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# Larval development of the blue crab *Callinectes sapidus*: effect of enriched rotifers as live food

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Understanding nutrition and its effect on growth is essential for developing diets in commercially important species. This study aimed to evaluate the larval development of *Callinectes sapidus* fed with rotifers (*Brachionus plicatilis*) enriched with *Chlorella pyrenoidosa* (Ch) as a main protein source and a lipid emulsion (Spr) rich in highly unsaturated fatty acids. The enriched compositions had seven ratios Ch/Spr (w/w): 100/0, 90/10, 80/20, 70/30, 60/40, 50/50, and 40/60. The enriched rotifers with each ratio were called diets. Lipids increased progressively between 100/0 and 40/60 (7.48 to 11.30 g 100 g<sup>-1</sup>), as well as energy (21.88 to 23.16 kJ g<sup>-1</sup>), but the protein level did not show a clear trend (63.93 and 67.50 g 100 g<sup>-1</sup>). The production response of blue crab larvae showed significant differences within diets ( $P < 0.05$ ). The highest survival (29 to 34%) was obtained in the 50/50 and 40/60 treatments. In the 40/60, 50/50, and 60/40 diets, the larval cycle was shorter ( $P < 0.05$ ), and the megalopa stage was reached between 49 and 52 days of culture. Metamorphosis to megalopa occurred between the fifth and eighth molts; its corresponding weight varied from 0.72 to 1.33 mg, the length varied from 2,632 to 2,926  $\mu\text{m}$ , and Le Cren's condition factor varied from 0.83 to 1.16. The most important factor in the production response was the diet lipid content, which was significantly correlated with the survival, weight, length, and condition factor of *C. sapidus* larvae. Findings of the present study can be applied in future efforts towards mass culture of blue crab larvae in the Caribbean and can also potentially be used to study other crustacean species with similar characteristics.

## KEYWORDS

*Callinectes sapidus*, blue crab larval development, rotifer enrichment, live food enrichment, rotifer proximal composition

## 1 Introduction

The blue crab, *Callinectes sapidus*, is a brachyuran crustacean of major interest to fisheries in subtropical waters of the Western Atlantic (Daly et al., 2021), but its commercial exploitation is rapidly increasing worldwide, including the Caribbean. Since the 1990s this species began to be captured in Colombia (South America) and soon became

an important commercial species; however, recent reports indicate that over 70% of capture was below the maturity size, affecting the reproductive capacity of natural populations (Maestre et al., 2022). One way to overcome the dependence on natural stocks is to develop aquaculture techniques.

After hatching, the blue crab goes through six to eight zoea stages (pelagic phase) until it reaches the last larval metamorphosis to the megalopa (MG) stage (benthic phase), which shares several characteristics with juveniles (Costlow and Bookhout, 1959; Anger, 2001; Anger, 2001; Bacab et al., 2002; Zmora et al., 2005). The time to reach the MG stage takes 26 to 65 days, depending on salinity and temperature conditions (Stuck et al., 2009; Maurer et al., 2017). Initial efforts to culture this species began in 1959, and culture protocols based on live food supplements were established (Costlow and Bookhout, 1959; Sulkin, 1975). Although survival rates above 30% have recently been obtained in waters of the Western Atlantic (Zmora et al., 2005; Maurer et al., 2017), little is known about its larval development from tropical regions specimens (Bacab et al., 2002; Stuck et al., 2009). Also, information about their nutritional requirements during larval development is lacking (Sulkin, 1975; Pletl, 1992; Maurer et al., 2017). The use of known nutritional composition diets has been extensively studied in some crustacean larval and juvenile cultures, with good results in shrimp (Brito et al., 2001; Pedroza-Islas et al., 2004; Calado et al., 2022; Zarantoniello et al., 2023), but information on commercially important brachyuran species is scarce and focused mainly on *Scylla* sp. and *Eriocheir* sp. larval cultures (Genodepa et al., 2004; Holme et al., 2007; Sui et al., 2007; Holme et al., 2009; Liu et al., 2021).

The rotifer *Brachionus plicatilis* has been widely used in the larval culture of marine fish and crustaceans; its morphological characteristics and size make it ideal for species that consume live prey in their early stages (Rodríguez et al., 1996; Li and Olsen, 2015). Zoeae consume live prey, but since the first larval culture of *C. sapidus* bioassays, it was determined that *B. plicatilis* use did not allow complete larval development to megalopa due to its nutritional deficiencies (Sulkin, 1975). However, enrichment of rotifers improves their nutritional profile and provides nutrients that, in their original state, are absent or present in low concentrations. Emulsions of oils rich in highly unsaturated fatty acids (HUFAs), which are decisive in the larval phases, have been used (Li et al., 2014; Li and Olsen, 2015; Waqalevu et al., 2019). Increased lipids (especially HUFAs) in rotifers used as live food promote improvements in the survival and morphogenesis of fish and crustacean larvae (Suprayudi et al., 2002; Hu et al., 2017; Jeffs and O'Rorke, 2020; Tian et al., 2020; Singh et al., 2023).

