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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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Abstract

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

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

Declarations

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Conflicts of interest/ Competing interests

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

Availability of data and material

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

Code availability

Not applicable

Author's contributions

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., D.B.R., J.A.P., and I.L. All the authors contributed to the preparation of the manuscript.

Ethics approval

The study meets the ethical standards for the care and handling of invertebrate species in Europe.

Consent to participate

Not applicable.

Consent for publication

Not applicable.

Introduction

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

Like other nephropid lobsters, *H. gammarus* has a relatively short planktonic phase, going through 3 stages (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 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. 1990) and the development of complementary teeth in the gastric mill (Charmantier and Aiken 1991) appear 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 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 early development. This is also supported by dietary shifts documented for the American lobster, *Homarus*

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

In crustaceans, there is a correlation between diet and digestive enzymes produced. In general, carnivorous species produce a wide range of proteases at high concentrations and are, therefore, capable of hydrolyzing high levels of dietary protein (Jonhston and Yellowless 1998). In contrast, herbivores and omnivores synthesize a greater variety and amount of carbohydrases according to their increased capacity to hydrolyze plant and animal dietary carbohydrates (Jonhston and Yellowless 1998). In this sense, ontogenetic changes in enzymatic activity may be indicative of shifts in the ability to hydrolyze different dietary components (Rodriguez et al. 1994). This has been investigated in several decapod species including shrimps (Lovett and Felder 1990; Lemos et al. 1999, 2002; Ribeiro and Jones 2000; Díaz et al. 2008), crabs (Andrés et al. 2010), spiny lobsters (Johnston 2003; Perera et al. 2008), and homarid lobsters (Biesiot and Capuzzo 1990a). Protease, carbohydrase, and lipase or esterase activities have been detected in all refereed species from hatching, suggesting the capacity to hydrolyze different dietary components and to exploit a variety of nutrient sources to meet nutritional requirements. In general, data indicate a higher activity of enzymes involved in lipid digestion at earlier stages (Perera et al. 2008; Andrés et al. 2010) with an increase in amylase activity after metamorphosis (Lovett and Felder 1990; Ribeiro and Jones 2000; Johnston 2003). The pattern for protease activity during development was not as clear, varying considerably between crustacean species. In *H. americanus*, total enzyme activities generally increase during early development 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 where a range of proteases (trypsin, elastase, leucine aminopeptidase, and carboxypeptidase *a* and *b*) (Glass and Stark 1994) and carbohydrases (amylase, maltase, α and ß -glucosidases) (Glass and Stark 1995) activities have been detected in the hepatopancreas of wild-caught adult specimens. More recently, Goncalves et al. (2021) demonstrated that digestive enzyme activities (trypsin, amylase, and lipase) in juvenile *H. gammarus* (stages VII – VIII) are affected by the dietary composition. However, there is no reported information about the ontogenetic variation in digestive enzyme activity in *H. gammarus* larvae and postlarvae.

Studies on the biochemical changes during early development might be indicative of the type of energy source used during ontogeny, and therefore, could be a valid approach to estimate nutritional requirements at each stage of development. In the *H. americanus*, protein is considered the main source of energy but lipids and carbohydrates also have an important contribution to the energy yield through larval stages I to III (Sasaki et al. 1986). After metamorphosis into stage IV, the dependence on lipids as an energy substrate decreases (Sasaki et al. 1986). The RNA:DNA index has been previously used to evaluate the effect of nutrient limitation on the growth of *H. gammarus* larvae (Schoo et al. 2014). In the cited study, the authors observed that imbalances in dietary nitrogen and phosphorus caused a decrease in the RNA:DNA ratio. Lipid utilization during embryogenesis has been previously studied in *H. gammarus* and results showed that neutral lipids are the main energy source during embryonic development, while polar lipids are not catabolized and play mainly a structural role (Rosa et al. 2005). Additionally, a close relationship between the physiological condition of the broodstock and their reproductive success has been demonstrated for marine organisms including lobsters (Agnalt 2008; Moland et al. 2010). More specifically, it was demonstrated that the inclusion of specific phospholipids in formulated feeds improved the nutritional status of the broodstock, gonad development, and egg and larvae quality (Navas et al. 1997; Rodríguez-García et 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*.

