



Effect of diet during larval development of *Menippe nodifrons* Stimpson, 1859 and *Callinectes danae* Smith, 1869

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Abstract

The worldwide decrease in fishery stocks in recent years is related to the degradation of coastal environments and to the increases in capture effort and extreme natural events. Restocking dilapidated populations with artificial hatchery-reared larvae has arisen in recent years as a viable technique for the reparation and maintenance of stocks of commercially exploited species. One of the first steps in larvae cultivation is the evaluation of an appropriate diet for rearing. We aimed to test viable diets for feeding zoea larvae of the crab *Menippe nodifrons* and the swimming crab *Callinectes danae*. We tested five live food treatments, three microalgae (*Tetraselmis gracilis*, *Chaetoceros calcitrans*, and *Thalassiosira weissflogii*), the rotifer *Brachionus plicatilis*, and *Artemia* sp. nauplii. The most suitable feeding protocol for rearing *M. nodifrons* larvae was a combined diet of enriched *B. plicatilis* up to the zoea III instar with the introduction of hatched *Artemia* sp. nauplii onwards to the megalopa stage. Larvae of *C. danae* showed high mortality rates and none achieved the zoea III phase on any of the diets tested. Although our results for *C. danae* rearing were not conclusive, the data provide additional information on improper rearing diets for this species for future research. Future studies testing other food sources are necessary to improve larvae production.

Keywords Larval survival · Brachyura · Fishery stocks · Food organisms · Microalgae

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Introduction

Globally, crab fishery is a vast industry, with ~ 180 million tons of global capture production in 2017 (FAO Fisheries and Aquaculture 2020). However, there is little information related to the real impact caused by industrial fishing on the stocks of exploited species (Da Nóbrega Alves and Nishida 2003; Becker 2008; Castilho-Westphal et al. 2008). Rodrigues et al. (2000) related the decline in the capture of the mangrove crab *Ucides cordatus* (Linnaeus, 1763) on the coast of São Paulo and Paraná states to a possible decrease of stocks. The same phenomenon is observed throughout the Brazilian coast, in the states of Pará, Paraíba, Bahia, and Santa Catarina (Da Nóbrega Alves and Nishida 2003; Souto 2007; Wunderlich et al. 2008; Freitas et al. 2015). As observed for *U. cordatus*, a decrease in the stock of many commercially exploited species is expected due to the rise in capture effort from market demand, together with habitat damage and extreme climate events in future decades (Da Nóbrega Alves and Nishida 2003; Bell et al. 2005; Becker 2008; Bell et al. 2008). The captive production of Brachyura can be rather scarce, due to difficulties throughout the species' life span. The low rate of larval survival is associated with a high predation rate combined with poor environmental tolerance (Silva 2002; Agh and Sorgeloos 2005).

Most marine Brachyuran species exhibit planktotrophic larvae (Pechenik 1999; Anger 2001; Vieira 2006). These species produce a greater number of offspring able to reproduce per unit energy invested, besides increased cannibalism, territorialism, and slow growth (Pechenik 1999). These characteristics can hinder the species' aquaculture economically and technically. The most common way of exploitation is direct gathering or fishing of wild individuals (Anger 2001; Carvalho 2010).

Due to decreases in the world's stocks, measures to enhance and conserve the populations of depleted species have been developed. Restocking by artificial hatchery-reared larvae has been emerging as a viable technology of population recovery and conservation of different coastal areas (Silva 2002; Silva 2007; Becker 2008; Bell et al. 2008). Stock recomposition is based on obtaining larvae from ovigerous females from the wild and growing cultures of zoea larvae to megalopa stage. The strong positive geotaxis of megalopa increases the success of larval recruitment, it being the suitable stage for the release of larvae (Silva 2007; Becker 2008). Additionally, release of juveniles and later stages can enhance restocking (Bell et al. 2005).

