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Research Article

Histological analysis of *Macrobrachium rosenbergii* (de Man) eggs incubated in different salinities

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ABSTRACT. *Macrobrachium rosenbergii* is the freshwater shrimp species of greatest economic interest, and its reproduction has been studied for commercial purposes. This study aimed to evaluate the effect of different salinities (0, 6, and 12 mg L^{-1}) on the embryogenesis of this species. The females were observed daily according to their reproductive behavior. Upon the first sign of spawning, when their eggs were attached to the abdomen, they were collected for egg collection and transferred to the incubators at the respectively tested salinities. The eggs were collected every five days for up to 23 days, separated according to their stage of development, histologically processed, and stained for visualization of structural changes during embryogenesis using an optical microscope. From the egg's histological images at different embryonic development stages, the egg hatching time was evaluated, the changes that occurred in the eggs during embryogenesis, the total area of the eggs, the vacuole area, and the ratio between the vacuole area and the total area of the eggs. Differences were observed in hatching time, egg size, and vacuole area and area ratio. The 6 g L^{-1} of salinity treatment was statistically different from the other in all parameters analyzed. Therefore, it was concluded that 6 g L^{-1} of salinity positively influenced embryogenesis, as it presented hatches from the 15th day of embryogenesis, demonstrating that there was a greater speed of migration of vitellogenin for the formation of the embryo, accelerating embryogenesis and hatching of *M. rosenbergii*.

Keywords: *Macrobrachium rosenbergii*; giant river prawn; embryonic development; oocytes; shrimp reproduction

INTRODUCTION

The genus *Macrobrachium* is the largest in number of species of the order Decapoda, and most species live in freshwater, at least in one stage of life. This genus can be found on all continents except Europe and Antarctica (New et al. 2010). The genus has most aroused commercial interest by breeders worldwide for cultivating in captivity and exploiting natural stocks (Chong-Carrillo et al. 2016). The demand for giant shrimp for consumption has increased, and with it comes the demand to improve breeding, hatching, and juvenile production techniques to supply grow-out farms (Kawan et al. 2019).

Among the species of this genus, *M. rosenbergii* is the preferred species for culture, popularly known as giant river prawn. Due to its fast-growing nature, robustness, low protein requirement in diets, good consumer market, and higher returns, this species is recommended for culture in many tropical and subtropical countries (Paul et al. 2016). Worldwide, this species represents 2.6% of the total crustacean production, totaling 294 million tons produced in 2020, almost 100 million more than the total produced in 2010, which was 193.1 million tons (FAO 2022).

M. rosenbergii lives in tropical freshwater environments with access to brackish water once its larval development occurs in low salinity environments (Ling

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& Merican 1961). When ovaries reach maturity, around the fifth-month-old, the females undergo the pre-nuptial molt, copulation (Valenti 1996), and spawning occurs about 24 h after copulation (Pinheiro & Hebling 1998). The eggs are approximately 0.6 mm in diameter, of orange color, and covered by a thin membrane. After spawning, the eggs begin the process of incubation or embryonic development, which lasts about 20 days. During this period, the eggs turn from orange to dark grey, making it possible to observe the eyes of the embryos due to the membrane transparency (Lobão 1996, Habashy et al. 2012).

The berried shrimp behavior characterizes the successful reproduction of some species of crustaceans, resulting in a high embryo survival rate. Females keep their eggs steadily attached next to the pleopods until hatching, allowing the monitoring of embryogenesis during incubation (Muller et al. 2004). Various morphological, physiological, and yolk content and distribution changes occur during egg development, ranging from smaller to larger eggs. Smaller volume eggs have less yolk and total cleavage, giving rise to autonomously swimming nauplii. When the amount of yolk is greater in the egg, and there is maternal incubation, partial cleavage takes place and hatching in the zoea form (Hertzler 2002).

