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Effect of salinity on embryo-larval development of yellow clam *Mesodesma mactroides* (Reeve, 1854) in laboratory

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Abstract: This study assessed the effect of salinity on embryonic development, larval growth and survival of the yellow clam *Mesodesma mactroides* in laboratory. Embryos and larvae of *M. mactroides* were submitted and maintained at four different salinities: 20, 25, 30 and 35 ppt, to determine optimal conditions for the species. Through descriptive analysis, the results showed that the embryos tolerate salinities between 25 - 35 ppt, presenting fast metamorphoses at salinities 30 and 35 ppt, during experimental period of 27 hours. The same tolerance pattern was observed in larval stage (25 - 35 ppt), showing a better development in salinity of 35 ppt. This result is verified in biometric analyzes of height and length of the shells and survival rate, with higher averages in treatments with salinity 35 ppt. The experimental period of this stage lasted 27 days, when the larvae were able to settle. These results indicate that embryos and larvae of *M. mactroides* tolerate salinities between (25-35 ppt), with the best growth and survival on high salinities being recommended to better yields in laboratory.

Key words: embryo development, growth, *M. mactroides,* salinity, survival.

INTRODUCTION

The sand mollusc *Mesodesma mactroides* (Reeve, 1854), also known as yellow clam, is a major component of intertidal communities of dissipative sandy beaches along the southwest coast of the Atlantic Ocean (Fiori et al. 2004). There are distribution reports of this species from Rio de Janeiro, Brazil, up to the south of Buenos Aires, Argentina (Rios 1994). This species is commercially exploited (Herrmann et al. 2011), having a historical value as a fishery resource (Coscarón 1959) for human feeding and fishing bait (Bastida et al. 1991). Besides, it has been considered a promising species in aquaculture due to its characteristics and socioeconomic importance (Santos et al. 2016).

In the last decades, mortality outbreaks have been affecting natural stocks of yellow clam throughout their geographic distribution (Ortega et al. 2012), which has motivated research aimed at understanding these phenomena. Studies that assess pathogen occurrence (Carvalho et al. 2013), the effects of anthropic action (Santos et al. 2016) and climate variability (Manta et al. 2017) have already been performed, although the real cause remains indefinite.

In order to relieve the pressure and increase natural populations of *M. mactroides*, handling and strategies programs for stocks improvement are being carried out (Gianelli et al. 2015), as well as defined techniques to embryonic and larval development of this species under controlled conditions in laboratory (Santos et al. 2020).

To mollusc farming development of a new species, it is important to know the biological and ecological requirements of environmental variables (Madrones-Ladja 2002, Huo et al. 2014). Salinity is an influential factor in biological activities of bivalves, directly affecting embryonic and larval development (Madrones-Ladja 2002, Huo et al. 2014, Fang et al. 2016), and is an important criterion in the selection of areas for growth (Madrones-Ladja 2002).

Salinity oscillations are common in some regions where yellow clam occurs (Odebrecht et al. 2010, Santos et al. 2016), and this may influence both development and reproduction. There is no information on the effect of salinity on embryonic and larval development of this species. Even juvenile and adult stages are classified as moderate euryhaline (Carvalho et al. 2015).

In addition, environmental tolerance studies in laboratory are essential, especially for more complex species life stages (Fang et al. 2016), considering as main reference growth rates presented to each observed salinity (Nell & Holliday 1988). This information can define optimal conditions for species survival and growth (Tan & Wong 1996) to improve seed production and eventually replenish stocks (Tan & Wong 1996, Fang et al. 2016).

Therefore, the objective of this work was to assess the effect of salinity on embryonic development, larval growth and survival of the yellow clam, *M. mactroides* in laboratory.

MATERIALS AND METHODS

Adult specimens of *M. mactroides* (N = 50) were collected in April 2017 in Rio Grande do Sul, Brazil (2 ° 3'10 "S 51 ° 59'26" W), stored in thermal boxes (without water and sand) at room temperature, and taken to the Marine Molluscs Laboratory, Laboratory at the Universidade Federal de Santa Catarina (LMM - UFSC), where the experiments were performed.

Laboratory procedures and spawning

After conditioning in laboratory (Santos et al. 2020), 10 specimens of yellow clam with a mean total length shell of 6.5 ± 4.43cm and total mean weight of 22.68 ± 4.87g were randomly at a 1:1 males to females ratio and induced to spawn using the "strip" technique (Helm et al. 2004). Female gametes (estimated at 11 x 10^6 oocytes) and male gametes were washed, sifted and mixed in filtered and UV-sterilized seawater (35ppt) (Santos et al. 2020).

