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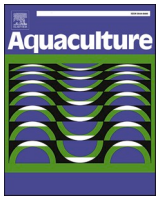

Impact of live feed substitution with formulated diets on the development, digestive capacity, biochemical composition, and rearing water quality of European lobster (*Homarus gammarus,* L.) larvae

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ABSTRACT

The lobster aquaculture industry, constrained by limited access to viable and cost-effective feed sources, prompted an investigation into the potential of replacing live feeds with formulated diets for *Homarus gammarus* larval culture, exploring alternative protein ingredients. Four diets were tested on newly-hatched *H. gammarus*: live *Artemia* nauplii (ART), an extruded control diet with krill meal and fishmeal as main protein sources (CTRL), and diets with 15% of dietary protein replaced by shrimp waste meal (SWM) or black soldier fly meal (BSF).

Results revealed significant diet-induced effects on digestive enzymes in *H. gammarus* larvae. ART-fed lobsters exhibited increased trypsin and amylase activities but lower lipase activity than those fed extruded diets, suggesting more efficient utilization of protein and carbohydrate in ART-fed larvae, while formulated diets compensated with efficient lipid utilization. BSF and SWM diets increased *exo-* and endochitinase activities, likely due to chitin presence. Additionally, CTRL and BSF-fed larvae exhibited elevated body glycogen content. ART-fed larvae displayed a significantly higher ratio of low DNA to high DNA bacteria in their rearing water, indicative of lower nutrient loading. Despite these diet-induced impacts, there was no dietary effect on growth and survival during *H. gammarus* larval development.

The study suggests successful formulated feed utilization by homarid larvae, even with alternative ingredients like BSF and SWM, offering a promising solution to reduce reliance on live feeds in lobster aquaculture. Further research is crucial to assess the long-term implications of the observed physiological responses to the different diets in later life stages.

1. Introduction

The European lobster, *Homarus gammarus*, holds a market value of €16,000 per ton and contributes to an annual market worth of €40 million in Europe (Hinchcliffe et al., 2022). Nevertheless, it encounters challenges stemming from the decline in wild capture fisheries during the 1960s and 1970s, resulting in stock collapses in specific regions, notably Norway (Ellis et al., 2015). Mitigation efforts involve hatchery production of juveniles for replenishing depleted fisheries (Agnalt et al., 2007) and aquaculture production for consumption (Drengstig and Bergheim, 2013). However, achieving successful larval rearing remains elusive, partly due to the reliance on live feeds with variable nutritional value and logistical challenges (Powell et al., 2017). The transition to artificial diets offers advantages, including the convenience of feed preparation and storage, cost reduction, controlled nutritional quality, and improved hygiene (Chong, 2022). Nonetheless, formulating diets that meet the complex feeding behavior and nutritional requirements of homarid lobster larvae remains a persistent challenge (Fiore and Tlusty, 2005). Moreover, the use of artificial diets may impact water quality, potentially compromising lobster health and welfare, leading to increased mortality rates (Hinchcliffe et al., 2022).

The success of transitioning from live to formulated feeds in decapod larvae is more evident in herbivorous compared to carnivorous species, possibly due to differences in their feeding strategies (Le Vay et al., 2001). While herbivorous decapod larvae exhibit a rapid and unselective feeding strategy, replenishing their gut frequently (5 to 7 times/h)

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(Kurmaly et al., 1990), carnivorous species like *H. gammarus* engage in selective feeding with longer gut residence times (Jones, 1998). Despite *H. gammarus* larvae's voracious feeding behavior, capturing most items that come into contact with their appendages, they display extended meal retention times (10 to 47 h) without a clear correlation between prolonged retention and enhanced digestibility (Kurmaly et al., 1990). So far, endeavors to replace live feeds in carnivorous decapod larvae, including *H. gammarus*, have encountered obstacles, such as the unbalanced nutrient composition and poor acceptance of the artificial diets, reflecting a limited understanding of their specific nutritional requirements (Jeffs and O'Rorke, 2020). and insufficient knowledge about how these animals digest and assimilate artificial feeds in comparison to fresh or live diets (Goncalves et al., 2021a).

A recent study by (Goncalves et al., 2022a) contributes to filling this knowledge gap by describing the variation in digestive capacity and biochemical composition during the early development of *H. gammarus*. The study suggests a high requirement for protein during both larval and postlarval development, emphasizing the importance of protein in supporting their growth and development. Furthermore, the research work highlights the significance of lipids, particularly phospholipids and cholesterol, compared to carbohydrates during the three larval phases. Interestingly, the nutritional priorities shift as the lobsters transition to the postlarval stage, with carbohydrates becoming more prominent and surpassing lipids in terms of significance. This shift in nutritional priorities may be attributed to changing energy demands and metabolic requirements as the lobsters grow.

In the present study, we investigated the feasibility of replacing live *Artemia* nauplii with formulated extruded feeds specifically designed for *H. gammarus* larval stages. The formulation of the extruded feeds was based on the recommendations outlined by (Goncalves et al., 2022a), incorporating a high protein content of approximately 55% and a relatively high lipid content of approximately 21%. The levels of cholesterol and phospholipids were also adjusted to meet the recommendations by incorporating cholesterol and utilizing krill oil and krill meal as ingredients in the formulation of the extruded feeds (Table 2). Furthermore, to explore more cost-effective and sustainable protein sources, we tested the replacement of 15% of dietary protein with black soldier fly meal and shrimp waste meal, while maintaining the same proximate composition of the formulated feeds. This resulted in four dietary treatments in our experimental setup: live *Artemia* nauplii (ART), a control formulated feed (CTRL), a formulated feed with 15% of dietary protein replaced by insect meal (BSF), and a third formulated feed with 15% of dietary protein supplied by shrimp waste meal (SWM).

The experimental diets were tested on *H. gammarus* larvae reared in standard common rearing tanks and individual chambers from hatching until metamorphosis to postlarval stage IV. Throughout the experiment, we assessed various parameters, including larval performance (growth and survival), digestive capacity, and biochemical composition. Additionally, we evaluated the impact of the different diets on the water quality within the rearing tanks. The overarching goal of this study was to provide valuable insights for the formulation of tailored and optimized artificial feeds specifically designed for *H. gammarus* larvae produced in aquaculture settings. The outcomes aim to support the growth, survival, and overall health of *H. gammarus* larvae, thereby contributing to the progress of sustainable lobster aquaculture practices.