The first step is the evaluation of an appropriate larval diet for rearing. The size of food in relation to larval feeding appendages (maxillipeds) is a key factor in proper feeding of larvae. Possible disproportionality can lead to a nutritional deficit, prolonged development time, and increased mortality rate (Harms and Seeger 1989; Anger 2001). Due to the absence of predators and steady conditions, larval survival reaches higher rates than those in nature. Food availability, which fluctuates seasonally in the environment, regulates larval hatching and growth. Decapod larvae hatch as lecithotrophs, although with a limited reserve. As such reserve ends, the now planktotrophic larvae must reach their optimum biomass. Although larvae can withstand periods of food deprivation, at a low feeding rate, larval development can be delayed, and growth and survival are reduced (Stanton and Sulkin 1991; Anger 2001). Low-cost larval production allied to a viable survival rate is the utmost intention (Bell et al. 2005; Becker 2008). This method will assert the supply of larvae to reach full potential, by attaining optimal stocking density of the species in later larval mass rearing.

Although larval culture already shows positive outcomes, there is no consolidation of its techniques, mainly in terms of successful feeding and breeding protocols of a range of species. The design of technically viable diets that provide the necessary nutritional requirements for

each species is a key factor in establishing successful population recovery methods (Becker 2008). Through established larval breeding protocols, restocking of estuarine/marine species with larval development may be possible. We aimed to evaluate the effect of different diets on the survival rate and development time of the stone crab *Menippe nodifrons* Stimpson, 1859 and the swimming crab *Callinectes danae* Smith, 1869, from zoea I to megalopa stage, clarifying which diet is the most suitable. We used three microalgae species, *Tetraselmis gracilis* (Kyllin) Butcher, 1959; *Chaetoceros calcitrans* (Paulsen) Takano, 1968; and *Thalassiosira weissflogii* Hustedt, 1926; the rotifer *Brachionus plicatilis* (Müller, 1786) (enriched with *T. gracilis*); and nauplii of *Artemia* sp. Leach, 1819 as a food source. We hypothesised that larval survival rate and time to develop in both species is directly linked to the most suitable diet. A microalgal and/or rotifer diet could be the most suitable for rearing both species, as similar results were found for closely related species (Sulkin 1975; Sorgeloos and Léger 1992; Abrunhosa et al. 2002; Brown 2002; Ruscoe et al. 2004; Waiho et al. 2018).

Menippe nodifrons inhabits rocky shores and banks of *Phragmatopoma* sp. and possesses great potential for exploitation due to its relatively large size and developed cheliped musculature (Melo 1996; Oshiro 1999; Fransozo et al. 2000; Rodrigues-Alves et al. 2013). The species is widely distributed and has potential economic importance. *Menippe nodifrons* exhibits five zoea phases (uncommonly six phases) and one megalopa stage (Scott 1979; Anger 2001). *Callinectes danae* inhabits estuarine waters near mangroves and open sea regions near sandy beaches (Sforza et al. 2010). The species displays a contiguous geographic distribution from Florida to the Brazilian state of Rio Grande do Sul (Melo 1996; Marochi et al. 2013). Actively exploited by artisanal fishery, the species is commonly captured as a bycatch of shrimp fishing (Costa and Negreiros-Fransozo 1998; Severino-Rodrigues et al. 2001; Marochi et al. 2013). The general larval development lasts six to eight zoea instars with a megalopa stage, with an optimal survival rate at salinity near 30 PSU (Costlow and Bookhout 1968; Sankarankutty et al. 1999).

Materials and methods

Collection area and conditioning of females

Seven ovigerous *M. nodifrons* females, mean carapace width 51.66 ± 3.78 mm, were manually sampled on a rocky shore in Itanhaém, São Paulo, Brazil ($24^{\circ} 12' 06.9''$ S $46^{\circ} 48' 41.3''$ W), and two ovigerous *C. danae* females, mean carapace width 89.5 ± 7.77 mm, were collected by trawling (23 mm fishnet mesh size) in São Vicente, São Paulo, Brazil ($23^{\circ} 58' 28.6''$ S $46^{\circ} 22' 57.1''$ W). We sampled only females with eggs in an advanced stage of embryo development, indicated by their greyish colour (Costa and Negreiros-Fransozo 1998; Oshiro 1999). The advanced period is preferred to avoid the interference of laboratory conditions on embryo development. The females were conditioned in glass aquariums ($290 \times 120 \times 185$ mm) with 500 ml of artificial marine water (salinity 30 PSU and 25 ± 1 °C) and a photoperiod of 12:12 h (light:dark). The artificial marine water was obtained by dissolving artificial refined sea salt (Blue Treasure Reef Sea Salt, Qingdao Sea-Salt Aquarium Technology Co., Ltd) in deionized water.

