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The salt tolerance of the freshwater snail *Melanoides tuberculata* (Mollusca, Gastropoda), a bioinvader gastropod

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Abstract: *Melanoides tuberculata* (Müller, 1774) (Gastropoda: Thiaridae) is a freshwater gastropod native to Africa and Asia. It is a bioinvader of remarkable ecological capabilities presenting euryoic and highly adaptable to eutrophic conditions, *M. tuberculata* has also been found in estuarine environments. The first occurrence of the species in South America was reported from Brazil, in the late 60's. The current literature documents a broader distribution of *M. tuberculata* in the rivers and reservoirs of the Brazilian north and northeast as well as in the Brazilian middle-west. The aim of this study is to analyze the salt tolerance of *M. tuberculata*, comparing the effects of salinity variation on adults and juveniles collected from a eutrophic lentic system in Bahia State (Brazil). Survival tests based on salinity exposure shows that the 50% survival salt concentration (salt LC50) for adults is 22.82‰ (CI= 20.46‰-25.19‰) and that the LC50 for juveniles was 21.56‰ (CI= 20.06‰-23.07‰). Activity tests show that the snails tested are motionless at salt concentrations of 30‰ or greater. This study provides new empirical information on the population characteristics of *M. tuberculata* in Brazil and also contributes to the understanding of physiological stress, ecological capabilities and dispersal strategies in bioinvader species.

Keywords:Thiaridae, euriotic organism, bioinvader, population behavior, salinity

Resumo: Tolerância à salinidade do caramujo de água doce *Melanoides tuberculata* (Müller, 1774) (Mollusca, Gastropoda), um gastrópode bioinvasor. *Melanoides tuberculata* (Müller, 1774) (Gastropoda: Thiaridae) é um gastropode nativo da África e Ásia. É um bioinvasor com importantes capacidades ecológicas, sendo eurióticos e apresentando alta capacidade de adaptação à condições eutróficas, *M. tuberculata* têm sido também encontrado em ambientes estuarinos. A primeira ocorrência dessa espécie na América do Sul foi reportada para o Brasil, no final da década de 60. A bibliografia atual relata uma ampla distribuição de *M. tuberculata* em rios e reservatórios do norte e nordeste do Brasil, assim como no centro-oeste. O objetivo do presente estudo foi avaliar a tolerância de *M. tuberculata* à salinidade, comparando os efeitos da variação salina em adultos e juvenis coletados em ambientes lênticos eutrofizados na Bahia (Brasil). Testes de sobrevivência baseado na exposição à salinidade mostram 50% de sobrevivência (Salinidade LC50) para adultos é 22.82‰ (CI= 20.46‰-25.19‰) e que o LC50 para os juvenis foi de 21.56‰ (CI= 20.06‰-23.07‰). Os teste de atividade mostram que os caramujos ficam imóveis em salinidades de 30‰ ou superior. Este estudo fornece novas informações empíricas sobre as características populacionais de *M. tuberculata* no Brasil, assim como contribui com o entendimento do estresse fisiológico, das capacidades ecológicas e das estratégias de dispersão de espécies bioinvasoras.

Palavras chave: Thiaridae, organism euriótico, bioinvasão, comportamento populacional, salinidade.

Introduction

The introduction of exotic species can occur naturally or can result from human activity. Exotic species can be assimilated into the ecological community without noticeable effects or can be directly responsible for drastic changes that may permanently affect native species (Townsend *et al.* 2010). In fact, bioinvasion is the second most important factor influencing the decline of the world's biodiversity, surpassed only by habitat degradation (Cain *et al.* 2011).

