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INSIGHTS INTO THE RESPONSE OF *Crassostrea rhizophorae* TO SALINITY STRESS: ENZYMATIC ACTIVITY AND ALLELIC FREQUENCIES FOR LEUCINE AMINOPEPTIDASE

Mauro Nirchio Tursellino[1,2*] & Ivette Arocha Vegas[1]

[1] *Escuela de Ciencias Aplicadas del Mar*, *Núcleo de Nueva Esparta*, *Universidad de Oriente*, *Porlamar*, *Isla de Margarita*, *Venezuela*
**autor de correspondencia*: *mauro.nirchio@gmail.com*. *https://orcid.org/0000-0001-7171-243*

[2] *Universidad Técnica de Machala*, *Av*. *Panamericana km 5.5*, *Vía Pasaje*, *Machala*, *El Oro*, *Ecuador*
ivettearochavegas@gmail.com. *https://orcid.org/0000-0002-6176-6259*

ABSTRACT: The effect of extreme salinities on survival, body water percentage, condition index, leucine aminopeptidase-I (LAP) enzyme activity and allelic frequencies at the LAP-2* and LAP-3* loci of *C. rhizophorae*, were studied. Sudden exposure caused 46.5% mortality at 5 PSU and 45.25% at 60 PSU after 96 hours. The highest values of body water were 82.18±0.16% at 5 PSU followed by 77.28±0.03% at 38 PSU and 74.25±0.10% at 60 PSU. The highest average value of enzymatic activity (U/mL of extract) was recorded at 60 PSU (576.160±33.135), the intermediate value at 5 PSU (432.178±26.705) and the lowest value at 38 PSU (364.146±16.115). Comparison of allelic frequencies among the surviving oysters at the three salinities tested revealed a significant dependence on salinity ($p < 0.05$) for both LAP-2* and LAP-3*. For LAP-2*, the frequency of allele 102 was significantly represented in extreme salinities, contributing 82.36% of the difference to the total value of χ^2, while for LAP-3* a proportional increase in the frequency of allele 104 and the decrease in the frequency of allele 100 was evidenced at 60 PSU and the contributions to the total value of χ^2 were 35.24% and 55.65%, respectively. The results provide important support for the hypothesis that LAP variability would be the result of the selective effect of environmental salinity in this mollusk and expand the evidence on the role of this enzyme to face salinity stress in marine bivalves.

Keywords: leucine aminopeptidase, oysters, cell volume regulation, adaptation, allele frequency

RESUMEN: Se estudió el efecto de salinidades extremas en la supervivencia, el porcentaje de agua corporal, el índice de condición, la actividad de la enzima leucil aminopeptidasa-I (LAP) y las frecuencias alélicas para los loci LAP-2* y LAP-3* de *C. rhizophorae*. La exposición repentina causó un 46,5% de mortalidad a 5 UPS y un 45,25% a 60 UPS, después de 96 horas. Los valores más altos de agua corporal fueron 82,18±0,16% a 5 UPS seguido de 77,28±0,03% a 38 UPS y 74,25±0,10% a 60 UPS. El valor promedio más alto de actividad enzimática (U/mL de extracto) se registró en 60 UPS (576,160 ± 33,135), el valor intermedio en 5 UPS (432,178 ± 26,705) y el valor más bajo en 38 UPS (364,146 ± 16,115). La comparación de las frecuencias alélicas entre las ostras supervivientes en las tres salinidades reveló una dependencia significativa de la salinidad ($p<0,05$) tanto para LAP-2* como para LAP-3*. Para LAP-2*, la frecuencia del alelo 102 estuvo significativamente representada en salinidades extremas, contribuyendo con el 82,36% de la diferencia en el valor total de χ^2, mientras que para LAP-3* se evidenció un aumento proporcional en la frecuencia del alelo 104 y la disminución en la frecuencia del alelo 100 a 60 UPS, y sus contribuciones al valor total de χ^2 fueron 35,24% y 55,65%, respectivamente. Los resultados brindan un apoyo importante a la hipótesis de que la variabilidad de LAP sería el resultado del efecto selectivo de la salinidad ambiental en este molusco y amplían la evidencia sobre el papel de esta enzima para enfrentar el estrés de salinidad en bivalvos marinos.

Palabras clave: leucil aminopeptidasa, ostras, regulación del volumen celular, adaptación, frecuencia alélica

INTRODUCTION

Allozymes are alternative forms of an enzyme encoded by different alleles of the same locus, which have been used as codominant markers to study genetic variation within and among populations of terrestrial and aquatic organisms (Allendorf 2017). Since the late 1970s, these molecular markers have been used to quantify genetic variation in natural populations of various species of pelecypod molluscs (Koehn & Mitton 1972; Boyer 1974; Koehn *et al*. 1976; 1980a, Beaumont 1982; Macleod *et al*. 1985; Nirchio *et al*. 1991; Pérez *et al*. 2011; Hmida *et al*. 2012; Voronoy & Zaslavskaya 2014; Bonner *et al*. 2019; Fassatoui *et al*. 2019), but with the development of DNA techniques since the late 1980s, allozymes were progressively less frequently used.

