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Survival rate of *Macrobrachium acanthurus* (Caridea: Palemonidae) larvae in laboratory conditions under different salinities and diets

MARIA MASCHIO RODRIGUES[1,3*], LUIS CARLOS FERREIRA DE ALMEIDA[2] & GIOVANA BERTINI[2,3]

[1] *Federal Institute of Education, Science and Technology of Espírito Santo (Ifes) Campus de Piúma-Rua Augusto Costa de Oliveira, 660 – Praia Doce – Piúma-ES – 29285-000, Brazil.*
[2] *Universidade Estadual Paulista "Júlio de Mesquita Filho", Campus de Registro, Rua Nelson Brihi Badur, 430 Registro-SP – 11900-000, Brazil.*
[3] *LABCRUST – Laboratório de Biologia e Cultivo de Crustáceos*

*Corresponding author: maria.maschio@hotmail.com

Abstract. *Macrobrachium acanthurus* is a native species from Brazil with a considerable economic interest, but there is no technology for its cultivation yet. Thus, the present work is aimed to determine the salinity conditions and the most effective diet for larval development. Zoea I were submitted to two experiments: the first one evaluated the effects of different salinities on survival time in starving larvae; the second experiment larvae were reared using five different diets (*Artemia*, microalgae, *Artemia* + microalgae, *Artemia* + inert diet and inert diet) using the salinity that showed higher survival period from the first experimental setting. Survival curves were calculated according to the Kaplan-Meier method and the multivariate Cox regression checked the effect of food types on larval survival rate. The lowest survival period occurred at a salinity of 0 and 33 gL^{-1} and the highest at a salinity of 15 gL^{-1}. *Artemia* and *Artemia* + inert diet were the most effective diets for larval development and survival, increasing by 281 and 199 times, respectively, the chances of survival compared to the inert diet alone. The results indicate that 15 gL^{-1} salinity and *Artemia* diet should be considered for future larviculture of *M. acanthurus*.
Keywords: Prawn larvae, starvation; inert diet; *Artemia*; Cinnamon river prawn.

Resumo. Sobrevivência larval de *Macrobrachium acanthurus* (Caridea: Palaemonidae) em condições laboratoriais sob diferentes salinidades e dietas. *Macrobrachium acanthurus* é uma espécie nativa brasileira que possui grande interesse econômico, mas ainda não possui tecnologia para seu cultivo. Assim, o presente trabalho visou determinar a salinidade e a dieta mais eficaz para sua larvicultura. As zoea I foram submetidas a dois experimentos: o primeiro avaliou os efeitos de diferentes salinidades no tempo de sobrevivência das larvas em inanição; no segundo as larvas foram cultivadas em cinco diferentes dietas (*Artemia*, microalgas, *Artemia* + microalgas, *Artemia* + dieta inerte e dieta inerte) utilizando a melhor salinidade encontrada. As curvas de sobrevivência foram calculadas de acordo com o método de Kaplan-Meier e a regressão multivariada de Cox verificou o efeito dos tipos de alimento na taxa de sobrevivência das larvas. Os menores tempos de sobrevivência ocorreram nas salinidades de 0 e 33 gL^{-1} e o maior na salinidade de 15 gL^{-1}. Os alimentos *Artemia* e *Artemia* + dieta inerte foram os mais efetivos para o desenvolvimento e sobrevivência das larvas, aumentando em 281 e 199 vezes, respectivamente, as chances de sobrevivência em relação à dieta inerte sozinha, respectivamente. Os resultados indicam que o uso da salinidade de 15 gL^{-1} e a alimentação composta por *Artemia* podem ser considerados em futuras larviculturas de *M. acanthurus*.

Palavras- chave: larvas de camarão; inanição; dieta inerte; *Artemia*; Camarão-canela.

Introduction

Freshwater prawn farming has been recognized as an essential alternative to cultivating crustaceans since they have characteristics favorable to farming such as independence of salt water in the final growth phase, production systems compatible with small properties and low environmental impact (New 2010).