Diet cultivation

The food source for Brachyuran larvae should meet specific criteria to be an appropriate diet, such as being of suitable size to be easily captured and digested, having a satisfactory

concentration (to supply enough food and avoiding excess feeding) and containing enough nutrients (Sui et al. 2008). The microalgae species strains were inoculated in culture medium (filtered seawater with nutrients) at 24 ± 1 °C, photoperiod 24:0 h (light:dark), and constant aeration, based on Guillard's F/2 protocols (for more detail, see Duerr et al. 1998). The chosen culture medium is the one most used in marine algae culture (Derner et al. 2006). The three microalgae species were chosen based on their previous application in the larval diet of closely related species and their widespread usage in aquaculture (Brown 2002; Becker 2008; Lober and Zeng 2009).

The rotifers were cultivated at the same temperature and photoperiod as the microalgae and were fed on *T. gracilis*. Microalgae, as prey, exhibit a positive influence on the reproduction and nutritional value of *B. plicatilis* and are considered a source of the rotifer enrichment (Duerr et al. 1998; Lubzens et al. 2001; Becker 2008). Although the rotifer *B. plicatilis* is not a natural prey for *Brachyura* larvae, it is commonly used in crustacean larvae culture (Shields and Lupatsch 2012). It exhibits slow swimming activity, an accelerated reproduction rate, and tolerance of a high culture density (Suantika et al. 2000). *Artemia* sp. has been used almost universally as a key zooplanktonic live prey since the 1960s in aquaculture hatchery, even that it is not a natural prey for *Brachyura* larvae (Abrunhosa et al. 2002; Shields and Lupatsch 2012). Dormant *Artemia* sp. eggs can be stored for long periods and then used, requiring only 24 to 48 h of incubation, which makes them the least labour-intensive live diet accessible for larval cultures (Shields and Lupatsch 2012).

Experimental design

After hatching, zoea I larvae from all females of each species were mixed in 500 ml of water in the same conditions as mentioned before (salinity 30 PSU and 25 ± 1 °C). This procedure was used to avoid the effect of maternal origin on the results (Simith et al. 2014). The zoea I larvae were transferred individually to 12-well cellular culture plates (Kasvi®) with 5 ml of water (salinity 30 PSU and 25 ± 1 °C). Hatchery in individual experimental units decreases cannibalism and competition between recently metamorphosed megalopae on other larvae still at zoea stage. At these controlled conditions, even larvae with developmental delay and slow growth can succeed in reaching further phases (Becker 2008). Five different diets were used as treatments: the microalgae *Tetraselmis gracilis* [T], *Chaetoceros calcitrans* [C], and *Thalassiosira weissflogii* [W]; the rotifer *Brachionus plicatilis* (enriched with *T. gracilis*) [R]; and freshly hatched *Artemia* sp. nauplii [A]. For each treatment, 96 zoea I (replica) larvae were used, totalling 480 *M. nodifrons* and *C. danae* larvae. After positive initial results in the *M. nodifrons* hatchery, a diet of *B. plicatilis* was tested on the first three zoea phases followed by the addition of *Artemia* sp. from then on [RA] using 96 zoea I larvae. The cellular culture plates with larvae were stocked in a germination chamber (model 347 CDG, FANEM Ltd.®) with temperature (25 ± 1 °C) and photoperiod (12:12 h light:dark) controlled. Every 24 h, the live larvae were relocated to a new well containing fresh saltwater and new food was provided. For feeding, we used the following concentrations: 400,000 ind/ml of microalgae, 40 ind/ml of rotifers, and 0.6 ind/ml of *Artemia* sp. nauplii (Becker 2008; Cottens et al. 2014). The contents of the old wells were then analysed in the search for ecdysis. Survival data and instar development were obtained daily, indicating the progress of larval development and the larval mortality rate. The experiment ended when all larvae died or reached the megalopa stage. To facilitate data analysis and discussion, the results are shown at two moments of development: from zoea I to zoea III and from zoea I to megalopa stage.