However, some single locus/enzymes are still considered very useful, due to their direct link to adaptation to environmental changes. Among these, leucine aminopeptidase (LAP) has been used in the screening of natural populations of the blue mussel, *Mytilus edulis* (Koehn & Mitton 1972; Levinton 1973; Mitton & Koehn 1973; Boyer 1974; Koehn *et al*. 1976; 1980b; Koehn 1978; Lassen & Turano 1978; Levinton *et al*. 1978; Gartner-Kepkay *et al*. 1980). LAP is an α-aminoacyl peptide hydrolase (LAP EC. 3.4.1.1) that catalyzes the release of neutral and aromatic amino acids from the N-terminal group from di-, tri- and tetrapeptides (Young *et al*. 1979) and has an important role in the degradation of intracellular proteins to provide the free amino acids required for the control of cell volume in marine bivalves (Moore *et al*. 1980; Koehn & Siebenaller 1981; Hilbish & Koehn 1985a). The enzymatic activity of LAP, *in vitro*, seems to be stimulated by the increase in environmental salinity and it has been found that the frequency of some alleles encoding this enzyme varies as a function of environmental salinity, suggesting that it would have adaptive importance in the eastern oyster *Crassostrea virginica* (Buroker 1983; Rose 1984); in the ribbed-mussel *Geukensia demissa* (Garthwaite 1986), hard clam *Mercenaria mercenaria* (Hilbish 2001) and in the blue mussel *Mytilus edulis* (Koehn 1991).

Earlier work on *Crassostrea rhizophorae*, reported that three loci of LAP were present. For the *LAP-1* locus, the allozymes only become evident after two hours of reaction with the substrate and the bands observed are barely perceptible, a situation that makes the analysis of this polymorphism very difficult; whereas *LAP-2** and *LAP-3**, show three and six electromorphs respectively. In *LAP-3**, four alleles are frequently observed and two are rare (<0.05% each) and the allele distribution in the populations seems to be associated with spatial heterogeneity in temperature and environmental salinity (Nirchio *et al*. 1987, 1991)

Due to the importance of oysters as food and as aquaculture commodities, understanding the potential impacts of low and high salinities is important to adapt and manage the long-term sustainability of this resource