Melanoides tuberculata (Müller, 1774) is a freshwater gastropod native to northeastern Africa and Southeast Asia (Gutiérrez Gregoric *et al.* 2007). It has been monitored worldwide due to its ability as bioinvasor and fast dispersion (Bogea *et al.* 2005, Bolaji *et al.* 2011). It is a bioinvader of remarkable ecological capabilities and is also the host for certain trematode parasites (Vaz *et al*. 1986). Euryoic and highly adaptable to eutrophic waters, *M. tuberculata* has also been found in estuarine environments (Bolaji *et al*. 2011). The ability of the species to tolerate variations in environmental conditions is noteworthy. Additionally, the females of *M. tuberculata* can reproduce parthenogenetically, supporting high local population densities (up to 17,000 individuals per m²) (Jesus *et al.* 2007).

Intentionally or accidentally, human activities may promote the introduction of exotic organisms. On the island of Martinique (Caribbean), for example, *M. tuberculata* was introduced in the 80's (Pointier 2001) to control the populations of *Biomphalaria glabrata* (Say, 1818) and *Biomphalaria straminea* (Dunker, 1848) (Gastropoda, Planorbidae), intermediate hosts of *Schistosoma mansoni* (Sambon, 1907). Unexpectedly, in addition to dominating the *Biomphalaria* species, the introduced thiarid became a new vector of schistosomiasis. The literature provides continuing warnings of the risks posed by *M. tuberculata* to human and animal health. In Brazil, it has been associated with the life cycle of *Clonorchis sinensis* (Looss, 1907) (the Chinese liver fluke) and *Philophthalmus gralli* (Mathis & Leger, 1910) (the Oriental avian eye fluke) (Vaz *et al.* 1986, Pinto & Melo 2010).

In South America, this exotic snail was first recorded in Brazil in the late 60's. The ornamental plant and fish trade in São Paulo State was hypothesized to be the source of the introduction (Vaz *et al.* 1986). Currently, the snail has spread northward and southward through major hydrographic basins, extending from inland reservoirs to lentic and lotic coastal systems and colonizing streams in an insular environment (Gutiérrez Gregoric *et al.* 2007, Santos & Eskinazi-Sant'Anna 2010, Souto *et al*. 2011).

Under experimental conditions, *M. tuberculata* is known to survive in extreme salinities, up to 45‰ (Bolaji *et al.* 2011). In view of these observations, it has been suggested that dispersal may occur through estuaries and may also involve drifting in the sea for brief periods. Accordingly, this study aims to analyze the salt tolerance of *M. tuberculata*, comparing the effects of salinity on adults and juveniles. The results are expected to provide further understanding of the population characteristics of *M. tuberculata* maintained experimentally under physiological stress. The study will also contribute to the understanding of the ecological capabilities and dispersal strategies of bioinvader species.

Materials and methods

Sampling site: The Parque Metropolitano de Pituaçu (12º06'24"S/38º24'22"W and 12º57'47"S/38º27'07"W) is located in the metropolitan area of Salvador City (Bahia State, Brazil). It was established on 4 September 1973 (State Decree nº 23.666), and recognized as a Conservation Unit (UC) in 1977 (Municipal Decree nº 5158) (Góes-Neto *et al.* 2012). Despite its ecological importance, the Parque Metropolitano de Pituaçu has been under continuous anthropogenic pressure from the dumping of sewage into the lagoon, illegal fishing activities, degradation of the riparian vegetation and introduction of wild animals (Oliveira-Alves *et al*. 2005).

Specimen sampling and storage: Samples were collected during the spring on 5 September and 12 November 2012. Specimens of *M. tuberculata* were collected adjacent to the riparian vegetation along the border of the lagoon with a hand net (23 x 17 cm, 1.0 mm mesh). Individuals were generally found buried in the sediment or sheltered under plants. Additionally, adults and juveniles were also observed attached to and feeding on almond leaves (*Terminalia catappa* L.). *In situ* water was collected for salt addition and animal maintenance. In the laboratory, the specimens were maintained in plastic containers with fragments of almond leaf for a one-week acclimatization period (synchronized to a 12h light- 12h dark cycle). The water was changed and additional leaves supplied at 48 h intervals.