The reproduction of *Macrobrachium* genus has been studied for commercial purposes for some decades (Daniels et al. 2000, Martins et al. 2007, David et al. 2018), and salinity has been observed influencing the development of eggs and larvae. According to Fukuda et al. (2017), the volume of *M. acanthurus* eggs showed a significant difference with different salinities, with lower salinities being adequate. Kawan et al. (2019) found that the highest egg production of parental groups of *M. rosenbergii* weighing between 40-50 g is obtained at a salinity of 5 g L^{-1}. The production of large-sized eggs in larger quantities makes it easier to follow embryogenesis during incubation (Odinetz-Collart & Rabelo 1996).

Embryogenesis in vitelliferous eggs allows visualization of all morphological changes occurring daily by macroscopic examination and through histological sections. However, some methodological approaches also allow the evaluation of development through measurements of some embryonic structures (Beltz et al. 1992), relating quantitative variables to morphological descriptions. By analyzing the empty areas (vacuoles), it is possible to observe the distribution or structural organization of the embryo, allowing us to predict that the protein present in the egg (consisting mainly of vitellogenin) was moved to nourish the embryo (Charniaux-Cotton 1985). In this way, it is possible to verify the effects of different factors on their development, such as salinity, through observation of the changes that occurred in the eggs during embryogenesis, the egg hatching time, the total area of the eggs, the area of the vacuole and the ratio between the area of the vacuole and the total area of the eggs.

Therefore, the present work aimed to verify the effects of different salinities on embryogenesis, embryonic development, hatching time, and possible relationships with the initial larval stages of *M. rosenbergii*.

MATERIALS AND METHODS

The experiment was conducted at the Shrimp Culture Laboratory at the Research and Development Center for Sustainable Aquaculture of Paraná Federal University (UFPR, by its Portuguese acronym) - Sector Palotina. For the study, females that presented distinguished gonadal maturation were selected, together with male specimens of *M. rosenbergii*, in the proportion of three females to one male, stocked in elevated tanks with a capacity of 1000 L, filled with aerated water. Shrimps were fed daily with a commercial diet (Guabitech Active 1.6 mm - Guabi Nutrição e Saúde Animal S.A.). Water quality parameters in the broodstock tanks, such as dissolved oxygen, temperature, and pH, were measured weekly using Hanna HI 9146 oximeter, digital thermometer, and Lucadema pH meter, 210, respectively.

Females were observed daily according to their reproductive behavior, and at the first spawning signal, when they presented their eggs adhered to the abdomen, they were selected for egg collection. Egg collection was performed according to the method described by Cavalli et al. (2001). Eggs were observed with the aid of a stereomicroscope. The method consists of removing all the eggs from the ovated female in a single collection and keeping them under controlled temperature and aeration conditions. The experiment was set up from these eggs, with three treatments and 15 replications.

The treatments consisted of a control, with fresh water without chlorine and artificial salinity, an intermediate treatment with water with an artificial salinity of 6 g L^{-1}, and another with water with an artificial salinity of 12 g L^{-1}. Salinities were obtained using a commercial artificial salt - Red Sea Salt®. Eggs were distributed in artificial incubators, with a capacity of 0.2 L, according to the treatments, and maintained at an average temperature of 28°C (Cavalli et al. 2001), in

a photoperiod of 12:12 h light:darkness, and lightly oxygenated with the aid of a mechanical aerator.

During the experimental period, the water quality parameters were also monitored: dissolved oxygen (oximeter Hanna HI 9146), temperature (mercury thermometer), salinity (analogic refractometer Rhb0-90), pH (pH meter Lucadema, 210). At the beginning and end of the experiment, water samples were collected from the incubators and sent to the Water Quality Laboratory of UFPR - Sector Palotina for analysis of alkalinity, hardness, total ammonia, and nitrite. These analyses were performed according to the methodologies proposed in APHA (2005).

Egg collection for histological analysis in each of the experimental units started on the same day the eggs were removed from the female (this was considered day zero for all treatments), and from day 5 of the experiment onwards, the eggs were collected every five days until day 20. The final collection was performed on the 23rd day; from hereafter, observing unhatched eggs was no longer possible. In each collection, 15 eggs were sampled per experimental unit to observe the development phases and allow the visualization of egg changes by histological technique.

