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Ontogenetic changes in digestive enzyme activity and biochemical indices of larval and postlarval European lobster (*Homarus gammarus*, *L*)

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1 Abstract

2 The currently limited knowledge on the nutritional requirements of the European lobster (Homarus 3 gammarus) remains a major obstacle to the improvement of growth and survival rates in lobster farming. 4 Therefore, digestive enzyme activity (trypsin, lipase, and amylase) and biochemical indices (RNA:DNA, 5 proximate and lipid class composition) of larval (I to III) and postlarval (IV) stages of H. gammarus fed 6 Antarctic krill (Euphausia superba) were determined to identify ontogenetic changes in digestive capacity 7 and hence potential nutritional requirements. Activity of the three digestive enzymes was detected in all 8 developmental stages examined, suggesting that *H. gammarus* is capable of exploiting a varied diet from 9 stage I onwards. Amylase activity increased significantly in postlarvae denoting a shift towards a diet richer 10 in carbohydrates after metamorphosis. Lipase activity increased progressively during the three larval stages 11 but not further, pointing to a higher relevance of dietary lipids before metamorphosis. The decrease from 12 32% to 24% DM of protein in postlarvae was partially compensated by an increase in ash (from 21% to 13 29% DM), reflecting the increased contribution of the exoskeleton to their total body mass. 14 Phosphatidylcholine (~20% total lipids), phosphatidylethanolamine (~14% total lipids), and cholesterol 15 ($\sim 20\%$ total lipids) were the most abundant lipid classes in the body composition of H. gammarus early 16 stages, implying high dietary requirements for these compounds. The results presented here provide new 17 insights into the metabolism and nutritional requirements of *H. gammarus* early stages, highlighting the 18 importance of lipids during larval development and the increased relative importance of carbohydrates after 19 metamorphosis.

Keywords: Trypsin, amylase, lipase, protein, carbohydrate, lipid, cholesterol, phospholipids, larvae,
 postlarvae.

22 **Declarations**

23 Funding

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- collaboration with the University of La Laguna (ULL) was partially funded by an ERASMUS + mobility
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28 **Conflicts of interest/ Competing interests**

- 29 The authors declare they have no known competing financial interests or personal relationships that could
- 30 have influenced the work reported in this paper.

31 Availability of data and material

- 32 The research data generated during the current study will be made public by the corresponding author via
- 33 the DTU data repository on reasonable request.

34 Code availability

35 Not applicable

36 Author's contributions

- 37 R.G., I.L., and M.G. conceived the research question and designed the study. R.G. carried out the study
- trial. Samples and data were analyzed by R.G. and D.B.R.. Findings were interpreted by R.G., M.G., C.R.,
- 39 D.B.R., J.A.P., and I.L. All the authors contributed to the preparation of the manuscript.

40 **Ethics approval**

- 41 The study meets the ethical standards for the care and handling of invertebrate species in Europe.
- 42 **Consent to participate**
- 43 Not applicable.
- 44 **Consent for publication**
- 45 Not applicable.

46 Introduction

47 The European lobster Homarus gammarus L. is an ecologically and economically important species 48 inhabiting coastal areas from Northern Norway to Morocco and the Western Mediterranean (Triantafyllidis 49 et al. 2005). During the 1960s and 1970s, there was a general decline in global catches with occasional 50 stock collapses among *H. gammarus* populations, which led to the development of juvenile production for 51 re-stocking purposes (Ellis et al. 2015). The cultivation of *H. gammarus* as an emerging species in 52 aquaculture is an increasingly realistic approach to assist in a sustainable market supply (Drengstig and 53 Bergheim 2013; Hinchcliffe et al. 2021) although, the low survival and growth rates are still major 54 bottlenecks hampering its successful cultivation (Hinchcliffe et al. 2020). A low survival rate is particularly 55 observed in the larval stages and has been often associated with intense cannibalism in communal rearing 56 tanks (Powell et al. 2017). The low growth rates can be, at least partially, due to inadequate nutrition in 57 early life stages because of a lack of knowledge about their digestive capabilities, digestive processes, and 58 nutrient requirements (Powell et al. 2017; Hinchcliffe et al. 2020).

59 Like other nephropid lobsters, H. gammarus has a relatively short planktonic phase, going through 3 stages 60 (I to III) over a period that can vary from 10 days to 2 months depending on the water temperature (Nicosia and Lavalli 1999; Anger 2001). The final larval stage (III) leads to metamorphosis into the postlarval (stage 61 62 IV), in which many of the morphological, anatomical, and physiological characteristics are considerably modified (Charmantier and Aiken 1991). The ability to use chemical cues to locate food (Kurmaly et al. 63 64 1990) and the development of complementary teeth in the gastric mill (Charmantier and Aiken 1991) appear 65 at stage IV. Additionally, postlarvae (stage IV) start to develop more complex swimming capabilities, becoming progressively more benthic, until this behaviour is completely established by the first juvenile 66 67 stage (stage V) (Ennis 1975). The aforementioned modifications point to important ontogenetic shifts in the digestive capability, feeding behavior, and dietary requirements of the homarid lobsters during their 68 69 early development. This is also supported by dietary shifts documented for the American lobster, Homarus

70 *americanus*. Both homarid species are generally classified as either omnivorous or carnivorous, feeding on 71 a variety of planktonic and benthic organisms present in their natural habitat. More specifically, stomach 72 content analysis in wild-caught specimens revealed that the natural diet of *H. americanus* larvae changes 73 from being predominantly composed of copepods, diatoms, and gastropods in stages I and II to including a 74 more substantial portion of decapod zoea and megalops in stage III (Nicosia and Lavalli 1999). Newly 75 settled postlarval and early juvenile H. americanus were described to feed mainly on mesoplankton that 76 can be found in suspension in their shelter habitats (Conklin 1995). Less is known about the natural diet or 77 feeding habits of *H. gammarus* because they are rarely observed in the wild (Linnane et al. 2001).

78 In crustaceans, there is a correlation between diet and digestive enzymes produced. In general, carnivorous 79 species produce a wide range of proteases at high concentrations and are, therefore, capable of hydrolyzing 80 high levels of dietary protein (Jonhston and Yellowless 1998). In contrast, herbivores and omnivores 81 synthesize a greater variety and amount of carbohydrases according to their increased capacity to hydrolyze 82 plant and animal dietary carbohydrates (Jonhston and Yellowless 1998). In this sense, ontogenetic changes 83 in enzymatic activity may be indicative of shifts in the ability to hydrolyze different dietary components 84 (Rodriguez et al. 1994). This has been investigated in several decapod species including shrimps (Lovett 85 and Felder 1990; Lemos et al. 1999, 2002; Ribeiro and Jones 2000; Díaz et al. 2008), crabs (Andrés et al. 86 2010), spiny lobsters (Johnston 2003; Perera et al. 2008), and homarid lobsters (Biesiot and Capuzzo 87 1990a). Protease, carbohydrase, and lipase or esterase activities have been detected in all refereed species 88 from hatching, suggesting the capacity to hydrolyze different dietary components and to exploit a variety 89 of nutrient sources to meet nutritional requirements. In general, data indicate a higher activity of enzymes 90 involved in lipid digestion at earlier stages (Perera et al. 2008; Andrés et al. 2010) with an increase in 91 amylase activity after metamorphosis (Lovett and Felder 1990; Ribeiro and Jones 2000; Johnston 2003). 92 The pattern for protease activity during development was not as clear, varying considerably between 93 crustacean species. In *H. americanus*, total enzyme activities generally increase during early development 94 as the number and length of the tubules comprising the hepatopancreas increases (Biesiot and McDowell

1995). The digestive enzyme activity of H. gammarus has only been reported in juvenile and adult stages 95 96 where a range of proteases (trypsin, elastase, leucine aminopeptidase, and carboxypeptidase a and b) (Glass 97 and Stark 1994) and carbohydrases (amylase, maltase, α and β -glucosidases) (Glass and Stark 1995) activities have been detected in the hepatopancreas of wild-caught adult specimens. More recently, 98 99 Goncalves et al. (2021) demonstrated that digestive enzyme activities (trypsin, amylase, and lipase) in 100 juvenile H. gammarus (stages VII – VIII) are affected by the dietary composition. However, there is no 101 reported information about the ontogenetic variation in digestive enzyme activity in H. gammarus larvae 102 and postlarvae.