Experimental design

The embryos was sifted in 18 µm mesh and randomly submitted to four salinities treatments possessing of 20, 25, 30 and 35 ppt, referring to embryonic development experiment. The remaining embryos were stocked in a 2,500L tank containing seawater (35 ppt) at a temperature of 23.4 °C. These were kept under these conditions until they metamorphosed into D-larvae. After development, D-larvae were then used for a growth and survival experiment, exposing them, as well the embryos, to treatments with salinities of 20, 25, 30 and 35 ppt. The water salinities were obtained by mixing freshwater to sea water. The determination of this parameter was performed with an optical, hand-held refractometer.

Embryonic development trial

Soon after spawning, embryos were distributed randomly in 20L containers with 15L of water and mild aeration, corresponding to the treatments of different salinities (20, 25, 30 and 35 ppt) with four replicates for each treatment.

For sample collection, the water in the containers was homogenized and then were taken by 2 mL pipettes for analysis of 10

(random) cell modifications per replicates. In the first hour, due to fast cell division rate at the beginning of the embryonic phase, samples were taken and analyzed every five minutes; every 30 minutes until the first 8 hours and then every 3 hours, according to the methodology used by Santos et al. (2020), until complete 27 hours post-fertilization. The experiment ended when the first embryos from on the treatments reached the complete metamorphosis to D-larva. In this period the temperature was analyzed presenting averages of 21.0 ± 0.9 °C, the processing time and embryos quality were analyzed by a light microscope and Sedgewick-Rafter chambers. The criteria to evaluate the quality and morphological patterns of the embryos and larvae (at the two experimental stages) was based on the study by Santos et al. (2020).

Larval Growth and Survival

The D-larvae were collected from the 2,500L tank after 24 hours of the spawning procedure. They were sifted in 35 µm mesh, calculating the density (of 5.9 x 10^6 larvae) and distributing them randomly (at 6 larvae per mL to 15L), following the same treatments as in the embryonic development trial (20L buckets with four different salinities and four replicates). Daily water renewal (100%) with the respective salinities of the treatments was carried out, followed by temperature measurements before and after handling (mean of 19.4 ± 1.4 °C) during the experimental period.

Handling and feeding

For larvae handling in the buckets were used sieves of: 35, 100 and 240 µm mesh. This sifting process simplified both cleaning of the treatments, caused by microalgae accumulation, as the samples collection to observe larval growth and survival, performed every 3 days until the end of the experiment. These collections consisted of accumulating the larvae in a 35 µm mesh sieve, thus using a 2 mL pipette, random samples were collected from each treatment for analysis of all biological material from this volume. After this stage, qualitative (morphology) and quantitative (percentages of larvae survival and growth in different treatments and replicates) analyzes were done measuring and recording images using LAS EZ Software and a light microscope. A daily feed with an algal mix composed of *Chaetoceros calcitrans*, *Isochrysis galbana* and *Pavlova lutheri* at an concentration of 2x10^4 cellsmL^{-1} (ratio: 0.67: 0.67: 0.66x10^4 cellsmL^{-1}, respectively), increasing such concentration to 3x10^4 cellsmL^{-1}, from 7th day of larvae culture (proportion of: 1: 1: 1x 10^4 cellsmL^{-1}) in the course of the experiment.

Statistical analysis

Linear regressions of *M. mactroides* larval growth exposed to different salinities were done, and their slopes were compared to determine differences in the development of biometric variables of height and length of the animals. An adjustment in the growth of the shellfish in salinity of 35 ppt to Von Bertalanffy model was also carried out in an attempt to describe the species development through a higher biologically representative asymptotic curve. Finally, to determine differences in survival, a time-repeated measure ANOVA (after determining time as a variation factor) and Tukey's post hoc analysis were used to establish differences between treatments.

For height and length statistical analyzes were tested the assumptions of data homoscedasticity and normality of residues generated before the application of parametric tests. A probability lower than 0.05 was considered as statistically significant.

RESULTS

Embryonic development

Through descriptive analyzes, as a function of time, the metamorphoses of *M. mactroides* embryos were observed under the effect of different salinities: 20, 25, 30 and 35 ppt described in Table I, totaling 27 hours until the end of this stage.