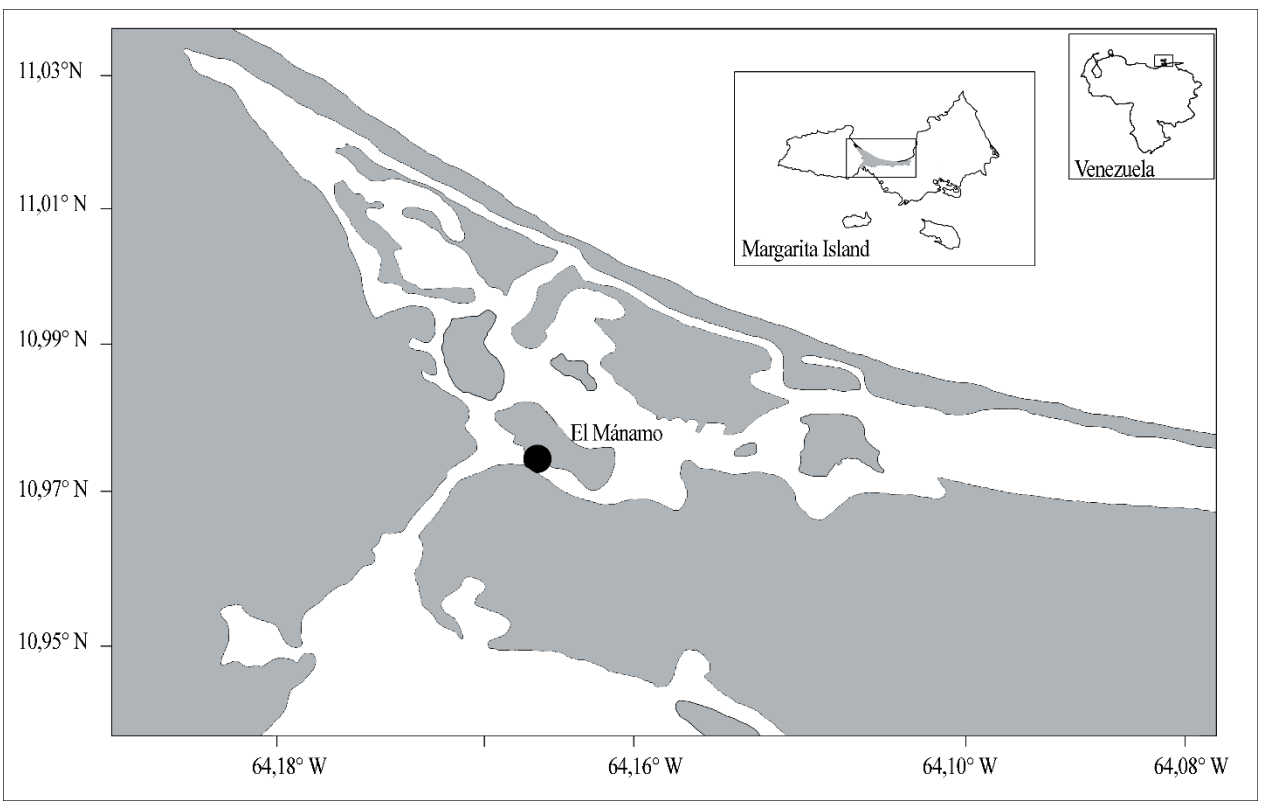


Figure 1. Location of oyster collection site in Laguna La Restinga, Margarita Island, Venezuela

(Funo *et al*. 2015; Lavaud *et al*. 2017; Mazon-Suastegui *et al*. 2017; Moussa 2018; Wang & Li 2018). In the present work we present the results of osmotic stress experiments conducted on *Crassostrea rhizophorae*, with the aim of analyzing the effect of extreme salinities on survival, percentage of body water, condition index, enzymatic activity of LAP and allelic frequencies at LAP-2* and LAP-3*.

MATERIALS AND METHODS

The oysters were collected from the roots of the red mangrove *Rhizohora mangle* L., in Laguna La Restinga (10°58'55.2" N 64°09'24.4" W), Isla de Margarita, Venezuela (Fig. 1). Epibionts and any remaining foreign material adhered to the valves were removed from each oyster by mechanical scraping. Two hundred-forty specimens between 6-7 cm were randomly selected and distributed in six circular aquariums (40 oysters per aquarium), representing 2 sets (one original and one replicate) with three different salinity 5 PSU, 38 PSU and 60 PSU. Each aquarium had 60 L capacity and was supplied with constant aeration. Salinity of 38 PSU was used as control, since it is similar to the annual average salt concentration in which the oysters are found in the collection area. The salinities of 5 PSU and 60 PSU correspond to the limits reported as the minimum and the maximum to which *C. rhizophorae* has been exposed in bioassays (Guimarães *et al*. 2008).

The different salinities were obtained from hypersaline seawater (180-210‰), conveniently diluted with distilled water. For the final adjustment of salinity, an Atago salinometer/refractometer was used, with 2 PSU precision. The dilutions were made one day before collection and the water was left to stand overnight in well-covered containers. The water in each aquarium was replaced entirely daily.

Once the oysters were simultaneously exposed to the different salinities, the study ran for 96 h, when about 50% mortality was reached in the two sets of the extreme salinity aquariums (5PSU and 60 PSU). Dead oysters, identified because they did not close their valves when touched with a glass rod, were extracted every two hours. During this period, oysters were fed daily with a suspension of *Tetraselmis* sp, 150 mL per aquarium, with a concentration of 10^9 cells/mL.

When the time limit established for the experience was reached, the total weight (tw), wet weight (ww), flesh wet weight (fww) and dry weight (fdw), weight of the valves (vw), percentage of body water (bw), condition index (ci), enzymatic activity (ea), specific enzymatic activity (sea), and genotype for LAP, were determined.

Condition Index

The intact oysters were blotted with absorbent paper and weighed. The flesh was immediately separated from the valves, placed on absorbent paper and weighed. The condition index was calculated using the formula IC = (DWx1000) / IV, where DW is the oyster dry weigh (g) and IV is the Intervalvar Volume (mL) (Lawrence & Scott 1982). Since the oysters that were genotyped for LAP, for obvious reasons, could not be dehydrated, the dry weight of each individual was estimated from its wet weight, using a linear regression equation (wet weight vs dry weight) established in a parallel trial for this purpose (see results).

Digestive gland homogenates

The digestive gland was dissected from each specimen, dried with absorbent paper, weighed and finally homogenized in Tris-HCl 0.05M. pH 7.00, in a ratio of 1: 3 (p: v). Homogenization was carried out cold, and the homogenate was centrifuged at 20,000 g at 4ºC for 30 min. The supernatant was used for the determination of the enzymatic activity and horizontal starch gel electrophoresis.