Additionally, protein sources from algae (frozen, powder, or paste), as well as probiotics, vitamins, and minerals, have been used to enrich rotifers (Hamre et al., 2008; Srivastava et al., 2011; Nordgreen et al., 2013; Le et al., 2017; Wang et al., 2019; Fu et al., 2021; Khoa et al., 2021; Louvado et al., 2023). Despite being freshwater microalgae, the genus *Chlorella* is widely used in marine species culture due to its high protein content and other essential nutrients, which are similar to those in the marine microalgae *Nannochloropsis* (Maruyama et al., 1997; Ashraf et al., 2011; Raja et al., 2018; Arguelles, 2021). The role of proteins as muscle and shell builders, digestive enzymes, and hormones is

crucial (Anger, 2001; Brucet et al., 2005; Lemos and Weissman, 2020). In *Callinectes sapidus*, *Maja brachydactyla*, and *Portunus trituberculatus*, the levels of most amino acids tend to increase during the early stages of zoeae and drop dramatically from the last zoea to megalopa stage (Tucker and Costlow, 1975; Andrés et al., 2010; Shi et al., 2019); consequently, diets with high protein digestibility allow greater survival from the larval to juvenile phase (Anger, 2001; Carter and Mente, 2014; Shi et al., 2019). This study aimed to evaluate the effect of rotifers (*B. plicatilis*) enriched with *Ch. pyrenoidosa* powder as a main protein source and a lipid emulsion as a main fatty acid source on the larval development (from the first zoea stage to MG) of *C. sapidus* from the Caribbean region.

## 2 Materials and methods

### 2.1 Rotifer enrichment

The primary rotifer culture (*B. plicatilis*) was developed in six 15 L conical tanks at a salinity of 30 g L<sup>-1</sup> with vigorous aeration in batch-type cultures, feeding them daily with live *Nannochloropsis* sp. microalgae (20 to 50 x 10<sup>3</sup> cells mL<sup>-1</sup>) and a mixture of dry yeast (Instant Success<sup>®</sup>), *Ch. pyrenoidosa* powder (Holistic Herbal Solutions<sup>®</sup>), and cod liver oil (Scott's Emulsion<sup>®</sup>) in proportions of 70, 10, and 20% at a rate of 0.5 g of mixture x 10<sup>6</sup> rot.

For the experiments, the rotifers were enriched with mixtures of *Ch. pyrenoidosa* (Ch) powder (Holistic Herbal Solutions<sup>®</sup>) as a main protein source and a lipid emulsion (S.presso Selco<sup>®</sup>) abundant in ω3 PUFAs at 150 mg g<sup>-1</sup> and 9 DHA/EPA (Spr), in seven different ratios Ch/Spr (w/w): 100/0, 90/10, 80/20, 70/30, 60/40, 50/50 and 40/60. The enrichment was carried out in 24 h periods in seven containers of 1.5 L with ~500 rot. mL<sup>-1</sup>. Each mixture was added to the respective flask at a rate of 2.0 g mixture/1 x 10<sup>6</sup> rotifers in four portions (at 0, 6, 12, and 18 h); after 24 h, the enriched batches were harvested and washed with sterile seawater to feed the *C. sapidus* larvae. The rotifers enriched with each of the mentioned mixtures were called diets.

To determine the proximal composition of the diets (enriched rotifers), ~200 rotifers mL<sup>-1</sup> were stocked in 120 L fiberglass tanks. In parallel, according to the previous description, each tank received the respective enrichment portion. This process was repeated until 5 g of the dry weight of rotifers was obtained by diet. Then, the collected batches were dried in a convection oven (Shel Lab) at 105°C for 12 h (A.O.A.C., 2005), and the resulting biomass was stored at -20°C and lyophilized (Labconco Free Zone 4.5) for later analysis.

### 2.2 Breeding process

Adult females of *C. sapidus* with ovigerous masses in an early stage of development (Hill et al., 1989) were collected at Ciénaga Grande de Santa Marta, Colombia. The organisms were transported to the Jorge Tadeo Lozano University aquaculture laboratory. The females were placed individually in 10 L containers and acclimatized to 30 g L<sup>-1</sup> salinity, previously recommended for the

larval development of the species (Zmora et al., 2005; Bembe et al., 2017; Maurer et al., 2017). Each container was maintained with sterilized seawater and vigorous aeration. Every other day, 85 to 90% of the water was changed. The temperature was maintained between 25 and 27°C, oxygen was at saturation ( $> 5 \text{ mg L}^{-1}$ ), and the photoperiod was 12 L:12 D. During this period, the females were fed once a day with fresh squid and were kept under permanent observation. When hatching was recorded, the zoeae were collected, immediately washed with sterile water at the same salinity, and placed in 3 L containers for acclimatization (described in the next section).

### 2.3 Acclimatization of larvae

The acclimatization period for the early zoeae lasted four days. The organisms were distributed in 14 containers of 3 L, at a density of  $\sim 30 \text{ larvae mL}^{-1}$ , with low aeration, a temperature of  $27 \pm 1^\circ\text{C}$ , and a photoperiod of 12 L:12 D. Beginning at this stage, artificial seawater (Premium Reef Salt Omega One<sup>®</sup>) previously filtered and autoclaved was used at  $30 \text{ g L}^{-1}$  salinity. As a preventive prophylactic treatment, chloramphenicol ( $10 \text{ mg L}^{-1}$ ) was added, according to Sulkin and Van Heukelem (1986). Daily, in the morning (7:30 h), each container received between 20,000 and 50,000 cells  $\text{mL}^{-1}$  live *Nannochloropsis* sp. to create a “green water” effect (Zmora et al., 2005; Shan and Lin, 2014; Basford et al., 2021), and in the afternoon (15:00 h), the respective diets were added ( $\sim 300 \text{ rot. mL}^{-1}$ ). Every other day, 90% of the water was changed to remove excess microalgae, rotifers, and dead larvae to maintain the water quality.

### 2.4 Experimental design

The experimental design consisted of seven treatments in quadruplicate (four larval batches). Each replicate had a total of 78 larvae by diet. To strictly control the development of the organisms, transparent tissue culture microplates with 24 wells (3 mL each) were used as experimental units; one larva was kept in each well (Maurer et al., 2017). Before stocking, each well received 1 mL of artificial seawater ( $30 \text{ g L}^{-1}$ ) that had been previously sterilized.

