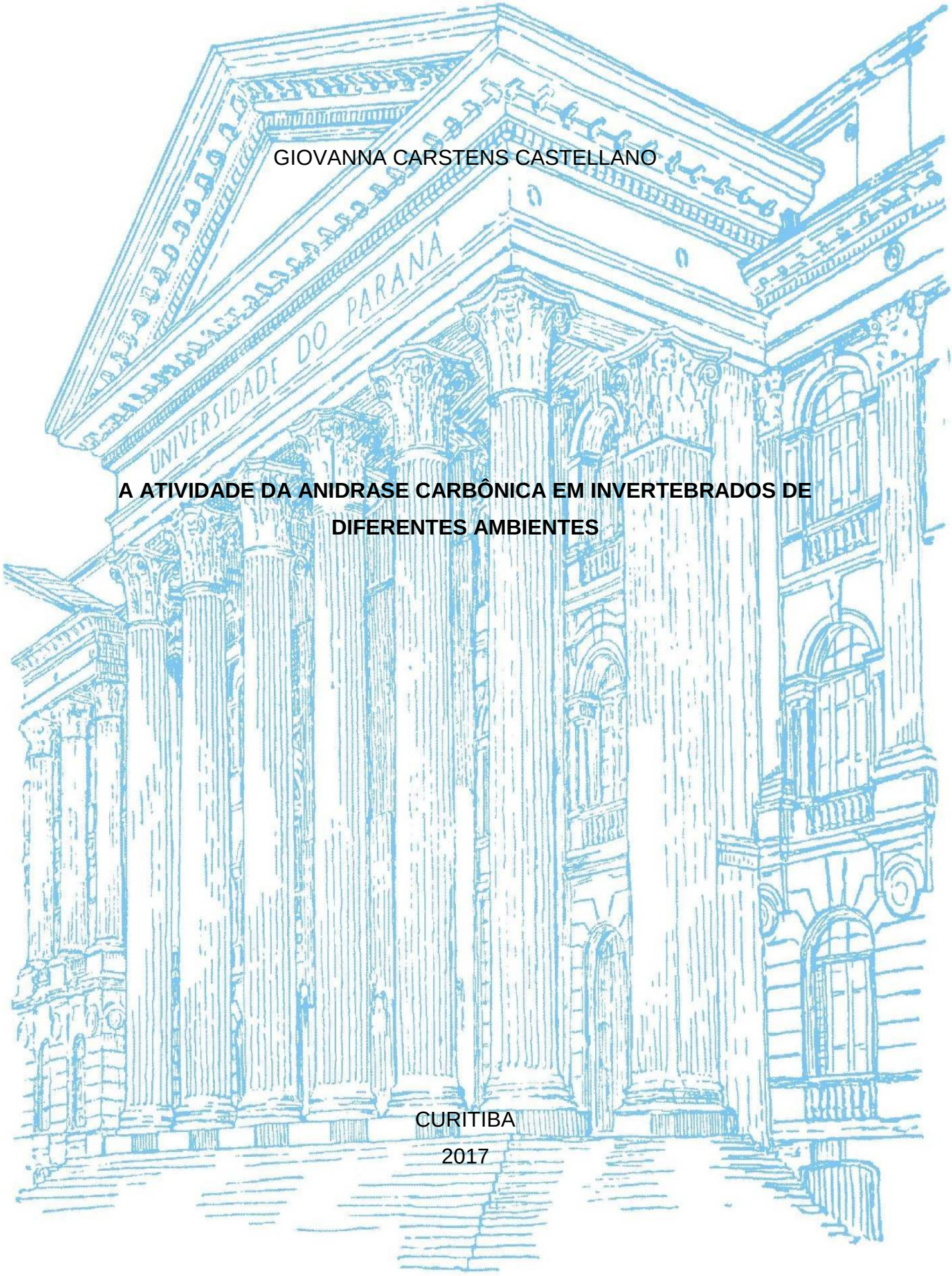
UNIVERSIDADE FEDERAL DO PARANÁ

GIOVANNA CARSTENS CASTELLANO



A ATIVIDADE DA ANIDRASE CARBÔNICA EM INVERTEBRADOS DE DIFERENTES AMBIENTES

CURITIBA

2017

GIOVANNA CARSTENS CASTELLANO

A ATIVIDADE DA ANIDRASE CARBÔNICA EM INVERTEBRADOS DE DIFERENTES AMBIENTES

Tese de doutorado apresentada como requisito parcial à obtenção do grau de Doutora em Zoologia, no Programa de Pós-Graduação em Zoologia, Setor de Ciências Biológicas, da Universidade Federal do Paraná,

Orientadora: Profª. Drª Carolina Arruda de Oliveira Freire

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TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em ZOOLOGIA da Universidade Federal do Paraná foram convocados para realizar a arguição da tese de Doutorado de **GIOVANNA CARSTENS CASTELLANO** intitulada: **A atividade da anidrase carbônica em invertebrados de diferentes ambientes**, após terem inquirido a aluna e realizado a avaliação do trabalho, são de parecer pela sua APROVAÇÃO.

Curitiba, 21 de Fevereiro de 2017.

CAROLINA ARRUDA DE OLIVEIRA FREIRE
Presidente da Banca Examinadora (UFPR)

ROSANA MOREIRA DA ROCHA
Avaliador Interno (UFPR)

MARTA MARQUES DE SOUZA
Avaliador Externo (FURG)

PAULO DA CUNHA LANA
Avaliador Interno (UFPR)

CARLOS RENATO REZENDE VENTURA
Avaliador Externo (UFRJ)

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RESUMO

Todos os invertebrados evoluíram no ambiente marinho e, posteriormente, alguns conquistaram os ambientes dulcícola e terrestre. Cada ambiente apresenta diferentes características de fatores abióticos, como salinidade e/ou disponibilidade de água. Neste contexto, o objetivo deste trabalho foi o de relacionar respostas fisiológicas de invertebrados à ocupação de novos ambientes (dulcícola e terrestre). No capítulo 1, espécies marinhas, estuarinas e dulcícolas de invertebrados foram expostas a estresse salino, sendo, equinodermos (apenas marinhas), moluscos, e crustáceos. Posteriormente foram realizadas análises de concentrações osmótica e iônicas de seus fluidos corporais, e de teor hídrico e atividade da anidrase carbônica (AAC) de seus tecidos. No capítulo 2, quatro espécies de caranguejos da família Sesarmidae com diferentes graus de terrestrialidade, eurihalinidade e atividade motora foram avaliados quanto à osmolalidade da hemolinfa e à atividade da anidrase carbônica das brânquias anteriores e posteriores. No capítulo 3, três espécies de poliquetas de diferentes ambientes foram estudados. A osmolalidade e a AAC constitutivas foram analisadas, e a capacidade de regulação de volume celular foi testada diante de choques osmóticos de 50% de intensidade com relação ao controle isosmótico. Nestes capítulos, buscou-se relacionar mecanismos fisiológicos (principalmente AAC) à conquista dos ambientes diluídos e terrestre. Resumidamente, os resultados demonstraram que a conquista de novos ambientes (não marinhos) demandam investimento energético em mecanismos fisiológicos que possibilitem a manutenção de gradientes osmóticos e/ou iônicos entre os meios interno (fluido corporal do animal) e externo (ambiente). A amplitude destes gradientes é proporcional ao grau de sucesso do grupo zoológico na conquista de novos ambientes (e.g. crustáceos apresentam maiores gradientes do que equinodermos). A manutenção de hidratação tecidual também é importante na conquista de novos ambientes, principalmente em espécies osmoconformadoras. A AAC tem diferentes magnitudes e funções em diferentes ambientes, sendo elevada nos ambientes marinho, dulcícola e terrestre, e baixa no estuarino. Em todos os ambientes a enzima provavelmente exerce função de equilíbrio ácido-base, mas a função osmorregulatória se restringe às espécies não marinhas. A novidade do trabalho foi a abordagem comparativa, relacionando AAC a diferentes grupos zoológicos de invertebrados, e ao seu grau de conquista de ambientes de água doce e terrestres. Além disso, AAC é muito pouco estudada nos grupos dos equinodermos e dos poliquetas. Então, sob essa visão altamente comparativa, foi possível detector os padrões fisiológicos que seguem: 1) AAC se relaciona ao ambiente, e tende a ser mais alta em habitantes marinhos, dulcícolas e de água muito diluídas do que em espécies estuarinas; 2) AAC e os gradientes osmóticos / iônicos são diretamente proporcionais à eurihalinidade e ao sucesso na conquista de novos ambientes por uma espécie, e por um grupo como um todo; 3) a capacidade de manutenção de volume celular / tecidual contribui para a tolerância à salinidade de osmoconformadores e para o grau de eurihalinidade de osmorreguladores.

Palavras-chave: crustáceos, equinodermos, moluscos, poliquetas, transição ambiental.

ABSTRACT

All invertebrates evolved in the sea and, later, some conquered freshwater and terrestrial environments. Each environment has different characteristics of abiotic factors such as salinity and/or water availability. In this context, the aim of this study was to relate physiological responses of invertebrates to the occupation of new environments (freshwater and terrestrial). In chapter 1, marine, estuarine and freshwater species of invertebrates were exposed to salt stress, being echinoderms (marine only), molluscs, and crustaceans. Then analyzes were performed on osmotic and ionic concentrations of their body fluids, water content and carbonic anhydrase activity (CAA) of their tissues. In Chapter 2, four species of Sesarmidae crabs with varying degrees of terrestriality, euryhalinity, and motor activity were evaluated for hemolymph osmolality and CAA in anterior and posterior gills. In chapter 3, three species of polychaetes from different environments were studied. The constitutive osmolality and CAA were analyzed, and the cell volume regulation capacity was tested under hypo and hyperosmotic shocks of 50% intensity in relation to the isosmotic control. In these chapters, we sought to relate physiological mechanisms (mainly CAA) to the conquer of freshwater and terrestrial environments, respectively. Briefly, the results have shown that the conquest of new environments (non marine)require energy investment in physiological mechanisms that enable the maintenance of osmotic and / or ionic gradients between the internal (body fluid of the animal) and external media (environment). The magnitude of these gradients is proportional to the degree of success of the zoological group in winning new environments (e.g. crustaceans have higher gradients than echinoderms do). Tissue hydration maintenance is also important in invading new environments, especially in osmoconformer species. CAA has different magnitudes and functions in different environments, being high in marine, freshwater and terrestrial environments, and low in the estuarines. In all environments, the enzyme probably exerts acid-base balancing, but osmoregulatory function is restricted to non-marine species. The novelty here was the comparative approach relating CAA to different zoological groups of invertebrates, and to their degree of conquest of freshwater and terrestrial environments. In addition, CAA is understudied in the groups of echinoderms and polychaetes. Thus, under this highly comparative view we could detect the following physiological patterns: 1) CAA is related to environment, and tend to be higher in dwellers of marine, freshwater, and very diluted waters than in estuarine species; 2) CAA and osmotic / ionic gradients are directly proportional to the euryhalinity and to the success of conquest of new environments of a species, and even of a group as a whole; 3) capacity of maintenance of cell / tissue volume contributes to the salinity tolerance of osmoconformers and to the degree of euryhalinity of osmoregulators.

Key-words: crustaceans, echinoderms, environmental transition, molluscs, polychaetes.

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SUMÁRIO

1 INTRODUÇÃO

A água do mar oceânica é um ambiente bastante estável com relação aos demais ambientes aquáticos, e contém cerca de 35 g de sal/ litro. Nas regiões mais rasas de contato com os continentes ou ilhas, encontram-se ambientes muito instáveis como a região de entremarés e os estuários. A região de entremarés apresenta variações bruscas de fatores abióticos, como salinidade e temperatura, durante a maré baixa, o que representa um desafio aos organismos que habitam esta região. Os invertebrados marinhos são em geral isosmóticos com relação à água do mar e osmoconformadores diante de variações osmóticas do ambiente (Vooys 1991; Péqueux 1995; Deaton 2008). Porém, a composição de solutos da água do mar e dos fluidos corpóreos destes animais podem ser diferentes, devido a uma certa capacidade de regulação iônica dos conformadores.

Estuários são áreas marinhas costeiras salobras que recebem aporte de água doce, e são caracterizados por grandes variações de salinidade, temperatura, oxigênio dissolvido, quantidade de nutrientes, marés, correntes marinhas e turbidez (Pritchard 1967). As variações destes fatores são provocadas principalmente pelo movimento das marés. Na maré vazante o ambiente recebe massas de água doce, o que ocasiona a diminuição da salinidade, por exemplo. Na maré enchente massas de água marinhas invadem o local, aumentando a salinidade, alterando novamente o ambiente. Mesmo com grandes desafios fisiológicos para o estabelecimento em estuários, uma grande diversidade faunística ocorre nestes ambientes, onde representantes de diversos filos estão presentes, como moluscos, crustáceos, poliquetas. O grau de tolerância a mudanças de salinidade (eurihalinidade) dos organismos é fator determinante na ocupação de ambientes diluídos como estuários (Evans 2008). Habitantes de estuários e águas continentais devem apresentar alguma capacidade osmorregulatória (regulação anisosmótica extracelular – RAE, e/ou regulação isosmótica intracelular RII ou regulação de volume celular), lidando com o excesso de água ou carência de sais (ou seja, variação rápida) nestes novos ambientes em relação ao ambiente marinho (Anger 2001; Evans 2008).

As águas continentais, ambientes dulcícolas, apresentam concentrações extremamente baixas e variadas de solutos dissolvidos. Os organismos que ocorrem nestes ambientes são necessariamente osmorreguladores (Freire et al. 2008), pois diluir seus fluidos corpóreos ao nível da água ambiental seria incompatível com a vida. Contudo, esta capacidade é bastante variável. Crustáceos decápodes são os invertebrados que sustentam os maiores gradientes em relação à água doce (Willmer et al. 2009; Freire et al. 2013).

O ambiente terrestre apresenta baixa disponibilidade de água e de sais, que devem ser obtidos via alimentação ou pelo aproveitamento das excretas (Willmer et al. 2009). Neste ambiente há a tendência de perda de água, entretanto, não há perda de solutos por difusão, como ocorre na água doce. A tendência à desidratação se acentua em animais com maior relação superfície / volume, ou seja, de tamanho reduzido, como formas jovens, por exemplo (Willmer et al. 2009). As concentrações corpóreas dos animais terrestres variam de acordo com sua ancestralidade. Por exemplo, os primeiros crustáceos eram marinhos, assim suas concentrações são altas, de 400 a 900 mOsm. As dos insetos, originalmente terrestres, têm concentrações mais baixas na hemolinfa, de 200 a 510 mOsm (Willmer et al. 2009).

As respostas osmorregulatórias dos invertebrados podem variar com o ambiente que as espécies habitam e com o grupo zoológico ao qual pertencem (Péqueux et al. 1995; Deaton et al. Willmer et al. 2009). Alguns exemplos podem ser observados na figura 1. Equinodermos são osmoconformadores, o que representa uma resposta osmorregulatória típica de invertebrados estritamente marinhos (Diehl 1986). O grupo dos moluscos também é tipicamente osmoconformador. Porém, espécies dulcícolas mantêm gradientes osmótico e iônico com relação à água ambiental em uma estreita faixa de variação de salinidade (Deaton 2008). Anelídeos marinhos e estuarinos mantêm pequenos gradientes com relação à água ambiental, enquanto os dulcícolas são osmorreguladores (Preston 2008). Crustáceos apresentam variados padrões de regulação osmótica e iônica, desde a osmoconformação em espécies marinhas, até osmorregulação, que pode ser observada em águas estuarinas e doces (Deaton 2008).

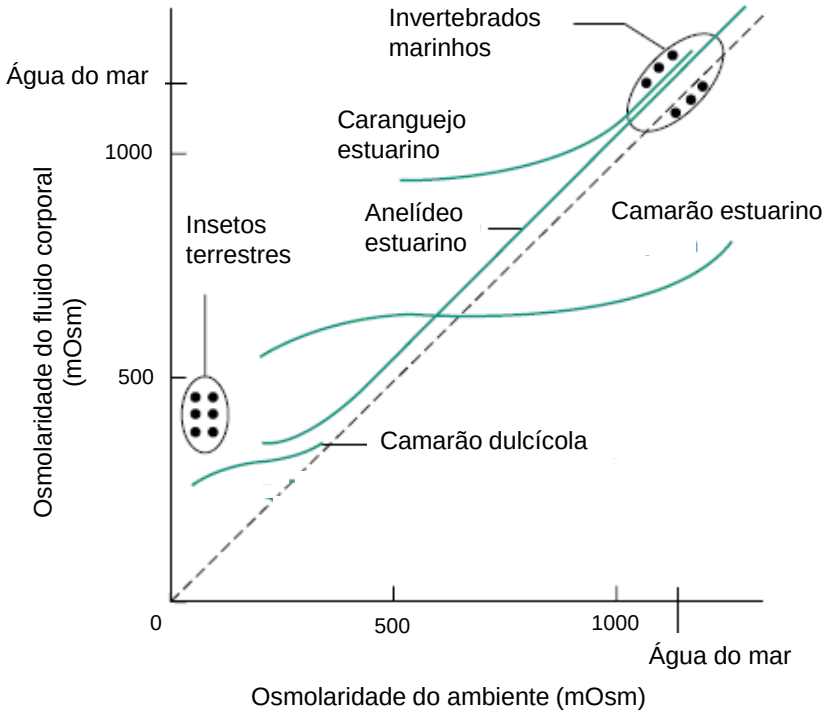

Figura 1. Exemplos de respostas osmorregulatórias de invertebrados. Adaptado de Willmer et al. (2009).