Morphometrics; The analyses focused on two distinct topics: the effects of increasing salt concentrations on 1) survival and 2) mobility. In both cases, it was recognized that the level of salt tolerance could vary among developmental stages. Following Okumura (2006), adults and juveniles were defined primarily in terms of the shell size: less than 10 mm in juveniles, 10 mm or more in adults. As the protoconch of the adult shell was often broken off, a Pearson correlation was calculated to determine whether the length of the opercular aperture varied in proportion to the overall shell size. The analysis was performed with GraphPad InStat software**.** Morphometric data were collected using a stereomicroscope (NIKON, model SMZ1000) supplied with a calibrated eyepiece and a digital camera (NIKON, model COOLPIX 995).

Survival tests: It was performed chronicle test over a period of one week (168 h). The snails were maintained in a germination chamber at 22ºC with a 12 h photoperiod. A methodology adapted from Bolaji *et al.* (2011) was used to test the tolerance of the organisms to various salt concentrations. After acclimatization, 500 individuals from the first sampling (total n= 986) were selected. Of these, 250 were considered juveniles and 250 adults. The individuals were separated into four treatment groups and a control group. Four different salt concentrations were used as treatments: 35‰, 27‰, 18‰ and 9.0‰, resulting from diluting natural seawater (35‰) in freshwater from Pituaçu's lagoon (Table 1). A control group was kept at 0‰. The salinity values were obtained with a manual refractometer (Mytutoyo Model RTS-101 ATC). Five replicates with ten individuals per container were tested for each concentration. Once a day, always at the same time, the survivors were counted. Individuals were considered to be alive if they moved within the containers, were attached to the walls or reacted to the touch of a needle. To confirm the number of survivors, the gastropods were removed from the mixed water and transferred into containers with ordinary freshwater from Pituaçu's lagoon. This protocol was adopted because it was observed that all individuals from the 35‰ salinity treatment remained motionless/inert during the counting procedure, compromising its accuracy. To calculate the 50% lethal concentration (LC50), a probit regression was performed using Statistica Software. To confirm the statistical significance of the values, an analysis of variance (ANOVA) and Tukey-Kramer multiple comparison test were performed with GraphPad InStat software.

Table I. Ratios of seawater dilution for distinct salt concentrations used in survival tests.

SAMPLE	SALINITY	RATIO	
		SEAWATER	FRESHWATER
A	35‰	100% (200 ml)	0% (0 ml)
B	27‰	75% (150 ml)	25% (50 ml)
C	18‰	50% (100 ml)	50% (100 ml)
D	9‰	25% (50 ml)	75% (150 ml)
E	0‰	0% (0 ml)	100% (200 ml)

Activity tests: The experiment was performed over a 24 h period. The individuals were maintained in a germination chamber at 22ºC and a 12 h photoperiod. After acclimatization, 500 individuals from the second sampling (total n= 1,223) were selected. These individuals were selected to furnish 250 juveniles and 250 adults, values identical to those used in the survival test. The individuals were subjected to four different salt concentrations (with values that differed from those used in the previous experiment in order to identificate the salinity in which individuals stay active): 33‰, 30‰, 26‰ and 21‰. Additionally, a control group was maintained at 0‰ salinity (Table 2). These salinity values were selected after it was found that individuals maintained at 35‰ were inactive until they were transferred to freshwater. Therefore, natural seawater (35‰) was gradually diluted by adding 20 ml (10% of total volume) of freshwater from Pituaçu's lagoon. The counting protocols followed the criteria adopted in the survival tests. To confirm the statistical significance of the values, an analysis of variance (ANOVA) and a Tukey-Kramer multiple comparison test were performed with GraphPad InStat software.

Table II. Ratios of seawater dilution for distinct salt concentrations used in activity tests.