2. Materials and method

2.1. Experimental diets

Three isoproteic (54%–55% crude protein) and isolipidic (19%–20% crude fat) experimental diets were formulated for *H. gammarus* larvae and compared to a reference live feed composed of *Artemia* nauplii (ART). Manufactured by SPAROS, Lda. (Olhão, Portugal), the experimental diets were extruded into 600–800 μm pellets. One of the experimental diets was formulated with fish meal, fish protein concentrate, squid meal, krill meal, and fish gelatin as protein sources (CTRL). Fish meal and krill meal were partially substituted with shrimp waste meal (SWM) or black soldier fly meal (BSF) at 15% of the dietary protein level in the other two experimental diets. The SWM and BSF ingredients were prepared at DTU Aqua facilities (Hirtshals, Denmark). SWM was made from a mixture of heads, appendages, and exoskeletons of commercially caught Northern shrimp *Pandalus borealis* (Launis A/S, Skagen, Denmark), following the methodology described in (Goncalves et al., 2022b). The BSF meal ingredient was produced from a batch of BSF larvae reared at ENORM Biofactory A/S (Flemming, Denmark). Details on the preparation of the BSF meal can be found in (Eggink et al., 2022).

Duplicate proximate analysis of the *Artemia* nauplii, extruded feeds, and the SWM and BSF ingredients was performed. For this, the *Artemia*, experimental diets, and ingredients were finely grounded in a Krups Speedy Pro homogenizer and analyzed for protein (i.e. Kjeldahl N x 6.25 (ISO 5983-2, 2005)), crude fat (Bligh and Dyer, 1959), dry matter, and ash (NMKL 23, 1991). The amino acid profile of the shrimp waste and black soldier fly meal ingredients was also analyzed in duplicate by Eurofins Steins Laboratory (Vejen, Denmark). Chitin content in the *Artemia* nauplii, SWM and BSF ingredients was determined spectrophotometrically using a modified method of (Tsuji et al., 1969) and (Guerreiro et al., 2020) described in (Eggink et al., 2022). Table 1 shows the proximate composition and amino acid profile of the SWM and BSF ingredients. Ingredient inclusion and proximate composition of the experimental extruded feeds and *Artemia* nauplii are presented in Table 2.

2.2. Larval rearing and sampling

The experiments were carried out at DTU Aqua facilities, Section for Aquaculture (Hirtshals, Denmark). Lobster larvae used in the study were obtained from a mixed parentage of 5 wild-caught females captured along the Skagerrak coast of North Jutland, Denmark. The study utilized twelve rearing tanks, with three tanks designated for each of the four dietary treatments. The tanks were seeded with 308 newly hatched

Table 1
Proximate and amino acid composition of the shrimp waste meal (SWM) and black soldier fly meal (BSF) ingredients.

Proximate composition (% content)			Amino acid composition (g 100 g^{-1})		
	SWM	BSF	*Essential*	SWM	BSF
Dry matter	95.1	97.0	Arginine	2.7	2.7
Crude protein [a]	43.3	57.3	Histidine	1.0	1.5
Corrected crude protein [b]	37.0	53.9	Isoleucine	1.8	2.4
Crude fat	6.1	18.3	Leucine	2.7	4.1
Ash	33.3	12.6	Lysine	2.6	3.1
Chitin	14.6	7.9	Methionine	0.9	0.9
			Phenylalanine	1.9	2.1
			Threonine	1.7	2.2
			Tryptophan	0.5	
			Valine	2.1	3.3
			ΣEAA	17.9	22.2
			Nonessential		
			Alanine	2.3	4.0
			Aspartate + asparagine	4.0	4.7
			Cysteine + Cystine	0.5	
			Glutamate + glutamine	5.4	5.5
			Glycine	2.1	3.0
			Proline	3.6	3.6
			Serine	2.0	2.5
			Tyrosine	1.6	3.7
			ΣNEAA	21.5	27.0
			EAA/NEAA	0.8	0.8

[a] Calculated using a nitrogen-to-protein conversion factor of 6.25%.
[b] Crude protein corrected for 6.9% nitrogen in pure chitin.

Table 2
Ingredients used and proximate composition of the formulated diets and *Artemia* nauplii.

	SWM	BSF	CTRL	ART
Ingredients (as used g 100 g^{-1})				
Live *Artemia* nauplii				100.0
Fish meal [a]	30.4	30.4	38.0	
Fish protein concentrate [b]	5.5	5.5	5.5	
Squid meal [c]	5.0	5.0	5.0	
Krill meal [d]	16.0	16.0	20.0	
Fish gelatin [e]	2.0	2.0	2.0	
Shrimp waste meal (SWM)	20.2			
Black soldier fly meal (BSF)		14.8		
Wheat gluten [f]	5.0	5.0	5.0	
Pea starch [g]	1.3	8.3	10.2	
Vitamin and mineral premix [h]	1.5	1.5	1.5	
Vitamin C [i]	0.1	0.1	0.1	
Vitamin E [j]	0.1	0.1	0.1	
Choline chloride [k]	0.2	0.2	0.2	
Antioxidant [l]	0.2	0.2	0.2	
Copper [m]	0.02	0.02	0.02	
Astaxanthin [n]	0.2	0.2	0.2	
L-Taurine [o]	1.6	1.6	1.6	
Cholesterol [p]	0.3	0.3	0.3	
Soy lecithin [q]	4.0	4.0	4.0	
Fish oil [r]	2.4	0.8	2.1	
Krill oil [s]	4.0	4.0	4.0	
Proximate composition (as fed g 100 g^{-1})				
Crude protein	54.8	54.5	54.0	7.4
Corrected crude protein [1]	53.5	54.0	54.0	
Crude fat	19.4	19.3	20.1	3.2
Moisture	6.5	6.9	6.4	79.5
Ash	13.9	9.5	9.5	1.2
Nitrogen-free extract [2] (NFE)	5.4	9.7	10.0	8.6
Gross energy [3] (KJ g^{-1} feed)	20.3	21.0	21.2	4.4
Chitin [4]	3.0	1.2	0	