In Brazil, the exotic species *Macrobrachium rosenbergii* is used in farming because the technology for its cultivation is relatively well developed (Valenti 1996). In general, native species have been understudied in Brazil and there is an urgent need to carry out research aimed at developing farming for alternative species.

In Brazil, the genus *Macrobrachium* is represented by 17 species (Pileggi & Mantelatto 2012), of which only three have an economic interest and a high potential for cultivation: *M. acanthurus, M. carcinus and M. amazonicum* (Bertini & Valenti 2010). Thus, this study aims to contribute with information towards the development of larviculture of the Cinnamon river prawn *M. acanthurus*, a common species of the western Atlantic (Holthuis 1980, Bond-Buckup & Buckup 1989, Melo 2003) found in estuaries and rivers up to ~300 km inland (Coelho 1963). Its occurrence is common in the Ribeira de Iguape basin (SP), where it has been heavily exploited by artisanal fishing, both for human consumption and for live baits market and sports fishing, an activity that is very common in the region (Bertini & Valenti 2010). These activities cause a strong stock reduction, compromising this fishing resource and affecting its reproductive cycle (Bertini *et al*. 2014, Bertini & Baeza 2014). However, despite the necessity to farm endemic species since the beginning of the 2000s (Kutty *et al*. 2000), there is no progress in *M. acanthurus* farming nowadays and no technology has been developed for its larviculture.

One of the critical points in freshwater shrimp farming is to know the most effective salinity for larviculture because they require brackish water for growth and survival (Guest & Durocher 1979, McNamara *et al*. 1983, Moreira *et al.* 1986, Cooper & Heinen 1991, Araújo & Valenti 2010). According to Cooper & Heinen (1991), starvation tests represent the best method to determine optimum salinity for larvae, since they have a lower energy expenditure for osmotic regulation, increased longevity and survival.

Another critical factor that affects freshwater prawn larvae is the feeding strategy, which must be adequate to the nutritional needs of the larvae (Loya-Javellana 1989). However, information on larval digestive capacity, digestive processes and nutritional requirements are still scarce. In addition, they go through several stages of development, and each stage has specific morphology, nutritional preferences and behavior (Sorgeloos & Léger 1992, Lavens *et al*. 2000). In this sense, some species of the genus *Macrobrachium* were investigated in relation to: microbound diet for the larval culture and acceptance of inert food items and *Artemia* for each larval stage of *M. rosenbergii* (Kovalenko *et al*. 2002, Barros & Valenti 2003); feeding strategy on larval development of *M. amazonicum* (Anger & Hayd 2010, Araújo & Valenti 2017); feeding and stocking density in *M. americanum* (Yamasaki-Granados *et al*. 2013); different foods and larval density in *M. equidens* (Gomes *et al*. 2014). However, little is known about the feeding habits of *M. acanthurus* larvae, except for Rodrigues *et al.* (2017), who investigated the supply of inert diet in an open system of cultivation.

Therefore, to improve our understanding of the initial requirements of the freshwater prawn *M. acanthurus* life cycle, we performed two experiments to determine the most effective salinity and diet for larval development, supposing that the determination of an adequate salinity and diet would increase the longevity and improve growth. These results may serve as a subsidy for aquaculture projects, to develop technologies for farming this native species of sizeable economic importance.

Materials and Methods

Ovigerous females of *M. acanthurus* were collected in Ribeira de Iguape River (S 23 ° 45 ', W 46 ° 45') located in the southern region of São Paulo State (Brazil), using a combination of minnow traps (1 m length, 30 cm diameter, and 8 mm mesh pore) and kick nets (0.5 m^2, 5 mm mesh). Immediately after collection, the ovigerous females were transferred to plastic buckets (20 L) with water (0 gL^{-1}) from the collection site, under constant aeration.

