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Ontogenetic development and nutritional requirements in early life stages of the European lobster (*Homarus gammarus*, L.)

Renata Gonçalves

PhD thesis

ONTOGENETIC DEVELOPMENT AND NUTRITIONAL REQUIREMENTS IN EARLY LIFE STAGES OF THE EUROPEAN LOBSTER (*Homarus gammarus*, L.)

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Submitted in fulfilment of the requirements for the Degree of Doctor of Philosophy (Ph.D.)

Technical University of Denmark

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Section for Aquaculture

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Preface

The present dissertation was prepared to fulfil the requirements for obtaining the degree of Doctor of Philosophy (Ph.D.). After several years working as a research assistant in the field of aquaculture nutrition, pursue a Ph.D. in this area was a missing step in my academic career. I am grateful for the opportunity to qualify as a Ph.D. candidate at the Technical University of Denmark (DTU).

This thesis consists of a synopsis and five manuscripts. The research work presented in the manuscripts was conducted at the Section for Aquaculture under the National Institute of Aquatic Resources (DTU Aqua) between September 2019 and August 2021. Within the same period, two external research stays took place at the Centre of Marine Sciences (CCMAR), Portugal, and at the University of La Laguna (ULL), Tenerife, Spain. The Ph.D. study was part of two projects: (1) Restocking of lobster at stone reefs in the sea at North West Jutland funded by "ENV"-Fonden and FLAG and (2) Nordic Centre of Excellence for Sustainability and Resilient Aquatic Production (SUREAQUA) supported financially by NordForsk.

I want to thank my supervisors Ivar Lund and Manuel Gesto for their constant support and encouragement. I am grateful to Peter Vilhelm Skov who supported the supervision of two experimental works included in this thesis. I would also like to thank all my colleagues from the Section for Aquaculture, in particular to Ulla Sproegel and Brian Møller for assistance with laboratory analyses, and to Rasmus Jensen, Ole Larsen, and Jens Nedergaard for help with the maintenance of the lobster systems. To Kim Gregersen and Tilo Pfalzgraff for sharing experiences, both successes and concerns, during the journey as Ph.D. students. To all the staff at CCMAR and ULL, a special thanks for their friendly collaboration and hospitality during both external research stays. Finally, a heartfelt thanks to my family and friends back home, for supporting the decision to move to Denmark and for, despite the distance, always providing me with valuable time.

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List of publications

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2 – Goncalves, R., Lund, I., Gesto, M., Skov, P.V., 2020. The effect of dietary protein, lipid, and carbohydrate levels on the performance, metabolic rate, and nitrogen retention in juvenile European lobster (*Homarus gammarus*, L.). Aquaculture 525, 735334. https://doi.org/10.1016/j.aquaculture.2020.735334.

3 – Goncalves, R., Gesto, M., Teodósio, M.A., Baptista, V., Navarro-Guillén, C., Lund, I., 2021. Replacement of Antarctic krill (*Euphausia superba*) by extruded feeds with different proximate compositions: effects on growth, nutritional condition, and digestive capacity of juvenile European lobsters (*Homarus gammarus*, L.). Journal of Nutritional Science 10(e36), 1-15. doi:10.1017/jns.2021.27.

4 – Goncalves, R., Lund, I., Sousa, D., Skov, P.V., 2021. Shrimp waste meal (*Pandalus borealis*) as an alternative ingredient in diets for juvenile European lobster (*Homarus gammarus*, L.). Under review, Aquaculture (major revisions requested, 9/08/2021).

5 – Goncalves, R., Lund, I., Gesto, M., 2021. Interactions of temperature and dietary composition on juvenile European lobster (*Homarus gammarus*, L.) energy metabolism and performance. Comparative Biochemistry and Physiology, Part A 260, 111019. https://doi.org/10.1016/j.cbpa.2021.111019.

Summary

The European lobster, *Homarus gammarus*, is an ecological and economically important species. Historically, it has been subjected to intense fishing pressure that caused the decline of several wild populations. To counteract the decrease in annual landings various stock enhancement programs have been launched around Europe. These initiatives are supported by hatchery-reared juvenile lobsters that are released into natural habitats. The commercial cultivation of the species is also attracting considerable attention and *H. gammarus* is currently considered an emerging aquaculture species. However, the economic viability of European lobster hatcheries is hampered by several drawbacks including low survival and growth rates, the strong cannibalistic nature of the species, and the dependence on live, fresh, and frozen diets.

The main purpose of this Ph.D. project was to increase knowledge on the metabolism and nutritional requirements of *H. gammarus* early stages and thus provide solutions to nutritional challenges faced by hatchery production units. Specific goals were: (1) to identify ontogenetic changes in the digestive capacity and potential nutritional requirements of larvae and postlarvae; (2) screen macronutrient levels in the design of formulated feeds for early juvenile stages; (3) assess the potential of alternative and more sustainable ingredients in formulated feeds for juvenile lobsters; and (4) evaluate the effect of dietary composition on the resilience of juvenile *H. gammarus* to environmental variation.

For that, five studies were conducted. In the first study, the digestive enzymatic activity (trypsin, amylase, and lipase) and biochemical indices (RNA:DNA ratios, proximate and lipid class composition) were measured in stages I, II, and III larvae, and stage IV postlarvae. Main results showed that lipase activity increased from stage I to stage III but not further, suggesting the increasing importance of lipids during the first three larval stages. Amylase activity increased significantly at stage IV pointing to a shift towards a carbohydrate-richer diet after metamorphosis. Cholesterol and phospholipids (phosphatidylcholine and phosphatidylethanolamine) were the most abundant lipid classes in larvae and postlarvae body tissues suggesting high dietary requirements for these compounds.

The second study examined the impact of formulated feeds on the metabolic cost of feeding (specific dynamic action, SDA), basal metabolism (standard metabolic rate, SMR), and nitrogen retention in juvenile *H. gammarus*. The metabolic cost of digesting and assimilating extruded feeds was comparable to that of digesting and assimilating a standard diet composed of thawed Antarctic krill. However, the basal metabolism and nitrogen retention decreased significantly in juveniles fed low protein (40%) and medium (1:2) to high (1:1) lipid : carbohydrate ratios likely caused by the low intake of these feeds. The higher level of carbohydrates (1:3 ratio) in low protein feeds (40%) enhanced feed intake, and consequently, the metabolic capacity and nitrogen retention. The basal metabolism and

nitrogen retention in juveniles fed feeds with high protein content (50%) was comparable to that of lobsters fed Antarctic krill, regardless of the lipid : carbohydrate ratio.

In study 3, the performance of juvenile *H. gammarus* fed formulated feeds was compared to the performance of lobsters fed Antarctic krill during 8-weeks. Growth, survival, and nutritional condition (evaluated through RNA:DNA ratio) were assessed and results showed that the most suitable experimental formulated feed was the 500MED with a macronutrient combination of 50% protein, 24% carbohydrates, and 12% lipids. Juveniles reared on this feed performed as well as those reared on Antarctic krill in all the performance indices. Additionally, among the experimental formulated feeds, the 500MED yielded the most efficient feed conversion ratio (FCR).

Study 4 tested the inclusion of shrimp waste meal (SWM) as an alternative protein source in formulated feeds for early juvenile *H. gammarus*. Growth, survival, nitrogen metabolism, and exoskeleton colouration were evaluated. The highest inclusion level of SWM (28% of dietary protein) did not affect growth nor nitrogen metabolism and enhanced survival of juvenile lobsters demonstrating its potential as an alternative ingredient. The highest SWM inclusion level was insufficient to improve exoskeleton colouration which was hampered by the lower, than initially projected, astaxanthin level in the alternative ingredient.

In the last study (study 5), the interaction effect between temperature and dietary composition on the energy metabolism and performance of juvenile lobster was evaluated. Juveniles fed with extruded feeds with either a high-protein (HP) or a high-carbohydrate (HC) content and thawed Antarctic krill (AK) were exposed to low temperatures of 13°C. Corresponding control groups for each diet were maintained at optimal rearing temperatures of 19°C. Lower temperatures induced a decrease in feed intake in all dietary groups but results showed that *H. gammarus* juveniles fed the HC feed were the least resilient to temperature variation. Results also disclosed that juvenile lobsters adapted their energy metabolism according to the diet received.

In conclusion, the results from this thesis provide new insights into the metabolism and nutritional requirements of the early stages of *H. gammarus*. Results demonstrate that protein is a key nutrient for all stages examined, while lipids are of particular importance during larval development and dietary carbohydrate requirements increase after metamorphosis. The potential protein-sparing effect of carbohydrates is of great interest and should be further studied. Lipid sources of richer phospholipid content might improve *H. gammarus* lipid digestion and assimilation and, therefore, its inclusion in feeds for *H. gammarus* early stages deserves further investigation.

Resumé

Den europæiske hummer, (*Homarus gammarus*), er en vigtig art i havenes økosystemer og har ligeledes en væsentlig økonomisk betydning. Historisk, har arten været genstand for intensivt fiskeri, som har forårsaget tilbagegang i mange populationer. For at forhindre tilbagegangen er der i Europa iværksat forskellige genudsætningsprogrammer med udsætning af hummeryngel på naturlige habitater men klækket og opdrættet i akvakultur. Kommerciel produktion af H. gammarus i akvakultur har pådraget sig stigende interesse og arten betragtes som en mulig akvakultur kandidat, dog er den økonomiske bæredygtighed herfor udfordret af en generel lav overlevelse, - en lav vækstrate, - artens høje tilbøjelighed til kannibalisme og ynglens afhængighed af enten levende – frisk eller frossent foder.

Hovedformålet i dette Ph.D projekt var at øge viden om såvel stofskifte – og ernæringsmæssige krav i de tidlige stadier af H. gammarus yngel og bidrage til løsninger på ernæringsmæssige udfordringer i opdræts produktions enheder. Specifikke mål var; (1) at identificere ontogenetiske ændringer i fordøjelses kapaciteten og potentielle ernæringsmæssige krav hos larver og post-larver; (2) at screene for optimale niveauer af makro-næringsstoffer med henblik på at kunne formulere foder til hummer yngel; (3) at undersøge potentialet af alternative og mere bæredygtige ingredienser i formulerede fodertyper til hummeryngel samt; (4) at evaluere indflydelse af foderets sammensætning på robusthed af juvenile hummeryngel, når udsat for miljømæssige ændringer.

For at undersøge ovenstående blev der foretaget 5 eksperimentalle undersøgelser. I den første undersøgelse blev larver i såvel stadie I, II og III og postlarver (stadie IV) analyseret for enzymatisk fordøjelses aktivitet (trypsin, amylase og lipase) samt biokemiske indikatorer (proximat sammensætning, RNA:DNA, og lipid klasse sammensætning). Overordnet viste resultaterne, at lipase aktiviteten steg fra stadie I til stadie III, men ikke yderligere til stadie IV, hvilket indikerede en stigende betydning af lipider gennem de første 3 larvestadier. Amylase aktiviteten steg signifikant for stadie IV postlarver, hvilket tyder på en øget betydning af kulhydrat-holdige diæter efter metamorfosen. Kolesterol – og fosfolipider (fosfatidylcholine og fosfatidylethanolamine) var de mest hyppigt forekommende lipid- klasser i væv hos larver og post-larver og peger på en væsentlig betydning af et højt indhold af disse lipid typer i foderet.

I undersøgelse nr 2 blev der testet indvirkning af fodring med tilvirkede (ekstruderede) fodertyper på juvenile hummeres stofskifte omkostninger herunder basal stofskifte (i.e. standard metabolic rate), og SMR specific dynamic action, SDA), samt deres evne til at tilbageholde kvælstof (nitrogen). Resultaterne viste, at metaboliske omkostninger til at fordøje og assimilere næringsstoffer for ekstruderede fodertyper var sammenlignelige med optøede Krill (lyskrebs) som blev benyttet som reference foder. Ikke desto mindre var basalstofskiftet og tilbageholdelse af nitrogen signifikant lavere for juvenile hummere fodret med et lavt indhold af protein (40%) og et samtidigt medium (1:2) - til højt

(1:1) lipid : kulhydrat forhold, som sandsynligvis skyldes det relative lave foderindtag for disse fodertyper. Et højere indtag af kulhydrater (1:3) i fodertyper med lavt protein indhold (40%) øgede foderindtaget og dermed de juvenile hummeres stofskifte kapacitet og deres tilbageholdelse af nitrogen. Basalstofskiftet og nitrogen tilbageholdelse i juvenile hummere fodret diæter med et højt proteinindhold (50%) var sammenligneligt med juvenile hummere fodret med Krill, uanset lipid : kulhydrat forholdet.

I undersøgelse nr 3, blev juvenile hummere (*H. gammarus*) fodret med ekstruderede fodertyper og deres performance over 8 uger sammenlignet med yngel fodret med Krill. Vækst, overlevelse og ernæringsmæssig kondition (RNA:DNA) blev undersøgt. Resultaterne viste, at den bedste ekstruderede fodertype var 500Med, (i.e. 50 % protein, 24 % kulhydrat og 12 % lipid). Juvenile yngel fodret med denne fodertype performede på alle parametre tilsvarende til yngel fodret med Krill. Yderligere viste 500MED at have den mest effektive foderkonvertering (FCR) af de ekstruderede fodertyper.

I undersøgelse 4 blev undersøgt, hvorvidt tilvirket mel af rejeaffald (Shrimp waste meal, SWM) er en mulig alternativ proteinkilde i formulerede fodertyper til tidlige juvenile hummer yngel (*H. gammarus*). Vækst, overlevelse og nitrogen stofskifte samt indvirkning på hummernes farve blev undersøgt. Den højeste inklusion af SWM (28%) havde ingen effekt på vækst eller nitrogen stofskifte, men bevirkede en højere overlevelse, og dermed SWMs mulige potentiale som alternativ råvare. Derimod opnåedes ingen gavnlig effekt på farven (exoskelettet), hvilket sandsynligvis var forårsaget af et lavere end forudset indhold af pigmentet astaxanthin i SWM råvaren.

I den sidste undersøgelse nr. 5 blev undersøgt samspillet mellem opdræts temperatur (13 °C vs 19°C); foderets sammensætning og indvirkning på energi stofskifte og performance hos juvenile yngel (*H. gam*marus). Juvenile yngel blev fodret med formulerede ekstruderede fodertyper med enten et højt protein indhold (HP), et højt kulhydratindhold (HC) eller optøede Krill og alle eksponeret til en lav temperatur på 13° C (tilsvarende referencekoder ved 19°C). Denne lavere temperatur forårsagede et lavere foderindtag for alle grupper, men viste, at yngel fodret med HC fodertypen var mindst påvirkede af temperatur ændringen. Resultaterne viste også, at den givne fodertype havde betydning for hummernes regulering af energi stofskiftet.

Som konklusion har resultaterne i denne afhandling givet ny viden og indblik i stofskifte processer og ernæringsmæssige krav hos tidlige stadier af *H. gammarus* yngel. Resultaterne viser, at protein er en nøgle komponent for alle de undersøgte stadier. Vigtigheden af lipider er højest i hummerens pelagiske larve stadier, mens krav til indhold af kulhydrat er højest efter metamorfosen. En mulig potentiel protein- sparende effekt af kulhydrat er en vigtig parameter og bør undersøges nøjere fremadrettet. Lipid kilder med et højt indhold af forfolipider sandsynliggør en højere lipid fordøjelighed og anvendelsen i fodertyper, udnyttelse og fysiologiske indvirkninger fordrer ligeledes nærmere undersøgelser.

SYNOPSIS

Early development, nutrition, and challenges in the cultivation of *Homarus gammarus*

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1. INTRODUCTION

The European lobster, *Homarus gammarus*, supports one of the most valuable commercial fisheries in the North Atlantic region (Nicosia and Lavalli, 1999). Variation in the annual landings of several natural stocks led to the implementation of stock enhancement programs that depend on the production of hatchery-reared juveniles to be released into natural habitats (Ellis et al., 2015). The high marketability of *H. gammarus* has attracted interest and is currently considered an emerging species for aquaculture (Drengstig and Bergheim, 2013).

Despite its economic potential, further development of *H. gammarus* aquaculture industry is hampered by high mortality rates that are particularly critical during larval development (Powell et al., 2017), low growth rates (Mente et al., 2001), and the dependence on live, fresh, or frozen diets (Hinchcliffe et al., 2020). In comparison to its counterpart the American lobster, *Homarus americanus*, the *H. gammarus* has received less attention over the years. However, owing to morphological, physiological, and behavioural similarities between both species, improvements to *H. gammarus* hatchery practices have been made largely based on previous knowledge acquired from research developed on the *H. americanus* (Nicosia and Lavalli, 1999). In any case, further research is required for the commercial viability of both *Homarus* species aquaculture industries.

Although poorly understood, the specific nutrient requirements of different larval stages and ontogenetic development in digestive capacity are among the most important aspects for the hatchery production of this species. Such information is of considerable importance for designing effective rearing and feeding protocols for the cultivation of larval stages (Johnston, 2003). Another major issue hampering the viability of *H. gammarus* commercial production is the lack of a suitable formulated feed capable of sustain, at least, comparable growth rates as conventional fresh or frozen diets (Powell, 2016). The effective transition from fresh or frozen diets to formulated feeds could support a more sustainable production by directly reducing feed costs, simplifying feed handling and storage, and offering a more consistent nutritional quality (Powell et al., 2017).

In this context, this Ph.D. project relates to two major challenges for the successful establishment of *H. gammarus* production: (1) improve the low survival rates observed in communal larval rearing systems and, (2) reduce the high costs associated with feeding during the cultivation of juvenile stages. To this end, experimental studies conducted within this thesis were directed to the early life stages of *H. gammarus*.

2. RESEARCH OBJECTIVES AND THESIS STRUCTURE

The main objective of this Ph.D. project was to advance the knowledge on the metabolism and nutritional requirements of *H. gammarus* larvae, postlarvae, and early juvenile stages. Such information can help answering research questions in relation to lobster early stages physiology and nutritional requirements. Additionally, it could, for instance, serve as a basis for the development of suitable formulated feeds specifically designed for early juvenile stages. In order to address the main objective, the following specific goals were defined:

i. Identify ontogenetic changes in the digestive capacity and nutritional requirements of larvae and postlarvae.
    a. Study 1: "Early ontogenetic changes in digestive enzyme activity and biochemical indices of the European lobster (*Homarus gammarus*, L.)".

ii. Screen dietary macronutrient requirements and understand the effect of feed composition on the metabolism of early juvenile stages using formulated extruded feeds.
    a. Study 2: "The effect of dietary protein, lipid, and carbohydrate levels on the performance, metabolic rate and nitrogen retention in juvenile European lobster (*Homarus gammarus*, L.)".
    b. Study 3: "Replacement of Antarctic krill (*Euphausia superba*) by extruded feeds with different proximate compositions: effects on growth, nutritional condition, and digestive capacity of juvenile European lobsters (*Homarus gammarus*, L.)".

iii. Investigate the feasibility of using alternative and more sustainable ingredients in the formulation of artificial feeds for early juvenile stages.
    a. Study 4: "Shrimp waste meal (*Pandalus borealis*) as an alternative ingredient in diets for juvenile European lobster (*Homarus gammarus,* L.)".

iv. Explore the effect of dietary composition on the resilience to environmental variation in early juvenile lobsters.
    a. Study 5: "Interactions of temperature and dietary composition on juvenile European lobster (*Homarus gammarus*, L.) energy metabolism and performance".

To adress these goals, the digestive enzyme activity (trypsin, amylase, lipase) and biochemical indices (RNA:DNA, proximate and lipid class composition) were assessed in *H. gammarus* larvae (stages I, II, III) and postlarvae (stage IV) to elucidate the potential role of ontogeny on their ability to digest and assimilate protein, carbohydrates, and lipids. Some of the issues related to the development of formulated feeds were addressed by performing different experiments with early juvenile *H. gammarus*. Different types of formulated feeds (semi-moist and extruded pellets) and the inclusion of an alternative ingredient (shrimp waste meal), a potential source of feeding attractant compounds (free-amino acids, nucleotides), were tested to explore possible improvements on feed intake. A study

targeting to evaluate the effect of formulated feeds composition on the specific dynamic action (SDA) of *H. gammarus* was conducted to assess their impact on the digestion process and appetite revival. The impact of formulated feed composition on digestive enzyme activities was evaluated. Experimental work comparing the energy metabolism and nitrogen excretion rates in *H. gammarus* juveniles fed a standard frozen diet (Antarctic krill) and formulated feeds of different compositions was performed to better understand the assimilation of the different types of food.

This thesis consists of a synopsis and five manuscripts. The synopsis summarizes the contribution of the main results from this Ph.D. project to the current state of the art on the topic and identifies areas where further studies are necessary for the improvement of European lobster production. The five manuscripts report and discuss the results obtained in each of the five studies conducted during the thesis.

3. HISTORY AND IMPORTANCE OF *HOMARUS GAMMARUS*

The European lobster, *Homarus gammarus*, is an ecological and economically important species distributed along the northeast Atlantic coast from Norway to Morocco and the Azores, including the northern coast of the Mediterranean and the Black sea (Nicosia and Lavalli, 1999). Together with its counterpart the American lobster, *Homarus. americanus*, they are the only representatives of the genus *Homarus* within the Family Nephropidae. Both support valuable commercial fisheries, even though there is a large difference in commercial catches between the two species. Global landings of *H. gammarus* have been historically lower than that of *H. americanus*, and currently, the difference became even more pronounced. The most recent available data indicates a 32-fold magnitude difference in the commercial catch of the American and European lobster (FIGURE 1. Panel A).

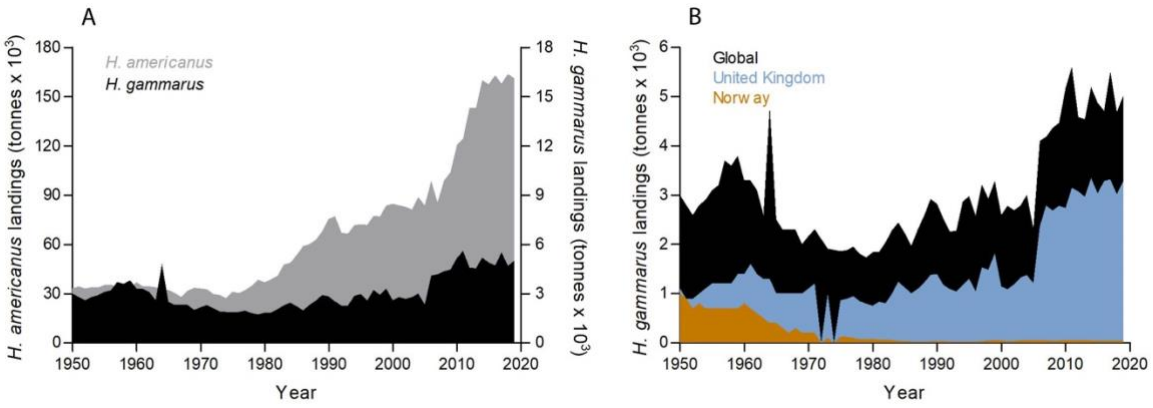

FIGURE 1. Commercial catch of the two *Homarus* species between 1950 and 2019 (panel A) and *Homarus gammarus* global landings, in the United Kingdom, and in Norway (panel B). Data from FishStatJ (FAO, 2021).

The global demand exceeding supply and high-market price for *H. gammarus* led to intense fishing efforts, in particular during the 1960s and 1970s, causing a decrease in the overall commercial catch

(FIGURE 1. Panel B). The implementation of protective measures in European lobster fisheries (e.g. closed seasons, trap limitations, minimum legal size, and the prohibition on possession of ovigerous females) was insufficient to prevent the decline of several wild populations (Kleiven et al., 2019; Whale et al. 2013). Norway was particularly affected – annual landings declined from ~ 800 to ~ 80 tonnes during the period between 1960 and 1980 and since then catches remain below 80 tonnes (FIGURE 1. Panel B). To counteract the decreasing trend in annual landings, multiple experimental stock-enhancement programs were launched across Europe including France (Latrouite and Lorec, 1991), the United Kingdom (Cook, 1995), Ireland (Browne and Mercer, 1998), Norway (Agnalt, 2004), and Germany (Schmalenbach et al., 2011). Since 2004, the global catch of the European lobster has increased considerably but almost exclusively due to the increase in landings in the United Kingdom (FIGURE 1. Panel B). In other areas, there is still no sign of stock recovery, despite the establishment of initiatives to enlarge the European lobster population (FAO, 2021).

4. DEVELOPMENT ASPECTS IN *HOMARUS* LOBSTERS

Knowledge about behavioural, morphological, and physiological developmental changes during ontogeny is necessary to design adequate rearing and feeding strategies. In the following sections, general aspects of the development and growth in homarid lobsters will be discussed with a special focus on the development of the digestive system.

4.1. MATURATION, FECUNDITY, AND HATCHING

The size at which *H. gammarus* females reach maturity ranges between 80 mm and 140 mm carapace length (Tully et al., 2001). Size at maturity in male lobsters is less evident because it is difficult to distinguish between gonadal and functional maturity. Mating usually occurs between a soft-shelled female and a hard-shelled male. The eggs are carried underneath the female abdomen for up to 12 months (Richards and Wickins, 1979). In general, *Homarus* females produce a clutch of eggs every second year, with moulting and spawning occurring in alternate years. However, temperature and age affect the reproductive cycle . For example, small *H. americanus* females inhabiting warmer water can moult and spawn in the same summer (Comeau and Savoie, 2002) and larger, older *Homarus* females (> 120 mm carapace length, CL) are able spawn in two consecutive years and moult in the third (Agnalt et al., 2007; Waddy et al., 1995).

In total, females can carry up to 15 clutches in a lifetime. A female that lives out its full reproductive life cycle might produce a total of 750000 eggs (Cobb and Castro, 2006). The number of eggs carried by a female (clutch size, CS) varies with the size of the individual. A relationship between carapace length (CL) and clutch size has been established for wild *H. gammarus* as CS = 0.0044 ×

$CL^{3.16}$ (Tully et al., 2001). However, egg loss during incubation can cause a significant reduction in the clutch size. For example, 15% to 50% egg mass loss was reported for *H. americanus* females (Savoie and Maynard, 1991). The main causes for egg mass loss are egg predators, fungal infection, and human handling of the ovigerous females (Cobb and Castro, 2006).

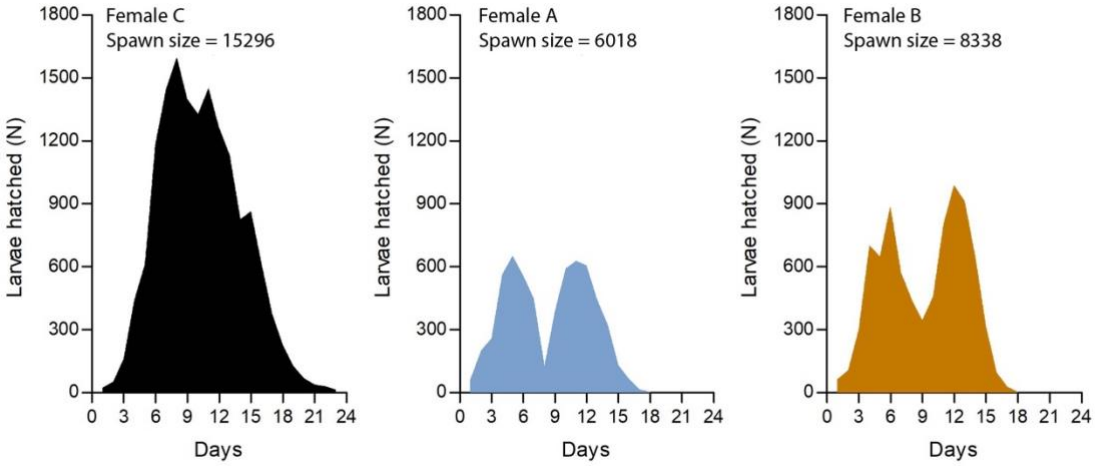

FIGURE 2. Spawning curve profile of three wild-caught *Homarus gammarus* females.

Within this thesis, the daily amount of larvae spawned was followed in three females of different sizes (study 1). Spawning curve profiles of each female are presented in FIGURE 2. All the three females spawned between July and August 2019. Results showed that spawning duration ranged from 18 to 23 days. The total number of larvae hatched varied greatly among females – from 6018 to 15296.

TABLE 1. Female weight, length, spawn size, estimated clutch size and egg loss in three wild-caught *Homarus gammarus* females held in captivity for approximately one month before spawning.

	♀C	♀A	♀B
Live weight	1700 g	1500 g	600 g
Carapace length	150 mm	143 mm	100 mm
Spawn size	15296	6018	8338
Clutch size (estimated)	33106	28465	9193
Egg loss (estimated)	54%	79%	9%

Maternal size alone could not explain the variation in spawn size. Genetic and condition-related effects are also important factors to be considered. Egg mass loss per female was extrapolated by applying the above-mentioned formula from Tully et al. (2001) to estimate the clutch size for each female based on their size (TABLE 1). Results suggest that egg mass loss can vary widely within females held in captivity (from 9% to 79%). However, without a more accurate estimation of egg mass in a larger number of females, the conclusions that can be drawn from these data are limited. A differential loss in egg mass during handling could not be ruled out.

4.2. LARVAL DEVELOPMENT

The larval development period in *H. gammarus* is relatively short. Individuals emerge from the egg as a prelarva that remains within the maternal pleopods for a few hours until moulting into stage I (Nicosia and Lavalli, 1999). As soon as prelarvae moult, individuals swim to the surface (Richards, 1979) maintaining the pelagic behaviour for the first three larval stages (Rötzer and Haug, 2015). The entire larval development can last from 10 days to 2 months, depending on external factors among which temperature has the main influence (Waddy et al., 1995). Within a temperature interval ranging from 20°C to 22°C, the larval development takes approximately 12 days (Rötzer and Haug, 2015). Other factors affecting larval development include water quality, salinity, light intensity and photoperiod, social interaction, food availability and quality (Beard et al., 1985).

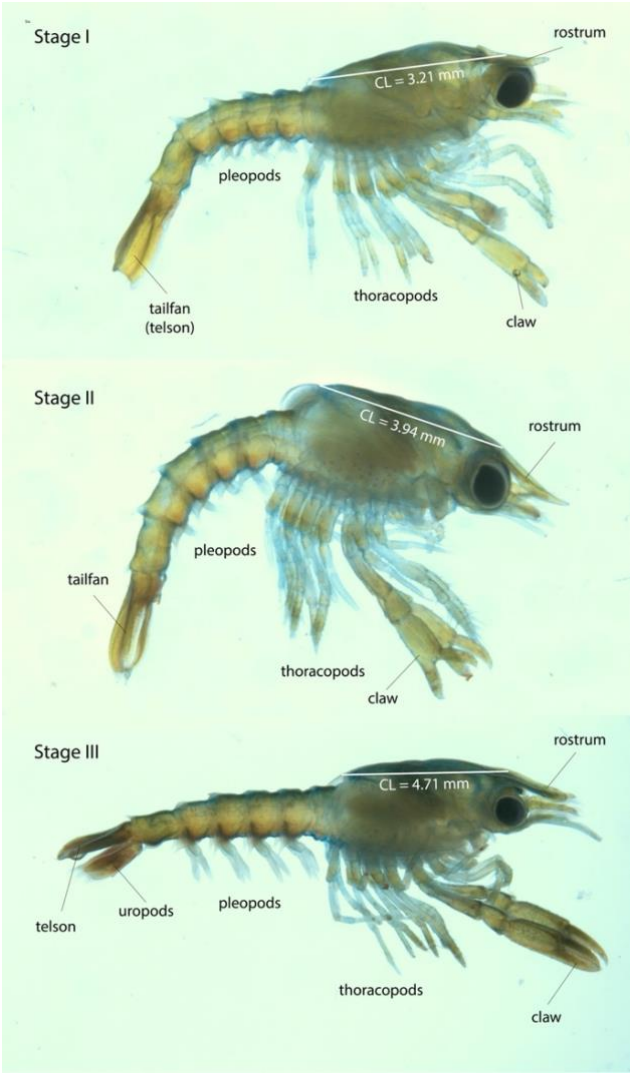

FIGURE 3. Larval stages (I to III) of *Homarus gammarus* in lateral view. The image scale differs within stages, for comparison note the carapace length measurement.
Larvae photographed under a stereomicroscope (MC125 C, Leica) equipped with a digital camera (MC190 HD, Leica) by the author.

In general, the larval size increases from stage to stage (I to III) but external morphology does not undergo dramatic changes as *H. gammarus* larvae develop (FIGURE 3). Main morphological

changes occur in the tailfan, rostrum, and appendages. No uropods are present in the tailfan of stages I and II, only the telson. In stage III larvae, the uropods develop with long setae. The rostrum develops from stage II to stage III, with stage III larvae presenting spines at its front. The antenna length increases slightly from stage II to stage III. Thoracopod and pleopod appendages enlarge in width and length, in particular, the distal parts forming the claws (Rötzer and Haug, 2015).

By the fourth stage, larvae metamorphosis into a postlarvae (FIGURE 4). The transition from stage III to IV is characterized by a marked change in morphology and behaviour in a short amount of time (Rötzer and Haug, 2015). At stage IV, the antenna length increases significantly. The chemoreceptive setae located in the antennules develop triggering the capacity to chemolocate food (Conklin, 1995). Stage IV *H. gammarus* develop functional pleopods and shift the function of swimming from the thoracic (thoracopods) to the abdominal (pleopods) appendages (Charmantier and Aiken, 1991). It is also at the metamorphosis that the lobsters move from the surface to the bottom changing from a planktonic to a benthic lifestyle (Rötzer and Haug, 2015) which is only fully established by stage V (Charmantier and Aiken, 1991).

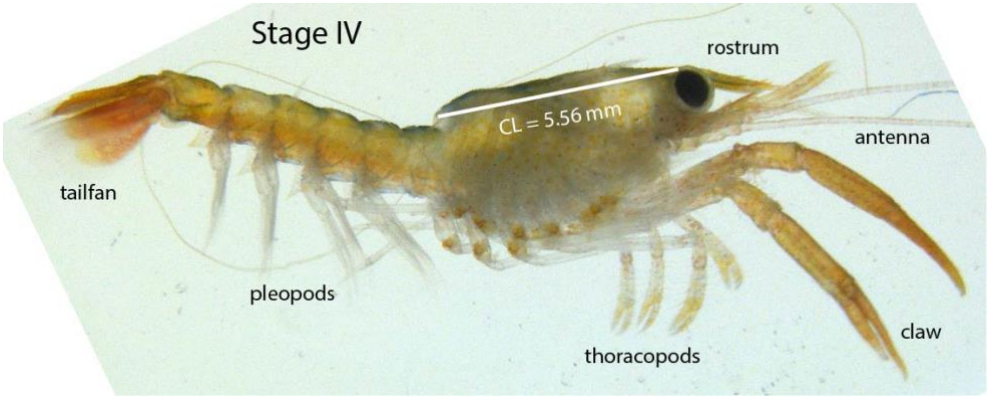

FIGURE 4. *Homarus gammarus* postlarvae stage IV in lateral view.
Postlarva photographed under a stereomicroscope (MC125 C, Leica) equipped with a digital camera (MC190 HD, Leica) by the author.

4.3. GROWTH AND MOULT

Unlike most other groups of animals, the growth of crustaceans, including the lobster *H. gammarus*, is not continuous. Changes in total and carapace length occur exclusively at ecdysis (FIGURE 5). Wet weight changes follow a basic pattern through the moult cycle with fast increases associated with rapid water uptake at ecdysis; further moderate gains associated with carapace mineralization and tissue growth in the postmoult period, and relative stabilization of fresh weight during the intermoult period until the onset of the successive ecdysis (Nguyen et al., 2014). The growth rate in crustacean species is, therefore, dependent on the intermoult period (time between moults) and the moult increment (increase in size after moulting) (FIGURE 5). The combination of extended intermoult periods and reduced moult increment result in decreased growth rates and vice versa (Nguyen et al., 2014).

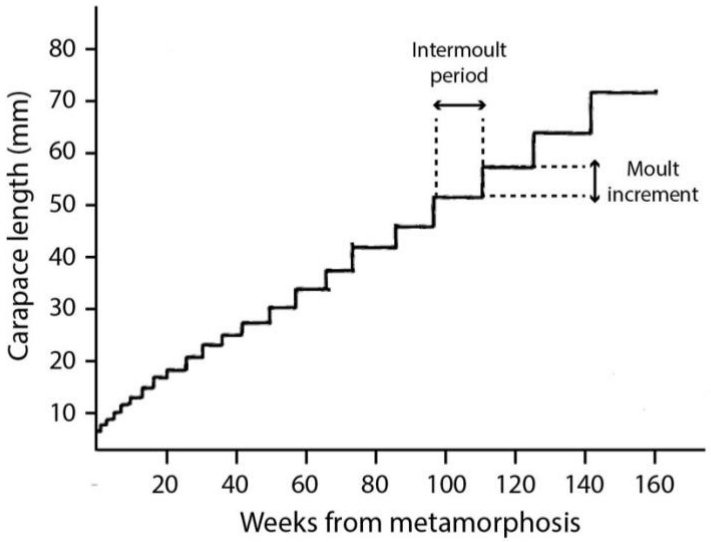

FIGURE 5. Individual growth diagram for cultured *Homarus gammarus* (Adapted from Richards and Wickins (1979)).

The moult cycle (FIGURE 6) comprises four periods (postmoult, intermoult, premoult, and ecdysis) divided into a sequence of five stages (A – E) and several substages during which the old exoskeleton separates from the epidermis while a new cuticle is synthesized. The postmoult period starts immediately after ecdysis and continues with the formation of the new cuticle. It is composed of stages A, B, and early stage C (C_1 to C_3). Stage C_4 represents the intermoult period and, at this stage, lobsters present a consolidated and hardened cuticle. The premoult period consists of stage D (D_0 to D_3) and starts with the separation of the cuticle from the epidermis (apolysis) followed by the development of a new cuticle underneath the old one. Stage E refers to the actual act of shedding the old exoskeleton (ecdysis) (Comeau and Savoie, 2001). The moulting stages can be determined roughly by the thickness of the carapace, or more accurately, by morphological changes in the epidermis and pleopods (Waddy et al., 1995).

The moult process is regulated by the endocrine system through the combined action of several hormones (FIGURE 6). During postmoult and intermoult, glands of the eyestalk produce two hormones – the moulting inhibiting hormone (MIH) and the crustacean hyperglycaemic hormone (CHH) – that regulate growth by suppressing the production of ecdysteroids in the Y-organs, located in the cephalothorax (Chang et al., 2001). By the end of the intermoult period, the concentration of MIH drops while the methyl farnesoate (MF) hormone rises, inducing the production of ecdysteroids in the Y-organs. This activates a complex physiological process that starts with premoult and ends in ecdysis (Chang et al., 2001). At the end of ecdysis, the levels of ecdysteroids and MF in hemolymph decrease (Chang et al., 2001).