Within 18minutes of the experiment, the development of the 1st polar corpuscle had begun in all treatments, with a more advanced stage in salinities 30 and 35 ppt. In this developmental stage, fast speed patterns were observed in cell divisions embryos maintained at 30 and 35 ppt and slower for 20 and 25 ppt, (Table I). From 39 to 55 minutes, occurred the cell processing from the 1st to the 2nd polar corpuscle, in which in 20 and 25 ppt salinities, only 50% of the cells sampled were with the 2nd corpuscle, while at 46 minutes in salinities of 30 and 35 ppt, all cells already presented the 2nd corpuscle.

At 1 hour and 22 minutes in 20 ppt salinity, all embryos were with the 2nd complete polar corpuscle, as in the other salinities the embryos were in the 1st cell division process. Within 1 hour and 45 minutes the cells were in the 1st and 2nd cell division in salinities of 20 and 25 ppt, with 50% in the 1st division, while in salinities 30 and 35 ppt, 50% in the 2nd division (Table I).

In 3 hours and 20 minutes samples, embryos in salinities 20 and 25 ppt presented 4-cells divisions or more cell characteristics, in proportion that the ones in salinity 30 and 35 ppt were in advanced divisions above four divisions. This division process followed actively and in 8 hours, salinities samples of 20 and 25

Table I. Embryonic development description of *Mesodesma mactroides* in different salinities: 20, 25, 30 and 35 ppt, in the period of 27 hours. Average number of individuals per treatment = 10. Mean of water temperature (± SD), during experimental period, 21.0 ± 0.9 °C.

Stage	Time to reach the stage (min/h)			
	Salinities (ppt)			
	20	25	30	35
1st polar corpuscle	18 – 39 min	18 – 39 min	18 – 39 min	18 – 39 min
1st 2nd polar corpuscle	39 – 55 min	39 – 55 min	39 – 55min	39 – 55 min
2nd complete polar corpuscle	1h 22 min	-	46 min	46 min
1st cell division	1h 45 min	1h 22 min	1h 22 min	1h 22 min
2nd cell division	1h 45 min	1h 45 min	1hr 45 min	1h 45 min
4-cells	3h 20 min	3h 20 min	-	-
Multiple division	4h 35min	4h 35 min	3h 20 min	3h 20 min
Round and active cells - swimming	8h	8 – 14h	8 – 14h	8 – 14h
Metamorphosis for Trochophore	-	17h	17h	17h
Trochophore	-	18 – 27h	18 – 24h	18 – 24h
D-larvae	-	-	27h	27h
Round cells - Defective	18 – 24h	-	-	-
All defective	27h	-	-	-

(-): indicate absence of information concerning the processing period.

ppt were in a transition process to swimming and active larvae, which were already present in upper salinities of this experiment (30 and 35 ppt). Between 8 and 17 hours, the swimming cells present in 20 ppt salinities became more static and began morphological deformities in their transformations (sizes, shapes and irregular cell motions), when compared to the cells of other treatments, which were metamorphosing into trochophore larvae (Table I).

Between 18 and 27 hours, the defect in cell formation and transformation was continuous in 20 ppt salinity samples, with no one cell being able to metamorphose into trochophore larvae. In 25 ppt salinity, all cells became trochophore larvae, with some showing morphological and motility modifications (when compared to normal ones). In 30 ppt salinities, the transition of active trochophore larvae to well-developed D-larvae was notorious. While in 35 ppt salinity, all biological material present in the samples was metamorphosed into D-larvae, ending this experiment stage in 27 hours (Table I).

Larval growth and survival

The larval development of *M. mactroides* in different salinities, 20, 25, 30 and 35 ppt, totaled 27 days when the first treatment presented larvae with settlement characteristics (seeds).

In the first days of this stage, the larvae developed homogeneously, with a singular growth pattern until the end of the experiment: slower growth in salinities of 20 and 25 ppt when compared with specimens that maintained at 30 and 35 ppt. Morphological changes in height, length (Figure 1a-c) and larvae survival (Figure 2), began to be more apparent on the ninth experimental day, which resulted in the assessment of this information from collected samples on this date.

On 9th day, the larvae maintained at 20 ppt were smaller and had higher mortality; when compared to larvae at 25 ppt that were of heterogeneous sizes and high survival percentage sampled and with salinities of 30 and 35 ppt, bigger homogeneous sizes, respectively (Figure 1a, b, Figure 2). In 12th day sampling, it was observed that the larvae in salinity 20 ppt, did not develop. While those maintained in 25 ppt salinity presented some adaptation, demonstrating that they were developing, well-fed, and with an active velum, similar to the larvae present in 30 and 35 ppt.