Grande parte da história evolutiva do planeta e o surgimento da vida na Terra ocorreram nos oceanos (Hill et al. 2008; Willmer et al. 2009). Há cerca de 30 filos animais, e todos evoluíram no ambiente marinho. Destes, cerca de 16 conquistaram a água doce, e apenas 7 a terra (Lee and Bell 1999). As transições entre ambientes estão associadas a mecanismos fisiológicos adaptativos às novas condições de salinidade e disponibilidade de água, relacionados à manutenção de gradientes osmóticos entre os meios interno (animal) e externo (ambiente), e à redução da permeabilidade do corpo a fluxos de água e de sódio (Lee and Bell 1999; Freire et al. 2003; Hill et al. 2008; Willmer et al. 2009).

A transição entre o ambiente marinho e os ambientes de água diluída ou doce oferece não apenas um gradiente salino, mas de outros fatores abióticos também, como pH, concentração de O_2 (Willmer et al. 2009). Por isso, a conquista de novos ambientes requer também mecanismos fisiológicos adaptativos relacionados a outras funções celulares e/ou do organismo (não apenas osmorregulatórias), como regulação ácido-base, e respiração. Todas

estas funções podem ser realizadas pela atividade da enzima anidrase carbônica. Esta catalisa a reação reversível de hidratação do CO_2, cujos produtos são os íons próton (H^+) e bicarbonato ($HCO3^-$) (Fig. 2 - Henry and Saintsing 1983; Henry 1988; Henry 1996). Estes íons são transportados para fora das células via trocadores iônicos, Na^+/H^+ e Cl^-/HCO_3^-. Ou seja, a eliminação de H^+ ocorre de forma acoplada à absorção de Na^+, e a eliminação de HCO_3^- acoplada à absorção de Cl^- (Fig. 2 - Henry 1996; Henry et al. 2012; Weihrauch and O'Donnell 2015). O transporte de H^+ e HCO_3^- causa alteração de pH interno dos animais, sendo relacionado ao equilíbrio ácido-base. A eliminação de H^+ eleva o pH interno, e o oposto ocorre para o HCO_3^- (Henry 1996; Henry et al. 2012). A função osmorregulatória desta enzima se relaciona ao transporte (absorção) de Na^+ e/ou Cl^-, que causa aumento da concentração interna do animal (Henry 1984; Lucu 1990; Henry 1996; Henry et al. 2012). Há diferenças funcionais entre a anidrase carbônica citoplasmática e a associada à membrana (Bundy 1977) e este processo de captação iônica se dá pela ação da anidrase carbônica citoplasmática das células branquiais (Henry 1988; Böttcher and Siebers 1993). Além disso, a reação reversa, de desidratação do HCO_3^-, tem como produtos CO_2 e água. O íon HCO_3^- é pouco permeável às membranas, então o CO_2 é eliminado das células na sua forma molecular não hidratada (CO_2 e não HCO_3^-). Esta é também uma demanda à anidrase carbônica (Bundy 1977; Farrelly and Greenaway 1994; Henry 1996; Henry et al.2012). Neste contexto, o objetivo deste trabalho foi o de relacionar a atividade da anidrase carbônica, gradientes osmóticos e iônicos e capacidade de manutenção de volume celular / tecidual de invertebrados à ocupação de novos ambientes (dulcícola e terrestre).

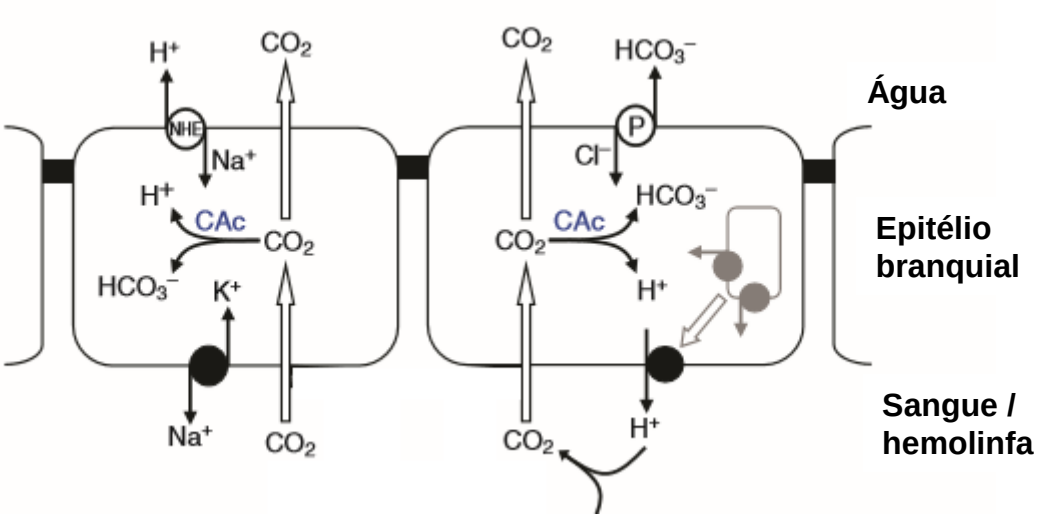

Figura 2. Esquema de funcionamento da enzima anidrase carbônica (CAc). Adaptado de Gilmour and Perry (2009).

REFERÊNCIAS

Anger, K. (2001). The biology of decapod crustacean larvae (Vol. 14, pp. 1-420). Lisse: AA Balkema Publishers.

Böttcher, K., & Siebers, D. (1993). Biochemistry, localization, and physiology of carbonic anhydrase in the gills of euryhaline crabs. Journal of Experimental Zoology, 265(4), 397-409.

Bundy, H. F. (1977). Carbonic anhydrase. Comparative Biochemistry and Physiology Part B: Comparative Biochemistry, 57(1), 1-7.

De Vooys, C. G. N. (1991). Anaerobic metabolism in sublittoral living *Mytilus galloprovincialis* in the Mediterranean—IV. Role of amino acids in adaptation to low salinities during anaerobiosis and aerobiosis. Comparative Biochemistry and Physiology Part A: Physiology, 100(2), 423-431.

Deaton, L. (2008). 4 Osmotic and Ionic Regulation in Molluscs. Osmotic and Ionic Regulation: Cells and Animals, 107.

Diehl, W. J. (1986). Osmoregulation in echinoderms. Comparative Biochemistry and Physiology Part A: Physiology, 84(2), 199-205.

Evans, D. H. (Ed.). (2008). Osmotic and ionic regulation: cells and animals. CRC Press.

Farrelly, C., & Greenaway, P. E. T. E. R. (1994). Gas exchange through the lungs and gills in air-breathing crabs. Journal of Experimental Biology, 187(1), 113-130.

Freire, C. A., Cavassin, F., Rodrigues, E. N., Torres, A. H., & McNamara, J. C. (2003). Adaptive patterns of osmotic and ionic regulation, and the invasion of fresh water by the palaemonid shrimps. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 136(3), 771-778.

Freire, C. A., Onken, H., & McNamara, J. C. (2008). A structure–function analysis of ion transport in crustacean gills and excretory organs. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 151(3), 272-304.

Freire, C. A., Souza-Bastos, L. R., Amado, E. M., Prodocimo, V., & Souza, M. M. (2013). Regulation of muscle hydration upon hypo-or hyper-osmotic shocks: differences related to invasion of the freshwater habitat by decapod crustaceans. Journal of Experimental Zoology Part A: Ecological Genetics and Physiology, 319(6), 297-309.

Gilmour, K. M., & Perry, S. F. (2009). Carbonic anhydrase and acid–base regulation in fish. Journal of Experimental Biology, 212(11), 1647-1661.

Henry, R. P. (1988). Multiple functions of carbonic anhydrase in the crustacean gill. Journal of Experimental Zoology, 248(1), 19-24.

Henry, R. P. (1996). Multiple roles of carbonic anhydrase in cellular transport and metabolism. Annual Review of Physiology, 58(1), 523-538.

Henry, R. P. (1984). The role of carbonic anhydrase in blood ion and acid-base regulation. American Zoologist, 24(1), 241-251.

Henry, R. P., Lucu, C., Onken, H., & Weihrauch, D. (2012). Multiple functions of the crustacean gill: osmotic/ionic regulation, acid-base balance, ammonia excretion, and bioaccumulation of toxic metals. Frontiers in physiology, 3.

Henry, R. P., & Saintsing, D. G. (1983). Carbonic anhydrase activity and ion regulation in three species of osmoregulating bivalve molluscs. Physiological Zoology, 274-280.

Hill, R. W., Wyse, G. A. & Anderson, M. (2008). Animal Physiology. Sinauer Associates Inc.

Lee, C. E., & Bell, M. A. (1999). Causes and consequences of recent freshwater invasions by saltwater animals. Trends in Ecology & Evolution,14(7), 284-288.

Lucu, Č. (1990). Ionic regulatory mechanisms in crustacean gill epithelia. Comparative Biochemistry and Physiology Part A: Physiology, 97(3), 297-306.

Pequeux, A. (1995). Osmotic regulation in crustaceans. Journal of Crustacean Biology, 15(1), 1-60.

Preston, R. L. (2008). 5 Osmoregulation in Annelids. Osmotic and Ionic Regulation: Cells and Animals, 135.

Pritchard, D. W. (1967). What is an estuary: physical viewpoint. American Association for the Advancement of Science.

Weihrauch, D., & O'Donnell, M. J. (2015). Links between osmoregulation and nitrogen-excretion in insects and crustaceans. Integrative and comparative biology, 55(5), 816-829.

Willmer, P., Stone, G., & Johnston, I. (2009). Environmental Physiology of Animals. John Wiley & Sons.

CAPÍTULO 1

The relationship between carbonic anhydrase activity and the occupation of diluted environments by invertebrates

Abstract

All animal phyla evolved in the sea, and some conquered freshwater. This change of environment represents the major evolutionary transition and one of the most drastic biological processes from the physiological point of view, and, therefore, required adaptive physiological mechanisms of the organisms. Carbonic anhydrase (CA) is an enzyme with several functions related to osmoregulation, respiration and acid-base balance, and as such, may represent an important research tool of the evolutionary trajectory mentioned above. The objective of this chapter was test if there is a relationship between the occupation of diluted environments by invertebrates, and carbonic anhydrase activity (CAA) and other osmoregulatory mechanisms in animals of different zoological groups. Marine, estuarine, and freshwater species of invertebrates from the groups Echinodermata (marine only), Mollusca, and Crustacea were exposed to salt stress. Subsequently, osmolality and ionic concentrations of sodium, chloride, magnesium and potassium were measured in their body fluids, and water content and CAA were measured in their cells and/or tissues. As expected, the lower osmotic and ionic gradients between body fluid and environmental water were found in marine species, followed by estuarine and by freshwater species. There is also a clear distinction between the magnitude of gradients among zoological groups, and the crustaceans maintained the widest gradients, followed by the mollusks and then by the echinoderms. The tissue water content was kept stable or was at least partially regulated in all species, but it should be noted that euryhaline conforming species require (and often have) greater efficiency in maintaining tissue hydration. Mechanisms of maintenance of gradients between the environmental water and the body fluids are primarily used than that related to tissue volume maintenance in the most successful zoological groups to conquer diluted environments. CAA was lower in the estuarine species in relation to the marine and freshwater ones of the same zoological group. The high enzymatic activity in marine species probably exerts an acid-base balance function. In estuarine and freshwater species, this activity may be associated to this same function, but also to osmoregulation, as their low permeability to water and ions allow maintenance of gradients generated. Thus, this comparative analysis led us to conclude that: 1) CAA is higher in dwellers of extreme salinities (marine and freshwater) than in the intermediate (estuarine); 2) osmotic / ionic

gradients are more frequent and intense in groups with success in the conquest of freshwater; 3) the maintenance of tissue volume is highly developed in osmoconformers, but is still present in osmoregulators.

1 Introduction

All 30 animal phyla evolved in the sea, and about 16 of them conquered freshwater (Lee and Bell 1999). This trajectory represents the biggest evolutionary transition, and one of the most drastic biological processes (Anger 1995; Lee and Bell 1999). This transition has been taking place along the macroevolutionary time, and even lately. Radiation and speciation of many animal groups began through the transition between marine and freshwater environments, in the shallow regions of continental borders (or island borders) (Lee and Bell 1999).

Freshwater populations may have arisen from various sources, from a unique source with subsequent dispersion, or by the combination of both. Freshwater invasion frequently occurs multiple and independently (e.g. in postglacial lakes), and has independent evolutionary trajectories. In addition similar evolutionary processes govern independent invasions and that events of gain and loss of characteristics occur in a same sequence in independent invasions (Lee and Bell 1999).

The occupation of transitional environments between sea and freshwater demanded osmoregulatory mechanisms that allowed the adaptation of the invertebrates to the new environment (Lee and Bell 1999; Hill et al. 2008; Willmer et al. 2009). These mechanisms result in energy waste, but they enable the maintenance of animal internal homeostasis, independently on the external (environmental) condition, and then they allowed the conquest of new environments, less stable than the sea (Lee and Bell 1999; Hill et al. 2008; Willmer et al. 2009). High degree of development of these mechanisms are found in osmoregulator animals, which maintain the internal concentrations (e.g. hemolymph) stable or constant and different from the environmental ones. Osmoregulation occur through the expenditure of metabolic energy to absorb salt from the diluted medium (active salt absorption against a concentration gradient), the reduction of permeability of epithelia to the influx of water and sodium efflux,

and the elimination of water excess via urine (Florkin 1962; Kirschner 1991; Lee and Bell 1999; Morris 2001; Freire et al. 2003; Deaton 2008; Freire et al. 2008; Hill et al. 2008; Willmer et al. 2009). In summary, current patterns of physiological response can reflect an evolutionary history of the species along an environmental gradient.

The transition between marine and diluted or freshwater environments offers, beyond a saline gradient, changes of other abiotic factors, such as pH, and O_2 concentration (Willmer et al. 2009). Therefore, the conquest of new environments also requires adaptive physiological mechanisms related to other cellular and / or organ functions (not just osmoregulatory), such as acid-base balance, and respiration. All these functions can be performed by the carbonic anhydrase (CA). This enzyme catalyzes the reversible CO_2 hydration reaction, whose products are proton (H^+) and bicarbonate (HCO_3^-) (Henry and Saintsing 1983; Henry 1988; Henry 1996). These ions are transported out of the cells through ion exchangers, Na^+/H^+ and Cl^-/HCO_3^-. The elimination of H^+ is coupled to Na^+ absorption, and the elimination of HCO_3^- is coupled to Cl^- absorption (Henry 1996; Henry et al. 2012; Weihrauch and O'Donnell 2015). The transport of H^+ and HCO_3^- causes changes of animal internal pH, being related to the acid-base balance. The secretion of H^+ results in a rise of internal pH, and the opposite occurs for HCO_3^- (Henry 1996; Henry et al. 2012). The osmoregulatory function of CA is related to the transport (absorption) of Na^+ and/or Cl^-, which causes increase of animal internal concentration (Henry 1984; Lucu 1990; Henry 1996; Henry et al. 2012). In addition, the reverse reaction, dehydration of H_2CO_3, results in CO_2 and water as products. Membranes are poorly permeable to HCO_3^- ion, thus it is eliminated from cells in its molecular format, as CO_2, and not in its hydrated form, as H_2CO_3. This is also a demand for CA (Bundy 1977; Farrelly and Greenaway 1994; Henry 1996; Henry et al. 2012).

Our aim here was to relate the occupation of diluted environments by invertebrates with the carbonic anhydrase activity (CAA) in animals of different zoological groups. It is known that CA exerts functions of osmoregulation (salt absorption) and acid-base balance (Henry 1984, 1988; Henry et al. 2012), and that the diluted environments have low concentration of salts, and present pH fluctuations (the high salt concentration acts as a buffer, and the opposite occurs in diluted waters) (Willmer et al. 2009). In this context, our hypothesis is that the

CAA would be higher in freshwater animals and would reduce along a growing saline gradient, with greater activities in zoological groups of greater success in the occupation of diluted environments.

2 Material and methods

2.1 Species and collection

Marine species of echinoderms, and marine, estuarine, and freshwater species of molluscs and crustaceans were studied (Table 1). IBAMA permission for collection, maintenance and manipulation of these animals is 20030-3, from March 22nd, 2013.

Table 1. Description of zoological group, environment, collection site, and collection method of studied species

Species	Zoological group	Environment	Collection site	Collection method
Arbacia lixula Linnaeus, 1758	Echinodermata	Marine	26°46'27"S 48°36'02"W Paciência Beach, Penha-SC	Manual
Echinometra lucunter Linnaeus, 1758	Echinodermata	Marine	26°46'27"S 48°36'02"W Paciência Beach, Penha-SC	Manual
Holothuria (Halodeima) grisea Selenka, 1867	Echinodermata	Marine	26°46'27"S 48°36'02"W Paciência Beach, Penha-SC	Manual
Stramonita brasiliensis (Claremont and Reid 2011)	Mollusca	Marine	26°46'27"S 48°36'02"W Paciência Beach, Penha-SC	Manual
Perna perna Linnaeus, 1758	Mollusca	Marine	26°46'27"S 48°36'02"W Paciência Beach, Penha-SC	Bought (farming in lantern)
Mytella charruana Orbigny, 1842	Mollusca	Estuarine	25°30'57"S, 48°29'57"W Paranaguá Yacht Club, Paranaguá-PR	Manual
Rhipidodonta charruana Orbigny,1835	Mollusca	Freshwater	26°15'10"S 51°03'36"W Pintado River, União da Vitória-PR	Sieved
Litopenaeus schmitti (Pérez-Farfante and Kensley, 1997)	Crustacea	Marine	25°41'32"S 48°27'55"W Leste Beach, Pontal do Paraná-PR	Bought
Hepatus pudibundus (Herbst, 1758)	Crustacea	Marine	25°40′27"S 48°27′02"W Ipanema Beach, Pontal do Paraná-PR	Manual – discard of the by-catch fauna from the trawling fishing

Species	Zoological group	Environment	Collection site	Collection method
Palaemon pandaliformis Stimpson, 1871	Crustacea	Estuarine	25°34′23”S 48°21′03”W Pontal do Sul Estuary, Pontal do Paraná-PR	Sieved
Macrobrachium amazonicum (Heller, 1862)	Crustacea	Freshwater		Farmed and provided by Unesp - Jaboticabal
Macrobrachium rosenbergii (De Man, 1879)	Crustacea	Freshwater		Framed and provided by Unesp - Jaboticabal
Macrobrachium potiuna Müller, 1880	Crustacea	Freshwater	25°31’015”S 49°00’30” W Piraquara River, Piraquara-PR	Sieved
Aegla parana (Schmitt, 1942)	Crustacea	Freshwater	26°15’10”S 51°03’36”W Pintado River, União da Vitória-PR	Subber

2.2 Transport and aclimation

Echinoderms and molluscs were wrapped with seaweed and transported in styrofoam boxes to keep the humidity and the temperature. Crustaceans were transported in gallons, with water from the collection sites. All specimens were taken to the Laboratory of Comparative Physiology of Osmoregulation, Universidade Federal do Paraná, where they were acclimated for about 5 days. Marine animals were maintained at 35 psu, estuarines at 15 psu, and freshwater at 0 psu (filtered tap water), all at 20°C ± 2, constant aeration and filtration, and natural photoperiod (~12 h light: 12 h dark). The animals were not fed during acclimation.