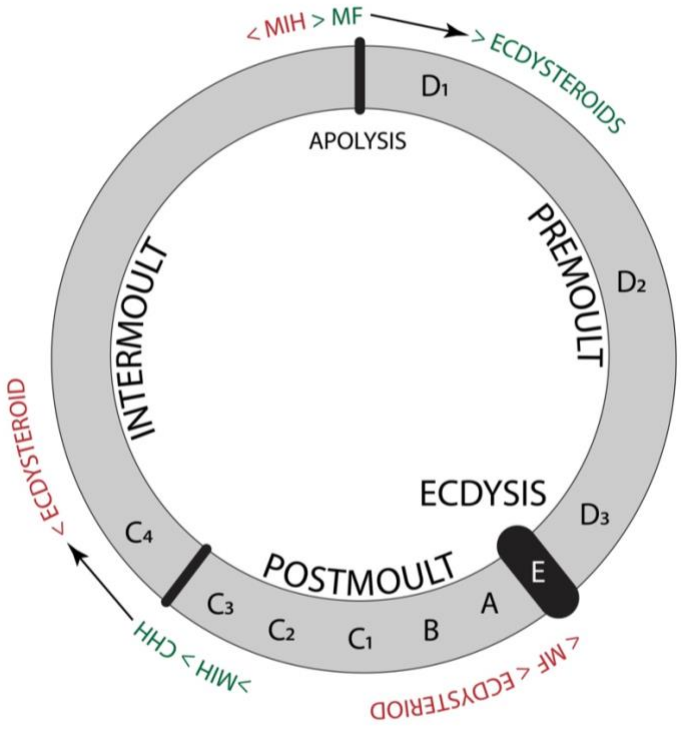

FIGURE 6. The moult cycle of *Homarus* lobster showing the moulting stage (A to E) and moulting phase (ecdysis, post-, inter-, and premoult). The hormones involved in the regulation of the moult cycle are shown adjacent to the moult stages where they were up (green) or down (red) regulated. MIH = moulting inhibiting hormone; CHH = crustacean hyperglycaemic hormone; MF = methyl farnesoate.

In captivity, under standard rearing conditions, *H. gammarus* live weight was reported to increase by up to 50% and the carapace length between 10% to 25% relative to the preceding moult (Richards and Wickins, 1979). Growth depends on both endogenous (genetics) and exogenous factors (Waddy et al., 1995). Among these factors, temperature is particularly important, but nutrition, appendages injury and age also play a significant role in growth. Generally, younger individuals moult more frequently than older lobsters. That is because the amount of energy invested in growth during the larval stages is greater compared to later stages (Wahle and Fogarty, 2006). Autotomy (regeneration of mutilated appendages) represents an energetic cost that could be, otherwise, invested in growth. In this way, injury, such as appendages loss, slows growth by decreasing moult increment (Cobb and Castro, 2006). *Homarus* growth enhancement is proportional to temperature increase within the thermal interval from 8°C to 25°C (Wahle and Fogarty, 2006). Temperatures above 25°C are physiological stressful and potentially lethal, whereas temperatures below 5°C inhibit the moulting process (Waddy et al., 1995).

The effect of low temperature on growth was assessed for *H. gammarus* in study 5. For that, early juvenile *H. gammarus* fed three different diets were exposed for 24 days to low temperatures (13°C) and their growth performance compared to a control group (maintained at 19°C), also divided into three dietary groups. Results showed that feed intake decreased at low temperatures for all dietary treatments. However, growth performance evaluated through specific growth rate (SGR) and

percentage of moult occurrence was significantly reduced only in the group of animals fed a high-carbohydrate extruded feed. Juveniles fed a high-protein feed or a standard diet composed of thawed Antarctic krill did not reduce significantly their growth in comparison to the respective dietary groups reared at 19°C. These findings demonstrated that the magnitude of the temperature effect can be modulated through the diet. The effect of diet composition and type on growth will be further discussed in sections 6 and 7.

4.4. DEVELOPMENT OF THE DIGESTIVE SYSTEM

The digestive system of *Homarus* lobster is divided into three major regions – foregut, midgut, and hindgut. The foregut comprises the mouth and the esophagus, the cardiac stomach, and the pyloric stomach. The midgut is composed of the intestine and three associated organs – hepatopancreas, anterior midgut caeca, and posterior midgut caecum. The hindgut comprises the rectum and the anus. Both the hindgut and foregut are coated with a thin layer of chitin that is continuous with the exoskeleton cuticle and shed through the mouth and the anus at each moult (Factor, 1995). The esophagus and the anus are equipped with tegumental glands responsible for secreting a lubricant mucus that facilitate the passage of food in both the esophagus and the rectum, and binding the feces in the rectum (Barker, 1977).

The foregut is relatively well developed in stage I larvae and, besides the obvious increase in size, most changes take place in the gastric mill (Factor, 1995). Rudimental gastric mill teeth first appear in the third larval stage but only in stage IV has the gastric meal a full complement of well-developed teeth (Factor, 1995). Some minor changes take place in the mouthparts where delicate larval teeth develop into heavier structures by stage IV (Factor, 1995).

The midgut is well developed from stage I larvae and is similar to those found in adults (Factor, 1995). Minor changes occur in the anterior midgut caeca and posterior midgut caecum. The anterior midgut caeca are more developed in larval stages I-III than in postlarval stage IV suggesting a reduction of these structures during development. Conversely, stage I larvae have no posterior midgut caecum which appears completely developed by stage III (Factor, 1995). The general form of the hepatopancreas in stage I is similar to that of the later stages but its complexity increases significantly until stage IV (Factor, 1995). In particular, the yolk is still present in the hepatopancreas of stage I larvae, and the number of tubules increases from stage I until stage IV (Biesiot and McDowell, 1995). All the different hepatopancreatic cells – E-, F-, B-, and R-cells – are present from the first stage. However, larval stages R-cells have fewer lipid vacuoles (maximum two, from resorbed yolk lipids) and R-cells do not attain the adult morphology before stage VI (Sasaki et al., 1986).

No major changes occur in the hindgut (rectum and anus) which is generally similar to those found in adult lobsters (Factor, 1995). The described changes in the morphology of the digestive system

coincide with the transition from planktonic to benthic life at stage IV and are likely to facilitate the gradual shift from a planktonic to a benthic diet.

The passage of food particles through the esophagus is facilitated by mucus secretions from the tegumental glands located in the walls of the esophagus. Larval lobster (stage I to III) does not possess a well-developed gastric meal and, therefore, much of the grinding of the food is accomplished externally using the mandibles (Lavalli and Factor, 1992). This likely limits the type of food larvae can use and should be considered in the development of artificial feeds for larvae. After metamorphosis into the fourth stage, postlarvae use the maxillipeds and mandibles to tear food into small pieces (Factor, 1995).

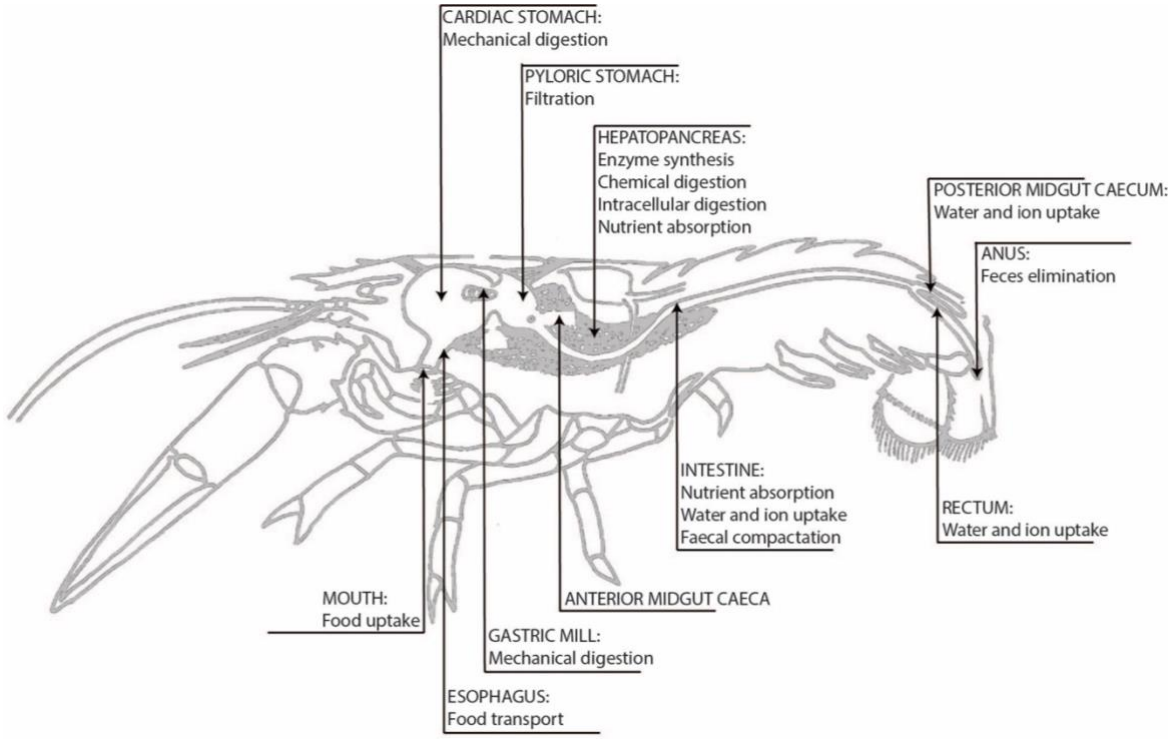

FIGURE 7. Digestive tract anatomy and function in homarid lobsters. (Adapted from Factor, 1995 and Conklin, 1995).

The chemical breakdown of food does not start until it enters the cardiac stomach as there is no evidence of digestive enzyme production by the oesophageal tegumental glands (Conklin, 1995). From stage IV onwards, upon entry in the cardiac chamber, ingested food particles are ground into fine particles by the action of the gastric meal (Conklin, 1995). Simultaneously, the food particles are combined with digestive enzymes secreted by the hepatopancreas (Conklin, 1995). Channel structures at the base of the cardiac stomach retain larger particles allowing only the finely grounded to pass through the pyloric stomach and subsequently to the hepatopancreas (Conklin, 1995).

The final phase of digestion and most the nutrient absorption occurs in the hepatopancreas (Conklin, 1995). This gland comprises four different types of cells: E cell (embryonic cell); R cells

(resorptive cells); F cells (fibrillar cells); and B cells (blister-like cells) (Biesiot and McDowell, 1995). Each cell type has a distinct function in the hepatopancreas. E cells mature into R and F cells; F-cells are responsible for the synthesis of digestive enzymes and subsequential differentiation into B-cells; B-cells secrete digestive enzymes into the hepatopancreas lumen; R-cells participate in the absorption of digested nutrients, intracellular digestion, and storage of lipid and glycogen reserves (Barker and Gibson, 1977; Biesiot and McDowell, 1995).

The undigested fraction from the hepatopancreas returns to the intestine to be combined with food particles that were retained at the base of the cardiac stomach (Conklin, 1995). The residual material is compacted into feces that pass through the intestine to the hindgut for eventual elimination (Conklin, 1995). As feces move through the intestine, water and ions are absorbed across the epithelial borders (Conklin, 1995).

A variety of proteases (trypsin, chymotrypsin, elastase, α- and β-carboxypeptidases, leucine aminopeptidase) have been identified in adult *H. americanus* (Brockerhoff et al., 1970) and *H. gammarus* (Glass and Stark, 1994) but the contribution of each specific enzyme in protein hydrolysis remains unclear. One of the identified proteases in both species was trypsin, which has been reported as the main protease for decapod crustaceans, accounting for 40% to 60% of total protease activity (Lemos et al., 1999). The lipase identified in the gastric fluid of adult homarid lobsters was determined to hydrolyse lipids into fatty acids, diglycerides, and monoglycerides (Brockerhoff et al., 1970). A range of carbohydrases (α-amylase, maltase, α and β-glucosidases, β-glucanase, α- and β-galactosidases) were detected in the hepatopancreas of adult *H. gammarus* (Glass and Stark, 1995). Chitin was found to be poorly digested by adult *H. gammarus* (Glass and Stark, 1995) but chitinase and chitobiase enzymes were identified in the digestive fluid of *H. americanus* (Lynn, 1990). In other decapod crustaceans, in particular, the shrimp *Penaeus setiferus* (Hood and Meyers, 1977) the hydrolyses of chitin occur through a combined action of endogenous and bacterial production of chitinase and chitobiase enzymes. Whether a similar chitin digestion process occurs in homarid lobsters it is not known as the microbiological contribution to digestive enzymes is yet to be determined. Overall, the enzymatic activity in *H. americanus* larvae tends to be constant during the day, most likely due to continuous ingestion of small amounts of food (Biesiot and Capuzzo, 1990a). On the contrary, adult *H. gammarus* undergo a cyclic secretory burst of enzymatic activity stimulated by feed intake reflecting the large intermittent meals of adults (Barker and Gibson, 1977).

In this thesis, trypsin, lipase, and amylase activities were detected in stage VII to VIII *H. gammarus* juveniles (study 3), stage I to III larvae, and stage IV postlarvae (study 1). Together, results showed that *H. gammarus* possess a wide range of digestive enzymes through their ontogeny, including the first stages, suggesting they can digest a wide variety of food items from early developmental stages. According to Biesiot and Capuzzo (1990a), there is an ontogenetic increase in digestive enzyme activities in *H. americanus* larvae. In the case of *H. gammarus*, the early ontogenetic development in

digestive enzyme activities differs, to some extent, from that described for *H. americanus*. In the first study of this thesis, it was determined that there is no clear trend for trypsin activity during *H. gammarus* larvae development and that the variation in trypsin through early development was affected by the broodstock. As for lipase, it was identified an increase in activity from stage I to III but not from stage III to IV. No changes were detected in amylase activity until metamorphosis. It was only at the fourth stage that a significant increase in amylase activity occurred. Together, results presented in study 1 and study 3 suggest that protein plays an important role in larvae, postlarvae, and early juvenile stages. Results point to an increased importance of lipids in the metabolism of larval stages while dietary carbohydrates become more important after metamorphosis.

5. PRESENT STATUS OF *HOMARUS GAMMARUS* PRODUCTION

The first *H. gammarus* hatcheries were built in France and Norway more than 150 years ago (Herrick, 1909). In the beginning, hatcheries aimed for hatching eggs and release early larvae to support stock-enhancement programs. However, the programs showed little measurable impact on wild production because of heavy mortality from the predation of larvae during the first weeks of planktonic life (Herrick, 1909). So, a new strategy was implemented aiming at the rearing of juveniles for longer periods before release. This new approach showed positive and encouraging results in the United Kingdom where a study using stage XII micro tagged *H. gammarus* juveniles demonstrated that released lobsters were able to grow to a commercial size and reproduce in the wild (Bannister et al., 1994) Another successful example was observed in Norway. Releasing cultured juveniles during 5 consecutive years led to a steady increase in commercial catches with released lobsters accounting for 50% to 60% of the landings (Agnalt, 2004).

At present, the culture of *H. gammarus* operates at a modest scale and is mainly targeted to support stock enhancement programs (Ellis et al., 2015). Commercial farming aiming the full grow-out of lobsters for consumption is gaining interest as an additional approach to increase market supply (Daniels et al., 2015; Drengstig and Bergheim, 2013). Developments have been made to improve lobster farming using sea-based culture container systems (Daniels et al., 2015; Halswell et al., 2018) and by employing advanced robotic systems in recirculating aquaculture systems (RAS) (Drengstig and Bergheim, 2013). Sea-based culture containers systems offer the advantages of lower energy costs, zero feed costs, and lobster environmental enrichment due to continuous exposure to natural variation in abiotic factors. Environmental enrichment is particularly advantageous for individuals reared to be released as part of stock enhancement programs (Daniels et al., 2015). However, sea-based container culture systems are currently restricted to the cultivation of juvenile stages. On the other hand, land-based farming can cover the production of all life stages. It allows higher control over feeding, harvesting, and disease, and the maintenance of constant optimal conditions for growth (Drengstig and Bergheim, 2013) but the

economic viability of intensive farming in RAS is not yet proved. Presently, most of *H. gammarus* production is accomplished in land-based farming units. Standard *H. gammarus* cultivation practices have been adapted from the experience with *H. americanus* culture. That is because available literature on the former species far exceeds that available for the *H. gammarus*. Moreover, the two species share many morphological, physiological, and behavioural similarities. Standard cultivation practices are described next.

5.1. REARING SYSTEMS AND STRATEGIES

As the life cycle under captivity is not yet closed, the supply of larvae depends on wild females. Female spawning schedules can be manipulated by temperature with warmer temperatures stimulating earlier spawns and colder temperatures retarding the time of hatching (Charmantier and Mounet-Guillaume, 1992). The fastest embryonic development is observed at 20 - 22°C whereas embryonic development retardation requires temperatures of 1°C to 3°C (Aiken, 1995). Spawning manipulation via temperature facilitates the supply of larvae year-round. Ovigerous females are maintained in individual tanks with sufficient area and water depth to moult and spawn and excessive handling or disturbance are avoided to minimize egg mass losses (Aiken, 1995).

After hatching, larvae are collected from the maternal tanks and stocked in larval rearing tanks. Significant age difference increases cannibalism, therefore, each tank is stocked with larvae hatched over a period of no more than three days (Richards and Wickins, 1979). The design of the tanks must ensure the maintenance of larvae in uniform suspension to reduce the risk of injury and cannibalism (Richards and Wickins, 1979). This is accomplished by supplying the tanks with bottom water inlet flow or strong aeration (Daniels et al., 2013; Middlemiss et al., 2015; Powell et al., 2017). Poor water quality promotes the proliferation of bacteria and fungus which increases mortality (Scolding et al., 2012). Flow-through systems with good water quality or RAS with effective water treatment and sterilization equipment are commonly used (Middlemiss et al., 2015; Scolding et al., 2012). Temperature is the primary factor regulating growth with moulting frequency increasing proportionally with temperate within the thermal interval of 6°C to 24°C (Aiken, 1995). Optimal rearing temperature and salinity range between 18°C to 22°C and 26 to 33 PSU, respectively (Richards and Wickins, 1979).

Larvae remain under these conditions until they reach stage IV (Beard et al., 1985) and begin the transition from pelagic to benthic life, which is fully established in stage V (Charmantier and Aiken, 1991). After metamorphosis to the fourth stage, lobsters are removed from communal rearing tanks and transferred to individual containers to minimize losses due to cannibalism. One important aspect to consider is the floor area of the individual chamber as it has a significant effect on growth. A minimum acceptable floor area of two times the total length (TL) – TL^2 – was estimated to produce 85% to 90% of the maximum yield in *H. gammarus* (Richards and Wickins, 1979). For *H. americanus*, the floor area

required to avoid significant growth reduction has been estimated at 15 × CL^2 (Aiken, 1995). The maintenance of individual chambers is expensive, labour intensive, and demands a large amount of space (Aiken, 1995) but due to their aggressive behaviour, homarid lobsters require to be kept isolated until harvest size is reached. Recently, a multi-layered tray RAS system – Aquahive® (Ocean on land, Northamptonshire, UK) – has been developed and demonstrated relative success in the rearing of postlarvae and early juvenile lobsters (Thorarinsdottir et al., 2017). The main advantage is that, in comparison to traditional mono-layer trayed-based systems, the Aquahive system operates in considerably smaller spaces.

5.2. CURRENT FEEDING PRACTICES

Homarid lobsters are opportunistic scavengers and are generally classified as omnivorous or carnivorous, feeding in a variety of planktonic and benthic organisms in their natural habitats. The type and amount of prey consumed depend on the developmental stage, temperature, prey availability, and moult cycle (Cobb and Castro, 2006). Stomach content analysis of *Homarus* lobster larvae showed that the natural diet is composed of copepods, diatoms, gastropods, and decapod larvae. The size of the prey increases as the larvae grow ranging from 210 μm to 610 μm and, at stage III, larvae preferentially consumed more decapod zoea and megalops larvae than copepods, diatoms, or gastropods (Nicosia and Lavalli, 1999). Newly settle and emergent juveniles of *H. americanus* were described to feed mainly on mesoplankton that can be found in suspension in their shelter habitats (Conklin, 1995). Early juvenile *H. gammarus* below ~10 cm total length are rarely observed in the wild and, therefore, little is known of their natural diet or feeding habits (Richards and Wickins, 1979). Mussels, crabs, polychaetes, and sea urchins were consistently identified and principal prey organisms in foraging juveniles and adults of homarid lobsters (Conklin, 1995). The dietary changes during the development of wild lobsters reflect different feeding strategies and also suggest changes in nutritional requirements during their ontogeny.

In captivity, lobsters are fed a variety of live, fresh, or frozen foods, including macroalgae, marine fish, molluscs, and other crustaceans. These types of foods produce satisfactory survival, growth, and normal carapace colouration (Floreto et al., 2001; Tlusty, 2005). However, standard fresh or frozen food items used in lobster hatcheries are expensive and prone to variations in supply and quality. Their optimal preservation and storage are also of major concern as it requires large cooling facilities. At a commercial scale, the use of standard fresh and frozen foods would be impractical.

The most widely used diet for larval *H. americanus* is live nauplii and adult brine shrimp (*Artemia* sp). Both have been shown to support reasonably good growth and survival during the three larval stages (Conklin, 1995). Live adult brine shrimp also serves postlarvae and early juvenile stages through the first three months after metamorphosis (Conklin, 1995). After the early benthic period,

Artemia no longer supports maximum growth presumably because energy spent to capture small prey exceeds the energy obtained from intake (Aiken, 1995). From this point onwards, juveniles and adult *H. americanus* are typically fed small shrimp and krill, or chopped mussels and squid (Conklin, 1995). Beyond nauplii and adult *Artemia*, frozen mysids are also used for rearing *H. gammarus* larvae. Comparatively to nauplii and adult brine shrimp *Artemia*, frozen mysids provide a more practical alternative, albeit at the expense of reduced survival (Richards and Wickins, 1979). After metamorphosis to stage IV, *H. gammarus* are usually fed with frozen mysids, chopped mussels, and shrimp (Richards and Wickins, 1979).

6. NUTRIENT REQUIREMENTS

The formulation of an efficient artificial feed for the culture of *H. gammarus*, like any other species, requires a comprehensible understanding of their nutritional requirements. This information coupled with data of the proximate composition of potential ingredients allows the formulation of nutritionally balanced feeds for adequate growth and survival of the animals. Knowledge on the nutrient requirements for *H. gammarus* species is sparse and, therefore, the larger amount of information available for *H. americanus* and for spiny lobster (Palinuridae) species can be useful references. Protein, carbohydrate, lipid, and micronutrient requirements are discussed in the following sections.

6.1. PROTEIN

Protein has an important role in promoting growth in crustacean species as it is the primary source of amino acids, which are essential building blocks (Mente, 2006). Insufficient dietary protein often leads to prolonged intermoult periods and decreased survival rates (Mente, 2006). On the other hand, excess protein may result in water deterioration from the degradation of protein surplus that is mostly excreted as total ammonia nitrogen (Aaqillah-Amr et al., 2021). Reported optimum protein levels for homarid lobsters reared on formulated feeds vary widely – from 30% to 60% (Conklin, 1995). The high variability among the results may be partially influenced by different experimental conditions, but protein quality and non-protein energy level in the feeds are likely to be the most significant factors (Conklin, 1995).

In this thesis, six extruded feeds were formulated to contain two protein levels (400 and 500 g kg^{-1}) with three lipid : carbohydrate ratios (LOW – 1:3, MED – 1:2, HIGH – 1:1) and used to screen appropriate macronutrient levels in feeds for *H. gammarus* juveniles. All six feeds were tested in studies 2 and 3. The 400LOW and 500LOW feeds were used in study 5, in which they are referred to as HP (500LOW) and HC (400LOW). Details on ingredient and proximate composition of the formulated feeds are provided in the respective papers. In general, the performance of juvenile *H. gammarus* was

higher with 50% crude protein content (500- feeds) than with 40% crude protein (400- feeds) (studies 2 and 3). However, within the three 500- feeds tested, the performance of the juvenile lobsters varied significantly according to the lipid : carbohydrate ratios in each feed. The best performance, even comparable to that obtained with the control diet composed of thawed Antarctic krill, was obtained with the 500MED formulated feed (study 3).

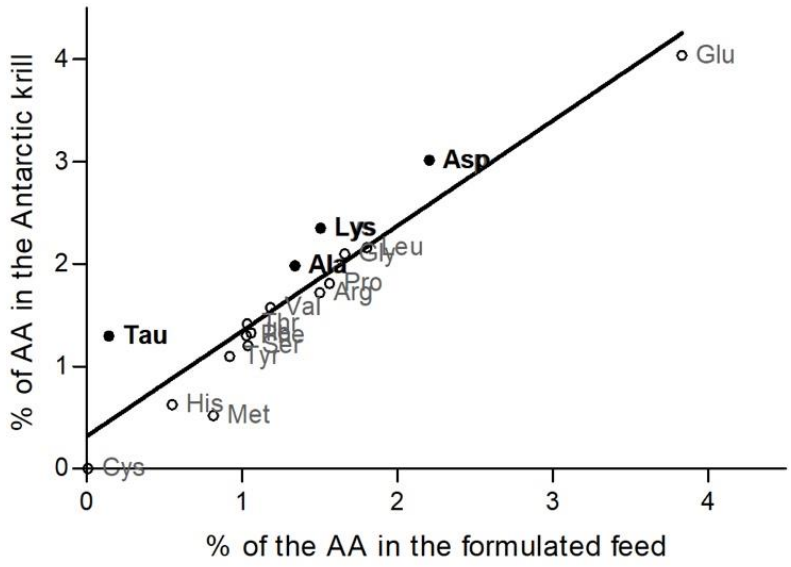

FIGURE 8. Amino acid profile of the control diet (Antarctic krill) and the best experimental formulated feed tested (500MED). Each amino acid percentage is expressed as % of the total protein in the 500MED feed and in the Antarctic krill diet. The line represent the best-fit.

Apart from total protein, it is also important to consider the amino acid requirements to satisfy lobster needs for good growth performance. Essential amino acids for *H. gammarus* are the same 10 typically required by other crustaceans – arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine (Mente et al., 2001). Methionine is particularly important for decapod species because it improves growth performance and feed conversion (Aaqillah-Amr et al., 2021). Additionally, some amino acids act as feed stimulants affecting the feeding behavioural response in decapods through chemical stimuli. The stimulus is triggered when there is sufficient leaching of attractant compounds from the feed after immersion in the water (Heinen, 1980). Taurine and glycine have been identified among the chemicals the elicit the greatest feed-attractant stimulus in lobsters, together with nucleotide adenosine 5-triphosphate and betaine (Tolomei et al., 2003; Williams et al., 2005).

The amino acid profile was analysed in the formulated feeds and Antarctic krill diet used during this thesis. Comparing the amino acid profile between the control diet (Antarctic krill) and the 500MED formulated feed (FIGURE 8), it was identified a shortage in taurine, lysine, asparagine, and alanine in the most suitable formulated feed tested. The requirements for alanine and asparagine in juvenile *H.*

gammarus are high (Mente et al., 2001). Taurine, as previously mentioned, acts as an important feed-attractant compound. The supplementation of formulated feeds with these amino acids could further improve the growth and survival rates of the juvenile lobsters.

Because protein is the most expensive component in formulated feeds, efforts have been directed to the use of other energy sources, aiming at the sparing of protein for growth (Aaqillah-Amr et al., 2021). However, inappropriate levels of non-protein energy components in the diet may result in inefficient use of protein. Excessive non-protein energy can limit feed intake and, consequently, decrease protein intake and limiting the growth potential. On the other hand, in case of insufficient non-protein energy, protein is partially used to make up the energy deficit, also reducing the growth potential (Cuzon and Guillaume, 1997). The protein-sparing potential for carbohydrates is discussed in more detail in the next section.

6.2. CARBOHYDRATES

Carbohydrates constitute the primary source of energy for crustaceans (Jimenez and Kinsey, 2015). The surplus of carbohydrates is accumulated in the hepatopancreas in the form of glycogen (Wang et al., 2016). During periods of limited feed intake, stored carbohydrates are the first energy reserves to be depleted, followed by lipids and proteins (Wang et al., 2016). Carbohydrates also have a fundamental role in the moulting process – glycogen reserves are used as precursors of chitin synthesis that, in turn, will be used in the formation of the new exoskeleton during ecdysis (Cuzon et al., 1994). Additionally, the accumulation of glycogen reserves is also important for the response to various stressors. During a stress event, crustacean decapods require additional energy, which is regulated by the crustacean hyperglycaemic hormone (CHH) (Chung et al., 2010). The hydrolysis of glycogen hepatopancreatic or muscle glycogen reserves is stimulated by the release of CHH, resulting in an increased concentration of glucose in the hemolymph (Chang, 2005).

As carbohydrate sources are less expensive than protein sources, the use of carbohydrates as an energy source can be advantageous in terms of profitability. However, the consumption of high amounts of carbohydrates can negatively affect lobster performance, leading to low growth and survival rates, as the lobster metabolic capacity to use carbohydrates is limited (Rodríguez-Viera et al., 2017). The prolonged high glucose level in the hemolymph of juvenile spiny lobsters *Jasus edwardsii* suggested that despite being efficiently digested and absorbed, carbohydrates are poorly utilized (Simon, 2009a). It was also demonstrated that the spiny lobster *Panulirus argus* cannot efficiently metabolize diets with more than 20% carbohydrate in their composition (Rodríguez-Viera et al., 2017).

Results presented in study 2 point to a potential protein-sparing effect of carbohydrates in juvenile *H. gammarus*, at least in the short-term period (32-day period growth trial). However, rearing *H. gammarus* juveniles with a carbohydrate-rich formulated feed (40% protein, 35% carbohydrate)

under lower temperature conditions (13°C) enhanced the negative impact of temperature on their growth and energy metabolism than a protein-rich formulated feed (50% protein, 26% carbohydrate) (study 5). Additionally, longer exposure to the same carbohydrate-rich formulated feed resulted in poorer performance (study 3) than initially expected (study 2). Nevertheless, in study 3, the performance of juveniles fed the high-carbohydrate feed was improved when compared to the performance of juveniles fed moderate- (31% carbohydrate) or low-carbohydrate (23% carbohydrate) feeds of equivalent protein content (40%). Taken together, the results suggest that there is some potential for protein-sparing using carbohydrates in formulated feeds for juvenile *H. gammarus*, but the efficient use of this macronutrient needs to be further explored.

6.3. LIPIDS

It is general accepted that crustacean requirements for lipids are lower than in fish (Williams, 2007). High dietary inclusion of lipids (> 10%) was associated with poor growth rates in juvenile spiny lobsters (Perera and Simon, 2015). This has been attributed to their poor capacity to utilize dietary lipids (Glencross et al., 2002). Results presented in studies 2 and 3 showed that the feed intake of juvenile *H. gammarus* fed a high lipid formulated feed (24% lipid) was severely reduced, leading to poor growth performance. Faster satiation due to high lipid content was pointed as a potential cause for the low feed intake, but lower palatability of the pellets could not be ruled out.

Lipids are, however, an important energy source throughout the early ontogenetic development of lobsters, in particular for the larval stages (Sasaki et al., 1986). Phospholipids play a dominant role in the early stages of spiny lobsters (Jeffs et al., 2002), while triacylglycerol fraction increases at older stages (Smith et al., 2004). In the first study of this thesis, it was observed that *H. gammarus* postlarvae (stage IV) presented the lowest polar lipid fraction and highest neutral fraction as compared with larvae (stage I to III). Polar lipids increased from stage I to stage II larvae and, from that point onwards, decreased to a minimum level in postlarvae stage IV. Whether phospholipid levels resume decreasing in the subsequent juvenile stages needs further investigation. Still, the results obtained suggest a more dominant role of phospholipids in larval than postlarval stages of *H. gammarus*, resembling the reported findings for spiny lobsters.

Highly unsaturated fatty acids (HUFA) including the arachidonic acid (ARA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) play an essential role in the survival and growth of decapod larvae (Conklin, 1995). A deficit of HUFA's often result in prolonged intermoult periods leading to poor growth and low survival (Glencross, 2009). ARA can be synthesized through a series of steps of elongation and desaturation of linoleic acid (LA), while EPA and DHA can be obtained from the elongation and desaturation of α-linoleic acid (ALA) (O'Connor and Gilbert, 1968). However, most decapod species depend on the diet to fulfil their requirements for HUFA's as they are not able to

biosynthesize them *de novo* (O'Connor and Gilbert, 1968), presumably due to a lack of desaturase enzymes (Chen et al., 2017).

The uptake and use of fatty acids by lobsters are facilitated if they are provided as phospholipids rather than triacylglycerols (Conklin, 1995). It has been previously demonstrated that the addition of soy lecithin (high content in phospholipids) to formulated diets significantly improved the survival rate of *H. americanus* juveniles (Conklin, 1980). Phospholipids, in particular phosphatidylcholine, play an essential role in the mobilization of cholesterol from the hepatopancreas to the hemolymph in *Homarus* lobsters (D'Abramo et al., 1982). Phosphatidylcholine was determined to be the dominant lipid class in the body tissues of *H. gammarus* larvae and postlarvae (study 1), corroborating the importance of this phospholipid for homarid lobster larvae. The second most abundant lipid class was cholesterol (study 1), corroborating the high requirement for cholesterol in *H. gammarus* early stages, as previously established for *H. americanus* (Castell and Covey, 1976).

TABLE 2. Macronutrient dietary content of formulated feed tested in homarid lobsters.

Macronutrient	Dietary level	Species	Feed type	Reference
Protein	53.9%	*H. gammarus*	Extruded pellet	500MED
	58.9%	*H. gammarus*	Dry pellet	Hinchcliffe et al. (2020)
	54.7%	*H. gammarus*	Dry pellet	Drengstig and Bergheim (2013)
	57.0%	*H. americanus*	Extruded pellet	Tlusty et al. (2005)
	43.3%	*H. americanus*	Dry pellet	Floreto et al. (2000)
	38.1%	*H. americanus*	Dry pellet	Castell and Kean (1989)
	38.8%	*H. americanus*	Dry pellet	Castell and Kean (1989)
Carbohydrate	26.1%	*H. gammarus*	Extruded pellet	500MED
	13.6%	*H. gammarus*	Dry pellet	Drengstig 2013
	12.0%	*H. americanus*	Extruded pellet	Tlusty et al. (2005)
	19.7%	*H. americanus*	Dry pellet	Floreto et al. (2000)
	20.0%	*H. americanus*	Dry pellet	Castell and Kean (1989)
	24.0%	*H. americanus*	Dry pellet	Castell and Kean (1989)
Lipid	13.1%	*H. gammarus*	Extruded pellet	500MED
	13.7%	*H. gammarus*	Dry pellet	Hinchcliffe et al. (2020)
	15.6%	*H. gammarus*	Dry pellet	Drengstig and Bergheim (2013)
	19.0%	*H. americanus*	Extruded pellet	Tlusty et al. (2005)
	14.3%	*H. americanus*	Dry pellet	Floreto et al. (2000)
	10.5%	*H. americanus*	Dry pellet	Castell and Kean (1989)
	12.9%	*H. americanus*	Dry pellet	Castell and Kean (1989)

Values refer to the formulated feed that provided best results within each study and are expressed as percent DW.

6.4. MICRONUTRIENTS

Apart from macronutrient sources, formulated feeds are generally supplemented with additives to satisfy micronutrient requirements (carotenoids, vitamins, and minerals). Carotenoids are a critical component in crustacean feeds because many species are not capable to synthesize carotenoids *de novo* and, therefore, depend on a dietary supply to satisfy their requirements (Liǹán-Cabello et al., 2002). Astaxanthin is the predominant carotenoid in fish and crustaceans (Williams, 2007). Beyond its role in

pigmentation, astaxanthin has antioxidant properties that have been considered beneficial for immunocompetence and tolerance to stress among aquatic species (Lim et al., 2018). No clearly defined requirement for vitamins and minerals has been reported for marine lobsters (Williams, 2007), and thus, information available for marine shrimp species is often used as a guide when formulating feeds for lobsters (Conklin, 1997). Calcium is of particular importance due to its role in the synthesis of a new exoskeleton at each moult cycle (Waddy et al., 1995). Copper also deserves particular attention. Crustaceans use hemocyanin, a copper-containing pigment, as the oxygen-carrying pigment that has the same role as haemoglobin in red-blooded animals (Davis and Gatlin, 1996). Marine lobsters are strict osmoconformers and, therefore, able to obtain minerals from the water via drinking, and by direct absorption via the gills and skin (Davis and Gatlin, 1996). But, whether their mineral requirements can be totally or partially satisfied through water absorption, remains unclear (Williams, 2007).

7. DEVELOPMENT OF FORMULATED FEEDS FOR LOBSTERS

The future of commercial lobster culture depends to a large extent on the development of a suitable formulated feed capable to sustain good growth and survival rates. Feed represents the largest cost in commercial crustacean hatcheries and, because of that, the development of cost-effective and nutritionally species-specific formulated feeds is fundamental for the sustainable and economic-viable production of a new aquaculture species (Perera and Simon, 2015). The use of a commercial formulated feed once *H. gammarus* enter the juvenile stages would allow the reduction of production costs by directly decreasing the feed costs, simplifying handling and storage, and offering a higher nutritional consistency in contrast to fresh and frozen foods routinely used in hatcheries (Powell et al., 2017).

The main challenges in the progress of the development of a suitable formulated feed are related to the consistently poor intake, digestion, and assimilation of these feeds in comparison to standard fresh and frozen diets. The difficulties in the transition from fresh or frozen diets to formulated feeds are shared among different lobster species. Not even the largest and most recent research effort with spiny lobsters (Palinuridae) (Perera and Simon, 2015) has solved all the challenges associated with the poor performance of spiny lobsters reared on formulated feeds. The development of a suitable formulated feed for the production of *H. americanus* was intensively pursued, in particular during the 1980s and 1990s (Aiken, 1995), but results were discouraging, showing that growth obtained was not as high as that obtained with fresh or frozen diets. The efforts for the development of formulated feeds for *H. gammarus* have been scarcer and reported results are similar to that observed in *H. americanus*, i.e., growth is superior in lobsters fed standard fresh or frozen diets as compared to those fed experimental formulated feeds (Hinchcliffe et al., 2020). More encouraging results were reported in Drengstig and Bergheim (2013). The authors estimated that plate-sized *H. gammarus* lobsters can be produced from stage IV to 300 g in less than 24 months using an optimum newly developed formulated

feed, even though no detailed results on growth performance were provided. The novel feed contained 54.7% protein, 13.6% carbohydrate, and 15.6% lipid (% of DW) (TABLE 2) and was supplemented with up to 200 mg/kg astaxanthin (% of DW). Details about the ingredient composition of the formulated feed were not specified.

Limited consumption of formulated feeds was related to a fast reduction of attractiveness through time (Williams et al., 2005). Thereby, the palatability and attractability of the feeds are decisive factors to be considered in the design of formulated feeds (Aaqillah-Amr et al., 2021). Palatability is related to the acceptance of the offered feed by the animal, and attractability, to the orientation towards the presence of feed in the tank (Suresh et al., 2011). More attractable and palatable pellets will be located and ingested faster, thus reducing the duration of the pellet's immersion and minimizing nutrient losses due to leaching processes (Tolomei et al., 2003). Another important aspect to take into account when developing formulated feeds is the ability of the pellet to maintain its integrity and nutrient content while in water. Pellets with high water stability are more effective in optimizing feed intake (Aaqillah-Amr et al., 2021). This is particularly relevant in crustaceans owing to their harsh handling and vigorous mastication of the feed (Volpe et al., 2012). External factors such as strong flow rates and aeration in the tanks can also accelerate pellet disintegration (Ruscoe et al., 2005). Pellet stability can be achieved by using suitable binding agents and finely ground ingredients which help to hold feed components together and minimize void spaces between particles when immersed in water (Ruscoe et al., 2005).