In the laboratory, the ovigerous females with eggs in the final stage of embryonic development (little yolk, well-developed eyes, chromatophores, and appendages) were separated under a stereomicroscope. These females were kept separately in glass aquaria (40 cm x 30 cm x 30 cm) containing freshwater from the collection site under

constant aeration and maintained at 29 ºC. The substrate was composed of a thin layer of coarse sand to simulate the natural environment. Black plastic covered the sides of the aquaria to avoid prawns interference. Females were fed daily with small pieces of squid; the food left over and the fecal matter was siphoned from the aquarium daily. Hatching always took place during the early hours of the night. The newly hatched larvae (zoea I) were attracted by a light bulb to one of the corners of the aquarium and were carefully siphoned into another vessel containing filtered freshwater and kept in this location for approximately 12 hours, according to the methodology of Choudhury (1971a). After this period, they were transferred to different experimental setups. Each experiment was conducted using a blend of larvae obtained from 2 females.

The seawater used for the experiments had a salinity of 33 gL^{-1} and was collected in the ocean, filtered and stored in gallons. From the collected seawater, we prepared a 5-liter gallon for each level of salinity to be tested in the experiments. Before being transferred to the culture vessels, the water in these gallons was again checked for salinity with a specific optical refractometer (ATAGO®, ATC-S/Mill-E; ± 1 gL^{-1}, with a built-in automatic temperature compensation system).

The temperature and the photoperiod were controlled by keeping the containers with the larvae in a BOD type incubator (ELETROLAB®, EL 202), where the temperature was maintained at 29 ± 1 ºC and the photoperiod 12 h light / 12 h dark. The luminous intensity of the BOD was reduced, keeping only two bulbs inside with 750 lux, according to New (2002). In addition, the containers with the larvae were wrapped in black paper. In all experiments, the containers containing the larvae were inspected daily for the detection of exudates, dead larvae and for complete water exchange.

For the determination of the salinity and the ideal diet during larval development of *M. acanthurus*, two experiments were carried out. The first experiment consisted of 7 treatments (salinity levels: 0, 5, 10, 15, 20, 25, 33 gL^{-1}) and 8 replicates using plastic containers of 150 mL with water at different salinities, containing 15 larvae each. The larvae were slowly acclimatized from freshwater to the different levels of salinity by the addition of saltwater in a known volume (2 L) of freshwater for a period of 4 to 6 hours (Choudhury 1971a). Subsequently, the larvae were transferred to the containers (150 ml) with different levels of salinity. The second experiment consisted of 5 treatments (types of diets: *Artemia*, microalgae (*Nannochloropsis oculata*), *Artemia* + microalgae, *Artemia* + inert diet and inert diet alone) and 4 replicates (plastic containers with 150 mL capacity with 15 larvae each). For the experiment, the salinity was fixed using the best survival rate obtained from the 1st experiment, that is, the salinity of 15 gL^{-1}. The acclimatization of the larvae was performed as previously described. Food was offered once a day in the morning.

For the second experiment, *Artemia* was offered at the newly hatched nauplii stage at a density of 5-15 $nauplii.mL^{-1}$ according to the most abundant larval stage found in the recipients (Valenti *et al*. 1998). The microalgae were cultivated in the laboratory from strains of *Nannochloropsis oculata* and were offered *ad libitum*. The exponential growth of algae was conducted using the *Guillard* medium until reaching a volume of 2,000 ml. The inert diet was elaborated according to the methodology of Mallasen & Valenti (1998) (Table I) and offered at a concentration of 0.07 a 0.20 $g.larvae^{-1}.day^{-1}$ as suggested by Valenti *et al*. (1998). It was frozen at -18 °C and at the time of feeding, it was separated into small pieces and passed through a fine-mesh sieve (250 - 425 μm), to obtain sizes suitable to the larvae.

Table I. Inert diet composition and proximate analysis, according to Mallasen & Valenti (1998).

Ingredients	**(%)**
Chicken egg	34.00
Squid flesh	10.00
Fish flesh	10.00
Dried milk	4.00
Wheat flour	2.00
Fish liver oil	0.80
Vitamin mix	0.70
Mineral mix	0.70
Water	37.80
Proximate analysis	**% dry weight**
Crude protein	45.07
Crude fat	22.55
Nitrogen-free extract	23.55
Ash	8.83
Original dry matter	18.29
Gross energy ($kcal.kg^{-1}$)	4,989.20

The separation of the different larval stages was done according to Choudhury (1970) and Quadros *et al*. (2004). The experiments were finished when all larvae died or when they reached the post-larvae stage.