LAP enzyme activity

The enzymatic activity of LAP was determined as Naphthylamidase activity (Smith & van Frank 1975; Koehn 1978; Young *et al*. 1979). The reaction mixture consisted of 2.0 mL of 0.2 M Tris-HCl buffer, pH 7.00 and 0.5 mL of a 0.005 M solution of L-leucyl-β-naphthylamide in 0.2 M Tris-HCl, pH 7.00 and 20% glycerol. The substrate was previously dissolved in two drops of dimethylformamide. The reaction mixture was chilled in an ice-water bath and then, 20 µL of a 1:20 dilution of the corresponding tissue extract was added and incubated at 37°C in a thermoregulated bath for 15 min, after which the reaction was stopped by adding 100 µL of a 0.5% solution of Fast Garnet GBC and 2% of Sodium Lauryl Sulfate. The solubilized Diazonium-Naphthylamine complex's absorbance was determined in a spectrophotometer at 510 nm and the activity was expressed in UAE/mL [UAE = unit of enzymatic activity, equivalent to 0.001 absorbance units at 510 nm, pH 7.00 and 37°C].

Electrophoresis

Electrophoresis was carried out on a horizontal 13% Starch gel (Sigma, Co. St. Louis, Mo., USA) in Tris-Citric Acid buffer, 0.008 M, pH 7.00. A Boric Acid-NaOH 0.3 M, pH 8.60 solution was used as a running buffer (Nirchio *et al*. 1991). Gels were run at 200 V for 3 h at 8-10°C. Then the gel was cut horizontally into two halves that were incubated in a 0.05M Tris-HCl buffer solution, pH 7.00 containing 10 mg of L-leucyl-B-naphthylamide-HCl and 40 mg of Fast Garnet GBC.

Nomenclature for loci and alleles followed the Nomenclature Committee of the Fish Genetics Section of the American Fisheries Society (Shaklee *et al*. 1990).

Statistics

To detect the existence of differences in each parameter between the salinities, a nested Analysis of Variance (ANOVA) was used, after checking compliance with the assumptions required for this test (Sokal & Rohlf 2009). In cases where the ANOVA revealed statistical differences, the SNK test was performed. Allele frequencies were given with standard error calculated according to p± (Levinton & Koehn 1976). The relative importance of specific alleles as causes of significant salinity differences was assessed by calculating the proportion contributed by each allele to the total χ^2 independence test.

RESULTS AND DISCUSSION

Survival and weight data are reported in TABLE 1. Mortality of 50% or higher occurred after 96 h in the groups corresponding to 5 PSU and 60 PSU. At 38 PSU, survival was 100%.

Total weight and weight of the valves did not differ significantly ($p > 0.05$) among the groups subjected to the different experimental salinities, indicating that the experimental groups were homogeneous for these variables, and allowing to rule out any effect of the size on the other variables analyzed (TABLE 1).

Figure 2 shows the linear regressions (wet weight vs dry weight) for each experimental salinity, established in a parallel trial carried out in order to estimate the dry weight required to calculate the condition index in the oysters that were genotyped for LAP.

Figure 3 shows the survival curves of the oysters that were used to establish the regression equations between wet weight and dry weight, to determine the condition index (see next section) and percentage of body water at each salinity. A wide range of salinity tolerance has been reported for *C. rhizophorae* between 5 PSU to 60 PSU, with an optimum between 28 and 32 PSU (Wedler 1983). However, under progressive acclimation to salinity changes, sublethal limits correspond to 2 PSU and 64 PSU, with lethal limits of 1PSU and 66 PSU, with

TABLE 1. Means ± standard deviation of total weight (tw), valve weight (vw), flesh wet weight (fww), flesh dry weight (fdw), percentage of body water (bw), index of condition (ci), and enzymatic activity (ea), F_{ANOVA} among salinities (Fs), F_{ANOVA} between original and replica ($F_{o\text{-}r}$), number of individuals (n), survival (s%).

	Salinity (PSU)				
Parameter	5	38	60	F_s	$F_{o\text{-}r}$
tw (g)	28.02 ± 1.59	26.47 ± 1.16	32.47 ± 1.77	4.89^{ns}	0.69^{ns}
vw (g)	17.68 ± 1.08	16.05 ± 0.76	20.02 ± 1.20	4.14^{ns}	1.01^{ns}
fww (g)	4.08 ± 0.23	2.75 ± 0.12	3.86 ± 0.21	64.77^{*}	0.23^{ns}
fdw (g)	0.74 ± 0.05	0.63 ± 0.03	0.99 ± 0.06	68.01^{*}	0.23^{ns}
bw (%)	82.18 ± 0.12	77.28 ± 0.03	74.25 ± 0.10	7.429^{**}	0.31^{ns}
ci	71.61 ± 2.91	60.72 ± 1.71	80.83 ± 2.47	27.26^{*}	0.87^{ns}
ea (UAE/ml)	432.18 ± 26.71	364.15 ± 16.12	576.16 ± 33.14	14.43^{*}	1.49^{ns}
$n_{(initial)}$	80	80	80		
$n_{(final)}$	38	80	37		
Survival (%)	47.5	100	46.3		

an interval within which no mortality is observed from 3 PSU to 55 PSU (Poza & Rodríguez 1987). Although the salinities of 5 PSU and 60 PSU used in the present study fall within the extremes of the tolerance range of *C. rhizophorae* collected from the same locality, there is a discrepancy that can be attributed to the fact that in our study we did not expose the specimens to a progressive variation in salinity, as Poza & Rodríguez (1987) did.