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

Materials and Methods:

Larval rearing and sampling

Experiments were conducted at the aquaculture facilities at the National Institute of Aquatic Resources, DTU Aqua, Section for Aquaculture, Hirtshals (Denmark). Larvae were obtained from three ovigerous wild-caught *H. gammarus* females (A, B, and C) captured along the Skagerrak coast of North Jutland, Denmark. Newly hatched larvae were collected from broodstock tanks and transferred to 46-L cylindroconical transparent acrylic tanks. Larvae of different females were reared separately and stocked into tanks over three consecutive days after hatching at an initial density of 9-11 larvae L^{-1}. Tanks were part of a flow-through seawater system composed of a 10 m^3 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 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: temperature 19.6 ± 0.7 °C, salinity 34 ± 1 PSU, and dissolved oxygen > 90%. Larvae were subjected to an 8h:16h light:dark photoperiod cycle. Thawed Antarctic krill (*Euphausia superba*) (Akudim A/S, Denmark) 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 13 DAH onwards, 5 g $tank^{-1}$ day^{-1}. Total daily amounts were evenly distributed three times per day (9:00h; 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.

Digestive enzyme activities

Total enzyme activity was measured in three individual larvae and postlarvae per stage and female (1 individual from each of the 3 freeze-dried pools). Whole-body individuals were mechanically homogenized in ice-cold Milli-Q water, centrifuged (10 min at 15800 *g*) and the supernatant used to assay enzymatic activity. Amylase activity was determined with a commercial kit (Ultra Amylase Assay kit E33651, Thermo Scientific, USA). Trypsin and lipase were assayed using the methods of Rotllant et al. (2008) modified as described in Goncalves et al. (2021). All enzyme activities are expressed as RFU (Relative Fluorescence Units) per individual. Results are expressed as total enzyme activity per individual because homogenates 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.

Nucleic acids determination

RNA and DNA were quantified in the freeze-dried abdominal section of three individual lobsters (1 individual from each of the 3 freeze-dried pools) for each developmental stage and female following procedures described previously (Goncalves et al. 2021). Briefly, the abdominal samples were chemically (cold sarcosyl Tris-EDTA extraction buffer) and mechanically homogenized in an ultrasonic homogenizer unit (4710 Series, Cole Parmer Instruments Co., USA). The concentration of RNA and DNA was quantified in the supernatant extract in analytical duplicates. A specific nucleic acid fluorochrome dye GelRED was

used for the fluorescent reading at 365 nm (excitation) and 590 nm (emission). Following the first scan to 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 from a standard curve of DNA-GelRED with known concentrations of λ-phagus DNA (Roche, Switzerland). The RNA fluorescence was calculated by subtracting the DNA fluorescence (second scan) from total fluorescence (first scan) and the concentration determined using a standard curve of 16S-23S *E. coli* RNA (Roche, Switzerland). The average ratio of DNA and DNA slopes (average ± SD) was 5.86 ± 0.01. The RNA/DNA ratios were standardized (sRD) using DNA and RNA slope ratios and the reference slope ratio of 2.4 (Caldarone et al. 2006). The concentration of nucleic acids is expressed as μg of RNA or DNA per mg of abdominal tissue DW.

Proximate chemical composition

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

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

was separated on a 10 × 10 cm HPTLC plates (Merck KGaG, Germany) by high-performance thin-layer chromatography (HPTLC) in a single-dimensional double-development using propanol/chloroform/methyl acetate/methanol/0.25% potassium chloride (5:5:5:2:1.8, v/v) as developing solvent for polar lipid classes, and hexane/diethyl ether/acetic acid (20:5:0.5, v/v) for the neutral fractions. The different LC were visualized by charring at 160ºC 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, Switzerland). Data of each lipid class were than transformed into absolute amounts (μg / mg WW) taking into account the samples total lipid contents and wet weight.