TABLE 1 Density and size of enriched rotifers supplied as live food during larval development of the blue crab *Callinectes sapidus* from hatching to the megalopa stage.

Days after hatching	Size of rotifers ( $\mu\text{m}$ )*	Density ( $\sim \text{rot. mL}^{-1}$ )
0-4 (Acclimatation)	$< 120$	300
5-15	$< 200$	60
16-30	$> 200$	80
31 – Molting to megalopa	$> 200$	100

\* The size of the rotifers was obtained by filtration through sieves with different mesh sizes.

### 2.5 Larval culture

After acclimatization, the larvae were fed daily with the respective diet (from 15:00 to 17:00 h), according to Table 1. In addition to maintaining water quality, during the first 10 days of the trial,  $200 \mu\text{L day}^{-1}$  *Nannochloropsis* sp. microalgae were added to each well (to maintain a density of  $\sim 50,000 \text{ cells mL}^{-1}$  as green water effect). The photoperiod was 12 L:12 D, and the temperature was  $27 \pm 1^\circ\text{C}$ . Every other day, the larvae were transferred to clean microplates with sterile seawater. The trials ended when the last larva reached the megalopa (MG) stage or died.

### 2.6 Production response of the larvae

Mortality and the presence of molts in the wells were recorded daily; each microplate was reviewed under a stereoscopic microscope (Nikon C-Leds) with 10X magnification. Microphotographs of each living MG were taken to determine the total length as the distance from the base of the rostrum to the lateral tip of the telson (Mantelatto et al., 2014). The images were processed with NIS-Elements Br software (Nikon). The weight was determined with an analytical balance (Precisa 321); previously, excess moisture was removed with absorbent paper. Le Cren's condition factor of the MG was also determined according to the equation:  $\text{Kn} = \text{W}/\text{Wc}$  (Le Cren, 1951), where W = weight of the larva at a given size and Wc = expected weight at the same length (calculated from a regression of weight and height of all the pooled treatments).

### 2.7 Proximal composition of the enriched rotifers (diets)

The composition of the diets was determined in the chemical and bromatological analysis laboratory of the National University of Colombia, Medellin Campus. Determinations were performed in triplicate. The protein content was determined with the Kjeldahl technique (A.O.A.C., 2000), the lipid content by the Soxhlet method with Velp Scientifica equipment (SER 148) (A.O.A.C., 2000), the ash content by calcination in a muffle furnace (A.O.A.C., 2000), the carbohydrate or total sugar content by the DuBois colorimetric method (DuBois et al., 1956), and the gross caloric value by Ika-Werke bomb calorimetry (C 2000 basic) (A.O.A.C., 2005).

### 2.8 Statistical analysis

With the collected data, normality (Shapiro–Wilks) and homoscedasticity (Barlett or Levene) tests were performed (Zar, 2010). One-way ANOVAs of the production response of the larvae were performed on final survival, the average time of molting, and the diet content of crude protein, total lipids, and carbohydrates. In cases where the statistical tests were significant ( $P < 0.05$ ), the Fisher's LSD post-test was applied. The nonparametric Kruskal

–Wallis test was applied to evaluate the ash and energy content of the diets, and Bonferroni's post-tests were used. A two-way ANOVA was also applied to MG length and Le Cren condition factor (fixed factor: diet; random factor: larval batch), with post-test LSD analysis.

Friedman's blocks test (diet as a categorical variable and larval batch as the block) was also performed on the duration of the larval cycle, MG weight, and percentage of MGs obtained in each molt, and a Conover's post-test was performed in cases of statistical significance ( $P < 0.05$ ). Finally, Spearman's linear correlation coefficients ( $r$ ) were determined between the diets' proximal composition and the larval production response variables. The information was processed with Statgraphics software (version 18) and R Studio 1.3.1093 software. In all tests, the significance level was  $P < 0.05$ .

## 3 Results

### 3.1 Survival and duration of the larval cycle

Survival and duration of the larval cycle of *C. sapidus* were related to the supplied diet. As the content of Spr increased, survival improved, and the duration of the larval cycle was reduced (Table 2). The highest survival (29 to 34%) was obtained with the 50/50 and 40/60 diets. Consistently, the duration of the larval cycle was shorter ( $P < 0.05$ ) with the 40/60, 50/50 and 60/40 diets, in which the MG stage was reached between 49 and 52 days of culture.

The survival curves show two critical stages. The first is at the beginning of the larval cycle, which comprised the first 13 days of the trial; at this time, the population of zoeae shrank by between 45 and 57%. The second critical stage, although with lower mortality, occurred prior to the MG phase, between days 43 and 46 (Figure 1).

TABLE 2 Survival and duration of the larval cycle of *Callinectes sapidus* larvae fed with enriched rotifers as live food (*Brachionus plicatilis* enriched for 24 h with *Chlorella pyrenoidosa*, Ch, and S.presso emulsion, Spr) in different proportions (Ch/Spr).