103 Studies on the biochemical changes during early development might be indicative of the type of energy 104 source used during ontogeny, and therefore, could be a valid approach to estimate nutritional requirements 105 at each stage of development. In the H. americanus, protein is considered the main source of energy but 106 lipids and carbohydrates also have an important contribution to the energy yield through larval stages I to 107 III (Sasaki et al. 1986). After metamorphosis into stage IV, the dependence on lipids as an energy substrate 108 decreases (Sasaki et al. 1986). The RNA:DNA index has been previously used to evaluate the effect of 109 nutrient limitation on the growth of *H. gammarus* larvae (Schoo et al. 2014). In the cited study, the authors 110 observed that imbalances in dietary nitrogen and phosphorus caused a decrease in the RNA:DNA ratio. 111 Lipid utilization during embryogenesis has been previously studied in *H. gammarus* and results showed 112 that neutral lipids are the main energy source during embryonic development, while polar lipids are not 113 catabolized and play mainly a structural role (Rosa et al. 2005). Additionally, a close relationship between 114 the physiological condition of the broodstock and their reproductive success has been demonstrated for 115 marine organisms including lobsters (Agnalt 2008; Moland et al. 2010). More specifically, it was 116 demonstrated that the inclusion of specific phospholipids in formulated feeds improved the nutritional status 117 of the broodstock, gonad development, and egg and larvae quality (Navas et al. 1997; Rodríguez-García et 118 al. 2015). Yet, to the best of our knowledge, there are no published studies on the ontogenetic changes in lipid class composition during larval development for H. gammarus. 119

- 120 The main objective of this study was to examine the digestive enzyme activity (trypsin, amylase, and lipase)
- 121 during *H. gammarus* early development (stage I IV) to elucidate the potential role of ontogeny on lobster's
- 122 ability to utilize proteins, carbohydrates, and lipids. Thawed Antarctic krill (*Euphausia superba*) was used
- 123 as feed in all stages. The RNA and DNA concentration, proximate composition, and lipid class profile were
- 124 determined to assess larval and postlarval energy metabolism and specific requirements.

125 Materials and Methods:

126 Larval rearing and sampling

127 Experiments were conducted at the aquaculture facilities at the National Institute of Aquatic Resources, 128 DTU Aqua, Section for Aquaculture, Hirtshals (Denmark). Larvae were obtained from three ovigerous 129 wild-caught H. gammarus females (A, B, and C) captured along the Skagerrak coast of North Jutland, 130 Denmark. Newly hatched larvae were collected from broodstock tanks and transferred to 46-L 131 cylindroconical transparent acrylic tanks. Larvae of different females were reared separately and stocked 132 into tanks over three consecutive days after hatching at an initial density of 9-11 larvae L^{-1} . Tanks were part 133 of a flow-through seawater system composed of a 10 m³ reservoir, a heat exchanger, and a header aeration tank. Each larval tank was equipped with a bottom seawater inlet at a constant flow rate of 40 L h⁻¹ and a 134 135 vertical outflow filter (0.7 mm mesh size). Strong aeration was provided from the bottom using air stones to maintain larvae in the water column. During the experiment abiotic conditions were kept constant: 136 137 temperature 19.6 \pm 0.7 °C, salinity 34 \pm 1 PSU, and dissolved oxygen > 90%. Larvae were subjected to an 138 8h:16h light:dark photoperiod cycle. Thawed Antarctic krill (*Euphausia superba*) (Akudim A/S, Denmark) 139 was supplied from hatching and onwards according to the following schedule: from 0 to 4 DAH (day after hatching), 15 g tank⁻¹ day⁻¹; from 5 to 8 DAH, 10 g tank⁻¹ day⁻¹; from 9 to 12 DAH, 8 g tank⁻¹ day⁻¹; from 140 13 DAH onwards, 5 g tank⁻¹ day⁻¹. Total daily amounts were evenly distributed three times per day (9:00h; 141 142 13:00h; 17:00h). The diet supplied 69% protein, 11% lipid, and 5% carbohydrate on a dry weight basis.

Six pools of lobster individuals (stages I-IV) were sampled per stage and per female following the sampling schedule of table 1. Larvae were collected from the rearing tanks before the first daily meal, lethally anesthetized in ice-cold seawater, rinsed in distilled water, and stored at -80°C until further analysis. Carapace length (CL) was measured for 20 individuals per stage and per female using a stereomicroscope (MC125 C, Leica, Germany) equipped with a digital camera (MC190 HD, Leica, Germany) to photograph the larvae and postlarvae. Carapace length, from the base of the eye socket to the posterior edge of the cephalothorax, was measured using Image J 1.52n software (University of Wisconsin, USA). Total dry weight (DW) was recorded for 20 freeze-dried individuals to the nearest 0.001 mg using a microbalance (Mettler Toledo, USA). Three sample pools collected per treatment (pools 1, 2, and 3) were freeze-dried and divided into sub-samples for digestive enzyme activities (1 individual larva/postlarva per pool), nucleic acids (1 individual larva/postlarva per pool), and proximate composition analysis (the remaining larvae/postlarvae per pool). The remaining three pools (pools 4, 5, and 6) were used fresh for the determination of lipid class composition.

156 **Digestive enzyme activities**

157 Total enzyme activity was measured in three individual larvae and postlarvae per stage and female (1 158 individual from each of the 3 freeze-dried pools). Whole-body individuals were mechanically homogenized 159 in ice-cold Milli-O water, centrifuged (10 min at 15800 g) and the supernatant used to assay enzymatic 160 activity. Amylase activity was determined with a commercial kit (Ultra Amylase Assay kit E33651, Thermo 161 Scientific, USA). Trypsin and lipase were assayed using the methods of Rotllant et al. (2008) modified as 162 described in Goncalves et al. (2021). All enzyme activities are expressed as RFU (Relative Fluorescence 163 Units) per individual. Results are expressed as total enzyme activity per individual because homogenates 164 from the whole body, rather than the hepatopancreas, were used for the digestive enzyme analysis given the small size of the lobsters used in this study. 165

166 Nucleic acids determination

167 RNA and DNA were quantified in the freeze-dried abdominal section of three individual lobsters (1 168 individual from each of the 3 freeze-dried pools) for each developmental stage and female following 169 procedures described previously (Goncalves et al. 2021). Briefly, the abdominal samples were chemically 170 (cold sarcosyl Tris-EDTA extraction buffer) and mechanically homogenized in an ultrasonic homogenizer 171 unit (4710 Series, Cole Parmer Instruments Co., USA). The concentration of RNA and DNA was quantified 172 in the supernatant extract in analytical duplicates. A specific nucleic acid fluorochrome dye GelRED was 173 used for the fluorescent reading at 365 nm (excitation) and 590 nm (emission). Following the first scan to 174 determine the total fluorescence of RNA and DNA, a ribonuclease A (Type-II A) solution was used to degrade RNA at 37°C for 30 min. A second scan measured the concentration of DNA, calculated directly 175 from a standard curve of DNA-GelRED with known concentrations of λ -phagus DNA (Roche, 176 177 Switzerland). The RNA fluorescence was calculated by subtracting the DNA fluorescence (second scan) 178 from total fluorescence (first scan) and the concentration determined using a standard curve of 16S-23S E. 179 *coli* RNA (Roche, Switzerland). The average ratio of DNA and DNA slopes (average \pm SD) was 5.86 \pm 180 0.01. The RNA/DNA ratios were standardized (sRD) using DNA and RNA slope ratios and the reference 181 slope ratio of 2.4 (Caldarone et al. 2006). The concentration of nucleic acids is expressed as μg of RNA or 182 DNA per mg of abdominal tissue DW.