Formulated feeds were reported to reduce appetite revival in the juvenile spiny lobster, *Jasus edwardsii* (Simon and Jeffs, 2013). However, results obtained within this thesis showed no evidence that the extruded feeds tested caused reduction in appetite revival of juvenile *H. gammarus*. In the second study, the metabolic cost of feeding, termed as specific dynamic action (SDA), was compared between juvenile lobsters fed the experimental extruded feeds and a standard diet composed of Antarctic krill. The duration of SDA was previously associated with appetite revival in other aquatic species (Fritz et al., 2013), i.e., shorter SDA allows more frequent feeding. Results from study 2 showed no significant differences between SDA duration of juvenile lobsters fed the extruded feeds or the Antarctic krill as long as meal energy was not significantly increased.

The digestion of formulated feeds in lobsters is, in general, more challenging than the digestion of fresh or frozen diets. It was previously demonstrated that the ingestion of formulated feeds is more prone to induce a rise in the pH of the gastric fluid than the ingestion of fresh diets (Simon, 2009b) probably due to increased uptake of seawater (pH 8) during feeding (Perera and Simon, 2015). This can compromise the optimal activity of digestive enzymes, for example, maximum protease and carbohydrase activities in the hepatopancreas of *H. gammarus* were determined at pH 5.8 (Glass and Stark, 1994), and pH 4.5 – 5.5 (Glass and Stark, 1995), respectively.

In study 3, digestive enzyme activities in juvenile lobsters fed formulated feeds and thawed Antarctic krill were evaluated. Results showed that amylase and trypsin activities were, in general, higher in animals fed the formulated feeds. It is suggested that the higher activity could have been a strategy to maximize the use of limited nutrients available in formulated feeds. Intracellular digestion and nutrient absorption may also be affected by the lower solubility of formulated feed particles that reduce appropriate pre-digestion of some particles before reaching the tubules of the hepatopancreas (Brunet et al., 1994). Nitrogen retention results from study 2 suggest that, even if at the expense of a greater enzymatic effort (study 3), the digestion and absorption of ingested protein was comparable between the 500MED (best tested formulated feed) and the Antarctic krill.

7.1. TYPES OF FORMULATED FEEDS

There are different types of pellet forms used in the hatchery of commercial crustacean species: dry-pellet (< 10% moisture); semi-moist pellet (~ 35% moisture), and moist pellet (62% - 82% moisture) (Aaqillah-Amr et al., 2021). In general, moist and semi-moist pellets are more effective in terms of feed efficiency and growth owing to their softer texture and higher palatability (Ruscoe et al., 2005). That, however, does not match the results obtained within this thesis. Juvenile *H. gammarus* fed semi-moist feeds performed poorer (study 4) than juveniles fed an extruded feed of equivalent macronutrient ratio (study 3). Results suggest that juveniles' uptake of the semi-moist diets was limited, possibly due to induced satiation from a mechanical expansion of the gut. Additionally, high moisture pellets tend to dissociate easily upon entering the water increasing the rate of nutrient leaching (Ruscoe et al., 2005). Moist and semi-moist formulated feeds are also more prone to deterioration from bacteria, yeast, or mould proliferation during storage (Coroller et al., 2001).

Dry pellets can be manufactured in a variety of forms: dry-sinking pellet, extruded sinking pellet, and extruded floating pellet. The main difference between dry and extruded pellets is that the extrusion method does not require any pellet binder to promote the adhesion of the feed ingredients (Misra et al., 2002). Instead, adhesion is achieved through starch gelatinization (Umar et al., 2013). Sinking pellets are more suitable for bottom feeders as is the case of juvenile lobsters while floating pellets are more appropriate for planktonic larvae stages (Aaqillah-Amr et al., 2021).

7.2. FEED INGREDIENTS

Fish meal is the main protein source used in formulated feeds for crustaceans (Cuzon et al., 1994) despite its less soluble protein fractions (Perera et al., 2010). Crustacean meals, in particular, shrimp and krill meals are good candidates. Shrimp meal usually produces good results, supporting higher growth rates at lower protein levels (Cuzon et al., 1994; Perera and Simon, 2015). Krill meal has a high solubility which facilitates its complete digestion (Nankervis and Southgate, 2006). In addition,

it is also rich in phospholipids (Barclay et al., 2006) which facilitates fatty acid uptake in lobsters (Conklin, 1995). Mussel meal is highly digestible due to its high content in soluble proteins (Nankervis and Southgate, 2006). Squid meal is often used in the formulation of crustacean feeds because of its attractiveness and growth-promoting effect (Mente, 2006). However, low digestibility for squid meal has been observed in spiny lobsters (Irvin and Williams, 2007; Perera et al., 2010; Ward et al., 2003). Taken together, results suggest that high inclusion of squid meal in formulated feeds may compromise the supply of amino acids to lobsters, but can promote growth when included at low levels (Perera and Simon, 2015).

The main source of carbohydrates in formulated feeds for lobsters is wheat starch. It is classified as a fairly well digested carbohydrate source among raw plant starches (Simon, 2009a). Dextrin, cooked starches, pregelatinized maize starch, and mussel glycogen had also shown good digestibility in spiny lobsters (Simon, 2009a). Apart from being a source of carbohydrates, wheat starch also provides pellet integrity and stability by promoting ingredients agglutination through starch gelatinization (Meyers, 2009). Other commonly used binder agents are agar, carrageenan, alginate, carboxymethyl cellulose, and gelatine. A higher digestibility of agar as compared with carrageenan or alginate has been reported for juvenile spiny lobster, *Jasus edwardsii* (Radford et al., 2007). The use of gelatine instead of agar and alginate produced better digestibility in juveniles of the same species (Simon, 2009a).

Fish oil has been used as the main lipid source in lobster feeds but its high content in triacylglycerols (Kutzner et al., 2017) might not be the most adequate as lobsters may be more efficient at digesting other sources, in particular, phospholipids (Conklin, 1995). Encouraging results were obtained with a diet developed for the spiny lobster, *Panulirus ornatus*, containing no additional lipid besides those provided by included marine animal meals (Barclay et al., 2006). The good performance of lobsters fed that diet was associated with the high content of phospholipids in krill meal in comparison to the high triacylglycerol content in fish oil (Barclay et al., 2006). Soybean lecithin is an excellent source of phospholipids, in particular, phosphatidylcholine. Its dietary inclusion has provided high survival and good growth in juvenile *H. americanus* × *H. gammarus* hybrids (Conklin et al., 1980). Dietary lecithin demonstrated improvements in lipid deposition in the hepatopancreas of *Jasus edwardsii* (Ward and Carter, 2009). Fatty aryl-taurine complexes were also determined to increase lipid digestion by their emulsifying and solubilizing action (Conklin, 1995).

Vitamin and mineral mixtures are often added to formulated feeds to supplement them with vitamins and minerals. Astaxanthin is commonly used as an additive to improve colouration in several commercially farmed aquatic species (Lim et al., 2018). The loss of natural colouration of the exoskeleton is a common response in lobsters reared on formulated feeds as compared to those reared on live, fresh, or frozen diets (Floreto et al., 2001). The supplementation of formulated feeds with astaxanthin showed encouraging results on the improvement of the exoskeleton colour of spiny lobsters (Crear et al., 2002).

7.3. ALTERNATIVE INGREDIENTS

The main sources of protein and lipid in formulated feeds for decapods are still fish meal and fish oil due to their high protein and lipid levels as well as other micronutrients such as amino acids and fatty acids (Aaqillah-Amr et al., 2021). Forage fish is the main raw material for the production of fish meal and fish oil. The growth of the aquaculture industry is imposing enormous pressure on natural fish stocks to fulfil the demand for formulated feeds (Hua et al., 2019). Thus, efforts have been directed to the reduction of fish meal and fish oil content in formulated feeds by substituting them with alternative ingredients. Plant-based materials, terrestrial animal-based materials, insect meal, food waste, and fishery and aquaculture by-products present potential as suitable alternatives (Hua et al., 2019).

The use of protein vegetable sources is gaining attention and represents the major source used in decapod feeds after the fish meal. However, the lack of attractants and reduced palatability of vegetable protein sources may promote poor feed intake and, consequently, retard growth (Silva-Neto et al., 2012). The digestibility of soybean meal has been reported to be only slightly lower (81%) when compared to fishmeal (84%) during in vivo digestibility tests performed in spiny lobsters (Irvin and Williams, 2007). Its relatively high digestibility has been linked to its high content in soluble proteins (Perera et al., 2010). Fish meal was replaced with soybean meal in formulated feeds for *H. americanus* juveniles without significant reduction in weight gain, as long as soybean levels did not exceed 50% of the dietary protein and by additional amino acid (arginine, leucine, methionine, and tryptophan) supplementation (Floreto et al., 2000).

Animal meat and bone meals have proven efficient fish meal replacements providing decapod crustaceans with adequate protein (Aaqillah-Amr et al., 2021). For example, good food conversion ratio (FCR) results were obtained in whiteleg shrimp fed 50% meat and bone meal diet (FCR = 1.70) in comparison to a control diet formulated with fish meal (FCR = 1.72) (Tazikeh et al., 2020). Nevertheless, some animal protein meals often present deficiencies in essential amino acids limiting their level of inclusion (Tantikitti, 2014). Poultry by-product meal is a good candidate as a substitute of fish meal because of its high protein content, low price, and consistent availability (Aaqillah-Amr et al., 2021).

Meanwhile, fishery and aquaculture by-products show high potential as protein sources (Hua et al., 2019). Generally, these products present a high protein content, a well-balanced amino acid profile (Cruz-Suárez et al., 1993), and high levels of astaxanthin (Shahidi and Synowiecki, 1991). The inclusion of shrimp waste meal in formulated feeds for juvenile *H. gammarus* was tested within this thesis (study 4). Results showed that the inclusion of shrimp waste meal up to 28% of the dietary protein had no negative effect on growth and improved the survival rate of the juvenile lobsters. Contrary to initial expectations, no significant improvements were observed in the exoskeleton colouration of the juvenile lobsters. Levels of astaxanthin were well below the reported in the literature (Dave et al., 2020),

and that might have affected the potential for exoskeleton colour enhancement. Degradation of carotenoids during the ingredient processing and feeds manufacturing were suggested as potential causes for the obtained results. In fact, the quality of the ingredients is highly dependent not only on the selection of raw materials but also on their production process (Cuzon et al., 1994).

8. PROSPECTS AND CONSTRAINTS IN *HOMARUS GAMMARUS* PRODUCTION

Despite the efforts devoted to progress the cultivation of homarid lobsters, its economically viable production is not yet established. On a commercial scale, the main constraint in developing the cultivation of *H. gammarus* is the low-profit margin. Although the marketability of this crustacean species is high, currently production costs do not offset the potential profit of the final product. Among several drawbacks, there are two major challenges for the successful establishment of *H. gammarus* farming: (a) decrease losses due to high mortality during larval rearing and (b) reduce the high costs associated with the cultivation of juveniles.

TABLE 3. Reported survival rates for *Homarus gammarus* larvae reared under experimental conditions.

	Rearing conditions			
Reference	Temperature	Initial density	Experimental period	Survival
Communal rearing				
Daniels et al. (2010)	18°C – 20°C	5 – 14 larvae L^{-1}	Hatching – stage IV	4% – 16%
Middlemiss et al. (2015)	17°C – 19°C	6 – 7 larvae L^{-1}	Hatching – 18 DAH	13% – 15%
Powell et al. (2017)	19°C – 20°C	10 larvae L^{-1}	Hatching – 14 DAH	4% – 16%
Scolding et al. (2012)	17°C – 19°C	15 – 20 larvae L^{-1}	Hatching – 18 DAH	12% – 16%
Individual rearing				
Attramadal et al. (2021)	19°C – 21°C	1 larvae per 208 mL	Hatching – stage IV	8% – 61%
Kurmaly et al. (1990)	25°C	1 larvae per 125 mL	Hatching – 16 DAH	36% – 80%
Powell et al. (2017)	19°C – 20°C	1 larvae per 100 mL	Hatching – 14 DAH	28% – 84%

Survival rates of *H. gammarus* larvae until metamorphosis to the fourth stage have not been reported to exceed 16% in communal rearing systems (TABLE 3). The low survival rates are, primarily, a consequence of the strong cannibalistic nature of this species (Powell et al., 2017). Potential nutritional imbalances provided under farming conditions have been also related to high larvae mortality (Powell et al., 2017; Schoo et al., 2014). A potential solution is the use of an appropriate artificial diet capable to meet the nutritional requirements at each larval developmental stage. Conducted studies testing the use of formulated feeds in *H. gammarus* larvae rearing have proven disappointing as survival rates are discouragingly poor (Kurmaly et al., 1990; Powell et al., 2017; Sá et al., 2014). Results showed that cultured lobsters reared on formulated diets were more prone to moulting difficulties, termed as the "moult-death syndrome" (MDS), resulting in increased mortality during or immediately after ecdysis. A deep understanding of the digestive capabilities and potential nutrient requirements during larval development is necessary before the potential of formulated feeds can be accurately evaluated.

The necessity to cultivate *H. gammarus* in individual compartments from metamorphosis onwards significantly increases land-based farming production costs. Important advances in automated rearing systems (Drengstig and Bergheim, 2013) and water quality (Middlemiss et al., 2015) have been recently made to improve *H. gammarus* hatchery production and cost-effectiveness. However, the lack of a species-specific formulated feed remains a major obstacle in the sustainable production of *H. gammarus*.

9. TECHNICAL ASPECTS OF EXPERIMENTS IN LOBSTER NUTRITIONAL STUDIES

Although laboratory trials allow the control of most of the environmental conditions (e.g. temperature, salinity, dissolved oxygen, photoperiod regime, light intensity), there are other factors that might interfere with the animal responses to the experimental diets tested in nutritional trials. This can lead to inconsistencies in the interpretation of the results obtained. Some of these factors, with particular focus on their impact on crustacean species, are discussed in the following sections.

9.1. GENETIC BACKGROUND OF THE EXPERIMENTAL ANIMALS

Unlike nutritional research performed with land-based animals (e.g. rats and chicken) or some fish species (e.g. zebrafish, *Danio rerio*) where it is possible to establish standard strains, lobster nutritional studies are usually conducted with wild captured animals or the offspring of wild broodstock. Thereby, it is not possible to exclude parental effects (Moland et al., 2010) on the variance in responses to different experimental diets. A solution to partially counter this issue is to use a sufficiently large number of animals derived from a considerable large number of females. However, it is not always possible to have a large number of lobster females with synchronized spawning at experimental scale units, as was the case during this thesis. In such circumstances, some authors choose to use sibling individuals derived from a single female to minimize any phenotypic effect and ensure that differences are mainly caused by the different dietary treatments (D'Abramo et al., 1982; Hinchcliffe et al., 2020; Tlusty, 2005). This approach is controversial as it raises concern regarding the representativeness of a single female. Regardless of the strategy followed, it is important to identify as accurately as possible the source of the stocks that are used in any nutritional studies (Castell, 1989).

Results from the first study conducted within this thesis disclosed significant differences in the weight of *H. gammarus* larvae (stages I, II, and III) and postlarvae (stage IV) hatched from different females as well as in the specific growth rate (SGR) (FIGURE 9). Results suggest a parental effect on the size of larvae, as previously demonstrated in *H. gammarus* (Moland et al., 2010), but there was also evidence of its effect on growth rates. Conclusions are, however, limited by the low number of females

examined and to developmental stages I to IV. Similar studies examining a larger number of females and assessing growth until later juvenile stages would be useful to broaden this issue.

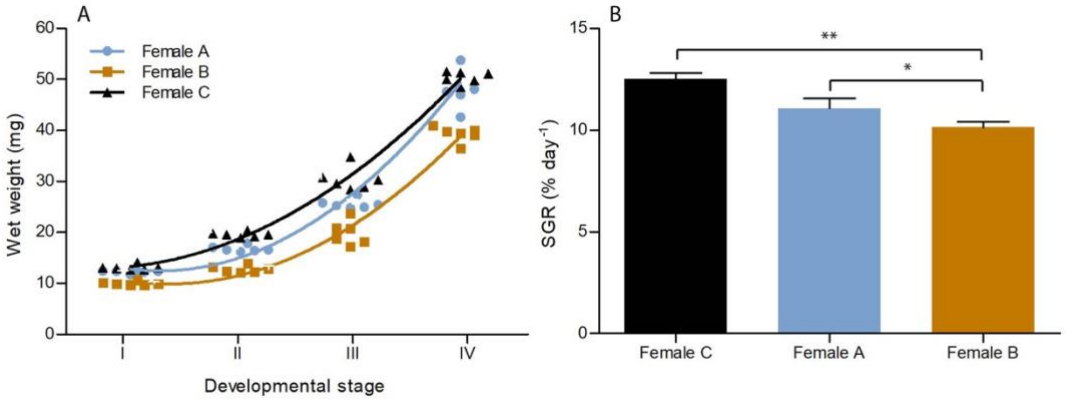

FIGURE 9. Wet weight variation in *Homarus gammarus* larvae (stage I to III) and postlarvae (stage IV) (panel A) and specific growth rate (SGR) until metamorphosis (stage IV) (panel B). SGR values are mean ± SD (N = 6). The symbols * ($p < 0.05$) or ** ($p < 0.01$) indicate significant differences identified by one-way ANOVA followed by *post hoc* Tukey test.

9.2. EVALUATION OF GROWTH RESPONSE

Crustaceans experience a very different growth pattern than most other animals. As previously mentioned (section 4.3.), growth in crustaceans is not a continuous process, with major increases in body size (WW and CL) occurring after each ecdysis. The application of classical methods (e.g. weight gain over time), commonly used in most other animals involved in nutrition research, may neglect some important information to interpret crustacean growth responses to nutritional differences among experimental diets (Castell, 1989). Thus, assessing variations in weight gain and carapace length per moult, as well as the duration of the intermoult period are standard outcomes in crustacean nutritional studies. That, however, implies that the duration of growth trials should be sufficient for the animals to complete, at least, one moult cycle. Moreover, even though gains in WW and CL are not continuous through the moult cycle, the increase in tissue mass is (Nguyen et al., 2014). In this sense, additional indirect methods such as RNA:DNA ratios can provide further information on the nutritional status of the animals and allow the estimation of growth potential even when a moult cycle is not yet completed (Wahle and Fogarty, 2006). Results from study 3 revealed a significant linear correlation between RNA:DNA ratios (at week 4) and SGR (at week 8) demonstrating that this index is a sensitive indicator of growth potential in *H. gammarus* juveniles. This is relevant for future nutritional studies in this species because it allows an accurate and faster evaluation of dietary effects on growth performance.

9.3. QUANTIFICATION OF FEED INTAKE

As previously mentioned in section 4.4., *Homarus*, like any other lobster genus, use their maxillipeds and mandibles to tear food into small pieces. This feeding behaviour complicates the accurate measurement of the feed intake in lobster trials (Perera and Simon, 2015). As a result, precise estimation of feed efficiency indices (e.g. nitrogen retention; feed conversion ratio, FCR) and apparent digestibility of feeds is often hampered (Smith and Tabrett, 2004). Non-dietary sources of nutrients can also interfere with experimental results. If not effectively removed, uneaten food particles may promote the growth of mould, fungus, or bacteria on the surfaces of rearing containers that, in addition to overall water quality degradation, can provide an uncontrolled source of nutrients to the experimental animals (Castell, 1989).

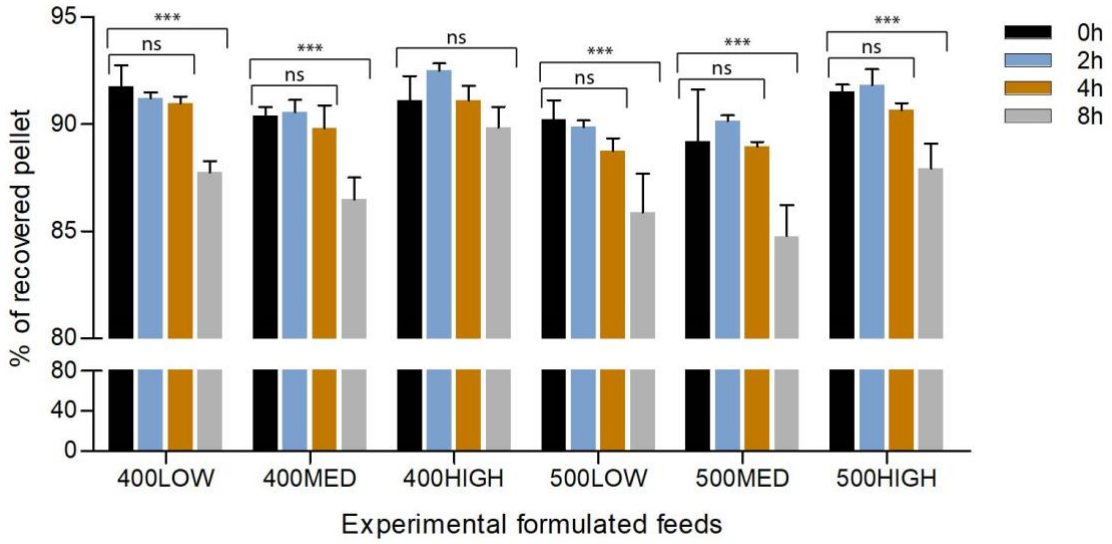

FIGURE 10. Percentage of recovered pellet after 0h, 2h, 4h, and 8h soaking in seawater. Values are mean ± SD (N = 3). The symbol *** ($p < 0.001$) indicates significant differences identified by one-way ANOVA followed by *post hoc* Tukey test. The letters "ns" indicate the no significant differences were detected.

Before the beginning of the experiments conducted in studies 2 and 3 of this thesis, a pilot study was performed to evaluate the stability of the experimental formulated pellets. Three pre-weighed pellets from each feed were placed for 2h, 4h, and 8h in the same individual containers used to reared the juvenile lobsters under the same conditions (temperature, salinity, flow rate). Additionally, a control group (0h) was composed of three pellets per feed that were not soaked in seawater. In the end, all pellets were dried (24h, 60°C) and weighed. The recovery percentage was estimated by comparing the initial and final weight of the pellets. Results showed that no significant weight losses occurred up to 4h soaking in all experimental feeds. The pilot study did not, however, allow any conclusions on the effect of lobster manipulation but it is conceivable that after break-down into small pieces, pellet losses increase (FIGURE 10).

In this thesis, feed intake was estimated with relative success following a method proposed by Nguyen et al. (2014). Briefly, a pre-weighted pellet or piece of krill was provided to each lobster (hold in individual containers) and, after a 4h meal, the uneaten fraction was removed, dried, and weighed. A detailed description of the methodology is provided in the material and methods section of studies 2 and 3. Despite labour intensive, the method was sufficiently accurate to allow the detection of significant differences among dietary (study 3 and 5) and temperature (study 5) treatments. Comparison between types of food largely different in terms of DM content was, however, of limited usefulness (study 3).

9.4. STANDARD EXPERIMENTAL CONDITIONS

Most often, as for other animals, nutritional trials performed in lobsters attempt to provide environmental conditions that deliver optimum growth and survival rates (Castell, 1989). Such conditions approximate the conditions under which the species would be cultured for commercial production. However, in the particular case of *H. gammarus*, currently mainly produced to support re-stocking programs, the evaluation of their performance under environmental conditions prevalent in their aquatic ecosystems is of great relevance. The acclimation to the prevailing conditions before release would allow a more accurate assessment of their ability to survive after release, and eventually, increase the chances for the success of the stock-enhancement programs (Daniels et al., 2015).

In this context, the development of formulated feeds for hatchery units targeting the production of juvenile *H. gammarus* for re-stocking programs would benefit if formulations were tested for resilience to environmental variation. Results from study 5 showed that juvenile lobsters use different strategies to adapt their metabolism in relation to the diet received and that the impact of low temperature was greater in lobsters fed a carbohydrate-rich formulated feed than those fed a high-protein formulated feed.

10. FINAL REMARKS AND CONCLUSIONS

This Ph.D. project has provided new insights into the metabolism and nutritional requirements of *H. gammarus* larvae, postlarvae, and early juvenile stages. The thesis focused on two major drawbacks hindering the efficient hatchery production of *H. gammarus*: (a) improve larval feeding protocols and (b) provide relevant information for the development of suitable pelleted feeds specifically formulated for early juvenile stages. While study 1 focused on the first issue (a), studies 2, 3, 4, and 5 focused on the second one (b).

Results from the first study highlight the increasing importance of lipids on the metabolism of *H. gammarus* larvae until stage III and the potential shift in dietary requirements at metamorphosis towards

a diet richer in carbohydrates. The high content in cholesterol and phospholipids in the body tissue of larvae and postlarvae, in particular phosphatidylcholine and phosphatidylethanolamine, points to high dietary requirements of these compounds.

The second study established that, in general, the metabolic cost for digesting and assimilating (SDA) formulated feeds was comparable to that of thawed Antarctic krill. The basal metabolism (SMR) and nitrogen retention of juvenile lobsters fed 40% protein feeds (400MED and 400HIGH) decreased, unless the protein was compensated with carbohydrates rather than lipids (400LOW). Protein deposition and metabolic capacity depression were closely related to the low feed intake of the 400MED and 400HIGH formulated feeds. The higher content of carbohydrates (35%) in the 400LOW feed enhanced feed intake, and consequently, the metabolic capacity and protein deposition. Results pointed to a potential protein-sparing effect of carbohydrates, at least in short-term periods (32-day).

Results from study 3 disclosed that the most successful formulated feed tested was the 500MED, composed of 50% protein, 24% carbohydrate, and 12% lipid. This macronutrient combination in a formulated extruded feed promoted growth and survival rates comparable to those obtained with a standard diet composed of Antarctic krill, at least for 8 weeks. Among the experimental feeds, the 500MED was also the most efficient in terms of FCR. The 400MED and 400HIGH produced the poorest performance, corroborating results from study 2. The potential protein-sparing effect of carbohydrates projected in study 2 based on the good results obtained with the 400LOW feed was not confirmed in the longer-term.

In study 4, the shrimp waste meal (SWM) showed a good potential as an alternative protein source in feeds for juvenile *H. gammarus*. The highest level of SWM inclusion (28% of dietary protein) showed no negative effect on growth and enhanced survival. Nevertheless, growth rates obtained in this study were lower than those reported in study 3 using a formulated extruded feed of equivalent macronutrient ratio. It is important to highlight that different types of feeds were tested. A semi-moist diet (40% DM) was used as an alternative to the extruded pellets (90% DM). It was initially hypothesized that a softer feed could improve feed intake but that does not seem to be the case. Results suggest that an extruded dry pellet feed might be more suitable than semi-moist feeds for juvenile *H. gammarus*.

Study 5 highlights the importance of evaluating the effect of novel products, in this case, the formulated feeds, on the resilience of the animals to environmental variation. As previously mentioned, this is of particular relevance in animals that are produced to support stock-enhancement programs. Results from this study revealed that juvenile lobsters adopted different metabolic strategies to cope with temperature variation concerning the composition of the diet received. Lobsters fed protein-rich feeds (500LOW / HP) were more resilient to low temperature than those fed a high-carbohydrate feed

(400LOW / HC). The potential protein-sparing effect of carbohydrates first observed in study 2 might be limited to optimal rearing temperatures (18°C to 22°C).

In conclusion, the overall results from this thesis contribute valuable insights into specific dietary requirements for *H. gammarus* early stages. The results provided can be used as guidelines for the future design and development of feeds for early European lobster stages.

11. FUTURE RESEARCH DIRECTIONS

While the results reported in this thesis are encouraging, there is still space for improvements towards the efficient hatchery production of *H. gammarus*. Potential dietary requirements of *H. gammarus* larvae for some key compounds, in particular lipids, are disclosed but its endogenous ability to metabolise different fatty acids is not completely understood yet. Further studies on this topic would provide important new insights on the capability of *H. gammarus* to biosynthesise different fatty acids and lipid classes containing essential fatty acids.

One of the most obvious results from this thesis was that feeds with high protein content (50%) were more suitable than low protein content (40%) for *H. gammarus* early juveniles. While there is a considerable amount of studies on protein requirements of different lobster species (Castell and Budson, 1974; Perera et al., 2010, 2005; Ward et al., 2003), few works addressed specific amino acids requirements (Mente et al., 2001). One important issue in such studies is the leaching of amino acids from formulated feeds. The application of encapsulating techniques would allow improvements in the precise determination of amino acid requirements.

The beneficial effect of high inclusion levels of carbohydrates in the low protein feeds should be further explored. The potential protein-sparing effect of carbohydrates is of great interest as carbohydrates are the least expensive energy source for aquatic animals (Wang et al., 2016). The most common source of carbohydrates in experimental formulated feeds for lobsters is starch, nonetheless, there might be other sources able to provide more efficient digestibility (Simon, 2009a). Explore alternative sources of carbohydrates such as dextrin, gelatinized products, or mussel glycogen may improve the potential for protein-sparing.

Results showed that high dietary lipids were not effective in formulated feeds for *H. gammarus* juveniles. The main lipid source used in the formulation of the extruded feeds tested in studies 2, 3, and 5 was fish oil which main lipid class is triacylglycerol (Kutzner et al., 2017). Lobsters can be more efficient at digest and use fatty acids from phospholipids (Conklin, 1995). Therefore, other lipid sources of richer polar lipid content should be considered in the formulation of future feeds.

As for other lobster species, vitamin and mineral requirements have been poorly studied for the *H. gammarus* (Williams, 2007). As a consequence, requirements found on shrimp species are commonly

the basis for the formulation of lobster experimental feeds (Conklin, 1997). Further studies in this topic are required.

This thesis focused on the nutritional aspects of *H. gammarus* early stages but there are other important variables influencing growth and survival rates. More studies on water quality and turbidity conditions, tank density and design, photoperiod regime, flow and aeration rates should be pursued in the future. In particular, the impact of using formulated feeds on the deterioration of water quality in the rearing tanks should be further investigated.

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STUDY 1

Early ontogenetic changes in digestive enzyme activity and biochemical indices of the European lobster (*Homarus gammarus*, L.)

Submitted, Marine Biology

Early ontogenetic changes in digestive enzyme activity and biochemical indices of the European lobster (*Homarus gammarus*, L.)

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Abstract

The lack of knowledge on the nutritional requirements and digestive capacity of the European lobster (*Homarus gammarus*) remains a major obstacle to the improvement of growth and survival rates. Digestive enzyme activity and biochemical indices (RNA:DNA, proximate and lipid class composition) of larvae and postlarvae of H. gammarus obtained from different females and fed Antarctic krill (Euphausia superba) were determined to identify ontogenetic changes in digestive capacity and potential nutritional requirements. Trypsin, lipase, and amylase activities were detected in all developmental stages examined suggesting that H. gammarus are capable of exploring a variety of food items from stage I. A significant interaction between stage and female suggests that analyzed trypsin levels can be affected by parental factors. Amylase activity increased significantly at stage IV denoting a shift in the dietary requirements at metamorphosis towards a diet richer in carbohydrates. Lipase activity raised progressively during the three larval stages stabilizing at stage IV. Results indicate an increasing relevance of dietary lipids from stage I to III. DNA content decreased faster than RNA content during larval development, pointing to a more important role of cell multiplication rather than protein synthesis in the metabolism of earlier stages. The decrease in protein content at metamorphosis was partially compensated by an increase in ash content, reflecting the increased contribution of the exoskeleton to the total body mass. Phosphatidylcholine, phosphatidylethanolamine, and cholesterol were the most abundant lipid classes in the body composition of H. gammarus early stages implying high dietary requirements for these compounds.

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

INTRODUCTION

The European lobster *Homarus gammarus* L. is an ecological and economical important species inhabiting coastal areas from Northern Norway to Morocco and Western Mediterranean (Triantafyllidis et al. 2005). A major stock decline during the 1960s and 1970s among several *H. gammarus* populations led to the development of juvenile production for re-stocking purposes (Ellis et al. 2015). The ongrowing cultivation of *H. gammarus* as an emerging species in commercial aquaculture has also attracted considerable attention (Drengstig and Bergheim 2013) although, the low survival and growth rates are still major bottlenecks hampering its successful cultivation (Hinchcliffe et al. 2020). The high

mortality during larvae development is particularly critical and it has been often associated with intense cannibalism in communal rearing tanks (Powell et al. 2017). The lack of knowledge about digestive capabilities and processes and requirements for specific nutrients has also been pointed out as major impediment to enhance the rearing success of *H. gammarus* lobster larvae (Powell et al. 2017; Hinchcliffe et al. 2020).

Like other nephropid lobsters, *H. gammarus* emerges from the egg as a "pre-larva" which remains among the pleopods of the female within the first few hours until it moults into the first larval stage, stage I (Nicosia and Lavalli 1999). Planktonic stage I larvae develop through two additional larval stages (II and III), each separated from the preceding by a moult, over a period that can vary from 10 days to 2 months depending on the water temperature (Nicosia and Lavalli 1999; Anger 2001). The moult from stage III to IV is a true metamorphosis in which many of the morphological, anatomical, and physiological characteristics are significantly modified (Charmantier and Aiken 1991). After metamorphosis, lobsters abandon planktonic life and start to develop more complex swimming capabilities such as horizontal swimming and crawling along the bottom. Towards the end of this stage, postlarvae become progressively more benthic, until this behaviour is completely established by stage V (Ennis 1975). 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) also appears at stage IV. In the American lobster *Homarus americanus*, total enzyme activities generally increase during early development as the number and length of the tubules comprising the hepatopancreas increase (Biesiot and McDowell 1995). Protein catabolism is considered the main source of energy but lipids and carbohydrates also have an important contribution to the energy yield through stage I to III. After metamorphosis, the dependence on lipids as energy substrate decreases (Sasaki et al. 1986).

The aforementioned modifications point to important ontogenetic shifts in the digestive capability and nutritional requirements of the homarid lobsters during early life stages. In fact, there is a close correlation between diet and digestive enzymes produced in crustaceans and, therefore, ontogenetic changes in enzymatic activity may be indicative of shifts in the ability to hydrolyze different dietary components (Rodriguez et al. 1994). The digestive enzyme activity of *H. gammarus* has been only 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) reported on trypsin, amylase, and lipase activities in juvenile *H. gammarus* (stage VII – VIII) demonstrating that enzyme activities 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.

The understanding of energy metabolism associated with growth and development at early stages is also necessary for the optimization of culture techniques (Lemos et al. 2002). RNA:DNA ratio in

crustaceans can be a proxy for nutritional condition and growth, which otherwise can be difficult to measure (weight and length) due to irregular moultings, and thus, sequential growth. While the amount of RNA increases with protein synthesis rate, the DNA remains fairly stable within the same cell (Chícharo and Chícharo 2008). Thus, lower protein synthesis, and consequently, lower growth rates, are generally associated with lower RNA:DNA ratios (Labh et al. 2014). This has been successfully demonstrated for juvenile *H. gammarus* where a positive correlation between specific growth rate and RNA:DNA has been observed (Goncalves et al. 2021). The same 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, more severe in stage I and II than in stage III.

Studies on the biochemical changes during early development might be indicative of the use of energy substrates during ontogeny, and therefore, a valid approach to estimate nutritional requirements at each stage of development. Lipid utilization during embryogenesis has been previously studied in *H. gammarus* where neutral lipids are considered the main energy source during embryonic development, while polar lipids are not catabolized and play essentially a structural role (Rosa et al. 2005). 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*. In this sense, a close relationship between broodstock physiological condition and reproductive success has been demonstrated for marine organisms including lobsters (Agnalt 2008; Moland et al. 2010). More specifically, some phospholipids have been highlighted by several authors to be included in formulated diets in order to improve the broodstock general nutritional status, gonad development, and egg and larvae quality (Navas et al. 1997; Rodríguez-García et al. 2015).

The main objective of this study was to examine the digestive enzyme activity (trypsin, amylase, and lipase) during *H. gammarus* larval development (stage I, II, III, and IV) to elucidate the potential role of ontogeny on larval ability to utilize proteins, carbohydrates, and lipids. Thawed Antarctic krill (*Euphausia superba*) was used as larval feed at all stages. Larval RNA and DNA concentration, proximate, and lipid class composition 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. 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 aeriation 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 the following mean values were kept constant, temperature 19.6 ± 0.7 °C, PSU 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.

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 of the six sample pools collected per treatment were freeze-dried and divided into sub-samples for digestive enzyme activities, nucleic acids, and proximate composition analysis. The remaining pools were used for the determination of lipid class composition.

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.

DIGESTIVE ENZYME ACTIVITIES

Total body enzyme activity was measured in three individual larvae and postlarvae per stage and female. Freeze-dried 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 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 performed 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 of 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 using a CAMAG TLC Visualizer (Camag, Switzerland).

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. 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 had body weights of 0.6 kg, 1.5 kg, and 1.7 kg. 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 1.86 ± 0.37 mg DW and 3.07 ± 0.16 mm CL (mean ± SD) to 7.01 ± 2.55 mg DW and 5.37 ± 0.32 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 addition, stage II and IV lobsters hatched from female B presented the lowest DW and CL.

TABLE 2. *Homarus gammarus* progenitor size and performance – body wet weight, larvae spawned, and spawning duration – and progeny size – body dry weight and carapace length – in the first four early life stages, I-IV.

Female	A	B	C	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}	$F_{2,59} = 88.76^{***}$
Stage II	2.77 ± 0.06^{a}	1.95 ± 0.08^{b}	2.92 ± 0.11^{a}	$F_{2,59} = 43.72^{***}$
Stage III	5.01 ± 0.23^{b}	3.69 ± 0.16^{c}	5.87 ± 0.15^{a}	$F_{2,59} = 35.71^{***}$
Stage IV	8.73 ± 0.59^{a}	5.12 ± 0.24^{b}	7.18 ± 0.51^{a}	$F_{2,59} = 17.68^{***}$
Progeny carapace length (mm)				
Stage I	3.06 ± 0.03^{b}	2.93 ± 0.02^{c}	3.21 ± 0.01^{a}	$F_{2,59} = 38.83^{***}$
Stage II	3.89 ± 0.04^{a}	3.40 ± 0.06^{b}	3.94 ± 0.03^{a}	$F_{2,59} = 41.32^{***}$
Stage III	4.54 ± 0.04^{b}	4.16 ± 0.06^{c}	4.71 ± 0.04^{a}	$F_{2,59} = 39.17^{***}$
Stage IV	5.44 ± 0.05^{a}	5.09 ± 0.07^{b}	5.56 ± 0.04^{a}	$F_{2,59} = 19.70^{***}$

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

DIGESTIVE ENZYME ACTIVITIES

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 batch B as compared to batch A and C.