Enriched rotifers (Diets; Ch/Spr)	Final survival (%) <sup>*</sup>	Duration of the larval cycle (days) <sup>**</sup>
100/0	6.7 ± 1.8 <sup>b</sup>	55.8 ± 6.3 <sup>ab</sup>
90/10	5.8 ± 1.7 <sup>b</sup>	56.0 ± 6.2 <sup>abc</sup>
80/20	10.6 ± 3.4 <sup>b</sup>	57.0 ± 6.1 <sup>ab</sup>
70/30	16.0 ± 3.0 <sup>ab</sup>	55.3 ± 4.8 <sup>abc</sup>
60/40	20.5 ± 5.4 <sup>ab</sup>	50.5 ± 3.1 <sup>bcd</sup>
50/50	28.8 ± 9.4 <sup>a</sup>	51.5 ± 6.4 <sup>cd</sup>
40/60	33.7 ± 12.0 <sup>a</sup>	48.8 ± 5.7 <sup>d</sup>

<sup>\*</sup> One-way ANOVA and a posteriori LSD test. <sup>\*\*</sup> Friedman test and a posteriori Conover test. Values are expressed as the mean ± SE of four replicates. Different letters in the same column indicate significant differences ( $P < 0.05$ ).

### 3.2 Molting cycle

In all diets, the first molt (M1) occurred at  $7.90 \pm 0.11$  days after hatching (the 4-day acclimatization phase is included in this period), and among M2, M3, and M4, the average time of molting was similar. However, in the subsequent molts, the periods began to show variations among diets, which were accentuated toward the end of the experiment (Figure 2). There were no significant differences in the average day on which the larvae molted between diets ( $P > 0.05$ ).

### 3.3 Number of molts to reach the megalopa stage

The zoeae required between 5 and 8 molts to reach the MG stage. The number of molts in which the organisms completed the larval cycle was related to the diet. In the 60/40, 50/50, and 40/60 diets, the first MGs were recorded beginning at the fifth molt (M5), while in the rest, they were recorded beginning at M6 (Figure 3). Extensive metamorphosis occurred at M7 (38.5–73.7%) and M8 (21.1–45.5%) in all diets, with no significant differences between them ( $P > 0.05$ ) but a significantly higher level occurred than in M5 ( $P < 0.05$ ; Figure 3).

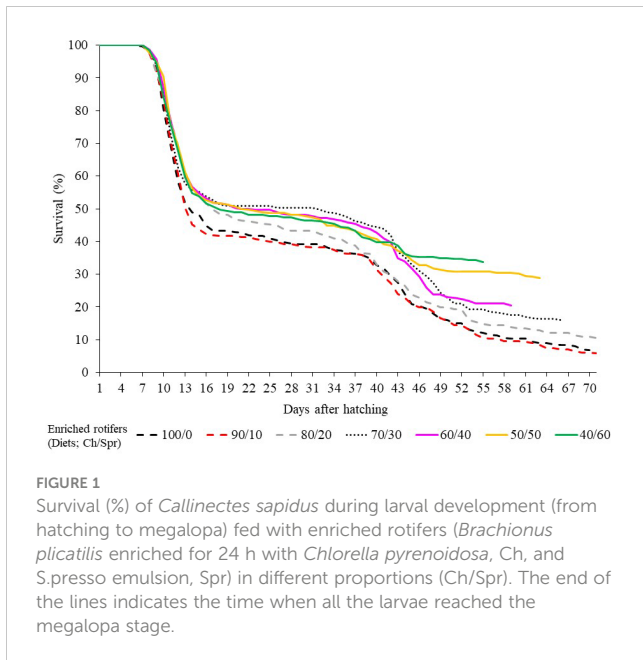
### 3.4 Production response of the larvae

The weight of the MG was between  $0.72 \pm 0.06$  and  $1.33 \pm 0.05$  mg ind.<sup>-1</sup>. The length varied from  $2,632 \pm 88.8$  to  $2,926 \pm 118.5$  μm ind.<sup>-1</sup>, while Le Cren's condition factor (Kn) showed an interval from  $0.83 \pm 0.11$  to  $1.16 \pm 0.12$ ; significant differences ( $P < 0.05$ ) were detected among diets (Table 3). The lowest values corresponded to the 100/0 diet, and the highest to the 40/60 diet. The difference in the weight of larvae fed the worst (100/0) and the best diets (40/60) was 46%, while that in size was 10% and that in Kn was 28%.

### 3.5 Proximal composition of the enriched rotifers (diets) and their relationship with the production response of larvae

The protein content varied from  $63.93 \pm 0.67$  to  $67.50 \pm 0.40$  g 100 g<sup>-1</sup> and was significantly lower ( $P < 0.05$ ) in the 90/10 diet (Table 4). Lipids were more abundant in diets with the highest proportion of Spr; the interval was between  $7.48 \pm 0.20$  and  $11.47 \pm 0.31$  g 100 g<sup>-1</sup>, with significant differences ( $P < 0.05$ ); the poorest diet was 100/0, and the richest was 50/50. Carbohydrates did not show a clear trend within diets (from  $0.81 \pm 0.11$  to  $1.38 \pm 0.05$  g 100 g<sup>-1</sup>); the lowest content was recorded in the 60/40 diet and the highest in the 50/50 diet ( $P < 0.05$ ).

The ash contents were superior in the diets with the highest proportion of Ch; the interval was between  $6.90 \pm 0.04$  and  $9.28 \pm 0.01$  g 100 g<sup>-1</sup>, with significant differences ( $P < 0.05$ ). The energy value was also significantly different ( $P < 0.05$ ), varying between



21.88 ± 0.04 and 23.38 ± 0.09 kJ g<sup>-1</sup>; the lowest level corresponded to the 100/0 diet and the highest to the 60/40 diet (Table 4).

