functions. However, since rRNA 445 - responsible for building protein - makes up the majority of total RNA, changes in total RNA 446 were assumed to primarily reflect changes in rRNA ⁽⁵⁹⁾. Thus, it was presumed that the reduction 447 in RNA reflected a decrease in protein synthesis, while the relative increase in DNA was associated 448 with an increase in the number of cells per tissue portion, however, of smaller dimensions ⁽⁶⁰⁾. 449 Thus, the results suggest that juveniles reared on the 400HIGH diet were not only affected by a 450 451 decreased capacity for protein synthesis but also by the mobilisation of proteins from the abdominal muscle to obtain energy, as previously suggested in starving fish larvae ⁽⁶¹⁾. Overall, 452 453 results showed that the sRD was positively correlated with growth performance and feed efficiency 454 indicators. Specifically, we demonstrated that sRD estimated from samples collected at week 4 were significantly correlated with SGR calculated at week 6. These observations suggest that sRD 455 456 is a sensitive indicator of the nutritional condition of European lobster juveniles. The use of this 457 estimate in crustaceans is of great applicability as it allows the assessment of recent growth and nutritional condition (33,62,63). Most of the nutritional studies performed on crustaceans considered 458 growth rate as gains in wet weight and length. However, the growth process in crustacean species 459 has an interrupted character because is connected to the moult cycle, whereas, somatic growth and 460

the accumulation of energy reserves in tissues are a continuous process ⁽⁴⁶⁾. We conclude that the
 nucleic derived indices are useful tool in future nutritional studies with European lobster juveniles

463 as it allows a faster evaluation when compared to conventional growth performance estimators.

464 *Digestive capacity*

The lowest activity levels of trypsin and amylase enzymes were detected in European lobster 465 466 juveniles reared on Antarctic krill while activities increased in animals offered the 400HIGH and 400MED extruded feeds. This is in contradiction to what was previously reported ⁽³⁹⁾. In that study, 467 was observed that spiny lobsters (Jasus edwardsii) reared on a formulated diet had a marked 468 469 decrease in trypsin and α -amylase activities of the foregut and digestive gland compared to juveniles fed on fresh mussels. A main difference, however, may be that in the present study, 470 enzymatic activity was estimated from the entire abdominal section and not from the foregut and/or 471 472 hepatopancreas. Thus, the activity detected in tissue homogenates may not necessarily represent the activity of enzymes that will be secreted into the digestive lumen. In crustaceans, proteases and 473 carbohydrases activity was found in tissues outside the gut ^(64,65). It may also be that the secretion 474 475 of large amounts of enzymes may maximize the use of limiting nutrients. Such elevated enzymatic activity could maximize hydrolysis and the resulting extraction of a dietary substrate that was 476 ingested in small amounts (66). Results from (67) point to the same hypothesis. In the cited study, the 477 authors reported a significant increase in the specific activity of α -amylase in J. edwardsii juveniles 478 479 fed diets with low starch inclusion level. Thus, based on this, the results suggest that trypsin and amylase activities increased to compensate for the low levels of protein and carbohydrates lobsters 480 481 could obtain when fed on the 400MED and 400HIGH feeds. For lipase the activity was lower for juveniles fed the 400HIGH and 400MED than for individuals reared on the 500LOW and 500MED 482 extruded feeds. While carbohydrates are the primary source of energy for crustaceans ⁽⁶⁸⁾, lipids 483 are the main energy reserve. European lobsters store the metabolized lipids in the R-cells of the 484 hepatopancreas ⁽⁶⁹⁾. Overall, the results point toward a combined effect of low protein, high lipid, 485 486 and low feed intake in the activity of trypsin and amylase. The increased activity of these enzymes in the group of animals fed the 400MED and 400HIGH feeds suggests the activation of a 487 488 mechanism to optimize the use of protein and carbohydrates as they seem to be the most important nutrients for European lobster juveniles. In contrast, lipase activity increased with protein level in 489 490 the diet, with significantly higher activity for the 500-protein lobster groups than the 400-protein groups. This suggests that the required supply of protein for growth is covered for the 500 group,
and, that energy reserves to support growth potential are ensured for the lobsters reared on the
500LOW and 500MED feeds.