For the histological procedure, eggs collected from the incubators were immediately fixed with Alfac solution for 12 h (Lopes & Malhão 2016), according to their stage of development. After this, eggs were kept in 70% alcohol until the dehydration process was carried out in an increasing series of 70, 80, 90%, and absolute alcohol, remaining for 15 min in each bath. Next to dehydration, the material was diaphanized in xylol in two 15-min baths. Subsequently, samples were embedded in paraffin for subsequent histological sectioning and staining. The obtained blocks were sectioned in 10 µ thickness, transferred to a glass slide, and progressively stained with hematoxylin and eosin solutions, leaving the sections in the hematoxylin only long enough for the nuclei to be stained. Then, these sections were stained with eosin, which distinguishes the cytoplasm of different cells and different types of tissue fibers. After staining, the slides were permanently mounted using Canada balsam (adapted from Lopes & Malhão 2016).

The slides with the sections were examined under a light microscope (Olympus BX50), with a digital camera (3CCD Pro-Series) attached, at 20x magnification, and images captured to subsequently analyze (Image Pro-Plus v. 4.5.1, Media Cybernetics®). The image analyzer was calibrated using a micrometer slide (Olympus®). The obtained images were colored in layers supported by the software (Image Pro-Plus v.4.5.1, Media Cybernetics®), which reads the different colors of images. Thus, the places of interest on the slide were selected, and then the total area and the area of empty spaces and embryo formation (µm) were measured.

At the end of the experimental period, it was evaluated the hatching time of the eggs, the changes occurring in the eggs during embryogenesis, the total area of the eggs, the vacuole area, and the ratio between the vacuole area and the total area of the eggs.

For the statistical analysis, the normality and homoscedasticity of the obtained data were verified, and they were subsequently submitted to ANOVA ($\alpha = 0.05$). The averages of the treatments that showed differences in the analysis of variance were submitted to the Tukey test ($\alpha = 0.05$) using the Statistica® software (Statsoft Inc. 2004).

RESULTS

The values measured of water quality parameters during the experimental period remained within the acceptable range for shrimp farming (dissolved oxygen: 5.9 ± 0.5 mg L^{-1}; temperature: 28.0 ± 2.0°C; pH: 7.9 ± 0.4; alkalinity: 45 mg L^{-1} of $CaCO_3$; hardness: 50 mg L^{-1} of $CaCO_3$; total ammonia: 0.2 mg L^{-1}; and nitrite: 0.05 mg L^{-1}). Significant differences in egg hatching time between treatments ($P < 0.05$). The eggs from the treatment containing 6 g L^{-1} of salinity showed hatching starting from the 15th day of incubation and full hatching before the 20th day at an average temperature of 28ºC. The treatments containing 0 and 12 g L^{-1} of salinity started to hatch after the 20th day, with total hatching after the 23rd incubation and an average temperature of 28ºC.

The generation of empty spaces and the developing embryo itself was verified in the histological sections of *M. rosenbergii* eggs at different evaluation periods (5, 10, 15, and 20 days, according to Figs. 1-4). Statistical differences were observed in the total areas of eggs from *M. rosenbergii* considering all the treatments and times evaluated ($P < 0.05$) (Table 1). At five days of incubation, the 6 g L^{-1} treatment showed significant differences compared to the 0 and 12 g L^{-1} salinity extremes (statistically equal), probably due to the increased water content. In the analysis of empty areas (vacuoles; Table 2), the embryo's greater distribution or structural organization can be observed (Figs. 1-4), being concentrated in the embryo. As for the area of the vacuoles, at five days of incubation, the highest value was observed in the treatment with 12 g L^{-1} of salinity ($P < 0.05$), and the lowest value was