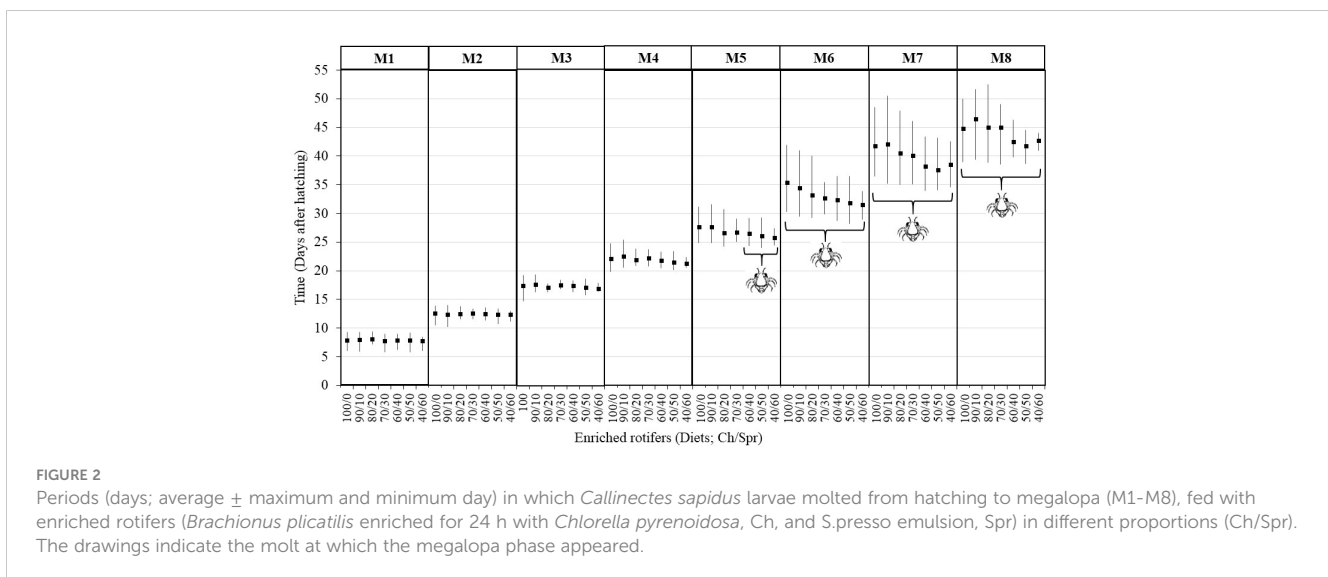
Total lipids had positive and significant correlation coefficients with survival, weight, and size of MGs, as well as with Kn (r between 0.81 and 0.85; Table 5). Additionally, diet energy was positively correlated with survival and MG size (r between 0.77 and 0.79) and negatively correlated with the duration of the larval cycle (r = -0.80). The ash content showed a significant negative correlation with survival and MG weight, size, and Kn (r between -0.84 and -0.91). The correlation coefficient was nonsignificant for the protein and carbohydrate contents with the production response of *C. sapidus* larvae (r between -0.44 and 0.42 and between -0.04 and 0.34, respectively).

## 4 Discussion

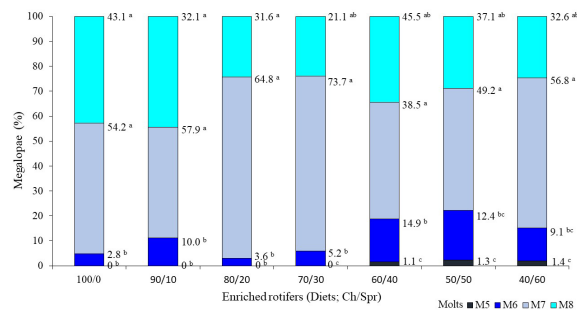
This work showed that the lipid content of enriched live rotifers was the most important factor in the production response of *C. sapidus* larvae reared in the laboratory. To conduct laboratory feeding studies, it is necessary to adapt the food size (prey) to the larvae. Previous reports from subtropical waters of the Western Atlantic recommended the use of *Artemia* nauplii for the larval culture of this species, but in this study, preliminary tests (unpublished) showed that *C. sapidus* larvae from the Colombian Caribbean region could not effectively capture and ingest them (size between 422 to 515 μm); even in later stages, they were only able to capture and consume rotifers (< 120 to > 200 μm). This prevented the use of previously reported feeding protocols (Zmora et al., 2005; Stuck et al., 2009; Maurer et al., 2017). This phenomenon has also been reported for *Callinectes similis* (Bookhout and Costlow, 1977) and *C. danae* (Guarizo et al., 2020).

### 4.1 Survival and duration of the larval cycle

The final survival percentages increased progressively from 6 to 34%, as the diet composition changed between 100/0 and 40/60, in correlation with the lipid content in the diets. In diets with lower lipid contents (100/0 to 70/30), the time to reach the MG phase was longer (> 55 days) compared to that in the rest of the treatments (< 51 days). The Spr product used to enrich rotifers contains fatty acids, including ω3 PUFAs and DHA/EPA; it has been demonstrated that HUFA incorporation into diets improves survival in crab larvae (Suprayudi et al., 2002; Hu et al., 2017; Shi et al., 2019). Previous studies of *C. sapidus* larval culture show that the time to reach the MG phase is variable. The origin of the larvae and other factors, such as temperature, salinity, photoperiod, culture tanks, and diet supplied, are involved and prevent







**FIGURE 3** Relationship of the total percentage of *Callinectes sapidus* larvae that reached the megalopa stage (in each enriched rotifer diet) with respect to the number of molts necessary to achieve it (M5, M6, M7 and M8). Different letters indicate significant differences in the diet (Friedman’s test and Conover’s post-test;  $P < 0.05$ ).

comparisons (Costlow and Bookhout, 1959; Sulkin, 1975; Sulkin et al., 1976; Bookhout and Costlow, 1977; Sulkin and Van Heukelem, 1986; Bacab et al., 2002; Zmora et al., 2005; Maurer et al., 2017).