2.3 Experiments

Animals were exposed to control (35 psu for marine animals, 15 psu for estuarine, and 0 psu for freshwater ones) or experimental salinities (30 psu for marine and 5 psu for estuarine and freshwater), at 20°C ± 2, with constant aeration, for 24 h. Experiments with marine and freshwater species were conducted with a narrow salinity challenge in relation to the estuarine species. We opted for this pattern of salinity range because estuarine species are more often exposed to salinity fluctuations in its environment than marine and freshwater species. After experiments the echinoderms, molluscs and *H. pudibundus* were crioanesthetized for 15 min, and the other crustaceans went through this process, but for a shorter period, ~2 to 5 min (see details in Table 2).

Waters for experiments were prepared from seawater, diluted by the addition of filtered tap water (no chlorine), or concentrated by the addition of sea salt. For the freshwater experiment, filtered tap water was used. Salinity was measured with a refractometer (Instrutherm), and osmotic and ionic concentrations of water were measured through the same methods described below for the body fluids of the animals.

Table 2. Experimental condition for each species.

Species	Average size	n° of individuals in each container / water volume in each container	Sampled material	Analysis (n)	Observations
A. lixula	Test diameter: 4 cm	1 / 3 l	Coelomic fluid	Osm (7-8), Na^+ (9), Cl^- (7), Mg^{++} (9), K^+ (9)	
			Esophagus	CAA (8)	
			Intestine	TWC (9), CA (9)	
			Coelomocytes	CAA (8)	Obtained through centrifugation (21380Xg for 5 min)
E. lucunter	Test largest diameter: 5.2 cm	1 / 3 l	Coelomic fluid	Osm (8-9), Na^+ (8-9), Cl^- (8-9), Mg^{++} (8-9), K^+ (8-9)	
			Esophagus	CAA (6)	
			Intestine	TWC (8-9), CAA (8-9)	
			Coelomocytes	CAA (6)	Obtained through centrifugation (21380Xg for 5 min)
H. (Halodeima) grisea	Weight: 55.3 g	1 / 3 l	Coelomic fluid	Osm (7-8), Na^+ (6-9), Cl^- (7-8), Mg^{++} (7), K^+ (6-9)	Body weight was adopted as a measure of body size because the length is variable in holothuroids

Species	Average size	n° of individuals in each container / water volume in each container	Sampled material	Analysis (n)	Observations
			Esophagus	CAA (8-9)	
			Intestine	TWC (8), CAA (8-9)	
			Coelomocytes	CAA (8-9)	Obtained through centrifugation (21380Xg for 5 min)
S. brasiliensis	Maximum shell length 3.5 cm	1 / 700 ml	Hemolymph	Osm (8), Na^+ (8), Cl^- (8), Mg^{++} (8), K^+ (6-7)	
			Foot muscle	TWC (8-9)	
			Gill	CAA (8-9)	
P. perna	Maximum shell length: 8.9 cm	1 / 700 ml	Hemolymph	Osm (8), Na^+ (8), Cl^- (8), Mg^{++} (8), K^+ (8)	
			Mantle cavity water[1]	Osm (7-8), Na^+ (8), Cl^- (7-8), Mg^{++} (7-8), K^+ (8)	
			Foot muscle	TWC (8)	
			Shell adutor muscle	TWC (8)	

Species	Average size	n° of individuals in each container / water volume in each container	Sampled material	Analysis (n)	Observations
			Gill	CAA (8)	
			Mantle edge	CAA (8)	
			Visceral mass	CAA (6-7)	
M. charruana	Maximum shell length: 4.4 cm	1 / 700 ml	Hemolymph	Osm (7-8), Na^+ (8), Cl^- (7-8), Mg^{++} (6-8), K^+ (8)	
			Mantle cavity water[1]	Osm (6-8), Na^+ (6), Cl^- (5-6), Mg^{++} (7-9), K^+ (6)	
			Foot muscle	TWC (8)	
			Shell adutor muscle	TWC (8)	
			Gill	CAA (8)	
			Mantle edge	CAA (8)	
R. charruana	Shell length: 3.3 cm	1 / 700 ml	Hemolymph	Osm (9-10), Na^+ (7-9), Cl^- (9-10), Mg^{++} (9-10), K^+ (7-9)	
			Mantle cavity water[1]	Osm (4-10), Na^+ (3-10), Cl^- (4-10), Mg^{++} (3-9), K^+ (4-10)	

Species	Average size	n° of individuals in each container / water volume in each container	Sampled material	Analysis (n)	Observations
			Foot muscle	TWC (10)	
			Gill	CAA (10)	
			Mantle edge	CAA (10)	
L. schmitti	Total length (rostrum to telson): 9.8 cm	1 / 3 l	Hemolymph	Osm (6), Na^+ (6), Cl^- (6), Mg^{++} (6), K^+ (6)	
			Abdominal muscle	TWC (6)	
			Gill	CAA (6)	
H. pudibundus	Test width: 5.1 cm	1 / 3 l	Hemolymph	Osm (8-9), Na^+ (8-10), Cl^- (9), Mg^{++} (9-10), K^+ (9-10)	
			Quelipod muscle	TWC (8)	
			Posterior gill	CAA (7-8)	
P. pandaliformis	Total length (rostrum to telson): 3.9 cm	4 / 700 ml	Hemolymph	Osm (8-9), Na^+ (6-7), Cl^- (6-9), Mg^{++} (4-8), K^+ (6-7)	Each hemolymph sample resulted from the pool of hemolymph from 4 individuals
			Abdominal muscle	TWC (10)	
			Gill	CAA (8)	

Species	Average size	n° of individuals in each container / water volume in each container	Sampled material	Analysis (n)	Observations
M. amazonicum	Total length (rostrum to telson): 5.7 cm	1 / 3 l	Hemolymph	Osm (7-8), Na^+ (3-6), Cl^- (8), Mg^{++} (7-8), K^+ (3-6)	
			Abdominal muscle	TWC (8)	
			Gill	CAA (8)	
M. rosenbergii	Total length (rostrum to telson): 10.7 cm	1 / 3 l	Hemolymph	Osm (8), Na^+ (8), Cl^- (7-8), Mg^{++} (8), K^+ (8)	
			Abdominal muscle	TWC (8)	
			Gill	CAA (8)	
M. potiuna	Total length (rostrum to telson): 4.1 cm	1 / 700 ml	Hemolymph	Osm (5-6), Na^+ (6-7), Cl^- (4-5), Mg^{++} (5), K^+ (6-7)	
			Abdominal muscle	TWC (10)	
			Gill	CAA (9-10)	
A. parana	Length (rostrum to abdomen fold): 3.4 cm	1 / 3 l	Hemolymph	Osm (7-8), Na^+ (8), Cl^- (8), Mg^{++} (8), K^+ (8)	
			Abdominal muscle	TWC (7-8)	

Species	Average size	n° of individuals in each container / water volume in each container	Sampled material	Analysis (n)	Observations
			Gill	CAA (8)	

Osm = osmolality dosage; Na^+ = sodium ion dosage; Cl^- = chloride ion dosage; Mg^{++} = magnesium ion dosage; K^+ = potassium ion dosage; TWC = tissue water content; CAA= carbonic anhydrase enzymatic activity measurement.

Samples of body fluids and of tissues for TWC were stored in freezer at -20°C. Samples of tissues for CAA were stored in freezer at -80°C.

[1]Mantle cavity water from bivalves were drained through a forced little opening of valves. Then, hemolymph was sampled through the insertion of an insulin syringe near the umbo.

2.4 Osmolality and ionic concentrations

Osmolality of body fluids was measured in vapour-pressure micro-osmometer (Wescor, VAPRO 5520), in non-diluted samples. Sodium and potassium dosages were performed in flame photometer (Micronal B462, Brazil), in samples diluted in deionized water. Chloride and magnesium dosages were conducted using colorimetric Labtest (Brazil) commercial kits, and absorbance was read in spectrophotometer (Ultrospec 2100 PRO Amersham Pharmacia Biotech).

2.5 Tissue water content (TWC)

For determination of TWC, each tissue fragment was weighted (Balance Bioprecisa, FA2104N, precision of 0.0001g) (wet weight), then dehydrated in stove at 60ºC for 24 h, and then weighted again (dry weight). TWC consists on the difference between wet and dry weights, expressed as a percentage of the initial weight (wet weight).

2.6 Carbonic anhydrase enzymatic activity (CAA)

Tissues were weighted and sonicated (20 s, at 1 pulse/s, in 50% amplitude - Fisher Scientific, Model FB120) in buffer (10% tissue weight/buffer volume). The homogenated was centrifuged at 2000xg for 5 min at 4°C (Hettich Zentrifugen Mikro 200R), and the supernatant was reserved for analysis. Carbonic anhydrase activity was determined through a protocol used by Vitale et al. (1999), and described by Souza-Bastos and Freire (2009). As a negative control of the method, one tissue type of one species of each zoological group (intestine of *H. (Halodeima) grisea*, gill of *R. charruana* and of *A. parana*) was used to conduct the same assay, but with acetazolamide, the specific enzymatic inhibitor. For this, samples were incubated in buffer with acetazolamide (100 µM final concentration in the sample – according to papers revised in Henry et al. 2012) for 10 min immediately prior the assay. The buffer used for both, samples homogenization and assay, is composed of mannitol 225 mM, sucrose 75 mM, Tris-phosphate 10 mM, and pH was adjusted for 7.4. Total protein concentration of each

homogenated sample, necessary to calculate carbonic anhydrase specific enzymatic activity, was measured through Bradford (1976) method.

2.7 Statistics

Each parameter (osm, Na^+, Cl^-, Mg^{++}, K^+, TWC, CAA) was compared in control and experimental salinities through t test (for data which achieved normality requirements), or through Mann-Whitney Rank Sum test (for data which did not achieve normality requirements). In addition, osm, Na^+, Cl^-, Mg^{++}, and K^+ were compared between internal fluids of animals and aquarium water through a 95% confidence interval. In bivalve molluscs these same tests were conducted to compare the concentrations (osm, Na^+, Cl^-, Mg^{++}, K^+) of hemolymph and mantle cavity water, for each salinity. CAA in different tissues of the same species, for each salinity, was compared through one way ANOVA with *post hoc* of Holm-Sidak (for data which achieved normality requirements) or through Kruskal-Wallis one way ANOVA *on Ranks* with *post hoc* of Dunn (for data which did not achieve normality requirements). CAA of each tissue in different species of each zoological group (Echinodermata, Mollusca, Crustacea) were compared, only for control salinity, through one way ANOVA with *post hoc* of Holm-Sidak or through Kruskal-Wallis one way ANOVA *on Ranks* with *post hoc* of Dunn. A Pearson correlation tested whether CAA is related to zoological group, species, habitat salinity, behavior of burrowing or shell closure (0 for animals which do not burrow or close shells, and 1 for animals which do it), osmotic change between salinity challenges (as a percentage of control value), and TWC change between salinity challenges (as a percentage of control value). Significance limit was always of 0.05.

3 Results

3.1 Osmolality and ionic concentrations

3.1.1 Osmolality

Osmolality in the coelomic fluid of the three species of echinoderms was lower in the experimental salinity (30 psu) than in the control (35 psu) (Fig. 1A). The same pattern was observed in the marine (*S. brasiliensis* and *P. perna*) and estuarine (*M. charruana*) molluscs. Osmolality of hemolymph of *R. charruana* was

higher in 5 psu than in 0 psu (Fig. 1B). In marine and estuarine crustaceans, hemolymph osmolality was lower in the saline challenge than in the control (35 psu and 15 psu, respectively), except for *L. schmitti*, which showed no differences between experimental and control salinities. Among freshwater species, only *M. rosenbergii* showed a higher hemolymph osmolality in 5 psu than in 0 psu. In the other freshwater species, hemolymph osmolality was not different in 5 and 0 psu (Fig. 1C).

With respect to the differences in the osmolality between animals and water, we observed that the values in animals were lower than in the water for *A. lixula* in 35 psu, and higher than in the water for *E. lucunter* in 30 psu (Fig. 1A). For molluscs, *S. brasiliensis* showed higher values than water in both salinities, and the same occurred for *P. perna* in 30 psu, for *M. charruana* in both salinities, and for *R. charruana* in 0 psu (Fig. 1B). For crustaceans, *L. schmitti* showed lower concentration than water in both salinities, in contrast with *H. pudibundus*, which showed higher concentration than water in 30 psu, and with the estuarine and the freshwater species, which showed higher concentration than in water for both salinities (Fig. 1C). In relation to differences between hemolymph and mantle cavity water (MCW) of bivalve molluscs, osmolality of hemolymph was higher than in the MCW in *M. charruana* at 15 psu, and in *R. charruana* at 0 psu (Table 3).

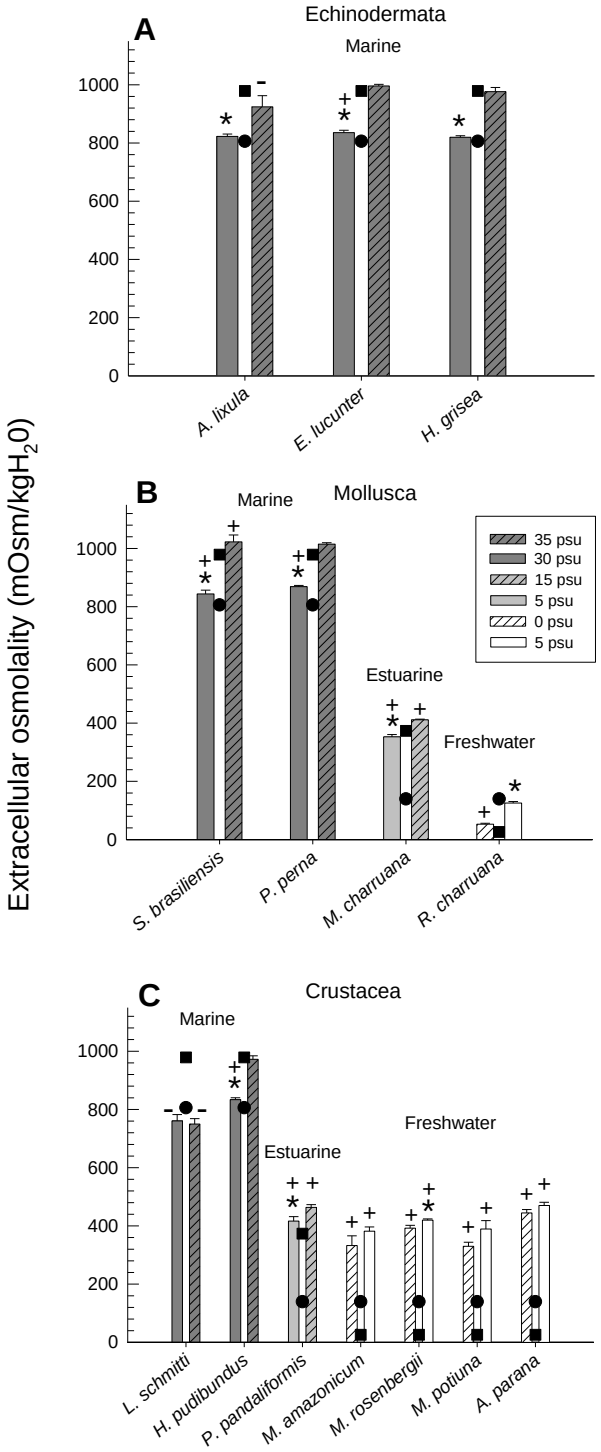

Figure 1. Osmolality of coelomic fluid of echinoderms (A), of hemolymph of molluscs (B), and of hemolymph of crustaceans (C). Marine species are represented by dark gray bars, estuarine by light gray bars, and freshwater by White bars. Experimental salinities (30, 5 and 5 psu, for marine, estuarine, and freshwater species, respectively) are represented by empty bars, and control salinities (35, 15 and 0 psu, for marine, estuarine, and freshwater species, respectively) by hatched bars. Black squares represent osmolality of water in control conditions, and black circles represent osmolality of environmental water in experimental conditions. Time of exposure to salinities was of 24 h. * = difference between experimental and control salinities. + = animal concentration > water concentration. - = animal concentration < water concentration. Error bars=standard error.

3.1.2 Na^+

Sodium concentration in coelomic fluid of echinoderms was lower in the experimental salinity (30 psu) than in the control (35 psu) (Fig. 2A). The same pattern was observed in marine (*S. brasiliensis* and *P. perna*) and estuarine (*M. charruana*) molluscs. In *R. charruana* hemolymph concentration of sodium was higher in 5 psu than in 0 psu (Fig. 2B). In marine and estuarine crustaceans, sodium concentration was lower in the experimental salinity (30 psu and 5 psu, respectively) than in the control (35 psu and 15 psu, respectively), except for *L. schmitti*, whose sodium concentration was not different between the saline challenge and the control. In *M. potiuna,* the concentration was higher in 5 psu than in 0 psu, but in the other freshwater crustaceans, sodium concentration was not different in the experimental salinity and in the control (Fig. 2C).