494 In the present study, we demonstrated that European lobster juveniles fed the 500MED experimental diet achieved a growth performance and nutritional condition statistically 495 comparable to that observed when animals were fed thawed Antarctic krill. This extruded feed 496 contained 500 g kg⁻¹ protein, 237 g kg⁻¹ carbohydrates, and 119 g kg⁻¹ lipids (as fed basis). The 497 results suggest that this composition is the most balanced to meet the overall nutritional 498 499 requirements of the European lobster juveniles among the tested extruded feeds. Results also 500 support what was previously observed for spiny lobster species: formulated feeds with a very similar L:CHO ratio (1:2) have been proven to provide the best balance of carbohydrate and lipid 501 502 when rearing juvenile J. edwardsii (70). Therefore, based on the results presented, we recommend that high protein and moderate inclusion of carbohydrates must be considered when formulating 503 feeds for *H. gammarus* juveniles. While relative growth benefits of lobsters fed the 500MED 504 experimental diet did not exceed that of lobsters fed the control diet, it is worth considering that 505 506 replacing fresh diets with extruded feeds can be advantageous in a cost-effective point of view. 507 However, from an economic point of view, the ingredients used in the formulation of experimental extruded feeds were not the most sustainable. Future studies, following some of the 508 recommendations presented here and using sustainable alternative ingredients (e.g. insect meal, 509 industrial by-products), will be of extreme importance for the establishment of European lobster 510 511 farming on a commercial scale.

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707

708 **Table 1.**

709 Formulation and chemical composition of experimental extruded feeds and Antarctic krill

710 (adapted from $^{(18)}$).

| Protein level | 400 g kg ⁻¹ | | | 500 g kg | | | |
|---|------------------------|-------|-------|----------|-------|-------|--------|
| L:CHO ratio | LOW | MED | HIGH | LOW | MED | HIGH | CTRL |
| Ingredients (g kg ⁻¹) | | | | | | | |
| Antarctic krill | | | | | | | 1000.0 |
| Fish meal ^a | 150.0 | 150.0 | 150.0 | 150.0 | 150.0 | 150.0 | |
| Squid meal ^b | 125.0 | 125.0 | 125.0 | 255.0 | 255.0 | 255.0 | |
| Krill meal ^c | 250.0 | 250.0 | 250.0 | 200.0 | 200.0 | 200.0 | |
| Wheat gluten ^d | 20.0 | 20.0 | 20.0 | 50.0 | 50.0 | 50.0 | |
| Wheat meal ^e | 172.5 | 172.5 | 172.5 | 172.5 | 172.5 | 171.5 | |
| Wheat starch ^f | 229.0 | 171.0 | 89.0 | 141.0 | 93.0 | 30.0 | |
| Fish oil ^g | 22.0 | 80.0 | 160.0 | 0.0 | 48.0 | 112.0 | |
| Soy lecithin ^h | 10.0 | 10.0 | 10.0 | 10.0 | 10.0 | 10.0 | |
| Vitamin & minerals premix ⁱ | 20.0 | 20.0 | 20.0 | 20.0 | 20.0 | 20.0 | |
| Astaxanthin ^j | 1.5 | 1.5 | 1.5 | 1.5 | 1.5 | 1.5 | |
| Proximate composition (g kg ⁻¹ as fed) | | | | | | | |
| Moisture | 78.0 | 81.0 | 82.0 | 86.0 | 81.0 | 71.0 | 916.1 |
| Ash | 68.1 | 68.0 | 66.2 | 68.70 | 68.2 | 66.3 | 11.6 |
| Crude protein | 400.0 | 397.0 | 385.0 | 497.0 | 495.0 | 481.0 | 58.2 |
| Total lipid | 107.0 | 147.0 | 233.0 | 85.8 | 119.0 | 172.0 | 9.6 |
| Carbohydrates ^x | 346.9 | 307.0 | 233.8 | 262.5 | 236.8 | 209.7 | 4.5 |
| L:CHO ratio | 0.3 | 0.5 | 1.0 | 0.3 | 0.5 | 0.8 | 2.1 |
| Gross energy (KJ. g ⁻¹) ^y | 19.0 | 19.8 | 21.6 | 18.7 | 19.5 | 20.8 | 1.8 |
| Protein/Energy (g MJ ⁻¹) | 21.0 | 20.1 | 17.8 | 26.5 | 25.4 | 23.1 | 32.6 |