Pelecypods are known to close their valves under stressing salinity conditions, and seal themselves inside the shell separating the internal fluid from the external environment for a short period of time (Lockwood 1976; Pourmozaffar *et al*. 2019), thus maintaining salinity inside the pallial cavity nearly constant (Chaparro *et al*. 2009). In this study, it was found that during the first 36 h of exposure and, unlike the individuals in the control

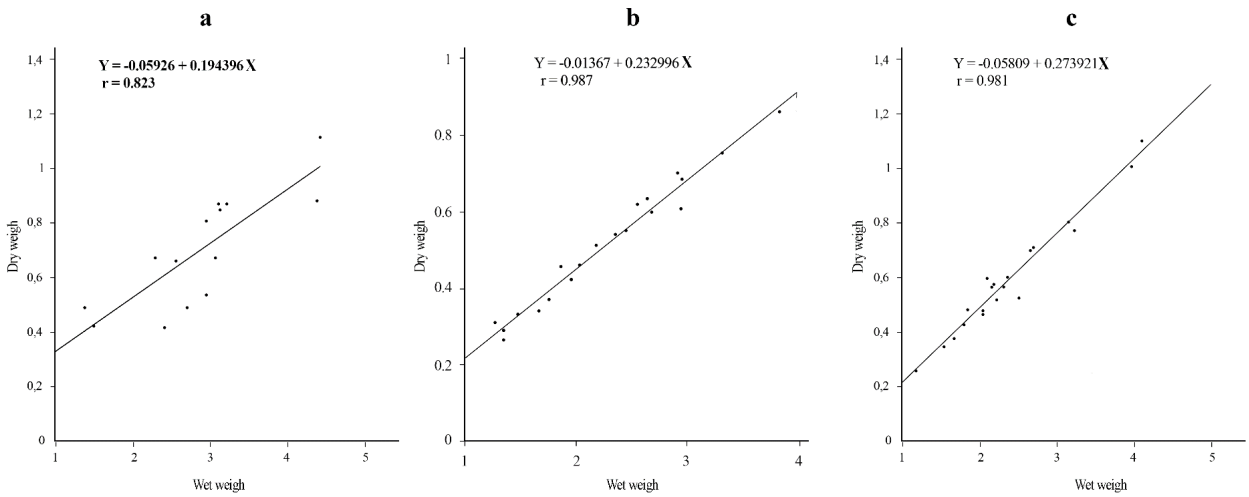


Figure 2. Regression between wet weight and dry weight of the flesh in *Crassostrea rhizophorae* at each salinity

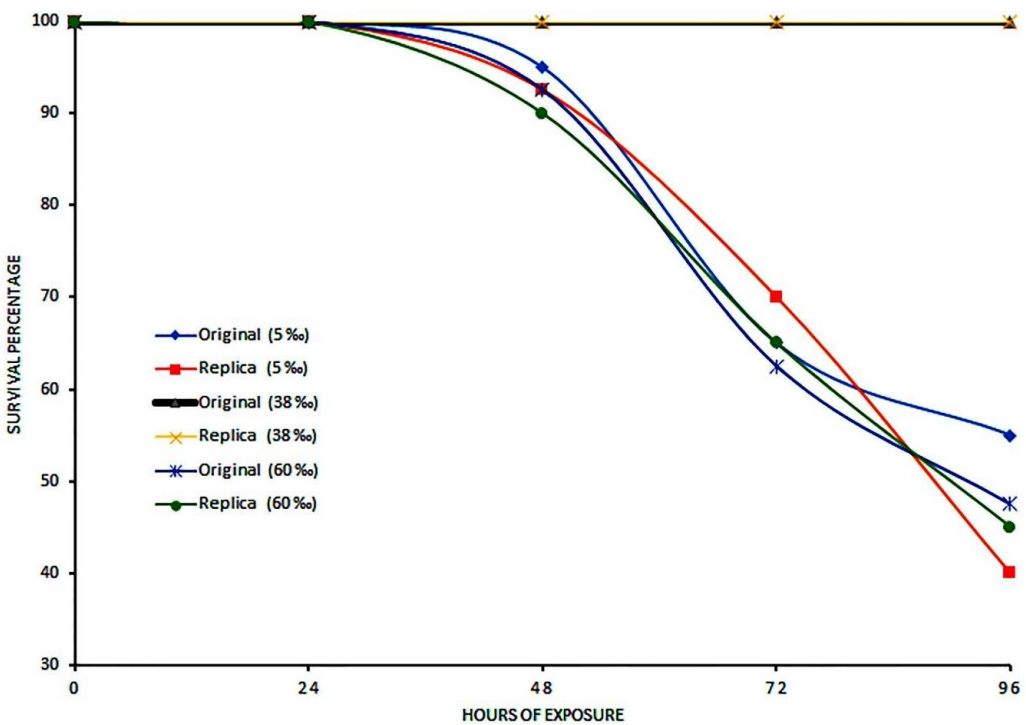


Figure 3. Survival of *Crassostrea*. *rhizophorae* in three experimental salinities