Echinoderms did not show differences in concentrations of sodium between coelomic fluid and water (Fig. 2A). *Perna perna* showed higher concentration than in water in 30 psu, similarly to *M. charruana* in 5 psu, and to *R. charruana* in 0 psu. In contrast, *R. charruana* showed lower concentration than in the water in 5 psu (Fig. 2B). For crustaceans, *L. schmitti* showed lower concentration in relation to water in both salinities. Conversely, *P. pandaliformis* showed higher concentration than in the water in 5 psu, as freshwater species did for both salinities (Fig. 2C). Sodium concentration was higher in the hemolymph than in MCW in the control of *M. charruana* and of *R. charruana* (15 psu and 0 psu, respectively) (Table 3).

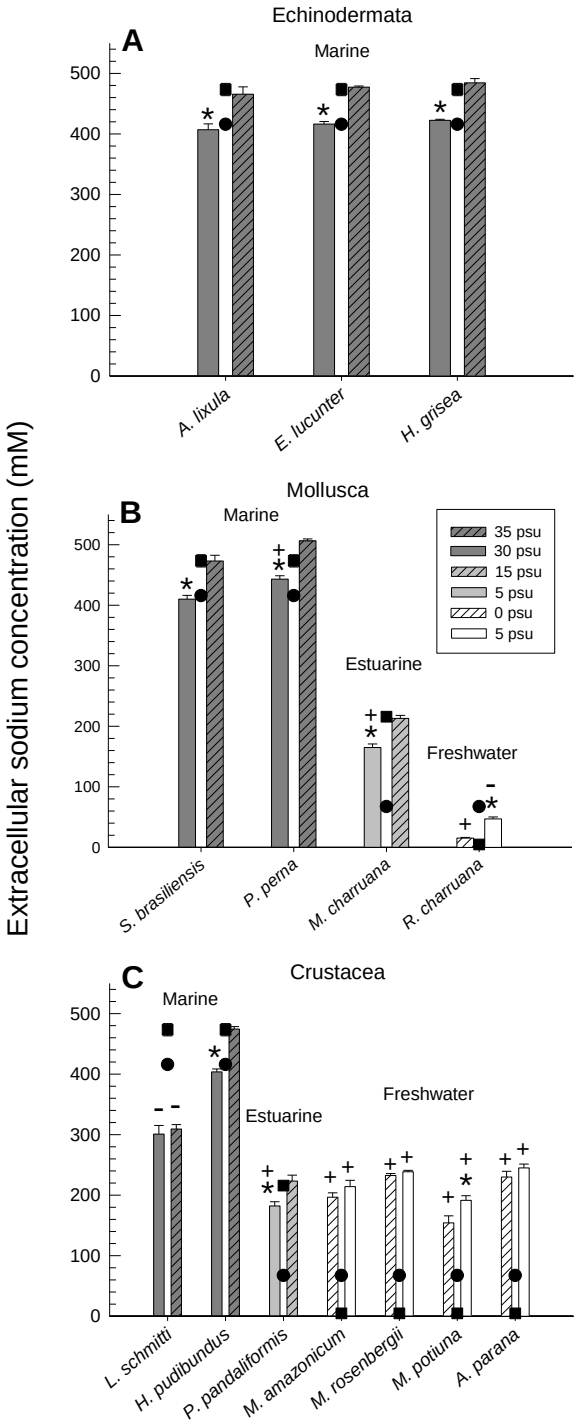

Figure 2. Sodium concentration of coelomic fluid of echinoderms (A), of hemolymph of molluscs (B), and of hemolymph of crustaceans (C). Marine species are represented by dark gray bars, estuarine by light gray bars, and freshwater by White bars. Experimental salinities (30, 5 and 5 psu, for marine, estuarine, and freshwater species, respectively) are represented by empty bars, and control salinities (35, 15 and 0 psu, for marine, estuarine, and freshwater species, respectively) by hatched bars. Black squares represent osmolality of water in control conditions, and black circles represent osmolality of environmental water in experimental conditions. Time of exposure to salinities was of 24 h. * = difference between experimental and control salinities. + = animal concentration > water concentration. - = animal concentration < water concentration. Error bars=standard error.

3.1.3 Cl^-

Chloride concentration of coelomic fluid of echinoderms was lower in the experimental salinity (30 psu) than in the control (35 psu) (Fig. 3A). The same pattern occurred for marine (*S. brasiliensis* and *P. perna*) and estuarine (*M. charruana*) molluscs. The freshwater mollusk showed a higher concentration in 5 psu than in 0 psu (Fig. 3B). The pattern verified in the echinoderms was also observed in the marine and in the estuarine crustaceans, except for *L. schmitti*, which showed no differences between experimental and control salinities. Among freshwater crustaceans, *M. rosenbergii* and *M. potiuna* showed higher concentration in 5 psu than in 0 psu. In the other freshwater crustaceans, chloride concentration was not different in experimental salinity (5 psu) and in the control (0 psu) (Fig. 3C).

Chloride concentration was lower in *A. lixula* than in the water in 30 psu, similarly to *H. (Halodeima) grisea* in both salinities (Fig. 3A). This pattern was also observed in *S. brasiliensis* and *P. perna* in 30 psu, and in *R. charruana* in 5 psu. The opposite occurred for *M. charruana* in 5 psu, and for *R. charruana* in 0 psu (Fig. 3B). The marine crustaceans (*L. schmitti* and *H. pudibundus*) showed lower concentration than water in both salinities, as also occurred for *P. pandaliformis* in 15 psu. The opposite pattern was observed for *P. pandaliformis* in 5 psu and in the freshwater species for both salinities (Fig. 3C). MCW of *P. perna* showed higher chloride concentration than hemolymph, in both salinities, 30 and 35 psu. In the other bivalve molluscs chloride concentration was not different in hemolymph and in MCW (Table 3).

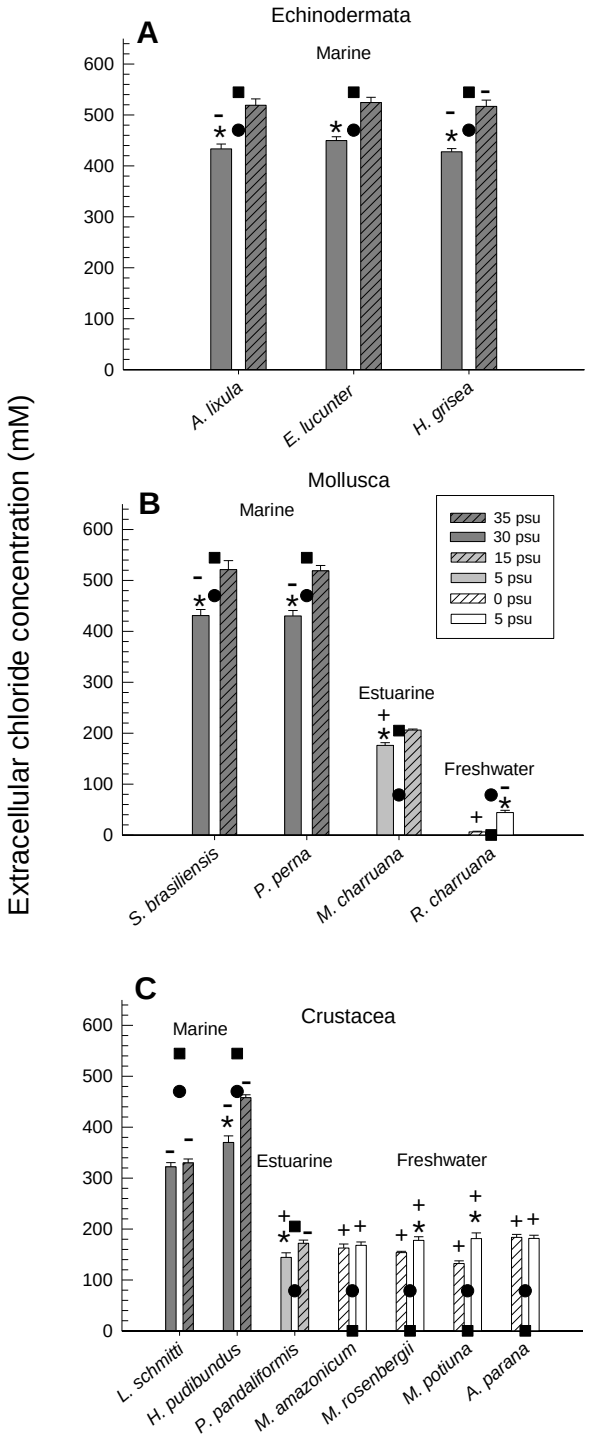

Figure 3. Chloride concentration of coelomic fluid of echinoderms (A), of hemolymph of molluscs (B), and of hemolymph of crustaceans (C). Marine species are represented by dark gray bars, estuarine by light gray bars, and freshwater by White bars. Experimental salinities (30, 5 and 5 psu, for marine, estuarine, and freshwater species, respectively) are represented by empty bars, and control salinities (35, 15 and 0 psu, for marine, estuarine, and freshwater species, respectively) by hatched bars. Black squares represent osmolality of water in control conditions, and black circles represent osmolality of environmental water in experimental conditions. Time of exposure to salinities was of 24 h. * = difference between experimental and control salinities. + = animal concentration > water concentration. - = animal concentration < water concentration. Error bars=standard error.

3.1.4 Mg^{++}

Magnesium concentration of the coelomic fluid of the echinoderms was lower in the saline challenge (30 psu) than in the control (35 psu) (Fig. 4A). Marine molluscs (*S. brasiliensis* and *P. perna*) followed the same pattern, but there were no differences between experimental and control salinities for *M. charruana*. *Rhipidodonta charruana* showed higher concentration in the experimental salinity (5 psu) than in the control (0 psu) (Fig. 4B). No differences in magnesium concentration were observed for *L. schmitti* between experimental and control salinities. *Hepatus pudibundus* and *P. pandaliformis* also followed the pattern of the echinoderms. Among freshwater crustaceans, only *M. potiuna* showed higher magnesium concentration in the experimental salinity (5 psu) than in the control (0 psu). In the other freshwater crustaceans, no differences were found between experimental and control salinities (Fig. 4C).

With respect to differences in magnesium concentration between animals and water, *A. lixula* and *E. lucunter* showed lower values than water in 30 psu (Fig. 4A). This same pattern was observed for the marine molluscs (*S. brasiliensis* and *P. perna*) exposed to 30 psu, for *M. charruana* in 15 psu, and for *R. charruana* in 5 psu. The opposite occurred for *M. charruana* in 5 psu and for *R. charruana* in 0 psu (Fig. 4B). Hemolymph concentration of *R. charruana* was higher than that of the MCW at 0 psu, and the opposite was observed at 5 psu (Table 3).

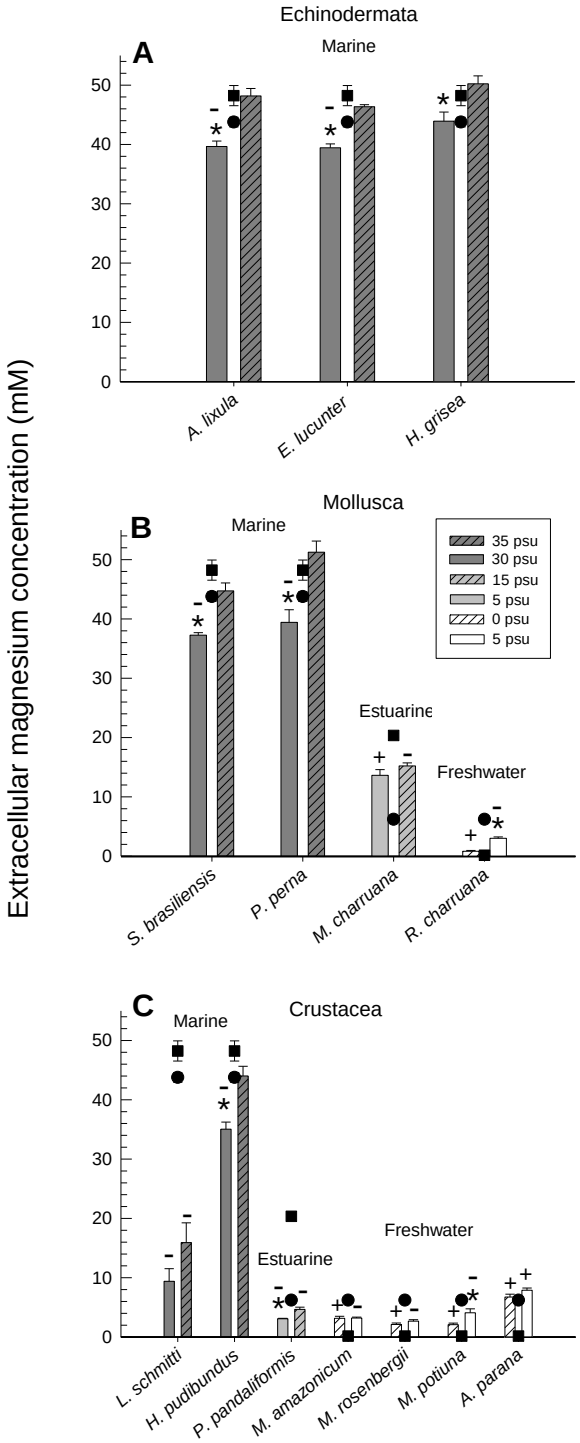

Figure 4. Magnesium concentration of coelomic fluid of echinoderms (A), of hemolymph of molluscs (B), and of hemolymph of crustaceans (C). Marine species are represented by dark gray bars, estuarine by light gray bars, and freshwater by white bars. Experimental salinities (30, 5 and 5 psu, for marine, estuarine, and freshwater species, respectively) are represented by empty bars, and control salinities (35, 15 and 0 psu, for marine, estuarine, and freshwater species, respectively) by hatched bars. Black squares represent osmolality of water in control conditions, and black circles represent osmolality of environmental water in experimental conditions. Time of exposure to salinities was of 24 h. * = difference between experimental and control salinities. + = animal concentration > water concentration. - = animal concentration < water concentration. Error bars=standard error.

3.1.5 K^+

Potassium concentration in the coelomic fluid of *A. lixula* and *H. (Halodeima) grisea* was lower in the experimental salinity (30 psu) than in the control (35 psu - Fig. 5A). The same was observed in the hemolymph of *P. perna*, no differences were found between experimental and control salinities for *S. brasiliensis* and *M. charruana*. The concentration was higher in 5 psu than in 0 psu for *R. charruana* (Fig. 5B). Potassium concentration of *L. schmitti* was not different between salinities 30 and 35 psu. However, in the hemolymph of *H. pudibundus* and *P. pandaliformis* this concentration was lower in the experimental salinities (30 and 5 psu, respectively) than in the controls (35 and 15 psu, respectively - Fig. 5C).

No differences were found between animal and water for echinoderms (Fig. 5A). For molluscs, *S. brasiliensis*, *P. perna*, and *M. charruana* showed higher concentration than water in both salinities (Fig. 5B). This same pattern was observed for *L. schmitti* in 30 psu, and for all other crustacean species in both salinities (Fig. 5C). Hemolymph concentration was higher than that of MCW for *P. perna* at 30 and 35 psu, and for *M. charruana* at 15 psu (Table 3).

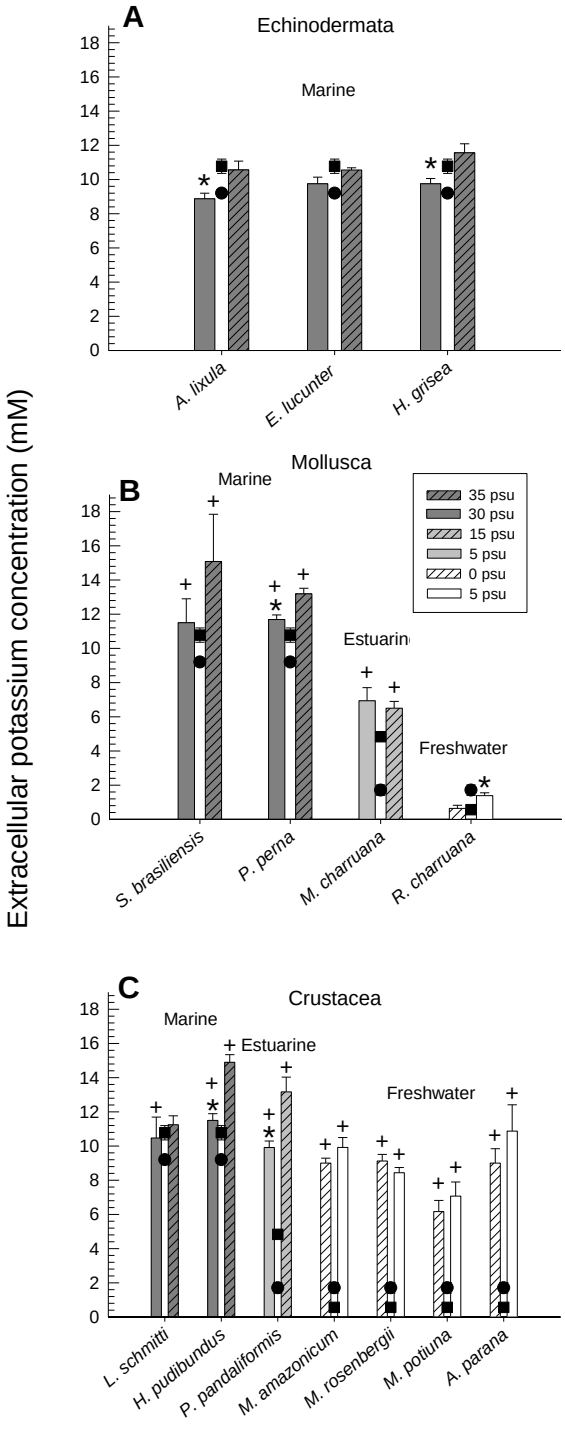

Figure 5. Potassium concentration of coelomic fluid of echinoderms (A), of hemolymph of molluscs (B), and of hemolymph of crustaceans (C). Marine species are represented by dark gray bars, estuarine by light gray bars, and freshwater by White bars. Experimental salinities (30, 5 and 5 psu, for marine, estuarine, and freshwater species, respectively) are represented by empty bars, and control salinities (35, 15 and 0 psu, for marine, estuarine, and freshwater species, respectively) by hatched bars. Black squares represent osmolality of water in control conditions, and black circles represent osmolality of environmental water in experimental conditions. Time of exposure to salinities was of 24 h. * = difference between experimental and control salinities. + = animal concentration > water concentration. - = animal concentration < water concentration. Error bars=standard error.