Statistical Analysis: Survival rates were calculated using the non-parametric Kaplan-Meier estimation method to describe the relationship between larvae survival and risk factors including salinity levels and diets. The specific test for Kaplan-Meier survival analysis, the LogRank test, was used to determine statistical differences between the survivorship curves distributions for each treatment (Colosimo & Giolo 2006, Kleinbaum & Klein 2012). Risk factors (salinity levels and diets) that significantly influenced overall survival were tested according to chi-square (x^2) and the p-value, using univariate analysis.

The hypothesis of equality between the curves was tested at the significance level $\alpha = 5\%$, corrected by the Bonferroni method, where significance is given by α/c, where c is the total number of comparisons performed in each experiment. This correction factor aims to control type I error due to a large number of comparisons between treatments (Kleinbaum & Klein 2012). Relationships of $p < p_{\alpha/c.}$ were considered significant.

Additionally, for the second experiment, a multivariate Cox regression was performed, using all diets to obtain proportional hazards values (Hazard Ratio - HR), through measuring the effects of each independent variable on survival rate. To obtain the final model we opted for the Stepwise Forward method where each significant variable is successively included in the model (Kleinbaum & Klein 2012). Survival analysis by these methods was also performed by Harper-Arabie *et al*. (2004), Ituarte *et al*. (2010) and Xia *et al*. (2015).

All analyses were performed using the Trial Software: SPSS Text Analytics for Surveys, version 22.0.

Results

The survivorship curves for *M. acanthurus* unfed larvae at different salinities can be visualized in Figure 1. Multiple comparison curves indicated significant differences between all salinities, with the longest survival period at 15 gL^{-1} salinity, where the larvae survived until the ninth day ($p<0.05$; $\alpha/c = 0.0023$). On the 8th day, 10 and 15 gL^{-1} salinities had live larvae with a survival rate of 34.9% and 84.7%, respectively. It was observed that in the 10 gL^{-1} salinity, survival decreased from day 5, indicating a lower chance of survival compared to the 15 gL^{-1} salinity.

The highest mortality occurred at salinities of 0 and 33 gL^{-1}, with larvae mortality of 100% at the end of the 2nd and 6th days, respectively (Fig. 1). In all treatments the larvae molted to the stage of zoea II, except for the treatment of 0 gL^{-1}, where there was no molting.

Based on Kaplan-Meier estimates, the survivorship curves were constructed for *M. acanthurus* larvae fed with different diets (*Artemia*, microalgae, inert diet, *Artemia* + microalgae, *Artemia* + inert diet) and maintained at 15 gL^{-1} salinity (Fig. 2). The results indicate that up to the 20th day of larval development, the estimated curve for the diets *Artemia* and *Artemia* + inert diet presented similar survival rate and after that, there was a drop in the survival, mainly for the *Artemia* + inert diet. In these treatments, nine larvae reached the post-larvae stage (five in the *Artemia* treatment and four in the *Artemia* + inert diet treatment) up to the 49th day, when the experiment was finished. The shortest survival period was obtained in the treatments with inert diet (9 days) and microalgae (10 days). Survival curves were compared through the LogRank test, where significant differences were observed between all diets ($p = 0.0001 < p_{\alpha/c} = 0.003$), except for the *Artemia* diet in relation to *Artemia* + inert diet ($p = 0.029 > p_{\alpha/c} = 0.003$) (Table II).

Hazard ratios (HR) for each risk factor, from the analysis of the multivariate Cox model, are listed in Table III. The treatment with inert diet was used as a reference, since this treatment showed the highest mortality in the shortest period (Fig. 2). All variables showed significant values of β ($p <0.0001$), which correspond to HR values greater than 1.0. Therefore, the diets that increased the chances of survival in relation to inert diet were *Artemia* (HR=281.01) and *Artemia* + inert diet (HR=199.59) (Table III).