183 **Proximate chemical composition**

184 Proximate composition was analysed in three of the six pools of whole-body samples collected per female 185 and developmental stage. Briefly, each pool was freeze-dried for dry matter (DM) determination. Further 186 biochemical analyses were performed on freeze-dried samples and corrected for the dry weight (DW). From 187 each pool, three subsamples of 40 mg, 40 mg, and 5 mg DW each were collected for the determination of 188 protein, lipid, and ash content, respectively. Protein was analysed spectrophotometrically at 750 nm using 189 a commercial Lowry-based micro-protein determination kit (BIO-RAD 500-0112, USA). Total lipids (TL) 190 were extracted with chloroform-methanol (2:1 by volume) according to the Folch method (Christie and Han 191 2010). The organic solvent was evaporated under a stream of nitrogen and lipid content was determined 192 gravimetrically. Ash was determined following the procedure described in (NMKL 23 1991).

193 Lipid class composition

Lipid classes (LC) were analysed in the lipid fraction of three pools (4, 5, and 6) of fresh whole-body samples per developmental stage and female. Total lipid was extracted by homogenization of approximately 150 mg lobster tissue wet weight as described above. Subsequently, an aliquot of 30 µg from the TL extract 197 was separated on a 10×10 cm HPTLC plates (Merck KGaG, Germany) by high-performance thin-layer 198 chromatography (HPTLC) in a single-dimensional double-development using propanol/chloroform/methyl 199 acetate/methanol/0.25% potassium chloride (5:5:5:2:1.8, v/v) as developing solvent for polar lipid classes, 200 and hexane/diethyl ether/acetic acid (20:5:0.5, v/v) for the neutral fractions. The different LC were 201 visualized by charring at 160°C after spraying with 3% (w/v) aqueous cupric acetate containing 8% (v/v) phosphoric acid and quantified as percentage (%) of TL using a CAMAG TLC Visualizer (Camag, 202 203 Switzerland). Data of each lipid class were than transformed into absolute amounts ($\mu g / mg$ WW) taking 204 into account the samples total lipid contents and wet weight.

205 Statistical analysis

206 The results are expressed as mean \pm SEM unless otherwise specified. Before analyses, the ANOVA 207 assumptions of normality of residuals and homogeneity of variances were tested using the Shapiro-Wilk 208 and Levene's test, respectively. In instances where assumptions were not met, data were square-rooted or 209 log-transformed. Carapace length and dry weight per developmental stage were compared in a one-way 210 ANOVA and whenever significant differences were detected (p < 0.05), comparisons between the different 211 batches were performed using the Tukey *post hoc* test. For the remaining analyzed parameters, comparisons 212 were performed using a two-way ANOVA considering stage and female as explanatory variables. When 213 differences were significant (p < 0.05), treatment means were compared using the Holm-Sidak *post hoc* test. 214 Additionally, a principal component analysis (PCA) was performed using the absolute amounts of each 215 lipid class, protein, and ash in whole body larvae (stages I, II, and III) and postlarvae (stage IV) H. 216 gammarus. The PCA analysis was carried out using R version 3.5.1 software (R Core Team 2018) and the 217 factoextra version 1.07 package (Kasambara and Mundt 2020). All statistical tests were performed using 218 the IBM SPSS Statistics 25.0 (IBM Corp., USA). Graphics were generated by GraphPad Prism 5.0 software 219 (GraphPad Software, USA).

220 **Results**

221 Female performance and larvae size

222 Broodstock size and spawning performance are summarized in Table 2. The three female breeders weighed 223 0.6 kg (\bigcirc B), 1.5 kg (\bigcirc A), and 1.7 kg (\bigcirc C). The largest female spawned over 15000 larvae during 23 days 224 of the spawning period, while the other two females had equal spawning duration (18 days) with a similar 225 number of spawned larvae (6000-8000). The mean individual dry weight and carapace length per 226 developmental stage of *H. gammarus* larvae and postlarvae are also summarized in Table 2. Stage I larvae grew from 1.87 ± 0.05 mg DW and 3.07 ± 0.05 mm CL to 7.01 ± 0.33 mg DW and 5.37 ± 0.04 mm CL at 227 228 stage IV postlarvae. The size of larvae at each developmental stage was significantly affected by the 229 broodstock. Thus, stage I and III larvae hatched from female C (1.7 kg) were the largest (DW and CL), 230 followed by larvae from female A (1.5 kg), and ultimately those from female B (0.6 kg) in which, stage II 231 and IV lobsters also presented the lowest DW and CL.

232 Digestive enzyme activities and ratios

The digestive enzyme activities of the three batches of larvae within each development stage are compared in Fig. 1. There was a trend towards an increase in amylase and lipase total activity during larval development whereas no clear trend was observed for trypsin.

236 Ontogenetic changes in trypsin were dependent on the broodstock as shown by the significant interaction stage \times female (F_{6,35} = 2.52, p = 0.049). No ontogenetic changes in trypsin activity were observed for 237 238 lobsters hatched from female A (Fig. 1). However, the trypsin activity increased significantly from stage II 239 to stage IV in individuals hatched from female B, and diminished from stage I to stage II in larvae from 240 female C. Within stages, the trypsin activity was significantly higher for female C progeny than for female 241 A progeny at stage I, while at stage IV, significantly higher trypsin was observed in the offspring from 242 female B than from female C. Amylase activity was significantly affected by the main factors stage ($F_{3,35}$ 243 = 10.25, p < 0.001) and female ($F_{2,35}$ = 12.86, p < 0.001). The amylase activity remained stable from stage

I to III and increased significantly from stage III to IV. Its overall activity was the lowest for lobsters hatched from female B. Lipase activity was also significantly affected by stage ($F_{3,35} = 32.81$, p < 0.001) and female ($F_{2,35} = 12.52$, p < 0.001). A significant increase was detected from stage I to II, and again from stage II to III, and remained stable after metamorphosis (stage IV). The overall lipase activity was also significantly lower for female B offspring as compared to A and C.