Table 3. Osmolality (mOsm.kgH_2O^{-1}) and ionic concentrations of Na^+, Cl^-, Mg^{++}, and K^+ (mM) assayed in the mantle cavity water of bivalve molluscs (average ± standard deviation). * animal fluids are different from water values.

	Salinity (psu)	Osmolality	Na^+	Cl^-	Mg^{++}	K^+
P. perna	30	878±43 n=8	443±19 n=8	521±38 n=8*	40±4.37 n=8	10±1.19 n=8*
	35	1042±41 n=7	488±43 n=8	616±27 n=7*	51±1.71 n=7	11±0.69 n=8*
M. charruana	5	337±25 n=6	177±22 n=5	163±41 n=5	14±3.47 n=7	5.58±2.46 n=6
	15	341±45 n=8*	175±34 n=6*	203±29 n=6	17±2.82 n=9	4.67±1.03 n=6*
R. charruana	0	39±8.23 n=4*	7.17±1.61 n=3*	2.58±1.12 n=4	0.18±0.10 n=3*	0.50±0.58 n=4
	5	124±24 n=10	45±9.65 n=10	55±13 n=10	5.39±1.17 n=9*	1.25±0.42 n=10

3.2 Tissue water content (TWC) determination

Intestinal TWC of echinoderms was not different in the saline challenge in relation to control (Fig 6A). Molluscan tissues (foot muscle, and shell adductor muscle) showed higher TWC in the low salinities than in the high salinities (Fig. 6B, C). In all crustacens species, TWC was not different in the experimental salinities in relation to the control (Fig. 6D).

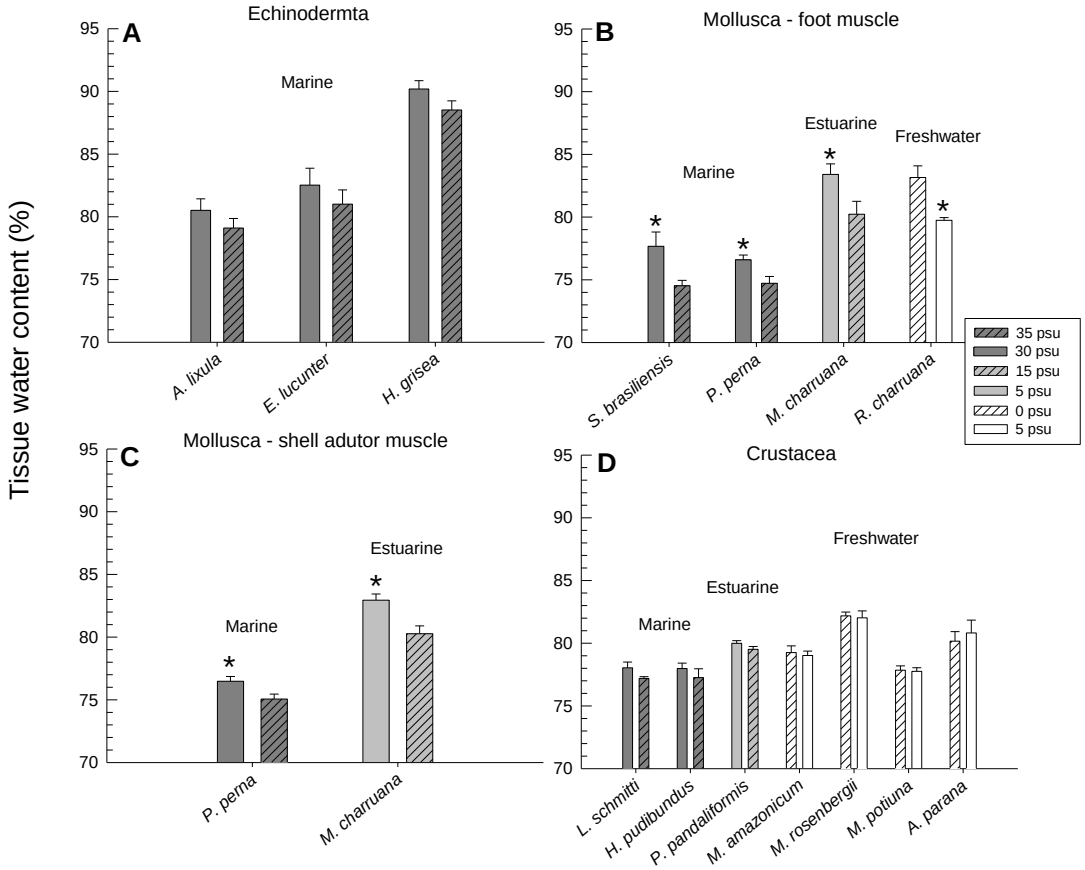

Figure 6. Tissue water content of intestine of echinoderms (A), foot muscle (B) and shell adutor muscle (C) of molluscs, and muscle of crustaceans (C – see details in Table 2). Marine species are represented by dark gray bars, estuarine by light gray bars, and freshwater by White bars. Experimental salinities (30, 5 and 5 psu, for marine, estuarine, and freshwater species, respectively) are represented by empty bars, and control salinities (35, 15 and 0 psu, for marine, estuarine, and freshwater species, respectively) by hatched bars. Time of exposure to salinities was of 24 h. * = difference between experimental and control salinities. Error bars=standard error.

3.3 Carbonic anhydrase enzymatic activity (CAA)

CAA, for all species and tissues, was not different in the saline challenge in relation to the control (Figs. 7, 8). Esophagus of *E. lucunter* showed higher CAA than the other tissues of this species(Fig. 7). Comparing level of CAA in control treatment of all species of each zoological group, we observed that in esophagus of echinoderms CAA was lower in *A. lixula* than in the other species (Fig. 7A). For intestine of echinoderms, *H. (Halodeima) grisea* showed higher

CAA than the other species (Fig. 7B). However, for coelomocytes, there were no differences of CAA between species (Fig. 7C).

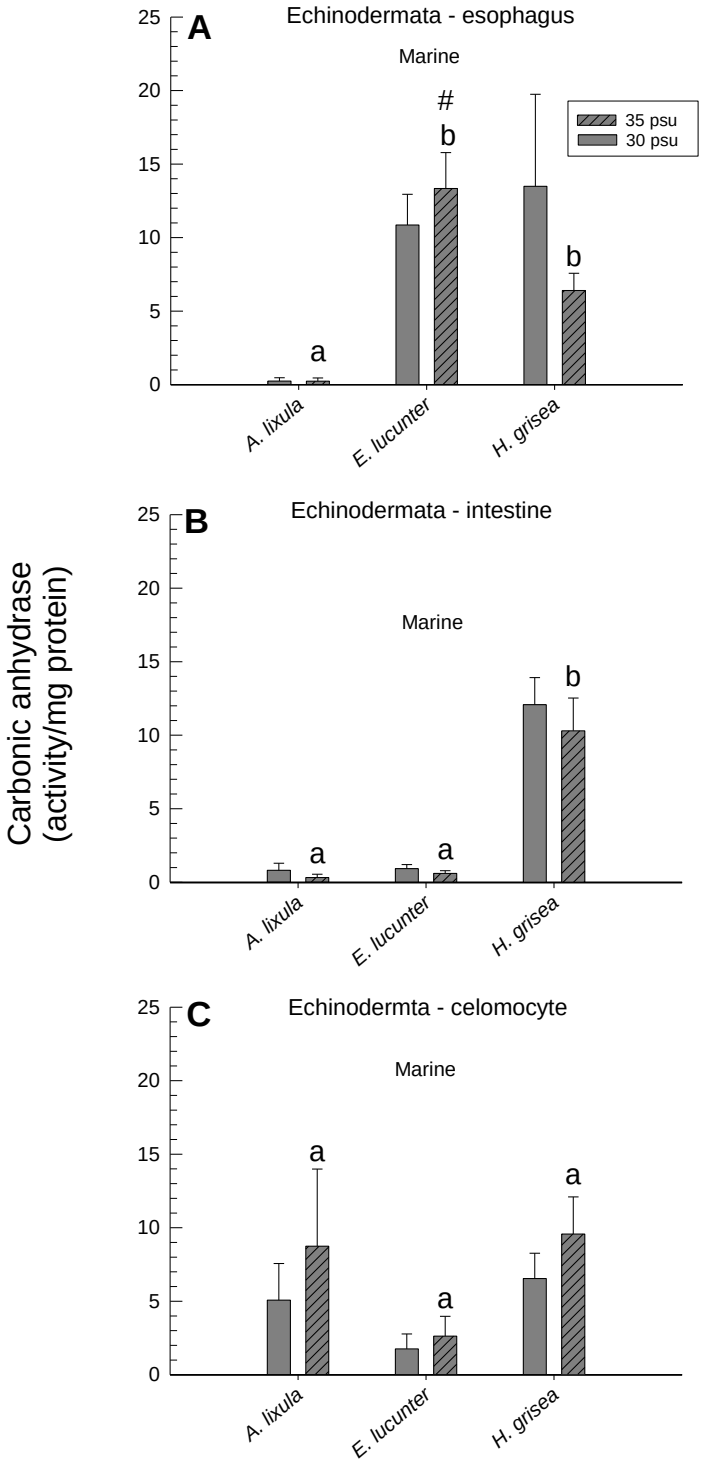

Figure 7. CAA in esophagus (A), intestine (B) and coelomocytes (C) of echinoderms exposed to experimental (30 psu, empty bars) and control (35 psu - control, hatched bars) salinities for 24 h. # = different from other tissues, within species. Lower case letters represent differences across species, for each tissue. Error bars=standard error.

In *R. charruana* CAA was higher in the gills than in the mantle. There were no differences between tissues in the other molluscan species (Fig. 8A, B). With respect to the molluscan gills, *S. brasiliensis* showed the highest CAA, followed by *R. charruana*, then by *M. charruana*, and finally by *P. perna* (Fig. 8A). There were no differences between species for mantle CAA (Fig. 8B). For crustaceans, the highest CAA was observed in *L. schmitti* and *A. parana*, followed for *H. pudibundus*, *M. rosenbergii*, and *M. potiuna*, and then by *P. pandaliformis* and *M. amazonicum* (Fig. 8C).

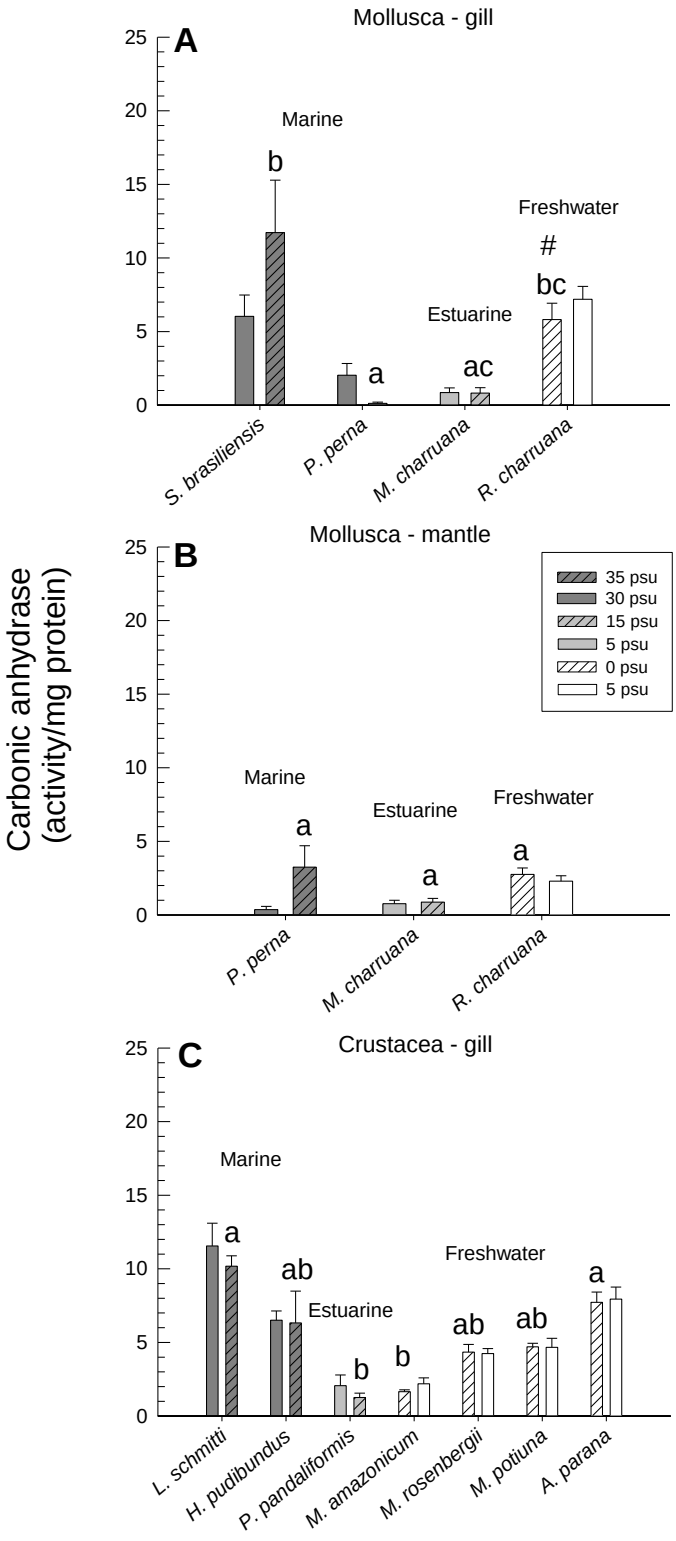

Figure 8. CAA in gills (A) and mantle (B) of molluscs, and of gills of crustaceans (C) in experimental (30, 5, and 5 psu for marine, estuarine, and freshwater species, respectively - empty bars) and control (35, 15, and 0 psu for marine, estuarine, and freshwater species, respectively - hatched bars) salinities. Time of exposure to salinities was of 24 h. # = different from other tissues, for each species. Lower case letters represent differences between species, for each tissue. Error bars=standard error.

CAA was inhibited in 87% for *H. (Halodeima) grisea*, 99.8% for *R. charruana*, and 98.7 for *A. parana*, in the presence of acetazolamide.

3.4 Pearson correlation

A positive correlation was identified between CAA and the behavior of burrowing or shell closure (correlation coefficient=0.413; $P=4.4x10^{-6}$), but no correlation was found with the other parameters.

4 Discussion

As expected, the lowest osmotic and ionic gradients between body fluid and environmental water were found in marine species (osmotic gradient of ~ 10.8% above or below water), followed by estuarine (osmotic gradient of ~ 77% above or below water) and then the broader gradients were observed in freshwater species (osmotic gradient of ~ 623% above water - ranging from 102% in *R. charruana* to 1598% in *A. parana*). TWC was kept stable or regulated, at least partially, in all species. With regard to CA, it was expected to find an ascending range of enzymatic activity in the species according to their habitat, from the sea (passing through the estuary) forward to freshwater, because the lower the concentration of salts the greater the demand for absorption of salts and for acid-base balance (high salt concentration acts as a buffer and maintains pH in a stable range) (Henry 1984, 1988; Willmer et al. 2009; Henry et al. 2012). However, the activity of this enzyme, in general, was low in the estuarine species in relation to the marine and freshwater species of the same zoological group. This result brings the following questions: what is the function of a high CAA in marine species? Why do estuarine species, inhabitants of a highly variable environment, need / use less carbonic anhydrase than marine species?