group (38 PSU), those exposed to 5 PSU and 60 PSU kept their valves closed. Just after that time, they began to open their valves possibly to allow the exchange of water and release of the toxic metabolites accumulated in the intervalvar water suffering the acute effects of the change in salinity and facing the problems of hydration and alteration of cell volume as a consequence of the variation in osmotic pressure of the surrounding water, which coincided with the appearance of the first dead specimens. After 96 h, 46.5% of mortality occurred at 5 PSU and 45.25% at 60 PSU, whereas at 38 PSU no mortality was recorded (TABLE 1). Experiments on survival of *C. rhizophorae* in response to sudden drops in salinity have shown 50% of mortality after 11 h of exposure at 4 PSU and 130 h at 6 PSU (Poza & Rodríguez 1987). Unfortunately, there are no available data for the species to compare the effect of the abrupt increase in salinity up to 60 PSU.

The analysis of variance showed significant differences ($p < 0.05$) for both, the wet weight and the dry weight of the flesh among salinities. The SNK test revealed the existence of two different groups. The mean wet weight of oysters kept at 38 PSU differed significantly from that of oysters kept at 60 PSU and 5 PSU, while between the averages at 60 PSU and 5 PSU there was no statistical difference.

The highest wet weight of the flesh was 4.08 ± 0.23 g at 5 PSU and the lowest was 2.75 ± 0.12 g at 38 PSU; at 60 PSU the wet weight of the flesh was 3.86 ± 0.21 g. Regarding the flesh dry weight, the mean values were 0.63 ± 0.03 g at 5 PSU, 0.74 ± 0.05 at 38 PSU and 0.99 ± 0.06 g at 60 PSU. For the flesh dry weight, the SNK test revealed significant differences among the three groups (TABLE l).

The analysis of variance showed the existence of significant differences for the percentage of body water ($p < 0.05$) among salinities and the SNK test revealed the existence of three different average values ($p < 0.05$) confirming an inverse relationship between the percentage of body water and salinity. The highest by value was 82.18 ± 0.16% at 5 PSU followed by 77.28 ± 0.03% at 38 PSU and 74.25 ± 0.10% at 60 PSU.

As expected, the sudden and pronounced change in salinity under experimental conditions, determined a significant variation in the percentage of body water ($p<0.05$). Indeed, in the location where oysters used in this study were collected, water has an average salinity of 38 UPS, with monthly fluctuations between 35.73 PSU and 42.88 PSU (Pineda 1978), thus, dehydration occurred at 60 PSU and hydration at 5 PSU (TABLE 1).

Osmoconformer animals, including bivalves, modify the concentration of free intracellular amino acids, in addition to other low molecular weight molecules, to regulate cell volume while facing changes in salinity of the water that surrounds them (Florking 1966; Pierce 1971; Koehn & Immermann 1981; Solan & Whiteley 2016). There are four main routes for the supply of free amino acids to the intracellular pool: (1) Protein degradation, (2) Transamination reactions, (3) *De novo* synthesis and (4) Direct absorption from the medium (Somero & Bowlus 1983).

The general role of LAP in protein catabolism for the supply of the cellular pool of free amino acids during regulation of cell volume has been recognized (Gharbi *et al*. 2016). Indeed, exposure of *Mytilus edulis* to hypersaline shock causes an approximately 10-15% decrease in intracellular protein concentration (Bishop *et al*. 1981), while increasing the enzymatic activity of peptidases, including LAP, concomitantly with an increase in the concentration of free amino acids (Levinton & Koehn 1976; Moore *et al*. 1980; Koehn & Immermann 1981; Koehn & Siebenaller 1981; Koehn 1983; Hilbish & Koehn 1985a, b; Koehn & Hilbish 1987). It has also been shown that the accumulation of free intracellular amino acids in the branchial tissue of *Geukensia demissa* is prevented by the action of lysosomal and protease inhibitors, which also supports the view that proteolysis is an important source of free amino acids used in the regulation of cell volume (Deaton 1987). Further, it has been shown that the concentration of free amino acids in the adductor muscle of *C. rhizophorae* increases proportionally with an increase in salinity (Nirchio 1995).