249 Nucleic acids

250 The ontogenetic variation in the concentration of the nucleic acids (RNA and DNA) and their standardized 251 ratio (sRD) is illustrated in Fig. 2. There was no significant effect of the main factor female or of the 252 interaction stage × female on the sRD ($F_{2,35} = 0.04$, p = 0.96 and $F_{6,35} = 1.68$, p = 0.17) RNA ($F_{2,35} = 2.82$, 253 p = 0.08 and $F_{6,35} = 2.09$, p = 0.09), and DNA ($F_{2,35} = 2.82$, p = 0.08 and $F_{6,35} = 1.68$, p = 0.17) content, respectively. In contrast, the main factor stage significantly affected the RNA ($F_{3,35} = 3,74$, p = 0.025) and 254 255 DNA ($F_{3,35} = 7,25$, p = 0.001) contents in the abdominal section of *H. gammarus* larvae and postlarvae. A 256 significant reduction in RNA concentration towards later stages was identified by the two-way ANOVA, 257 although the Holm Sidak *post hoc* test failed in identifying differences between stages. Similar but more 258 pronounced was the reduction for the DNA concentration which gradually and significantly decreased from 259 stage I to IV.

260 **Proximate and lipid class composition**

The proximate biochemical and lipid class composition (% TL) of the whole-body larvae and postlarvae *H. gammarus* is summarized in Table 3. In addition, Table 4 displays the statistics (two-way ANOVA) on the effect of the stage, female, and their interaction on the body composition of *H. gammarus*. The DM content of larvae and postlarvae was affected by the interaction stage × female. Significant ontogenetic changes in DM were only observed in larvae hatched from female C, for which the DM content of stage IV postlarvae was significantly lower as compared to stage I and III. Within stages, DM was the highest in stage I larvae hatched from female C and the lowest in stage III larvae hatched from female B. The DM content in stage IV postlarvae hatched from female A was significantly lower than in postlarvae hatched from females B and C. The ash content was also significantly affected by the interaction between stage and female and was significantly higher after metamorphosis as compared to the first three pelagic stages for female A and C offspring, while for female B progeny the ash content was significantly higher in stage IV as compared to stage I and III. There was a trend towards a significant reduction in protein content during ontogenetic development for which protein level was significantly lower in stage IV postlarvae than in stage I larvae.

274 Phosphatidylcholine (PC, 16.7% – 22.9% of TL) and phosphatidylethanolamine (PE, 11.9% – 16.6% of 275 TL) were the dominant phospholipids in *H. gammarus* larvae and postlarvae, while the major neutral lipid 276 component was cholesterol (CHO, 17.0% to 22.5%) (Table 3). Overall, the polar lipid fractions remained 277 fairly stable regardless of larval stage and female whereas variations within the neutral lipid profile were 278 more evident. Thus, the only significant differences found between phospholipids were the higher 279 phosphatidylserine portion for stage II larvae than for stage I and III, and the higher phosphatidylglycerol 280 content in lobsters hatched from female C than from female A. Interestingly, the free fatty acids (FFA) level 281 significantly decreased from stage III to IV. In addition, both triacylglycerol (TAG) and sterol esters (SE) 282 levels were significantly affected by the interaction stage \times female. The TAG content tended to increase 283 with age (stage III and IV) for larvae hatched from females A and B, but no significant changes among 284 stages were detected in lobsters from female C. Only minor differences within each particular stage existed 285 in TAG and SE contents of lobster larvae (Table 3 and 4). Ontogenetic changes for the SE levels were only 286 observed in batch C, for which SE content was significantly higher in stage III larvae as compared to stage 287 IV postlarvae. The total polar lipid content was significantly affected larval stage being significantly higher 288 for stage II larvae than for stage IV postlarvae and, as expected, the inverse was observed for the overall neutral lipids content. 289

A principal component analysis (PCA) was also carried out using the absolute amounts (µg per mg of wet weight) of each lipid class, protein, and ash in whole body larvae and postlarvae as variables to show the differences between developmental stage and female regarding biochemical composition (Fig. 3). Two 293 principal components (PC) accounted for 65.4% of the variability (PC1 52.0% and PC2 13.4%). From all 294 variables, the phospholipids PI, PS, and PE showed the highest loadings in the PC1 (-0.358, -0.352, and -295 0.351, respectively). Protein (+0.589), ash (-0.443), and the neutral lipid TAG (-0.411) showed the highest 296 loadings in the PC2. In panel A, samples were clustered by developmental stage. Stage IV postlarvae 297 formed a cluster to the bottom right area of the plot indicative of a positive correlation with ash and TAG 298 contents and a negative correlation with protein level. Stage I, II, and III are distributed in a more central area of the PCA plot. Panel B clustered samples by female and no clear separation was detected among the 299 300 three different groups.

301 **Discussion:**

302 Female performance and larvae size

303 Both the body DW and CL of H. gammarus varied significantly between the three batches of larvae 304 throughout the ontogenetic development. Results suggest a positive correlation between size of broodstock 305 and size of offspring as previously demonstrated by Moland et al. (2010), which could be related to the fact 306 that smaller, and presumably, younger H. gammarus females are more likely to moult between each 307 spawning as compared to older females. Consequently, ovigerous females will allocate an important amount 308 of energy to the moulting process that could, otherwise, be invested in embryonic development (Agnalt 309 2008). Also observed was a large variation in the numbers of hatched larvae, probably related to genetic 310 and condition-related effects but the loss of different egg mass volume among the three females during 311 handling at captured cannot be completely ruled out in the present study.

312 Digestive enzymes ontogenetic trends

313 No clear variation pattern was identified for the activity of the protease trypsin during larval development. 314 This corroborates the results obtained in previous studies for other decapod species. For example, Lemos 315 et al. (2002) observed that trypsin activity was highest in nauplius IV and protozoea I and III of the white 316 shrimp Litopenaeus schmitii but no clear patterns could be established for trypsin variation during 317 development. Further, the trypsin activity variation within developmental stages seemed to be more 318 dependent on the broodstock as demonstrated by the significant interaction stage \times female found. One 319 possible explanation for the observed broodstock-specific variation on the trypsin activity profile might be 320 the use of yolk reserves accumulated in the midgut during the early life stages (Biesiot and Capuzzo 1990b), 321 assuming that different females were providing different quantities and qualities of volk reserves to their 322 progeny. The significant increase in amylase activity after metamorphosis as compared to the previous three 323 larval stages is in agreement with previous observations in the white shrimp *Penaeus setiferus* (Lovett and 324 Felder, 1990) and may indicate an increased capacity of H. gammarus postlarvae stage IV to hydrolyze

325 dietary carbohydrates (Johnston 2003). An increased capacity for carbohydrate dietary digestion and 326 assimilation in postlarvae, juvenile, and adult specimens as compared to larvae has been previously 327 demonstrated for different species of lobster, including the H. gammarus (Radford et al. 2008; Simon 2009; 328 Rodríguez-Viera et al. 2014; Goncalves et al. 2021). The total lipase activity increased progressively from 329 stage I to III but no further increase was observed in the transition from larvae to postlarvae, supporting the 330 idea of lipid having increasing importance as an energy source during larvae development, but the 331 dependence on lipids as a substrate is reduced after metamorphosis (Sasaki et al. 1986). The greater 332 importance of lipids as an energy source in earlier stages has been previously confirmed by analyses of 333 lipase and esterase in the spider crab Maja brachydactyla (Andrés et al. 2010) and the spiny lobster 334 Panulirus argus (Perera et al. 2008). This is also corroborated by the decreasing trend in the TAG contents 335 and the correlated increasing trend in the FFA levels until stage III, after which TAG increases and FFA 336 drops significantly when metamorphosis into stage IV takes place (Table 3). In decapod crustaceans, 337 elevated FFA results from dietary or depot lipid degradation by lipase and esterase (O'Connor and Gilbert 338 1968). Subsequently, the released FFA might be mainly incorporated into cellular membrane 339 phospholipids, and the excess diverted to energy for growth during larval development (Nates and 340 Mckenney 2000).