Although CAA have been observed in marine species, the osmotic and ionic gradients were rarely found in these animals, and their magnitude was low, which is expected for marine invertebrates, typically conformers (Robertson 1949; Evans 2008). As previously mentioned, it is known that the referred enzyme

exerts various functions, related not only to osmoregulation, but also to acid-base balance and respiration (Henry 1984, 1988; Henry et al. 2012). The CAA of these species may be related to the acid-base balance function of this enzyme (Henry et al. 2012). Most of the marine species here studied occupy microenvironments of their habitat, and this may be the reason to the rejection of the initial hypothesis. A possible explanation for this pattern, but specifically for echinoderms, is related to the catch connective tissue. This tissue has the property of changing its viscosity / rigidity. The variation in viscosity occurs under fluctuations in ionic composition and pH of the environment (e.g. Hayashi and Motokawa 1986; Motokawa 1994). Thus, a high CAA in echinoderms can be a resource to regulate the catch connective tissue viscosity. In relation to studied marine species of the three zoological groups, *Echinometra lucunter* inhabits crevices and burrows of rocks in the intertidal region (Lawrence and Kafri 1979, Santos-Gouveia and Freire 2007; Santos et al. 2013), *H. (Halodeima) grisea* buries itself in the sandy bottom of this same region (Castellano et al. 2016), *S. brasiliensis* and *P. perna*, also intertidal species, can occupy tide pools (Zardi et al. 2006; Veiga et al. 2016), and the marine crustaceans *L. schmitti* and *H. pudibundus* bury themselves in the sandy bottom of the sublittoral region (Dall et al. 1990; Magalhães et al. 2012; Furlan et al. 2013). This argument is consistent with the result of Pearson correlation. *A. lixula* represents an exception to this rule because it dwells the sublittoral adhered to the rocks. Microenvironments, such as tidal pools, may offer fluctuating conditions of abiotic factors, such as hypoxia (Henry et al. 2012). The microenvironments of all these species are likely to receive less water circulation and consequently a different oxygenation than the adjacent marine environment as a whole (out of the microenvironments) (Kristensen and Kostka 2005; Fluck et al. 2007). By being isolated or less exposed, animals are likely to have fluctuating respiratory rates (Truchot 1990), resulting in an accumulation of CO_2 during the period of insulation or of less exchange with environmental water. This CO_2, when hydrated, results in the production of carbonic acid and then, in the drop of internal pH of the animals (intra and extracellular) (Henry 1996; Gilmour and Perry 2009; Henry et al. 2012). This situation requires mechanisms of acid-base balance and, in this case, we hypothesize that the high CAA in marine studied species is associated with this role in the cells and tissues. This hypothesis is further supported by the fact that pH is stable in the sea, differently from the

estuary and freshwater (Willmer et al. 2009), thus marine species are not commonly challenged and nor accustomed with pH fluctuations. Then, in the microenvironments, they have a demand for acid-base regulation. In addition, CAA may be associated with the rapid elimination of CO_2 after periods of isolation, as already recorded in acid-base balance of oysters (Nielsen and Frieden 1972). This enzyme catalyzes CO_2 hydration / dehydration reactions, with formation of H^+ and HCO_3^-, and these ions can be secreted from the cells, adjusting intracellular pH (Henry 1996; Gilmour and Perry 2009; Henry et al. 2012). In accordance to our data, CAA was found to be similar in a marine and a freshwater flounders, which also bury themselves (Sender et al. 1999). *A. lixula* does not show this great demand to acid-base balance, because it dwells the sublittoral and does not occupy microenvironments, thus, supposedly because of it, its enzymatic activity is low. Although *P. perna* can isolate itself from the environment within its valves, the species presented low CAA. Probably because it occurs not only in the marine environment, but also in the estuarine (Davenport 1995; Abessa et al. 2005), *P. perna* presents a pattern of enzymatic activity similar to that of estuarine species, which will be discussed below. It is important to consider that the majority of marine species tested here dwells the intertidal region, which also offers challenges with fluctuations on abiotic factor. Anyway, the acid-base balance function of CA is coupled to the ionic transport, due to the elimination of H^+ and HCO_3^- ions, respectively, through the exchangers Na^+/H^+ and Cl^-/HCO_3^- (Henry 1996; Gilmour and Perry 2009). However, marine animals have high permeability to water and ion fluxes, so even if there is ionic absorption, the gradients are quickly dissipated (Diehl 1986; Rasmussen and Andersen 1996; Foster et al. 2010; Henry et al. 2012). In addition, invertebrates, even osmoregulators (e.g. crustaceans), tend to osmoconform in high salinities (35 psu in this case – Pèqueux 1995; Henry et al. 2012). Therefore, the high CAA in marine species studied is probably associated with acid-base balance, due to fluctuation in the respiratory rate of animals occupying microenvironments.

The relatively low CAA in estuarine animals may be related to behavioral strategies of avoidance, allied to the physiological mechanisms used by the species when they are in hostile salinities. The estuarine species here studied can, hypothetically, in the short term (e.g. tidal cycle), tolerate changes of abiotic factors, and/or minimize diffusive fluxes between their internal medium (i.e.

hemolymph) and the environmental water. It was observed in *M. acanthurus* (Freire et al. unpublished data), through reduction of branchial perfusion, and we can hypothesize that *P. pandaliformis* can also perform it. In *M. charruana* and *P. perna,* it occurs through valves closure, as already reported for other bivalve molluscs (Davenport and Wond 1986; Berger and Kharazova 1997; Mirjana 2006). Although the low CAA, it was enough to generate the osmotic and ionic gradients observed between external (environmental water) and internal (body fluid) media in the estuarine species. Perhaps the gradients were generated not only by CAA, but also by other osmoregulatory mechanisms, such as Na^+, K^+-ATPase activity and also the avoidance behaviors cited above, both not tested here. Thus, behavioral strategies (avoidance), not just physiological (osmoregulatory mechanisms), allow species to inhabit the estuary, a variable and challenging environment.

The freshwater species, as expected, showed high enzymatic activity, accompanied by wide osmotic and ionic gradients between the external and internal environments of the animals. These data are consistent with each other, since a high activity results in salt absorption and then in gradients (Henry 1996; Henry et al. 2012). The role of CA in acid-base balance is probably also being performed in these freshwater species because, as mentioned earlier, the osmoregulatory and acid-base balance functions are coupled (Henry 1996; Gilmour and Perry 2009). In addition, freshwater animals tend to be less permeable to water and ions flow, and thus, unlike marine species, the osmotic and ionic gradients are maintained (Florkin 1962; Kirschner 1991; Rasmussen and Andersen 1996; Freire et al. 2008; Willmer et al. 2009).

Data of TWC showed that all species have high capacity to regulate tissue hydration. Echinoderms and crustaceans kept the TWC unchanged in the salt challenge (dilution) in relation to control. However, the molluscs also had the capacity to maintain tissue volume, as an average change of 3.34% was observed in the TWC under saline challenge in relation to the control. In addition, molluscs are typically osmoconformer organisms (marine and estuarine species) or weak regulators (freshwater species) (Henry and Saintsing 1983; Deaton 2008), therefore their tissues are subject to wide variations in hemolymph osmotic concentration (17% in *S. brasiliensis*, 14% in *P. perna*, 17% in *M. charruana*, 137% in *R. charruana*) in the saline challenge in relation to the control. Then, it is

noteworthy that the TWC variation (1.41-3.41%) in the saline challenge in relation to control was proportionally lower than the osmotic alteration of the hemolymph, which demonstrates the good tissue hydration maintenance capacity of the group. The corresponding osmotic changes observed in echinoderms (~ 11 to 16%) and crustaceans (~ 6 to 18%) were lower than those of mollusks, which indicates that maintenance of tissue hydration is a somewhat less expensive task for these two groups than it is for molluscs.

Osmoregulator species, especially those adapted to freshwater, use physiological mechanisms related to maintenance of osmotic and ionic gradients more than mechanisms of regulation / maintenance of tissue / cellular hydration. The opposite is observed for the osmoconformer species, which occupy saline waters (sea and estuary) (Augusto et al. 2007a, b; Freire et al. 2008; Foster et al. 2010). Marine invertebrates (and some estuarine, as molluscs) are osmoconformers and, therefore, their internal medium (i.e. hemolymph, coelomic fluid) change when animals face environmental saline fluctuations, thus their tissues and cells are osmotically challenged (Diehl 1986; Pèqueux 1995; Deaton 2008; Foster et al. 2010). Osmoregulators, oppositely, maintain their internal medium in a relatively constant concentration even under salinity variations in the external water, and then they have lower demand to mechanisms related to tissue volume regulation (Péqueux 1995; Foster et al. 2010; Freire et al. 2013). However, it is important to remember that the mechanisms of volume regulation are the most primitive and contribute to the euryhalinity of the species and conquest of new environments (Florkin and Schoffeniels 1969 apud Freire et al. 2013; Diehl and Lawrence 1985; Péqueux 1995; Freire et al. 2013). This pattern of response is also observed in our data. We observed capacity to maintain tissue hydration in all species, but osmotic and ionic gradients were wider in freshwater species than in the estuarines. In marine species, these gradients were narrow and rarely observed. Moreover, this tendency is also present between zoological groups of studied species, according to the general osmoregulatory pattern and the degree of conquest of diluted waters of each group. Groups with greater success in the conquest of the freshwater environment use mechanisms to maintain osmotic gradients, and whether this conquest has occurred long evolutionary time, the mechanisms of tissue volume regulation tend to be supplanted by osmotic and ionic regulation (Augusto et al. 2007a). This difference

between zoological groups can be observed through the amplitude of the osmotic and ionic gradients maintained by the species studied. The largest gradients between extracellular fluid and environment were observed in crustaceans, followed by mollusks and, finally, by echinoderms. Coherently, crustaceans presents a higher percentage of freshwater species (16%) compared to molluscs (13%) and echinoderms (0%) (Ruppert et al. 2005; Grosberg et al. 2012).

CAA does not follow the pattern mentioned above. We can hypothesize that this is due to the fact that this enzyme probably evolved as an enzyme of facilitated transmembrane transport of CO_2 (respiratory function). Then, later in the evolution of the metazoan, the enzyme assumed a metabolic role, providing HCO_3^- to other regulatory and synthetic pathways (related to osmoregulation and metabolism) (Henry 1996). Then the activity of this enzyme does not reveal patterns of freshwater conquest by different groups of invertebrates, but reveals the different functions of this enzyme in each environment, already mentioned above. The great novelty of this study is the hard comparative view focused in osmoregulation of various zoological groups, to understand the physiological mechanisms related to the trajectory of conquest of diluted waters.

5 Conclusion

The comparative analysis conducted here is a novelty for literature, as a wide approach in terms of zoological groups and types of environments have not been reported before. Thus, this chapter led us to conclude that: 1) CAA is higher in dwellers of extreme salinities (marine and freshwater) than in the intermediate (estuarine), but with different functions; 2) osmotic / ionic gradients are more frequent and intense in groups with success in the conquest of freshwater; 3) the maintenance of tissue volume is highly developed in osmoconformers, but is still present in osmoregulators. In light of these conclusions, the initial hypothesis was partially confirmed, as crustaceans (the zoological group of greatest success in the occupation of diluted environments) and freshwater species, as a whole, showed high CAA.

Literature cited

Abessa, D. M. D. S., Zaroni, L. P., Sousa, E. C. P. M. D., Gasparro, M. R., Pereira, C. D. S., Rachid, B. R. D. F., ... & King, R. S. (2005). Physiological and cellular responses in two populations of the mussel *Perna perna* collected at different sites from the coast of São Paulo, Brazil. Brazilian Archives of Biology and Technology, 48(2), 217-225.

Anger, K. (1995). The conquest of freshwater and land by marine crabs: adaptations in life-history patterns and larval bioenergetics. Journal of Experimental Marine Biology and Ecology, 193(1), 119-145.

Augusto, A., Greene, L. J., Laure, H. J., & Mcnamara, J. C. (2007) a. Adaptive shifts in osmoregulatory strategy and the invasion of freshwater by brachyuran crabs: evidence from *Dilocarcinus pagei* (Trichodactylidae). Journal of Experimental Zoology Part A: Ecological Genetics and Physiology, 307(12), 688-698.

Augusto, A., Greene, L. J., Laure, H. J., & McNamara, J. C. (2007) b. The ontogeny of isosmotic intracellular regulation in the diadromous, freshwater palaemonid shrimps, *Macrobrachium amazonicum* and *M. olfersi* (Decapoda).Journal of Crustacean Biology, 27(4), 626-634.

Berger, V. J., & Kharazova, A. D. (1997). Mechanisms of salinity adaptations in marine molluscs. In Interactions and Adaptation Strategies of Marine Organisms (pp. 115-126). Springer Netherlands.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical biochemistry, 72(1-2), 248-254.

Bundy, H. F. (1977). Carbonic anhydrase. Comparative Biochemistry and Physiology Part B: Comparative Biochemistry, 57(1), 1-7.

Castellano, G. C., Santos, I. A., & Freire, C. A. (2016). Maintenance of ionic gradients and tissue hydration in the intertidal sea cucumber *Holothuria grisea* under hypo-and hyper-salinity challenges. Journal of the Marine Biological Association of the United Kingdom, 1-8.

Dall, W. H. B. J., Hill, B. J., Rothlisberg, P. C., & Sharples, D. J. (1990). The biology of the Penaeidae. Advances in marine biology, 27.

Davenport, R. (1995). *Perna perna* enters the bays. Texas Conchologist, 31, 92.

Davenport, J., & Wong, T. M. (1986). Responses of the blood cockle *Anadara granosa* (L.)(Bivalvia: Arcidae) to salinity, hypoxia and aerial exposure. Aquaculture, 56(2), 151-162.

Deaton, L. (2008). 4 Osmotic and Ionic Regulation in Molluscs. Osmotic and Ionic Regulation: Cells and Animals, 107.

Diehl, W. J. (1986). Osmoregulation in echinoderms. Comparative Biochemistry and Physiology Part A: Physiology, 84(2), 199-205.

Diehl, W. J., & Lawrence, J. M. (1985). Effect of salinity on the intracellular osmolytes in the pyloric caeca and tube feet of *Luidia clathrata* (Say)(Echinodermata: Asteroidea). Comparative Biochemistry and Physiology Part A: Physiology, 82(3), 559-566.

Evans, D. H. (Ed.). (2008). Osmotic and ionic regulation: cells and animals. CRC Press.

Farrelly, C., & Greenaway, P. E. T. E. R. (1994). Gas exchange through the lungs and gills in air-breathing crabs. The Journal of experimental biology, 187(1), 113-130.

Foster, C., Amado, E. M., Souza, M. M., & Freire, C. A. (2010). Do osmoregulators have lower capacity of muscle water regulation than osmoconformers? A study on decapod crustaceans. Journal of Experimental Zoology Part A: Ecological Genetics and Physiology, 313(2), 80-94.

Florkin, M. (1962). La régulation isosmotique intracellulaire chez les invertébrés marins euryhalins. Académie royale.

Flück, M., Webster, K. A., Graham, J., Giomi, F., Gerlach, F., & Schmitz, A. (2007). Coping with cyclic oxygen availability: evolutionary aspects. Integrative and comparative biology, 47(4), 524-531.

Freire, C. A., Cavassin, F., Rodrigues, E. N., Torres, A. H., & McNamara, J. C. (2003). Adaptive patterns of osmotic and ionic regulation, and the invasion of fresh water by the palaemonid shrimps. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 136(3), 771-778.

Freire, C. A., Onken, H., & McNamara, J. C. (2008). A structure–function analysis of ion transport in crustacean gills and excretory organs. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 151(3), 272-304.

Freire, C. A., Souza-Bastos, L. R., Amado, E. M., Prodocimo, V., & Souza, M. M. (2013). Regulation of muscle hydration upon hypo-or hyper-osmotic shocks: differences related to invasion of the freshwater habitat by decapod crustaceans. Journal of Experimental Zoology Part A: Ecological Genetics and Physiology, 319(6), 297-309.

Furlan, M., Castilho, A. L., Fernandes-Goes, L. C., Fransozo, V., Bertini, G., & COSTA, R. C. (2013). Effect of environmental factors on the abundance of decapod crustaceans from soft bottoms off southeastern Brazil. Anais da Academia Brasileira de Ciências, 85(4), 1345-1356.

Gilmour, K. M., & Perry, S. F. (2009). Carbonic anhydrase and acid–base regulation in fish. Journal of Experimental Biology, 212(11), 1647-1661.

Grosberg, R. K., Vermeij, G. J., & Wainwright, P. C. (2012). Biodiversity in water and on land. Current Biology, 22(21), R900-R903.

Hayashi, Y., & Motokawa, T. (1986). Effects of ionic environment on viscosity of catch connective tissue in holothurian body wall. Journal of Experimental Biology, 125(1), 71-84.

Henry, R. P. (1988). Multiple functions of carbonic anhydrase in the crustacean gill. Journal of Experimental Zoology, 248(1), 19-24.

Henry, R. P. (1996). Multiple roles of carbonic anhydrase in cellular transport and metabolism. Annual Review of Physiology, 58(1), 523-538.

Henry, R. P. (1984). The role of carbonic anhydrase in blood ion and acid-base regulation. American Zoologist, 24(1), 241-251.

Henry, R. P., & Cameron, J. N. (1982). The distribution and partial characterization of carbonic anhydrase in selected aquatic and terrestrial decapod crustaceans. Journal of Experimental Zoology, 221(3), 309-321.

Henry, R. P., Lucu, C., Onken, H., & Weihrauch, D. (2012). Multiple functions of the crustacean gill: osmotic/ionic regulation, acid-base balance, ammonia excretion, and bioaccumulation of toxic metals. Frontiers in physiology, 3.

Henry, R. P., & Saintsing, D. G. (1983). Carbonic anhydrase activity and ion regulation in three species of osmoregulating bivalve molluscs. Physiological Zoology, 274-280.

Hill, R. W., Wyse, G. A. & Anderson, M. (2008). Animal Physiology. Sinauer Associates Inc.

Kirschner, L. B. (1991). 2 Water and Ions. Comparative Animal Physiology, Environmental and Metabolic Animal Physiology, 1, 13.

Kristensen, E., & Kostka, J. E. (2005). Macrofaunal burrows and irrigation in marine sediment: microbiological and biogeochemical interactions. Interactions between macro-and microorganisms in marine sediments, 125-157.

Lawrence, J. M., & Kafri, J. (1979). Numbers, biomass, and caloric content of the echinoderm fauna of the rocky shores of Barbados. Marine Biology, 52(1), 87-91.

Lee, C. E., & Bell, M. A. (1999). Causes and consequences of recent freshwater invasions by saltwater animals. Trends in Ecology & Evolution,14(7), 284-288.

Lucu, Č. (1990). Ionic regulatory mechanisms in crustacean gill epithelia. Comparative Biochemistry and Physiology Part A: Physiology, 97(3), 297-306.

Magalhães, C. A., Taniguchi, S., Cascaes, M. J., & Montone, R. C. (2012). PCBs, PBDEs and organochlorine pesticides in crabs *Hepatus pudibundus* and *Callinectes danae* from Santos Bay, State of Sao Paulo, Brazil. Marine pollution bulletin, 64(3), 662-667.