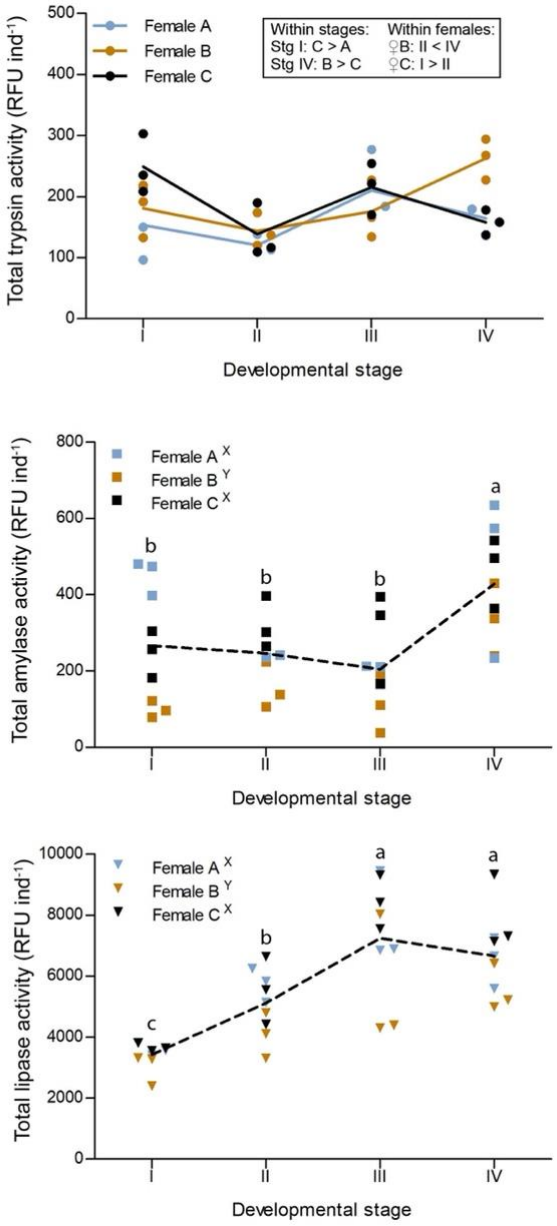

FIGURE 1. Total activity of trypsin, amylase, and lipase for developmental stages of *Homarus gammarus*, obtained from whole-body homogenates (N=3). Data points represent each replicate. Activity expressed as Relative Fluorescence Units (RFU) per individual. Different letters "x, y" and "a, b, c" indicate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

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

neither in the interaction stage × female. On the contrary, 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.

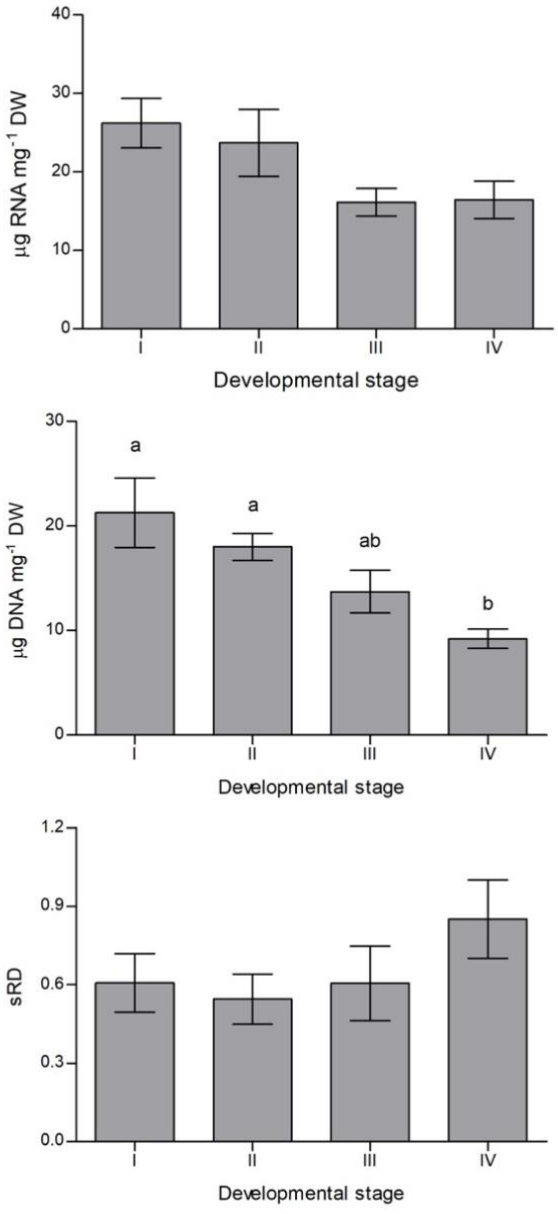

FIGURE 2. Changes in RNA concentration, DNA concentration, and standardized RNA/DNA ratio, sRD for developmental stages of *Homarus gammarus*, obtained from abdominal tissue homogenates (n=9). Bars represented as mean ± SEM. Different letters "a, b" indicate significant differences between developmental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

PROXIMATE AND LIPID CLASS COMPOSITION

The proximate biochemical and lipid class composition 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.

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}**

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

DISCUSSION

FEMALE PERFORMANCE AND LARVAE SIZE

Both the body DW and CL of *H. gammarus* throughout the ontogenetic development significantly varied between the three batches of larvae. Results suggest a broodstock influence on the size of the offspring as previously stated by (Moland et al. 2010). In the mentioned study, the authors suggested that the general larger-sized larvae hatched from larger females 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, moulting ovigerous females will allocate an important amount of energy to the moulting process that could, otherwise, be invested in embryonic development (Agnalt 2008). It was observed an important variability in hatched larvae numbers, probably related to genetic and condition-related effects but the loss of different egg mass volume among the three females during manipulation cannot be completely ruled out in the present study.

DIGESTIVE ENZYMES ONTOGENETIC TRENDS

No clear variation trend was identified for the activity of the protease, trypsin during larval 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 possibility to explain 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 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 that the dependence on lipids as a substrate is reduced after metamorphosis (Sasaki et al. 1986). This is 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 trypsin has been described as the main protease for decapod crustaceans, accounting for 40% to 60% of total protease (Lemos et al. 1999), 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 temporal genetic regulation of enzyme synthesis. Moreover, the uneven increase among the different enzyme types suggests that protein becomes more important as *H. gammarus* develop 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 more severe 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 related to 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.

BIOCHEMICHAL COMPOSITION

The major changes observed in terms of proximate composition in the carcass 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. These variations in ash and protein are most 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. Cholesterol content was not estimated in that study. 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 resulting, therefore, in its dependence on dietary supply to meet larvae 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 shrimps *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 relevance during larval development until stage III but not further. By contrast, no clear trend was observed for trypsin total activity suggesting similar importance of dietary protein throughout the first four development stages. Early *H. gammarus* larvae metabolism seems to be more dependent on cell multiplication processes rather than on protein synthesis and the overall protein synthesis capacity was not affected during larval development. Although it was not the primary goal of this study, a parental influence not only on larvae weight and size but also on the digestive enzyme activity and on the biochemical composition of *H. gammarus* larvae and postlarvae was evidenced. 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, as well as the dietary inclusion of phospholipids such as PC and PE, and cholesterol for the culture of *H. gammarus* larvae.

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STUDY 2

The effect of dietary protein, lipid, and carbohydrate levels on the performance, metabolic rate, and nitrogen retention in juvenile European lobsters (*Homarus gammarus*, L.)

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The effect of dietary protein, lipid, and carbohydrate levels on the performance, metabolic rate, and nitrogen retention in juvenile European lobster (*Homarus gammarus*, L.)

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Abstract

Releasing hatchery-reared juveniles in the wild can mitigate the general decline in the natural stocks of European lobster, *Homarus gammarus*, L. However, growth and survival rates in lobster culture are low, presumably due to suboptimal nutrition and feeding. With the aim of determining appropriate nutrient levels, we tested different formulated extruded feeds for the culture of juvenile European lobster. Baseline metabolism (standard metabolic rate, SMR), in combination with the metabolic cost of feeding (specific dynamic action, SDA), and nitrogen retention during digestion and assimilation was investigated for six experimental diets. Diets were formulated to contain two different levels of protein (400 and 500 g kg^{-1}), with three lipid to carbohydrate (L:CHO) ratios (low, medium, and high). These experimental diets were tested over a 32-day period, against a conventional control diet (Antarctic krill, *Euphausia superba*). During this period, the growth performance of the juveniles was assessed as molting frequency, increments in carapace length and whole body wet weight. At the end of the growth performance trial, oxygen consumption (MO_2) and nitrogen excretion rates of individual lobsters were determined prior to and following the ingestion of a single meal. Molting occurred more frequently in juveniles fed with krill and krill resulted in a significantly higher specific growth rate than experimental dry feeds except for the 500-low diet. However, lobsters fed any of the three 500 and the 400-low diets had carapace length increments, SMR, SDA, and nitrogen retention similar to those fed the krill diet. Results suggest that protein is an important macronutrient for juveniles of this species and must be included above 40 %. Also, lobsters have a dietary requirement for carbohydrates ranging from 24% to 35% probably related to the need for glycogen in chitin synthesis. The lower the protein content, the higher the requirement in carbohydrates.

Keywords: Formulated feeds; Antarctic krill, Nitrogen retention, Standard metabolic rate; Specific dynamic action; Growth.

INTRODUCTION

The European lobster (*Homarus gammarus*) is an economically important decapod crustacean distributed from Northern Norway to Morocco and Eastern Mediterranean (Triantafyllidis et al., 2005). Commercial landings of this species are declining and efforts to enhance natural populations have been made by restocking with hatchery-reared juveniles (Agnalt et al., 2007). Hatchery production of

European lobster aims to enhance growth and survival rates by securing optimal water quality, reducing predation, and by improving access to nutritional rich diets (Powell, 2016). Research efforts have been devoted mainly towards improving water quality conditions and the development of novel rearing systems (Daniels et al., 2013, 2015; Drengstig and Bergheim, 2013; Halswell et al., 2016; Middlemiss et al., 2015), but less so towards the development of a species-specific formulated diet. The transition from live or frozen feeds to the use of dry formulated diets may be one way to support a simpler and more sustainable production through the ease of application, reduced cost, and a more consistent nutritional quality (Powell et al., 2017).

The development of a nutritionally balanced formulated feed requires species-specific information on nutritional requirements. A considerable research effort on formulated feed development for spiny lobsters has been made in the last 30 years, the results of which indicate a dietary demand for high protein (> 45%), low lipid (<10%), and moderate carbohydrate (~20%) (Williams, 2007). However, the performance of spiny lobsters fed on formulated feeds remains poor, partially as a consequence of a lack of understanding of how they digest and assimilate this type of feeds (Perera and Simon, 2015). There is less literature available for homarid species than for spiny lobsters, and while some nutritional studies have been conducted on American and European lobster, appropriate nutritional levels are still to be defined. Based on the idea that diets should match the dry matter biochemical composition of an organism (Dall et al., 1991), the proximate composition content of *Homarus americanus* post-larvae suggests that an appropriate diet for this species should contain 53% protein, 4% lipid, and 12% carbohydrates (Haché et al., 2015). The observation that European lobster possesses a variety of carbohydrases (Glass and Stark, 1995), is indicative of a digestive capacity of different carbohydrate sources. Furthermore, Powell et al. (2017) identified glycogen deficiencies in the biochemical composition of *H. gammarus* larvae reared on formulated dry feed. Taken together, these findings suggest that the development of a formulated dry feed for European lobster should consider carbohydrates as a potential non-protein energy source.

The standard metabolic rate (SMR) represents the minimum energy expenditure of an ectotherm animal (Rosewarne et al., 2016). Although individuals with higher SMR have higher maintenance metabolism, previous studies also suggest this is indicative of an increased growth potential (Álvarez and Nicieza, 2005; Auer et al., 2015; Reid et al., 2012; Van Leeuwen et al., 2012). The magnitude of the postprandial metabolism, commonly referred to as the specific dynamic action (SDA), depends largely on the size and nutritional composition of a meal. It provides information on the cost and duration of the nutritional processes, including the energy expended towards food handling, absorption and storage of nutrients, deamination of amino acids, protein and lipids synthesis for growth, and synthesis of excretory products (Jobling, 1993). In crustaceans, the mechanical costs of digestion are calculated to be 5% to 8% while protein synthesis accounts for 20% to 37% of SDA (Whiteley et al., 2002). Therefore, SDA determination is a useful performance parameter in nutritional studies. In

principle, the higher the fraction of the meal energy allocated to SDA, the less energy will be retained to fuel locomotion, growth, and reproduction (Stieglitz et al., 2018). The present information on the metabolism in European lobster is sparse. Whiteley et al,. (1990) compared aquatic and aerial rates of MO_2 (mass-specific oxygen consumption) of this species at a temperature range 10-20˚C. Later, Drengstig (2017) reported data of standard metabolism in *H. gammarus* at 20˚C. However, to our knowledge, no studies on the effects of dietary composition on SMR or the SDA of European lobster have been performed.

Lobsters excrete the majority of the end product of protein metabolism across the gill epithelium in the form of ammonia (Burger, 1957), while a smaller part of nitrogenous waste is converted into urea in the antennal and maxillary glands (Binns and Peterson, 1969). Wickins (1985) investigated the effect of feeding on ammonia excretion by *Homarus gammarus* and observed that lobsters exhibited a significant increase in ammonia excretion after a meal. The efficiency with which dietary amino acids are deposited as new tissue can be estimated by the quantification of nitrogen excretion during digestion (Ming, 1985). Amino acids that are deaminated for *de novo* lipogenesis and glycogenesis, or oxidized for fuel, turn into nitrogenous waste (Skov et al., 2017). Therefore, high nitrogen excretion rates are indicative of reduced protein retention.

This study aimed to investigate the role of protein inclusion levels combined with different non-protein energy sources (lipids and carbohydrates) in the metabolism and growth of European lobster. For that purpose, the present work compared the respiration and nitrogen excretion rates of European lobster juveniles (< 1 g) reared on Antarctic krill or experimental extruded dry feeds with different inclusion levels of protein, lipids, and carbohydrates. SMR, SDA response, nitrogen retention, and growth performance were determined and discussed.

MATERIALS AND METHODS

EXPERIMENTAL ANIMALS

Experiments were conducted at the aquaculture facilities at the Technical University of Denmark, Section for Aquaculture, Hirtshals. All experimental animals were hatched from eggs obtained from wild European lobster females caught along the Skagerrak coast of North Jutland, Denmark. Experimental lobster juveniles were reared individually in cassette systems consisting of 200 mL compartments. Cassettes were placed in raceways supplied by a flow-through semi-closed seawater system at a constant flow rate of 330 L h^{-1} (18 ± 0.5˚C temperature, 34 ± 1 PSU salinity, > 90% dissolved oxygen, <0.1mg L^{-1} ammonia-N), subjected to a photoperiod cycle of 8h light: 16h dark. Lobsters were fed once daily with thawed Antarctic krill, *Euphausia superba* (Akudim A/S, Denmark).

GROWTH TRIAL

Animals were held under the above-described conditions for four months from settling, after which they were randomly divided into seven treatment groups (N=10, per diet) while ensuring animals were of similar size across all treatments (0.86 ± 0.06 g wet weight, mean ± SEM). An experimental period of 32 days before respirometry and nitrogen excretion trials was used for evaluation of growth performance. During this period, each juvenile was individually fed its respective diet in excess each morning, and allowed to feed for 4h before uneaten food was removed. Additionally, juveniles were allowed to feed on their molted exoskeletons. Molt occurrences were recorded daily. At the beginning and end of the experimental period, lobsters were gently blotted dry with a paper towel and body wet weight was recorded to the nearest 0.01g. Carapace length was recorded with a vernier caliper from the base of the eye socket to the posterior edge of the cephalothorax. The following formulas were used:

$$Cumulative\ molting\ (CM,\%) = \sum_{i=0}^{n} (Mi \times Lob^{-1}) \times 100$$

where: i = the day; Mi = number of molts on the day i; Lob = number of lobsters in each treatment.

$$Specific\ growth\ rate\ (SGR,\%\ day^{-1}) = \left[ln\left(BW_f\right) - ln(BW_i)\right] \times days^{-1} \times 100$$

where: BW_f = final wet body weight, BW_i = initial wet body weight.

$$Carapace\ length\ increment\ (iCL,\%) = (CL_f - CL_i) \times CL_i^{-1} \times 100$$

where: CL_f = final carapace lentgh; CL_i = initial carapace length.

EXPERIMENTAL DIETS

Six formulated dry diets were evaluated using Antarctic krill as a reference diet. The six experimental dry diets were formulated to have two fixed protein levels (400 or 500 g kg^{-1}), and for each protein level, three L:CHO ratio levels (low: 0.3, medium: 0.5, and high: 0.8-1.0). Different protein, lipid, and carbohydrate contents were achieved by altering squid meal, wheat gluten, wheat starch, and fish oil inclusion levels. Experimental diets were extruded as 4 mm pellets and were manufactured by SPAROS Lda (Olhão, Portugal). Proximal analysis of krill and experimental diets were performed in duplicate. Briefly, the diets were finely ground using a Krups Speedy Pro homogenizer and analyzed for crude protein, (i.e. Kjeldahl N × 6.25, ISO 5983-2 (2005), crude fat (Bligh and Dyer, 1959), dry matter and ash (NMKL 23, 1991). Formulation and proximate composition are presented in Table 1.

TABLE 1. Formulation and chemical composition of experimental diets.

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

[a] Micronorse: 70.9% CP, 8.7% CF, Tromsø Fiskeindustri AS, Norway.
[b] Squid meal: 83% CP, 4% CF, Sopropêche, France.
[c] Krill meal: 61.1% CP, 17.4% CF, Aker Biomarine, Norway.
[d] VITAL: 80.4% CP, 5.6% CF, Roquette, France.
[e] Wheat meal: 11.7% CP, 1.6% CF, Molisur, Spain.
[f] Meritena 200: 0.4% CP, 0.1% CF, 90% starch, Tereos, France.
[g] Fish oil: 98.1% CF, 16% EPA, 12% DHA, Sopropêche, France.
[h] P700IPM, Lecico GmbH, Germany.
[i] Vitamins (IU or mg kg^{-1} diet): DL-alpha tocopherol acetate, 200 mg; sodium menadione bisulphate, 50 mg; retinyl acetate, 40000 IU; DL-cholecalciferol, 4000 IU; thiamine, 60 mg; riboflavin, 60 mg; pyridoxine, 40 mg; cyanocobalamin, 0.2 mg; nicotinic acid, 400 mg; folic acid, 30 mg; ascorbic acid, 1000 mg; inositol, 1000 mg; biotin, 6 mg; calcium pantothenate, 200 mg; choline chloride, 2000 mg, betaine, 1000 mg. Minerals (g or mg kg^{-1} diet): copper sulphate, 18 mg; ferric sulphate, 12 mg; potassium iodide, 1 mg; manganese oxide, 20 mg; sodium selenite, 0.02 mg; zinc sulphate, 15 mg; sodium chloride, 800 mg; excipient wheat gluten, Premix Lda., Portugal.
[j] Carophyll Pink 10% CWS, 10% astaxanthin, DSM Nutritional Products, Switzerland.
[x] Carbohydrate (%) = 100 – (Crude protein % + crude lipid % + moisture % + ash %)
[y] Gross energy (MJ kg^{-1}) = Protein content × 21.3 kJ g^{-1} + Lipid content × 39.5 kJ g^{-1} + Carbohydrate content × 17.6 kJ g^{-1}) / 1000 kJ MJ^{-1} (Cuzon and Guillaume, 1997)

RESPIROMETRY TRIAL

Measurements were performed on 10 intermolt lobsters per dietary treatment. All animals used were fasted for 48h prior to respirometry measurements to ensure a post-absorptive state. Experiments were performed in 75 mL respirometers supplied with temperature-controlled aerated seawater, using 8 chambers at a time. The bottom of each chamber was equipped with a perforated base plate, under which a magnetic stirrer ensured water mixing in the chamber. Oxygen content was registered every 15

sec using a sensor connected to an optical oxygen meter (FireSting O2, Pyro Science GmbH, Aachen, Germany) installed in each chamber. MO_2 measurements were performed by computerized intermittent flow, in loops of 60 min (consisting of a 5 min flushing period, followed by a 55 min closed period). The oxygen consumption rate was determined by linear regression of the decline in oxygen content during the closed period. The mass-specific oxygen consumption (MO_2) was calculated based on the slope of the regression according to Steffensen (1989) as:

$$MO_2 = \alpha \times V_{resp} \times \beta \times BW^{-1}$$

where: α = slope ($\Delta pO2 \times \Delta t^{-1}$), V_{resp} = volume of the chamber minus the volume of the lobster (using a lobster density of 1), β = oxygen solubility at the experimental temperature, and BW = wet body weight of the lobster.

Standard metabolic rate (SMR) was estimated from the first 48h MO_2 measurements, which was calculated as described by Skov et al. (2011). Briefly, MO_2 measurements were grouped in frequency classes, and SMR was calculated from the most frequently occurring bins and their relative contribution. Following SMR measurements, chambers were opened and a pre-weighed piece of thawed krill or a feed pellet was offered to each lobster. During feeding, chambers were kept unsealed, the flushing pump was stopped, and external aeration was provided. After a 2h feeding period, the remaining krill or pellet was carefully removed and MO_2 postprandial measurements resumed for 48h for estimation of SDA response. The uneaten feed fraction was collected, filtered, and dried for voluntary feed intake (VFI) estimation employing the following formula (Nguyen et al., 2014):

$$VFI = dF - uF - L$$

where: dF = distributed feed, uF = unconsumed feed, L = leaching after 2h. Leaching was estimated by placing a pre-weighed quantity of each diet in the chambers under the same conditions as in the feeding period but in this case, without animals.

For calculation of SDA variables digestion was determined to be completed when MO_2 postprandial measurements plotted over time fell within 15% of the SMR previously recorded for that chamber (Jordan and Steffensen, 2007). According to Secor (2009), the following SDA variables were calculated to describe the postprandial MO_2: SDA_{dur} (h) is the time from feeding until MO_2 converged with the SMR + 15%. The SDA_{cost} (μg O_2 g^{-1}) is the post-feeding integrated excess MO_2 above SMR. The SDA_{peak} (μg O_2 g^{-1} h^{-1}) is the maximum value of MO_2 above SMR during the SDA course and SDA_{ttp} (h) is the time from feeding to SDA_{peak}. SDA_{coef} (%) is the SDA_{cost} converted to energy using an oxycalorific coefficient of 14.06 J mg^{-1} O_2 (Dejours, 1981) and divided by the energy content of the meal which was calculated from the estimated feed intake. The scope is the SDA_{peak} divided by the SMR.

NITROGEN EXCRETION TRIAL

Following oxygen consumption measurements, each lobster was transferred to a 130 mL seawater container supplied with aeration. Water samples of 15mL were collected manually from individual chambers at time 0h and 48h for baseline screening of total ammonia and nitrogen excretion rates. After this period, lobsters were offered a pre-weighed pellet or krill piece for 2h. After the meal, lobsters were transferred into containers with fresh seawater. Water samples were manually collected at time 0h and 48h for the determination of total postprandial ammonia and nitrogen excretion rates. Collected water samples were filtered (0.2µm, Filtropur Sarstedt, Numbrecht, Germany) and stored at 0°C until analysis. Total nitrogen and ammonia nitrogen of collected water samples were determined in duplicate according to ISO 11905-1 (1997) and DS (1975), respectively. The voluntary feed intake was calculated from the uneaten fraction that was collected, filtered, and dried. N intake was calculated as 16% of protein intake (Chibnall et al., 1943). Total postprandial nitrogen excretion was calculated using the following formulas:

$$N_{pre-feeding} = [(N_{48h} \times V_{48h}) - (N_{0h} \times V_{0h})] \times BW^{-1}$$

$$N_{post-feeding} = [(N_{48h} \times V_{48h}) - (N_{0h} \times V_{0h})] \times BW^{-1}$$

$$N_{excreted} = N_{post-feeding} - N_{pre-feeding}$$

where: $N_{excreted}$ = total postprandial nitrogen excreted (mg total nitrogen per mg wet weight), $N_{pre\text{-}feeding}$ = pre-feeding nitrogen excretion (mg total nitrogen per mg wet weight), $N_{post\text{-}feeding}$ = post-feeding nitrogen excretion (mg total nitrogen per mg wet weight), N_{48h} = total nitrogen concentration at time 48h (mg total nitrogen per mL); N_{0h} = total nitrogen concentration at time 0h (mg total nitrogen per mL), V_{48h} = volume of the chamber at time 48h (mL), V_{0h} = volume of the chamber at time 0h (mL), BW = wet body weight of the lobster. Nitrogen retention was expressed as percentage of total N intake.

DATA ANALYSIS AND STATISTICS

Data are expressed as means ± SEM unless otherwise specified. All dietary treatments were subjected to a one-way ANOVA to test the experimental formulated dry feeds against the control diet (krill). Whenever significant differences were identified, comparisons against the krill diet were conducted using the Dunnett t-test. Data from experimental formulated dry feed treatments were subsequently subjected to a two-way ANOVA, considering protein level and L:CHO ratio as variables. Following a two-way ANOVA and whenever significant differences were identified, means were compared by the Holm-Sidak post hoc test. Data were checked for normal distribution and homogeneity of variances and, when necessary, log-transformed. Data expressed as percentage were arcsin transformed. Carapace length increment was log(x+2) transformed due to a high frequency of null observations. Statistical significance was set at $p \leq 0.05$. All statistical tests were performed using the

IBM SPSS Statistics 25.0 and graphics were generated by GraphPad Prism version 5.0 software package. The linear regression of the decline in oxygen content was computed using R version 3.5.1 software (R Core Team, 2018).

RESULTS

GROWTH PERFORMANCE

Observation during feeding showed that *H. gammarus* juveniles were attracted to all experimental diets and actively manipulated the offered feed. Minimum cumulative molting during the 32-day growth trial was recorded as 10% for the group of animals fed the 400-high diet. Maximum cumulative molting (90%) was observed for lobsters fed the krill diet (Figure 1). SGR was significantly higher ($F_{6,69} = 4.90$, $p < 0.001$) in krill-fed lobsters in comparison to lobsters fed experimental dry diets, with the exception of the 500-low diet (Figure 2A). Among experimental diets, SGR was unaffected by protein level ($F_{1,59} = 2.63$, $p = 0.11$), L:CHO ratio ($F_{2,59} = 2.62$, $p = 0.08$), or the interaction of the two variables ($F_{2,59} = 1.05$, $p = 0.36$). Nevertheless, protein content caused a significant positive effect on the carapace length increment (iCL) increment (Figure 2B). The iCL increment in lobsters fed the 500-high diet was significantly higher compared to animals fed the 400-high diet ($F_{1,59} = 6.12$, $p = 0.02$). The animals fed the 400-medium and 400-high experimental diets presented a significantly lower iCL in opposition to the krill-fed animals ($F_{1,69} = 2.89$, $p = 0.02$).

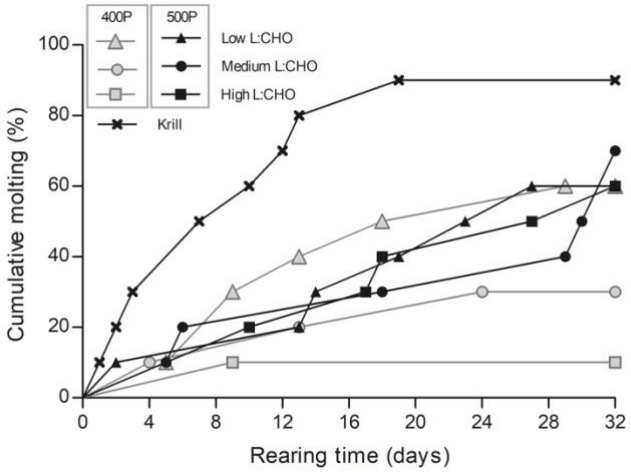

FIGURE 1. Cumulative molting of European lobster juveniles 8% of initial numbers) fed the different diets.

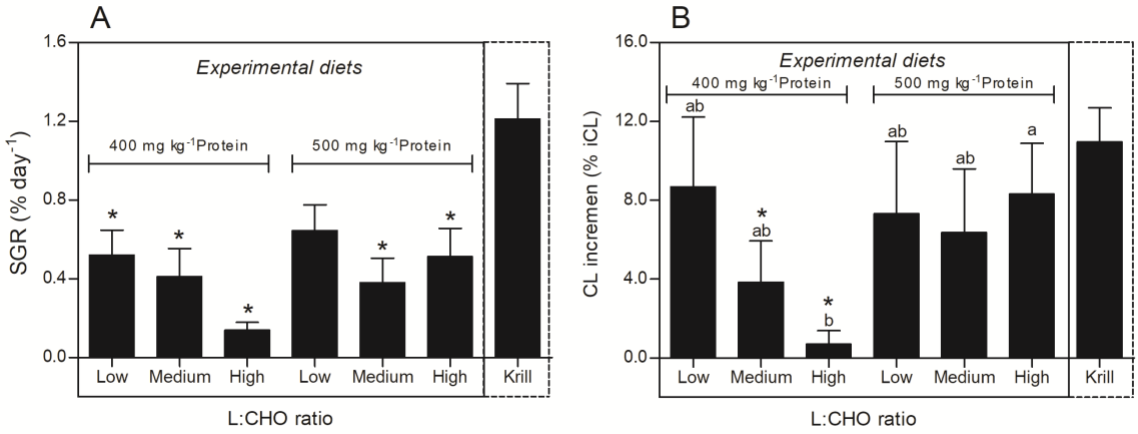

FIGURE 2. Specific growth rate (A) and carapace length increment (B) after a 32-day period for European lobster juveniles fed on different diets. Data represents the mean ± SEM of 10 animals per treatment. Dietary treatments that were significantly different from control (krill) are marked with an asterisk. Different letters indicate significant differences between experimental formulated diets.

Voluntary feed intake expressed as dry weight was higher ($F_{6,71}$= 3.19, p = 0.01) for the 400-low and 500-medium diets compared to the krill. No significant effect on feed intake for protein or L:CHO ratio was detected on experimental diets. However, the interaction of both was statistically meaningful ($F_{2,62}$ = 3.33, p = 0.04). The L:CHO ratio did not affect the 500 group of diets but in the 400 group, the 400-medium diet had a significantly lower VFI compared to the 400-low (Figure 3).

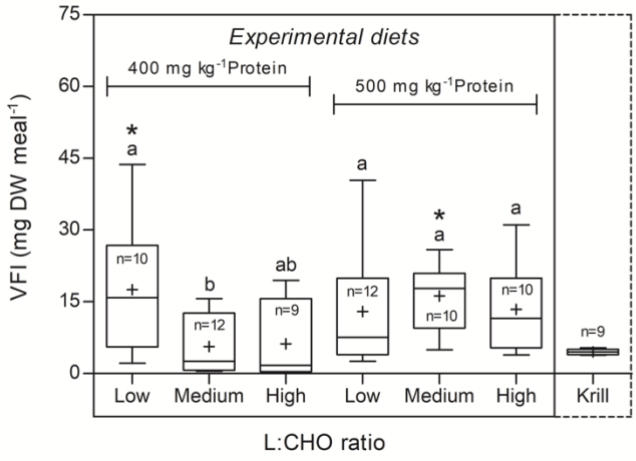

FIGURE 3. The effect of dietary treatment on voluntary feed intake. Estimated individual feed intake values from both respirometry and nitrogen excretion trials were pooled. The box includes observations from the 25th to the 75th percentile and the whiskers above and below the box indicate the 10th and 90th percentiles. The horizontal line within the box represents the median value and the symbol (+) indicates the mean. Dietary treatments significantly different from control (krill) are marked with an asterisk. Different letters indicate significant differences among experimental formulated diets.

TABLE 2. Overview of pre and postprandial metabolism responses for European lobsters fed six different experimental and one control diet.

Protein ($g.kg^{-1}$)	L:CHO	SMR ($\mu g\ O_2\ g^{-1}\ h^{-1}$)	SDA_{peak} ($\mu g\ O_2\ g^{-1}\ h^{-1}$)	Scope	SDA_{ttp} (h)	SDA_{cost} ($\mu g\ O_2\ g^{-1}$)	SDA_{dur} (h)	SDA_{coef} (%)	Meal energy (J)	N
	Low	75.6 ± 10.4 ^{ab}	249.4 ± 44.6	3.3 ± 0.4 ^{*,a}	9.0 ± 1.5	2079.6 ± 554.4	23.6 ± 0.5 ^{*,a}	3.9 ± 1.5	580.0 ± 130.2 *	5
400	Medium	51.2 ± 6.1 ^{*,bc}	137.6 ± 16.6	2.7 ± 0.2 ^{ab}	10.3 ± 0.9	704.2 ± 291.3	19.0 ± 1.7 ^{ab}	6.1 ± 2.5	245.1 ± 55.3	6
	High	42.7 ± 5.0 ^{*,c}	135.1 ± 27.2	3.2 ± 0.5 ^{*,a}	10.8 ± 1.3	581.9 ± 229.6	20.0 ± 2.2 ^{ab}	4.8 ± 3.6	278.9 ± 99.1	5
	Low	97.9 ± 11.9 ^{a}	214.0 ± 33.1	2.2 ± 0.2 ^{b}	9.8 ± 0.7	1099.3 ± 247.0	18.0 ± 0.4 ^{b}	3.5 ± 0.6	445.1 ± 160.9	6
500	Medium	80. 7 ± 4.0^{a}	224.2 ± 45.5	2.7 ± 0.5 ^{b}	11.3 ± 1.2	1683.8 ± 750.5	20.7 ± 0.8 ^{ab}	3.4 ± 1.2	473.0 ± 42.7 *	6
	High	73.1 ± 6.5 ^{a}	149.5 ± 22.5	2.0 ± 0.2 ^{b}	9.6 ± 0.7	621.0 ± 233.7	17.2 ± 1.3 ^{ab}	2.5 ± 1.0	438.7 ± 110.1	5
Control (Krill)		107.3 ± 19.3	186.3 ± 17.6	1.9 ± 0.2	7.6 ± 0.2	779.7 ± 229.5	17.2 ± 1.4 ^{ab}	7.0 ± 1.7	103.6 ± 5.6	5
[1] One-Way ANOVA		$F_{6,37}=5.27^{**}$	$F_{6,37}=1.99$	$F_{6,37}=2.83^{*}$	$F_{6,37}=1.50$	$F_{6,37}=1.45$	$F_{6,37}=2.90^{*}$	$F_{6,37}=0.58$	$F_{6,37}=2.76^{*}$	
[2] Two-Way ANOVA										
P		$F_{1,32}=17.70^{***}$	$F_{1,32}=0.63$	$F_{1,32}=7.69^{**}$	$F_{1,32}=0.39$	$F_{1,32}=0.39$	$F_{1,32}=4.50^{*}$	$F_{1,32}=0.51$	$F_{1,32}=1.19$	
L:CHO		$F_{2,32}=6.79^{**}$	$F_{2,32}=3.39^{*}$	$F_{2,32}=0.08$	$F_{2,32}=2.82$	$F_{2,32}=2.82$	$F_{2,32}=1.38$	$F_{2,32}=0.21$	$F_{2,32}=0.50$	
P x L:CHO		$F_{2,32}=0.15$	$F_{2,32}=1.73$	$F_{2,32}=1.95$	$F_{2,32}=1.36$	$F_{2,32}=1.36$	$F_{2,32}=4.21^{*}$	$F_{2,32}=0.13$	$F_{2,32}=2.57$	

Values are mean ± standard error.
[1] Superscript * indicate dietary groups significantly different from control (Krill).
[2] Means in the same column with a different superscript letter are significantly different.
* $p < 0.05$; $^{**}p<0.01$; $^{***}p<0.001$

METABOLIC RATES

The pre- and postprandial metabolic data for lobsters fed control and experimental diets are presented in Table 2. Individuals that did not ingest sufficient feed to induce a clear postprandial metabolic response were omitted from the analyses. Comparing the results of all treatments, animals fed the 400-medium and 400-high diets had significantly lower SMR compared to the krill-fed animals (Table 2, Figure 5). Within experimental diets, SMR was significantly affected by protein level and L:CHO ratio, but unaffected by the interaction of protein × L:CHO. With the exception of the 400-low, animals fed diets with 40% protein were observed to have significantly lower SMR values compared to the 50% protein diets (Table 2, Figure 5). The amount of ingested energy was significantly higher for lobsters fed on the 400-low and 500-medium diets compared to the krill-fed group (Table 2). No significant differences in meal energy were observed between experimental diets. SDA_{dur} was significantly longer in lobsters fed 400-low when compared to krill-fed lobsters. Within experimental diets, duration of SDA was affected by protein level and the interaction of protein × L:CHO, but was unaffected by L:CHO ratio (Table 2, Figure 4). Results showed lobsters fed the 500-low diet had a shorter SDA response compared to the animals fed on the 400-low diet.

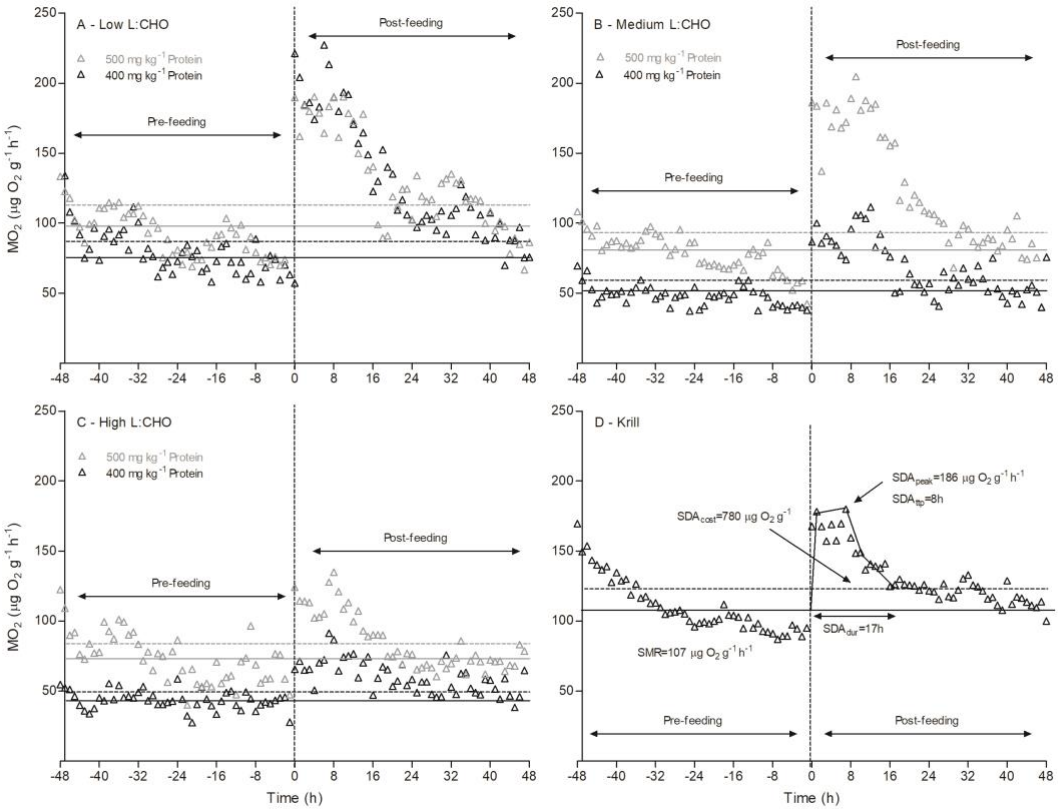

FIGURE 4. Representative plots of pre and post-feeding metabolic rates (µg O2 g^{-1} h^{-1}) over time in lobsters fed experimental (A, B, and C) and control (D) diets. The solid line represents SMR and the dashed line represents SMR + 15%. The lobsters were fed at 0 h (vertical dashed line). The SDA variables accounting for MO2 postprandial metabolism are visually explained in panel D.