[a] Norvik: 71.9% CP, 6.8% CF, Sopropêche, France.
[b] CPSP90: 82.6% CP, 9.6% CF, Sopropêche, France.
[c] Squid meal: 83% CP, 4% CF, Sopropêche, France.
[d] Krill meal: 61.1% CP, 17.4% CF, Aker Biomarine, Norway.
[e] Fish gelatin: 94% CP, WEISHARDT International, Slovakia.
[f] VITAL: 80.4% CP, 5.8% CF, Sopropêche, France.
[g] NASTAR, 0.3% CP, 0.1% CF, 90% starch; Roquette, France.
[h] Vitamins (IU or mg/Kg diet): DL-alphatocopherol acetate, 100 mg; sodium menadione bisulphate, 25 mg; retinyl acetate, 20,000 IU; DL-cholecalciferol, 2000 IU; thiamine, 30 mg; riboflavin, 30 mg; pyridoxine, 20 mg; cyanocobalamin, 0.1 mg; nicotidin acid, 200 mg; folic acid, 15 mg; ascorbic acid, 1000 mg; inositol, 500 mg; biotin, 3 mg; calcium panthotenate, 100 mg; betaine, 500 mg. Minerals (g or mg/kg diet): cobalt carbonate, 0.65 mg; copper sulphate, 9 mg; ferric sulphate, 6 mg; potassium iodide, 0.5 mg; manganese oxide, 9.6 mg; sodium selenite, 0.01 mg; zinc sulphate. 7.5 mg; sodium chloride, 400 mg; calcium carbonate, 1.86 g; excipient wheat middling's. Premix Lda., Portugal.
[i] ROVIMIX Stay C35, DMS Nutritional Products, Switzerland.
[j] ROVIMIX E50, DMS Nutritional Products, Switzerland.
[k] Choline chloride 60, ORFFA, The Netherlands.
[l] VERDILOX, Kemin Europe NV, Belgium.
[m] Availa Cu100, 10% copper, Zinpro, USA.
[n] Carophyll Pink 10% CWS, 10% astaxanthin, DMS Nutritional Products, Switzerland.
[o] L-Taurine, 98% Tau, ORFFA, The Netherlands.
[p] Cholesterol SF, CARBOGEN AMCIS B.V., The Netherlands.
[q] P700IPM, LECICO GmbH, Germany.
[r] Fish oil, 98.1% CF, 16% EPA, 12% DHA, Sopropêche, France.
[s] QRILL Antarctic Phospholipid Oil, 98% CF, 37% phospholipids, Aker Biomarine, Norway.
[1] Crude protein corrected for 6.9% nitrogen in pure chitin (Liu et al., 2012).
[2] Nitrogen-free extract calculated as: 100% - crude protein % - crude fat % - ash % - moisture %.
[3] Coefficients for energy concentration: 21.3 kJ, 39.5 kJ, and 17.6 kJ for protein, lipids, and carbohydrates (NFE), respectively (Cuzon et al., 1994).
[4] Estimated from chitin content in the SWM and BSF and assuming they are the only ingredients containing chitin.

larvae collected from the broodstock tanks over three consecutive days, maintaining the same daily distribution among the tanks. The hatchery tanks system set-up was described previously in (Goncalves et al., 2022a), and included twelve 46 l cylindroconical transparent acrylic tanks, which were part of a flow-through system. Each tank was equipped with a bottom up-flow seawater inlet kept at a constant flow of 40 l h^{-1} and an outflow filter (0.7 mm mesh size). The larvae were maintained in the water column through strong aeration provided from the bottom using air stones. Throughout the trial period, water temperature was maintained at 19.0 ± 0.4 °C, salinity at 34 ± 1 PSU, and dissolved oxygen at 99.5 ± 0.5%. The larvae were offered the assigned diet until apparent satiation three times per day (at 9:00; 13:00; and 17:00). Each meal involved the manual delivery of 0.2 mg of formulated diet per mg of wet body weight (adjusted every fourth day after survival and weight estimation) or 7 *Artemia* nauplii per milliliter of water volume in the tank.

Initially, 10 newly hatched individual larvae were measured and weighed after lethally anesthetized in ice-cold water. The larvae were then rinsed with ultrapure water and stored in an ultrafreezer at −80 °C for further analysis. From this point onwards and every fourth day, six individuals per tank were collected for growth measurements and stored as described above. Additionally, the rearing tanks were emptied, cleaned with fresh hot water, and the larvae were counted for survival determination. At the end of the experiment, all surviving larvae were counted, collected, rinsed, and stored at −80 °C until further analysis. Carapace length was estimated by measuring the distance from the base of the eye socket to the posterior edge of the cephalothorax using a stereomicroscope (MC125 C, Leica, Germany) equipped with a digital camera (MC 190 HD, Leica, Germany). The body weight was recorded to the nearest 0.1 mg using a balance (Mettler Toledo, USA) after gently blotting dry each individual larva with a paper towel.

Simultaneously, an individual rearing experiment was conducted with 160 larvae (40 per dietary treatment) placed in individual chambers within a raceway tank supplied with seawater from a semi-recirculated system described in (Goncalves et al., 2021a). The 160 individual chambers consisted of 30 ml cylindroconical transparent plastic containers equipped with a bottom inlet and a top outlet comprising 8 drilled apertures (1.5 mm diameter) covered with a mesh filter (size 0.7 mm). The larvae were fed the assigned diet to apparent satiation three times daily (9:00; 13:00; 17:00). The amounts per mg of wet body weight of formulated diet or *Artemia* nauplii per milliliter of water volume in each chamber remained consistent with those in the communal rearing trial described above. Any uneaten food, waste, and dead larvae were removed before each meal by siphoning the bottom of each chamber with a plastic air tube. The sole purpose of this trial was to assess survival rates, excluding cannibalism; thus, no samples were collected for subsequent analysis from this system. The duration of the individual (15 DPH) and communal rearing (12–14 DPH) experiments slightly differed due to the individual trial being conducted with larvae hatched on the same day, whereas the communal rearing trial involved larvae hatched over three consecutive days.

2.3. Energetic reserves of the whole-body tissue

Protein, lipid, glucose, and glycogen content in the whole-body tissue of *H. gammarus* were assessed at the end of the trial (12–14 DPH). To determine these parameters, two pools of 3 individuals per tank ($n = 6$ per treatment) were homogenized by ultrasonic disruption with 1 ml of ice-cold ultrapure water. The resulting homogenate was centrifuged (10 min at 13000*g*) and the supernatant was used to assay protein, glucose, and glycogen. Soluble protein was quantified using a commercial Bradford-based reagent from Sigma (B6916, St. Louis, USA) by spectrophotometrically measuring the absorbance at 595 nm. Glucose levels were assessed by measuring glucose with a colorimetric assay kit (10,009,582, Cayman Chemical, USA). Glycogen levels were determined by measuring glucose before and after glycogen degradation by

α-amyloglucosidase (Keppler and Decker, 1974).

To determine the total lipid content, another 2 pools of 3 individuals per tank ($n = 6$ per treatment) were used. Total lipids were extracted from the whole-body tissue using a 2:1 chloroform methanol solution. After extraction, the organic solvent was evaporated under a stream of nitrogen and the lipid content was determined gravimetrically (Christie and Han, 2010).