SAMPLE	SALINITY	RATIO	
		SEAWATER	FRESHWATER
F	33‰	90% (180 ml)	10% (20 ml)
G	30‰	80% (160 ml)	20% (40 ml)
H	26‰	70% (140 ml)	30% (60 ml)
I	21‰	60% (120 ml)	40% (80 ml)
J	0‰	0% (0 ml)	100% (200 ml)

Results

A correlation analysis confirmed that the total length grows in proportion to the opercular aperture ($r = 0.9650$, $P < 0.0001$). The opercular aperture is a reliable morphometric trait for distinguishing the adults from the juveniles in *M. tuberculata* (Figure 1). For the adults, the mean size of the opercular aperture was 4.34 mm (SD=1.26; Min. = 2.72 and Max. = 9.6), and the total length was 13.38 mm (SD = 3.65; Min. = 10.08 and Max. = 25.92). For the juveniles, the mean size of the opercular aperture was 2.24 mm (SD = 0.47; Min. = 0.8 and Max. = 3.2), and the total length was 7.3 mm (SD = 1.46; Min. = 2.56 and Max. = 9.76).

During the acclimatization of the specimens for the survival tests, 127 deaths were observed (out of 986 organisms). After 96 h of exposure, the LC50 of the adults was 22.82‰ (CI= 20.46‰-25.19‰), whereas the LC50 of the juveniles was 21.56‰ (CI= 20.06‰-23.07‰). The period of 96 h was specified as the exposure duration for an acute toxicity test, but the observations extended to 168 h. The daily survival data are plotted in Figures 2A and B.

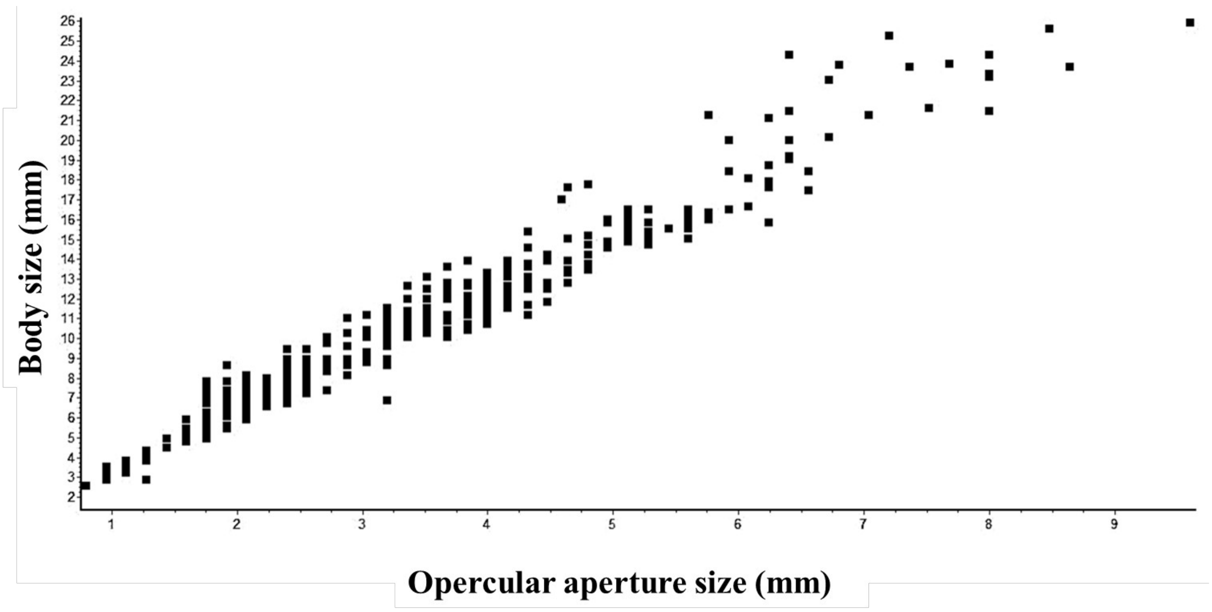

Figure 1. Scatterplot (total length vs. opercular aperture size). Positive correlation (r = 0.9650).