In samples on 15th day, there was no survival in treatment of 20 ppt salinity, but there was size heterogeneity in larvae (alive and dead) present in samples of 25 ppt salinities; and the same pattern of growth and survival in larvae of 30 and 35 ppt (Figure1a, b, Figure2). This pattern was kept in the samples on the 18th day for 25, 30 and 35 ppt treatments.

On 21st and 24th day, an abrupt decrease in survival was observed for at 30 ppt, although the live larvae were well developed and active (pre - seeds). In the last experimental day (27th), larvae at 25 ppt had pre-seed characteristics whereas those at 35 ppt had completely metamorphosed into seeds (Figure 1 a, b, Figure 2), i.e., with complete velum loss and active feet to search for a substrate.

As the best salinity assertion to *M. mactroides* species growth, the height values of conditioned larvae to this salinity, throughout time, fit in von Bertalanffy statistical model (Figure 1c).

DISCUSSION

Embryonic development is an important phase that needs to be carefully controlled in laboratory (hatchery) to increase the process yield into veliger D-larvae (Huo et al. 2014).

The present results indicate that *M. mactroides* embryos have the best development

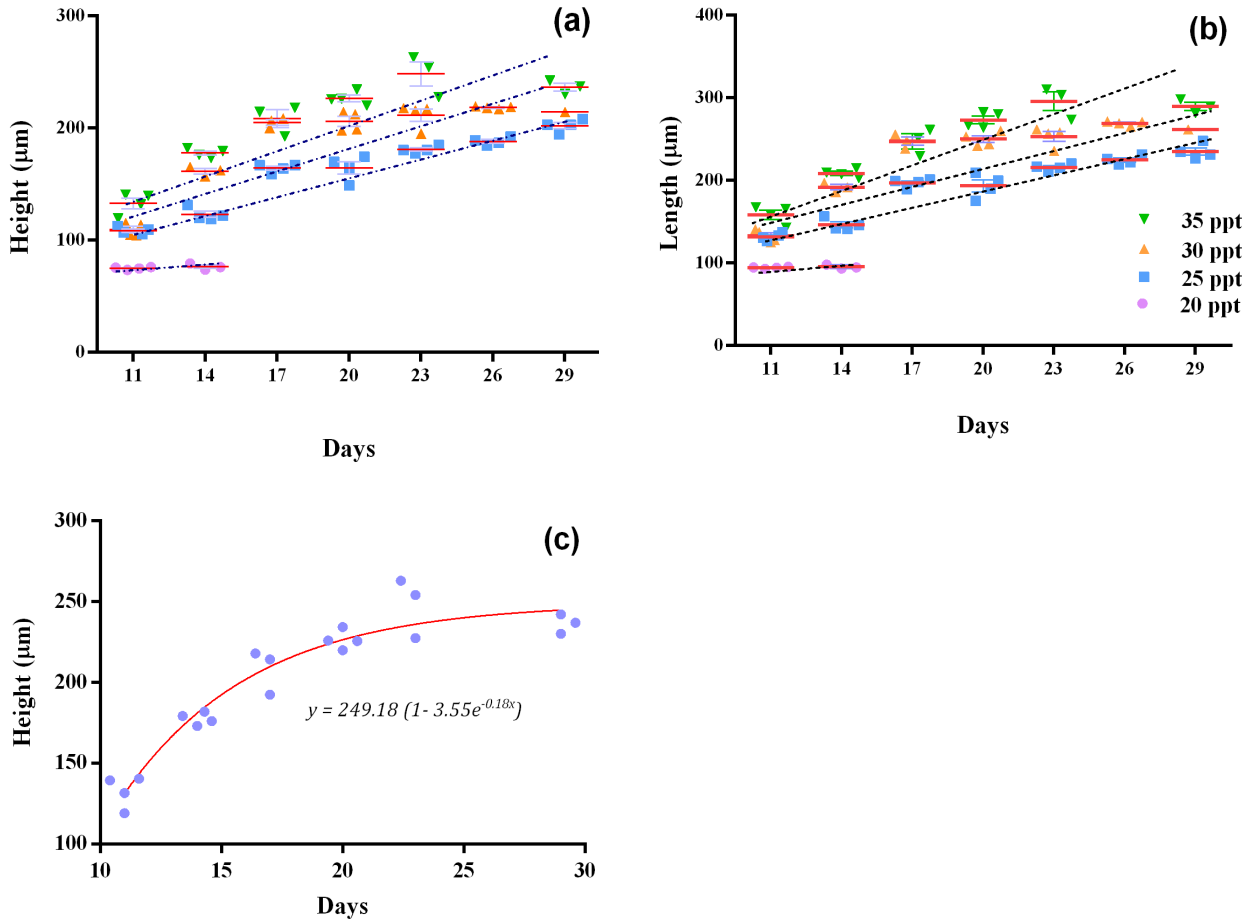

Figure 1. (a-c) - Mean height **(a)** and length **(b)** mean of *Mesodesma mactroides* larvae exposed to different salinities: 20, 25, 30 and 35 ppt in laboratory, with 27 days duration of culture. **(c)** Mean height of larvae as a function of time in a Von Bertalanffy model. Mean (± SD) temperature was 19.4 ± 1.4 °C.

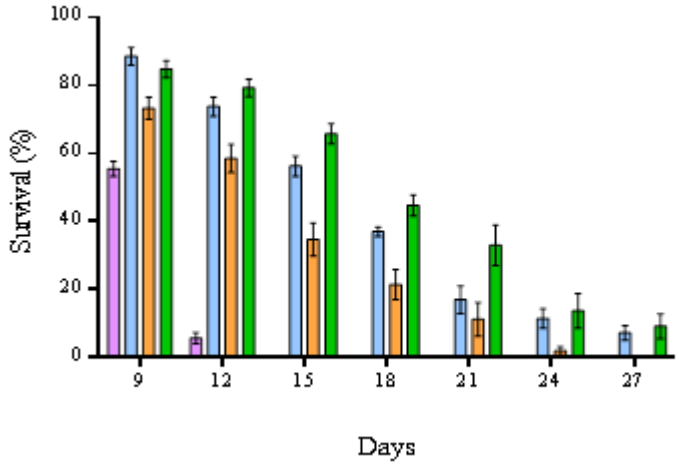

Figure 2. Survival rate (%) of *Mesodesma mactroides* larvae reared in the laboratory for 27 days and exposed to different salinities (20, 25, 30 and 35 ppt) at a mean (± SD) temperature of 19.4 ± 1.4 °C.

in veliger D-larvae in a salinity range of 30 and 35 ppt, as found by Santos et al. (2020). These described the embryonic development of this species in 24 hours period, with a salinity of 32 ppt. It was also observed tolerance of the embryos exposed to a salinity of 25 ppt, showing slower metamorphoses and some deformations, but in 20 ppt salinities all the embryos presented deformations. However, in both salinities, it was not possible to observe the complete metamorphosis for D-larvae from 27 hours of observation time (Table I).