2.4. Digestive enzyme activities

Trypsin, lipase, α-amylase, *exo*- and endochitinase activities were assayed in 2 pools of 3 individuals per tank (n = 6 per treatment) collected on days 0, 4, 8, and 12 after stocking. Each pool of larvae was homogenized, as described in the previous sub-chapter, and the resulting supernatant was used for the assays. Trypsin and lipase were assayed following the methods of (Rotllant et al., 2008), modified as described in (Goncalves et al., 2021a). Alpha-amylase activity was assayed using a commercial kit (Ultra Amylase Assay kit E33651, Thermo Scientific, USA). Exochitinase β-*N*-acetylglucosaminidase and endochitinase β-D-N,N′,N″triacetylchitotrioside activities were assayed using a modified procedure based on the method initially described by (McCreath and Gooday, 1992) and later modified by (Eggink et al., 2022). All enzyme activities were expressed as relative fluorescence units (RFU) per individual, representing the total activity.

2.5. Rearing water sampling and analysis

On the eighth day of the rearing trial, water samples were collected from each tank as described in (Goncalves et al., 2023). A 1-l sample was collected near the outlet filter of each tank before the first feeding, at 8:30. Each sample was divided into homogeneous subsamples for bacterial community analysis, ultraviolet transmission (UVT), total ammonia-nitrogen (TAN), and total chemical oxygen demand (COD) measurements. All analyses were performed in duplicate. The bacterial community analysis was performed using flow cytometry. A 500 μl water sample from each tank was filtered through a 40 μm cell strainer (FisherBrand, Thermo Fisher Scientific, USA), labeled with 5 μl of SYBR Green (100×, MilliporeSigma, Germany), and incubated at 37 °C for 10 min. The abundance of total and live cells, as well as low and high DNA bacteria (cells ml^{-1}), was determined using a BD Accuri C6 Plus flow cytometer (Becton, Dickinson and Company, USA). Ultraviolet transmission was measured with an UV/Vis spectrophotometer (Beckman DU® 530 Life Science, Beckman Coulter Inc., USA). Total ammonia-nitrogen was determined using the method described in (DS, 1975), and total chemical oxygen demand was quantified in unfiltered water samples according to the (ISO 6060, 1989) standard methods.

2.6. Statistical analysis

The results are expressed as mean ± SD, unless otherwise stated. Data expressed as percentage was square-root transformed before analysis. Normality of residuals and homogeneity of variances were assessed using Shapiro-Wilk and Levene's tests, respectively. If the assumptions were not met, the data were log-transformed. In cases where the parametric assumptions were still not met after transformation, the statistical tests were conducted with significance levels of $\alpha = 0.01$ instead of $\alpha = 0.05$. Survival in individual chambers was analyzed using the Kaplan-Meier procedure and compared with the Log-rank (Mantel-Cox) test. Survival in communal rearing tanks was compared for each sampling point using a one-way ANOVA, and if significance was detected, means were compared using the Tukey post hoc test. Water quality parameters were compared between dietary treatments using the same strategy. All the other parameters were analyzed using a two-way ANOVA considering age (days post hatching, DPH) and dietary treatment as explanatory variables. In instances where differences were detected, treatment means were compared using the Holm-Sidak post hoc test. All statistical analyses were performed using IBM SPSS 25.0 (IBM Corp., USA). Graphs were generated using GraphPad Prism 10.0 software (GraphPad Software, USA).

3. Results

3.1. Larvae growth performance and survival

The effect of diet on the survival of *H. gammarus* larvae was not significant, whether the larvae were reared individually ($\chi^2 = 5.66$, df = 6, $p = 0.129$) or in communal rearing tanks ($F_{3,11} = 0.69$, $p = 0.585$). Survival rates at the end of the trials ranged from 2.5% to 22.5% in individual chambers and 5.1% to 8.6% in communal rearing tanks. Despite the comparable low survival rates at the conclusion of the communal (12–14 DPH) and individual (15 DPH) experiments, noteworthy high mortality rates occurred much earlier in the communal rearing tanks compared to the individual chambers. For instance, in the communal rearing tanks, the average survival rate was 47% at 4–6 DPH and 20% at 8–10 DPH, whereas in the individual chambers, the average survival rate ranged between 71 and 78% at 4–6 DPH and between 52 and 66% at 8–10 DPH. (Fig. 1). Additionally, there was no effect of diet on lobster larval development during the experiment (Fig. 2). At DPH 4–6, between 44% and 83% of the larvae reached the second stage, while 17% to 56% remained in the first stage of development. By DPH 8–10, 56% to 83% of lobster larvae remained in the second stage, and 17% to 44% developed to stage III. At DPH 12–14, the tanks of all dietary treatments were dominated by postlarvae stage IV (56% to 72%), 22% to 44% of the larvae reached the third stage, and a small percentage remained in the second stage (6% to 17%). Overall, there was a significant increase in weight and carapace length during development from 0 DPH to 12–14 DPH but the dietary treatment produced no significant differences in weight gain or carapace length throughout larval development (Table 3).

3.2. Digestive enzyme activities

The diet had a significant effect on total trypsin activity ($F_{3,71} = 13.17$, $p < 0.001$), whereas age had no effect ($F_{2,71} = 1.97$, $p = 0.148$). Lobster larvae fed on *Artemia* nauplii showed higher trypsin activity levels than those fed on any of the extruded diets (Fig. 3). In contrast, total lipase activity was higher in larvae reared on the extruded diets than in those fed the *Artemia* nauplii ($F_{3,71} = 12.18$, $p < 0.001$), and it increased as lobster larvae aged in all dietary treatments ($F_{2,71} = 131.76$,

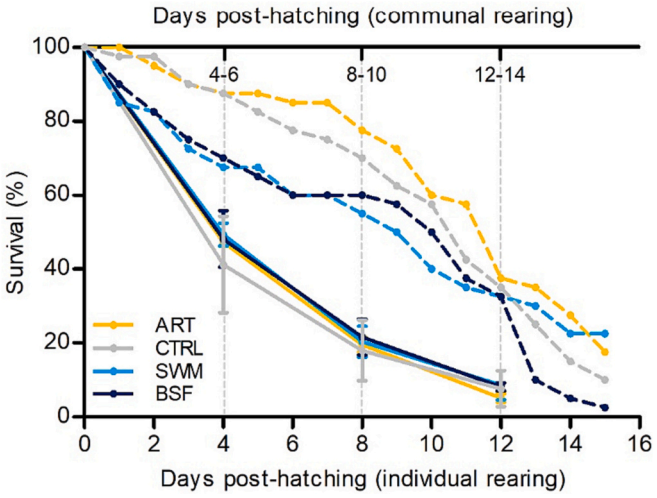

Fig. 1. Survival of *Homarus gammarus* larvae (% of initial numbers) fed on the different diets and reared in individual chambers (dashed lines) and in communal tanks (solid lines).