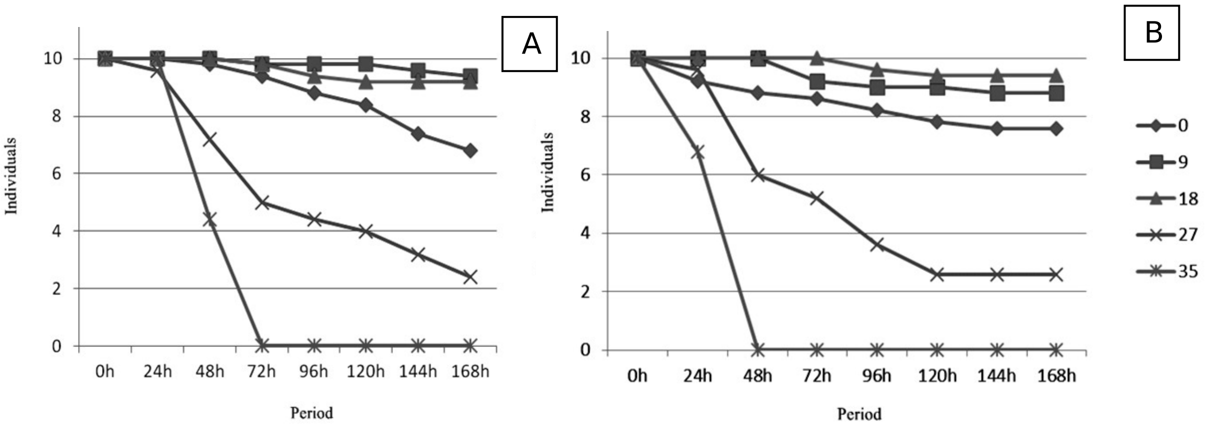

Figure 2. Average daily survival for adults (A) and juviniles (B). Lines represent salinity (‰).

The analysis of variance (ANOVA) performed to compare survival rates between distinct salinity concentrations showed that the salt tolerance of the adults and juveniles differed (P <0.0001) (Tables 3 and 4). Similarly, the Tukey-Kramer test (using pairwise comparisons) showed that the pairwise differences among the samples were statistically significant (P< 0.001). Based on the majority of the pairwise comparisons, the number of survivors was variable, especially at the highest salt concentrations (27‰ and/or 35‰) (Tables 5 and 6).

During the analysis of the survival tests, it was observed that individuals exposed to 35‰ (i.e., seawater) remained completely inert. Based on this observation, the individuals were considered dead, but this interpretation was questionable. To obtain valid observations of mortality and to ensure the accuracy of the tests, the samples were transferred into a container holding only pond water. After approximately 30 min, healthy individuals started extending their bodies from the shell and reacting to the touch of a needle. This behavior was also conspicuous during the activity tests. Adults and juveniles remained motionless when exposed to new salinities, particularly when they were immersed in the 33‰ and 30‰ concentrations. However, no measurable differences in activity were observed at the other salinities (26‰ and 21‰); these individuals were proportionally active in lower salt concentrations as well as in pond water from the capture site, with higher activity for adults in the 33‰ or 30‰ salinity concentration (Figures 3A and B). A Tukey-Kramer test showed that the activity values at the two higher salt concentrations did not differ significantly. Both salinities produced the same result by inhibiting the activity of 100% of the individuals. However, if the 33‰ or 30‰ concentration was compared against all other samples, the difference was significant (P<0.001), showing that the highest concentrations most likely affected the activity of the organisms (Tables 7 and 8).

Table III. Survival test for adult individuals of *M. tuberculata* under different salinity concentrations. Survival rate per replicate (R1, R2, R3, R4 and R5) and total.