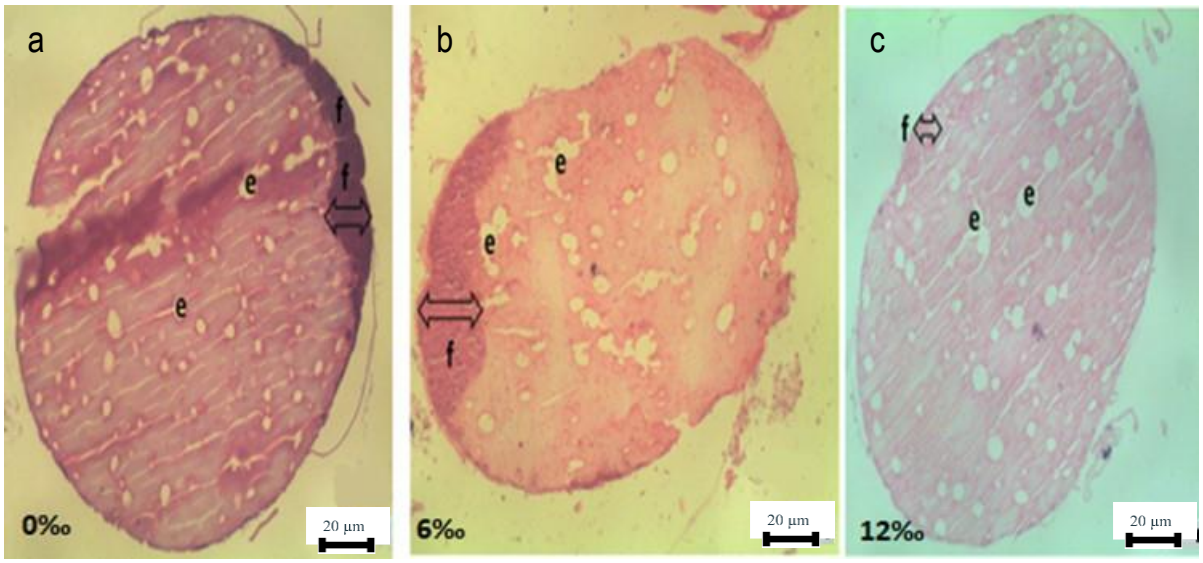

Figure 1. Histological sections of eggs five days after spawning (E1) at different salinities. a) Egg in 0% salinity, showing spaces (e); b) egg in 6% salinity showing several spaces allowing the development of the embryo to be seen (f) and an electron-dense field showing the region of embryo formation (double arrow); c) egg at 12‰ salinity, where the embryo formation region (e) does not present an electron-dense appearance (double arrow), but the location of the embryo is evident. HE staining 20X.

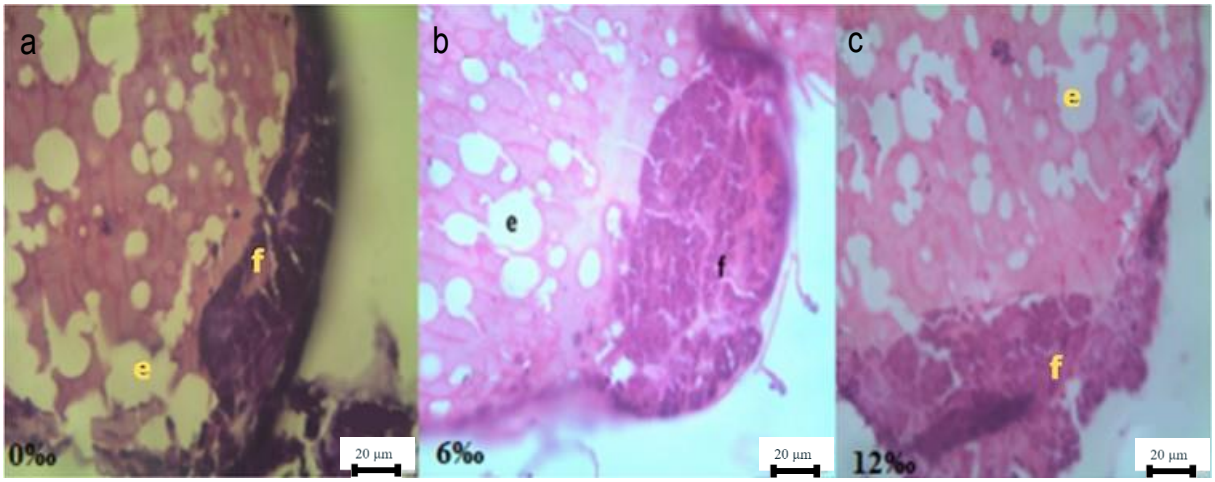

Figure 2. Histological sections of eggs 10 days after spawning (E2) at different salinities. a) Egg in salinity 0% with presence of empty spaces (e) and increase in the space occupied by the formation of the embryo (f), characterizing the development of the embryo (e) in the images; b) egg in salinity 6% with presence of empty spaces (e) and increase in the space occupied by the formation of the embryo (f), characterizing the development of the embryo (e) in the images; c) egg in salinity 12% with presence of empty spaces (e) and increase in the space occupied by the formation of the embryo (f), characterizing the development of the embryo (e) in the images. HE staining 40X.