In all diets, the highest mortality (43 to 55%) was recorded during the first 13 days after hatching, between the first and second molts (M1 to M2). This phenomenon has been reported in *C. sapidus* during the first three zoea instars (Z1 to Z3), with ages of 6 to 16 days, and mortalities vary between 30 and 95% (Costlow and Bookhout, 1959; Costlow, 1967; Sulkin et al., 1976; Bacab et al., 2002). These events seem to be associated with the poor ability of the larvae to detect, capture and consume prey. Upon hatching, *C. sapidus* larvae consumed 45 to 61% of the lipids and 35 to 100% of the initially available proteins (Amsler and George, 1984; Li et al., 2012). A density of ~50 rotifers mL<sup>-1</sup> is recommended to feed the first six instars of zoeae; in this way, abundant food is guaranteed, and the additional energy expenditure to search for prey is avoided (Maurer et al., 2017). In this study, the food density supplied was not considered a limiting factor (60 to 100 rot. mL<sup>-1</sup>); despite this, high mortality was recorded in this period (M1 to M2). This supports the hypothesis that there is no hunting behavior in the early stages. In several decapods, the zoeae move their maxillipeds to swim and generate currents that allow fortuitous encounters with possible prey (Epelbaum and Borisov, 2006; Caracappa and

Munroe, 2018; Almeida et al., 2021). From M3 (days 15-19), feed intake may have increased and was associated with survival curve stabilization (Figure 1).

### 4.2 Molting cycle

The molting cycle was related to the composition of the diet; the periods were generally longer in low-lipid diets. Considering that the larvae do not feed from the late premolt period to the early postmolt period (Anger, 2001; Lemos and Weissman, 2020), they must accumulate enough nutrients and energy to reach the reserve saturation point during the intermolt period; this guarantees that larvae will be able to complete the molt cycles (Gore, 1995; Anger, 2001). In this study, more than 55% of the zoeae survived the first molting event (M1), which took place between 6 and 9 days after hatching. For this to occur, it was assumed that the necessary energy could come from yolk reserves (Gore, 1995), supplied diets, or a combination of both, as occurred in other decapod larvae (Gore 1985; Anger, 1987; Anger and Spindler, 1987). To achieve subsequent molts (M2 to MG), the larvae will depend at some point only on the availability of nutrients in the diets and their ability to capture food.

**TABLE 3** Production response of *Callinectes sapidus* larvae fed whit enriched rotifers as live food (*Brachionus plicatilis* enriched for 24 h with *Chlorella pyrenoidosa*, Ch, and S.presso emulsion, Spr) in different proportions (Ch/Spr).

Enriched rotifers (Diets; Ch/Spr)	Megalopa weight (mg)*	Megalopa length (µm)**	Condition factor (Kn)**
100/0	0.72 ± 0.06 <sup>e</sup>	2,632 ± 88.8 <sup>c</sup>	0.83 ± 0.11 <sup>c</sup>
90/10	0.92 ± 0.07 <sup>d</sup>	2,833 ± 134.4 <sup>ab</sup>	0.88 ± 0.10 <sup>bc</sup>
80/20	1.02 ± 0.05 <sup>cd</sup>	2,876 ± 105.6 <sup>ab</sup>	0.97 ± 0.08 <sup>bc</sup>
70/30	0.96 ± 0.08 <sup>cd</sup>	2,769 ± 120.8 <sup>bc</sup>	0.92 ± 0.13 <sup>b</sup>
60/40	1.08 ± 0.15 <sup>bc</sup>	2,832 ± 104.0 <sup>ab</sup>	0.93 ± 0.11 <sup>b</sup>
50/50	1.29 ± 0.07 <sup>ab</sup>	2,900 ± 115.9 <sup>a</sup>	1.11 ± 0.11 <sup>a</sup>
40/60	1.33 ± 0.05 <sup>a</sup>	2,926 ± 118.5 <sup>a</sup>	1.16 ± 0.12 <sup>a</sup>

\*Friedman test and a posteriori Conover test. \*\*Two-way ANOVA and a posteriori LSD test. Values are expressed as the mean ± SE of four replicates. Different letters in the same column indicate significant differences ( $P < 0.05$ ).

TABLE 4 Nutritional composition of enriched rotifers used as live food (*Brachionus plicatilis* enriched for 24 h with *Chlorella pyrenoidosa*, Ch, and S.presso emulsion, Spr) in different proportions (Ch/Spr).