Germination Chamber Temperature: 22ºC Period: 96h							
						Survival Rate	
Concentration (‰)	R1	R2	R3	R4	R5	Total	%
35	0	0	0	0	0	0	0
27	2	3	2	4	1	12	24
18	7	10	9	10	10	46	92
9	8	10	10	10	9	47	94
Control (0)	8	9	5	4	8	34	68

Table IV. Survival test for juvenile individuals of *M. tuberculata* under different salinity concentrations. Survival rate per replicate (R1, R2, R3, R4 and R5) and total.

Germination Chamber Temperature: 22ºC Period: 96h							
						Survival Rate	
Concentration (‰)	R1	R2	R3	R4	R5	Total	%
35	0	0	0	0	0	0	0
27	6	3	2	1	1	13	26
18	8	10	9	10	10	47	94
9	10	9	7	9	9	44	88
Control (0)	5	8	8	9	8	38	76

Discussion

Body size is a useful parameter in the study of ecology and structure population; additionally, according to Rocha (1983), body size is a fundamental property of the organism, providing clues to the nature of physiological processes, primarily during environmental toxic stress. In this study, the body length (represented by the shell size) was identified as an important parameter for separating developmental stages and served to define two population categories: adults and juveniles. Based on Okumura (2006), who found that the size at sexual maturation of *M. tuberculata* in Brazil is approximately 9.97-10.28 mm, it was assumed that a shell length as great as 10.00 mm would be used as the criterion for separating adults from juveniles. Similar values have been found for populations in Hong Kong (10.8-11.5 mm *in* Dudgeon 1989) and Malaysia (8.3-9.5 mm *in* Berry & Kadri 1974). These differences are most likely due to genetic variability and environmental constraints or to differences among the measurement methods used.

As a result of the tendency of the snails to bury themselves in the sediment, the protoconch may be severely eroded. This feature may bias the morphometric analysis of the shell. Bolaji *et al.* (2011) performed a correlation analysis of the number of whorl occurring on the shell. According to these authors, juveniles would have three whorls, whereas adults would have eight to eleven whorls, on average. In fact, a Pearson test identified a significant correlation between the shell length and the number of whorls (r^2 = 0.625, $P < 0.01$). However, depending on the extent of damage on the distal protoconch, older whorls may also be affected. Thus, the reliability of the whorl counts for morphometry is not clear. Alternatively, in the present study, a Pearson test demonstrated a strong positive correlation (r^2 = 0.9650, $P < 0.0001$) between the shell length and the opercular aperture. Of these two characters, the latter is assumed to be more reliable for aging.

The distribution and abundance of organisms depend on a range of factors such as temperature, humidity and luminosity. According to Maia *et al.* (2011), the distribution of *Melampus coffeus* (Linnaeus, 1758), a pulmonate gastropod of the family Ellobiidae typically found in estuarine regions, is primarily determined by salinity, sediment characteristics and desiccation stress (alternating time of exposure and submersion). Data from Kock & Wolmarans (2009) verify that *M. tuberculata* attains greater densities in long-lived stagnant water bodies with temperatures ranging from 21°C to 25°C and in muddy or sandy substrates. Abílio *et al.* (2006) noted that changes in water quality (e.g., due to rainfall) could affect the population densities of *M. tuberculata*. According to Araújo *et al*. (2003), the greatest rainfall period at the Parque Metropolitano de Pituaçu occurs between March and July. The samples for the present study were collected between September and November, a period of lower rainfall. A temporal analysis could provide important information on the population fluctuations of *M. tuberculata* during the year, additionally indicating probable changes in densities and age structure. Compared with previous findings, the results of this study show no evident discrepancies in the value of LC50. For example, the tolerance response found in adults was closer to that found by authors that did not verified the age of individuals (Bolaji *et al*. 2011, Silva & Barros 2015) than was for juveniles, that presented lower survivorship., who did not age the individuals. Field and laboratory observations have demonstrated the ability of *M. tuberculata* to retract into the shell and seal the opercular aperture, floating on the water due to surface tension (Fell 2006). Gastropod retraction and shell enclosure commonly occur in response to a variety of disruptive factors (e.g., predators, desiccation, changes in water quality, starvation). These responses improve survival and contribute to the overall ecological competence of the organism. However, whether floating can be used to move from one estuary to another remains unclear.