observed in the treatment with 6 g L^{-1} of salinity, which remained with the lowest values until the 20th day of evaluation (Table 2).

In the 6 g L^{-1} of salinity treatment, there was a lower relationship ($P < 0.05$) in the size of the observed spaces occupied by vitellogenin with the total size of the egg, in comparison with the 0 and 12 g L^{-1} of treatments, which maintained a very close relationship, indicating statistical similarity (Table 3).

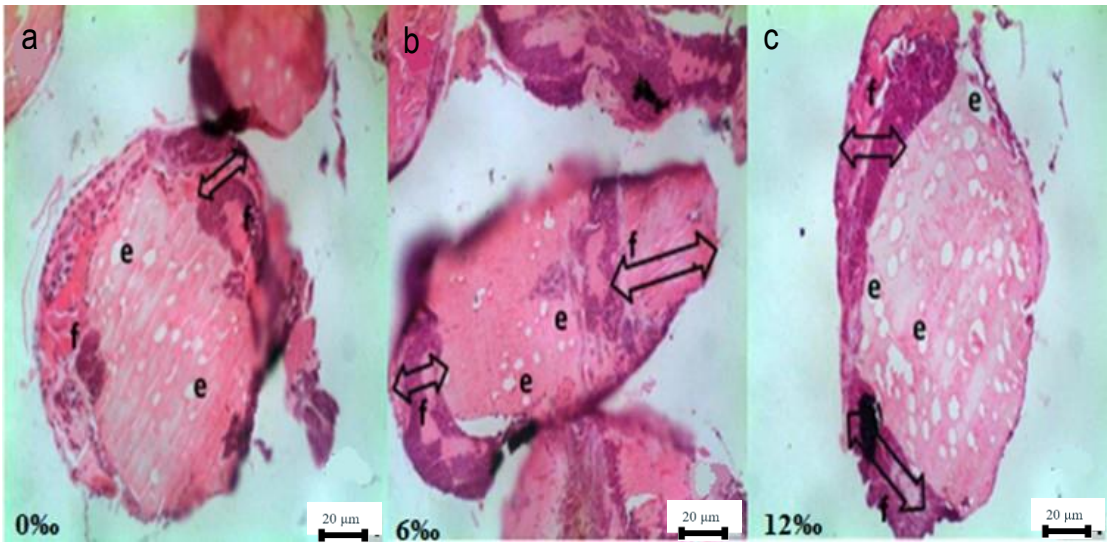

Figure 3. Histological section of eggs 15 days after spawning (E3) at different salinity concentrations. a) Egg in salinity 0% with decrease in empty spaces (e) and increase in embryo development (f) shown by more electron-dense marking (double arrow) in treatments with salinity concentrations; b) egg in salinity 6% with decrease in empty spaces (e) and increase in embryo development (f) shown by more electron-dense marking (double arrow) in treatments with salinity concentrations; c) egg in salinity 12%, we can see the egg with the embryo filling (f) occupying the entire lateral end of the egg (arrowhead). HE staining 20x.