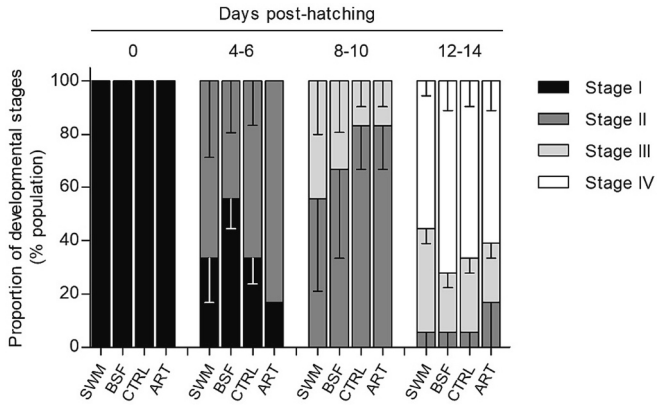

Fig. 2. *Homarus gammarus* larval development rate throughout the experimental period in the communal rearing tanks.

Table 3
Growth performance of *Homarus gammarus* larvae fed the formulated diets and live *Artemia* nauplii.

Days post-stocking (DPH)	0	4–6	8–10	12–14
Larvae wet weight (mg)				
SWM	[1]	10.77 ± 2.55	16.23 ± 4.31	20.81 ± 4.24
BSF	[1]	10.48 ± 2.89	14.97 ± 4.59	21.65 ± 4.52
CTRL	[1]	12.22 ± 3.08	13.63 ± 3.92	20.28 ± 5.22
ART	[1]	11.82 ± 1.99	14.76 ± 3.77	20.34 ± 4.51
Overall mean	**9.80 ± 1.70^{d}**	**11.32 ± 2.70^{c}**	**14.90 ± 4.17^{b}**	**20.77 ± 4.57^{a}**
Two-Way ANOVA				
DPH	$F_{3,254} = 110.44$, $p < 0.001$*			
Diet	$F_{3,254} = 0.13$, $p = 0.941$ ns			
DPH × Diet	$F_{9,254} = 0.94$, $p = 0.493$ ns			
Larvae carapace length (mm)				
SWM	[1]	3.29 ± 0.44	3.70 ± 0.33	4.25 ± 0.33
BSF	[1]	3.11 ± 0.50	3.84 ± 0.40	4.34 ± 0.23
CTRL	[1]	3.42 ± 0.54	3.70 ± 0.33	4.25 ± 0.33
ART	[1]	3.56 ± 0.30	3.72 ± 0.26	4.27 ± 0.36
Overall mean	**2.70 ± 0.14^{d}**	**3.34 ± 0.47^{c}**	**3.81 ± 0.38^{b}**	**4.28 ± 0.30^{a}**
Two-Way ANOVA				
DPH	$F_{3,254} = 193.24$, $p < 0.001$*			
Diet	$F_{3,254} = 0.50$, $p = 0.681$ ns			
DPH × Diet	$F_{9,254} = 2.46$, $p = 0.011$ ns[2]			

Values are means ± SD of 10 replicates for DPH 0 and 18 replicates per dietary treatment for subsequent DPH's. Different superscript letters "a, b, c" within the same row indicate significant differences between age groups (DPH).

[1] Ten individuals were collected and measured for growth before the initial distribution of larvae among the experimental tanks and their average weight and carapace length was assumed to be representative and the same for the four dietary treatments at the beginning of the trial.

[2] When parametric assumptions were not met, the significance level was set at $p < 0.010$ instead of $p < 0.050$.

$p < 0.001$) (Fig. 3). Changes in α-amylase were affected by the interaction of diet and age ($F_{6,71} = 22.58$, $p < 0.001$). While α-amylase activity increased significantly with age in larvae fed on *Artemia* nauplii, the activity of this enzyme remained stable in those fed on the extruded diets (Fig. 3).

The total exochitinase and endochitinase activities were also influenced by the interaction between diet and age ($F_{6,71} = 3.15$, $p = 0.009$; $F_{6,71} = 4.99$, $p < 0.001$, respectively). The increase in exochitinase activity by 12–14 DPH was more pronounced in the SWM and BSF dietary

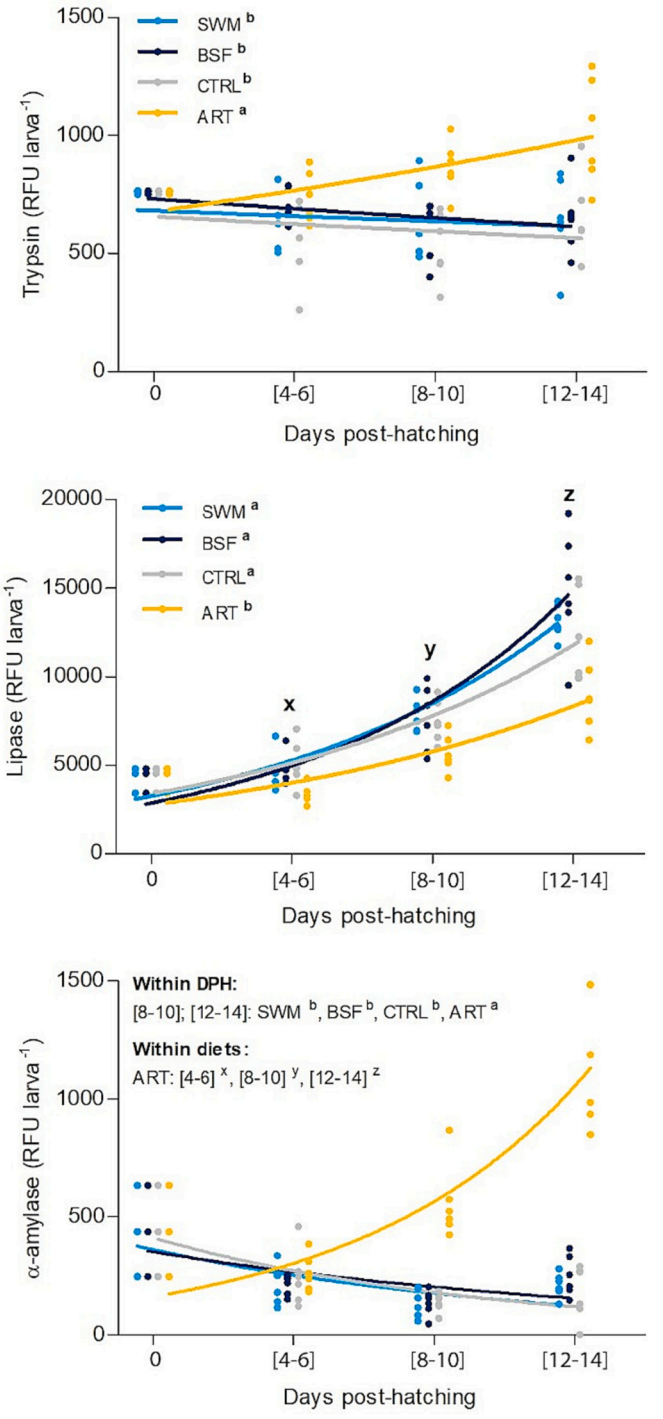

Fig. 3. Temporal change in total (RFU larva^{-1}) activities of trypsin, lipase, and α-amylase in *Homarus gammarus* larvae. Each dot ($n = 3$ at 0 DPH and $n = 6$ onwards) represents a pooled sample of 3 individuals. Different letters "a, b, c" and "x, y, z" indicate significant differences between dietary treatments and age (DPH), respectively.

treatments (Fig. 4). Total endochitinase activity also increased with age in all dietary treatments, with the strongest increase seen in the BSF and SWM, followed by the CTRL and finally the ART group (Fig. 4).