Statistical analysis

Normality (Shapiro–Wilk) and variance homogeneity (Bartlett) tests were performed. The effects of diet on the survival rate and development duration were evaluated through a permutational analysis of variance (PERMANOVA) with a pairwise post hoc PERMANOVA test. The rate of larval survival (number of larvae alive), time for metamorphosis, and maximum time for mortality were used as response variables, and the diet treatments as explanatory variables. Survival curves were obtained using the Kaplan–Meier test. For all analysis, data were analysed at two different moments of development: from zoea I to zoea III and from zoea I to megalopa stage. Analyses were performed in the R environment (R Development Core Team 2018) using the ‘survival’ (Therneau 2017) and ‘vegan’ (Oksanen 2018) packages. The PERMANOVA post hoc test was performed using PRIMER (Anderson et al. 2008).

Results

Diet affected *M. nodifrons* larvae survival and development time from zoea I to zoea III ($p < 0.05$), except for [T] × [C] ($p = 0.43$). Only larvae fed [R], [A], and [RA] diets developed to zoea III. Forty-one larvae on the [R] diet reached this phase, with a survival rate of 40% and development time of 10 ± 2.64 days. Five larvae on the [A] diet survived to ZIII, with a survival rate of 4.8% and development time of 14.75 ± 3.70 days. The [RA] treatment showed a 55.2% larval survival rate and development time of 12.65 ± 1.18 days (Fig. 1 and Table 1). The longevity of larvae fed a microalgae diet was short. Only [T] treatment larvae advanced to ZII. Survival rate dropped 45% on the third day and all larvae died on the eighth day. The [W] and [C] treatments ended on the seventh and sixth days, respectively. The survival rate of larvae on both diets dropped on the fifth (45.84%) and fourth (52.08%) days. Metamorphosis from ZI to ZII started on the fourth day for all diets.

Diet also affected *M. nodifrons* larval survival and development time from zoea I to megalopa stage ($p < 0.05$) (Fig. 2 and Table 1). Only larvae on the [A] and [RA] diets reached the megalopa stage. On the [A] diet, two larvae (2.08%) moulted to megalopa, in an average time of 29.5 ± 0.70 days (Fig. 2 and Table 1). On the [RA] diet, three larvae (5.88%) reached the megalopa stage, in an average time of 34.33 ± 8.39 days and with a mean survival rate of 15.33% (Fig. 2 and Table 1).

Diet also affected the survival of *C. danae* ($p < 0.05$). No larvae reached the zoea III stage. On the [T], [C], [W], and [A] diets, the mean larval survival time was 3.56 ± 1.22 days overall, without any larvae reaching ZII. Only two larvae fed the [R] diet reached the ZII phase, with a mean survival time of 6.59 ± 2.69 days (Fig. 3 and Table 2).

Discussion

The survival and duration of development from zoea I to megalopa stage of *M. nodifrons* and *C. danae* are affected by diet. Our results with *M. nodifrons* suggest that diets using different organisms at different life stages are ideal for larval development. We accept our initial hypothesis for *M. nodifrons* that the most suitable diet for larval development also shows a similar development time. On the other hand, none of the diets tested was effective for