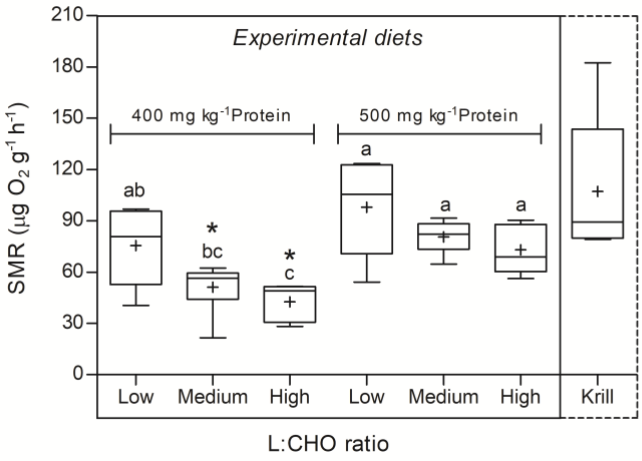

FIGURE 5. The effect of dietary treatment on the standard metabolic rate of European lobster juveniles. The box includes observations from the 25th to the 75th percentile and the whiskers above and below the box indicate the 10th and 90th percentiles. The horizontal line within the box represents the median value and the symbol (+) indicates the mean. Dietary treatments significantly different from control (krill) are marked with an asterisk. Different letters indicate significant differences among experimental formulated diets.

TABLE 3. Dietary effects on nitrogen budgets in juvenile European lobster.

Protein ($g.kg^{-1}$)	L:CHO	N_{intake} ($\mu g.mg\ WW^{-1}$)	$N_{excreted}$ ($\mu g.mg\ WW^{-1}$)	$N_{retention}$ (%)	N
	Low	806.44 ± 269.46 [a]	65.80 ± 43.32	86.02 ± 9.54 [ab]	5
400	Medium	65.32 ± 23.35 [*,b]	30.41 ± 9.63	41.58 ± 15.47 [b]	6
	High	42.73 ± 8.50 [*,b]	55.17 ± 25.91	-22.08 ± 38.85 [*,c]	4
	Low	839.33 ± 153.47 [a]	71.02 ± 13.42	90.85 ± 1.36 [a]	6
500	Medium	1166.83 ± 387.53 [a]	72.78 ± 11.34	91.98 ± 2.17 [a]	4
	High	873.94 ± 298.23 [a]	127.34 ± 71.63	87.12 ± 4.49 [a]	5
Control (Krill)		657.18 ± 74.53	190.85 ± 87.83	73.30 ± 12.11	4
[1] One-Way ANOVA		$F_{6,33}=17.14^{***}$	$F_{6,33}=1.00$	$F_{6,33}=6.96^{***}$	
[2] Two-Way ANOVA					
P		$F_{1,29}=52.21^{***}$	$F_{1,29}=2.71$	$F_{1,29}=19.03^{***}$	
L:CHO		$F_{2,29}=9.49^{**}$	$F_{2,29}=0.07$	$F_{2,29}=6.68^{**}$	
P x L:CHO		$F_{2,29}=9.64^{**}$	$F_{2,29}=0.38$	$F_{2,29}=5.79^{**}$	

Values are mean ± standard error.
[1] Superscript * indicate dietary groups significantly different from control (krill).
[2] Means in the same column with different superscript letter are significantly different.
* $p < 0.05$; ** $p<0.01$; *** $p<0.001$

NITROGEN RETENTION

The effects of dietary treatment on nitrogen budgets are shown in Table 3. As for the respirometry trials, animals that did not feed sufficiently were not included in the analysis. Within the experimental dietary groups, protein level, L:CHO ratio, and the interaction of the two, all significantly affected nitrogen intake. N intake was generally higher for the 50% protein diets with no effect of L:CHO. Among the 40% protein diets, N intake was highest for the 400-low diet. Animals fed the 400-

medium and 400-high diets presented a lower N intake in comparison to krill. No significant differences were observed for the total nitrogen excreted. Comparing all the dietary treatments, nitrogen retention was significantly lower in animals fed the 400-high diet against lobsters fed krill. Within the experimental diets the % total N retention was significantly affected by protein level, L:CHO ratio, and the interaction of protein × L:CHO ratio. Nitrogen retention was higher for the 50% protein diets with no effect of L:CHO. The lowest N retention was observed in the 40% protein, with a significant decrease with increasing L:CHO ratios (Table 3). The contribution of ammonia to the total nitrogen excreted varied between 64% and 88% among dietary treatments (data not presented) but no statistical differences were found ($F_{6,33} = 1.37$, $p = 0.27$).

DISCUSSION

The present study demonstrates a limited successful growth of European lobster juveniles fed on formulated dry feeds. Results indicate that SGR of European lobster juveniles fed the 500-low diet (50% protein, 9% lipid, 26% carbohydrate) was not significantly lower than the control group fed on krill. However, the cumulative molting rate was lower in all treatment groups fed formulated experimental diets. Results suggest that the poor growth performance of *H. gammarus* fed on formulated feeds remains one of the principal obstacles in the development of sustainable aquaculture of this species. Further optimization of formulated diets in terms of mechanical and chemical digestion is imperative. Improving pellet size, format, and texture, and supplementing feeds with additives (digestible binders, pH buffers, and exogenous enzymes) need to be addressed in future research.

Results from this study establish that the dietary regime affects the SMR in European lobster juveniles. Animals fed the 400-medium (40% protein, 15% lipid, and 31% carbohydrate) and 400-high diets (40% protein, 23% lipid, and 23% carbohydrate) showed the lowest SMR. According to Biro and Stamps (2010) a higher SMR is associated with a larger metabolic capacity. In the same study, the authors suggested that individuals with high metabolism were able to process larger meals. Therefore, under this hypothesis, SMR is expected to produce a positive impact on performance (Burton et al., 2011). Our results showed that the juveniles fed the 400-medium and 400-high experimental diets presented the poorest performance in terms of cumulative molting, SGR, and CL increment, confirming Biro and Stamps (2010) hypothesis. The voluntary feed intake for the group of animals fed these two diets was also the lowest, which most likely contributed to the poorest growth performance. Protein synthesis, a crucial process in growth, is strongly affected by the feed intake. Previous studies in several crustacean species have demonstrated that protein synthesis rates generally decrease in starved or less frequently fed animals (Carter and Mente, 2014). The reason why the feed intake was lower for the 400-medium and 400-high diets remains unclear but it might be related to lower palatability of these two diets, or that high lipid levels cause faster satiation. The growth compensation for animals fed the 400-

low diet, i.e., low lipid (11%) and high carbohydrate (35%) is in agreement with the effect observed between SMR and L:CHO ratio. This result supports our initial hypothesis that carbohydrate represent an important macronutrient for *H. gammarus* especially in diets with reduced protein content. In fact, carbohydrates are important for crustacean species as glycogen is an essential precursor of chitin synthesis, serving a critical role during the molt cycle (Wang et al., 2016).

Studies on the SDA in lobster species are scarce, and, to our knowledge, there is no information for European lobsters. In this work, we observed that feeding caused a rise in oxygen consumption 2 to 3 times above SMR levels in European lobster juveniles fed the different diets. In a previous study, in 3.2 g *Homarus americanus* fed on formulated diets, Koshio et al. (1992) reported an SDA scope of 1.5. The smaller size of the lobsters tested in this study can explain the difference in the SDA scope, as the animal size is known to influence the SDA variables (McCue, 2006). In the present study, the time to achieve the SDA peak ranged between 8h to 11h, with elevated oxygen consumption rates lasting for 17 to 24h. The SDA duration was significantly longer for the 400-low experimental diet compared to krill, which is likely related to the higher meal energy or protein intake (Secor, 2009). SDA duration in southern rock lobster (*Jasus edwardsii*) fed squid was longer than what we observed in European lobster juveniles. Crear and Forteath (2000) reported that 750 g *J. edwardsii* took 42h to return to the pre-feeding oxygen consumption level, while Radford et al. (2004) observed that SDA response in 16 g animals of the same species lasted 30h. In this study, SDA coefficient results showed that juveniles fed on the tested diets spend between 3.4% to 7.0% of the meal energy on digestive processes. These results are in agreement with the findings by Crear and Forteath (2000) who reported an SDA coefficient of 6.6% in *J. edwardsii*. Nevertheless, the amount of meal energy allocated to SDA reported for crustacean species is highly variable even within the same species. For example, Houlihan et al. (1990) observed an SDA_{coef} of 13.3% for 37 g *Carcinus maenas* while Wallace (1973) reported an SDA_{coef} of 3.4% in 10 g individuals of the same species. Collectively, these findings suggest that the duration of the SDA response and the SDA coefficient increases with increasing body sizes.

In this study, we observed that juveniles excreted the majority of the nitrogenous waste in the form of ammonia (64-88%). Results reported here suggest that the mechanism for nitrogen excretion in *H. gammarus* is similar to other aquatic crustacean species, namely, the *H. americanus* (Burger, 1957) and *J. edwardsii* (Binns and Peterson, 1969). Total nitrogen budget results showed that the nitrogen retention of juveniles fed the 400-high diet (40% protein, 23% carbohydrate, and 23% lipid content) was significantly lower than in juveniles fed the other experimental or control diets. The severely reduced N intake in this group of animals induced a negative nitrogen balance, i.e., nitrogen excretion exceeded the nitrogen intake. The incapacity of this group of animals for nitrogen retention suggested that, rather than protein deposition, animals were periodically undergoing tissue protein catabolism (Guo et al., 2012).

Results from this study suggest that European lobster juveniles with low SMR and low nitrogen retention have a reduced growth capacity. Nevertheless, the estimated SMR and nitrogen retention were highly affected by the feed intake of the different tested diets. Therefore, the results presented in this study should be interpreted with caution. Moreover, the growth performance indices were calculated over a 32-day period, which could be considered relatively short, particularly in the case of crustacean species. In these animals, wet weight changes follow a typical pattern through the molt cycle. The highest increase occurs in the brief period of rapid water uptake at ecdysis. Further moderate gains are related to carapace mineralization and tissue growth. Finally, during the intermolt period, there is a relative stabilization of fresh weight until the onset of the successive ecdysis (Nguyen et al., 2014). As follows, nutritional studies targeting evaluation of growth performance should allow at least one complete molt cycle per individual.

SMR and nitrogen retention results from this study corroborate the hypothesis that juvenile *H. gammarus* perform better fed on 500 against 400 g kg^{-1} protein content in their diet. This level agrees with the protein content (52% DM) in European lobster larvae reported by Powell et al. (2017) supporting the idea that diets should meet the organism's biochemical composition. The results of this study show that protein is a fundamental nutrient in formulated dry feeds. However, its inclusion can potentially be reduced when compensated with appropriate carbohydrate levels. Carbohydrates are the least expensive energy source for aquatic animals (Wang et al., 2016) and therefore, this is an important opportunity for the production of sustainable and economically viable formulated feeds for this species.

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STUDY 3

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

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

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Abstract

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

Keywords: Digestive enzymatic activity; Feed efficiency; Macronutrients; Nucleic acid indices.

INTRODUCTION

The European lobster (*Homarus gammarus*, L.) is an economically important decapod crustacean geographically ranging from Northern Norway to Morocco and the Eastern Mediterranean (Triantafyllidis et al., 2005). The increased fishing pressure from 1930 to 1970 led to the collapse of several European lobster populations throughout Europe (Agnalt et al., 1999). To counteract the decline, multiple experimental stock enhancement programs have been launched to release large numbers of

juveniles to lobster grounds in France (Latrouite and Lorec, 1991), the United Kingdom (Cook, 1995), Ireland (Browne and Mercer, 1998), Norway (Agnalt, 2004), and Germany (Schmalenbach et al., 2011). Commercial aquaculture is gaining interest as an additional approach. However, in comparison with other aquaculture sectors, lobster farming still faces several constraints for their commercial production to be economically sustainable. Among them are the high mortality rates, energy and feeding costs (Powell, 2016). Significant advances in automated rearing systems and water quality have been made in recent years that improve European lobster hatchery production and cost-effectiveness (Daniels et al., 2015, 2013; Drengstig and Bergheim, 2013; Halswell et al., 2018; Middlemiss et al., 2015). Nevertheless, the lack of a balanced artificial feed that is affordable, with better nutritional value, and easier to handle and store compared to live, fresh, or frozen foods remains a major drawback in the sustainable farming of *H. gammarus* (Gamble et al., 2015; Powell et al., 2017).

Fresh, live mussels (Crear and Forteath, 2002; Floreto et al., 2000) and frozen krill (Burton, 2003; Goncalves et al., 2020) are commonly used as preferred reference diets, as they appear to perform consistently well as food for several lobster species. However, the future success of lobster aquaculture will depend on the ability to replace live, fresh, or frozen diets by more practical and cost-effective artificial feeds. The transition to specifically formulated, dry pelleted feeds has been applied with relative good success to several crustacean species within the Penaeidae (Wickins and Lee, 2002). While considerable research efforts in the last three decades have been devoted to spiny lobster species (Palinuridae), the transition to extruded feeds has proven to be challenging (Perera and Simon, 2015; K.C. Williams, 2007). Results indicate that protein is the most important macronutrient for optimal growth. Carbohydrates are readily digested and absorbed but their subsequent fate is poorly understood, while lipids appear to be poorly digested, absorbed and used. These findings are in agreement with the chemical composition of the preferred prey of several spiny lobster species which presents high protein, low lipid, and moderate to high carbohydrate content (Kevin C. Williams, 2007).

The feasibility of using artificial feeds for the production of juvenile American lobster (*Homarus americanus*) was extensively investigated (Castell and Budson, 1974; Conklin et al., 1977; Floreto et al., 2000; Lim et al., 1997; Tlusty et al., 2005) but growth obtained was not as high as animals that are fed live/fresh food. The efforts on the development of artificial feeds for the European lobster have been less and only a few published studies refer to the potential application of formulated feeds for juvenile *H. gammarus* farming (Goncalves et al., 2020; Hinchcliffe et al., 2020). Reported results are similar to what has been observed for the *H. americanus*, i.e., lower growth performance in lobsters fed the experimental artificial diets. The relatively limited success of formulated feeds for lobster juveniles can be partially explained by the lack of information on how lobsters digest and assimilate formulated feeds (Perera and Simon, 2015). Additionally, the higher growth and feeding rates of lobsters fed natural diets has been generally related to: (a) their superior water stability and attractiveness and (b) a nutrient profile approximating closely to their body composition (Tacon, 1996).

Most nutritional studies performed with crustacean species address weight and length gains as growth performance indicators. However, maximum size *per se* does not necessarily imply the best condition. This is particularly relevant in decapod crustacean species since their body weight is highly influenced by the water content and moulting stage (Albalat et al., 2019). The use of biochemical indicators can aid in a more comprehensive evaluation of the nutritional status of the reared animals. The ratio of RNA:DNA concentrations has been previously used as an indicator of recent growth and nutritional condition in a variety of crustacean species including the *H. gammarus* (Parslow-Williams et al., 2001; Sacristán et al., 2019; Schoo et al., 2014; Yun et al., 2016). The main premises are that the amount of RNA in a cell varies in proportion to protein synthesis, whereas the amount of DNA remains fairly constant (Chícharo and Chícharo, 2008). Thus, theoretically, poor nutritional condition contributes to low protein synthesis and slow growth, resulting in a low RNA:DNA ratio (Labh et al., 2014).

Increasing the knowledge on the activity of digestive enzymes of European lobsters is essential to establish a better understanding of their digestive ability. Analysing the digestive response might help revealing why some diets show better performance than others might. There are few published studies referring to the digestive capacity of *H. gammarus.* A range of carbohydrases (Glass and Stark, 1995) and proteases (Glass and Stark, 1994) have been detected in the hepatopancreas and gastric juice of wild caught adults. However, changes in the digestive enzyme activity with type of food or nutritional composition have not yet been determined in European lobster. To the best of our knowledge, only one such study on lobsters has been published (Simon, 2009) but on a different species. In this cited work, the rearing of juvenile spiny lobsters (*Jasus edwardsii*) on a formulated diet for six months resulted in a pronounced decrease in the enzymatic activity (protease, trypsin, α-amylase, and α-glucosidase) of the foregut and digestive gland compared to juveniles reared on fresh mussels.

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

MATERIALS AND METHODS

EXPERIMENTAL ANIMALS

Juveniles of *H. gammarus* were obtained from a wild European lobster female caught in the Skagerrak coast of North Jutland, Denmark. Animals used in this experiment were procured from the same female to reduce phenotypic variation. The fertility/fecundity of the chosen female, as well as the

survival and apparent quality of her progeny was observed to be as usual in our facilities. From hatching and until settling, larvae were reared in 500-l rectangular tanks installed at the North Sea Oceanarium, Hirtshals. Each tank was equipped with a top seawater inflow (50 l h^{-1}) and a vertical outflow filter (1mm mesh diameter). Temperature and salinity were kept at 18 ± 1 ºC and 34 ± 1 PSU, respectively. Strong aeration was provided from the bottom of the tank using airstones to maintain larvae in the water column. Larvae were stocked sequentially into tanks over three consecutive days after hatching at an initial density of 5-7 larvae l^{-1} and daily fed thawed Antarctic Krill, Euphausia superba (Akudim A/S, Denmark). The bottom of the tanks was syphoned every week to remove debris. Upon metamorphosis to stage IV (Rötzer and Haug, 2015), post-larvae were transferred to the aquaculture facilities at the Technical University of Denmark, Section for Aquaculture, Hirtshals. Animals were individually reared in 3D printed PolyLacticAcid (PLA) bioplastic cassette systems of 200 ml compartments with perforated grids for water in- and outlet. Cassettes were distributed and immersed in raceway tanks of 80-l capacity supplied by recirculation seawater system at a constant flow rate of 330 l h^{-1} (18 ± 0.5 ºC temperature, 34 ± 1 PSU salinity, > 90% dissolved oxygen, < 0.1 mg l^{-1} ammonia-N) subjected to a photoperiod cycle of 8h light:16 h dark. Lobsters were adapted to the trial conditions for one month, during which they were daily fed thawed Antarctic krill.

EXPERIMENTAL EXTRUDED FEEDS AND CONTROL DIET

Details of the experimental feeds and control diet are reported in (Goncalves et al., 2020). Briefly, experimental extruded feeds were formulated to contain 400 or 500 g kg^{-1} protein, with lipid contents ranging from 86 to 233 g kg^{-1} and carbohydrates from 210 to 347 g kg^{-1} resulting in three ratios of lipid to carbohydrate: low (1:3), medium (1:2), and high (approx. 1:1). This resulted in six experimental feeds referred as 400LOW, 400MED, 400HIGH, 500LOW, 500MED, and 500HIGH. The different protein, carbohydrate, and lipid contents were achieved by altering squid meal, wheat gluten, wheat starch, and fish oil inclusion levels. Experimental feeds were extruded as 4 mm pellets and were manufactured by SPAROS, Lda. (Olhão, Portugal). Thawed Antarctic krill was used as control feed (CTRL). Proximate analysis of the Antarctic krill and extruded feeds were performed in duplicate. Feeds and krill were finely ground using a Krups Speedy Pro homogenizer and analysed for crude protein (i.e. Kjeldahl N × 6.25 (ISO 5983-2, 2005)), total lipid (Bligh and Dyer, 1959), dry matter, and ash (NMKL 23, 1991). Likewise, amino acid analyses was performed in duplicate by use of hydrolysed feed samples (Rutherfurd, 2009). The amino acid content was determined by HPLC (Larsen et al., 2012). The ingredients and proximate chemical composition of the experimental extruded feeds and control diet are shown in Table 1. The amino acid profiles of the extruded feeds and control diet are listed in Table 2.

TABLE 1. Formulation and chemical composition of experimental extruded feeds and Antarctic krill (adapted from (Goncalves et al., 2020)).

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

[a] Micronorse: 70.9% CP, 8.7% CF, Tromsø Fiskeindustri AS, Norway.
[b] Squid meal: 83% CP, 4% CF, Sopropêche, France.
[c] Krill meal: 61.1% CP, 17.4% CF, Aker Biomarine, Norway.
[d] VITAL: 80.4% CP, 5.6% CF, Roquette, France.
[e] Wheat meal: 11.7% CP, 1.6% CF, Molisur, Spain.
[f] Meritena 200: 0.4% CP, 0.1% CF, 90% starch, Tereos, France.
[g] Fish oil: 98.1% CF, 16% EPA, 12% DHA, Sopropêche, France.
[h] P700IPM, Lecico GmbH, Germany.
[i] Vitamins (IU or mg kg^{-1} diet): DL-alpha tocopherol acetate, 200 mg; sodium menadione bisulphate, 50 mg; retinyl acetate, 40000 IU; DL-cholecalciferol, 4000 IU; thiamine, 60 mg; riboflavin, 60 mg; pyridoxine, 40 mg; cyanocobalamin, 0.2 mg; nicotinic acid, 400 mg; folic acid, 30 mg; ascorbic acid, 1000 mg; inositol, 1000 mg; biotin, 6 mg; calcium pantothenate, 200 mg; choline chloride, 2000 mg, betaine, 1000 mg. Minerals (g or mg kg^{-1} diet): copper sulphate, 18 mg; ferric sulphate, 12 mg; potassium iodide, 1 mg; manganese oxide, 20 mg; sodium selenite, 0.02 mg; zinc sulphate, 15 mg; sodium chloride, 800 mg; excipient wheat gluten, Premix Lda., Portugal.
[j] Carophyll Pink 10% CWS, 10% astaxanthin, DSM Nutritional Products, Switzerland.
[x] Estimated by difference: Carbohydrates (%) = 100 - (Crude protein + Total lipid + Ash).
[y] Gross energy (MJ kg^{-1}) = Protein content × 21.3 kJ g^{-1} + Lipid content × 39.5 kJ g^{-1} + Carbohydrate content × 17.6 kJ g^{-1}) / 1000 kJ MJ^{-1} (71)

TABLE 2. Amino acid profile (g per 100 g as is) of experimental extruded feeds and Antarctic krill.

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

GROWTH TRIAL

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

Individual lobsters were hand-fed in excess a pre-weighed amount of thawed krill or extruded pellet each morning, and allowed to feed for 4h. Thus, all lobsters had equal access to feed for a limited amount of time per day. Additionally, lobsters were permitted to consume their shedded exoskeletons. After each meal, the uneaten feed was siphoned off and stored in 50 ml tubes at -20 º C. To estimate feed intake (FI), each treatment was divided into subgroups of 10 individuals. The uneaten feed fraction of each subgroup (minus sampled or dead animals, when applicable) was collected daily, accumulated over 6 weeks, and stored in a single tube, allowing triplicate values to be obtained for FI estimation. At the end of the trial each tube content was filtered, dried (24h at 60 ºC), and weighed for FI estimation using the following formula (Nguyen et al., 2014):

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

where: dF = distributed feed, uF = unconsumed feed, L = leaching after 4 h, BW_i = initial body weight, Δt = number of days during which uneaten feed was collected (42 days). Leaching was estimated by placing a pre-weighed quantity of each diet in the cassette compartments under the same conditions as in the trial but in this case, without animals. FI was expressed in percent of dry matter ingested per

initial body weight per day. Energy intake was calculated by multiplying the daily individual intake (mg) by the gross energy content of each diet.

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

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

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

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

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

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

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

JUVENILE LOBSTERS PROXIMATE CHEMICAL COMPOSITION

At the end of the trial lobsters were lethally anesthetized in ice-cold seawater, weighed, measured, rinsed in distilled water, and stored at -80ºC for proximate chemical composition analysis. Proximate analysis of the juveniles was performed in analytical triplicates per dietary treatment. Briefly, the pool of six to twelve individuals per treatment were finely ground using a Krups Speedy Pro homogenizer and analysed for dry matter and ash (NMKL 23, 1991). Protein was determined spectrophotometrically at 750 nm using a commercial Lowry-based, micro-protein determination kit (BIO-RAD 500-0112). Lipids were extracted with chloroform - methanol (2:1 by volume) according to the Folch method (Christie and Han, 2010) and lipid content determined gravimetrically.

NUCLEIC ACID DETERMINATIONS

At week 4, six juveniles per dietary treatment were collected for nucleic acid analysis 24h after being fed. Lobsters were lethally anesthetized in ice-cold seawater, weighed, measured, and rinsed in distilled water before being frozen at -80ºC. Lobsters were freeze-dried and weighed (± 1 µg dry weight [DW]) on an electronic microbalance (Sartorius M5P). The concentration of nucleic acids (RNA, DNA) was quantified in approx. 1 mg of dry abdominal muscle tissue from each individual lobster following procedures described previously (Caldarone et al., 2001). Briefly, the muscle samples were chemically (cold sarcosyl Tris-EDTA extraction buffer) and mechanically homogenized in an ultrasonic homogenizer unit (4710 Series, Cole Parmer Instruments Co.). The homogenate was centrifuged for 15 min at 1,200 g, 4 ºC, and the supernatant extract was used for the analysis of RNA and DNA. The supernatants (30 µl) were placed into fluorescent microplate wells with Tris buffer (140 µl). Finally, a specific nucleic acid fluorochrome dye GelRED (30 µl) was added into each well for the fluorescent reading of nucleic acids. Fluorescence was measured on a microplate reader (Biotek Synergy HT model SIAFRTD – BioTek Instruments, Inc., Vermont, USA) with an excitation wavelength of 365 nm and an emission wavelength of 590 nm. Following the first scan to determine total fluorescence of RNA and DNA, a ribonuclease A (Type-II A) solution (30 µl) was added to each well and activated by incubating the fluorescent plates at 37ºC for 30 minutes. The plates were read again, and the concentration of DNA was calculated directly from the standard curve described below. The RNA fluorescence was calculated subtracting the DNA fluorescence (second scan) from total fluorescence (first scan). Concentrations were determined by running standard curves of DNA-GelRED and RNA-GelRED with known concentrations of ƛ-phagus DNA (0.25 µg μl^{-1}) and 16S-23S E. coli RNA (4 µg μl^{-1}) (Roche). The average ratio of DNA and RNA slopes (average ± SD) was 5.85 ± 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 physiological condition of the juveniles was estimated with nucleic acid derived indices: RNA (µg mg^{-1} DW), DNA (µg mg^{-1} DW), and sRD (standardized RNA/DNA ratios).

DIGESTIVE ENZYME ACTIVITIES

At week 6 nine lobsters per treatment were collected 4h to 5h after being fed for the analysis of digestive enzyme activities. Individuals were rinsed in distilled water and stored at -80 ºC. To prepare the enzyme extracts, juveniles were previously freeze-dried. Rostrum, chelipeds, legs, pleopods, uropods, and telson were removed and the abdominal section of each individual was mechanically homogenized in 1 ml distilled water. The homogenate was centrifuged for 10 min at 15,800 g, 4 ºC, and the supernatant extract was used for the analysis of trypsin, amylase, and lipase activities. All samples

were kept in ice all times to avoid enzymes denaturation or damage. Enzyme extract aliquots were stored at -80 ºC until analysis.

For trypsin analysis, the fluorogenic substrate Boc-Gln-Ala-Arg-7-methylcoumarin hydrochloride (BOC-SIGMA B4153) was diluted in dimethyl sulfoxide (DMSO), to a final concentration of 20 µM. For analysis, 5 µl of this substrate, 190 µl of 50mM Tris + 10 mM $CaCl_2$ buffer (pH 8.5) and 15 µl of the enzyme extract were added to the microplate (Rotllant et al., 2008). Fluorescence was measured at 355 nm (excitation) and 460 nm (emission).

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

Lipase activity was assayed using 4-methylumbelliferyl heptanoate (M2514, Sigma-Aldrich). The substrate was dissolved in phosphate buffer pH 7.0 to a final concentration of 0.4 mM, modified method from (Rotllant et al., 2008). Fifteen µl of the lobster extract was added to the microplate and mixed with 250 µl of 0.4 mM substrate for the analysis. Fluorescence was measured at 355 nm (excitation) and 460 nm (emission).

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

STATISTICAL ANALYSES

The amino acid profiles of control diet and experimental feeds were analysed by principal component analysis performed using R version 3.5.1 software (R Core Team, 2018) and the factoextra version 1.0.7 package (Kasambara and Mundt, 2020). Survival and moult occurrence data were analysed by a Kaplan-Meier procedure. Log-rank (Mantel-Cox) test was used to determine significance ($p<0.05$). Whenever significance was detected, a Chi-square table with multiple comparisons was generated to identify differences among dietary treatments. All other parameters shown are expressed as means ± 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 root or log-transformed. A second order polynomial regression with time was used as an estimate of the average growth, both in terms of carapace length and body weight. The models were validated via residual plot analysis and generated using R version 3.5.1 software (R Core Team, 2018). Curve coefficients were compared in a one-way ANOVA and whenever significant differences were detected, the coefficients from

experimental feed curves were tested against the CTRL using the Dunnett t-test. For the remaining estimated growth parameters, RNA and DNA concentrations, sRD, and digestive enzyme activities, the dietary treatments were subjected to a one-way ANOVA to test the experimental formulated dry feeds against the CTRL diet. Whenever significant differences were identified, comparisons against the CTRL diet were conducted using the Dunnett t-test. Data from experimental extruded feed treatments were subsequently subjected to a two-way ANOVA, considering protein level and L:CHO ratio as explanatory variables. Following the two-way ANOVA and, whenever significant differences were identified, means were compared by the Holm-Sidak post hoc test. Standardized RNA/DNA ratio was expected to reflect changes in growth performance parameters (Grimm et al., 2015). Thus, a simple linear regression was calculated to predict sRD based on SGR. Apart from the principal component analysis and the second order polynomial regression for carapace length and body weight, all statistical tests were performed using the IBM SPSS Statistics 25.0 and graphics were generated by GraphPad Prism version 5.0 software package.

RESULTS

DIETS AMINO ACID COMPOSTION

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

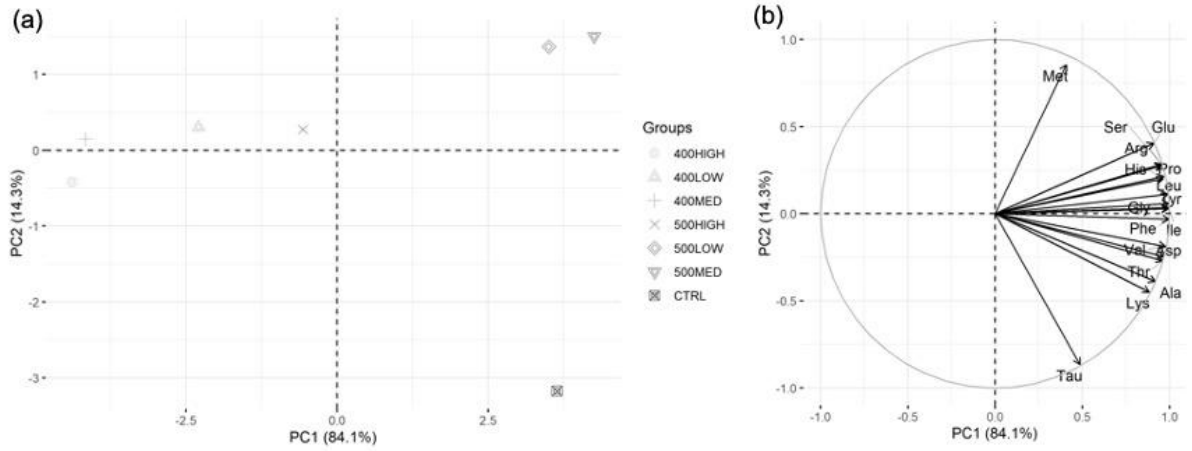

FIGURE 1. Graphical representation of principal components analysis of amino acid profiles from tested diets. The two panels are complementary to one another. (a)Biplot of the first two principal components (PC1 and PC2) of tested diets amino acid profiles. PC1 separated the amino acid profiles horizontally andexplained 84·1% of the variance. The amino acid profiles of 500MED, 500LOW and CTRL diets formed a succinct group to the right of the plot and were positively correlated to PC1.The 400HIGH, 400MED, 400LOW and 500HIGH diets were negatively correlated to PC1. PC2 separated the amino acid profiles vertically and explained 14·3%ofthe variance. The 500LOW and 500MED diets were positively correlated, while the CTRL was negatively correlated to PC2. (b) Variables (amino acids) used toconstruct the principal components. The circle in this plot is the correlation circle, the stronger the correlation of an amino acid to PC1 or PC2 the closer its arrowheadto the circle. The arrows indicate how the amino acids contributed to the formation of PC1 and PC2 and thus the formation of plot (a).

SURVIVAL, GROWTH, AND FEED UTILIZATION

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

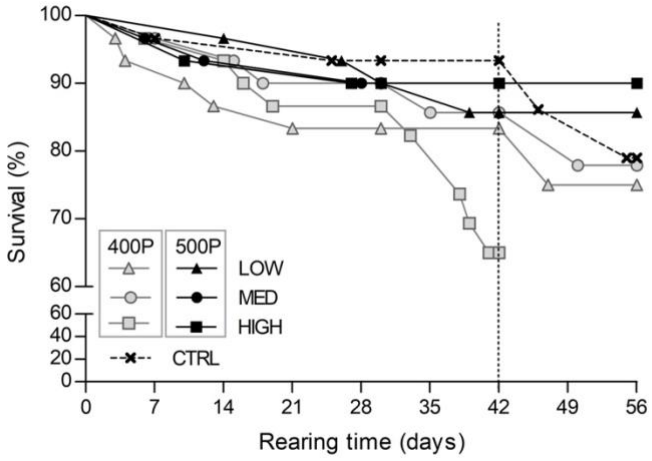

FIGURE 2. Survival of *H. gammarus* juveniles (% of initial numbers) fed on the dif-ferent diets. The dashed vertical line indicates the rearing time limit (42 d) con-sidered in the statistical comparison of the curves.

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

Changes in weight and carapace length throughout the trial were evaluated by second order polynomial regression with time. Regression curves are represented for all dietary treatments in Fig. 4. However, because the trial ended at week 6 for the 400HIGH treatment, the curve coefficients from this group were not statistically compared with the CTRL. During the 8-week experimental period, lobsters grew from an initial mean weight of 90 mg (6.5 mm carapace length) to mean weights ranging from 134 to 254 mg (7.9 – 9.5 mm carapace length). The juveniles fed the 500MED and CTRL diets sustained an increase in carapace length and body weight over the 8-week growth trial. The remaining groups reached a growth plateau, both in terms of length and weight, after 6 weeks (Fig. 4). The 400MED and 500LOW experimental feeds had a significant lower carapace length increase over time compared to the CTRL (Fig 4, panels a and b). In terms of body weight gain over the trial period, all the experimental dietary groups except the 500MED and 400LOW showed a significantly slower growth compared to the reference group (CTRL) (Fig 4, panels c and d).

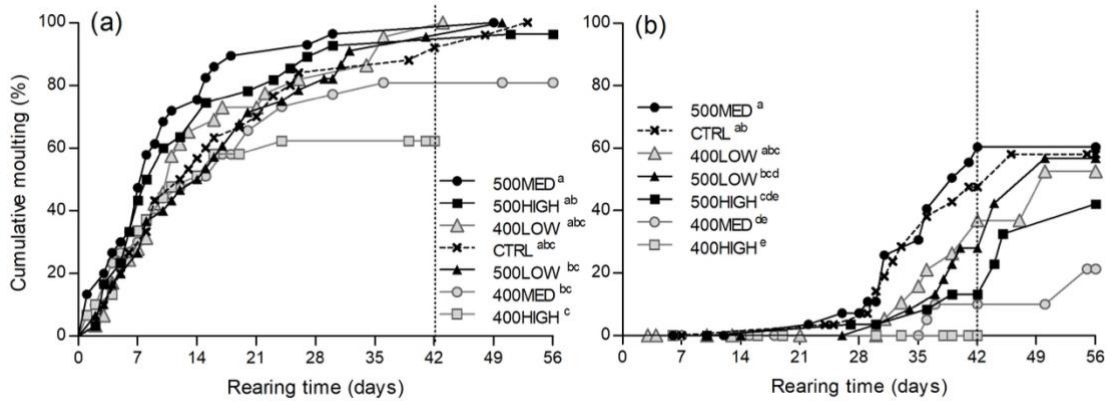

FIGURE 3. Cumulative moults of *H. gammarus* juveniles (% of initial numbers) fed on different diets. Panel (a) refers to the first moult occurred after the beginning of thegrowth trial and panel (b) refers to the second moult occurrence. Dietary treatments are indicated in the legend in ascending order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

The growth performance indices - SGR and iCL- were determined for a 6 weeks period in all treatments (Table 3). A significantly higher SGR was observed for krill-fed lobsters in comparison to lobsters fed the extruded feeds, except for the 500MED diet (Table 3). Within the extruded feeds, SGR was significantly affected by protein level, L:CHO ratio and the interaction of protein × L:CHO ratio. Results showed that for the 400-protein group, juveniles fed the 400LOW diet grew faster than the ones fed the 400MED and 400HIGH feeds. Within the 500-protein level, a significantly faster growth was observed in the 500MED treatment. The protein content did not affect the SGR in the LOW L:CHO ratio category while in the MED and HIGH groups juveniles fed the 500 g kg^{-1} protein feeds grew significantly faster (Table 3). Juveniles fed the 400MED, 400HIGH, and 500HIGH feeds had a significantly lower iCL than the CTRL group. The protein content, L:CHO ratio, and protein × L:CHO all had a significant effect on the iCL. Among the 400-protein level, the highest iCL was achieved for

the 400LOW and the lowest for the 400HIGH group. Within the 500 level, the highest iCL was observed for the 500MED and the lowest for the 500HIGH treatment. The protein content only had a significant effect on iCL at the intermediate level of L:CHO for which the 500MED were larger than the 400MED group (Table 3).