Significant differences in the enzymatic activity of LAP among the groups of oysters exposed to the three experimental salinities were evident (TABLE 1). Indeed, the SNK test showed three different group ($p<0,05$), being the highest average value 576.160 ± 33.135 at 60 UPS, the intermediate value 432.178 ± 26.705 at 5 UPS, and the lowest value 364.146 ± 16.115 at 38 UPS.

An increase of LAP enzymatic activity can occur due to the modification of the catalytic properties of the enzyme or by an increase of its concentration (Koehn 1978). Thus, the need for free amino acids in

the intracellular medium to increase the internal osmotic pressure would determine the increase in enzyme activity in individuals maintained at 60 PSU (TABLE 1). Nonetheless, in the group maintained at 5 PSU there was also a significant increase in the enzymatic activity of LAP, compared to those maintained at 38 PSU.

When oysters are transferred to hypohaline conditions, their intracellular osmotic pressure is higher than the external one and swelling of the cell occurs due to the influx of water. To prevent lysis of the cell and to be able to recover its volume, the release of amino acids from the cell pool to the hemolymph must occur, which, in turn, are excreted directly to the surrounding medium or as ammonia (Pierce 1971, Bishop 1976). In this regard, a study on *C. rhizophorae* kept under hypohaline conditions (18 PSU), shows a significant increase in the level of ammonia excretion (compared to individuals kept at 38 PSU) (Gaviria 1989). Another study conducted to determine the effect of environmental salinity on the concentration of intracellular free amino acids in the muscle of *Crassostrea virginica*, revealed that in the salinity range between 3 PSU and 26.7 PSU, a small set of amino acids (glycine, proline, taurine, leucine and isoleucine) are found in minimal concentrations, but most amino acids, in addition to ammonia, remain at levels relatively constant (Lynch & Wood 1966). This amino acid excretion would represent a substantial loss of carbon and, consequently, degradation of intracellular proteins due to an increase in the enzymatic activity of LAP. In extreme salinities, it would involve a considerable energy expenditure that could affect the physiological state of the organism (Hilbish & Koehn1985).

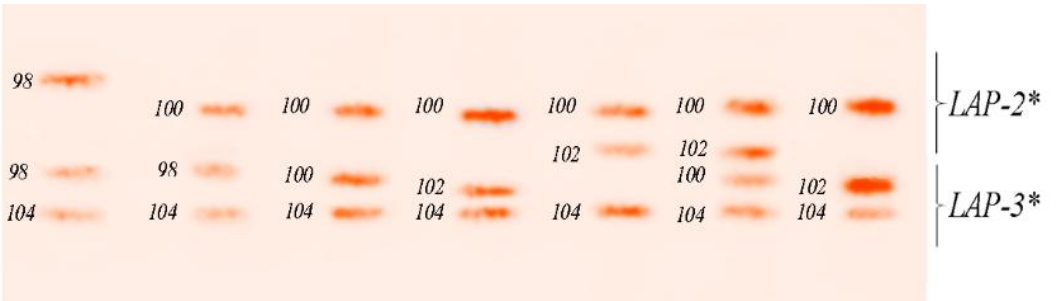
The condition index (CI) reflects the physiological state in bivalves (Galtsoff 1964). Calculation of CI is based on a relationship between the dry weight of the flesh and the volume of the internal cavity of the shell, and it can be affected by numerous factors, including the age of the oysters, temperature, seasonal changes, stage of gonadal development, depth, salinity, nature of the substrate, population density, and food availability, as well as associated organisms and contaminants (Lawrence & Scott 1982).

The analysis of variance revealed significant differences in CI ($p<0.05$) among the groups maintained at the different salinities. The SNK test showed statistical differences ($p<0.05$) among the three average values (TABLE 1), with an average value of 80.83 ± 2.47 at 60 PSU followed by 71.81 ± 2.91 at 5 PSU and 60.72 ± 1.71 at 38 PSU. It was to be expected that the values obtained in the extreme salinities would be lower than those obtained in the control salinity, since the increase in the enzymatic activity of LAP would impose considerable energy expenditure. Thus, although it seems contradictory, the averages of the condition index were significantly higher at extreme salinities than at 38 PSU (TABLE 1). Since these values correspond to the surviving individuals, the highest averages of CI in extreme salinities would reflect the selective action of salinity that would favor those individuals with greater availability of energy reserves, including intracellular proteins, which would allow them to maintain increased levels of LAP activity without significant detriment to their physiological condition. Thus, at 38 PSU, the mean value of the CI was lower simply because the mean would include those oysters with characteristics similar to those that did not survive in extreme salinities. In fact, this was confirmed by ordering the values of the condition index obtained in the control group (38 PSU) and dividing them into two subgroups with the same number of individuals, clearly differentiable, one with a low condition index (49.94 ± 3.02) and the other with a high condition index (71.82 ± 1.58), the latter resembling the condition index corresponding to the individuals who survived in extreme salinities.