Statistical analysis

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

Results

Female performance and larvae size

Broodstock size and spawning performance are summarized in Table 2. The three female breeders weighed 0.6 kg (♀B), 1.5 kg (♀A), and 1.7 kg (♀C). The largest female spawned over 15000 larvae during 23 days of the spawning period, while the other two females had equal spawning duration (18 days) with a similar number of spawned larvae (6000-8000). The mean individual dry weight and carapace length per 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 stage IV postlarvae. The size of larvae at each developmental stage was significantly affected by the broodstock. Thus, stage I and III larvae hatched from female C (1.7 kg) were the largest (DW and CL), followed by larvae from female A (1.5 kg), and ultimately those from female B (0.6 kg) in which, stage II and IV lobsters also presented the lowest DW and CL.

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.

Ontogenetic changes in trypsin were dependent on the broodstock as shown by the significant interaction stage × female ($F_{6,35}$ = 2.52, p = 0.049). No ontogenetic changes in trypsin activity were observed for lobsters hatched from female A (Fig. 1). However, the trypsin activity increased significantly from stage II to stage IV in individuals hatched from female B, and diminished from stage I to stage II in larvae from female C. Within stages, the trypsin activity was significantly higher for female C progeny than for female A progeny at stage I, while at stage IV, significantly higher trypsin was observed in the offspring from female B than from female C. Amylase activity was significantly affected by the main factors stage ($F_{3,35}$ = 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.

Nucleic acids

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

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.

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

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

principal components (PC) accounted for 65.4% of the variability (PC1 52.0% and PC2 13.4%). From all variables, the phospholipids PI, PS, and PE showed the highest loadings in the PC1 (-0.358, -0.352, and -0.351, respectively). Protein (+0.589), ash (-0.443), and the neutral lipid TAG (-0.411) showed the highest loadings in the PC2. In panel A, samples were clustered by developmental stage. Stage IV postlarvae formed a cluster to the bottom right area of the plot indicative of a positive correlation with ash and TAG 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 three different groups.

Discussion:

Female performance and larvae size

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

Digestive enzymes ontogenetic trends

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

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

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

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

Ontogenetic change in DNA and RNA content

The whole-animal concentrations of RNA and DNA in *H. gammarus* decreased gradually from stage I to IV suggesting that the overall metabolic activity decreased during larval development. The steeper decrease in DNA than in RNA points out to a higher dependence of early stages metabolism on cell multiplication rather than on cellular protein synthesis. That is because, while an increase in RNA reflects an increase in protein synthesis capacity, the increase in DNA is associated with an increase in the number of cells per tissue portion (Olivar et al. 2009). Despite the sharper decrease in DNA than in RNA content, no significant differences were found between stages based on the standardized RNA:DNA ratio – sRD. Results suggest that the cellular protein synthesis capacity was not affected throughout larval development. Laubier-Bonichon et al. (1977) examined the RNA and DNA concentrations in the prawn *Penaeus japonicus* and concluded that both the rates of cell multiplication and protein synthesis were at maximum levels during larval development, but then drop after the transition to postlarvae. Later, Lovett and Felder (1990)

suggested that the reduced metabolic activity of *Penaeus setiferus* during the critical postlarvae period after metamorphosis was associated with low digestive enzyme activity triggered by the limited nutrient uptake during the transformational period of morphogenesis in the gut. Several prawn and shrimp species, including the *P. japonicus* and *P. setiferus*, undergo complex modifications in their digestive system after metamorphosis. In particular, the anterior midgut caeca degenerates into the vestigial anterior midgut diverticulum (Lovett and Felder 1990). Although there is some development of the hepatopancreas with *H. gammarus* growth, particularly the increase in size and number of tubules within the hepatopancreas tissue (Biesiot and McDowell 1995), transformations during metamorphosis seem to be much less dramatic than in prawns and shrimps which can partially explain the lack of significant changes in sRD between development stages.

Biochemical composition

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

Phospholipid was the predominant lipid class while only traces of TAG (< 0.1%) were present in wild-caught pueruli of the spiny lobster *Jasus edwardsii* (Jeffs et al. 2001). In the same work, the authors showed that phospholipid reserves are primarily used during this important phase in the life cycle and that diacylglycerol plays a minor secondary role. Likewise, the major lipid classes identified in the early larval stages of the western rock lobster, *Panulirus cygnus*, were polar lipids followed by sterols (mainly cholesterol) (Liddy et al. 2004). The authors observed that polar lipid was the main lipid class catabolized by starved larvae whereas its content increased in fed larvae, with no significant changes in sterol content (Liddy et al. 2004).