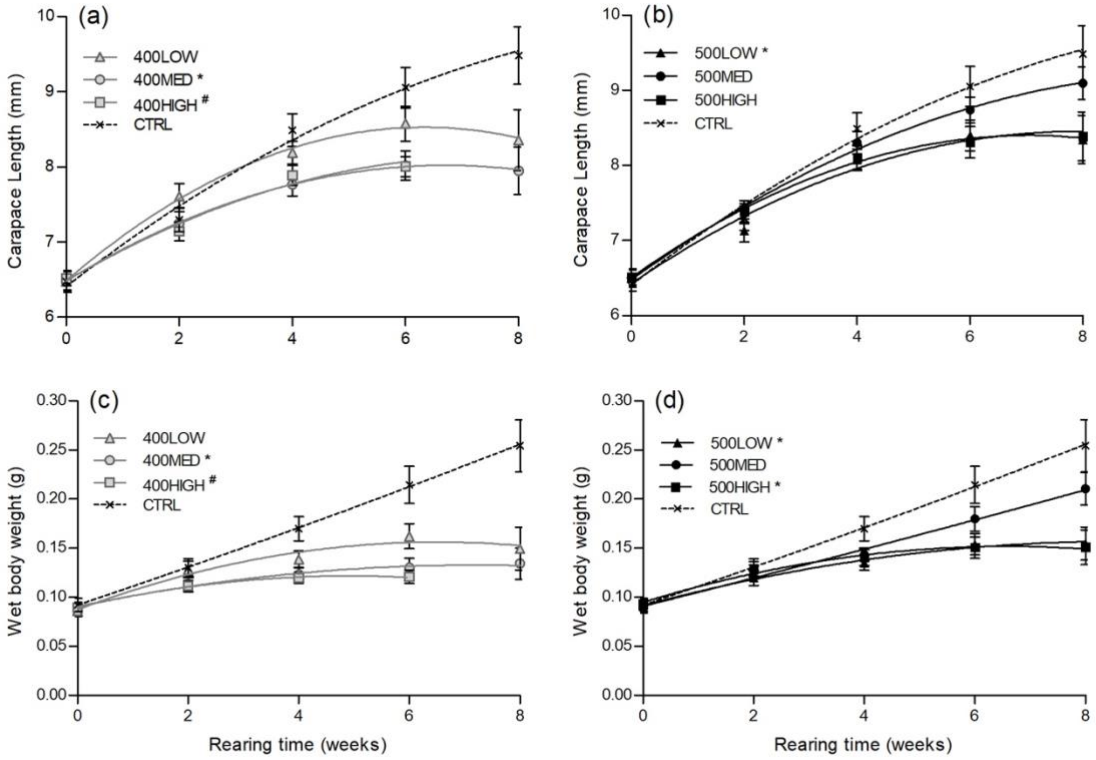

FIGURE 4. Second order polynomial model fit to average carapace length (upper panels (a) and (b)) and wet body weight (lower panels (c) and (d)) of *H. gammarus* juveniles fed on different diets throughout the growth trial period. Data points represented as mean ± SEM. Dietary treatments that were significantly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

As for SGR and iCL, the feed efficiency indices - energy intake, FI, and FCR - were calculated for the first 6 weeks period in all treatments (Table 3). Feed intake was significantly higher for all experimental feeds compared to the CTRL except for the 400HIGH. Within the experimental feeds L:CHO ratio and the interaction of protein × L:CHO had a significant effect on the FI. A higher feed intake in extruded feeds with higher carbohydrate content was observed for both protein levels examined (400 and 500 mg kg^{-1}) with most pronounced differences in feeds with a lower protein content. As such, lobsters fed LOW feeds presented higher FI than HIGH with intermediate levels observed in the MED treatments (Table 3). Intake expressed in terms of energy followed the same trend as FI. The FCR was significantly lower for the CTRL diet compared to the experimental extruded feeds except for the 500MED group. There was a significant effect on FCR from the L:CHO ratio among the experimental dry feeds, for which FCR in juveniles reared on MED feeds was significant lower compared to LOW feeds (Table 3).

TABLE 3. Growth performance, feed efficiency, and whole body composition of juvenile *H. gammarus* fed the experimental extruded feeds and Antarctic krill.

Protein level	400 g kg^{-1}			500 g kg^{-1}				One-Way ANOVA	Two-Way ANOVA		
L:CHO ratio	LOW	MED	HIGH	LOW	MED	HIGH	CTRL		P	L:CHO	P X L:CHO
SGR (% d^{-1})	1.28 ± 0.13$^{\#,ax}$	0.77 ± 0.09$^{\#,bx}$	0.50 ± 0.11$^{\#,bx}$	1.29 ± 0.13$^{\#,Bx}$	1.72 ± 0.13^{Ay}	1.06 ± 0.07$^{\#,By}$	1.89 ±0.13	$F_{6,137} = 17.10^{***}$	$F_{1,115} = 29.17^{***}$	$F_{2,115} = 11.29^{***}$	$F_{2,115} = 8.86^{***}$
iCL (% CL$_i$)	29.0 ± 2.3^{ax}	21.2 ± 2.4$^{\#,abx}$	19.3 ± 2.8$^{\#,bx}$	30.4 ± 2.2^{ABx}	35.2 ± 2.5^{Ay}	25.4 ± 1.6$^{\#,Bx}$	37.5 ± 2.7	$F_{6,137} = 8.04^{***}$	$F_{1,115} = 14.63^{***}$	$F_{2,115} = 5.60^{**}$	$F_{2,115} = 4.00^{*}$
N	19	20	15	20	21	21	22				
FI (%BW$_i$ d^{-1})	10.1 ± 0.6$^{\#,ax}$	4.6 ± 0.8$^{\#,bx}$	2.7 ± 0.2^{cx}	7.3 ± 0.3$^{\#,Ay}$	5.3 ± 0.2$^{\#,Bx}$	5.1 ± 0.3$^{\#,By}$	2.7 ± 0.2	$F_{6,20} = 34.74^{***}$	$F_{1,17} = 1.59$	$F_{2,17} = 53.11^{***}$	$F_{2,17} = 15.64^{***}$
EI (J d^{-1})	179.2 ± 8.8$^{\#,ax}$	89.1 ± 16.3$^{\#,bx}$	56.6 ± 3.9 ^{bx}	135.7 ± 5.5$^{\#,Ay}$	100.4 ± 3.8$^{\#,By}$	106.6 ± 6.2$^{\#,ABy}$	53.3 ± 4.7	$F_{6,20} = 29.37^{***}$	$F_{1,17} = 0.72$	$F_{2,17.} = 44.52^{***}$	
FCR	4.3 ± 0.1$^{\#}$	3.9 ± 0.4$^{\#}$	3.3 ± 0.5$^{\#}$	4.2 ± 0.7$^{\#}$	2.0 ± 0.2	3.2 ± 0.3$^{\#}$	0.7 ± 0.1	$F_{6,20} = 10.45^{***}$	$F_{1,17} = 4.15$	$F_{2,17} = 4.94^{*}$	$F_{2,17} = 2.77$
N	3	3	3	3	3	3	3				
Proximate composition (% of DM)											
Dry matter	24.5 ± 0.4	22.7 ± 1.1	19.9 ± 1.2	25.3 ± 0.4	23.7 ± 1.8	24.2 ± 0.6	29.1 ± 0.5				
Ash	34.7 ± 0.6	36.8 ± 0.3	42.8 ± 1.1	35.0 ± 0.4	34.0 ± 0.3	34.7 ± 0.5	32.3 ± 0.5				
Soluble protein	50.4 ± 3.1	47.8 ± 4.1	42.2 ± 1.8	40.6 ± 1.4	42.9 ± 2.4	49.8 ± 3.4	45.4 ± 1.7				
Total lipid	6.3 ± 1.3	6.4 ± 0.4	3.5 ± 0.8	7.0 ± 0.7	5.5 ± 1.0	5.2 ± 0.2	5.4 ± 1.1				
Carbohydrates †	8.6 ± 3.1	9.0 ± 4.0	11.5 ± 0.7	17.5 ± 2.3	17.6 ± 1.6	10.4 ± 3.9	17.0 ± 2.9				

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

Values are means ± SEM.

Superscript $^{\#}$ indicate dietary groups significantly different from CTRL (Krill).

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

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

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

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

PROXIMATE COMPOSITION

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

NUCLEIC ACID DERIVED INDICES

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

In comparison to the CTRL group the 400HIGH treatment presented a significant increase ($F_{6,41} = 5.08$, $p = 0.001$) in DNA content. Among the experimental extruded feeds both protein ($F_{1,35} = 10.41$, $p = 0.003$) and L:CHO ratio ($F_{2,35} = 3.75$, $p = 0.035$) significantly affected the DNA concentration in the muscle. Feeds within the 400-protein group supported a significant increase in DNA content compared to the 500-protein group. The DNA content of the abdominal muscle of juveniles reared on the L:CHO MED category feeds was significantly lower compared to the HIGH groups (Fig. 5 b).

The standardized ratio (sRD) was significantly affected by dietary treatment. The sRD was significantly lower ($F_{6,41} = 7.87$, $p < 0.001$) in animals fed the 400MED, 400HIGH and 500HIGH feeds when compared to the CTRL. Within the experimental feeds, sRD was significantly affected by protein ($F_{1,35} = 7.63$, $p = 0.010$), L:CHO ratio ($F_{2,35} = 7.09$, $p = 0.003$) and the interaction protein × L:CHO ($F_{2,35} = 5.83$, $p = 0.007$). For the 400-protein level, juveniles fed the 400MED and 400HIGH feeds showed a significantly lower ratio compared to the lobsters fed the 400LOW diet. For the 500 level the 500MED showed higher sRD than the 500HIGH group. Protein level had a significant effect within the MED L:CHO category, for which the 500 resulted in a higher sRD than the 400 group (Fig. 5 c). A significant regression equation was found between sRD and SGR ($F_{1,40} = 49.05$, $p < 0.001$), with an R^2

of 0.55. The predicted sRD for juvenile lobsters was equal to $-0.143 + 0.644$ (SGR) when SGR is estimated in % d^{-1} (Fig. 6).

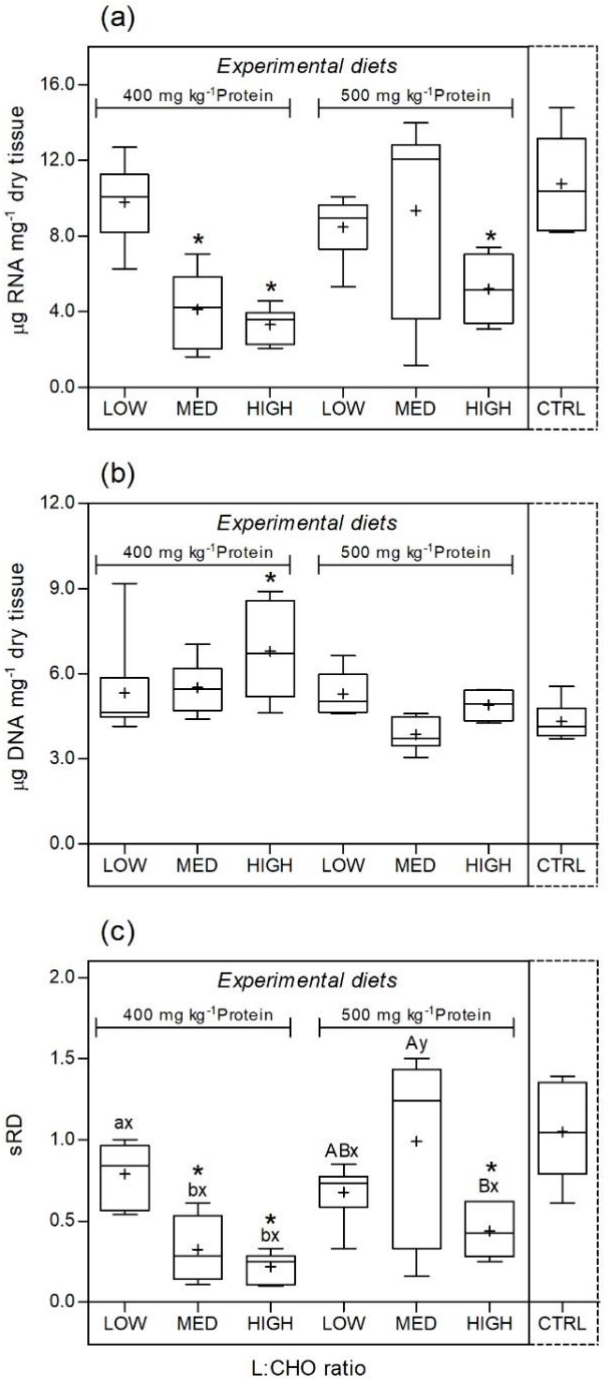

FIGURE 5. Changes in RNA concentration (a), DNA concentration (b) and standardized RNA/DNA ratio, sRD (c) of abdominal muscle tissue of *H. gammarus* juveniles fed on different diets (n=6). The box includes observations from the 25th to the 75th percentile and the whiskers above and below the box indicate the 10th and 90th percentiles. The horizontal line within the box represents the median value and the symbol (+) indicates the mean. Dietary treatments that were significantly different from CTRL are marked with an asterisk (*). Different letters "a, b" or "A, B" indicate significant differences within the 400 or 500-protein level, respectively. Different "x or y" indicate significant differences within the same L:CHO ratio category.

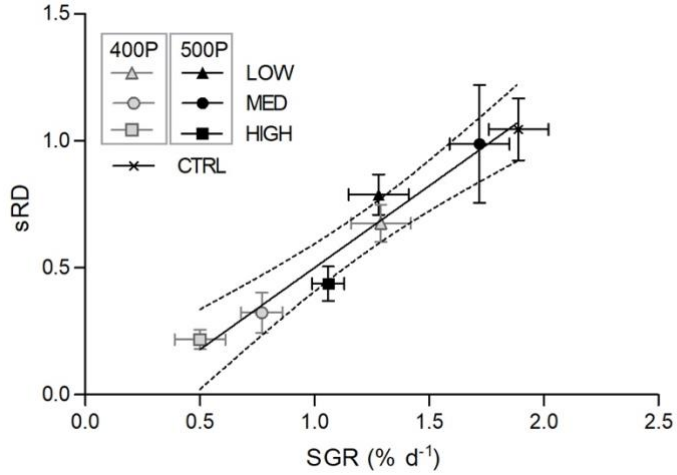

FIGURE 6. Fitted linear regression model of standardized RNA/DNA ratio (sRD) vs. specific growth rate (SGR). Data points per dietary treatment represented as mean ± SEM. The solid line displays the average estimates of the predicted sRD and the dashed lines the 95% confidence limits. Simple linear regression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

DIGESTIVE ENZYME ACTIVITIES

Trypsin activity was significantly higher for the animals fed the experimental feeds than for the CTRL group, except for the 400LOW treatment ($F_{6,62} = 8.37$, $p < 0.001$). Within the experimental feeds trypsin activity was affected by the L:CHO ratio ($F_{2,53} = 9{,}86$, $p < 0.001$) and the interaction protein x L:CHO ($F_{2,53} = 6.49$, $p = 0.003$). No differences were observed among the three feeds in the 500-protein level but in the 400 group the 400HIGH treatment promoted a significant increase in trypsin activity compared to the 400LOW. The protein content significantly affected the trypsin activity for the LOW (higher for the 500 level) and HIGH (higher for the 400 level) categories (Fig. 7 a).

As for trypsin, a similar trend was observed for the activity of amylase. When compared to the CTRL, amylase activity was significantly higher in the 400MED and 400HIGH treatments ($F_{6,62} = 5.00$, $p < 0.001$). Protein ($F_{1,53} = 28.91$, $p < 0.001$), L:CHO ratio ($F_{2,53} = 12.79$, $p < 0.001$), and the interaction of both factors ($F_{2,53} = 4.97$, $p = 0.011$) had a significant effect on amylase activity. No significant differences were observed within the 500-protein level. For the 400-protein group amylase activity was significantly higher in juveniles fed the 400MED and 400HIGH feeds. Within the MED and HIGH categories, amylase activity increased for the 400-protein level (Fig. 7 b).

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

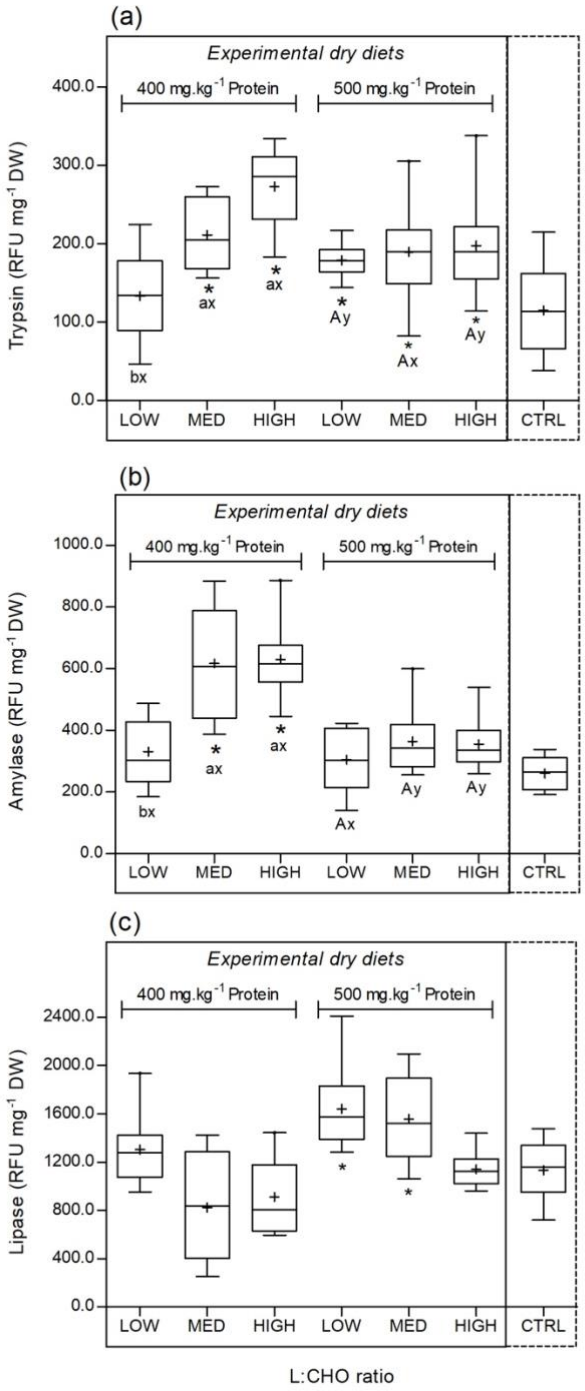

FIGURE 7. Changes in trypsin (a), amylase (b), and lipase (c) activity of *H. gammarus* juveniles fed on the different tested diets (n=9). The box includes observations from the 25th to the 75th percentile and the whiskers above and below the box indicate the 10th and 90th percentiles. The horizontal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

DISCUSSION

GROWTH PERFORMANCE

Results obtained indicate that the overall performance of European lobster juveniles reared on the 500MED extruded feed for an 8-week period was comparable to the performance of juveniles fed thawed Antarctic krill (CTRL). Previous nutritional studies carried out on H. gammarus (Hinchcliffe et al., 2020), H. americanus (Tlusty et al., 2005), and several Palinuridae species (Perera and Simon, 2015) showed that the growth rate was generally poorer in lobsters fed formulated compared to fresh diets. A recent study (Goncalves et al., 2020), investigated the effect of the same feeds that are used here on the performance, metabolic rate, and nitrogen retention in European lobster juveniles. The results indicated that H. gammarus grew faster when fed the 500 g kg^{-1} compared to the 400 g kg^{-1} protein feeds. The results also suggested a potential protein sparring capacity in the formulated feeds, when compensated by the inclusion of carbohydrates, given that the growth obtained for the 400LOW was comparable to that of the three 500 feeds. In the present study, we found that the growth rate of juveniles fed the 400LOW, 500LOW, and 500HIGH feeds was lower than previously projected. This is most likely due to the too short growth trial period in the previous study. We thus confirm what had already been hypothesized in the previous work, i.e., a period of 4 weeks is not long enough to assess the effects of experimental feeds on the growth of juvenile European lobsters. Even the present study is arguably a little too short. As growth in crustaceans, as opposed to fish, rely on sequential moulting (Simon et al., 2015), nutritional trials performed in crustacean species aiming growth performance as the primary outcome must consider a minimum duration that allows individuals to complete two moults.

The poorest overall performance observed in the group of lobsters reared on the 400HIGH diet is most likely related to the low feed intake. The reason for such low intake in this dietary group remains unclear but it might be associated with a lower palatability, different smell, or that the higher lipid content caused faster satiation. Results from a feeding trial carried out with European lobster juveniles of similar size (Mente et al., 2001) showed that animals fed 5% dry feed of body mass grew significantly less than juveniles fed 10%. In our study, only lobsters fed the 400LOW diet were close to the 10% level recommended (Mente et al., 2001) but a direct comparison between the current and the mentioned study is not possible since both test diets and feeding duration were different. In the cited study (Mente et al., 2001), juveniles were allowed to feed from the afternoon until the next morning, while only 4 hours in our set up. Nonetheless, the fact that low intake compromised growth was validated in both studies. In animals fed the 400HIGH diet, not only growth but also survival was compromised. Results suggest that juveniles were not taking enough feed to support growth, nor even enough to sustain the minimum standard metabolic rate (SMR). In our previous study using the same feeds (Goncalves et al., 2020), a significant decrease was observed in the SMR of European lobster juveniles reared on the

400MED and 400HIGH feeds for a 32-day period. Another likely reason for such a low performance on this diet may be related to a low tolerance to high lipid diets by crustaceans (Nelson et al., 2006). It has been previously suggested (Cuzon et al., 1994) that high dietary lipids reduce the capacity for amino acid absorption due to an increase in bile acid concentration in response to a high-fat diet.

FEED EFFICIENCY

Although the CTRL group grew faster with the lowest FI among all treatments, the low FI observed for this group is masked by the fact that calculations were performed on a dry weight basis. The CTRL diet composed of frozen Antarctic krill had a much lower dry matter (DM) content (approx. 11%) than the experimental extruded feeds (DM approx. 90%) which complicates FI comparisons. Also, the energy intake was lower in lobsters fed with krill compared to the extruded feeds. Moreover, the form in which krill is presented is so different from an extruded feed, which may cause differences in the overall nutrient absorption and digestibility. The highest intake observed for the experimental feeds in the LOW L:CHO category suggests that feeds with high carbohydrate content are more attractive to European lobster juveniles. This is supported by previous studies showing that the composition of the preferred prey of spiny lobster is generally high in protein content, moderate to high in carbohydrate content, and low in lipid content (Kevin C. Williams, 2007). In the present study, we observed that the CTRL group was the most efficient in terms of FCR but highly influenced by the low DM content of the thawed Antarctic krill. When FCR is estimated on a wet weight basis, the trend inverts. For example, FCR increases from 2.0 to 2.2 and from 0.7 to 6.0 in the 500MED and CTRL groups, respectively. More meaningful is the comparison of FCR values among the experimental extruded feeds. Results indicate a lower FCR in diet 500MED and points to a better utilization of this diet. The general best performance of animals fed the 500MED when compared to the other extruded feeds could also be related to a more balanced amino acid composition. This diet, together with the 500LOW, showed the most similarity to the Antarctic krill among the experimental extruded feeds. Still, the apparent lower level of taurine in all the extruded feeds might have limited the growth of juveniles. This amino acid is known to elicit a feed-attractant stimulus in crustaceans (Perera and Simon, 2015). A potential solution to increase the performance of lobsters fed on artificial feeds could be the incorporation of taurine to formulated feeds to increase feed intake of lobsters (Floreto et al., 2000). The 500 mg kg^{-1} protein level in the experimental feeds was achieved by increasing the proportion of squid meal. This ingredient is often used in crustaceans' diets to increase growth and attractiveness of artificial feeds and the replacement of fish by squid meal has been proven beneficial for growth in several crustacean species (Mente, 2006). However, since the amino acid profiles of fish and squid meal are similar (Nelson et al., 2006), it is unlikely that the dissimilarities observed among the experimental extruded feeds were caused by the different inclusion levels of this ingredient.

NUCLEIC ACID DERIVED INDICES

The decrease in sRD for the 400MED and 500HIGH dietary groups was mainly caused by a reduction in the RNA content , while for the 400HIGH group, results show that the low sRD was a combined effect of decreased RNA and increased DNA concentrations in the muscle tissue. The quantified RNA includes ribosomal RNA (rRNA), messenger RNA (mRNA), and transfer RNA (tRNA) which respond in different ways and have different functions. However, since rRNA - responsible for building protein – makes up the majority of total RNA, changes in total RNA were assumed to primarily reflect changes in rRNA (Buckley et al., 1999). Thus, it was presumed that the reduction in RNA reflected a decrease in protein synthesis, while the relative increase in DNA was associated with an increase in the number of cells per tissue portion, however, of smaller dimensions (60). Thus, the results suggest that juveniles reared on the 400HIGH diet were not only affected by a decreased capacity for protein synthesis but also by the mobilisation of proteins from the abdominal muscle to obtain energy, as previously suggested in starving fish larvae (61). Overall, results showed that the sRD was positively correlated with growth performance and feed efficiency indicators. Specifically, we demonstrated that sRD estimated from samples collected at week 4 were significantly correlated with SGR calculated at week 6. These observations suggest that sRD is a sensitive indicator of the nutritional condition of European lobster juveniles. The use of this estimate in crustaceans is of great applicability as it allows the assessment of recent growth and nutritional condition (33,62,63). Most of the nutritional studies performed on crustaceans considered growth rate as gains in wet weight and length. However, the growth process in crustacean species has an interrupted character because is connected to the moult cycle, whereas, somatic growth and the accumulation of energy reserves in tissues are a continuous process (Nguyen et al., 2014). We conclude that the nucleic derived indices are useful tool in future nutritional studies with European lobster juveniles as it allows a faster evaluation when compared to conventional growth performance estimators.

DIGESTIVE CAPACITY

The lowest activity levels of trypsin and amylase enzymes were detected in European lobster juveniles reared on Antarctic krill while activities increased in animals offered the 400HIGH and 400MED extruded feeds. This is in contradiction to what was previously reported (Simon, 2009). In that study, was observed that spiny lobsters (Jasus edwardsii) reared on a formulated diet had a marked decrease in trypsin and α-amylase activities of the foregut and digestive gland compared to juveniles fed on fresh mussels. A main difference, however, may be that in the present study, enzymatic activity was estimated from the entire abdominal section and not from the foregut and/or hepatopancreas. Thus, the activity detected in tissue homogenates may not necessarily represent the activity of enzymes that will be secreted into the digestive lumen. In crustaceans, proteases and carbohydrases activity was found

in tissues outside the gut (64,65). It may also be that the secretion of large amounts of enzymes may maximize the use of limiting nutrients. Such elevated enzymatic activity could maximize hydrolysis and the resulting extraction of a dietary substrate that was ingested in small amounts (66). Results from (67) point to the same hypothesis. In the cited study, the authors reported a significant increase in the specific activity of α-amylase in J. edwardsii juveniles fed diets with low starch inclusion level. Thus, based on this, the results suggest that trypsin and amylase activities increased to compensate for the low levels of protein and carbohydrates lobsters could obtain when fed on the 400MED and 400HIGH feeds. For lipase the activity was lower for juveniles fed the 400HIGH and 400MED than for individuals reared on the 500LOW and 500MED extruded feeds. While carbohydrates are the primary source of energy for crustaceans (68), lipids are the main energy reserve. European lobsters store the metabolized lipids in the R-cells of the hepatopancreas (69). Overall, the results point toward a combined effect of low protein, high lipid, and low feed intake in the activity of trypsin and amylase. The increased activity of these enzymes in the group of animals fed the 400MED and 400HIGH feeds suggests the activation of a mechanism to optimize the use of protein and carbohydrates as they seem to be the most important nutrients for European lobster juveniles. In contrast, lipase activity increased with protein level in the diet, with significantly higher activity for the 500-protein lobster groups than the 400-protein groups. This suggests that the required supply of protein for growth is covered for the 500 group, and, that energy reserves to support growth potential are ensured for the lobsters reared on the 500LOW and 500MED feeds.

CONCLUDING REMARKS

In the present study, we demonstrated that European lobster juveniles fed the 500MED experimental diet achieved a growth performance and nutritional condition statistically comparable to that observed when animals were fed thawed Antarctic krill. This extruded feed contained 500 g kg^{-1} protein, 237 g kg^{-1} carbohydrates, and 119 g kg^{-1} lipids (as fed basis). The results suggest that this composition is the most balanced to meet the overall nutritional requirements of the European lobster juveniles among the tested extruded feeds. Results also support what was previously observed for spiny lobster species: formulated feeds with a very similar L:CHO ratio (1:2) have been proven to provide the best balance of carbohydrate and lipid when rearing juvenile J. edwardsii (70). Therefore, based on the results presented, we recommend that high protein and moderate inclusion of carbohydrates must be considered when formulating feeds for H. gammarus juveniles. While relative growth benefits of lobsters fed the 500MED experimental diet did not exceed that of lobsters fed the control diet, it is worth considering that replacing fresh diets with extruded feeds can be advantageous in a cost-effective point of view. However, from an economic point of view, the ingredients used in the formulation of experimental extruded feeds were not the most sustainable. Future studies, following some of the

recommendations presented here and using sustainable alternative ingredients (e.g. insect meal, industrial by-products), will be of extreme importance for the establishment of European lobster farming on a commercial scale.

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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 analysed by R.G. and V.B.. Findings were interpreted by R.G., I.L., M.G., C.N. and M.A.T.. All authors contributed to the preparation of the manuscript.

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STUDY 4

Shrimp waste meal (*Pandalus borealis*) as an alternative ingredient in diets for juvenile European lobster (*Homarus gammarus*, L.)

Under review, Aquaculture

Shrimp waste meal (*Pandalus borealis*) as an alternative ingredient in diets for juvenile European lobster (*Homarus gammarus*, L.)

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Abstract

The use of sustainable ingredients in the formulation of aquafeeds remains ever-important. The European lobster (*Homarus gammarus*) is an emerging species for aquaculture, the success of which relies on knowledge of its nutritional requirements, and the selection of dietary ingredients that facilitate a viable production and future commercialization. In this study, we investigated the feasibility of using shrimp waste meal (SWM) as an ingredient in formulated diets for juvenile lobsters (~ 164 mg) during an 8-week feeding experiment. The experimental diets were isoproteic (~ 54% crude protein) and isolipidic (~ 11% crude fat), in which either 7%, 14%, 21%, or 28% of the dietary protein was supplied by SWM. Experimental diets were benchmarked against a control diet without SWM (SWM 0%). Diet performance was evaluated based on survival, body mass gain, carapace length increment, and moulting cycle duration. Nitrogen metabolism was evaluated from nitrogen excretion rates of individual lobsters, determined before, and following the ingestion of a single meal. The exoskeleton colouration of individual lobsters was assessed using digital colour analysis at the end of the growth trial. The results indicate that SWM can be used as an ingredient in lobster diets at least up to 28% protein inclusion, without adverse effects on growth performance and nitrogen metabolism. Further, the inclusion of SWM at 28% protein significantly enhanced survival with no effect on the exoskeleton colouration. Based on enhanced survival combined with no negative effect on growth and nitrogen excretion, the use of SWM at least up to 28% of dietary protein appears promising in the formulation of future commercial feeds for juvenile *H. gammarus*.

Keywords: Dietary protein; Growth performance; Nitrogen excretion; Exoskeleton colour

INTRODUCTION

A global demand exceeding supply and high market prices for European lobster *Homarus gammarus*, L. makes this species an excellent candidate for aquaculture. At present, the farming of *H. gammarus* operates at a modest scale within two complementary segments: as a fisheries remediation measure to enhance wild stocks by releasing hatchery-reared juveniles into natural habitats, and as an emerging sector of commercial on-growing for consumption (Powell et al., 2017). Developments have been made to improve land-based farming by using advanced robotic systems in RAS (Drengstig and Bergheim, 2013) and at sea by employing sea-based container culture systems (Daniels et al., 2015; Halswell et al., 2018). However, the farming of *H. gammarus* has yet to overcome several major

challenges. In particular, slow growth rates and high mortalities associated with cannibalistic behaviour (Powell et al., 2017; Scolding et al., 2012) lead to high production costs (Drengstig and Bergheim, 2013). The replacement of fresh or frozen diets, currently being used in hatcheries, with formulated feeds specifically designed for *H. gammarus* would improve the economic viability of land-based lobster farming by reducing the feeding costs (Powell et al., 2017). Further economic benefits could be gained from the use of sustainable ingredients (Cottrell et al., 2020; Naylor et al., 2021), such as industrial by-products or waste streams, in the formulated feeds (Hinchcliffe et al., 2020).

The Northern shrimp, *Pandalus borealis*, is the most commercially important Caridean species in the North Atlantic, and accounts for 10% of the global shrimp production (Paterson, 2003). Global landings of *P. borealis* amounted to ~295.000 tonnes annually between 2010 and 2016 (FAO, 2021). The processing of *P. borealis* leads to large amounts of waste, in the form of heads, appendages, and exoskeletons, which accounts for at least 50% of total production (Dave et al., 2020), and which are mostly disposed of as biological waste or discarded at the sea. That may lead to environmental concern since the rate of biodegradation of the discarded waste is low (Shahidi and Synowiecki, 1991). Valorising shrimp processing waste as an ingredient for lobster rearing represents not only an opportunity to reduce feed costs but has also the potential to reduce environmental pollution.

The idea that shrimp waste meal (SWM) represents a potential protein source in aquafeeds is based on its high protein content, and a well-balanced amino acid profile (Cruz-Suárez et al., 1993). A protein content of 44.5% (dry weight basis) and high levels of arginine and lysine has previously been reported for *P. borealis* processing waste (Heu et al., 2003). Arginine and lysine, together with leucine, have been identified as the main amino acids in whole-body tissue of *H. gammarus* juveniles (Mente et al., 2001), indicating a high requirement of these three amino acids for the species. Additionally, the SWM is a natural source of astaxanthin, which is frequently used as a feed additive in the formulation of aquafeeds to improve the colouration of many aquatic species, including lobsters (Barclay et al., 2006). Previous studies on homarid lobsters revealed that individuals reared on experimental formulated diets frequently display a different and deviating exoskeleton colouration compared to those fed natural diets (Floreto et al., 2001) which can interfere with survival at restocking or with the valorisation of the final product when produced for consumption. The inclusion of astaxanthin in formulated diets can successfully revert exoskeleton colour alterations (Crear et al., 2002). Astaxanthin also presents high antioxidant characteristics, and is considered to be health beneficial among aquatic species (Lim et al., 2018).

Yet, the SWM contains high levels of chitin, known to limit nutrient digestibility in fishes (Karlsen et al., 2017), although the effects of chitin on the digestive performance of crustaceans is more controversial. Chitinase, a digestive enzyme required to break down chitin, has been documented in most, if not all crustacean species examined, and facilitates digestion of prey skeletons, as well as own exoskeleton following ecdysis (Ceccaldi, 1989). Although the metabolic cost of moulting cannot be

precisely quantified, ecdysis is associated with large losses of nitrogen and carbon (Zoutendyk, 1988), which must be recovered from the diet. For crustaceans, it may conceivably be advantageous to use chitin rich diets, if intermediate products in the chitin degradation pathway can be used in exoskeleton synthesis, rather than being synthesized *de novo* from monosaccharide and amino acid precursors. This is supported by previous research showing a beneficial effect of dietary chitin or its derivatives on the growth performance or survival of the white leg shrimp, *Litopenaeus vannamei*; the black tiger shrimp, *Penaeus monodon*; and the shore crab, *Carcinus maenas* (Niu et al., 2013, 2011; Powell and Rowley, 2007). However, increasing levels of dietary chitin has also been reported to have no effect on growth or survival of the black tiger shrimp (Fox, 1993).

Taken together, reported findings suggest that *P. borealis* processing waste is a protein source with considerable potential in the formulation of diets for *H. gammarus*. In this context, this study was conducted to investigate the feasibility of using meal from *P. borealis* processing waste (heads, appendages, and exoskeletons) as a protein source in formulated feeds for *H. gammarus* early juveniles. Five diets were formulated to include different levels of SWM (from 0% to 28% of total dietary protein) while maintaining equal lipid and protein content. The 28% SWM inclusion level was the maximum possible to sustain a total protein content of 54% (dry weight basis) previously reported as optimal for juvenile *H. gammarus* (Goncalves et al., 2021a). The diets were fabricated as soft semi-moist diets, to explore the effect on feed intake, as extruded pellets may be too hard (personal observation, Goncalves et al., 2021a). Diet performance was evaluated by examining juvenile growth, survival, feed efficiency, proximate composition, nitrogen metabolism, and exoskeleton colouration reared on the experimental diets during an 8-week period.

MATERIALS AND METHODS

EXPERIMENTAL DIETS

Experimental diets (semi-moist, ~ 40% DM) were formulated to contain 22% total crude protein – CP (54% of DM) and 4.3% total crude fat – CF (11% of DM). Diets were formulated by combining different proportions of fishmeal (South America, CP 68.1%, CF 8.9%, Ash 15%) and shrimp waste meal (SWM - heads, appendages, and exoskeletons from Northern shrimp *Pandalus borealis*, CP 47.2%; CF 6.1%, Ash 33.5%). A fixed amount of squid meal (CP 77.2%, CF 4.4%, Ash 8%) was kept in all the experimental diets. Five inclusion levels of SWM (0%, 7%, 14%, 21%, and 28% of total dietary protein) were tested. Shrimp waste meal (SWM) and experimental diets were prepared at DTU Aqua facilities (Hirtshals, Denmark). A mixture of heads, appendages, and exoskeletons from commercially caught Northern shrimp *Pandalus borealis* (Launis A/S, Skagen, Denmark) were oven-dried at 60ºC until constant weight. Dried SWM was pre-grounded to a particle size < 5mm (Krups

Speedy Pro homogenizer) and then milled to a final particle size of 120 μm using an ultra-centrifugal mill (ZM 200, Retsch GmbH, Dusseldorf, Germany). The obtained SWM was stored at -20ºC until used. The dry ingredients of each diet (except the carrageenan) were thoroughly mixed and heated to 85ºC. Carrageenan, krill oil, and water were mixed and heated to 85ºC to create a carrageenan emulsion. The dry ingredients were stirred with the carrageenan emulsion until a homogeneous paste was obtained. Water loss by evaporation (estimated by weight difference) was compensated by adding additional heated water (85ºC). The fluid viscous mixture was poured into a steel tray, evenly distributed in a thin layer, and cooled down until the mixture solidified. Finally, moist pellets with a size of 4 × 4 × 4 mm were obtained by pushing a 3D printed plastic grid (Prusa i3 MK2, Czech Republic) into the solidified mixture. The proximate composition, carotenoid and chitin content as well as the amino acid composition of the SWM is shown in Table 1. Ingredient inclusion and proximate composition of experimental diets are presented in Table 2. Amino acid profiles of the experimental diets are presented in Table 3.

TABLE 1. Proximate and amino acid composition of the shrimp waste meal (SWM).

Proximate composition (% content)		Amino acid composition (g 100 g^{-1} SWM)	
Dry matter	97.2	*Essential*	
Crude protein	47.8	Arginine	2.7
Crude fat	6.1	Histidine	1.0
Ash	33.5	Isoleucine	1.8
Chitin	16.0	Leucine	2.7
		Lysine	2.6
		Methionine	1.0
		Phenylalanine	1.9
		Threonine	1.8
		Tryptophan	0.5
		Valine	2.1
		ΣEAA	18.1
Carotenoid contents (mg kg^{-1})		*Nonessential*	
Esterified astaxanthin	69.0	Alanine	2.3
Free astaxanthin	3.6	Aspartic acid	4.0
Canthaxanthin	nd	Cysteine + Cystine	0.5
Lutein	nd	Glutamic acid	5.4
Zeaxanthin	nd	Glycine	2.1
Beta-carotene (cis + trans)	nd	Proline	3.6
		Serine	2.0
		Tyrosine	1.6
		ΣNEAA	21.5
		EAA/NEAA	0.84

nd, not detected

EXPERIMENTAL ANIMALS

All experimental animals were hatched from eggs obtained from wild ovigerous females (body weight 500-700 g) caught in the Limfjord (North Jutland, Denmark). After hatching, lobster larvae were communally reared following the same procedure previously described in (Goncalves et al., 2021a). Upon metamorphosis to stage IV (Rötzer and Haug, 2015), post-larvae were transferred to individual

compartments in a raceway system. The system consisted of 3D printed plastic cassette systems with 200 mL perforated compartments to allow water flow. The cassettes were distributed into a raceway (250 × 35 × 6.5 cm, 57 L), supplied by a saltwater recirculation system. The raceway was supplied with water at a constant flow rate of 330 L h^{-1} (19ºC ± 1 ºC temperature, 34 ± 1 salinity, > 90% dissolved oxygen, < 0.1 mg L^{-1} ammonia-N). The photoperiod cycle was fixed at 8h light: 16h dark by fluorescent lamps. Light intensity at the water surface was 7-8 lux. Post-larvae were fed daily with thawed Antarctic krill (Euphausia superba) and kept under these conditions for approximately 8 weeks during which individuals developed into stage > VI.