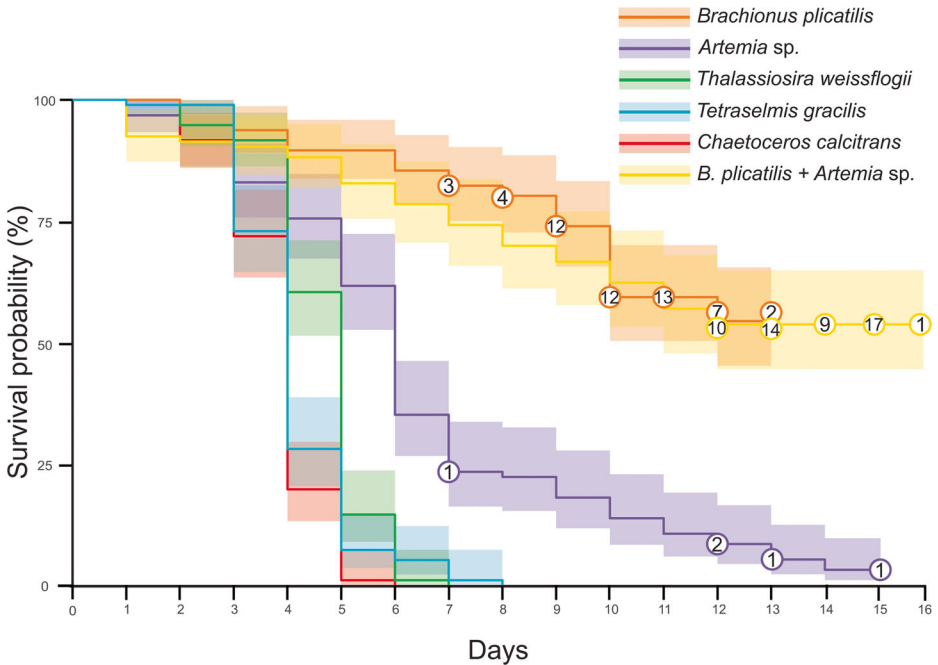


Fig. 1 Survival curves for *Menippe nodifrons* zoea I to zoea III. Survival percentage of 576 larvae until ZIII metamorphosis or death on six diets. Lighter-toned areas denote confidence intervals. Circles with numbers denote the number of events (metamorphosis to ZIII)

C. danae larvae. The most suitable feeding protocol for rearing *M. nodifrons* larvae is a combined diet of *B. plicatilis* up to the zoea III instar, with the introduction of hatched *Artemia* sp. nauplii onwards to the megalopa stage. The [RA] diet should be also tested for mass culture rearing of *M. nodifrons*. Although our results for rearing *C. danae* were not conclusive, the data provide additional information on improper diets for rearing the species for future research.

The most suitable diet for larval development until zoea III of *M. nodifrons* is the enriched *B. plicatilis* [R] diet. The nutritional needs of larvae alter at each stage as a function of their development (Souza and Costa 2006). An inadequate food source can generate disturbances in

Table 1 Number of zoea III and megalopa larvae, survival rate, and development time of *Menippe nodifrons* obtained for six diet treatments

Species	Treatment	Number of zoea III	Survival rate	Development time (in days)	Number of megalopae	Survival rate	Development time (in days)
<i>Menippe nodifrons</i>	<i>Tetraselmis gracilis</i>	-	-	-	-	-	-
	<i>Chaetoceros calcitrans</i>	-	-	-	-	-	-
	<i>Thalassiosira weissflogii</i>	-	-	-	-	-	-
	<i>Brachionus plicatilis</i>	41	40%	10 ± 2.64	-	-	-
	<i>Artemia</i> sp.	5	4.8%	14.75 ± 3.70	2	2.08%	29.50 ± 0.70
	<i>B. plicatilis</i> and <i>Artemia</i> sp.	51	55.20%	12.65 ± 1.18	3	5.88%	34.33 ± 8.39

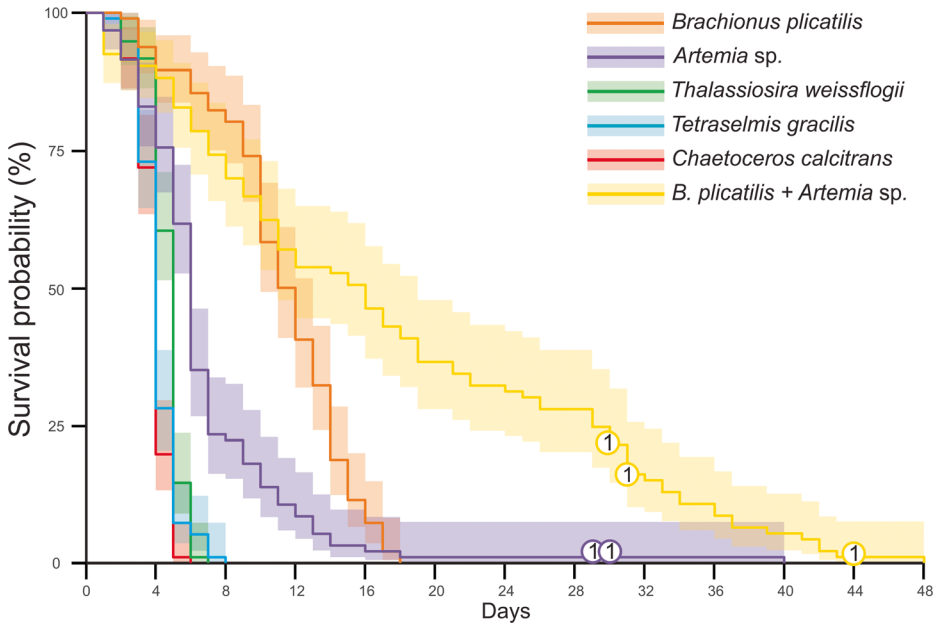


Fig. 2 Survival curves for *Menippe nodifrons* zoea I to megalopa stage. Survival percentage of 576 larvae until megalopa metamorphosis or death on six diets. Lighter-toned areas denote confidence intervals. Circles with numbers denote the number of events (metamorphosis to megalopa)