Table V. Tukey-Kramer multiple comparison test for samples of adult individuals of *M. tuberculata* tested for survival. Sample A (35‰), Sample B (27‰), Sample C (18‰), Sample D (9‰) and Sample E (0‰). *** = significant, ns = not significant.

Comparison	Mean Diference	Statistical significance	P value
Sample A vs Sample B	-4.400	***	$P < 0.001$
Sample A vs Sample C	-9.400	***	$P < 0.001$
Sample A vs Sample D	-9.800	***	$P < 0.001$
Sample A vs Sample E	-8.800	***	$P < 0.001$
Sample B vs Sample C	-5.000	***	$P < 0.001$
Sample B vs Sample D	-5.400	***	$P < 0.001$
Sample B vs Sample E	-4.400	***	$P < 0.001$
Sample C vs Sample D	-0.4000	ns	$P > 0.05$
Sample C vs Sample E	0.6000	ns	$P > 0.05$
Sample D vs Sample E	1.000	ns	$P > 0.05$

Table VI. Tukey-Kramer multiple comparison test for samples of juvenile individuals of *M. tuberculata* tested for survival. Sample A (35‰), Sample B (27‰), Sample C (18‰), Sample D (9‰) and Sample E (0‰). *** = significant, ns = not significant.

Comparison	Diferença Média	Statistical significance	P value
Sample A vs Sample B	-3.600	***	$P < 0.01$
Sample A vs Sample C	-9.600	***	$P < 0.001$
Sample A vs Sample D	-9.000	***	$P < 0.001$
Sample A vs Sample E	-8.200	***	$P < 0.001$
Sample B vs Sample C	-6.000	***	$P < 0.001$
Sample B vs Sample D	-5.400	***	$P < 0.001$
Sample B vs Sample E	-4.600	***	$P < 0.001$
Sample C vs Sample D	0.6000	ns	$P > 0.05$
Sample C vs Sample E	1.400	ns	$P > 0.05$
Sample D vs Sample E	0.8000	ns	$P > 0.05$

The natural dispersal of the snail over all major Brazilian basins has not been inferred. The expansion of the snail's distribution has been consistently and circumstantially attributed to human activity. In fact, the activity tests showed that at salinities of 30‰ and above, the mobility of all individuals was reduced by not inhibited, especially in adults. Thus, the ability to retract into the shell and adhere to the surface of the water could represent an important survival strategy for overcoming stressful environments – for example, avoiding unfavorable salinities in an estuarine system. This could be confirmed by Silva & Barros (2015), they results indicate the ability of *M. tuberculata* to adapt to euryhaline conditions.

In our study, the survivorship of *M. tuberculata* in salinity 0 was lower than in treatment with 9‰ and 18‰ salinity concentrations, opposite to what was expected. We believe that due to the use of water from the place where snails were collected, a polluted lake, the laboratory conditions could beneficiate microorganisms in the water that could became harmful for *M. tuberculata.* In treatments where salinity was increased, these harmful microorganisms could not proliferate and influence the survivorship of *M. tuberculata*. In this way, future evaluations should investigate also microbiological activity.