3.3. Metabolites content in the body tissues of lobster larvae

The diet had no effect on the energy content or on the protein, lipid, and glucose reserves. However, there was a significant effect of the diet

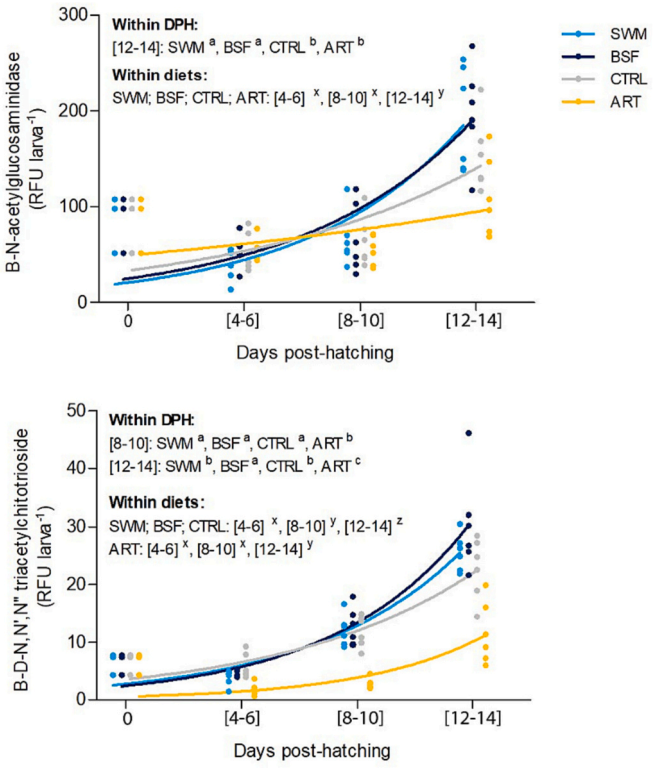

Fig. 4. Temporal change in total (RFU larva^{-1}) activities of exochitinase B-*N*-acetylglucosaminidase and endochitinase B-D-N, N′, N″ triacetylchitotrioside in *Homarus gammarus* larvae. Each dot (n = 3 at 0 DPH and n = 6 onwards) represents a pooled sample of 3 individuals. Different letters "a, b, c" and "x, y, z" indicate significant differences between dietary treatments and age (DPH), respectively.

on the level of glycogen accumulated in the body tissues. The larvae fed the BSF and CTRL diets accumulated more glycogen than larvae fed the SWM and ART diets (Table 4).

3.4. Microbial abundance and physico-chemical water quality

The dietary treatment had no effect on the total cell abundance, live cell abundance, or proportion of dead cells. However, a significant effect was observed on the LNA/DNA ratio, with higher ratios observed in tanks where larvae were fed *Artemia* nauplii compared to tanks with larvae fed any of the three formulated diets. No significant effect of the diet was observed on the concentrations of either TAN or total COD. UV transmission was significantly lower in the tanks of the SWM treatment compared to those of the other dietary treatments (Table 5).

4. Discussion

In this study, we demonstrated that the utilization of extruded formulated feeds resulted in comparable growth and survival rates in *H. gammarus* during their larval development, suggesting their potential as a viable alternative to live feeds. However, we did identify noteworthy differences in the digestive capacity of lobsters based on the diet they received.

4.1. Homarus gammarus larvae performance

As anticipated, our study confirmed that providing individual rearing conditions had a positive impact on survival rates by delaying mortality and reducing cannibalism, at least in the early stages, regardless of the dietary treatment. Furthermore, our results indicate that the formulated feeds tested in this study had no significant effect on the survival, development rate, and growth of *H. gammarus* larvae when compared to those fed live *Artemia* nauplii. Studies conducted on crabs, including *Lithodes santolla*, *Portunus pelagicus*, and *Scylla paramamosain* have shown that suboptimal diets can result in reduced growth, prolonged molting cycle, and increased cannibalism (Møller et al., 2008; Sotelano et al., 2018; Zhang et al., 2022). The increased cannibalism is often triggered as a compensatory mechanism to address nutritional deficiencies and low feed palatability (Romano and Zeng, 2017). Similarly, previous research on *Penaeus indicus* larvae using microencapsulated diets as the sole diet reported lower survival rates, inferior total length at metamorphosis, and a delay in larval development by 1.5 to 2 days compared to live feed (Kumlu and Jones, 1995a). In contrast, our study's findings indicated that the formulated feeds used did not trigger any compensation mechanism for nutritional deficiencies or low feed palatability, as evidenced by the absence of dietary effects on performance and survival.

Table 4
Energetic reserves (protein, lipid, glucose and glycogen) and energy content in the whole body tissues of *Homarus gammarus* larvae reared for 12–14 days on the formulated diets and live *Artemia* nauplii.

	SWM	BSF	CTRL	ART	One-Way ANOVA
Protein (ug mg^{-1}ww)	18.82 ± 1.27	21.59 ± 3.56	20.18 ± 2.39	19.74 ± 5.07	$F_{3,23}$ = 0.70, p = 0.565
Lipid (ug mg^{-1}ww)	10.08 ± 4.12	10.17 ± 5.30	11.90 ± 6.36	8.93 ± 3.52	$F_{3,23}$ = 0.37, p = 0.776
Glucose (ug mg^{-1}ww)	0.557 ± 0.098	0.729 ± 0.157	0.708 ± 0.132	0.830 ± 0.387	$F_{3,23}$ = 1.47, p = 0.252
Glycogen (ug mg^{-1}ww)	**0.008 ± 0.003^{b}**	**0.013 ± 0.003^{a}**	**0.014 ± 0.002^{a}**	**0.008 ± 0.003^{b}**	**$F_{3,23}$ = 11.96, p < 0.001**
Free carbohydrates (ug mg^{-1}ww)	0.565 ± 0.098	0.742 ± 0.158	0.722 ± 0.132	0.838 ± 0.388	$F_{3,23}$ = 1.52, p = 0.241
Biochemical energy (J mg^{-1}ww)	843.3 ± 180.9	912.4 ± 154.3	947.4 ± 232.2	819.6 ± 237.5	$F_{3,23}$ = 0.51, p = 0.682

Values are mean ± SD of 6 replicates per dietary treatment.