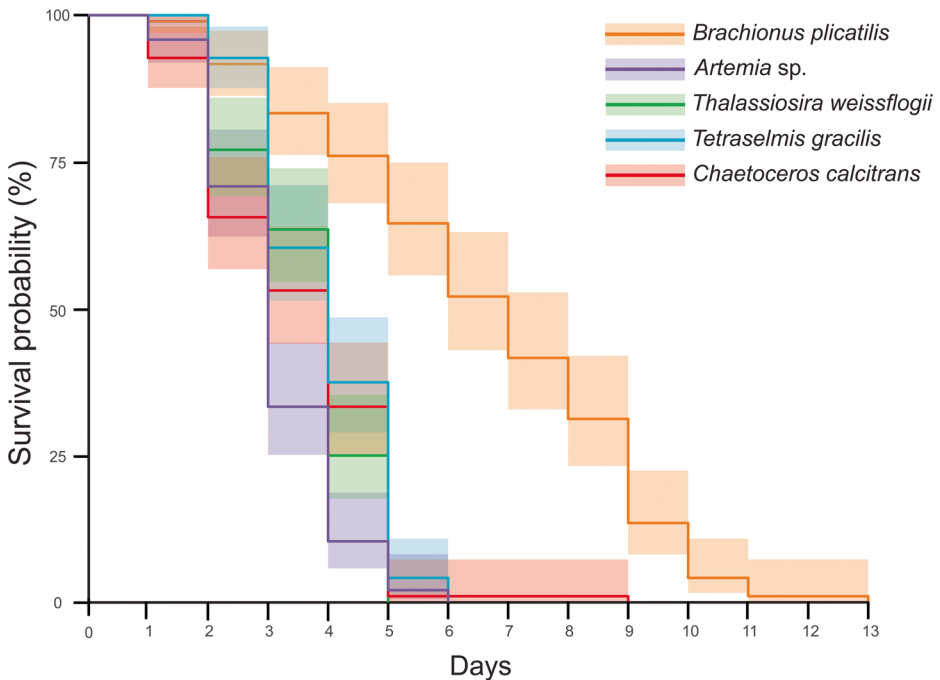


Fig. 3 Survival curves for *Callinectes danae* larvae. Survival percentage of 480 larvae on five diets. Lighter-toned areas denote confidence intervals

Table 2 Number of zoea II larvae, survival rate, and development time of *Callinectes danae* obtained for five diet treatments

Species	Treatment	Number of zoea II	Survival Rate	Development time (in days)
<i>Callinectes danae</i>	<i>Tetraselmis gracilis</i>	-	-	-
	<i>Chaetoceros calcitrans</i>	-	-	-
	<i>Thalassiosira weissflogii</i>	-	-	-
	<i>Brachionus plicatilis</i>	2	1.92%	6.59 ± 2.69
	<i>Artemia</i> sp.	-	-	-

both larval development time and survival (Carvalho 2010). For the [R] diet, the minor mortality rate in the first days was related to fast development until the zoea III stage. The rotifer's size (~250 to 300 µm) compared with the size of zoea I–zoea III *M. nodifrons* larvae (~0.55 to 0.80 mm) and easy laboratory culture makes it a fit diet for pre-zoea III larvae (Scotto 1979; Harvey and Epifanio 1997; Waiho et al. 2018). The rotifer diets were not suitable for feeding *C. danae* larvae. While other factors are also significant, the disparity in size between larvae and prey is also likely the key factor in this case. Sulkin (1975) suggested that *Callinectes sapidus* first-stage larvae are not able to ingest rotifers (> 120 µm) due to the difficulty in breaking down the prey into pieces. The same can be inferred for small *C. danae* larvae.