341 Our results differ, to some extent, from those describing the ontogenetic change in total digestive enzyme 342 activities for H. americanus (Biesiot and Capuzzo 1990a) where authors reported a gradual increase in 343 protease, amylase, and lipase during early development within each successive stage. Lipase activity profile 344 better matches with the results reported by these authors, at least from stage I to III. The differences between 345 both studies might be related to the use of different feeding regimes during larvae rearing. While Biesiot 346 and Capuzzo (1990b) fed larvae with frozen adult brine shrimp Artemia (43% protein, 10% lipid, and 17% 347 carbohydrate), in this study we used thawed Antarctic krill (69% protein, 11% lipid, and 5% carbohydrate). 348 Further, in the former study, the authors estimated general protease activity whereas, in the present study, 349 we measured the activity of a specific protease – trypsin. Although the endoprotease trypsin has been

described as the main protease for decapod crustaceans, accounting for 40% to 60% of total protease (Lemos et al. 1999; Perera et al. 2010, 2015), other endoproteases (elastase) and exoproteases (α - and β carboxypeptidases) are also involved in the digestion of protein in adult *H. gammarus* (Glass and Stark 1994).

354 The digestive enzyme activity has been previously determined in *H. gammarus* juveniles (stage VII-VIII) 355 using the same methodologies described here (Goncalves et al. 2021). Trypsin, amylase, and lipase total 356 activities of juveniles fed the same diet used in this study (i.e. thawed Antarctic krill) were $66 \times 13 \times$, and 357 $8 \times$ higher as compared to postlarvae stage IV, respectively. Results suggest that the ontogenetic change in 358 H. gammarus digestive capabilities and nutritional requirements may reflect a developmentally cued 359 regulation of enzyme synthesis (Ribeiro and Jones 2000). Moreover, the uneven increase among the 360 different enzyme types suggests that protein becomes more important as H. gammarus develops compared 361 to carbohydrates, and even more to lipids.

362 Ontogenetic change in DNA and RNA content

363 The whole-animal concentrations of RNA and DNA in H. gammarus decreased gradually from stage I to 364 IV suggesting that the overall metabolic activity decreased during larval development. The steeper decrease 365 in DNA than in RNA points out to a higher dependence of early stages metabolism on cell multiplication 366 rather than on cellular protein synthesis. That is because, while an increase in RNA reflects an increase in 367 protein synthesis capacity, the increase in DNA is associated with an increase in the number of cells per 368 tissue portion (Olivar et al. 2009). Despite the sharper decrease in DNA than in RNA content, no significant 369 differences were found between stages based on the standardized RNA:DNA ratio - sRD. Results suggest 370 that the cellular protein synthesis capacity was not affected throughout larval development. Laubier-371 Bonichon et al. (1977) examined the RNA and DNA concentrations in the prawn Penaeus japonicus and 372 concluded that both the rates of cell multiplication and protein synthesis were at maximum levels during 373 larval development, but then drop after the transition to postlarvae. Later, Lovett and Felder (1990) 374 suggested that the reduced metabolic activity of *Penaeus setiferus* during the critical postlarvae period after 375 metamorphosis was associated with low digestive enzyme activity triggered by the limited nutrient uptake 376 during the transformational period of morphogenesis in the gut. Several prawn and shrimp species, 377 including the *P. japonicus* and *P. setiferus*, undergo complex modifications in their digestive system after 378 metamorphosis. In particular, the anterior midgut caeca degenerates into the vestigial anterior midgut 379 diverticulum (Lovett and Felder 1990). Although there is some development of the hepatopancreas with H. 380 gammarus growth, particularly the increase in size and number of tubules within the hepatopancreas tissue 381 (Biesiot and McDowell 1995), transformations during metamorphosis seem to be much less dramatic than 382 in prawns and shrimps which can partially explain the lack of significant changes in sRD between development stages. 383

Biochemical composition

385 The major changes observed in terms of proximate composition in whole-body homogenates of H. 386 gammarus throughout larval development were a significant increase in ash content at stage IV in relation 387 to stage I to III, along with a significant decrease in protein content from stage I to postlarvae stage IV. 388 Interestingly, the decrease in protein at stage IV was not accompanied by significant variation in trypsin 389 activity but, as previously mentioned, adjustment in the activity of other proteases cannot be ruled out. The 390 observed variations in ash and protein are likely related to each other and associated with the development 391 of a more heavily calcified exoskeleton after metamorphosis (Charmantier and Aiken 1991). Lipids, 392 however, suffer slighter variations with compensated processes of catabolism and anabolism of lipid 393 classes.

The most abundant lipid classes identified in the body tissues of *H. gammarus* at early stages were PC and cholesterol, followed by PE, suggesting a high requirement for structural lipids in *H. gammarus* larvae. Rosa et al. (2005) identified PC as one of the most abundant lipid classes in *H. gammarus* eggs, even though PE and FFA were slightly higher at the last stage of embryonic development. A high requirement for PC to satisfy metabolic demands in juvenile *H. americanus* has been previously reported (D'Abramo et al. 1982). 399 Phospholipid was the predominant lipid class while only traces of TAG (< 0.1%) were present in wild-400 caught pueruli of the spiny lobster Jasus edwardsii (Jeffs et al. 2001). In the same work, the authors showed 401 that phospholipid reserves are primarily used during this important phase in the life cycle and that 402 diacylglycerol plays a minor secondary role. Likewise, the major lipid classes identified in the early larval 403 stages of the western rock lobster, *Panulirus cygnus*, were polar lipids followed by sterols (mainly 404 cholesterol) (Liddy et al. 2004). The authors observed that polar lipid was the main lipid class catabolized 405 by starved larvae whereas its content increased in fed larvae, with no significant changes in sterol content 406 (Liddy et al. 2004).

407 Phospholipids are considered essential nutrients for the early stages of many decapod crustacean species 408 because of their important role in cell membrane structure and function (Nicolson 2014), and in the 409 digestion and transport of lipids, as well as for the low rates for their endogenous biosynthesis (Landman 410 et al. 2021). Particularly, PC is regarded as the main reservoir of choline, up to 95% of its pool, in animal 411 tissues (Zeisel and Blusztajn 1994; Zeisel et al. 2003) whereas PE stands out by its key role in neural tissues 412 and retina development (Calzada et al. 2016). Both phospholipids (PC and PE) are particularly rich in DHA 413 (22:6n-3), a highly polyunsaturated fatty acid (Lands 2017) physiologically essential for decapod larvae 414 (Conklin 1995). Cholesterol is also classified as an essential nutrient for crustaceans since they are not 415 capable of its biosynthesis, therefore, resulting in their dependence on dietary supply to meet nutritional 416 requirements (Nates and Mckenney 2000).

The most obvious effect of the development stage on the lipid classes composition of *H. gammarus* larvae tissues was the significant reduction in the FFA content after metamorphosis, coinciding with the minimum level of the total polar lipid fraction and the maximum total neutral lipid proportion, mainly supported by an increment in TAG levels. Neutral lipids are the main depot of energy reserves for many marine crustaceans (Anger 1998). Thus, both the lower FFA levels and the higher neutral lipid fraction in stage IV *H. gammarus* postlarvae compared to stage I-III larvae suggests that there is a priority for development and growth over the accumulation of reserves during the first three planktonic larvae stages. This strategy changes once the true metamorphosis and the associated morphological, anatomical, and physiological
variations (Charmantier and Aiken 1991) have taken place in stage IV *H. gammarus* postlarvae. Similar
findings have been shown in other decapod crustacean larvae including the shrimp species *Pandalus borealis* (Ouellet et al. 1995) and *Lepidophtalmus louisianensis* (Nates and Mckenney 2000).