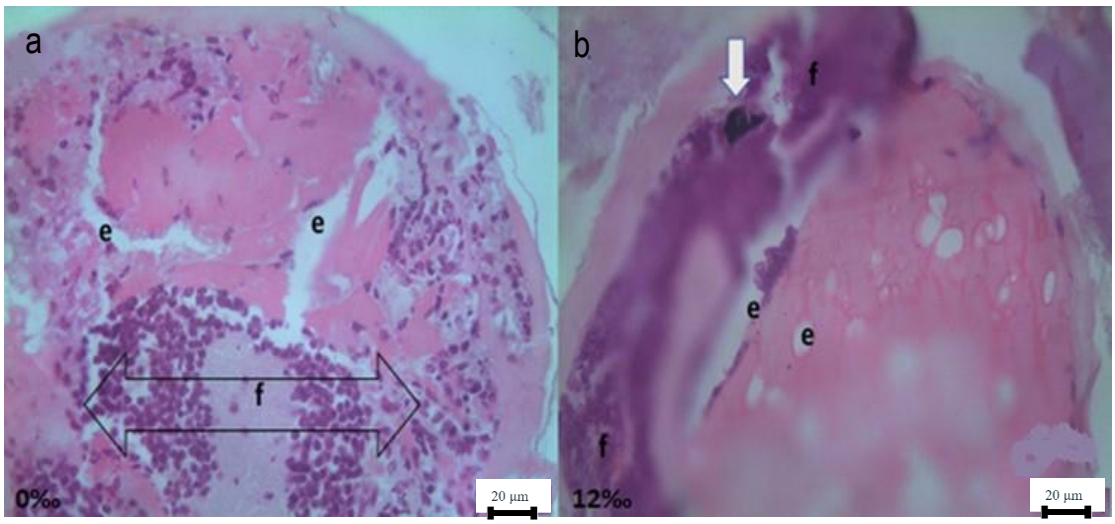

Figure 4. Histological section of eggs 20 days after spawning (E4) at different salinity concentrations. a) Egg in salinity 0%, we observe a significant reduction in empty space (e) and consequently the development of the embryo (f) and the occupation of the embryo (double arrow) inside the egg; b) egg in salinity 12%, we observe a significant reduction in empty space (e) and consequently the development of the embryo (f), and can see the beginning of eye development in the embryo (white arrow). HE staining 40X.

DISCUSSION

The values measured of water quality parameters during the experimental period remained within the acceptable range for shrimp farming (Correia et al. 1986, Valenti 1998, New 2002, Mallasen & Valenti 2008). The water was supplied in the experimental units to maintain the volume of 2,000 mL, according to the established salinity of the treatments.

Table 1. Egg area (μm^2) of *Macrobrachium rosenbergii* at different evaluation times. *Different letters in the same column indicate significant differences.

Treatment (g L^{-1} salinity)	Evaluation time (days)				
	5	10	15	20	23
0	49063^{a}	45725^{a}	38302^{ab}	45470^{a}	36983^{a}
6	29964^{b}	41540^{b}	28555^{b}	hatched	hatched
12	45913^{a}	42245^{ab}	45150^{a}	32092^{b}	44245^{a}

Table 2. Area (μm^2) of *Macrobrachium rosenbergii* egg vacuoles at different evaluation times. *Different letters in the same column indicate significant differences.

Treatment (g L^{-1} salinity)	Evaluation time (days)			
	5	10	15	20
0	6982^{b}	13373^{a}	6198^{a}	10003^{a}
6	4011^{c}	5587^{c}	520^{c}	hatched
12	10823^{a}	7498^{b}	5281^{b}	6418^{b}

Table 3. Relationship between the area of vacuoles and the total area of *Macrobrachium rosenbergii* eggs at different evaluation times. *Different letters in the same column indicate significant differences.

Treatment (g L^{-1} salinity)	Evaluation time (days)			
	5	10	15	20
0	0.14^{b}	0.28^{a}	0.16^{a}	0.22^{a}
6	0.13^{b}	0.13^{b}	0.01^{c}	hatched
12	0.24^{a}	0.17^{b}	0.11^{b}	0.20^{a}

The significant differences in egg hatching time between treatments agree with other studies, such as those of Habashy et al. (2012), who observed a significant increase in egg diameter during the embryonic development of *M. rosenbergii*. The same study observed that *M. rosenbergii* completely developed in 20 days, but the incubation period varied from 18 to 24 days for different female sizes. It was also observed that the number of eggs and hatched larvae is directly proportional to the size of the shrimp parent (Habashy 2013).