Similar responses in terms of salinity tolerance range and optimal level for embryonic development were reported for *Crassostrea rhizophorae* (Dos Santos & Nascimento 1985), *Placuna placenta* (Madrones-Ladja 2002) and *Panopea japonica* (Huo et al. 2017) (Table II). In other studies with species of the genus *Crassostrea*, the bivalves presented more estuarine characteristics, since the embryos can tolerate and develop at lower salinities, such as *Crassostrea belcheri* (Tan & Wong 1996), *Crassostrea hongkongensis* (Huo et al. 2014) and *Crassostrea iredalei* (Fang et al. 2016) (Table II). According to Davis (1958), an important aspect to determine the tolerance of embryos is the salinity range in which the breeding and the spawning site are conditioned. The yellow clam breeding used in this research were collected from the environment and acclimatized in laboratory at the same salinity of 35 ppt (Santos et al. 2020), characterizing an optimum salinity for adults of this species (Carvalho et al. 2015), but more studies are necessary to understand if the conditioning effect also applies to embryos of these animals. These findings provide background information for improving the production and yield of *M. mactroides* D-larvae in laboratory.

In the present study, the larval development of *M. mactroides*, characterized by the metamorphosis of veliger D-larvae in pediveliger, showed that different salinities had effects on survival and growth of this species. The results revealed that the yellow clam larvae were able to tolerate salinities between 25 - 35 ppt. On the other hand, the larvae metamorphosis to seed was completed between 30 and 35 ppt. With a faster development in 35 ppt, as well as higher

Table II. Optimum salinities and tolerance limit for the development of eggs to normal D-larvae of some bivalve species.

Species	Salinity (ppt)		References
	Tolerance limit	Optimum	
Mesodesma mactroides	25-35	30-35	This study
Crassostrea rhizophorae	16-40	25-37	Dos Santos & Nascimento 1985
Crassostrea belcheri	12-30	24-30	Tan & Wong 1996
Placuna placenta	22-34	22-34	Madrones-Ladja 2002
Crassostrea hongkongensis	15-30	15-23	Huo et al. 2014
Crassostrea iredalei	10-30	15-30	Fang et al. 2016
Panopea japonica	25-32	32	Huo et al. 2017

growth and survival during the experimental period of 27 days (Figure 1a-c, Figure 2).