428 Conclusions

429 In this study we demonstrated that the ontogenetic shifts of total enzyme activity are developmentally cued 430 in *H. gammarus* larvae, pointing to a temporal genetic regulation. Dietary amylase becomes more important 431 after metamorphosis, while lipids gradually gain importance during larval development until stage III but 432 not further. By contrast, no clear trend was observed for total trypsin activity suggesting a similar importance of dietary protein throughout the first four development stages. The ontogenetic changes in 433 434 digestive enzyme activity identified in this study can be indicative for changes in dietary composition for culture of H. gammarus. In particular, results point to an increase in carbohydrate content after 435 436 metamorphosis and higher lipid content during larval stages. The phospholipids PC and PE and cholesterol 437 were identified as the most abundant lipid classes in *H. gammarus* larvae and postlarvae suggesting high 438 dietary requirements for these compounds. Although it was not the primary goal of this study, a parental 439 influence was observed on size, digestive enzyme activity, and biochemical composition of H. gammarus 440 larvae and postlarvae. Results suggest that, while often disregarded, the background of larvae used in 441 nutritional studies might have a considerable impact on the results obtained. Therefore, we recommend that 442 the effect of broodstock should be considered in future nutritional experiments performed on H. gammarus 443 early stages.

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641	

642 Tables:

Stage	Ι			II			III			IV		
Female	A	В	С	A	В	С	A	В	С	A	В	С
DAH	2	2	2	5-6	5-6	5-6	11-12	11-12	10-12	11-18	13-18	12-14
N (pools)	6	6	6	6	6	6	6	6	6	6	6	6
N (per pool)	50	50	50	40	40	40	30	30	30	15	15	15
N (total)	300	300	300	240	240	240	180	180	180	90	90	90

643 **Table 1.** Sampling time and number of *Homarus gammarus* larvae collected per stage and female lobster.

644 DAH: days after hatching.

Table 2. *Homarus gammarus* females' size and performance (body wet weight, larvae spawn, and spawning
 duration) and larvae / postlarvae growth (body dry weight and carapace length).

Female	A	В	С	Mean per stage	One-way ANOVA
Progenitor size and performance					
Body weight (kg)	1.5	0.6	1.7		
Larvae spawned (N)	6018	8338	15296		
Spawning duration (days)	18	18	23		
Progeny dry weight (mg)					
Stage I	1.94 ± 0.05 ^b	1.44 ± 0.04 c	2.22 ± 0.03 $^{\rm a}$	1.87 ± 0.05	$F_{2,59} = 88.76^{***}$
Stage II	2.77 ± 0.06 $^{\rm a}$	1.95 ± 0.08 ^b	2.92 ± 0.11 ^a	2.55 ± 0.07	$F_{2,59} = 43.72^{***}$
Stage III	5.01 ± 0.23 ^b	$3.69\pm0.16~^{c}$	$5.87\pm0.15^{\text{ a}}$	4.86 ± 0.16	$F_{2,59} = 35.71^{***}$
Stage IV	8.73 ± 0.59 ^a	5.12 ± 0.24 ^b	7.18 ± 0.51 a	7.01 ± 0.33	$F_{2,59} = 17.68^{***}$
Progeny carapace length (mm)					
Stage I	3.06 ± 0.03 ^b	2.93 ± 0.02 c	3.21 ± 0.01 a	3.07 ± 0.02	$F_{2,59} = 38.83^{***}$
Stage II	3.89 ± 0.04 ^a	3.40 ± 0.06 ^b	3.94 ± 0.03 ^a	3.74 ± 0.04	$F_{2,59} = 41.32^{***}$
Stage III	$4.54\pm0.04~^{b}$	$4.16\pm0.06~^{c}$	4.71 ± 0.04 a	4.47 ± 0.04	$F_{2,59} = 39.17^{***}$
Stage IV	5.44 ± 0.05 a	5.09 ± 0.07 ^b	5.56 ± 0.04 ^a	5.37 ± 0.04	$F_{2,59} = 19.70^{***}$