During the development of *M. rosenbergii*, changes occur inside the egg, which can be visualized in the histological sections. In these sections, the formation of uncolored spaces can be easily perceived from the initial stages at five days (Fig. 1) to the final stage of hatching at 15 days (Fig. 3) and 20 days (Fig. 4), indicating the movement of biochemical compounds. This displacement of the yolk content occurs in the construction and supply of energy to the developing embryo. According to Clarke et al. (1990), during the stages of embryonic development, eggs have an approximate composition of 31.1 µg of protein per gram and 14.4 µg of lipids per gram after one day. In the following days, the amount of protein varied as the embryo developed, with a slightly high value maintained until day 16. At the same time, lipids decreased about day one compared to protein, presenting 9.4 µg after 16 days.

In a biochemical analysis of *M. rosenbergii* eggs, the higher levels of lipids and proteins may be the reason for the long larval stages in the embryonic development of this species (Habashy et al. 2012). It also revealed that the biochemical changes in eggs reflect changes in morphogenesis during embryonic development (Habashy et al. 2012), such as those observed in this study, and that carbohydrate is the main energy source in the early stages of embryonic development. Lipid also serves as an energy source, and protein primarily serves as a structural substance (Habashy et al. 2012). The egg retains a little yolk until hatching, which ensures the first successful molt and favors independence from the external energy resource during external feeding (Yao et al. 2006).

The histological analysis shows cell differentiation during development. It exhibits the clusters of embryonic cells that will give rise to the embryo and the organization of cells for the morphogenesis of the future shrimp. It is also possible to visualize the evidence of eye formation (Fig. 4) in a rudimentary way, corroborating the results obtained by Caceci et al. (1996).

Statistical differences were observed in the total areas of eggs from *M. rosenbergii* considering all the treatments and times evaluated. Water provides a liquid environment for the embryo and greater pressure, which allows the embryo to rupture the egg membrane in preparation for hatching (Yao et al. 2006). This indication is reinforced, considering the eggs from the 6 g L^{-1} of salinity treatment hatched faster than the others. Egg measurements were not taken from days 20 and 23 in the 6 g L^{-1} of salinity treatments, as the remaining eggs hatched between day 15 and day 20 of this treatment.

In the analysis of empty areas (vacuoles; Table 2), the embryo's greater distribution or structural organization can be observed concentrated in the embryo (Figs. 1-4). It is estimated that a good percentage of the protein in the egg has been moved for the nutrition of the embryo, mostly constituted by vitellogenin, which is a precursor of vitellin and is present in the yolk and has a fundamental role in shrimp growth (Charniaux-Cotton 1985).

As for the area of the vacuoles, from the fifth until the 20th day of incubation, the lowest value was observed in the treatment with 6 g L^{-1} of salinity, as well as, in the 6 g L^{-1} of salinity treatment, there was a lower relationship in the size of the observed spaces occupied by vitellogenin with the total size of the egg. Therefore, it was inferred that the treatment with salinity 6 g L^{-1} allowed for a greater growth response in the eggs in the same period, with hatching taking less time.

It correlates with the developmental stage and serves as an indicator of energy content (Herring 1974). Generally, species with large-sized eggs contain more yolk nutrients, and their embryonic development is longer. Habasy et al. (2012) show that the egg size of *M. rosenbergii* gradually increased during embryogenesis from 563-710 µm (narrow side) and from 682-797 µm (broad) and can be considered small, typical of *M. rosenbergii*.

CONCLUSIONS

During the embryonic development of *M. rosenbergii*, changes occur inside the egg, which can be visualized in histological sections. Already in the initial phases of 5 days, the formation of non-stained spaces can be easily seen until the final hatching phase of 15-20 days (depending on salinity), which indicates the displacement of the vitelline content in the supply of energy and embryo development. The salinity of the 6 g L^{-1} treatment allowed greater egg growth, a smaller vacuole area, and a lower ratio between egg size and vacuole area. It resulted in the start of hatching on the 15th day of the experimental period and total hatching on the 20th day at an average temperature of 28ºC. Also, it demonstrates that low salinity levels may influence the speed of vitellogenin migration for embryo formation, accelerating both the embryogenesis and the hatching of *M. rosenbergii*.

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