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

Conclusions

In this study we demonstrated that the ontogenetic shifts of total enzyme activity are developmentally cued in *H. gammarus* larvae, pointing to a temporal genetic regulation. Dietary amylase becomes more important after metamorphosis, while lipids gradually gain importance during larval development until stage III but 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 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 metamorphosis and higher lipid content during larval stages. The phospholipids PC and PE and cholesterol were identified as the most abundant lipid classes in *H. gammarus* larvae and postlarvae suggesting high dietary requirements for these compounds. Although it was not the primary goal of this study, a parental influence was observed on size, digestive enzyme activity, and biochemical composition of *H. gammarus* larvae and postlarvae. Results suggest that, while often disregarded, the background of larvae used in nutritional studies might have a considerable impact on the results obtained. Therefore, we recommend that the effect of broodstock should be considered in future nutritional experiments performed on *H. gammarus* early stages.

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Tables:

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

Stage	I			II			III			IV		
Female	A	B	C	A	B	C	A	B	C	A	B	C
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

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	B	C	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^{a}	1.87 ± 0.05	$F_{2,59} = 88.76^{***}$
Stage II	2.77 ± 0.06^{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 ± 0.16^{c}	5.87 ± 0.15^{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 ± 0.04^{b}	4.16 ± 0.06^{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	$F_{2,59} = 19.70^{***}$

Values are means ± SEM of 20 replicates. Different superscript letters "a, b, c" within the same row indicate significant differences between females. *** $p < 0.001$.