- ^a Micronorse: 70.9% CP, 8.7% CF, Tromsø Fiskeindustri AS, Norway.
- 712 ^b Squid meal: 83% CP, 4% CF, Sopropêche, France.
- ^c Krill meal: 61.1% CP, 17.4% CF, Aker Biomarine, Norway.
- 714 ^d VITAL: 80.4% CP, 5.6% CF, Roquette, France.
- ^e Wheat meal: 11.7% CP, 1.6% CF, Molisur, Spain.
- 716 ^f Meritena 200: 0.4% CP, 0.1% CF, 90% starch, Tereos, France.
- 717 ^g Fish oil: 98.1% CF, 16% EPA, 12% DHA, Sopropêche, France.
- 718 ^h P700IPM, Lecico GmbH, Germany.

¹ Vitamins (IU or mg kg⁻¹ diet): DL-alpha tocopherol acetate, 200 mg; sodium menadione bisulphate, 50 mg; retinyl acetate, 40000

720 IU; DL-cholecalciferol, 4000 IU; thiamine, 60 mg; riboflavin, 60 mg; pyridoxine, 40 mg; cyanocobalamin, 0.2 mg; nicotinic acid,

400 mg; folic acid, 30 mg; ascorbic acid, 1000 mg; inositol, 1000 mg; biotin, 6 mg; calcium pantothenate, 200 mg; choline chloride,

722 2000 mg, betaine, 1000 mg. Minerals (g or mg kg⁻¹ diet): copper sulphate, 18 mg; ferric sulphate, 12 mg; potassium iodide, 1 mg;

manganese oxide, 20 mg; sodium selenite, 0.02 mg; zinc sulphate, 15 mg; sodium chloride, 800 mg; excipient wheat gluten, Premix
 Lda., Portugal.

725 ^j Carophyll Pink 10% CWS, 10% astaxanthin, DSM Nutritional Products, Switzerland.

- 726 ^x Estimated by difference: Carbohydrates (%) = 100 (Crude protein + Total lipid + Ash).
- 727 ^y Gross energy (MJ kg⁻¹) = Protein content \times 21.3 kJ g⁻¹ + Lipid content \times 39.5 kJ g⁻¹ + Carbohydrate content \times 17.6 kJ g⁻¹) / 1000
- 728 kJ MJ^{-1 (71)}

729 **Table 2.**

Amino acid profile (g per 100 g as is) of experimental extruded feeds and Antarctic krill.