We chose an enriched rotifer diet to improve the overall larval survival rate. Rotifer enrichment maintains the general health and development of Brachyuran larvae (Ben-Amotz et al. 1987; Baylon 2009; Waiho et al. 2018). *Brachionus plicatilis* are non-selective filter feeders, ingesting any particle up to 12–15 µm, ideal for the bioencapsulation of any fatty acid-rich algae (Becker 2008). The alga used to enrich our [R] treatment (*T. gracilis*) showed a high level of essential fatty acids (EFAs). EFAs such as highly unsaturated fatty acids (HUFAs) or polyunsaturated fatty acids (PUFAs) are crucial to the growth and survival of marine fish and crustacean larvae (Lourenço et al. 1997; Selvakumar and Umadevi 2014). The use of EFA enrichment in a high-density rotifer diet at initial stages minimizes metamorphosis time, reduces larval development time, and standardizes the larval growth rate (Becker 2008).

The mixed rotifer plus *Artemia* sp. diet [RA] (400–500 µm) was the most suitable for rearing *M. nodifrons* from zoea III onwards to megalopa stage. Although *Artemia* nauplii are an extremely convenient source of food, they are naturally low in EFAs. Thus, larval survival is likely enhanced on an *Artemia* diet combined with other fatty acid-enriched food sources (Agh and Sorgeloos 2005; Beder et al. 2018). Ruscoe et al. (2004) suggested that larvae fed an *Artemia* mono-diet take longer to reach megalopa and endure higher levels of mortality. The diet is also not suitable for rearing *C. danae*. In previous studies on the rearing of *Callinectes sapidus* and *Callinectes similis*, early-stage larvae were able to consume only parts of the *Artemia* nauplii and individuals no larger than 110 µm (Sulkin and Epifanio 1975; Bookhout and Costlow 1977). Therefore, *Artemia* nauplii are only appropriate live food in terms of size and swimming speed for post-ZIII instars of *M. nodifrons* larval development.

Our data suggest a non-exclusive microalgae diet for both *M. nodifrons* and *C. danae* larvae. Both species suffered a high larval mortality rate allied to a lack of development for all microalgae treatments. Our main hypothesis is related to the disproportion of the size of microalgae and larval feeding appendages. *Tetraselmis gracilis*, *C. calcitrans*, and *T. weissflogii* are small (~9.59 µm, ~7 µm, and 4–32 µm, respectively; Olenina et al. 2006; Da Silva Gorgônio et al. 2013) in comparison to *M. nodifrons* (~0.55 mm) and

C. danae (~ 0.40 mm) zoea I larvae. Likely, the larvae were not able to feed on the [W] and [C] diets, dying by the end of their reserves, at ZI. It is unlikely that the food quantity was insufficient because we used a high concentration commonly used in larvae aquaculture (Becker 2008; Cottens et al. 2014). The [T] treatment was the only one under which larvae developed into the ZII phase. The genus *Tetraselmis* exhibits increased mobility due to its flagellated morphology and was the largest microalga used (Bicudo and Menezes 2006). The predatory behaviour of larvae and their preference for mobile food (Anger 2001) may stimulate them to feed, leading to ZII development under this treatment. Prior research showed only improved outcomes in treatments with a combination of an algal and non-algal diet (Sulkin 1975). Brown mono-microalgal diets may also cause a deficiency of some essential nutrients, decreasing growth and general survival (Harms and Seeger 1989; Seixas 2016). Our survey brings new information about a combination of a non-algal diet.

The most suitable feeding protocol for rearing *M. nodifrons* larvae is a combined diet of *B. plicatilis* up to the zoea III instar with the introduction of hatched *Artemia* sp. nauplii onwards to the megalopa stage. The [RA] diet can be also tested for mass culture rearing of *M. nodifrons*. Although our results on *C. danae* rearing were not conclusive, the data provide additional information on unsuitable diets for rearing the species for future research. Based on widespread species production, different microalga genera with a high concentration of fatty acids can be tested, such as *Pavlova*, *Skeletonema*, *Chlorella*, *Isochrysis*, and *Porphyridium* (Muller-Feuga 2000; Brown 2002), or other holoplanktonic and meroplanktonic organisms.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed by the authors. All biological samples collected for the present study complied with the current laws of the Brazilian Federal Government and experiments were conducted with the permission of the Brazilian Institute of Environment and Renewable Natural Resources (IBAMA) (Authorization # 60095-1-DIFAP/IBAMA, 12 September 2017).

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