TABLE 2. Ingredients used (g 100 g^{-1} diet) and proximate composition of the experimental diets.

	Level of SWM as % of dietary protein				
	0	7	14	21	28
Ingredients (as used g 100 g^{-1})					
Fish meal	19.1	17.2	15.5	13.6	11.8
Shrimp waste meal	0	3.5	6.4	9.8	13.2
Squid meal	7.1	7.3	7.3	7.4	7.4
Krill oil	2.6	2.6	2.6	2.6	2.5
Maize starch	1.7	1.6	1.4	1.2	1.0
Potato starch	1.7	1.6	1.4	1.2	1.0
Wheat starch	3.5	3.1	2.8	2.4	2.1
Vitamin premix	1.2	1.2	1.2	1.2	1.2
Carrageenan	1.6	1.6	1.6	1.6	1.6
Copper supplement	0.005	0.005	0.005	0.005	0.005
Proximate composition (as fed g 100 g^{-1})					
Crude protein	23.5	22.2	21.4	20.7	22.5
Crude fat	4.6	4.4	4.2	4.0	4.4
Moisture	59.5	59.7	58.9	60.6	56.5
Ash	5.4	6.0	6.7	7.2	8.8
NFE [1]	7.0	7.7	8.8	7.5	7.8
Gross energy [2] (KJ g^{-1})	8.1	7.8	7.8	7.3	7.9
Chitin [3] (% of DM)	0	1.4	2.5	4.0	4.9

[1] Nitrogen-free extract (NFE), calculated as: 100% - crude protein% - crude fat% - ash% - moisture%.
[2] Coefficient for energy concentration: 21.3 kJ, 39.5 kJ, and 17.6 kJ for protein, lipid, and carbohydrate (NFE), respectively (Cuzon and Guillaume, 1997).
[3] Estimated from chitin content on SWM (16%) and assuming SWM is the only ingredient containing chitin.

GROWTH TRIAL

At the beginning of the experiment, lobsters were individually weighed and carapace length measured. Five groups of 15 individuals (stage VI-VII, initial weight of 164 ± 3 mg; carapace length 8.2 ± 0.1 mm, mean ± SD) were randomly allocated to the dietary treatments. The same 3D cassette system described in the previous paragraph was used for the feeding trial. Individual lobsters in each group were hand-fed one pellet (55 mg average weight) of the assigned diet each morning and allowed to feed for 4h. Shed exoskeletons during moulting were left in the compartments for the newly moulted juveniles to consume. The presence of shed exoskeletons and mortalities were recorded daily. Lobsters were individually weighed and measured every second week. Body weight (BW) was recorded to the nearest 0.001 g after gently blotted dry each individual with a paper towel. Carapace length (CL) was

measured to the nearest 0.1 mm with a vernier caliper from the base of the eye socket to the posterior edge of the cephalothorax. The following formulas were used to determine growth performance:

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

Where: SGR = specific growth rate; BW_f = final body weight; BW_i = initial body weight; Δt = duration of the growth trial (56 days).

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

Where: iCL = increment in carapace length; CL_f = final carapace length; CL_i = initial carapace length.

At the end of the trial juveniles were lethally anesthetized in ice-cold seawater, weighed, measured, individually photographed, rinsed in distilled water, and stored at -80ºC until further analysis.

TABLE 3. Amino acid profile of the experimental diets (g 100 g^{-1} diet)

	Level of SWM as % of dietary protein				
	0	7	14	21	28
Essential					
Arginine	1.2	1.1	1.1	1.1	1.2
Histidine	0.7	0.5	0.5	0.5	0.6
Isoleucine	0.9	0.8	0.8	0.8	0.9
Leucine	1.6	1.4	1.4	1.4	1.5
Lysine	1.5	1.4	1.4	1.4	1.4
Methionine	0.6	0.5	0.5	0.5	0.5
Phenylalanine	0.8	0.7	0.8	0.8	0.8
Threonine	0.9	0.8	0.8	0.9	0.9
Valine	1.0	0.9	0.9	0.9	1.0
ΣEAA	9.1	8.0	8.4	8.4	8.9
Nonessential					
Alanine	1.3	1.1	1.2	1.2	1.3
Aspartic acid	1.8	1.7	1.8	1.9	2.0
Cysteine + Cystine	0.2	0.2	0.2	0.2	0.2
Glutamic acid	2.7	2.5	2.6	2.6	2.8
Glycine	1.4	1.2	1.2	1.2	1.3
Proline	1.0	0.9	1.0	0.9	1.1
Serine	0.8	0.7	0.8	0.8	0.9
Tyrosine	0.8	0.6	0.7	0.7	0.7
Tryptophan	0.2	0.2	0.2	0.2	0.3
Hydroxyproline	0.3	0.3	0.0	0.0	0.0
ΣNEAA	10.5	9.4	9.7	9.7	10.6
EAA/NEAA	0.86	0.85	0.87	0.86	0.84

ANALYTICAL METHODS

Proximate composition and amino acid profile of the diets and SWM, as well as carotenoid content of the SWM, were analyzed by Eurofins Steins Laboratory (Vejen, Denmark). The SWM chitin content was determined spectrophotometrically following the method described in (Tsuji et al., 1969). Proximate composition analysis of lobster juveniles was performed for three individuals per dietary treatment. Whole body juvenile lobsters were freeze-dried and subsequently combusted for 16h at 500 ºC (NMKL 23, 1991) for dry matter and ash content determination, respectively. Protein was

determined spectrophotometrically at 750 nm using a commercial Lowry-based, micro-protein determination kit (BIO-RAD 500-0112). Lipids were extracted with chloroform – methanol (2:1 by volume) according to the Folch method (Christie and Han, 2010) and the lipid content determined gravimetrically.

NITROGEN EXCRETION

Pre- and postprandial total ammonia nitrogen (TAN) excretion was determined for each individual lobster between the second and fourth week of the growth trial in 130 mL chambers, using 15 chambers at a time. After a 24h fasting period, each lobster was transferred to a chamber supplied with aeration. Water quality conditions were maintained within the same limits as in the growth trial (19ºC ± 1 ºC temperature, 34 ± 1 salinity, > 90% dissolved oxygen). Water samples of 15 mL were collected manually from individual chambers at time 0h, 2h, 6h, 12h, and 24h for baseline screening of TAN excretion rates. After this period, lobsters were offered a pre-weighed pellet for 4h. At the end of the meal, juveniles were transferred into chambers with new seawater. Water samples were manually collected at time 0h, 2h, 6h, 12h, and 24h for the determination of postprandial TAN excretion rates. Collected water samples were filtered (0.2 μm, Filtropur Sarstedt, Numbrecht, Germany) and stored at 4 ºC until analysis. TAN of collected water samples was determined in duplicate according to (DS, 1975). TAN excretion rate was determined by adapting the formula often used to calculate oxygen consumption rate (Frisk et al., 2013):

$$TAN\ (\mu g\ h^{-1} g\ BW^{-1}) = V_{ch} \times \Delta[TAN] \times \Delta t^{-1} \times BW^{-1}$$

Where: V_{ch} = volume of the chamber, Δ[TAN] = the change in TAN-concentration over time, Δt = time period between sample collection, BW = body weight.

Feed intake was estimated from the uneaten feed fraction after collection, filtration and drying, employing the formula:

$$FI\ (\%\ BW^{-1} day^{-1}) = (dF - uF - L) \times BW^{-1} \times 100\%$$

Where: FI = feed intake, dF = distributed feed, uF = unconsumed feed, L = leaching after 4h, BW = body weight. Leaching was estimated by placing a pre-weighed pellet in the chamber under the same conditions as in the feeding period but without animals. Leaching was estimated for 6 pellets of each diet. Nitrogen intake was calculated as 16% of protein intake estimated from total amino acid content in each diet (Chibnall et al., 1943). The TAN excretion profile was analyzed following a similar approach as in (Frisk et al., 2013), using curve fitting and integrals of the area under the curve (AUC) produced with GraphPad Prism version 5.0 software package. From the TAN profile, we quantified standard TAN excretion rate (STR, μg N h^{-1} g^{-1} BW) estimated by averaging the pre-feeding TAN excretion rates; postprandial peak (μg N h^{-1} g^{-1} BW) - maximum value of TAN excretion rate curve

during the postprandial course; time to peak (TTP, h) - the time from feeding to the postprandial peak; factorial postprandial scope - the peak divided by the STR; postprandial duration (h) - the time from feeding until TAN excretion rate converged with the STR; TAN excretion - the post-feeding integrated excess TAN excretion rate above STR. A graphical representation of the above-mentioned variables is provided in Fig. 3.

EXOSKELETON COLOURATION

Exoskeleton colouration was evaluated following a similar procedure described in (Tlusty, 2005). Briefly, each lobster was photographed with a digital camera (Canon PowerShot G15). The camera was set with a focal length of 6.1 mm and pictures were taken at an exposure time of 1/30 s with an aperture of F2.8. Lobsters were prepared for the photograph by wiping excess water off the exoskeleton and placed on the same white background. Pictures were stored in a 4000 × 2664 pixel format. Each image was analyzed for RGB scores using ImageJ 1.52n software (Abramoff, 2004). RGB scores were measured for each individual at 3 different body locations, including the telson (TEL), the first abdominal segment (FAS) and the dorsal carapace (DC) (Fig. 4). The white background was used to correct for deviation for pure white (100, 100, 100) by standardizing the exoskeleton RGB measurements by the values obtained for the white surface.

STATISTICAL ANALYSIS

Survival data was analyzed by a Kaplan-Meier procedure. Log-Rank (Mantel-Cox) test was used to determine significance ($p < 0.05$). Whenever significance was detected, a Chi-square table with multiple comparisons was generated to identify differences among dietary treatments. All other analyzed parameters are expressed as mean ± SEM, unless otherwise specified. Before analysis, parametric assumptions of normality of residuals and homogeneity of variances were tested using the Shapiro-Wilk and the Levene's test, respectively. In instances where assumptions were not met, data were square root or log-transformed. Comparisons between dietary treatments were performed using one-way ANOVA followed by the Tukey post hoc test, whenever significance differences were identified. A second order polynomial regression with time was used to estimate the average growth in terms of body weight gain for each dietary treatment. The models were validated via residual plot analysis and generated using R version 3.5.1 software (R Core Team, 2018). Curve coefficients were compared in a one-way ANOVA. Apart from the second order polynomial regression for body weight gain, all statistical tests were performed using the IBM SPSS Statistics 25.0 and graphics were generated by GraphPad Prism version 5.0 software package.

RESULTS

Results for specific growth rate (SGR), carapace length increment (iCL), duration of the moult cycle, and proximate composition of the juveniles are presented in Table 4.

SURVIVAL, GROWTH, AND FEED UTILIZATION

The survival of juvenile lobsters varied from 47% to 87% and was significantly affected by the dietary treatment (Fig. 1). Feeding lobsters with the SWM 28% diet resulted in a significantly better survival than that for the SWM 7% diet ($\chi^2 = 5.44$, $p = 0.02$). During the experimental period, lobsters grew from an initial mean weight of 164 mg (8.2 mm carapace length) to mean weights ranging from 308 to 362 mg (10.2 to 11.2 mm carapace length). The estimated growth curves of the juveniles are presented in Fig. 2. Despite the trend for faster growth in juveniles fed the SWM 28% compared to those fed the SWM 0% ($p = 0.07$), no significant differences were detected among the dietary treatments. The SGR and the iCL were not significantly affected by the dietary treatment: SGR varied from 1.05 to 1.25 and the iCL between 24% and 35% (Table 4). Juveniles completed on average 1.7 moulting cycles for the duration of the experiment. The intermoult period was not affected by the dietary treatment and varied between 26 and 31 days (Table 4).

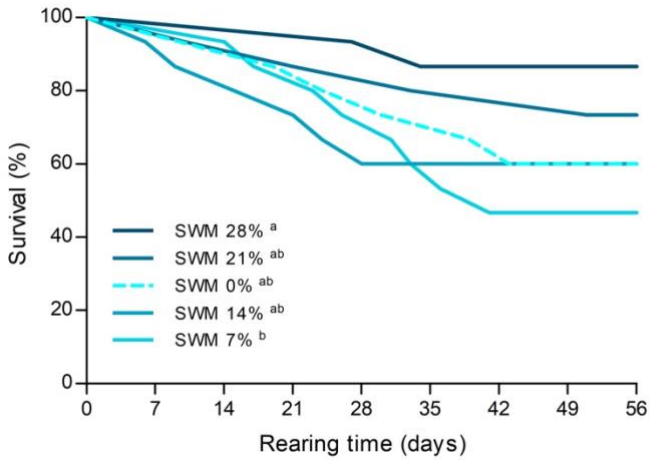

FIGURE 1. Survival of *Homarus gammarus* juveniles (% of initial numbers, N=15 per group) fed experimental diets containing different levels of shrimp waste meal over an eight-week period. Different letters denote statistically significant difference ($p < 0.05$) determined by Log-rank test.

PROXIMATE COMPOSITION

There was no effect of diet on the proximate composition of the juvenile lobsters (Table 4). The protein content ranged between 24% and 32% and lipid content between 5% and 8%. The dry matter content varied from 24% to 29% and the ash content between 31% and 35%.

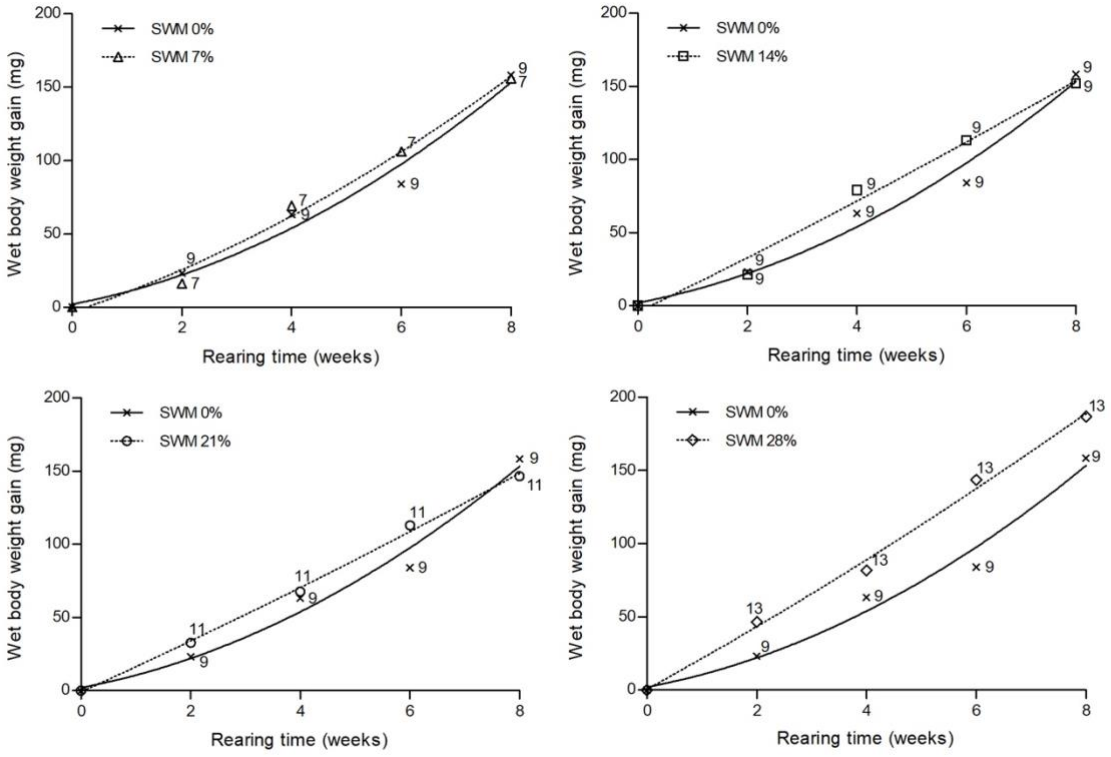

FIGURE 2. Second order polynomial model fit to average wet body weight gain of *Homarus gammarus* juveniles fed the experimental diets over an eight-week period. Data points represented as mean. Point label indicates sample size.

NITROGEN EXCRETION

Pre- and postprandial TAN excretion values are summarized in Table 5. No statistical difference was observed in the STR between treatments. The postprandial course of TAN excretion rate was identical among dietary groups with an increase during the first 5h to 6h followed by a decline during the following 9h to 11h (Fig. 3). The postprandial peak, scope, time to peak, and duration of TAN excretion rate was similar between diets. Diet significantly affected the N-intake, however, a post hoc Tukey test failed in identifying differences among dietary treatments. The N-excretion / N-intake ratio was between 12% and 19% and no differences were observed between diets.

TABLE 4. Growth performance and proximate composition of juvenile *Homarus gammarus* fed the experimental diets over an eight-week period.

	Level of SWM as % of dietary protein					
	0	7	14	21	28	One-Way ANOVA
Bioassay						
BW_i (mg)	168 ± 18	162 ± 12	164 ± 16	160 ± 12	167 ± 16	$F_{1,74} = 0.05$
CL_i (mm)	8.2 ± 0.3	8.1 ± 1.1	8.2 ± 0.2	8.2 ± 0.3	8.4 ± 0.3	$F_{1,74} = 0.24$
N	15	15	15	15	15	
BW_f (mg)	342 ± 40	315 ± 26	309 ± 54	308 ± 44	362 ± 47	$F_{1,48} = 0.31$
CL_f (mm)	11.2 ± 0.5	10.7 ± 0.3	10.2 ± 0. 6	10.3 ± 0.5	10.8 ± 0.4	$F_{1,48} = 0.68$
SGR (% d^{-1})	1.14 ± 0.19	1.24 ± 0.19	1.25 ± 0.21	1.05 ± 0.14	1.25 ± 0.13	$F_{1,48} = 0.31$
iCL (% CL_i^{-1})	34.9 ± 4.7	33.6 ± 5.7	26.9 ± 3.7	24.1 ± 4.3	26.0 ± 2.9	$F_{1,48} = 1.31$
N	9	7	9	11	13	
Moult cycle (days)	29.3 ± 1.6	26.0 ± 2.4	28.3 ± 2.0	30.7 ± 2.1	30.9 ± 1.8	$F_{1,37} = 1.03$
N	7	8	7	7	9	
Proximate composition (% of DM)						
Dry matter	23.9 ± 1.2	26.4 ± 2.5	28.8 ± 1.4	26.2 ± 1.3	24.4 ± 2.7	$F_{1,14} = 1.00$
Protein	29.1 ± 2.2	24.0 ± 4.1	30.6 ± 2.2	32.2 ± 1.1	31.5 ± 1.7	$F_{1,14} = 1.74$
Lipid	5.2 ± 1.0	6.2 ± 0.8	6.7 ± 1.0	7.8 ± 0.7	6.3 ± 0.2	$F_{1,14} = 1.41$
Ash	34.4 ± 0.4	31.6 ± 2.3	35.4 ± 4.9	32.9 ± 2.9	31.2 ± 1.7	$F_{1,14} = 0.40$
N	3	3	3	3	3	

BW_i = initial wet body weight; CL_i = initial carapace length; BW_f = final wet body weight; CL_f = final carapace length; SGR = specific growth rate; iCL = carapace length increment; FI = dry feed intake; FCR = dry feed intake / wet weight gain. Values are means ± SEM.

TABLE 5. Overview of pre and postprandial total ammonia nitrogen excretion (TAN) of juvenile *Homarus gammarus* fed the experimental diets.

	Level of SWM as % of dietary protein					
	0	7	14	21	28	One-Way ANOVA
STR (μg h^{-1} g^{-1}BW)	11.6 ± 1.3	9.6 ± 1.4	8.4 ± 0.7	8.7 ± 0.9	8.2 ± 0.7	$F_{4,62} = 1.66$
Postprandial peak (μg h^{-1} g^{-1} BW)	44.5 ± 5.4	36.8 ± 5.0	42.5 ± 7.2	43.0 ± 4.2	43.1 ± 3.4	$F_{4,62} = 0.34$
Factorial postprandial scope	4.2 ± 0.5	4.4 ± 0.7	5.3 ± 1.0	5.3 ± 0.5	5.8 ± 0.7	$F_{4,62} = 0.99$
Time to peak (h)	4.8 ± 0.5	5.2 ± 0.5	5.7 ± 0.6	4.8 ± 0.5	4.9 ± 0.5	$F_{4,62} = 0.53$
Duration (h)	13.9 ± 1.6	13.5 ± 1.8	12.8 ± 1.3	16.2 ± 1.6	14.5 ± 1.7	$F_{4,62} = 0.64$
TAN excretion (μg g^{-1} BW)	195.3 ± 37.7	164.2 ± 29.1	191.3 ± 40.1	247.3 ± 33.8	244.9 ± 37.4	$F_{4,62} = 1.03$
N-intake (μg g^{-1} BW)	1310 ± 255	1323 ± 177	1116 ± 335	1880 ± 197	2046 ± 264	$F_{4,62} = 2.65^{*}$
N_{exc} / N_{int} (%)	12.3 ± 3.8	12.2 ± 2.0	18.5 ± 4.3	13.5 ± 1.8	11.8 ± 1.6	$F_{4,62} = 1.45$
N	13	12	11	13	14	

Visual explanation of the different variables showed in Fig 3. STR = Standard ammonium nitrogen excretion rate; N_{int} = nitrogen intake. Values are means ± SEM. The symbol (*) indicates statistical significant effect of dietary treatment at $p < 0.05$ measured by one-way ANOVA. The absence of superscript letters indicates that no significance differences were detected by the Tukey post hoc test followed by one-way ANOVA.

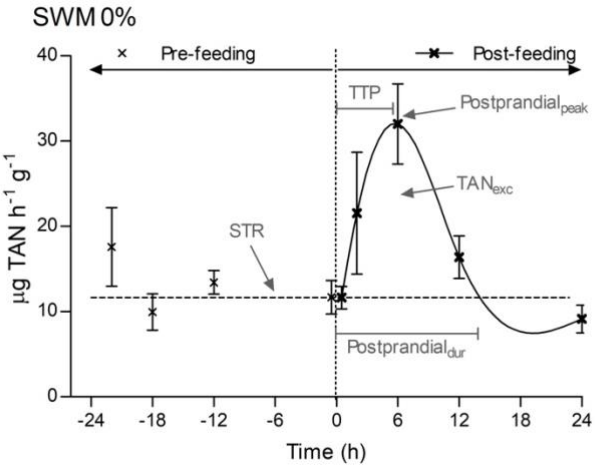

FIGURE 3. Representative plot of pre and post-feeding total ammonia nitrogen excretion rates (ug TAN h-1 g $^{-1}$ BW) over time in *Homarus gammarus* juveniles fed one of the experimental diets (SWM 0%). The juveniles were fed at 0h (vertical dashed line). The dashed horizontal line represents the standard TAN excretion rate (STR). The postprandial TAN excretion variables mentioned on table 4 are visually explained. Data points represented as mean ± standard error.

EXOSKELETON COLOURATION

Two individuals representing the extremes on RGB scores – the lightest and the darkest individuals among all individuals analyzed – are presented in Fig. 4 for comparison. High RGB scores translate into lighter colour while low scores translate into a darker colouration of the exoskeleton. Despite tendencies towards lower scores in all three colours (Red, Green, and Blue) with increasing SWM dietary content, in particular for the FAS and DC body locations, no significant differences were observed between dietary treatments (Fig. 5).

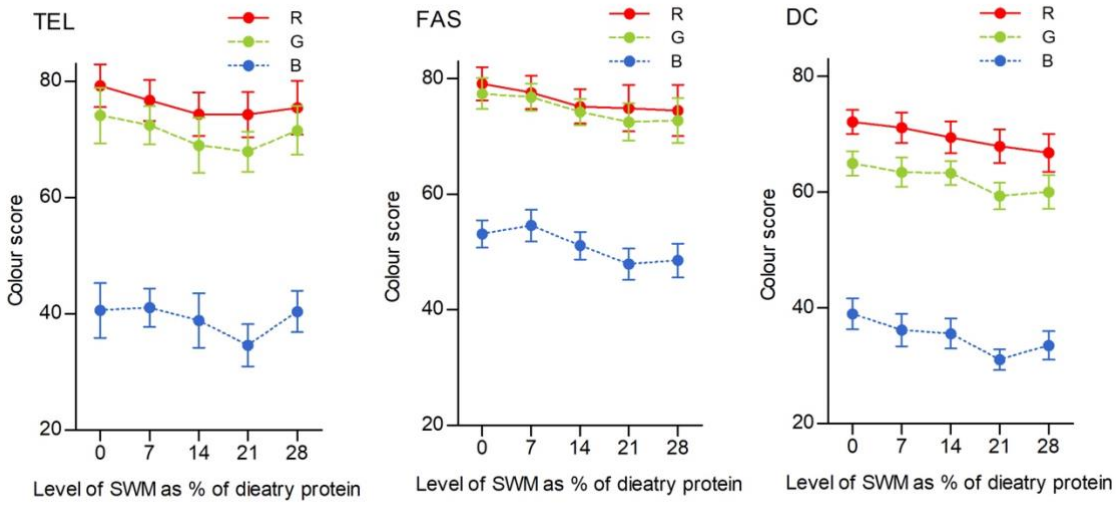

FIGURE 5. RGB colour scores at three different body locations of *Homarus gammarus* juveniles fed experimental diets containing different levels of shrimp waste meal over an eight-week period. Body locations are as identified in Fig. 4. Data points represented as mean ± standard error.

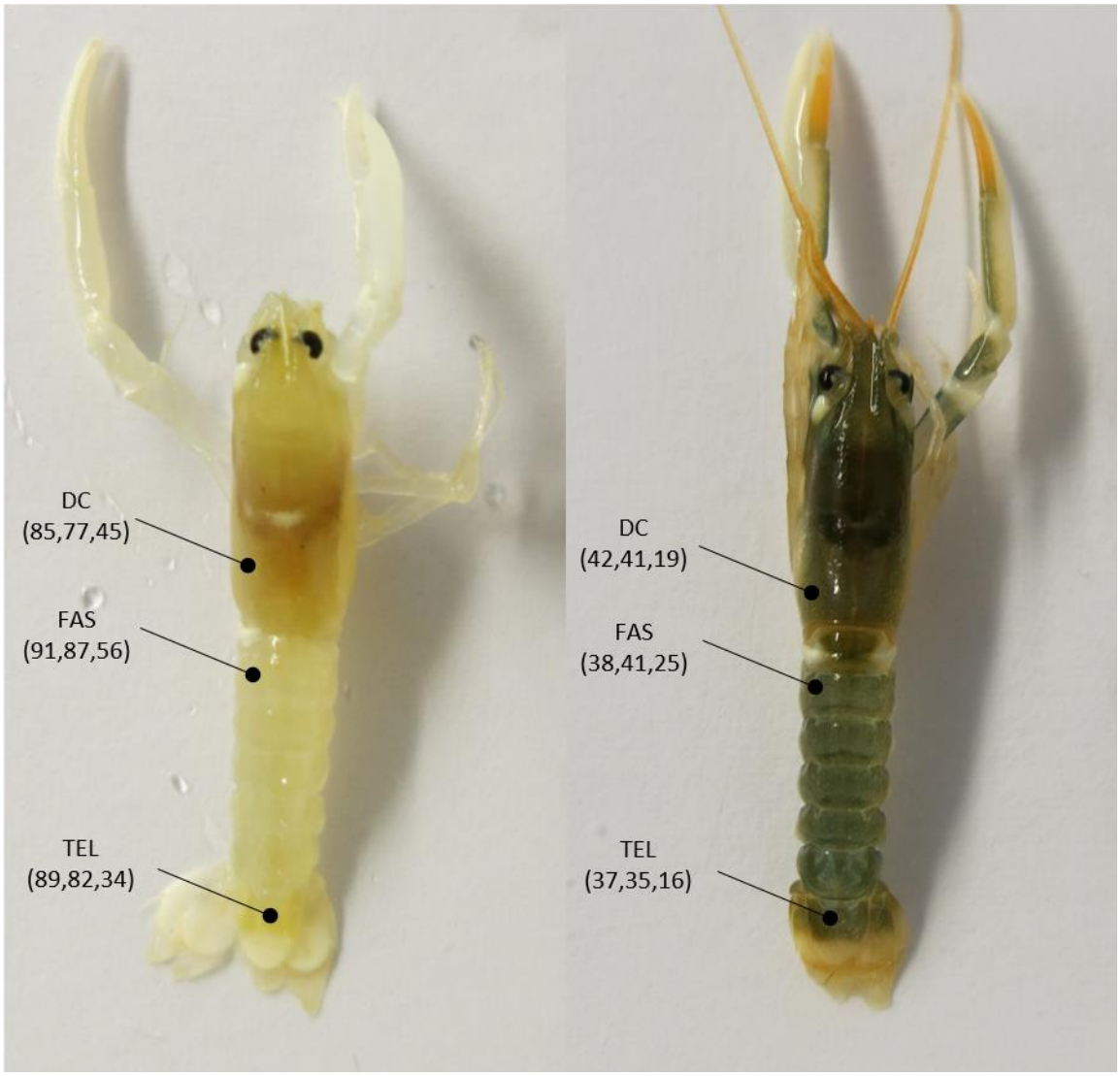

FIGURE 4. Body locations of *Homarus gammarus* juveniles examined for digital colour analysis. TEL = telson; FAS = first abdominal segment; DC = dorsal carapace. Example of two specimen; a light colour and a dark colour juveniles and respective (R,G,B) scores.

DISCUSSION

The main findings from this study were that the dietary inclusion of SWM had a significant positive effect on the survival of *H. gammarus* juveniles, and neither negatively nor positively influenced growth performance. The highest survival (87%) was observed for the group of lobsters fed the SWM 28% while the lowest survival (47%) was recorded for the SWM 7% dietary group. This finding suggests that the chitin or astaxanthin content in the SWM is potentially nutritionally beneficial for *H. gammarus*, since the SWM was the only source of both chitin and astaxanthin in the diets. Calculating dietary chitin levels (from 0% to 4.9%) this shows a positive correlation between SWM inclusion and chitin content in the experimental diets (Table 2).

Dietary incorporation of chitin and its derivatives has been previously reported to increase survival in crustaceans (Niu et al., 2013; Powell and Rowley, 2007). Chitin is a natural polymer present in the exoskeletons of crustaceans. Chitinase (EC 3.2.1.14), NAGase (EC 3.2.1.30), chitobiase (EC 3.2.1.29), and lysozymes (EC 3.2.1.17) degrade chitin into glucosamine (Stoykov et al., 2015), that can be used for the synthesis of the new exoskeleton during the moulting process (Fox, 1993). The moulting process is a critical period for lobsters where increased mortality is particularly likely (Anger et al., 1985; Goncalves et al., 2021b). Hence, the potentially higher bioavailability of glucosamine in lobsters fed the SWM 28% could partly explain the lower mortality observed in this dietary group. Additionally, it has been demonstrated that chitin has an immune stimulatory activity (Tsai and Hwang, 2004) and can remove potentially pathogenic bacteria from the gut via attachment of these microorganisms to chitin-binding proteins (Holt et al., 2019; Vaaje-Kolstad et al., 2005). In fact, it has been observed that crabs (*Carcinus maenas*) fed 5% chitin supplemented diets had a lower number of bacteria in the hepatopancreas (Powell and Rowley, 2007). The authors concluded that enhanced survival was related to a bacteria purge from the gut caused by dietary chitin.

The increased survival observed for the SWM 28% group may also be related to a strong antioxidant capacity of astaxanthin. Astaxanthin is present in the exoskeleton of several crustaceans, including the shrimp *P. borealis* (Dave et al., 2020), the species used to manufacture the SWM and incorporated in our experimental diets. The antioxidant capacity of astaxanthin is caused by its strong electron-donating capability enabling protection of the cells from free oxygen radicals (Lim et al., 2018).

Despite the trend for faster growth in lobsters fed the SWM 28% diet compared to those fed the SWM 0%, results showed no significant improvement on growth, neither in terms of SGR nor carapace length increment. In this study the SGR ranged between 1.05 % day^{-1} and 1.25 % day^{-1} among dietary treatments which was slightly lower than previously observed for *H. gammarus* (1.72 % day^{-1}) in a study using an extruded feed of similar macronutrient ratios (Goncalves et al., 2021a). One possible explanation for the difference in growth rates is the use of a younger stage and smaller initial size (stage V to VI, ~ 90 mg wet body weight) animals in the former study as compared to individuals in this study (stage VI – VII, ~ 164 mg wet body weight). The higher SGR observed by Goncalves et al. (2021a) could also be related to a higher dry mass feed intake of the juveniles fed the extruded feed (5.3% BW day^{-1}) compared to the semi-moist diets used here (1.6% to 2.9% BW day^{-1}). Despite the different methodologies to estimate feed intake between both studies, the observed differences are most likely related to the higher DM content of the former extruded feed (92%) compared to the semi-moist diets used here (~ 40%). It was initially hypothesized that lobsters may be thrashing and tearing feed more easily when reared on diets with a softer consistency than the extruded feeds previously used, and that this may result in increased feed intake. However, it appears instead that lobster juveniles fed the semi-

moist diets reduced their feed intake, possibly due to induced satiation from a mechanical expansion of the gut.

Although the dietary inclusion of shrimp waste meal has been shown to improve growth and feed conversion ratio of different shrimp species (*P. vannamei* and *P. monodon*) (Cruz-Suárez et al., 1993; Fox et al., 1994), this was not the case for *H. gammarus* in this study. One possibility to explain the dissimilar results could be the different species or source (heads, appendages, exoskeletons) and the processing methods used to obtain the shrimp waste meal ingredient. For example, (Tacon, 1996) reported that shrimp heads contained higher levels of protein and lipids than exoskeletons, whereas exoskeletons had higher levels of ash and crude fibre. Another possibility to explain the lack of significant growth improvement could be the restriction in energy intake caused by the semi-moist diets due to the above-mentioned mechanical satiation. Such restriction may have limited their ability to unfold the growth potential, which could potentially be overcome by feeding the juveniles multiple times a day.

Postprandial total ammonia nitrogen excretion was not affected by the dietary treatment suggesting a similar capacity for protein utilization within the experimental groups (Frisk et al., 2013). That is because the amount of postprandial nitrogen excreted as ammonia depends largely on the amount of protein assimilated after feeding (Ponce-Palafox et al., 2017). The estimated ratio N-excreted / N-intake varied between 12% - 19% corresponding to 81% - 88% N-retention. A slightly higher N-retention (92%) was estimated for juvenile *H. gammarus* tested under the same conditions and fed an extruded feed of similar protein, lipid, and carbohydrate ratios (Goncalves et al., 2020). However, in the latter, N-retention was estimated from the excretion of total nitrogen instead of TAN, which justifies the slightly higher observed retention. A decreasing trend in the STR (pre-feeding TAN excretion rates) with increasing SWM inclusion suggests a potential for lower amino acid catabolism of metabolic origin and, consequently, a higher availability of proteins that can be used for growth (Rosas et al., 2001). However, despite the trend, no significant differences were detected among dietary treatments which can also explain the lack of significant effect of SWM inclusion on lobsters growth performance.

It was hypothesized that SWM, as a natural source of astaxanthin, would affect the exoskeleton colouration of the juvenile lobsters. However, despite the decreasing trend in RGB scores (lower scores indicating darker and more natural colour), improvements in exoskeleton colouration of juvenile *H gammarus* in response to SWM inclusion did not occur. It has been previously observed that the use of formulated diets with no astaxanthin supplementation to grow lobsters induces a shift towards a progressively lighter colour (Barclay et al., 2006; Crear et al., 2002; Floreto et al., 2001). Whether in commercial on-growing for consumption or as a fisheries remediation measure, the loss of natural colour in hatchery-reared lobsters is undesirable. It decreases the commercial value of the final product and interferes with survival in their natural habitat by decreasing camouflage from predators (Tlusty, 2005). Therefore, it is of great interest if the supplementation of diets with additives can boost body

pigmentation, and astaxanthin being the most commonly carotenoid used among several aquatic species (Lim et al., 2018). A carotenoid level of 115 mg kg^{-1} was required in formulated diets to produce juvenile lobster *Jasus edwardsii* of similar colour to wild-caught individuals (Crear et al., 2002) while a specification for juvenile *Panulirus ornatus* of 80 mg kg^{-1} total carotenoid (50 mg kg^{-1} free astaxanthin) was considered appropriate to ensure good colouration (Barclay et al., 2006). If the requirement for juvenile *H. gammarus* is within the same range, than the carotenoid content in the tested diets was clearly insufficient. The estimated total carotenoid content in the processed SWM was 73 mg kg^{-1} (of which only 3.9 mg kg^{-1} was free astaxanthin), considerably lower than previously reported values for *P. borealis* by-products (284 mg kg^{-1}, Dave et al., 2020). Astaxanthin has a highly unsaturated molecular structure and hence, a high sensitivity to heat, light, and oxidative conditions (Lim et al., 2018). Consequently, the exposure of the raw shrimp waste to elevated temperature during processing as well as during pellet formulation (85°C) may have caused damage to, or losses of, astaxanthin.

In conclusion, this study showed that SWM can be included at least up to 28% of dietary protein in formulated diets for juvenile *H. gammarus* without a decrease of growth. Rather, the inclusion of SWM at the highest level (28%) improved survival. The question remains which component in the SWM was responsible for improving survival, but the high chitin content in the SWM, and low astaxanthin content, points to a major role of the former, although further studies should explore this hypothesis. The high moisture content (~60%) in our experimental feeds might have limited, to some extent, the growth potential of the lobster juveniles. Further investigations on the incorporation of SWM in feeds with a higher dry matter content would be relevant, although our results clearly demonstrate the quality of SWM as a protein source for *H. gammarus* juveniles. Results are encouraging for the future valorisation of shrimp waste as an ingredient for aquafeeds. There is still room to improve the potential of SWM, especially concerning the levels of astaxanthin in the processed ingredient. Future studies should consider pre-treatment techniques so that the preservation of carotenoid levels in the SWM and diets can be maximized. In addition, the application of techniques during raw material processing that allow the concentration of protein in the SWM would enable its dietary incorporation at higher levels. Overall, we conclude that the inclusion of SWM into formulated diets for *H. gammarus* has a tremendous potential and may, therefore, contribute to the economic viability of European lobster farming.