| Protein level | 400 g kg ⁻¹ | | | 500 g kg | | | |
|------------------------------------|------------------------|-------|-------|----------|-------|-------|-------|
| L:CHO ratio | LOW | MED | HIGH | LOW | MED | HIGH | CTRL |
| Amino acids(g 100 g ¹) | | | | | | | |
| Essential | | | | | | | |
| Arg | 2.16 | 2.00 | 2.02 | 2.80 | 2.77 | 2.41 | 2.49 |
| His | 0.74 | 0.66 | 0.65 | 0.98 | 1.02 | 0.84 | 0.91 |
| Ile | 1.54 | 1.43 | 1.44 | 1.91 | 1.96 | 1.65 | 1.92 |
| Leu | 2.61 | 2.42 | 2.42 | 3.24 | 3.33 | 2.80 | 3.13 |
| Lys | 2.29 | 2.01 | 1.99 | 2.69 | 2.78 | 2.30 | 3.41 |
| Met | 1.15 | 1.09 | 0.87 | 1.44 | 1.51 | 0.99 | 0.76 |
| Phe | 1.48 | 1.39 | 1.39 | 1.89 | 1.90 | 1.61 | 1.89 |
| Thr | 1.48 | 1.39 | 1.40 | 1.86 | 1.91 | 1.61 | 2.05 |
| Val | 1.75 | 1.64 | 1.64 | 2.11 | 2.19 | 1.85 | 2.28 |
| ∑EAA | 15.18 | 14.01 | 13.79 | 18.91 | 19.35 | 16.04 | 18.82 |
| Nonessential | | | | | | | |
| Ala | 2.01 | 1.85 | 1.84 | 2.40 | 2.48 | 2.10 | 2.88 |
| Asp | 3.31 | 3.03 | 3.06 | 3.95 | 4.07 | 3.47 | 4.37 |
| Cys | 0.04 | 0.03 | 0.03 | 0.04 | 0.02 | 0.03 | |
| Glu | 5.36 | 4.86 | 4.81 | 6.99 | 7.07 | 5.99 | 5.85 |
| Gly | 2.41 | 2.24 | 2.23 | 2.97 | 3.07 | 2.66 | 3.04 |
| Pro | 2.17 | 2.02 | 2.02 | 2.76 | 2.89 | 2.49 | 2.63 |
| Ser | 1.46 | 1.38 | 1.40 | 1.93 | 1.92 | 1.63 | 1.74 |
| Tau | 0.24 | 0.21 | 0.20 | 0.29 | 0.27 | 0.19 | 1.88 |
| Tyr | 1.30 | 1.22 | 1.23 | 1.64 | 1.70 | 1.41 | 1.59 |
| ∑NEAA | 18.28 | 16.81 | 16.79 | 22.97 | 23.46 | 19.96 | 23.97 |
| $\overline{\Sigma}$ TAA | 33.46 | 30.82 | 30.58 | 41.88 | 42.81 | 36.00 | 42.78 |

731

732 **Table 3.**

Growth performance, feed efficiency, and whole body composition of juvenile *H. gammarus* fed the experimental extruded feeds and
 Antarctic krill.