Regarding the electrophoretic results, three electromorphs (*LAP-2*^{*98}, *LAP-2*^{*100}, *LAP-2*^{*102}) for *LAP-2**, and four for *LAP-3** (*LAP-3*^{*98}, *LAP-3*^{*100}, *LAP-3*^{*102} and *LAP-3*^{*104}) were detected in the sample (Fig. 4). All possible genotypes for the *LAP-2** were observed whereas for *LAP-3**, only eight of the ten possible genotypes were represented.

The comparison of allele frequencies among the surviving oysters in the three salinities assayed revealed a significant dependence ($p<0.05$) both for *LAP-2** and *LAP-3** with salinity (TABLE 2). For *LAP-2**, frequency of allele 102 was significantly represented in extreme salinities, contributing with 82.36% of the difference to the total χ^2 value whereas for *LAP-3** an increase in the frequency of allele *LAP-2*^{*104}, proportional to the decrease in the frequency of allele *LAP-2*^{*100}, was evidenced at 60 PSU contributing to total χ^2 value with 35.24% and 55.65%, respectively. In a population genetic study on *C. rhizophorae*, significant differences were found in allele frequencies for *LAP-3** between two natural populations from localities that differ in

Figure 4. Electrophoretic phenotypes of LAP-2* and LAP-3* loci for the Leucyl Aminopeptidase isozyme system in *Crassostrea. rhizophorae*.



salinity and temperature: the frequency of the *LAP-3*98* allele was higher where the average salinity had larger values, whereas frequency of *LAP-3*100* was increased in the locality with the lowest average salinity (Nirchio *et al*. 1991), suggesting that *LAP-3*100* could provide a selective advantage where the salinity was lower.

Like in *C. rhizophorae*, the relationship between the variation of allele frequencies at *LAP* loci with salinity and temperature has also been evidenced in *Crassostrea virginica*. For both species, the allele frequencies of *LAP-3** locus in *C. rhizophorae* and Lap 1 in *C. virginia*, were not in Hardy Weinberg (H-W) equilibrium, whereas for *LAP-2**, the allele frequencies fitted to the expected H-W frequencies and, coincidentally, the factors altering the H-W equilibrium seem to operate in the more anodic electrophoretic forms of the isozymes (Nirchio *et al*. 1991).

In two natural populations of *Mytilus edulis*, one in southern Cape Cod and the other a few kilometers north in the Gulf of Maine, the allele *LAP94* is present with a higher frequency in the oceanic regions, whereas in estuaries, where salinity is lower, the frequency of this allele drops with an increase in the *LAP96* and *LAP98* (Koehn 1983).

This concomitant variation of allelic frequencies with environmental salinity in LAP coding loci in *Mytilus edulis*, *C. virginica* and *C. rhizophorae*, provides important support for the hypothesis that variability of this enzyme would be the result of the selective effect of salinity. It is important to note that sequencing of the LAP gene region of the Pacific oyster, *C. gigas*, has revealed many more (at least 34 alleles) than the 6 alleles previously predicted by allozyme

TABLE 2. Allelic frequencies for LAP-2* and LAP-3* loci in the surviving individuals (N) of *Crassostrea rhizophorae* in each salinity and values of Chi-square independence test (χ^2). Proportion contributed by each allele to the total χ^2 value is indicated; d.f (degrees of freedom); p (probability).

		Salinity (PSU)			
Locus	Allele	5	38	60	Contribution
LAP-2*	98	0.096 ± 0.04	0.090 ± 0.03	0.043 ± 0.03	2.88 %
	100	0.692 ± 0.06	0.861 ± 0.03	0.522 ±0.07	14.75 %
	102	0.211 ± 0.06	0.049 ± 0.02	0.435 ±0.07	82.36 %
		χ^2= 36.773; d.f= 4; p= 2.006 x 10^{-7}			
LAP-3*	98	0.231 ± 0.06	0.303 ± 0.04	0.283 ±0.07	5.27 %
	100	0.442 ± 0.07	0.328 ± 0.04	0.196 ± 0.07	35.24 %
	102	0.288 ± 0.06	0.328 ± 0.04	0.370 ± 0.07	3.83 %
	104	0.038 ± 0.03	0.041 ± 0.02	0.152 ± 0.05	55.65 %
		χ^2= 12.877; d.f= 6; p= 0.045			
	N	38	80	37	

analysis and predictions of the structure and protein stability indicates that many of these changes likely affect enzyme function (Wharam *et al*. 2008). Since to the fact that *C*. *rhizohorae* is a congeneric species and therefore it could also have a higher polymorphism for LAP, it is necessary to deepen this line of research to have a better understanding of the adaptive importance of aminopeptidase I in this bivalve.

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