Discussion

Most freshwater prawns require an estuarine environment to complete their life cycle and the knowledge of ideal salinity is of high relevance for freshwater shrimp farming. Brackish water requirements for larval development of *M. acanthurus* was observed in the present study and by Choudhury (1971a) and Ismael & Moreira (1997). Congeneric species also require water with variable levels of salinity for an appropriate larval development such as *M. carcinus* (14 -17.5 gL^{-1})

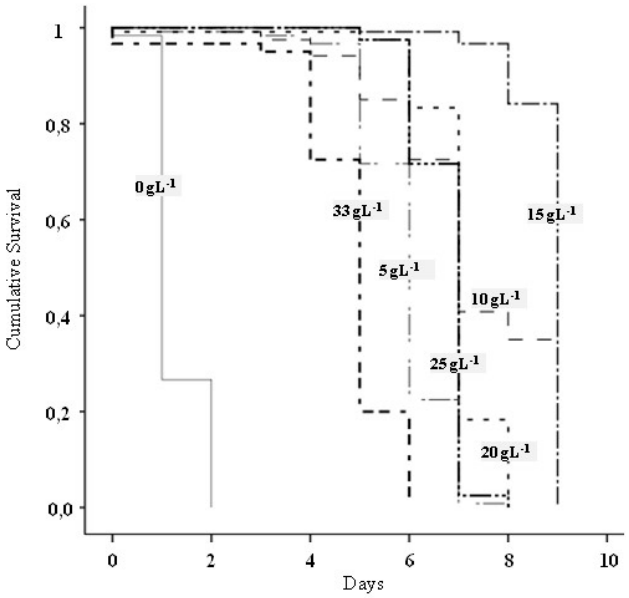

Figure 1. Survival curves estimated by the Kaplan-Meier method relating the cumulated survival with time (days) for *Macrobrachium acanthurus* unfed larvae at different salinities (0, 5, 10, 15, 20, 25, 33 gL-1).

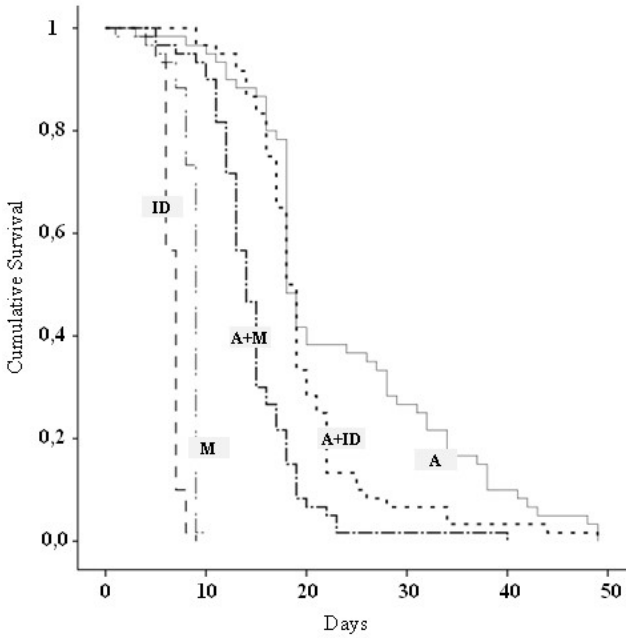

Figure 2. Survival curves estimated by the Kaplan-Meier method relating the cumulative survival with survival period (days) for *Macrobrachium acanthurus* larvae fed with different diets at a salinity of 15 gL^{-1}. (A- *Artemia*, ID- inert diet, M-microalgae). See the statistical differences in table II.

(Choudhury 1971b), *M. amazonicum* (12-18 gL^{-1}) (Moreira *et al*. 1986), *M. rosenbergii* (10-15 gL^{-1}) (Agard 1999), *M. ohione* (15 gL^{-1}) (Bauer & Delahoussaye 2008), among others.

The highest survival rates for crustacean larviculture requires optimal salinities due to lower energy expenditure, especially for osmoregulation (Cooper & Heinen 1991). Inadequate environmental conditions lead to physiological stress, increasing the energy expenditure for homeostasis (Agard 1999). Therefore, 15 gL^{-1} salinity was shown to be ideal for *M. acanthurus* initial larval development.