| Protein level | 400 g kg ⁻¹ | | | 500 g kg ⁻¹ | | | | One-Way ANOVA | Two-Way ANOVA | | |
|---------------------------------|--------------------------------|--------------------------------|-------------------------|---------------------------|-------------------------|----------------------------|----------------|-----------------------------------|------------------------------------|-----------------------------------|-----------------------------------|
| L:CHO ratio | LOW | MED | HIGH | LOW | MED | HIGH | CTRL | | Р | L:CHO | P X L:CHO |
| SGR (% d ⁻¹) | $1.28 \pm 0.13^{\text{\#,ax}}$ | $0.77 \pm 0.09^{\text{\#,bx}}$ | $0.50 \pm 0.11^{\#,bx}$ | $1.29 \pm 0.13^{\#,Bx}$ | 1.72 ± 0.13^{Ay} | $1.06 \pm 0.07^{\#,By}$ | 1.89 ±0.13 | $F_{6,137} = 17.10^{***}$ | $F_{1,115} = 29.17^{\ast\ast\ast}$ | $F_{2,115} = 11.29^{***}$ | $F_{2,115} = 8.86^{***}$ |
| iCL (% CLi) | 29.0 ± 2.3^{ax} | $21.2\pm2.4^{\#,abx}$ | $19.3\pm2.8^{\#,bx}$ | $30.4\pm2.2^{\text{ABx}}$ | 35.2 ± 2.5^{Ay} | $25.4 \pm 1.6^{\#,Bx}$ | 37.5 ± 2.7 | $F_{6,137} = 8.04^{\ast\ast\ast}$ | $F_{1,115} = 14.63^{\ast\ast\ast}$ | $F_{2,115} = 5.60^{\ast\ast}$ | $F_{2,115} = 4.00^{\ast}$ |
| Ν | 19 | 20 | 15 | 20 | 21 | 21 | 22 | | | | |
| $FI (\% BW_i d^{-1})$ | $10.1\pm0.6^{\text{\#,ax}}$ | $4.6\pm0.8^{\#,bx}$ | 2.7 ± 0.2^{cx} | $7.3\pm0.3^{\#,Ay}$ | $5.3\pm0.2^{\#,Bx}$ | $5.1\pm0.3^{\#,By}$ | 2.7 ± 0.2 | $F_{6,20} = 34.74^{***}$ | $F_{1,17} = 1.59$ | $F_{2,17} = 53.11^{\ast\ast\ast}$ | $F_{2,17} = 15.64^{\ast\ast\ast}$ |
| EI (J d ⁻¹) | $179.2\pm8.8^{\#,ax}$ | $89.1\pm16.3^{\text{\#,bx}}$ | $56.6\pm3.9~^{bx}$ | $135.7 \pm 5.5^{\#,Ay}$ | $100.4 \pm 3.8^{\#,By}$ | $106.6 \pm 6.2^{\#,ABy}$. | 53.3 ± 4.7 | $F_{6,20} = 29.37^{\ast\ast\ast}$ | $F_{1,17} = 0.72$ | $F_{2,17} = 44.52^{***}$ | |
| FCR | $4.3\pm0.1^{\#}$ | $3.9\pm0.4^{\#}$ | $3.3\pm0.5^{\#}$ | $4.2\pm0.7^{\#}$ | 2.0 ± 0.2 | $3.2\pm0.3^{\#}$ | 0.7 ± 0.1 | $F_{6,20} = 10.45^{\ast\ast\ast}$ | $F_{1,17} = 4.15$ | $F_{2,17} = 4.94^{\ast}$ | $F_{2,17} = 2.77$ |
| Ν | 3 | 3 | 3 | 3 | 3 | 3 | 3 | | | | |
| Proximate composition (% of DM) | | | | | | | | | | | |
| Dry matter | 24.5 ± 0.4 | 22.7 ± 1.1 | 19.9 ± 1.2 | 25.3 ± 0.4 | 23.7 ± 1.8 | 24.2 ± 0.6 | 29.1 ± 0.5 | | | | |
| Ash | 34.7 ± 0.6 | 36.8 ± 0.3 | 42.8 ± 1.1 | 35.0 ± 0.4 | 34.0 ± 0.3 | 34.7 ± 0.5 | 32.3 ± 0.5 | | | | |
| Soluble protein | 50.4 ± 3.1 | 47.8 ± 4.1 | 42.2 ± 1.8 | 40.6 ± 1.4 | 42.9 ± 2.4 | 49.8 ± 3.4 | 45.4 ± 1.7 | | | | |
| Total lipid | 6.3 ± 1.3 | 6.4 ± 0.4 | 3.5 ± 0.8 | 7.0 ± 0.7 | 5.5 ± 1.0 | 5.2 ± 0.2 | 5.4 ± 1.1 | | | | |
| Carbohydrates [†] | 8.6 ± 3.1 | 9.0 ± 4.0 | 11.5 ± 0.7 | 17.5 ± 2.3 | 17.6 ± 1.6 | 10.4 ± 3.9 | 17.0 ± 2.9 | | | | |

735 SGR = specific growth rate; iCL = increment in carapace length; FI = dry feed intake; FCR = dry feed intake / wet weight gain; EI = energy intake, N = number of

replicates per treatment.

737 Values are means \pm SEM.

738 Superscript [#] indicate dietary groups significantly different from CTRL (Krill).

739 Means in the same row with a different superscript "a, b, c" or "A, B, C" are significantly different within the 400 or 500-protein level, respectively.