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

Stage	I			**Stage I**	II			**Stage II**	III			**Stage III**	IV			**Stage IV**
Female	A	B	C	**mean**	A	B	C	**mean**	A	B	C	**mean**	A	B	C	**mean**
Proximate composition																
Dry matter	15.7 ± 0.3^{xy}	14.3 ± 0.3^{y}	16.8 ± 0.3^{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 ± 0.4^{x}	14.5 ± 0.3^{y}	17.5 ± 0.4^{a,x}	**16.4 ± 0.5**	18.0 ± 1.7^{x}	12.9 ± 0.6^{y}	13.5 ± 0.5^{b,y}	**14.8 ± 1.0**
Ash	20.4 ± 0.2^{b}	23.3 ± 1.1^{b}	21.0 ± 0.6^{bc}	**21.6 ± 0.6**	21.7 ± 1.1^{b}	24.0 ± 2.1^{ab}	24.9 ± 0.8^{b}	**23.5 ± 0.8**	21.9 ± 1.0^{b,x}	23.7 ± 0.6^{b,x}	17.3 ± 1.4^{c,y}	**21.0 ± 1.1**	28.6 ± 0.9^{a}	28.6 ± 1.4^{a}	29.8 ± 1.6^{a}	**29.0 ± 0.7**
Protein	33.0 ± 3.9	32.2 ± 1.8	29.6 ± 2.6	**31.6 ± 1.5^{A}**	33.9 ± 4.7	30.0 ± 5.7	27.7 ± 2.3	**30.5 ± 2.4^{AB}**	34.3 ± 1.6	26.3 ± 3.7	32.1 ± 0.9	**30.9 ± 1.7^{AB}**	23.1 ± 2.5	23.0 ± 1.5	25.8 ± 3.9	**24.0 ± 1.5^{B}**
Total lipid content	6.1 ± 1.6	4.4 ± 0.4	6.9 ± 1.6	**5.8 ± 0.8**	4.8 ± 0.6	5.1 ± 0.6	5.6 ± 0.7	**5.2 ± 0.4**	4.8 ± 1.3	5.9 ± 0.7	4.1 ± 1.1	**4.9 ± 0.6**	3.9 ± 0.3	4.2 ± 1.1	5.1 ± 1.7	**4.4 ± 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	**0.1 ± 0.0**
Sphingomyelin	2.0 ± 0.5	2.6 ± 0.7	1.8 ± 0.4	**2.1 ± 0.3**	2.5 ± 0.8	3.1 ± 0.9	2.8 ± 0.4	**2. 8 ± 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	**2.4 ± 0.4**
Phosphatidylcholine	20.4 ± 1.5	20.9 ± 1.0	20.0 ± 0.5	**20.4 ± 0.6**	22.9 ± 0.7	21.4 ± 2.1	21.1 ± 0.8	**21. 8 ± 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 ± 0.4	3.9 ± 0.1	**4.4 ± 0.2^{B}**	5.2 ± 0.2	5.1 ± 0.2	5.1 ± 0.4	**5.1 ± 0.1^{A}**	3.7 ± 0.3	4.0 ± 0.1	4.6 ± 0.2	**4.1 ± 0.2^{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	**4.1 ± 0.2**	4.5 ± 0.3	4.1 ± 0.3	4.7 ± 0.3	**4.5 ± 0.2**	3.7 ± 0.2	3. 9 ± 0.2	4.6 ± 0.3	**4.1 ± 0.2**	3.3 ± 0.2	4.0 ± 0.3	4.3 ± 0.2	**3.9 ± 0.2**
Phosphatidylglycerol	4.7 ± 0.9	4.6 ± 0.2	4.5 ± 0.4	**4.6 ± 0.3**	4.1 ± 1.0	4.8 ± 0.1	5.8 ± 0.4	**4.9 ± 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 ± 0.2^{y}	**4.7 ± 0.3**
Phosphatidylethanolamine	13.1 ± 0.7	13.9 ± 0.3	13.6 ± 0.9	**13.5 ± 0.4**	16. 6 ± 0.6	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	**0.8 ± 0.2**	1.1 ± 0.5	0.7 ± 0.4	1.0 ± 0.1	**1.0 ± 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 ± 1.2^{AB}**	56.9 ± 1.5	53.6 ± 3.1	55.1 ± 1.6	**55.2 ± 1.2^{A}**	46.2 ± 2.7	46. 1 ± 1.5	54.7 ± 4.5	**49.0 ± 2.1^{AB}**	42.5 ± 3.9	48.6 ± 3.2	53.4 ± 2.4	**48.2 ± 2.3^{B}**
Monoacylglycerols + Diacylglycerols	6.0 ± 0.7	3.1 ± 0.7	2.6 ± 0.6	**3.9 ± 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	**3.0 ± 0.5**
Cholesterol	22.0 ± 1.6	19.2 ± 0.7	21.0 ± 1.0	**20.7 ± 0.7**	22.5 ± 1.1	21.4 ± 1.0	18.8 ± 1.2	**20.9 ± 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	**20.0 ± 0.9**
Free Fatty Acids	8.7 ± 1.5	10.1 ± 0.5	7.4 ± 1.3	**8.7 ± 0.7^{AB}**	8.5 ± 1.6	11.9 ± 1.2	7.9 ± 1.5	**9.5 ± 1.0^{AB}**	10.7 ± 1.9	13.5 ± 0.8	10.1 ± 4.1	**11.4 ± 1.4^{A}**	6.6 ± 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	**8.8 ± 0.9**	4.4 ± 0.2^{b}	2.6 ± 0.7^{b}	5.1 ± 0.6	**4.1 ± 0.5**	17.9 ± 1.6^{a,x}	8.4 ± 1.5^{ab,y}	5.9 ± 1.2^{y}	**10.7 ± 2.0**	25.3 ± 4.3^{a,x}	14.9 ± 4.5^{a,y}	10.2 ± 3.3^{y}	**16.8 ± 3.0**
Sterol Esters	6.7 ± 1.0	5.5 ± 1.4^{ab}	5.7 ± 0.4	**5.9 ± 0.5**	3.2 ± 1.4^{y}	5.1 ± 2.9^{ab,xy}	7.9 ± 1.2^{x}	**5.4 ± 1.2**	3.4 ± 0.8^{y}	8.9 ± 0.9^{a,x}	4.3 ± 1.2^{y}	**5.5 ± 1.0**	2.6 ± 0.3	4.0 ± 0.3^{b}	3.4 ± 0.1	**3.3 ± 0.2**
UK$_{(b)}$	0.7 ± 0.7	2.3 ± 0.3	2.8 ± 0.9	**1.9 ± 0.5**	0.4 ± 0.4	1.9 ± 0.7	2.1 ± 0.4	**1.5 ± 0.4**	2.3 ± 0.5^{a}	0.0 ± 0.0^{b}	1.4 ± 1.1^{ab}	**1.2 ± 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 ± 1.1^{AB}**	43.1 ± 1.5	46.4 ± 3.1	44.9 ± 1.6	**44.8 ± 1.2^{B}**	53.8 ± 2.7	53.9 ± 1.5	45.3 ± 4.5	**51.0 ± 2.1^{AB}**	57.5 ± 3.9	51.4 ± 3.2	46.6 ± 2.4	**51.8 ± 2.3^{A}**