Table 5
Water quality characteristics in the rearing tanks of *Homarus gammarus* larvae fed the formulated diets and live *Artemia* nauplii.

	SWM	BSF	CTRL	ART	One-Way ANOVA
Total cells (×10^{5} cells ml^{-1})	2.16 ± 0.59	2.12 ± 0.08	2.37 ± 0.29	1.46 ± 0.50	$F_{3,11}$ = 2.69, p = 0.117
Live cells (×10^{5} cells ml^{-1})	1.36 ± 0.48	1.37 ± 0.09	1.44 ± 0.15	0.94 ± 0.45	$F_{3,11}$ = 1.36, p = 0.322
Proportion of dead cells (%)	37.97 ± 5.14	35.10 ± 6.50	38.17 ± 11.24	38.03 ± 9.61	$F_{3,11}$ = 0.09, p = 0.963
LNA/HNA cell ratio	**2.37 ± 1.07^{b}**	**2.40 ± 0.70^{b}**	**1.85 ± 0.27^{b}**	**6.73 ± 1.41^{a}**	**$F_{3,11}$ = 16.79**, p = 0.001**
UVT (% transmission)	**91.83 ± 1.63^{b}**	**96.28 ± 0.27^{a}**	**94.88 ± 0.38^{a}**	**96.34 ± 0.21^{a}**	**$F_{3,11}$ = 18.27**, p = 0.001**
TAN (μg NH_4-N l^{-1})	1.20 ± 1.06	5.87 ± 1.97	3.60 ± 2.62	3.07 ± 3.59	$F_{3,11}$ = 1.79, p = 0.227
Total COD (mg O_2 l^{-1})	16.99 ± 1.00	15.75 ± 2.26	15.68 ± 1.81	15.41 ± 2.15	$F_{3,11}$ = 0.43, p = 0.739

Values are means ± SD of 3 replicates per treatment. Different superscript letters "a, b, c" within the same row indicate significant differences between dietary treatments.

4.2. Biochemical composition and energy

The results of this study revealed that *H. gammarus* larvae were predominantly composed of proteins (19–22 μg mgWW^{-1}), followed by lipids (9–12 μg mgWW^{-1}), with only a minor fraction of carbohydrates (0.6–0.8 μg mgWW^{-1}), which aligns with the general proximate composition of decapod larvae described in previous studies (Anger, 2001). The dominance of protein over lipids and the relatively low percentage of carbohydrates in the body tissues of decapod larvae is attributed to their energetic metabolism. Decapods can rapidly accumulate or catabolize lipids, while proteins have a slower turnover rate, becoming significant as an energy reserve only during prolonged starvation (Sacristán et al., 2020). Given the critical role of glucose in the energy metabolism of decapods, free carbohydrates exhibit rapid turnover, resulting in a relatively small fraction compared to proteins and lipids (Anger, 2001).

In our study, no significant dietary effects were observed on the protein, lipid, and energy content in the body tissue of *H. gammarus* larvae. However, we cannot rule out potential alterations in terms of amino acid or fatty acid composition. For instance, a study by Ma et al. (2023) revealed that megalopa of the mud crab *Scylla paramamosain*, when fed live *Artemia*, exhibited higher levels of DHA and EPA compared to those fed compound diets. These long-chain polyunsaturated fatty acids play a crucial role in crab molting, metamorphosis rate, and survival. Additionally, we observed that lobster larvae fed the CTRL and BSF displayed significantly higher glycogen content. Travis (1955) previously observed that spiny lobsters (*Panulirus argus*) can convert lipids stored in the hepatopancreas into glycogen during late premolt to support chitin synthesis after ecdysis. The elevated lipase activity and increased glycogen levels observed in lobsters fed the formulated feeds CTRL and BSF may indicate a similar mechanism at work in *H. gammarus* larvae, although it remains difficult to explain why the same phenomenon was not evident in the SWM group. Another plausible explanation for the elevated glycogen levels in larvae fed the CTRL and BSF feeds may be related to the lower carbohydrate content in the extruded diets, amounting to 4 to 5 times less than that in live *Artemia* on a dry matter basis. It has been previously demonstrated that decapods, including homarid lobsters, have the capacity to adapt their metabolism according to the carbohydrate content in their diet, by relying more on glycogen mobilization to sustain homeostasis when fed carbohydrate-rich diets (Goncalves et al., 2021b; Vinagre et al., 2020). Consequently, larvae fed CTRL and BSF diets might have mobilized fewer glycogen reserves than those fed the ART diet due to the lower carbohydrate content. The reason for the absence of a similar response in the SWM group remains unclear.

4.3. Digestive enzymes activity

The response of digestive enzyme activities to the ingested diet remains inconclusive in decapods. On one hand, some studies validate the concept that increasing amounts of ingested nutrients lead to enhanced secretion or activation of enzymes, while malnutrition induces a reduction of enzyme activity (Anger, 2001). On the other hand, other studies support the idea that enzyme secretion increases when dietary nutrient supply is scarce or of poor quality and decreases when nutrients are digestible and readily available (Jones, 1998). Overall, we observed a strong correspondence between larval enzyme activities and dietary treatments, which is somewhat unexpected considering the substantial level of cannibalism likely contributing considerably to the intake of all surviving individuals. In the following paragraphs, we thoroughly discuss the observed changes in the total activity of trypsin, lipase, α-amylase, *exo*- and endochitinase enzymes in response to the dietary treatment and development stage.

In this study, we did not detect changes in total trypsin activity during larval development regardless of the dietary treatment. This finding aligns with previous research conducted on *H. gammarus*, which also reported no significant changes in trypsin activity within the first four stages of development (Goncalves et al., 2022a). Interestingly, we observed that trypsin activity was generally higher in lobster larvae fed live *Artemia* compared to those fed the three formulated feeds tested. A similar response has been observed in shrimp larvae of *Penaeus indicus*, which exhibited lower trypsin activity when fed microencapsulated diets compared to larvae fed algae or co-fed microencapsulated diets with algae (Kumlu and Jones, 1995a). Conversely, the same authors reported enhanced trypsin activity in response to artificial diets in caridean shrimp larva of *Palaemon elegans* and *Macrobrachium rosenbergii* (Kumlu and Jones, 1995b). While one could argue that the elevated trypsin activity in the ART group might be attributed to enzymes present in the prey, previous studies have demonstrated that enzymes from live *Artemia* make only a minimal contribution to the endogenous larval enzymes in decapod larvae (Kamarudin et al., 1994). It is more plausible that the autolysis of ingested live *Artemia* nauplii provides the larvae with a readily available supply of pre-digested, high-energy protein, resulting in the observed increase in trypsin activity. This interpretation aligns with findings from an in vitro protein digestibility study, which tested different feed raw materials using crude enzyme extracts from larvae of the crab *Portunus pelagicus*, revealing live *Artemia* as one of the most favorable protein sources for decapod larvae (Chamchuen et al., 2014). Our results suggest that artificial feeds for *H. gammarus* larvae must be formulated with a high level of predigested protein, similar to that found in autolyzed *Artemia* or supplemented with enzymes that can autodigest the feed once it is ingested.