740 Means in the same row with a different superscript "x or y" are significantly different within the same L:CHO ratio category.

741 † Estimated by difference: Carbohydrates (%) = 100 - (Soluble protein + Total lipid + Ash).

742 * p < 0.05, ** p < 0.01, *** p< 0.001.



Fig. 1. Graphical representation of principal components analysis of amino acid profiles from tested diets. The two panels are complementary to one another. (a) Biplot of the first two principal components (PC1 and PC2) of tested diets amino acid profiles. PC1 separated the amino acid profiles horizontally and explained 84.1% of the variance. The amino acid profiles of 500MED, 500LOW and CTRL diets formed a succinct group to the right of the plot and were positively correlated to PC1. The 400HIGH, 400MED, 400LOW and 500HIGH diets were negatively correlated to PC1. PC2 separated the amino acid profiles vertically and explained 14.3% of the variance. The 500LOW and 500MED diets were positively correlated to PC2. (b) Variables (amino acids) used to construct the principal components. The circle in this plot is the correlation circle, the stronger the correlation of an amino acid to PC1 and, or PC2 the closer its arrowhead to the circle. The arrows indicate how the amino acids contributed to the formation of PC1 and PC2 and thus the formation of plot (a).



Fig. 2. Survival of *H. gammarus* juveniles (% of initial numbers) fed on the different diets. The dashed vertical line indicates the rearing time limit (42 days) considered in the statistical comparison of the curves.



Fig. 3. Cumulative moults of *H. gammarus* juveniles (% of initial numbers) fed on different diets. Panel (a) refers to the first moult occurred after the beginning of the growth trial and panel (b) to the second moult occurrence. Dietary treatments are indicated in the legend in ascending order of cumulative moults. The dashed vertical line indicates the rearing time limit (42 days) considered in the statistical comparison of the curves. Different letters superscript letters indicate significant differences between dietary treatments.



Fig. 4. Second order polynomial model fit to average carapace length (upper panels (a) and (b)) and wet body weight (lower panels (c) and (d)) of *H. gammarus* juveniles fed on different diets throughout the growth trial period. Data points represented as mean \pm SEM. Dietary treatments that were significantly different from CTRL are marked with an asterisk (*). # indicates that curve coefficients from 400HIGH group were not statistically compared with CTRL.



Fig. 5. Changes in RNA concentration (a), DNA concentration (b) and standardized RNA/DNA ratio, sRD (c) of abdominal muscle tissue of *H. gammarus* juveniles fed on different diets (n=6). The box includes observations from the 25^{th} to the 75^{th} percentile and the whiskers above and below the box indicate the 10^{th} and 90^{th} percentiles. The horizontal line within the box represents the median value and the symbol (+) indicates the mean. Dietary treatments that were significantly different from CTRL are marked with an asterisk (*). Different letters "a, b" or "A, B" indicate significant differences within the same L:CHO ratio category.



Fig. 6. Fitted linear regression model of standardized RNA/DNA ratio (sRD) vs. specific growth rate (SGR). Data points per dietary treatment represented as mean \pm SEM. The solid line displays the average estimates of the predicted sRD and the dashed lines the 95% confidence limits. Simple linear regression model: y = 0.644x - 0.143 ($R^2 = 0.55$).



Fig. 7. Changes in trypsin (a), amylase (b), and lipase (c) activity of *H. gammarus* juveniles fed on the different tested diets (n=9). The box includes observations from the 25^{th} to the 75^{th} percentile and the whiskers above and below the box indicate the 10^{th} and 90^{th} percentiles. The horizontal line within the box represents the median value and the symbol (+) indicates the mean. Dietary treatments that were significantly different from CTRL are marked with an asterisk (*). Different letters "a, b" or "A, B" indicate significant differences within the 400 or 500-protein level, respectively. Different "x or y" indicate significant differences within the same L:CHO ratio category.