	Enriched rotifers (Diets; Ch/Spr)						
	100/0	90/10	80/20	70/30	60/40	50/50	40/60
Crude protein (g 100 g <sup>-1</sup> ) *	66.93 ± 1.62 <sup>ab</sup>	63.93 ± 0.67 <sup>c</sup>	66.27 ± 0.60 <sup>ab</sup>	66.90 ± 0.20 <sup>ab</sup>	67.50 ± 0.40 <sup>a</sup>	65.90 ± 0.44 <sup>b</sup>	67.10 ± 0.56 <sup>ab</sup>
Total lipids (g 100 g <sup>-1</sup> ) *	7.48 ± 0.20 <sup>c</sup>	8.80 ± 0.26 <sup>d</sup>	10.10 ± 0.20 <sup>c</sup>	10.24 ± 0.13 <sup>c</sup>	11.07 ± 0.03 <sup>b</sup>	11.47 ± 0.31 <sup>a</sup>	11.30 ± 0.12 <sup>ab</sup>
Carbohydrates (g 100 g <sup>-1</sup> ) *	1.05 ± 0.04 <sup>b</sup>	0.95 ± 0.03 <sup>bc</sup>	1.00 ± 0.04 <sup>bc</sup>	0.91 ± 0.08 <sup>cd</sup>	0.81 ± 0.11 <sup>d</sup>	1.38 ± 0.05 <sup>a</sup>	0.95 ± 0.11 <sup>bc</sup>
Ash (g 100 g <sup>-1</sup> ) **	9.28 ± 0.01 <sup>a</sup>	8.63 ± 0.07 <sup>ab</sup>	7.74 ± 0.30 <sup>ab</sup>	7.64 ± 0.02 <sup>ab</sup>	7.84 ± 0.19 <sup>ab</sup>	7.02 ± 0.24 <sup>b</sup>	6.90 ± 0.04 <sup>b</sup>
Energy (kJ g <sup>-1</sup> ) **	21.88 ± 0.04 <sup>b</sup>	22.46 ± 0.00 <sup>ab</sup>	22.61 ± 0.04 <sup>ab</sup>	22.67 ± 0.08 <sup>ab</sup>	23.38 ± 0.09 <sup>a</sup>	23.06 ± 0.15 <sup>ab</sup>	23.16 ± 0.15 <sup>ab</sup>

\* One-way ANOVA and a posteriori LSD test. \*\* Kruskal–Wallis test and a posteriori Bonferroni test. Values are expressed as the mean ± SD of three replicates. Different letters in the same row indicate significant differences ( $P < 0.05$ ).

Between M1 and M4, the zoeae molted approximately every five days, regardless of diet; subsequently, differences were found: Diets with compositions between 100/0 and 70/30 showed the widest intervals, and these treatments can be considered deficient in supplying the nutritional requirements necessary to molt. In each molting cycle, during temporary starvation, the blister-like cells absorb the nutrients discharged by the resorptive cells in the hepatopancreas to support the organism, which causes a notable decrease in the weight of this organ (Jones et al., 1997; Anger, 2001), and these losses can only be replaced when the larvae feed (Gore, 1995; Anger, 2001). Therefore, if the nutrients supplied are not those required or do not have the appropriate concentrations, the larvae will need more time to replenish the lost biomass and reach the necessary threshold to supply their energy reserves. In *Hyas araneus*, *Carcinus maenas* and other decapods, this phenomenon delays the production of the molting hormone and the onset of integumentary apolysis (Anger and Dawirs, 1981; Dawirs, 1984; Anger, 1987; Anger and Spindler, 1987; Suprayudi et al., 2002; Gimenez and Anger, 2005).

### 4.3 Number of molts to reach the megalopa stage

Although the ontogenetic development of crustaceans is genetically determined, several decapod species exhibit some degree of plasticity through the addition or omission of stages

(Maurer et al., 2017), especially in carideans and brachyurans, which present numerous larval stages (Oliphant and Thatje, 2013; Sugumar et al., 2013). In crustaceans, molts are used to define successive larval stages because changes in size or morphology usually occur; however, in some cases, molting is not associated with significant modifications and sometimes not even with size increments (Anger, 2001; Moller et al., 2020).

In this study, up to eight zoea instars were recorded, and metamorphosis to MG occurred between M5 and M8. In the diets with higher lipid contents (60/40, 50/50, and 40/60), MG were observed beginning in M5, although at very low percentages (< 1.4%). This early presence was also reported by Maurer et al. (2017), although at a higher proportion (10 to 40%). Studies carried out for *C. sapidus* reported between six and nine stages for zoeae to reach the MG stage, although the highest occurrence was recorded in M7 and M8 (Costlow and Bookhout, 1959; Costlow, 1965; Sulkin, 1975; Sulkin et al., 1976; Bacab et al., 2002; Zmora et al., 2005; Stuck et al., 2009; Maurer et al., 2017), as occurred in the present bioassay.

Each diet produced a specific physiological response, which was reflected in the final length and weight of MGs. Both variables were combined in Le Cren's condition factor (Kn), which can be used as an indicator of health (Le Cren, 1951). This study showed that the best Kn values coincided with the highest inclusion of Spr (40/60 and 50/50), however it would have enhanced the study to observe the extended viability of MG under conditions of starvation as an additional indicator of their fitness to complement Kn. This condition factor has been used in a few species of adult brachyurans, but there are no

TABLE 5 Pearson's linear correlation coefficients ( $r$ ) between the enriched rotifer composition (diets), and the production response of *Callinectes sapidus* larvae (significant values  $P < 0.05$ , are in bold).

Larval variables	Crude protein (g 100 g <sup>-1</sup> )		Total lipids (g 100 g <sup>-1</sup> )		Carbohydrates (g 100 g <sup>-1</sup> )		Ash (g 100 g <sup>-1</sup> )		Energy (kJ g <sup>-1</sup> )	
	$r$	P value	$r$	P value	$r$	P value	$r$	P value	$r$	P value
Survival (%)	0.42	0.35	<b>0.85</b>	<b>0.01</b>	0.27	0.56	<b>-0.88</b>	<b>0.01</b>	<b>0.79</b>	<b>0.03</b>
Duration of the larval cycle (days)	-0.44	0.32	-0.72	0.07	-0.04	0.94	0.65	0.11	<b>-0.80</b>	<b>0.03</b>
Megalopa weight (mg)	0.09	0.85	<b>0.84</b>	<b>0.02</b>	0.34	0.46	<b>-0.91</b>	<b>0.00</b>	0.74	0.06
Megalopa length (μm)	-0.16	0.73	<b>0.83</b>	<b>0.02</b>	0.16	0.73	<b>-0.84</b>	<b>0.02</b>	<b>0.77</b>	<b>0.04</b>
Megalopa condition factor (Kn)	0.16	2.70	<b>0.81</b>	<b>0.03</b>	0.41	0.37	<b>-0.91</b>	<b>0.00</b>	0.68	0.09

reports for larvae (Amaro and Fiscarelli, 2009; Fassatoui et al., 2021). It has been proposed that this type of morphometric measurement, complemented with lipid content, can be used as a first step to assess the availability of energy reserves in larvae (Lloret et al., 2013).