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STUDY 5

Interactions of temperature and dietary composition on juvenile European lobster (*Homarus gammarus*, L.) energy metabolism and performance

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Interactions of temperature and dietary composition on juvenile European lobster (*Homarus gammarus*, L.) energy metabolism and performance

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Abstract

Optimal rearing temperatures for European lobster *Homarus gammarus* in aquaculture differ from those prevalent in their aquatic ecosystems and acclimating juveniles to the prevailing temperatures before release may aid in the success of re-stocking programs. As the dietary nutritional composition is important for optimal performance of *H. gammarus*, in this study we aimed to investigate whether juvenile growth and energy metabolism responses to temperature variation could be modulated by the diet. Prior to the trial start, the juveniles were divided into two groups. One was maintained at 19°C and the other gradually adapted to 13°C. From this point and for a 24-day period, juveniles (~ 100 mg) within each temperature group were assigned one of two experimental diets: a carbohydrate-rich (HC) or a protein-rich (HP) extruded feed. Antarctic krill (AK) was used as a control diet within each temperature group. Feed intake, growth, glycogen, glucose, lactate, and protein concentrations of *H. gammarus* in each group were evaluated. Regardless the dietary treatment, feed intake, cephalothorax protein and glucose, and abdominal glycogen and glucose levels decreased at colder temperature. The effect of lower temperature on growth (SGR and moulting rate declines) and energy metabolism (reduction on cephalothorax glycogen and protein) was more severe in HC-fed lobsters. Results showed that the impact of lower temperature on juvenile *H. gammarus* can be modulated by diet highlighting the importance of designing optimized diets not only for growth and feed efficiency but also for resilience to environmental variation.

Keywords: Extruded feeds; Carbohydrate; Protein; Glycogen; Glucose

INTRODUCTION

The European lobster *Homarus gammarus* is a commercially important crustacean widely distributed from northern Norway to Azores and Morocco (Triantafyllidis et al., 2005). Because of its high market price, the species has been subjected to high fishing pressure causing decreases in annual landings particularly during the 1960's and 1970's (Ellis et al., 2015). As a mitigation measure, several experimental stock enhancement projects have been launched across Europe (Agnalt, 2004; Browne and Mercer, 1998; Cook, 1995; Latrouite and Lorec, 1991; Schmalenbach et al., 2011). Despite the relative success of some programs in recovering European lobster natural stocks (Agnalt, 2004), the

hatchery-rearing of larvae and juvenile lobsters is not yet a sustainable practice (Beal et al., 2002). The lack of suitable artificial diets capable of reducing feeding costs and simplifying production practices remains as a major issue, together with high mortalities associated to cannibalistic behavior (Hinchcliffe et al., 2020; Powell, 2016; Powell et al., 2017).

The substitution of natural diets for artificial feeds has been investigated in both the American lobster, *Homarus americanus* and the *H. gammarus* (Floreto et al., 2000; Goncalves et al., 2021, 2020; Hinchcliffe et al., 2020; Tlusty et al., 2005). Together, the results establish that juveniles of both species have a high requirement for protein, a moderate requisite for carbohydrates, and a poorer utilization of lipids. The high requirement for protein is also reflected in the fact that, in their natural habitats, homarid lobsters mainly feed on mollusks, crustaceans and polychaetas (Ali and Wickins, 1994). Protein is the main building block for tissues in crustaceans, and therefore, fundamental for somatic growth (Castell and Budson, 1974). In instances of insufficient non-protein energy in the diet, crustaceans will use protein for energy instead of growth (Ward et al., 2003). Hence, an efficient diet must provide adequate non-protein energy to allow the more costly protein sources to be spared for growth (Nelson et al., 2006).

Unlike fishes, crustaceans can make use of high carbohydrate in their diet to meet energy requirements as it is a readily available source of energy for most species (Wang et al., 2017). An efficient digestibility and subsequent use for energy of polysaccharides (starch and dextrin) have been demonstrated for several crustaceans including the spiny lobsters *Panulirus argus* and *Jasus edwardii* (Rodríguez-Viera et al., 2017; Simon, 2009). (Goncalves et al., 2021, 2020) observed that the increase of carbohydrate content in a formulated extruded feed of 40% protein (as fed) improved *H. gammarus* growth performance. It was also observed for the prawn *Penaeus monodon* that the dietary protein content could be lowered from 50% to 40% without significant effect on growth, if the energy level of the diet was kept constant (Bautista, 1986). Since carbohydrates are in general less expensive than animal protein ingredients (Wang et al., 2016) it is economically attractive to increase the proportion of carbohydrates in formulated feeds for lobsters.

While the mentioned studies provide relevant information for the development and optimization of formulated feed for *H. gammarus*, little information is available on the dietary effects on animal resilience to environmental change. This is particularly relevant when developing feeds for hatcheries targeting the production of juveniles for re-stocking purposes. Even if the optimal rearing conditions for dissolved oxygen, pH, and salinity lie within the prevalent conditions at sea, that is not the case for temperature (Kristiansen et al., 2004). Recommended aquaculture rearing temperatures between 18°C and 22°C (Wickins and Lee, 2002) are justified by maximum growth rates within this thermal window (Thomas et al., 2000). However, and at least for releases in the North Atlantic and North Sea region where temperatures oscillate between 11°C to 17°C from May to August (van der Meeren et al., 2000), temporarily rearing juvenile *H. gammarus* at colder temperatures before release

would allow a more precise evaluation of their ability to survive in the sea, find out whether improvements are needed, and eventually increase the likelihood for successful restoration.

Beyond growth performance, changes in temperature can also alter the energy utilization in crustaceans causing changes in the metabolite levels of their most important depots – the hepatopancreas and muscle (Jimenez and Kinsey, 2015). These shifts in energy storage may reflect changes not only due to a direct effect of temperature, but also to indirect adjustments, for example, in activity level and feed intake (Jimenez and Kinsey, 2015). In fact, coupled with metabolic rate depression, the decrease in feed intake is a common response to decreased temperature in crustaceans and, depending on its magnitude, the energy metabolism might be affected (Sacristán et al., 2017). Further, it has also been demonstrated that, in instances of feed intake restriction, the energy metabolism can be modulated by dietary composition (Vinagre and Silva, 1992).

In this context, we aimed to evaluate the ability of juvenile *H. gammarus* to cope with the effects of temperature variation while fed different formulated experimental diets. Therefore, the impact of a high protein feed or a carbohydrate-rich extruded feed were evaluated in relation to a control diet of Antarctic krill (*Euphausia superba*) on the performance and energy metabolism of juvenile *H. gammarus* held at different temperatures. To this end, we monitored growth (moulting rates, specific growth rates, and carapace length increments) and measured glycogen, glucose, lactate, and protein concentrations in juvenile *H. gammarus* held at 13°C and 19°C and fed the different diets.

MATERIALS AND METHODS

EXPERIMENTAL ANIMALS

Juvenile lobsters were obtained from wild females caught in the Limfjord (North Jutland, Denmark). After hatching, pelagic larvae were communally reared in 500 mL squared tanks, supplied with fresh seawater in a flow through system (17°C temperature, 33-35 PSU salinity, > 90% dissolved oxygen, < 0.1 $mg.L^{-1}$ ammonia-N). From hatching, larvae were daily fed with thawed Antarctic krill, *Euphausia superba*, Akudim A/S, Denmark, a common diet used in lobster hatchery farming units (Burton, 2003). When the animals reached the postlarval stage IV, they were transferred to individual compartments in a raceway system. The system consisted of 3D printed PolyLacticAcid (PLA) bioplastic cassette systems (Prusa i3 MK2, Czech Republic) with individual compartments of 200 mL. The cassettes were placed in the raceway that was supplied by a semi-closed recirculation seawater system at a constant flow rate of 330 L h^{-1} (19 ± 1°C temperature, 34 ± 1 PSU salinity, > 90% dissolved oxygen, < 0.1 $mg.L^{-1}$ ammonia-N). The photoperiod was set at 8h light: 16h dark. The early juveniles were fed daily with thawed Antarctic krill and kept under these conditions for approximately six weeks during which individuals developed into stage > V. Prior to the commencement of the experiment, the

juveniles were divided in two groups of 27 individuals each. One group was maintained in the same raceway at 19 ± 1°C temperature. The other was moved to an identical raceway system and adapted to a lower temperature (13 ± 1°C) by decreasing 1°C per day during six days. The chosen acclimation period was within the thermal acclimation interval (3-14 days) suggested by (Camacho et al., 2006) for adult *H. americanus*.

EXPERIMENTAL PROCEDURE

At the beginning of the experiment, all lobsters were individually weighted and measured for carapace length. Three homogeneous groups of nine individuals per temperature (initial weight of 101 ± 37 mg per lobster; carapace length of 7 ± 1 mm, mean ± SD) were randomly allocated to the dietary treatments. The same 3D printed cassette system described above was used for the feeding trial. Hence, as each lobster was held separately, the experimental unit in the present study was the individual lobster. Juvenile lobsters were fed Antarctic krill - AK, a carbohydrate-rich - HC (40% protein and 35% carbohydrate) extruded feed or a protein-rich - HP (50% protein and 26% carbohydrate) extruded feed. The AK was used as a control group. The extruded feeds (Sparos Lda., Portugal) were formulated to be isoenergetic and were extruded as 4 mm pellets (Goncalves et al., 2020). Details on the experimental feed ingredients and proximate composition are provided in Table 1. Each individual lobster was daily fed a pre-weighted pellet (~ 45 mg) or krill piece (~ 40 mg). Juveniles were allowed to eat for 4h, from 9:00 to 13:00. At the end of each meal, uneaten feed was removed and stored at -20°C for feed intake estimation. The daily uneaten feed fraction from groups of three lobsters (minus eventual dead) was stored in the same vial until the end of the trial, allowing triplicates per dietary × temperature treatment. Each vial content was then filtered, dried and weighed. The feed intake was estimated applying the formula:

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

Where: FI = feed intake, dF = distributed feed, uF = unconsumed feed, L = leaching after 4h, BWi = initial body weigth, Δt = number of days during which uneaten food was collected. Details on the procedure are described in (Goncalves et al., 2021). The occurrence of new moults and deaths was daily inspected. Dead individuals were daily counted and removed. Moulted exoskeletons were left in the compartments, so the juveniles were allowed to eat them upon moulting. After 24 days kept under the above mentioned conditions, each lobster was lethally cold anesthetized, measured and weighted. Individual juveniles were rinsed with Milli-Q water and stored at -80°C until further analysis. The following formulas were used to determine growth performance:

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

Where: SGR = Specific growth rate, BW_f = final wet body weight, BW_i = initial wet body weight, Δt = duration of the trial (24 days).

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

Where, iCL = increment in carapace length, CL_f = final carapace length, CL_i = initial carapace length.

TABLE 1. Formulation and chemical composition of experimental diets (adapted from Goncalves et al., 2020).

	AK	HC	HP
Ingredients (g 100 g^{-1}as fed)			
Antarctic krill	100		
Fish meal		15.0	15.0
Squid meal		12.5	25.5
Krill meal		25.0	20.0
Wheat gluten		2.0	5.0
Wheat meal		17.3	17.3
Wheat starch		22.9	14.1
Fish oil		2.2	0.0
Soy lecithin		1.0	1.0
Vitamin & minerals premix		2.0	2.0
Astaxanthin [a]		0.15	0.15
Proximal composition (g 100 g^{-1} as fed)			
Moisture	91.6	7.8	8.6
Ash	1.2	6.8	6.9
Crude protein	5.8	40.0	49.7
Crude fat	1.0	10.7	8.6
Carbohydrates [x]	0.5	34.7	26.3
Gross energy (KJ g^{-1}) [y]	1.8	19.0	18.7
Protein/Energy (g MJ^{-1})	32.6	21.0	26.5

[a] Carophyll Pink 10% CWS, 10% astaxanthin, DSM Nutritional Products, Switzerland.
[x] Estimated by difference: Carbohydrates (%) = 100 – (Crude protein + Crude fat + Ash).
[y] Gross energy (MJ kg^{-1}) = (Protein content × 21.3 kJ g^{-1} + lipid content × 39.5 kJ g^{-1} + Carbohydrate content × 17.6 kJ g^{-1}) / 1000 kJ MJ^{-1} (Cuzon and Guillaume, 1997)

BIOCHEMICAL ANALYSES

After removal of the pleopods, legs, chelipeds, antennae and antennules, each individual juvenile was divided in two different sections – cephalothorax and abdomen. The separation was performed to distinguish different target tissues, since the small size of the animals did not allow for the dissection of specific organs (i.e. hepatopancreas) nor the collection of hemolymph. Thus, it was assumed that the cephalothorax would better represent the metabolite levels in the hepatopancreas and hemolymph and the abdomen would represent the metabolites in the muscle. The frozen cephalothorax and abdomen of each lobster were minced on an ice-cold Petri dish, homogenized by ultrasonic disruption with 10 and 20 volumes of ice-cold Milli-Q water, respectively, centrifuged (10 min at 13000 *g*) and the supernatant used to assay tissue metabolites. Lactate levels were determined with a colorimetric kit (K-Late 06/18, Megazyme, Ireland). Tissue homogenate glucose was analyzed with colorimetric kit from Merck Millipore (CBA086, Germany). Glycogen levels were assessed by measuring glucose before and after glycogen breakdown by α-amyloglucosidase (Keppler and Decker,

1974). Soluble protein was determined spectrophotometrically at 595 nm using a commercial Bradford-based reagent from Sigma (B6916, St. Louis, USA).

STATISTICAL ANALYSIS

Data are expressed as means ± SEM unless otherwise specified. Before analysis, parametric 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 root transformed. Metabolite levels, protein concentration, SGR, iCL, and FI were subjected to a two –way ANOVA, considering temperature and diet as explanatory variables. Whenever significant differences were identified, means were compared by the Holm-Sidak *post hoc* test. Principal component analysis – PCA – was performed using the metabolite levels. Moult occurrence was analyzed by using a Kaplan-Meier procedure. Significance was tested using the Log-rank (Mantel-Cox) test. Whenever significance was detected, a Chi-square table with multiple comparisons was generated to identify differences among treatments. Differences were considered significant when $p < 0.05$. The PCA analysis was performed using R version 3.5.1 software and the factoextra version 1.07 package. All other statistical analysis were performed using the IBM SPSS Statistics 25.0 and graphics were generated by GraphPad Prism version 5.0 software package.

RESULTS

GROWTH PERFORMANCE

At the end of the 24-day experimental period, the cumulative moulting for the HC13 (11%) group was significantly lower compared to all the other treatments ($\chi^2 = 6.16$, $p = 0.01$), except for the HC19 (Fig. 1). Table 2 summarizes the effect of temperature, diet, and their interaction on growth, feed intake, and survival of early juvenile *H. gammarus*. During the experimental period, juveniles grew from an initial mean weight of 101 mg (7.0 mm carapace length) to mean weights ranging from 100 mg to 138 mg (7.6 mm to 8.5 mm carapace length) among treatments. The SGR was significantly affected by the main factor diet – the SGR in lobsters fed the HC feed (0.1 ± 0.2 % d^{-1}) was 10-fold lower compared with the AK-fed lobsters (1.0 ± 0.2 % d^{-1}). The negative SGR (-0.2 % d^{-1}) observed for the HC13 group had a major contribution for the overall lower SGR in the HC-fed juveniles. No significant effects were detected for the carapace length increment. The feed intake varied between 2 % BW_i d^{-1} and 12 % BW_i d^{-1} and was significantly affected by both main factors - temperature and diet. The dry mass feed intake was higher for both extruded feeds (HP and HC) when compared to the AK diet but the reverse trend was observed when intake was estimated from wet mass (data not shown). Low temperature caused a significant decrease in feed intake in all dietary treatments. The survival of

juvenile lobsters fluctuated between 56% (HC13) and 100% (HP13), with most deaths being observed during the moulting process.

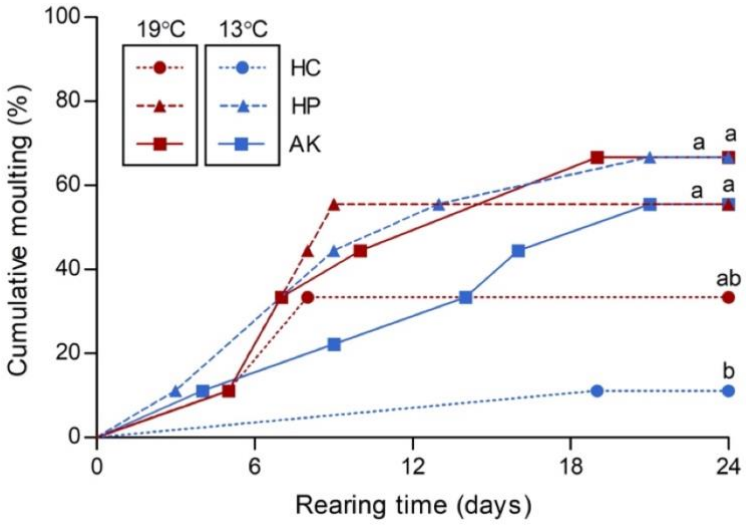

FIGURE 1. Cumulative moults of *Homarus gammarus* (% initial numbers) fed on different diets and maintained at different temperatures over a 24-day period. Different letters denote statistically significant difference between moult curves at $p < 0.05$.

METABOLITES

The effect of temperature, diet, and the interaction of both factors on the content of metabolites is summarized in Table 3. In general, glycogen and lactate levels were higher in the abdomen than the cephalothorax while glucose and protein contents varied within a similar range in both body sections. There was a significant main effect of the diet on the glycogen content in the cephalothorax, which was lower in the HC-fed lobsters (8 ± 2 μmol g^{-1}) than in the AK-fed group (40 ± 9 μmol g^{-1}). In the abdomen, the level of glycogen was significantly affected by temperature being higher at 19°C (183 ± 44 μmol g^{-1}) than at 13°C (77 ± 23 μmol g^{-1}). There was a main effect of temperature on glucose content in both cephalothorax and abdomen, which was higher at 19°C than at 13°C. No significant differences among treatments were observed for the lactate levels in neither the cephalothorax nor the abdomen. Both the temperature and the interaction temperature × diet affected the protein content in the cephalothorax of early juveniles. Thus, within the HC dietary treatment, a significant reduction of 51% in protein content was detected in the cephalothorax of juveniles held at 13°C compared to those maintained at 19°C. Within the 13°C temperature group, the cephalothorax protein content was significantly lower (10 ± 1 mg g^{-1}) for the HC-fed juveniles compared to those fed on the AK diet (20 ± 1 mg g^{-1}).

TABLE 2. Growth, feeding and survival of *Homarus gammarus* after held for 24 days at two different temperatures and for each temperature fed three different diets.

Temp	Diet	CL_i (mm)	BW_i (mg)	N	CL_f (mm)	BW_f (mg)	iCL (% CL_i)	SGR (% d^{-1})	N	FI (% BW_i d^{-1})	N	Survival (%)
13°C	HC	6.9 ± 0.3	99.7 ± 11.0	9	7.7 ± 0.3	114.7 ± 3.5	3.6 ± 3.6	-0.22 ± 0.12	5	6.9 ± 1.4	3	55.6
	HP	6.8 ± 0.3	96.7 ± 9.5	9	7.6 ± 0.3	99.5 ± 8.5	12.5 ± 3.8	0.16 ± 0.18	9	7.0 ± 0.9	3	100
	AK	7.3 ± 0.3	104.3 ± 12.1	9	8.5 ± 0.3	138.2 ± 13.9	12.9 ± 3.2	0.78 ± 0.29	8	1.7 ± 0.1	3	88.9
19°C	HC	7.1 ± 0.4	105.4 ± 15.1	9	8.1 ± 0.4	121.9 ± 23.8	11.9 ± 5.4	0.37 ± 0.33	6	10.8 ± 0.7	3	66.7
	HP	6.9 ± 0.3	100.2 ± 15.2	9	8.0 ± 0.7	122.1 ± 26.5	13.1 ± 3.1	0.58 ± 0.33	6	11.9 ± 1.5	3	66.7
	AK	7.1 ± 0.3	99.7 ± 13.6	9	8.2 ± 0.6	131.1 ± 23.8	15.4 ± 4.3	1.18 ± 0.38	7	2.4 ± 0.3	3	77.8
Two-Way ANOVA												
Temp		$F_{1,53} = 0.00$	$F_{1,53} = 0.02$		$F_{1,40} = 0.22$	$F_{1,40} = 0.24$	$F_{1,40} = 1.32$	$F_{1,40} = 3.74$		$F_{1,17} = 15.57^{**}$ (19°C > 13°C)		
Diet		$F_{2,53} = 0.69$	$F_{2,53} = 0.06$		$F_{2,40} = 0.85$	$F_{2,40} = 0.91$	$F_{2,40} = 1.27$	$F_{2,40} = 4.91^{*}$ (AK > HC)		$F_{2,17} = 35.92^{***}$ (HC, HP > AK)		
Temp × Diet		$F_{2,53} = 0.24$	$F_{2,53} = 0.09$		$F_{2,40} = 0.40$	$F_{2,40} = 0.34$	$F_{2,40} = 0.44$	$F_{2,40} = 0.06$		$F_{2,17} = 2.57$		

CL_i = initial carapace length; CL_f = final carapace length; iCL = increment in carapace length; BW_i = initial body weight; BW_f = final body weight; SGR = specific growth rate; FI = dry mass feed intake.

Values are means ± standard error.

* $p < 0.050$; ** $p < 0.010$; *** $p < 0.001$

TABLE 3. Concentration of glycogen, glucose, lactate and protein in the cephalothorax and abdominal muscle tissues of *Homarus gammarus* after held for 24 days at two different temperatures and for each temperature fed three different diets.

Temp	Diet	Glycogen (glycosil units, μmol.g^{-1})	Glucose (μmol.g^{-1})	Lactate (μmol.g^{-1})	Protein (mg.g^{-1})	N
Cephalothorax						
13°C	HC	3.0 ± 0.3	7.9 ± 2.1	36.3 ± 2.6	10.0 ± 1.0 ^{b,x}	5
	HP	23.8 ± 9.6	49.1 ± 18.5	63.2 ± 15.6	14.5 ± 1.7 ^{ab}	9
	AK	45.7 ± 12.3	51.8 ± 12.5	46.1 ± 7.4	19.9 ± 2.0 ^{a}	8
19°C	HC	13.9 ± 1.7	84.8 ± 19.1	52.2 ± 5.0	20.4 ± 2.4 ^{y}	5
	HP	30.5 ± 12.0	103.5 ± 25.4	81.7 ± 20.2	18.5 ± 1.2	6
	AK	30.5 ± 14.7	65.4 ± 28.1	51.1 ± 13.0	18.1 ± 2.5	5
Two-Way ANOVA						
Temp		$F_{1,37} = 0.32$	$F_{1,37} = 8.79^{**}$ (19°C > 13°C)	$F_{1,37} = 1.49$	$F_{1,37} = 7.05^{*}$ (19°C > 13°C)	
Diet		$F_{2,37} = 4.21^{*}$ (AK > HC)	$F_{2,37} = 1.18$	$F_{2,37} = 2.21$	$F_{2,37} = 1.85$	
Temp × Diet		$F_{2,37} = 1.48$	$F_{2,37} = 1.23$	$F_{2,37} = 0.17$	$F_{2,37} = 4.54^{*}$	
Abdomen						
13°C	HC	6.7 ± 3.2	9.1 ± 2.7	105.5 ± 22.8	14.2 ± 2.2	5
	HP	70.1 ± 37.1	25.9 ± 7.3	146.1 ± 42.6	12.6 ± 2.0	9
	AK	129.4 ± 42.5	31.0 ± 12.2	131.2 ± 23.5	15.7 ± 2.2	8
19°C	HC	151.8 ± 60.1	60.4 ± 18.9	204.6 ± 66.7	16.8 ± 3.5	5
	HP	202.5 ± 62.6	47.0 ± 9.0	217.3 ± 57.8	15.8 ± 1.8	6
	AK	191.0 ± 116.5	35.4 ± 10.9	143.9 ± 51.5	14.4 ± 3.8	5
Two-Way ANOVA						
Temp		$F_{1,37} = 8.23^{**}$ (19°C > 13°C)	$F_{1,37} = 10.89^{**}$ (19°C > 13°C)	$F_{1,37} = 1.90$	$F_{1,37} = 0.52$	
Diet		$F_{2,37} = 1.63$	$F_{2,37} = 0.29$	$F_{2,37} = 0.34$	$F_{2,37} = 0.13$	
Temp × Diet		$F_{2,37} = 1.56$	$F_{2,37} = 2.40$	$F_{2,37} = 0.48$	$F_{2,37} = 0.48$	

Values are means ± standard error.
* $p < 0.050$; ** $p < 0.010$; *** $p < 0.001$
Means in the protein column with a different superscript “a” or “b” are significantly different within the 13°C temperature group. A different superscript “x” or “y” indicates significantly differences between temperatures within the HC dietary treatment.

To obtain an overall picture of the nutrient partitioning of the lobsters at the end of the trial, metabolite levels measured in the cephalothorax were subject to principal component analysis – PCA (Fig. 2). Two principal components accounted for 83.4% of the variability (PC1 60.9% and PC2 22.5%). From all parameters, glucose (0.58) showed the highest loading in the PC1 while the protein (-0.84) showed the highest loading in the PC2. The clearest separation was observed between the HC fed group at 13°C and all the other groups. While all other groups were evenly distributed in the plot, the HC13 formed a distinct cluster to the upper left area of the plot indicative of a negative correlation mainly with glucose and protein levels.

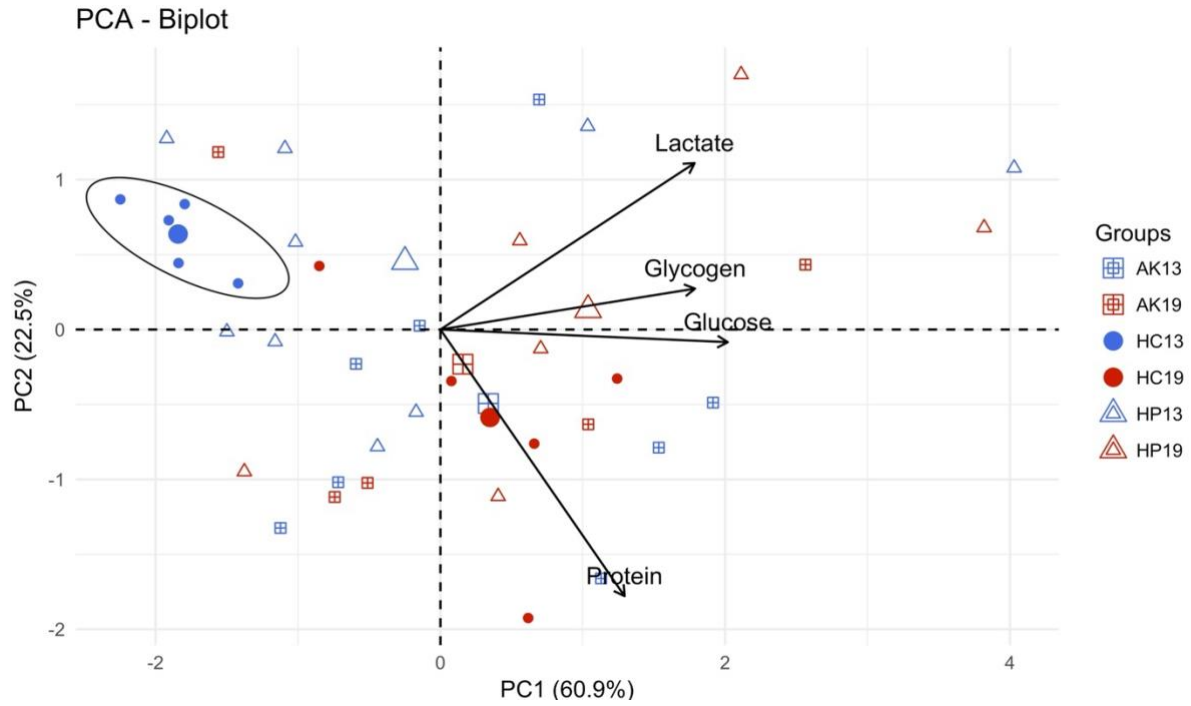

FIGURE 2. Principal component analysis of parameters measured in the cephalothorax of *Homarus gammarus* after held for 24 days under different temperature conditions and fed different diets (n = 5 to 9, see table 2). The PC1 separated the metabolites and protein levels horizontally and explained 60.9% of the variance. The PC2 separated the variables vertically and explained 22.5% of the variance. The contribution of the variables (metabolites and protein) are represented by the arrows and the stronger the correlation of a variable to PC1 and PC2 the longer the arrow.

DISCUSSION

This study demonstrated that both temperature and diet type had a significant impact on *H. gammarus* juvenile growth performance and energy metabolism. Lobsters reared on krill (AK) performed generally better compared to those fed on the HC extruded feed while no significant differences were observed between the HP and the AK diets. Results presented here compare well with a previous study performed in juvenile *H. gammarus* reared under similar conditions as our temperature control (19°C) and using the same diets (Goncalves et al., 2020). In the cited work, the authors also observed a significant reduction on the SGR of juveniles fed the HC feed compared to those fed the AK diet, whereas the SGR of HP-fed lobsters was not different from either AK- or HC-fed juveniles. Moreover, the assessed SGR by Goncalves et al. (2020) – 0.5 % day^{-1} for the HC feed, 0.6 % day^{-1} for the HP feed, and 1.2 % day^{-1} for the AK diet – agrees well with the estimated SGR in this study: 0.4 % day^{-1}, 0.6 % day^{-1}, and 1.2 % day^{-1} for the HC, HP, and AK diet reared at 19°C, respectively. The lack of significant differences in cumulative moultings within dietary treatments in lobsters reared at 19°C also corroborates the results reported in Goncalves et al. (2020). However, a significant lower moulting rate (complete moults), caused by the high incidence of deaths during the moulting process, was observed for the HC13 group. The surprisingly negative SGR (-0.22 % d^{-1}) recorded for the HC13 group

is probably a consequence of this. While the observed negative SGR goes against the well-accepted hypothesis that crustaceans body weight is modulated by water content (Nguyen et al., 2014), it was previously observed that juvenile *H. americanus* lost some weight prior to moulting (Floreto et al., 2000). Our results suggest that juveniles fed the HC feed while held at 13°C could have been progressing until pre-moult stage but most of them did not succeed further in the moulting process. Also, it seems that the higher incidence of "moult death syndrome" – MDS – in the HC13 group might have been caused by suboptimal feeding. High mortalities by entrapment in the enxuviae – MDS – previously reported for *H. gammarus* juveniles reared on experimental feeds have been associated to potential nutritional imbalances in those feeds (Hinchcliffe et al., 2020). So, taken together, results suggest an adaptive response to the lower protein content in the HC extruded feed, in particularly when reared at lower temperatures.

It has been previously established that some protein-sparing effect of carbohydrates exists in crustaceans. For example, (Conklin, 1995) suggested that the protein requirement for *H. americanus* could be as low as 30%, given an appropriate protein source and sufficient non-protein energy in the diet. More specifically, Bautista (1986) demonstrated that the protein content in a diet used for *P. monodon* could be reduced from 50% to 40% without compromising growth by increasing carbohydrate, as long as the energy level was maintained. For the spiny lobster *Panulirus argus*, dietary protein could also be replaced by carbohydrates but was limited by their metabolic capacity to use up to 20% of carbohydrates in their diet (Rodríguez-Viera et al., 2017). If a similar limit applies to juvenile *H. gammarus*, than the HP diet (26% carbohydrate content) was already above their metabolic capacity. Hence, increasing carbohydrates to 35% in the HC feed at the expense of protein would not provide any advantage in terms of protein-sparing. In this study, the protein-sparing effect potential of the HC feed remains unclear. The lack of a dietary effect in the cephalothorax and abdomen protein content in lobsters held at 19ºC points to a protein-sparing effect of the HC feed at this temperature. However, the more severe impact of low temperature on the growth and cephalothorax protein level in lobsters fed the HC feed suggests no protein-sparing potential at lower rearing temperatures.

In the present study, the feed intake was calculated on a dry weight basis, which explains the general lower intake recorded for the AK diet compared to both – HC and HP – feeds. That is because the AK diet had a much lower dry matter content (~ 11%) than the extruded feeds (~ 90%). Nevertheless, we observed a reduction in feed intake for all dietary groups at 13ºC. The decrease in feed intake is a common response of poikilothermic animals to lower temperatures (Thomas et al., 2000; Tully et al., 2000) and it has been previously demonstrated for *H. gammarus* juveniles (Small et al., 2016). Unlike the HC-fed lobsters, juveniles fed the AK diet and the HP feed were able to sustain moulting at the same rate when held at both temperatures even if a higher moulting rate would imply a higher metabolism and, therefore, greater energy demand to maintain homeostasis (Sacristán et al., 2017). Hence, the similar degree in decreasing intake at 13°C for lobsters fed both extruded feeds

coupled with the lower moulting rate observed for lobsters fed the HC feed and held at 13ºC corroborates the previously mention direct effect of the diet composition in the energy metabolism (Vinagre and Silva, 1992).

The lower glycogen level at both temperatures in the cephalothorax tissue homogenates of juveniles fed the HC feed support the hypothesis of a higher energy demand for the HC feed digestion or a limited nutrient assimilation, which may require the mobilization of more glycogen reserves. Hepatopancreatic glycogen is the primary source of energy for crustaceans (Vinagre and Silva, 1992). It can be rapidly converted into glucose to generate energy (Sacristán et al., 2017). Lipids can also be mobilized as an additional source of energy, but more frequently during prolonged periods of food deprivation (Watts et al., 2014). Conversely, the dietary treatment did not affect the glycogen levels in the abdominal tissues. Lower glycogen levels in the muscle of animals in poorer condition, such as the juveniles fed the HC diet, would be expected but that was not the case. It has been previously suggested that decapod crustaceans do not mobilize the tail muscle energetic resources in the same degree as the hepatopancreas (Sacristán et al., 2017). The authors suggested that the observed tail muscle glycogen preservation may reflect its utility as a fuel in searching for food and/or tail flip escape reaction. We observed that glycogen levels were 2 to 3 times and 6 to 11 times higher in the abdomen compared to the cephalothorax, at 13°C and 19°C respectively, supporting the above-mentioned hypothesis. Despite the lack of dietary effect on the abdominal glycogen reserves, there was a temperature effect. Glycogen reserves in the abdomen decreased 96%, 60%, and 32% at the low temperature for lobsters fed the HC feed, the HP feed and the AK diet, respectively. The decrease of glycogen level in the abdomen may be the result of lower feed intake at 13°C. Previous research has shown that a glycogen drop in adult *H. gammarus* (Albalat et al., 2019) and *H. americanus* (Stewart et al., 1972) held at lower temperatures reflected lower feed intake.

Glucose levels were reduced in both – cephalothorax and abdominal – tissues at lower temperature, following a similar trend to the glycogen levels in the abdomen. In the cephalothorax, the glucose content decreased by 91%, 53%, and 21%, while the abdominal glucose levels were reduced by 85%, 45%, and 12% for the HC, HP, and AK groups, respectively. Results pointed towards a more pronounced glucose reduction in *H. gammarus* fed the HC feed, followed by the HP feed, and ultimately the AK diet, even if this trend was not clearly reflected in statistically significant differences. The reduction in glucose levels at lower temperatures is likely related to the decrease in feed intake observed in animals held at 13°C. Another possibility is the increased mobilization of glucose from glycogen to sustain the higher metabolism at higher temperatures (Thomas et al., 2000). Yet, the higher impact of low temperature in glycogen and glucose reserves of juveniles fed the HC in comparison to HP cannot be explained by feed intake differences since they were fairly similar for both extruded feeds. In crustaceans, the response of carbohydrate metabolism to feed intake restriction can be modulated by the diet composition (Vinagre and Silva, 1992). Glucose and glycogen levels were marginally affected

by food deprivation in the crab *Chasmagnathus granulata* previously fed a protein rich diet, while glycogen was hardly detectable and glucose was reduced 57% in crabs previously fed a high-carbohydrate diet (Oliveira et al., 2004). Similar results were obtained for the crab *Neohelice granulata* (Sarapio et al., 2017). The same hypothesis was also demonstrated for the shrimp *Litopenaeus vannamei*: when fed a low carbohydrate diet it was observed an increase in PEPCK activity, an enzyme that allows synthesis of glucose from pyruvate derived from amino acid metabolism (Rosas et al., 2002). Taken together, results suggest that, during feed restriction, gluconeogenesis and glyceroneogenesis are the main pathways involved in metabolic homeostasis in individuals previously fed a high-protein diet (Sarapio et al., 2017). On the other hand, glycogen mobilization might be more important for crustaceans adapted to carbohydrate-rich diets (Vinagre et al., 2020). Our results suggest that juvenile lobster *H. gammarus* might use a similar strategy to adapt their carbohydrate metabolism in relation to the diet received. Additionally, the lack of dietary effect on the protein levels of lobsters held at 19ºC coupled with no alterations in the lactate concentration reinforces this hypothesis – the HC-fed animals were consuming their carbohydrate reserves to sustain aerobic metabolism and preserving their proteins.

Protein content in the cephalothorax was lower at 13°C suggesting a correlation between protein reserves exhaustion and feed intake. In instances of poor condition, crustaceans can consume the main protein present in the hemolymph – haemocyanin – as energy resource (Watts et al., 2014). The significant interaction effect between the diet and temperature points to a more severe effect of low temperature on the HC group. In fact, results showed a significant decline of 51% in the HC group and a non-significant reduction of 22% in HP fed lobsters held at 13°C compared to 19°C. No reduction was detected in AK fed juveniles. The depletion of protein in the cephalothorax of *H. gammarus* fed the carbohydrate rich feed reflects the lower dietary protein content in the HC feed. It might also be that *H. gammarus* suppress protein synthesis to reduce the costly cellular ATP consumption as previously shown in cichlid fish *Astronotus ocellatus* (Lewis et al., 2007). Neither the diet nor the temperature affected the protein content of the abdominal tissues of the juvenile *H. gammarus*. The abdomen in most lobster species is a muscular structure that supports swimming movement (Duffy, 2007). It has been previously observed that food restriction did not cause extensive degradation of myofibrillar protein (actin and myosin) in the tail of *H. americanus* (D'Agaro et al., 2014). A prudent utilization of muscle protein in less severe nutrient restriction state may be an adaptive strategy to avoid the usage of high costly macromolecules, which could represent an energetic saving in case of prolonged periods without food (Sánchez-Paz et al., 2007).

CONCLUSION

This study showed that the resiliency of juvenile *H. gammarus* to the effects of temperature variation on growth and energy metabolism can be modulated by the dietary composition. The more pronounced effect of low temperature on growth and energy metabolism of the HC fed lobsters compared to those fed on krill may be related, at least partially, with the adaptation to a new dietary type. Yet, the more severe impact of low temperature on HC fed individuals compared to HP, suggests that protein-rich feeds may offer some advantage in comparison to high-carbohydrate feeds. Despite the trend for decreased growth and a more pronounced decline in glycogen, glucose, and protein reserves at lower temperatures in lobsters fed the HP feed than those fed the AK diet, we did not find significant differences between both diets. Further studies considering long adaptation to the extruded feeds before exposure to low temperature are required to broaden this point. There is, however, statistical evidence that animals fed the HP feed were more resilient to low temperature than HC fed animals, as suggested by the difference in moulting rates and the PCA analysis, which identifies a distinct cluster for the HC13 group indicative of a negative correlation with metabolites, in particularly, protein and glucose. Although wheat has been identified as one of the best potential carbohydrate sources for the spiny lobster *Jasus edwardsii* (Simon and Jeffs, 2011), future studies should consider other sources (e.g. dextrin, cooked and pregelatinized starches, mussel glycogen) in formulated feeds for *H. gammarus* to further explore better carbohydrate assimilation and hence, improve the potential for protein-sparing. Findings from this study highlight the importance of using well-optimized diets, not only for growth and feed efficiency but also for resilience to environmental change. This is particularly relevant when developing feed products for hatchery units targeting the production of juvenile *H. gammarus* for re-stocking programs.

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