During our study, we observed a general trend of increased total lipase activity with age, consistent with previous findings in *H. gammarus* during larval development (Goncalves et al., 2022a). Notably, the lipase activity was higher in larvae fed the three formulated diets compared to those fed live *Artemia*. True lipase (triacylglycerol hydrolases) are responsible for digesting medium and long-chain triglycerides, requiring emulsification of the substrates in the hepatopancreas for effective hydrolysis (Lopes et al., 2011; Perera and Simon, 2015). Emulsifiers coat the lipid droplets, allowing lipase enzymes to act more efficiently (Rodríguez-Viera et al., 2022). Crustaceans have the capability of endogenous production of fatty acyl-taurine, an emulsifier-like compound (Dall and Moriarty, 1983), but the inclusion of dietary lipid emulsifiers such as soy lecithin can further improve lipid digestion (Khan et al., 2018). In fact, studies have shown that a mixture of fish oil with soybean lecithin (1:3) significantly promoted more lipid deposition in the hepatopancreas of the spiny lobster *Jasus edwardsii* than fish oil alone (Ward and Carter, 2009). Moreover, the use of crustacean oils, including krill oil as utilized in our study, also demonstrated high digestion rates. The abundance of phospholipids in krill oil contributes to the improved solubility and digestibility of dietary lipids, thereby enhancing their utilization (Perera and Simon, 2015). The higher lipase activity in lobster larvae fed formulated feeds observed in our study may have promoted a greater proportion of ingested proteins being channeled towards growth (Perera et al., 2008), which would explain why these larvae were able to maintain similar growth rates compared to the ART group even when protein digestibility was lower in the artificial feeds.

We observed that amylase activity increased with age in lobster larvae fed live *Artemia*, which aligns with previous findings in *H. gammarus* larvae fed thawed Antarctic krill, *Euphausia superba* (Goncalves et al., 2022a). However, the larvae reared on the three formulated diets did not show an age-related increase in amylase activity and displayed lower activity levels of this digestive enzyme compared to lobster larvae fed *Artemia* from DPH 8–10 onwards. It has been previously demonstrated that under conditions of high food availability, digestive enzyme levels in decapod larvae are reduced as energy requirements are met without the need for highly efficient digestion. Conversely, in situations of lower food or specific nutrient availability, higher digestive enzyme content may be observed (Le Vay et al., 2001). Interestingly, in this study, the higher amylase activity in

Artemia-fed larvae suggests that enzyme activities did not act as a compensatory mechanism in response to a deficient diet, as previously observed for juvenile *H. gammarus* (Goncalves et al., 2021a). Instead, the increased activity of amylase in larvae fed the live diet (ART) indicates that *Artemia* glycogen may offer higher nutritional value than the starch present in the formulated feeds for *H. gammarus* larvae. This finding is consistent with Simon (2009) observations in spiny lobsters (*Jasus edwardsii*), where those fed fresh mussels presented increased amylase activity compared to animals fed artificial feeds.

Perera et al. (2008), demonstrated that lower amylase activity in spiny lobsters (*Panulirus argus*) reared with formulated feeds was partially compensated with higher chitinase activities, which increases glycogen availability for chitin synthesis after molt as animals grow. In our study, we observed that total *exo*- and endochitinase activities increased with age for all dietary treatments but the increase was greater for the BSF and SWM groups, followed by the CTRL group, and the ART group showed the lowest increase, in line with Perera et al. (2008) findings. The maximum exo- and endochitinase activities in the BSF and SWM dietary treatments are most likely related to the higher chitin content in these two formulated feeds. These results suggest that the dietary content of chitin can influence chitinase activities in lobster larvae, which may have implications for chitin synthesis and subsequent molting as the larvae develop.

4.4. Water quality

Monitoring the dietary impact on water quality is crucial for the health and welfare of aquatic organisms, including decapod species like *H. gammarus*, particularly in early developmental stages when molting is more frequent. Ecdysis is critical for decapod larvae, and exposure to unfavorable water quality conditions can lead to a dramatic increase in mortality as soon as molting is approached (Anger, 2001). In this study, the diet had a notable impact on microbial parameters, specifically the LNA/HNA ratio of bacteria detected by flow cytometry (Liu et al., 2013). The ART treatment showed a higher LNA/HNA ratio compared to the tanks with larvae fed with formulated feeds. LNA bacteria thrive in nutrient-limited environments with minimal contamination, while HNA bacteria are more dynamic and responsive to environmental changes, often associated with nutrient-rich conditions (Santos et al., 2019). Thus, the higher LNA/HNA ratio observed in the ART group tanks indicates improved water quality with lower nutrient loading, contrasting the tanks where larvae were provided with artificially formulated feeds. Nonetheless, despite the enhanced water quality in the ART treatment, this factor did not affect the larvae's ability to reach metamorphosis more successfully in comparison to those fed formulated feeds.

5. Conclusion

Our study has demonstrated the potential of extruded formulated feeds as a viable alternative to live feeds for *H. gammarus*, evidenced by comparable growth and survival rates. However, further investigations are needed to optimize the nutritional content and formulation of the feeds to better support the digestive capacity, particularly concerning proteins and carbohydrates. Among the strategies worth investigating are the inclusion of enzymes in the formulation and the exploration of different carbohydrate sources to enhance nutrient digestibility and utilization. Furthermore, it is essential to reassess other factors, such as pellet format, size, and texture, which could substantially contribute to improved feed digestion and assimilation. Addressing these aspects holds the key to lessening the reliance on live feeds and propelling the progression of larval aquaculture practices towards a more advanced and ecologically responsible direction.

CRediT authorship contribution statement

Renata Goncalves: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. **Tilo Pfalzgraff:** Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – review & editing. **Ivar Lund:** Conceptualization, Formal analysis, Funding acquisition, Methodology, Project administration, Resources, Supervision, Validation, Writing – review & editing.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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