Macrobrachium acanthurus larvae did not reach the stage of zoea II in freshwater (0 gL^{-1}), indicating that the yolk reserve used for metamorphosis had to be redirected to maintain homeostasis. As observed in this study and for *M. ohione* by Bauer & Delahoussaye (2008), although embryos may hatch in freshwater, they are unable to undergo ecdysis to stage II of zoea, which is the first planktotrophic stage that enables the larvae to complete its development. Thus, the present study indicates that the first larval stage uses all its yolk reserves for metabolic requirements, therefore surviving only two full days.

Amphidromous species of the genus *Macrobrachium*, such as *M. acanthurus*, usually do not feed in the first stage of zoea, initiating feeding with *Artemia* nauplii after the stage II of zoea. This observation is confirmed by Rocha *et al*. (2017), who verified that the first larval stage of *M. acanthurus* has rudimentary mouthparts and a small number of setae, indicating limited functionality of the mouthparts. Moreover, the intestine is rudimentary and non-functional, showing no differentiation between cardiac and pyloric chambers, lacking setae and pyloric filter, all indicating obligatory lecithotrophic feeding behavior. Therefore, offering *Artemia* nauplii must start from stage II of zoea, since only after this stage the larvae can process the food. In *M. rosenbergii* the functional morphology of the foregut indicates facultative lecithotrophy from zoea I stage onwards, with exogenous nutrients only being required from zoea stage III onwards (Abrunhosa & Melo 2002).

According to Bertini *et al*. (2014), *M. acanthurus* females do not have a migratory behavior towards the estuaries to release the larvae, and consequently the larvae hatch in freshwater and take about 1.54 days (37 hours) to be carried by passive transport to the estuarine region of the Ribeira de Iguape River. Thus, the results presented here suggest that the larvae can survive in freshwater using the yolk reserves to be able to migrate to the estuary.

The experiment related to the survival time of larvae submitted to different diets indicated that,

Table II. LogRank test for comparison of survival curves of *Macrobrachium acanthurus* larvae submitted to different diets at 15 gL^{-1} salinity. Values of probability below α/c = 0.003 for comparisons between treatments indicate significant differences.

Treatments (diets)	*Artemia*	Inert diet	Microalgae	*Artemia* + inert diet
Inert diet	0.000			
Microalgae	0.000	0.000		
Artemia + inert diet	0.029	0.000	0.000	
Artemia + microalgae	0.000	0.000	0.000	0.000

Table III. Cox multivariate regression model with Hazard Ratio (HR) and 95% Confidence Interval (CI) for different treatments (risk factors).

Treatments	β[a]	HR[b]	95% CI	p value
Artemia	5.64	281.01	120.64-654.55	0.000
Microalgae	1.62	5.07	3.19-8.06	0.000
Artemia + microalgae	4.42	83.34	37.07-187.38	0.000
Artemia + inert diet	5.30	199.59	86.56-460.20	0.000

[a] β - represents regression coefficients of the corresponding variable and the value from parameter estimates, which indicates the factors affecting larvae survival in the treatments. Higher absolute values of β indicate a larger positive impact on the survival of larvae prawns. β=0 indicates that the corresponding variable is considered as the baseline.

[b] HR values were from eβ, which estimates the hazard ratio relative to baseline, where HR = 1 indicates no effect, and HR > 1 indicates an increased rate of survival.

when the larvae were fed exclusively with microalgae and inert diet, these were inefficient for *M. acanthurus* as they resulted in delayed development and low survival rate. Although it is well known that microalgae are widely used in penaeid larviculture, for example for *Litopenaeus vannamei* (Kent *et al*. 2011), the results here suggest that *M. acanthurus* larvae do not feed directly on the microalgae *Nannochloropsis oculata*, because the longevity found under the microalgae treatment was similar to that for starving larvae from the first experiment. Probably, this green microalga does not have all the necessary nutrients for *M. acanthurus* larval development, confirming the carnivorous behavior of this species. Similar results were found for *Macrobrachium equidens* by Gomes *et al.* (2014), where larvae fed exclusively with the microalgae *Chaetocerus gracilis* showed low longevity as well, due to the low amount of nutrients found in these algae.