There was also, in the beginning, expressive mortality in treatment with the salinity of 20 ppt (Figure 2), suggesting that these larvae are sensitive to low salinities. While the mortalities at the end of the experimental period in treatments with salinity 30 ppt may be related to the larvae ready to settle, although unsuccessful in substrate absence, magnified by saline stress caused by a lower salinity than the optimum (personal observation).

In principle, the mechanisms under the effect of salinity on mollusc larvae are not fully clarified yet (Huo et al. 2014, 2017, Wang et al. 2018), conversely these results agree with previous studies that when the larvae are exposed to extreme levels of tolerance, results in slower growth or may affect the survival rate (Madrones-Ladja 2002, Huo et al. 2017, Wang et al. 2018). As well as other marine bivalves, the yellow clam is an osmoconformer (Lopes et al. 2011). Wang et al. (2018) observed as a defense mechanism the shells closure of *Crassostrea nippona* larvae when kept in stressful salinities of 14 and 34 ppt, while, in present study, 20 and 25 ppt were considered to be stressfulto *M. mactroides*. This mechanism tends to generate energy expenditure in osmoregulatory processes, interfering in food intake and growth (Huo et al. 2017) and consequently in survival. The slower growth in this study was observed at salinity 25 ppt, because the larvae with lower growth (compared to treatments 30 and 35 ppt) were able to adapt and survive in these conditions until the end of the experiment.

Despite the different effects of salinity on bivalve species, studies show that even larvae with the ability to tolerate variations, exhibit higher growth and survival at optimal salinity (Huo et al. 2014). This also supports an observation reported by Tan & Wong (1996), when they claim that most hatcheries are located near to the marine environment, where seawater is collected to larvae culture processing (high salinities), which may be may be inappropriate for the production of desired species. Table III summarizes the range of tolerant and optimal salinity, in addition to the best larvae survival range of some bivalve species.

Salinity variations are commonplace in some areas where yellow clams are naturally found, with records of 14 to 38 ppt (Odebrecht et al. 2010). Santos et al. (2016) related that the possible decrease of natural stocks of this species are associate to anthropic actions or are the influence of environmental variables such as salinity and temperature, since the most

Table III. Optimum salinity levels and tolerance limit for larval growth and survival of *Mesodesma mactroides* and other bivalve species.

Species	Salinity (ppt)			References
	Tolerance limit	Optimum		
		Growth	Survival	
Mesodesma mactroides	25-35	35	35	This tudy
Crassostrea belcheri	12-30	12-24	12-24	Tan & Wong 1996
Placuna placenta	16-34	16-34	22-34	Madrones-Ladja 2002
Panopea japonica	25-32	32	32	Huo et al. 2017

affected regions in their study were near Laguna dos Patos (32 ° 09'S, 52 ° 06'W) mouth, which bring on changes in immunological system of the animals. In this premise, the present study shows that the embryos and larvae of this species do not tolerate salinities below 20 ppt, demonstrating that may be the main factor for the reproductive success of this species in these regions.

Studies proved that salinity may also interact directly with other physicochemical factors e.g. in temperature tolerance of bivalve larvae (Davis & Calabrese 1964, Cain 1973). Thus, it is relevant to investigate, in future studies, the effect of this variable on embryonic and larval development of *M. mactroides* species in laboratory.

CONCLUSION

The results in this study showed that embryos and larvae of *M. mactroides* larvae can tolerate a salinity range between 25 and 35 ppt. However, the individuals presented greater growth and survival in salinities of 35 ppt. The use of salinity range in embryonic and larval development should allow the seed production of this species in captivity.

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Juan Jethro Silva Santos performed the animal collection and all the experimental steps, as well, prepared figures and tables and wrote the paper. Juliana Portella Bernardes participated in the experimental stages in laboratory, morphological analysis and manuscript writing. Juan Rafael Buitrago Ramírez conducted the statistic, data analysis and reviewed drafts of the paper. Carlos Henrique Araujo de Miranda Gomes (co-advisor), participated and monitored all the experimental stages, contributing also to the writing of the manuscript. Luis Alberto Romano conducted the orientation of the activities, as well as contributed to the writing of the final manuscript. All authors revised the manuscript and approved the final version.