#### 4.4 Proximal composition of the enriched rotifers (diets) and their relationship with the production response of larvae

The positive correlation between the lipid content in the diets and survival and MG weight, length, and Kn reinforces the importance of lipids (particularly HUFAs) in *C. sapidus* larval development. The negative correlation ( $r = -0.80$ ) between the duration of the larval cycle and the energy content of the diets is related to the accumulation of reserves and their conversion to energy, factors involved in the endocrine regulation of the molt cycle (Anger, 1987). Although the content of fatty acids was not determined in this research, it is assumed that the positive effect was associated with the content of lipids indicated in the Spr datasheet ( $150 \text{ mg g}^{-1}$   $\omega 3$  PUFAs and 9 DHA/EPA).

In this experiment, the amount of lipids was between  $7.48 \pm 0.20$  and  $11.47 \pm 0.31 \text{ g } 100 \text{ g}^{-1}$ , and it was the most important factor in the production response of *C. sapidus* larvae. Similar concentrations have been tested in *Scylla* sp. and *Portunus* sp. larval cultures (6.8 to 16%); in those studies, it was shown that HUFA-unbalanced diets produced low survival rates and a high incidence of “death by molting syndrome” (Wu et al., 2014; Hu et al., 2017; Shi et al., 2019). On the other hand, it was considered that the enrichment period of the rotifers was adequate since Rodríguez et al. (1996) and Li and Olsen (2015) indicated that allowing 24 h to enrich *B. plicatilis* maximizes the incorporation of lipids into the tissues and allows them to remain stable until one day later.

The protein concentration requirements in decapod larval diets oscillate from 30 to 80%, depending on species, stage, food source, and digestibility (Holme et al., 2007; Shi et al., 2019; Jeffs and O’Rourke, 2020). The microalga *Chlorella* is often used to cultivate fish and crustacean larvae but is usually complemented with products rich in DHA and EPA to meet the needs of n-3 HUFAs (Taufik et al., 2016; Waqalevu et al., 2019), as was done in this investigation. In previous studies, the proximal composition of *Chlorella*-fed rotifers showed a wide range of protein (27.6 to 67.3%) and lipid (9.5 to 12%) contents (Dan and Hamasaki, 2011; Hamre, 2016; Waqalevu et al., 2019). The variations may be due to each experiment’s particular conditions and the enrichment period.

The Ch fractions used during the enrichment of the rotifers yielded protein levels between  $63.93 \pm 0.67$  and  $67.50 \pm 0.40 \text{ g } 100 \text{ g}^{-1}$  within the required range, but the protein content in the diets did not show significant correlation coefficients with the production response. These results lead us to hypothesize that this variable was not limiting. Nevertheless, other studies could delve into the absorption, metabolism, and function of its amino acids in nutrition since there are few studies of this type in crustacean larval stages (Carter and Mente, 2014; Li et al., 2021).

It may be possible that lipid levels higher than those tested in the present study could further improve survival and growth

rates of blue crab larvae. For example, the enrichment of rotifers with another commercial emulsion (EPA28<sup>®</sup>, Nippon Kagaku Shiryo Co., LTD, Japan) to feed other brachyuran species (Takeuchi et al., 1999; Suprayudi et al., 2002) showed that insertions between 10 to  $40 \mu\text{L L}^{-1}$  resulted in higher rotifer lipid content (15.4 to 20.3%) than the present study (11.47%). With the results showing that the highest lipid levels 50/50 and 40/60 performed best, and protein levels were not limited, further investigations should focus on higher lipids and less or no proteins in enrichment formulas.

Although culture assays under laboratory conditions in *C. sapidus* larval have been developed (Zmora et al., 2005; Stuck et al., 2009; Maurer et al., 2017), the proximal composition of live prey and its relation with their larval development had not been covered until this work. The present study is the first to establish the preliminary bases on enriched live food nutritional content with the main variables of the larval development of *C. sapidus*, setting up the essential foundations of diets of known biochemical composition as a supply for *C. sapidus* larvae to parameterize their production response.

In conclusion, the evidence presented here indicates that *B. plicatilis* enrichment for 24 h with different Ch/Spr ratios significantly affects the larval development of *C. sapidus* in terms of survival, growth, and molting cycle. The diets with the highest lipid content (40/60 and 50/50) showed the best survival, duration of the larval cycle, size and weight of the megalopae, and short molting periods. The correlation coefficients indicated a significant relationship between the diet’s lipid, ash, and energy content and the larvae’s production response variables. Findings of the present study can be applied in future efforts towards mass culture of blue crab larvae in the Caribbean and can also potentially be used to study other crustacean species with similar characteristics.

#### Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors upon request, without undue reservation.

#### Author contributions

GO-S: Funding acquisition, laboratory assembly and maintenance, fieldwork, experimental design and development, data analysis, original manuscript writing, reviewing and editing. SZ: Funding acquisition, administration, experimental design, advice on statistical analysis, supervision, and reviewing. AM-B: Experimental design, supervision, writing, reviewing and editing. All authors read and approved the final version of the manuscript.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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