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Stage	Ι			Stage I	II			Stage II	III			Stage III	IV			Stage IV
Female	A	В	С	mean	A	В	С	mean	А	В	С	mean	A	В	С	mean
Proximate composition													· ·			
Dry matter	15.7 ± 0.3^{xy}	$14.3\pm0.3^{\rm y}$	$16.8\pm0.3^{a,x}$	15.6 ± 0.4	15.6 ± 0.7	14.7 ± 0.1	15.0 ± 0.3^{ab}	15.1 ± 0.3	$17.2\pm0.4^{\rm x}$	$14.5\pm0.3^{\text{y}}$	$17.5\pm0.4^{a,x}$	16.4 ± 0.5	18.0 ± 1.7^{x}	$12.9\pm0.6^{\text{y}}$	$13.5\pm0.5^{\text{b},\text{y}}$	14.8 ± 1.0
Ash	20.4 ± 0.2^{b}	23.3 ± 1.1^{b}	21.0 ± 0.6^{bc}	$\textbf{21.6} \pm \textbf{0.6}$	21.7 ± 1.1^{b}	24.0 ± 2.1^{ab}	$24.9\pm0.8^{\text{b}}$	$\textbf{23.5} \pm \textbf{0.8}$	$21.9\pm1.0^{\text{b},\text{x}}$	$23.7\pm0.6^{\text{b},\text{x}}$	$17.3\pm1.4^{c,y}$	$\textbf{21.0} \pm \textbf{1.1}$	28.6 ± 0.9^{a}	28.6 ± 1.4^{a}	$29.8 \pm 1.6^{\rm a}$	$\textbf{29.0} \pm \textbf{0.7}$
Protein	33.0 ± 3.9	32.2 ± 1.8	29.6 ± 2.6	$31.6 \pm \mathbf{1.5^A}$	33.9 ± 4.7	30.0 ± 5.7	27.7 ± 2.3	$30.5\pm2.4^{\rm AB}$	34.3 ± 1.6	26.3 ± 3.7	32.1 ± 0.9	$30.9 \pm 1.7^{\rm AB}$	23.1 ± 2.5	23.0 ± 1.5	25.8 ± 3.9	$24.0 \pm 1.5^{\scriptscriptstyle B}$
Total lipid content	6.1 ± 1.6	4.4 ± 0.4	6.9 ± 1.6	$\textbf{5.8} \pm \textbf{0.8}$	4.8 ± 0.6	5.1 ± 0.6	5.6 ± 0.7	$\textbf{5.2} \pm \textbf{0.4}$	4.8 ± 1.3	5.9 ± 0.7	4.1 ± 1.1	$\textbf{4.9} \pm \textbf{0.6}$	3.9 ± 0.3	4.2 ± 1.1	5.1 ± 1.7	$\textbf{4.4} \pm \textbf{0.6}$
Lipid classes																
Lysophosphatidylcholine	0.1 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.0	0.2 ± 0.0	0.3 ± 0.1	0.6 ± 0.4	0.3 ± 0.1	0.1 ± 0.1	0.2 ± 0.1	0.0 ± 0.0	$\textbf{0.1} \pm \textbf{0.0}$
Sphingomyelin	2.0 ± 0.5	2.6 ± 0.7	1.8 ± 0.4	$\textbf{2.1} \pm \textbf{0.3}$	2.5 ± 0.8	3.1 ± 0.9	2.8 ± 0.4	$\textbf{2.8} \pm \textbf{0.4}$	2.0 ± 0.5	1.5 ± 0.5	2.5 ± 0.4	2.0 ± 0.3	2.4 ± 0.9	2.0 ± 0.7	2.9 ± 0.3	$\textbf{2.4} \pm \textbf{0.4}$
Phosphatidylcholine	20.4 ± 1.5	20.9 ± 1.0	20.0 ± 0.5	$\textbf{20.4} \pm \textbf{0.6}$	22.9 ± 0.7	21.4 ± 2.1	21.1 ± 0.8	$\textbf{21.8} \pm \textbf{0.7}$	19.1 ± 1.1	17.3 ± 1.5	20.9 ± 3.1	19.1 ± 1.2	16.7 ± 1.2	18.7 ± 1.7	19.8 ± 1.7	18.4 ± 0.9
Phosphatidylserine	4.7 ± 0.1	$4.\ 5\pm0.4$	3.9 ± 0.1	$4.4\pm0.2^{\text{B}}$	5.2 ± 0.2	5.1 ± 0.2	5.1 ± 0.4	$5.1\pm0.1^{\rm A}$	3.7 ± 0.3	4.0 ± 0.1	4.6 ± 0.2	$4.1\pm0.2^{\rm B}$	4.0 ± 0.6	4.8 ± 0.6	4.4 ± 0.1	4.4 ± 0.3^{AB}
Phosphatidylinositol	4.1 ± 0.2	4.4 ± 0.3	3.9 ± 0.3	$\textbf{4.1} \pm \textbf{0.2}$	4.5 ± 0.3	4.1 ± 0.3	4.7 ± 0.3	$\textbf{4.5} \pm \textbf{0.2}$	3.7 ± 0.2	3.9 ± 0.2	4.6 ± 0.3	$\textbf{4.1} \pm \textbf{0.2}$	3.3 ± 0.2	4.0 ± 0.3	4.3 ± 0.2	$\textbf{3.9} \pm \textbf{0.2}$
Phosphatidylglycerol	4.7 ± 0.9	4.6 ± 0.2	4.5 ± 0.4	$\textbf{4.6} \pm \textbf{0.3}$	4.1 ± 1.0	4.8 ± 0.1	5.8 ± 0.4	$\textbf{4.9} \pm \textbf{0.4}$	4.3 ± 0.3	5.0 ± 0.4	5.7 ± 0.6	5.0 ± 0.3	3.5 ± 0.2^{x}	4.9 ± 0.3^{xy}	$5.7\pm0.2^{\rm y}$	4.7 ± 0.3
Phosphatidylethanolamine	13.1 ± 0.7	13.9 ± 0.3	13.6 ± 0.9	13.5 ± 0.4	$16.\ 6\pm0.6$	14.2 ± 0.5	14.4 ± 0.5	15.1 ± 0.5	12.4 ± 1.0	13.3 ± 1.0	14.9 ± 1.3	13.5 ± 0.7	11.9 ± 0.9	13.7 ± 1.4	15.5 ± 1.2	13.7 ± 0.8
UK _(a)	0.5 ± 0.5	0.9 ± 0.2	0.8 ± 0.5	$\textbf{0.8} \pm \textbf{0.2}$	1.1 ± 0.5	0.7 ± 0.4	1.0 ± 0.1	$\textbf{1.0} \pm \textbf{0.2}$	0.8 ± 0.4	0.9 ± 0.9	1.1 ± 0.4	0.9 ± 0.3	0.5 ± 0.5	0.4 ± 0.4	0.8 ± 0.5	0.6 ± 0.2
Total polar lipids	49.7 ± 2.5	51.8 ± 2.3	48.6 ± 1.3	$50.0\pm1.2^{\rm AB}$	56.9 ± 1.5	53.6 ± 3.1	55.1 ± 1.6	$55.2\pm1.2^{\rm A}$	46.2 ± 2.7	$46.\ 1\pm1.5$	54.7 ± 4.5	$49.0\pm2.1^{\rm AB}$	42.5 ± 3.9	48.6 ± 3.2	53.4 ± 2.4	$48.2\pm2.3^{\rm B}$
Monoacylglycerols +																
Diacylglycerols	6.0 ± 0.7	3.1 ± 0.7	2.6 ± 0.6	$\textbf{3.9} \pm \textbf{0.6}$	4.0 ± 0.6	3.5 ± 0.5	3.0 ± 0.5	3.5 ± 0.3	2.7 ± 0.7	3.6 ± 0.4	3.2 ± 0.1	3.1 ± 0.3	3.2 ± 1.5	3.4 ± 0.4	2.5 ± 0.4	$\textbf{3.0} \pm \textbf{0.5}$
Cholesterol	22.0 ± 1.6	19.2 ± 0.7	21.0 ± 1.0	$\textbf{20.7} \pm \textbf{0.7}$	22.5 ± 1.1	21.4 ± 1.0	18.8 ± 1.2	$\textbf{20.9} \pm \textbf{0.8}$	17.0 ± 1.0	19.5 ± 1.3	20.4 ± 3.0	19.0 ± 1.1	18.4 ± 0.8	20.5 ± 2.0	21.3 ± 1.9	$\textbf{20.0} \pm \textbf{0.9}$
Free Fatty Acids	8.7 ± 1.5	10.1 ± 0.5	7.4 ± 1.3	$8.7 \pm \mathbf{0.7^{AB}}$	8.5 ± 1.6	11.9 ± 1.2	7.9 ± 1.5	$9.5\pm1.0^{\rm AB}$	10.7 ± 1.9	13.5 ± 0.8	10.1 ± 4.1	11.4 ± 1.4^{A}	$6.6\ \pm 0.5$	8.0 ± 0.7	7.1 ± 0.6	7.2 ± 0.4^{B}
Triacylglycerols	6.3 ± 0.3^{b}	8.2 ± 0.8^{ab}	11.9 ± 1.0	$\textbf{8.8} \pm \textbf{0.9}$	4.4 ± 0.2^{b}	2.6 ± 0.7^{b}	5.1 ± 0.6	4.1 ± 0.5	$17.9 \pm 1.6^{\text{a},\text{x}}$	$8.4 \pm 1.5^{\text{ab},\text{y}}$	$5.9\pm1.2^{\rm y}$	$\textbf{10.7} \pm \textbf{2.0}$	$25.3\pm4.3^{a,x}$	$14.9\pm4.5^{a,y}$	$10.2\pm3.3^{\text{y}}$	$\textbf{16.8} \pm \textbf{3.0}$
Sterol Esters	6.7 ± 1.0	5.5 ± 1.4^{ab}	5.7 ± 0.4	5.9 ± 0.5	$3.2\pm1.4^{\rm y}$	$5.1\pm2.9^{ab,xy}$	$7.9 \pm 1.2^{\rm x}$	$\textbf{5.4} \pm \textbf{1.2}$	$3.4\pm0.8^{\rm y}$	$8.9\pm0.9^{\text{a},\text{x}}$	$4.3\pm1.2^{\rm y}$	$\textbf{5.5} \pm \textbf{1.0}$	2.6 ± 0.3	$4.0\pm0.3^{\rm b}$	3.4 ± 0.1	$\textbf{3.3} \pm \textbf{0.2}$
UK _(b)	0.7 ± 0.7	2.3 ± 0.3	2.8 ± 0.9	$\textbf{1.9} \pm \textbf{0.5}$	0.4 ± 0.4	1.9 ± 0.7	2.1 ± 0.4	$\textbf{1.5} \pm \textbf{0.4}$	2.3 ± 0.5^{a}	0.0 ± 0.0^{b}	1.4 ± 1.1^{ab}	$\textbf{1.2} \pm \textbf{0.5}$	1.5 ± 0.4	0.6 ± 0.6	2.1 ± 0.5	1.4 ± 0.3
Total neutral lipids	50.3 ± 2.5	48.2 ± 2.3	51.4 ± 1.3	$50.0 \pm 1.1^{\rm AB}$	43.1 ± 1.5	46.4 ± 3.1	44.9 ± 1.6	$44.8 \pm 1.2^{\scriptscriptstyle B}$	53.8 ± 2.7	53.9 ± 1.5	45.3 ± 4.5	$51.0\pm2.1^{\rm AB}$	57.5 ± 3.9	51.4 ± 3.2	46.6 ± 2.4	$51.8\pm2.3^{\rm A}$