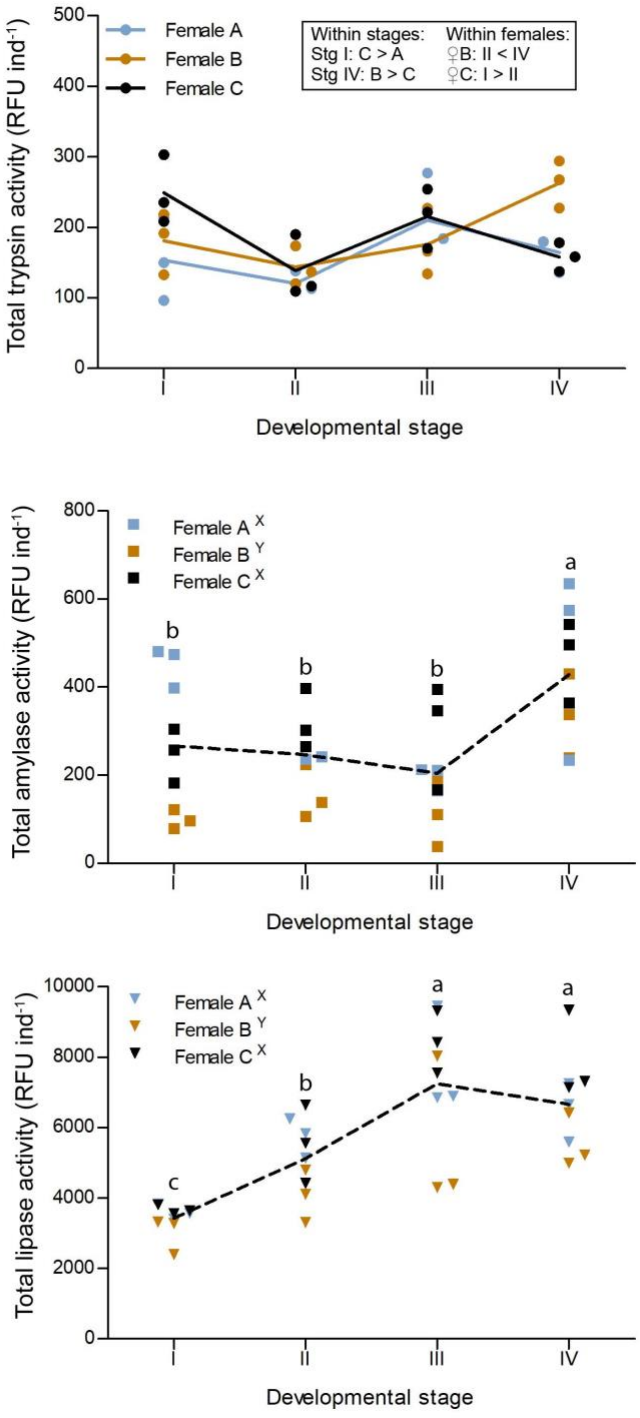
650 Values are means ± SEM of three replicates per treatment. UK, unknown.
651 Means in the same raw with a different superscript “x,y” are significantly different within the same stage.
652 Means in the same raw with a different superscript “a,b,c” are significantly different within the same female.
653 Overall stage means in the same raw with a different superscript “A,B,C” are significantly different

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

	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**
$UK_{(b)}$[1×2]	$F_{3,35}$ = 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

[1] Significant effect of main factor stage.
[2] Significant effect of main factor female.
[1×2] Significant effect of interaction stage × female.
UK, unknown.