Mirjana, H. B. (2006). The basket shell, *Corbula gibba* Olivi, 1792 (Bivalve Mollusks) as a species resistant to environmental disturbances: A review. Acta adriatica, 47(1), 49-64.

Morris, S. T. E. P. H. E. N. (2001). Neuroendocrine regulation of osmoregulation and the evolution of air-breathing in decapod crustaceans. Journal of Experimental Biology, 204(5), 979-989.

Motokawa, T. A. T. S. U. O. (1994). Effects of ionic environment on viscosity of Triton-extracted catch connective tissue of a sea cucumber body wall. Comparative Biochemistry and Physiology Part B: Comparative Biochemistry, 109(4), 613-622.

Nielsen, S. A., & Frieden, E. (1972). Carbonic anhydrase activity in molluscs. Comparative Biochemistry and Physiology Part B: Comparative Biochemistry, 41(3), 461-468.

Oglesby, L. C. (1969). Salinity—stress and desiccation in intertidal worms. American Zoologist, 9(2), 319-331.

Péqueux, A. (1995). Osmotic regulation in crustaceans. Journal of Crustacean Biology, 15(1): 1-60

Rasmussen, A., & Andersen, O. (1996). Apparent water permeability as a physiological parameter in crustaceans. The Journal of experimental biology, 199(12), 2555-2564.

Robertson, J. D. (1949). Ionic regulation in some marine invertebrates. Journal of Experimental Biology, 26(2), 182-200.

Ruppert, E. E., Fox, R. S., Barnes, R. D. Zoologia dos Invertebrados – Uma Abordagem Funcional-Evolutiva. São Paulo: Roca, 2005; 1145 pp

Santos, I. A., Castellano, G. C., & Freire, C. A. (2013). Direct relationship between osmotic and ionic conforming behavior and tissue water regulatory capacity in echinoids. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 164(3), 466-476.

Santos-Gouvea, I. A., & Freire, C. A. (2007). Effects of hypo-and hypersaline seawater on the microanatomy and ultrastructure of epithelial tissues of *Echinometra lucunter* (Echinodermata: Echinoidea) of intertidal and subtidal populations. ZOOLOGICAL STUDIES-TAIPEI-, 46(2), 203.

Sender, S., Böttcher, K., Cetin, Y., & Gros, G. (1999). Carbonic anhydrase in the gills of seawater-and freshwater-acclimated flounders *Platichthys flesus*: purification, characterization, and immunohistochemical localization. Journal of Histochemistry & Cytochemistry, 47(1), 43-50.

Souza-Bastos, L. R., & Freire, C. A. (2009). The handling of salt by the neotropical cultured freshwater catfish *Rhamdia quelen*. Aquaculture, 289(1), 167-174.

Truchot, J. P. (1990). Respiratory and ionic regulation in invertebrates exposed to both water and air. Annual review of physiology, 52(1), 61-74.

Veiga, M. P. T., Gutierre, S. M., Castellano, G. C., & Freire, C. A. (2015). Tolerance of high and low salinity in the intertidal gastropod *Stramonita brasiliensis* (Muricidae): behaviour and maintenance of tissue water content. Journal of Molluscan Studies, eyv044.

Vitale, A. M., Monserrat, J. M., Castilho, P., & Rodriguez, E. M. (1999). Inhibitory effects of cadmium on carbonic anhydrase activity and ionic regulation of the estuarine crab *Chasmagnathus granulata* (Decapoda, Grapsidae).

Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology, 122(1), 121-129.

Weihrauch, D., & O'Donnell, M. J. (2015). Links between osmoregulation and nitrogen-excretion in insects and crustaceans. Integrative and Comparative Biology, 55(5), 816-829.

Willmer, P., Stone, G., & Johnston, I. (2009). Environmental physiology of animals. John Wiley & Sons.

Zardi, G. I., Nicastro, K. R., McQuaid, C. D., Rius, M., & Porri, F. (2006). Hydrodynamic stress and habitat partitioning between indigenous (*Perna perna*) and invasive (*Mytilus galloprovincialis*) mussels: constraints of an evolutionary strategy. Marine Biology, 150(1), 79-88.

CAPÍTULO 2

Carbonic anhydrase: terrestriality, euryhalinity and refuges in Sesarmidae crabs

Abstract

Brachyuran lineages evolved in the sea and some conquered the land or live in semi terrestrial environments. This transition between environments demanded adaptive physiological mechanisms. Carbonic anhydrase (CA) is an enzyme with various physiological functions (osmoregulation, respiration, acid-base balance), being a tool to understand the relationship between physiology and change between environments. We aimed to relate physiological mechanisms to the degrees of terrestriality, euryhalinity and activity of the semi-terrestrial Sesarmidae crabs *Sesarma rectum* (Sr), *Armases rubripes* (Ar), *Aratus pisonii* (Ap), and *Armases angustipes* (Aa). We hypothesized that the most terrestrial, euryhaline and active species would have the highest carbonic anhydrase activity (CAA) and osmotic gradient between environment and hemolymph (OG). Crabs were collected and acclimated in 25 psu for constitutive analysis of hemolymph osmolality and of branchial CAA. Ar sustained the highest OG and displayed the highest CAA in posterior gills (osmoregulatory function), being one of the most aquatic, the most euryhaline and oligohaline among the four species. Ar and Ap showed the highest CAA in anterior gills (breathing and acid-base balance functions), probably because they are less protected from predation and temperature fluctuations than Sr and Aa. These results partially confirm our initial hypothesis, and, in summary, CAA and OG tend to be higher in aquatic, euryhaline and active species. The physiological focus is a novelty for the studied species. Our data were associated to ecological issues, which also brings a broad contribution to literature.

1 Introduction

Life arose in the sea, and all animal phyla (~ 30) evolved in this environment (Hill et al. 2008; Willmer et al. 2009). Among these phyla, only about seven conquered the terrestrial environment (Lee and Bell 1999). The transition between marine and terrestrial environments is a drastic process, and, among the main invertebrate taxa, decapod crustaceans have been successful in this process (Anger 1995). Within this group, Sesarmidae is one of the most successful families in the invasion of the terrestrial environment (and also the freshwater) (Anger 1995). Sesarmidae was previously classified as a subfamily, Sesarminae, within the Grapsidae family, became recognized as a family since

the publication of Schubart et al. 2000. These crabs frequently dwell transitional environments between the sea and freshwater or land, where they represent much of the diversity (Anger and Charmantier 2000; Schubart et al. 2000; Thiercelin 2015).

The marine environment is abundant in water and salts, and, conversely, the land is scarce in both resources (Bliss 1968; Willmer et al. 2009). Thus, on terrestrial environment there is a tendency for loss of water and salts (Bliss 1968; Wolcott 1992; Willmer et al. 2005). Therefore, osmoregulatory, morphological, behavioral, and physiological mechanisms were necessary to this environmental transition (Bliss 1968; Burggren and McMahon 1988). These mechanisms cover strategies related to the minimization of water loss, and absorption of salts against a concentration gradient. The loss of water is minimized by morphological adaptations, such as reduction of gill size, which results in reduction of the surface of exchange with the environment, with potential for transpiration (Bliss 1968; Burggren and McMahon 1988). In addition, exoskeleton of terrestrial crabs are less permeable to water flow than exoskeleton of aquatic species, which results in reduced water loss through transpiration through the carapace (Herreid 1969; Bliss 1979). The physiological mechanisms include salt absorption with consequent water conservation, and acid-base balance (Bliss 1979; Wheatly and Henry 1992). Salt can be obtained through diet, or reabsorbed from excreta, or absorbed from the aquatic environment (in semi-terrestrial species) (Bliss 1968; Burggren and McMahon 1988). In addition, since air has a higher concentration of oxygen than water, a reduction in the respiratory rate in terrestrial species in relation to the aquatic ones is observed. Then, a high efficiency to eliminate CO_2 is required and, acid-base regulation is necessary (Bliss 1979; Wheatly and Henry 1992).

Species can reach terrestrial and semi-terrestrial environments through water bodies of different salinities, from marine or even hypersaline, until freshwater (Burggren and McMahon 1988). The more terrestrial the species, the more variable will be the salinity (and other abiotic factors such as pH and O_2 concentration) of the available water supply, since it should use any water it finds in its environment (Bliss 1968). This is coherent with the success of sesarmid crabs in land conquest, as they belong in an euryhaline group, which occur from

0 to 60 psu, in permanent or temporary water bodies (Anger 1996). These situations also represent a demand for physiological mechanisms.

Among the physiological mechanisms, the carbonic anhydrase enzyme (CA) may be considered as a key element due to its functions in osmoregulation, respiration and acid-base balance. This enzyme catalyzes the reversible reaction of CO_2 hydration, whose products are the proton (H^+) and bicarbonate (HCO_3^-) ions (Henry and Saintsing 1983; Henry 1988; Henry 1996). These ions can be removed from the cells by ion exchangers, Na^+/H^+ and Cl^-/HCO_3^-. Thus, elimination of H^+ is coupled to Na^+ absorption, and elimination of HCO_3^- is coupled to Cl^- absorption. (Henry 1996; Henry et al. 2012; Weihrauch and O'Donnell 2015). The elimination of H^+ and HCO_3^- ions leads to changes in the internal pH of the animals, and the elimination of the first ion increases the internal pH, and the opposite occurs for the second ion (acid-base balance function) (Henry 1996; Henry et al. 2012). In addition, absorption of Na^+ and / or Cl^- leads to an increase in the internal concentration of the animal (osmoregulatory function) (Henry 1984; Lucu 1990; Henry 1996; Henry et al. 2012). Finally, the reverse reaction, dehydration (of H_2CO_3), results in CO_2 and water. As membranes are poorly permeable to HCO_3^-, CO_2 is eliminated from the cells (through simple diffusion) in their non-hydrated molecular form (CO_2 and not HCO_3^-), which also demands the catalytic action of CA (Bundy 1977; Farrelly and Greenaway 1994; Henry 1996; Henry et al. 2012).

The aim of this study was to relate physiological mechanisms to the different degrees of terrestriality and euryhalinity of four species of crabs of the family Sesarmidae. The null hypothesis was that the higher the degrees of terrestriality and / or euryhalinity of the species, the greater the carbonic anhydrase enzymatic activity (CAA) and, therefore, the greater the osmotic gradient between environmental water and hemolymph (OG).

2 Material and methods

2.1 Species

Four species of sesarmidae crabs were studied: *Sesarma rectum* Randall, 1840 (carapace width ~2.16 cm), *Armases rubripes* (Rathbun, 1897) (carapace

width ~1.07 cm), *Aratus pisonii* (H. Mile Edwards, 1837) (carapace width ~ 1.92 cm), and *Armases angustipes* Dana, 1852 (carapace width ~ 1.45 cm).

Sesarma rectum is found in the intertidal/upper shore, in riverbanks, estuaries, and mangroves (Anger 1995). The species occur in burrows, with water in the bottom (de Arruda Leme 2006; da Silva Castiglioni et al. 2011), and in environments with salinities between 13 and 37 psu (Schwamborn et al. 2001; Anger and Moreira 2004; da Silva Castiglioni et al. 2011). This species is also observed in salinities as low as ~5 psu, but they are mostly abundant between 15 and 25 psu (Marochi, MZ, personal communication December, 2016).

Armases rubripes occurs in the intertidal/upper shore of mangroves, on the substrate in bromeliads, in burrows made by themselves, between roots. The species is found in resting areas, near rivers outfalls, mainly associated to *Scirpus californicus* and to *Spartina* sp. in marshes (Capítoli et al. 1977; Anger 1995; Fischer et al. 1997; Lima et al. 2006). It occurs in salinities from 2 until 30 psu (Diaz and Ewald 1968; Capítoli et al. 1977; Monte et al. 1990; Luppi et al. 2003), but large individuals and abundances are observed in the range of 2-9 psu (Diaz and Ewald 1968; Capítoli et al. 1977; Monte et al. 1990; Marochi, personal communication).

Aratus pisonii dwells the upper shore of estuaries, especially in aerial portions of mangrove trees (mainly *Rizophorae mangle*), such as roots, trunks and branchs (Warner 1967; Diaz and Conde 1988; Anger 1995). The adults occur in salinities between 5 and 38 psu (Diaz and Conde 1989; Schwamborn et al. 2001; Marochi, personal communication), but the species reaches the highest abundances in the rage of 15-25 psu (Marochi, personal communication).

Armases angustipes is also an estuarine species, found in the stratum up the upper shore, in sandy, mudy and rocky substrata in mangrove margin, and near to river outfalls. It also occur in the edge of vegetation (mainly of mangroves) under dry leaves, in bromeliads, and under vegetation in the margin of rocky coasts (Abele 1992; De Melo 1996; Anger 1995). This species occur in salinities of ~18-37 psu (Schwamborn et al. 2001), but is also found, in lower abundance, in salinity ~5 psu (Marochi, personal communication), and also associated to the water of bromeliads (Cumberlidge et al. 2005). The species reaches high abundances when associated to water bodies of ~15-25 psu (Marochi, personal communication).

2.2 Collection, transport and acclimation

Animals (only males) were manually collected during low tide in a mangrove located in Guaratuba Bay, Guaratuba-PR (25°49’23”S 48°35’19”W), and transported in plastic containers, to the aquarium room of the Zoology Department of the Federal University of Paraná, Curitiba-PR. They were then individually acclimated for 9 days, in plastic containers containing about 150 ml of previously aerated saline water (25 psu), a straw and a rock so that the animals could remain out of the water. Crabs were fed daily, with carrot, leaves of *Rizophorae mangle*, or *Tenebrio*, alternately. Total water change was performed in this same frequency. The ambient temperature was maintained at 25°C ± 2.

2.2.1 Sampling

After acclimation period, animals were removed from containers, cryoanesthetized for 5 min, and samples were collected for analysis. Hemolymph was collected with the assistance of an insulin syringe, for osmolality dosage (n = 6-8 individuals of each species). Anterior (n = 5-7 individuals of each species, 3 pairs of gills) and posterior gills (n = 6-7 individuals of each species, 3 pairs of gills) were collected separately for the measurement of CAA. Some samples from containers waters were randomly collected for osmolality dosage (total n=4). Hemolymph and water samples from the aquaria were stored in a freezer at -20°C, and the gill samples were kept in a freezer at -80°C.

2.3 Osmolality

Osmolality of water and hemolymph samples was measured in a vapour-pressure micro-osmometer (Wescor, VAPRO 5520) in undiluted samples.

2.4 Carbonic anhydrase enzymatic activity (CAA)

Gills (anterior and posterior, separately) were weighed and sonicated (20 s, at 1 pulse/s, in 50% amplitude - Fisher Scientific, Model FB120) in buffer at a ratio of ~10% tissue weight/ buffer volume (with the exception of *A. rubripes* samples, which were sonicated in a standardized buffer volume of 100 µl, because they did not reach the minimum mass required). Then the homogenate

was centrifuged at 2000xg for 5 min at 4°C (Hettich Zentrifugen Mikro 200R), and the supernatant was reserved for analysis. CAA was determined through the protocol established by Vitale et al (1999), and described in Souza-Bastos and Freire (2009). As a negative control of the technique, the same assay was also performed, but with samples preincubated for 10 min in buffer with acetazolamide addition (final concentrations of 100 nM and 100 μM in the sample - concentrations used, respectively, by Mitsunaga et al., 1986; Henry et al., 2012), the inhibitor of CA (n = 5-7 of each species and of each acetazolamide concentration). 100 nM acetazolamide was used only in samples of *S. rectum* and *A. pisonii*, since the samples of the other species were not sufficiently abundant to conduct assay with two concentrations of the inhibitor. The use of acetazolamide represents a validation of the method, as a drop in pH (observed in the assay) is, solely, so vague to infer enzymatic activity. The homogenization buffer is composed of 225 mM mannitol, 75 mM sucrose, 10 mM Tris-phosphate, with pH adjusted to 7.4. The total protein concentration of each homogenate, required to calculate CAA, was obtained through the method of Bradford (1976).

2.5 Statistics

Osmolality data were compared between species through 1-way ANOVA, with Holm-Sidak *post hoc*. The osmolality values of the water were compared to those of the hemolymph of each species by 95% confidence interval. CAA data from each set of gills (anterior and posterior) were compared between species through 1-way ANOVA with Holm-Sidak *post hoc* (when normality requirements were met), or through Kruskal-Wallis with *post hoc* of Dunn (when normality requirements were not met). Differences in CAA between anterior and posterior gills of each species were analyzed through t-test (when the normality requirements were met), or through Mann-Whitney Rank Sum test (when the normality requirements were not met). T-test or Mann-Whitney Rank Sum test were performed to compare acetazolamide concentrations in each gill and species. Mann-Whitney Rank Sum test was performed to compare anterior and posterior gills in each species and acetazolamide concentration. 1-way ANOVA was performed to compare species in each gill and acetazolamide concentration. Significance limit was always of 0.05.

3 Results

Hemolymph osmolality was lower in *A. rubripes* than in the other species. The osmolality of hemolymph was higher than the water osmolality of all species, except for *A. rubripes*, whose hemolymph had lower osmolality than water (Fig. 1A). CAA of anterior gills was higher in *A. rubripes* and *A. pisonii* than in *S. rectum* and *A. angustipes*. The activity of this enzyme in the posterior gills was higher in *A. rubripes* than in the other species. The enzymatic activity was higher in the posterior than in the anterior gills in all species (Fig. 1B).

CAA was inhibited in the anterior and posterior gills of the four species under exposure to both tested acetazolamide concentrations, 100 μM (all species) and 100 nM (*S. rectum* and *A. pisonii*). The highest degrees of inhibition were observed under the highest inhibitor concentration, and generally in the posterior gills relative to the anterior ones (Table 1).