The use of the inert diet by *M. acanthurus* larvae as the only source of food since the beginning of the feeding stage (zoea II stage) was also inadequate. Some authors indicate that, although the inert diet has adequate consistency and palatability, other factors can lead to low survival rates, such as: low production of digestive enzymes by the larvae, low dietary digestibility, incomplete nutritional requirements in the diet and inadequate feeding frequency (Kamarudin *et al.* 1994, Maciel & Valenti 2014). In addition, factors related to the possible effects of by-products from the degradation of the inert diet may cause stress to the larvae, which may alter feeding, growth and survival rates (Rodrigues *et al.* 2017).

The complete larval development and the longest survival time for *M. acanthurus* larvae occurred with both *Artemia* and *Artemia* + inert diet diets, showing, however, increased chances of survival when fed only with *Artemia*. Some studies have shown that this food was not adequate for the last larval stages of *M. rosenbergii* due to insufficient nutrients (Alam *et al.* 1995, Valenti *et al.* 2010), and the small size of nauplii (Barros & Valenti 2003). Recently, Araújo & Valenti (2017) concluded that the best survival and productivity for *M. rosenbergii* were seen with a combination of *Artemia* nauplii and inert diet from stage II onwards. However, in the present work, the addition of the inert diet to *Artemia* did not increase the production of post-larvae in comparison to the diet composed of *Artemia* only, suggesting that *M. acanthurus* larvae can metamorphose into post-larvae when fed with *Artemia* nauplii as the only source of food. These

results agree with the studies of Lavens *et al*. (2000) for *M. rosenbergii* and of Gomes *et al.* (2014) for *M. equidens*, showing that larvae could be reared successfully on *Artemia* nauplii as their only source of food.

The larviculture of many native species of economic importance is still not well established due to an absence of adequate technologies and basic studies about the nutritional requirements for larval development in the laboratory, as is the case for *M. acanthurus*. Kutty *et al.* (2000) emphasized the need for the development of a larviculture for *M. acanthurus*, since the farming for this species is complicated and there is no technology to achieve adequate survival yet. Recently, Rodrigues *et al*. (2017) carried out the larviculture of this species and did not have high survival rates, indicating that more studies are necessary for farming of this native species of economic interest.

One of the difficulties observed in *M. acanthurus* larviculture was an irregular metamorphosis in the stage of zoea VI, that is, the larvae molted but we could not characterize as a new larval stage (VII). In this case, new structures were not present, such as biramous pleopods with exopod and endopod without setae, therefore extending the larval development period and ending up in death. According to Valenti (1985), the larval development of *M. acanthurus* lasts for 30 - 40 days, however, both in the present study and observations by Rodrigues *et al.* (2017) showed an extended period of 49 days and 42 days, respectively. The extension of the larval period may be related to the type of food offered, temperature, salinity, parental population, among other factors (Sandifer & Smith 1979, Melo & Brossi-Garcia 2005). Besides, the low larval survival found in the 2nd experiment suggests that the larvae are under stress, probably through alterations in the optimum salinity requirements along the larval development, since the amphidromous species begin their return to fresh water in the early juvenile stages. Many studies have shown that several species of decapods can alter the ability of osmoregulation during ontogeny, through anatomical and physiological modifications, allowing the species to migrate to regions of different salinities (Charmantier 1998, Charmantier *et al*. 1998, Charmantier & Anger 1999, Charmantier & Anger 2011, Boudour-Boucheker *et al*. 2013, Soeiro *et al*. 2016).

The results from this work represent a further step towards farming techniques for *M. acanthurus*. However, to increase the survival rates and obtain a higher number of successful post-larvae, it would be necessary to carry out further experiments on a large-scale larviculture in tanks with recirculating water system involving a combination of different foods and salinities along the larval development.

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