Figure captions:



661

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

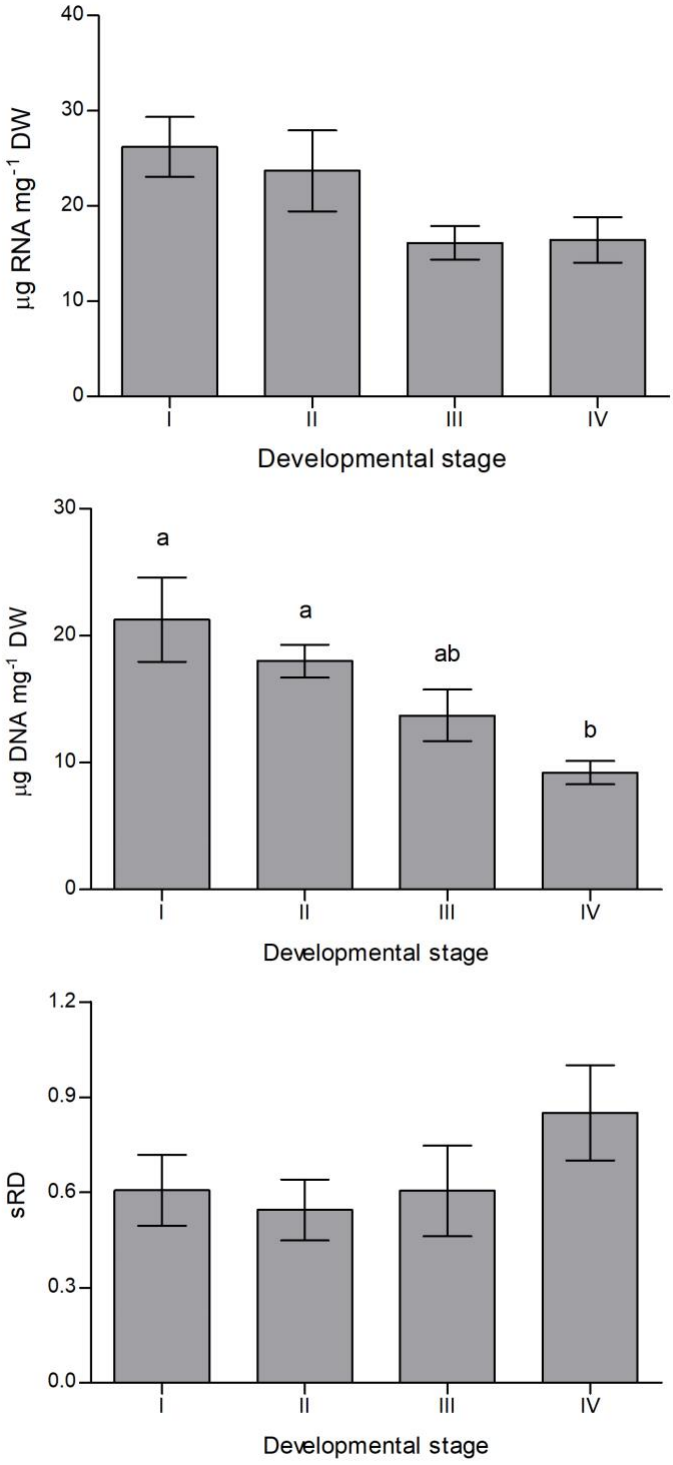


Fig. 2. Changes in RNA concentration, DNA concentration, and standardized RNA/DNA ratio, sRD for developmental stages of *Homarus gammarus*, obtained from abdominal tissue homogenates (n=9). Bars represented as mean ± SEM. Different letters "a, b" indicate significant differences between developmental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