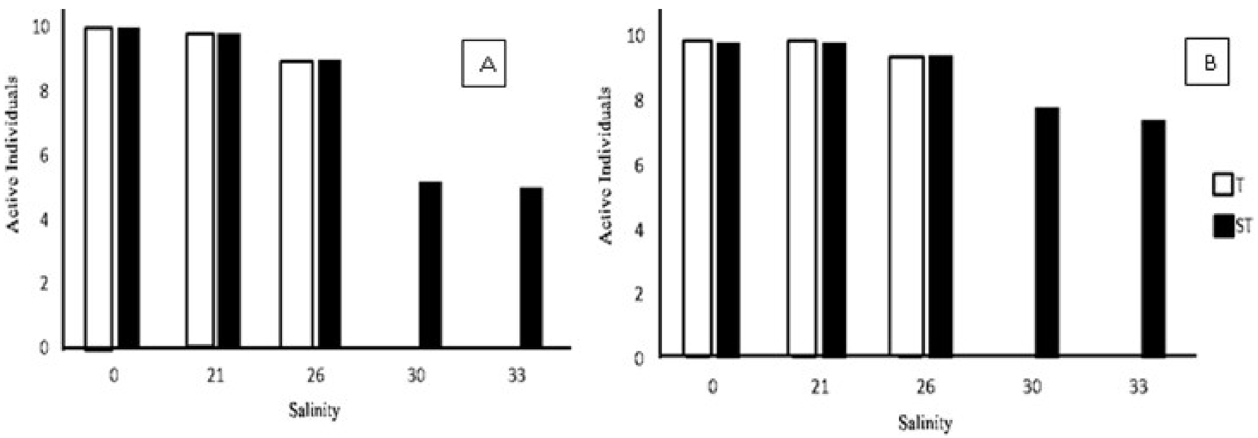

Figure 3. Activity tests for juveniles (A) and adults (B) (T = under salinity influence, ST = only fresh water).

Table VII. Tukey-Kramer multiple comparison test for samples of adult individuals of *M. tuberculata* tested for activity. Sample F (33‰), Sample G (30‰), Sample H (26‰), Sample I (21‰) and Sample J (0‰). *** = significant, ns = not significant.

Comparison	Mean Difference	Statistical Significance	P Value
Sample F vs Sample G	0.000	ns	P>0.05
Sample F vs Sample H	-9.400	***	P < 0.001
Sample F vs Sample I	-9.800	***	P < 0.001
Sample F vs Sample J	-9.800	***	P < 0.001
Sample G vs Sample H	-9.400	***	P < 0.001
Sample G vs Sample I	-9.800	***	P < 0.001
Sample G vs Sample J	-9.800	***	P < 0.001
Sample H vs Sample I	-0.4000	ns	P > 0.05
Sample H vs Sample J	-0.4000	ns	P > 0.05
Sample I vs Sample J	0.000	ns	P > 0.05

Given that the individuals collected and analyzed in the present work came from freshwater environments, it is probable that different results would be found with individuals collected from estuaries and, most likely, exposed to greater variations in salinity. For this reason, further modifications of the protocols used to evaluate the influence of salinity on physiology and adaptive behavior should reveal the mechanisms that have allowed the bioinvader *M. tuberculata* to spread successfully worldwide.

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Table VIII. Tukey-Kramer multiple comparison test for samples of juvenile individuals of *M. tuberculata* tested for activity. Sample F (33‰), Sample G (30‰), Sample H (26‰), Sample I (21‰) and Sample J (0‰). *** = significant, ns = not significant.

Comparison	Mean Difference	Statistical Significance	P Value
Sample F vs Sample G	0.000	ns	P > 0.05
Sample F vs Sample H	-9.000	***	P < 0.001
Sample F vs Sample I	-9.800	***	P < 0.001
Sample F vs Sample J	-10.000	***	P < 0.001
Sample G vs Sample H	-9.000	***	P < 0.001
Sample G vs Sample I	-9.800	***	P < 0.001
Sample G vs Sample J	-10.000	***	P < 0.001
Sample H vs Sample I	-0.8000	ns	P > 0.05
Sample H vs Sample J	-1.000	ns	P > 0.05
Sample I vs Sample J	-0.2000	ns	P > 0.05

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