649 **Table 3.** Proximate composition (% DW basis) and lipid class composition (% TL) of larvae and post larvae *Homarus gammarus*.

650 Values are means ± SEM of three replicates per treatment. UK, unknown.

651 Means in the same raw with a different superscript "x,y" are significantly different within the same stage.

652 Means in the same raw with a different superscript "a,b,c" are significantly different within the same female.

653 Overall stage means in the same raw with a different superscript "A,B,C" are significantly different

-	Two-Way ANOVA		
	Stage	Female	Stage × Female
Proximate composition			
Dry matter 1, 2, 1×2	$F_{3,35} = 3.71^*, p = 0.03$	$F_{2,35} = 16.28^{***}, p < 0.001$	F _{6,35} = 4.76 ^{**} , p < 0.01
Ash ^{1,1×2}	$F_{3,35} = 29.34^{***}, p < 0.001$	$F_{2,35} = 2.77, p = 0.08$	$F_{6,35} = 3.05^*, p = 0.02$
Protein ¹	$F_{3,35} = 3.60^*, p = 0.03$	$F_{2,35} = 1.03, p = 0.37$	$F_{6,35} = 0.69, p = 0.66$
Lipid	$F_{3,35} = 0.83, p = 0.49$	$F_{2,35} = 0.33, p = 0.72$	$F_{6,35} = 0.71, p = 0.65$
Lipid classes			
Lysophosphatidylcholine	$F_{3,35} = 2.02, p = 0.14$	$F_{2,35} = 0.21, p = 0.82$	$F_{6,35} = 0.67, p = 0.68$
Sphingomyelin	$F_{3,35} = 0.96, p = 0.43$	$F_{2,35} = 0.20, p = 0.82$	$F_{6,35} = 0.58, p = 0.74$
Phosphatidylcholine	$F_{3,35} = 2.72, p = 0.07$	$F_{2,35} = 0.32, p = 0.73$	$F_{6,35} = 0.79, p = 0.59$
Phosphatidylserine ¹	F _{3,35} = 5.75 ^{**} , p < 0.01	$F_{2,35} = 0.33, p = 0.72$	$F_{6,35} = 1.76, p = 0.15$
Phosphatidylinositol	$F_{3,35} = 2.61, p = 0.08$	$F_{2,35} = 3.20, p = 0.06$	$F_{6,35} = 2.14, p = 0.09$
Phosphatidylglycerol ²	$F_{3,35} = 0.32, p = 0.81$	F _{2,35} = 6.31 ^{**} , p < 0.01	$F_{6,35} = 1.09, p = 0.40$
Phosphatidylethanolamine	$F_{3,35} = 2.09, p = 0.13$	$F_{2,35} = 1.61, p = 0.22$	$F_{6,35} = 2.15, p = 0.08$
UK _(a)	$F_{3,35} = 0.45, p = 0.72$	$F_{2,35} = 0.30, p = 0.74$	$F_{6,35} = 0.15, p = 0.99$
Total polar lipids ¹	$F_{3,35} = 4.04^*, p = 0.02$	$F_{2,35} = 2.41, p = 0.11$	$F_{6,35} = 1.88, p = 0.13$
Monoacylglycerols +			
Diacylglycerols	$F_{3,35} = 0.96, p = 0.43$	$F_{2,35} = 2.99, p = 0.07$	$F_{6,35} = 2.05, p = 0.10$
Cholesterol	$F_{3,35} = 1.02, p = 0.40$	$F_{2,35} = 0.07, p = 0.93$	$F_{6,35} = 1.58, p = 0.20$
Free Fatty Acids ¹	$F_{3,35} = 3.38^*, p = 0.04$	$F_{2,35} = 3.20, p = 0.06$	$F_{6,35} = 0.21, p = 0.97$
Triacylglycerols ^{1, 2, 1×2}	F _{3,35} = 17.07 ^{***} , p < 0.001	F _{2,35} = 6.94 ^{**} , p < 0.01	$F_{6,35} = 5.11^{**}, p < 0.01$
Sterol Esters ^{1×2}	$F_{3,35} = 2.76, p = 0.07$	$F_{2,35} = 2.64, p = 0.09$	$F_{6,35} = 2.61^*, p = 0.04$
$\mathrm{UK}_{\mathrm{(b)}}^{1 imes 2}$	$F_{3,35} = 0.71, p = 0.55$	$F_{2,35} = 3.08, p = 0.07$	$F_{6,35} = 2.66^*, p = 0.04$
Total neutral lipids ¹	$F_{3,35} = 4.04^*, p = 0.02$	$F_{2,35} = 2.41, p = 0.11$	$F_{6,35} = 1.88, p = 0.13$

654 Table 4. Summary of statistics for two-way ANOVA testing the effect of stage, female, and interaction 655 stage × female on the proximate and lipid class composition of larvae and post larvae Homarus gammarus.

656

657

 1 Significant effect of main factor stage.

 2 Significant effect of main factor female.

 $1\times^2$ Significant effect of interaction stage × female.

 658

659 UK, unknown.

Figure captions: 660



661

Fig. 1. Total activity of trypsin, amylase, and lipase for developmental stages of *Homarus gammarus*, obtained from
 whole-body homogenates (N=3). Data points represent each replicate. Activity expressed as Relative Fluorescence
 Units (RFU) per individual. Different letters "x, y" and "a, b, c" indicate significant differences between females and
 developmental stages, respectively.



666

Fig. 2. Changes in RNA concentration, DNA concentration, and standardized RNA/DNA ratio, sRD for developmental stages of *Homarus gammarus*, obtained from abdominal tissue homogenates (n=9). Bars represented as mean \pm SEM. Different letters "a, b" indicate significant differences between developmental stages.





Fig. 3. Principal component analysis (PCA) of biochemical composition for developmental stages of *Homarus gammarus*. The amounts (μ g / mg WW) of each lipid class, protein, and ash were used to form the principal components. Panel A clusters individuals per developmental stage and panel B per female. The PC1 separated the biochemical composition horizontally and explained 52.0% of the variance. The PC2 separated the variables vertically and explained 13.4% of the variance. The contribution of the variables (lipid classes, protein, and ash) is represented

by the arrows, and the stronger the correlation of a variable to PC1 or PC2 the longer the arrow is.