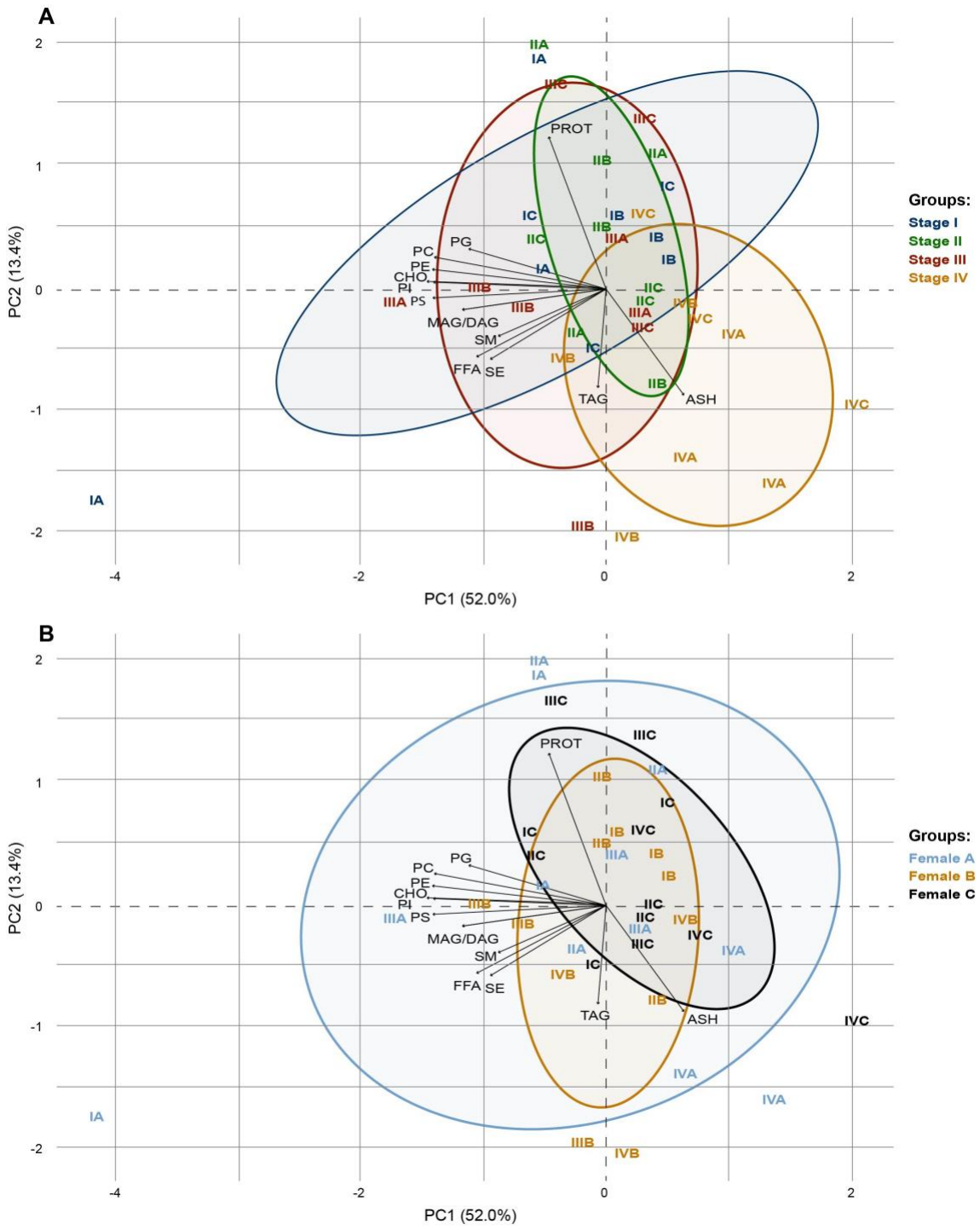


Fig. 3. Principal component analysis (PCA) of biochemical composition for developmental stages of *Homarus gammarus*. The amounts (μg / mg WW) of each lipid class, protein, and ash were used to form the principal components. Panel A clusters individuals per developmental stage and panel B per female. 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.