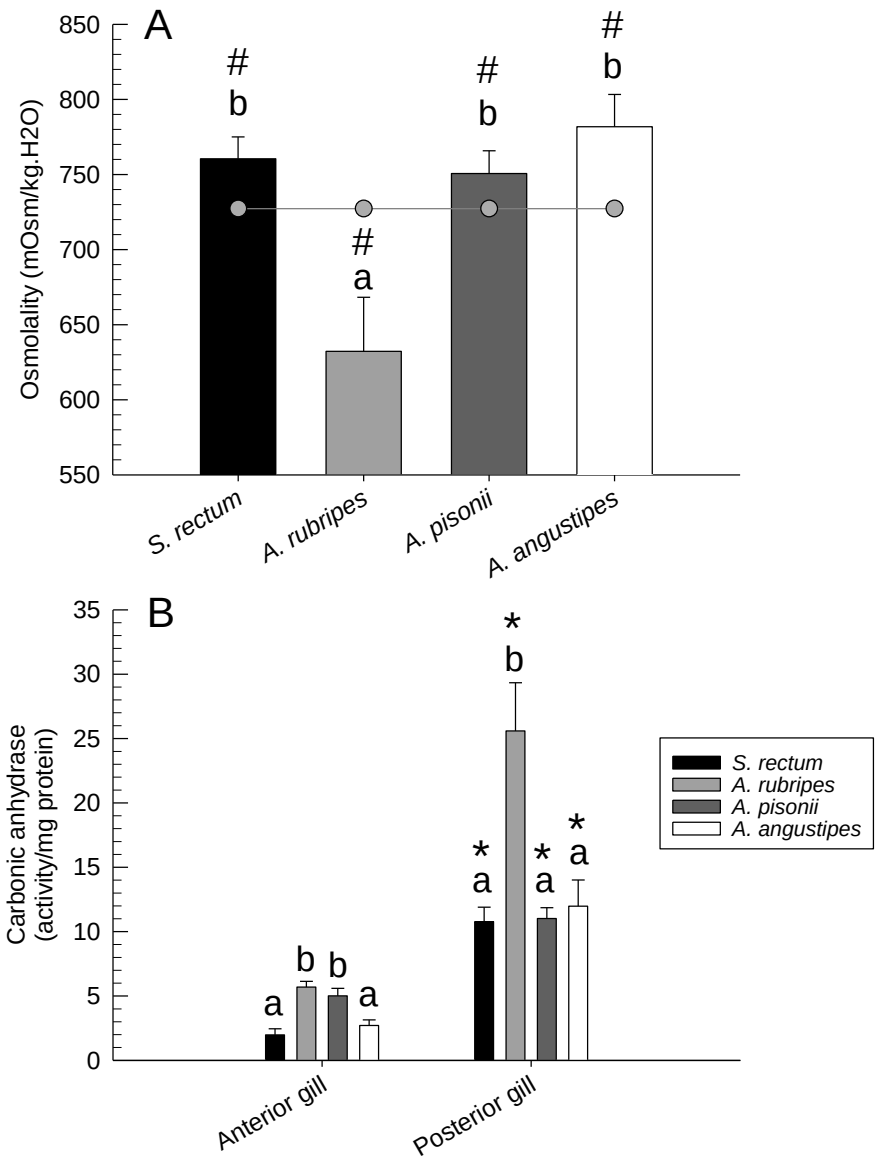

Figure 1. Osmolality of container water (line and scatter, n=4) and of hemolymph (bars, n=6-8) (A), and CAA in anterior and posterior gills (B) of *S. rectum* (black bars), *A. rubripes* (light gray bars), *A. pisonii* (dark gray bars), and *A. angustipes* (white bars), after 9 days of acclimation in 25 psu. Letters represent differences between species for each parameter (osmolality, CAA in anterior gills, CAA in posterior gills). # represent difference between osmolality of water and hemolymph. * represent difference between CAA in anterior and posterior gills (n=5-7). Error bars of water osmolality are smaller than the plotted symbols. Error bars=standard error.

Table 1. Inhibition of CAA in anterior and posterior gills of *Sesarma rectum, Armases rubripes, Aratus pisonii*, and *Armases angustipes*, under incubation with acetazolamide (100 μM and 100 nM).

	S. rectum		*A. rubripes*		*A. pisonii*		*A. angustipes*	
	Anterior gills	Posterior gills	Anterior gills	Posterior gills	Anterior gills	Posterior gills	Anterior gills	Posterior gills
Acetazolamide 100 μM	91.2%	99.5%[b]	96.3%	99.0%	100.0%	100.0%[b]	97.4%	98.5%
Acetazolamide 100 nM	67.9%	90.0%[a]	--	--	97.5%	94.1%[a]	--	--

Lower case letters represent differences between acetazolamide concentrations in the same gill. No differences were found between acetazolamide concentrations in anterior gills, nor between anterior and posterior gills in a same acetazolamide concentration, neither between species for each gill and acetazolamide concentration. --: not done.

4 Discussion

The initial hypothesis, according to which the higher the degrees of terrestriality and / or euryhalinity of the species, the greater the carbonic anhydrase enzymatic activity (CAA) and, therefore, the greater the osmotic gradient between environmental water and hemolymph (OG), was rejected in relation to terrestriality, and confirmed in relation to euryhalinity. All studied species maintained some osmotic gradient in relation to water (OG). The broadest gradient was that of *A. rubripes*, which maintained hemolymph 95 mOsm/kg.H_2O below the water concentration. In contrast, the other species maintained their internal concentration (hemolymph) above the external one (water), with lower gradients, of 23 mOsm/kg.H_2O in *A. pisonii*, 33 mOsm/kg.H_2O in *S. rectum*, and 55 mOsm/kg.H_2O in *A. angustipes*. The four species are very euryhaline (Anger 1995). However, unlike *A. rubripes*, the other three species are almost isosmotic to ambient water. This pattern probably occurred due to acclimation salinity, 25 psu, which is very close to the threshold (~26 psu) between osmoregulation and osmoconforming for most species (Henry 1996; Henry et al. 2003). That is, in salinities below ~26 psu species tend to keep their internal concentrations stable, even under environmental saline changes (osmoregulate), and, at salinity above this, they tend to remain isosmotic to water (osmoconform) (Henry 1996; Henry et al. 2003). Interestingly, *A. rubripes* shows a pattern of hyporegulation, that is, maintenance of hemolymph concentration below the water concentration, still at 25 psu. This indicates a greater osmoregulatory capacity of *A. rubripes* in relation to the other species. Perhaps *A. rubripes* is also less tolerant to the increase in the concentration of its internal environment, since it is often found in great abundance in waters of lower salinity (2-9 psu) in relation to those of the other species, frequently associated to *Scirpus californicus* (Diaz and Ewald 1968; Capítoli et al. 1977; Conde and Diaz 1989; Monte et al. 1990; Conde et al. 2000; Kowalkzuc and Masunari 2000; Luppi et al. 2003; Marochi, personal communication). In addition, a low internal concentration (i.e. hemolymph osmolality) represent a low cost of osmoregulation for dwellers of diluted waters, because just a short gradient must be sustained (Freire et al. 2003), as seem to be the case of *A. rubripes*.

CAA of posterior gills was higher in *A. rubripes* than in the other species. This enzyme has an osmoregulatory function in the posterior gills of decapod

crustaceans (Henry and Cameron 1983; Lucu 1990; Henry 1996; Henry et al. 2012). In general, CAA is associated with the absorption of Na^+ and Cl^- ions, and therefore a high activity results in high concentration of hemolymph (Henry 1984; Lucu 1990; Henry 1996; Henry et al. 2012). However, data presented indicate exactly the opposite: the highest CAA associated with the lowest osmolality value seems to mean that the enzyme may have a secretory function. A similar hypothesis has already been suggested when *Gecarcinus lateralis* was submitted to inhibition of CA (with acetazolamide). Its osmotic, sodium, chloride and calcium concentrations increased, and there was a high mortality rate (Henry and Cameron 1983). In *Aratus pisonii* females, the osmolality and the CAA have also shown inversely proportional magnitudes (high CAA associated with low osmolality - Marochi et al. unpublished data). Other protocols involving saline stress and induction of CAA and other enzymes involved in the osmoregulatory physiology of brachyurans would be necessary to support the hypothesis that CA is related to salt secretion in *A. rubripes*. However, the high CAA in *A. rubripes* in relation to *S. rectum*, *A. pisonii*, and *A. angustipes* may represent a demand of the first species due to its small size and, therefore, very high surface/volume ratio. This relationship favors changes with the environment, which, in an environment of very diluted waters, represent the loss of salts and water gain (Van Horn and Tolley 2009; Henry et al. 2012). Smaller individuals tend to lose more salts and gain more water than larger ones (Bliss 1968; Buggren and McMahon 1988; Van Horn and Tolley 2009; Willmer et al. 2009; Henry et al. 2012). Thus, the high CAA observed in *A. rubripes* may represent the possibility of active salt absorption when in very dilute media, and/or secretion of salt in high salinities.

CAA in anterior gills is usually related to the functions of respiration and acid-base balance (Henry et al. 2012; Rivera-Ingraham et al. 2016). The enzymatic activity in these gills was higher in *A. rubripes* and *A. pisonii* than in *S. rectum* and *A. angustipes*. These data seem to be associated with the pattern of species refuges observed in the field. The first species is often observed on the sediment of the mangrove (Capitoli et al. 1977; Marochi, personal communication) and *A. pisonii* occupy mostly the tree trunks (Warner 1967; Diaz and Conde 1988). Conversely, *S. rectum* dwells burrows (Anger and Moreira 2004; Castiglioni et al. 2011; Ribeiro et al. 2012), and *A. angustipes* inhabits a

great variety of habitats, including under leaves and litter (Abele 1992; De Melo 1996). Predation occurs more often in the bottom and in the trees than in burrows and refuges (Warren 1990; Eshky et al. 1995). In addition, fluctuations in temperature are narrower in the burrows and refuges than out of them (Smith and Miller 1973; Eshky et al. 1995; Masunari 2006). Thus, *S. rectum* and *A. angustipes*remain protected from predation and also from steep fluctuations of temperature in their habitat. In contrast, the other two species are exposed to these factors. *A. rubripes* is mainly exposed to predation, as the bottom offers higher risk than the trees (Wilson 1989). Moreover, *A. pisonii* is very exposed to high temperatures, mainly in the canopy (Wilson 1989). Protected in their refuges, *S. rectum* and *A. angustipes* probably do not have to perform active escape from predators as frequently as the other two species must do. In addition, the reduced fluctuation in environmental, and hence, in the body temperature prevents these species from variations in the breathing rate, as this parameters varies with temperature (Ivleva 1980; Riisgård et al. 2015). Thus, the activity and heating, and therefore, the demand for oxygen consumption and consequent CO_2 elimination, is higher in *A. rubripes* and *A. pisonii*, which explains its high enzymatic activity in relation to the other species. Finally, greater enzymatic activity was observed in the posterior gills of all species than in the anterior ones, which is typically observed in species of aquatic and semi-terrestrial osmoregulator brachyurans (Henry and Cameron 1982; Henry 1991; Péqueux 1995; Henry et al. 2003; Freire et al. 2008; Henry et al. 2012; Rivera-Ingraham et al. 2016). The patterns of osmolality and enzymatic activity of the species are high compared to aquatic species of crustaceans (Maraschi et al. 2015; Castellano et al. unpublished data). High osmolality values probably represent a strategy for the conservation of body water in terrestrial and semi-terrestrial species, since high internal concentrations (hemolymph) favor the absorption of water from the environment (Bliss 1979; Henry 1984). The high enzymatic activity in the posterior gills represent an osmoregulatory mechanism (Henry and Cameron 1983; Lucu 1990; Henry 1996; Henry et al. 2012), and, then, offers to the species, the possibility to use available environmental water, in a wide range of salinity (Bliss, 1968). In different salinities a pH variation also occurs, with higher values being found in the sea, intermediate in estuaries, and lower in freshwater bodies. In addition, contact with air in the terrestrial environment, and

the possibility of aerial respiration among the Sesarmidae (Mangum 1994; Cumberlidge et al. 2005; Giomi et al. 2014), even in other species, demand CAA on the catalysis of dehydration from bicarbonate to CO_2 so that it can be eliminated (Bundy 1977; Farrelly and Greenaway 1994; Henry 1996; Henry et al. 2012). Thus, high CAA values in the anterior gills may also be related to the acid-base regulation requirement of these species, since they are euryhaline and semi-terrestrial. These high values of osmolality and enzymatic activity are consistent with the semi-terrestrial habit of the four species. The greatest novelty of this study was the evaluation of physiological parameters related to habitat, in a comparative focus, in semi-terrestrial crabs.

5 Conclusion

The null hypothesis was confirmed, as the most euryhaline (and oligohaline) species, *A. rubripes*, showed the highest CAA and the greatest osmotic gradient between environmental water and hemolymph. In summary, the pattern of high osmolality in the semi-terrestrial Sesarmidae crabs may represent a strategy of water body conservation, and the high levels of CAA represents a mechanism of osmotic and acid-base regulation. In posterior gills, CAA is inversely related to salinity occupied for species, thus a high CAA is associated to low salinity habitats. This physiological focus is a novelty for the studied species, as just four studies relating CAA with terrestriality were found for crabs, and not for species studied here.

Literature cited

Abele, L. G. (1992). A review of the grapsid crab genus *Sesarma* (Crustacea: Decapoda: Grapsidae) in America, with the description of a new genus (No. 527). Smithsonian Institution Press.

Anger, K. (1995). The conquest of freshwater and land by marine crabs: adaptations in life-history patterns and larval bioenergetics. Journal of Experimental Marine Biology and Ecology, 193(1), 119-145.

Anger, K. (1996). Salinity tolerance of the larvae and first juveniles of a semiterrestrial grapsid crab, *Armases miersii* (Rathbun). Journal of experimental marine biology and ecology, 202(2), 205-223.

Anger, K., & Charmantier, G. (2000). Ontogeny of osmoregulation and salinity tolerance in a mangrove crab, *Sesarma curacaoense* (Decapoda: Grapsidae). Journal of experimental marine biology and ecology, 251(2), 265-274.

Anger, K., & Moreira, G. S. (2004). Biomass and elemental composition of eggs and larvae of a mangrove crab, *Sesarma rectum* Randall (Decapoda: Sesarmidae) and comparison to a related species with abbreviated larval development. Scientia Marina, 68(1), 117-126.

Bliss, D. E. (1979). From sea to tree: saga of a land crab. American Zoologist, 19(2), 385-410.

Bliss, D. E. (1968). Transition from water to land in decapod crustaceans. American Zoologist, 8(3), 355-392.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical biochemistry, 72(1-2), 248-254.

Bundy, H. F. (1977). Carbonic anhydrase. Comparative Biochemistry and Physiology Part B: Comparative Biochemistry, 57(1), 1-7.

Burggren, W. W., & McMahon, B. R. (1988). Biology of the land crabs. Cambridge University Press.

Capitoli, R. R., Benvenuti, C. E., & Gianuca, N. M. (1977). Ocorrência e observações bioecológicas do caranguejo *Metasesarma rubripes* (Rathbun) na região estuarina da Lagoa dos Patos. Atlântica, 2(1), 50-62.

Conde, J. E., & Díaz, H. (1989). The mangrove tree crab *Aratus pisonii* in a tropical estuarine coastal lagoon. Estuarine, Coastal and Shelf Science, 28(6), 639-650.

Conde, J. E., Tognella, M. M. P., Paes, E. T., Soares, M. L. G., Louro, I. A., & Schaeffer-Novelli, Y. (2000). Population and life history features of the crab *Aratus pisonii* (Decapoda: Grapsidae) in a subtropical estuary. Interciencia-Caracas-, 25(3), 151-158.

Cumberlidge, N., Fenolio, D. B., Walvoord, M. E., & Stout, J. (2005). Tree-climbing crabs (Potamonautidae and Sesarmidae) from phytotelmic microhabitats in rainforest canopy in Madagascar. Journal of Crustacean Biology, 25(2), 302-308.

da Silva Castiglioni, D., de Oliveira, P. J. A., da Silva, J. S., & Coelho, P. A. (2011). Population dynamics of *Sesarma rectum* (Crustacea: Brachyura: Grapsidae) in the Ariquindá River mangrove, north-east of Brazil. Journal of the Marine Biological Association of the United Kingdom, 91(07), 1395-1401.

de Arruda Leme, M. H. (2006). Seasonal changes in reproductive traits of the crab *Sesarma rectum* (Grapsoidea: Sesarmidae) on the northern coast of São Paulo State, Brazil. Journal of Crustacean Biology, 26(2), 141-147.

De Melo, G. A. S. (1996). Manual de identificação dos Brachyura (caranguejos e siris) do litoral brasileiro. Editora Plêiade; Fundação de Amparo à Pesquisa do Estado de São Paulo.

Díaz, H., & Conde, J. E. (1988). On the food sources for the mangrove tree crab *Aratus pisonii* (Brachyura: Grapsidae). Biotropica, 20(4), 348-350.

Díaz, H., & Conde, J. E. (1989). Population dynamics and life history of the mangrove crab *Aratus pisonii* (Brachyura, Grapsidae) in a marine environment. Bulletin of Marine Science, 45(1), 148-163.

Diaz, H., & Ewald, J. J. (1968). A comparison of the larval development of *Metasesarma rubripes* (Rathbun) and *Sesarma ricordi* H. Milne Edwards (Brachyura, Grapsidae) reared under similar laboratory conditions. Crustaceana. Supplement, 225-248.

Eshky, A. A., Atkinson, R. J. A., & Taylor, A. C. (1995). Physiological ecology of crabs from Saudi Arabian mangrove. Marine Ecology Progress Series, 126, 83-95.

Farrelly, C., & Greenaway, P. E. T. E. R. (1994). Gas exchange through the lungs and gills in air-breathing crabs. The Journal of experimental biology, 187(1), 113-130.

Fischer, E. A., Duarte, L. F., & Araujo, A. C. (1997). Consumption of bromeliad flowers by the crab *Metasesarma rubripes* in a Brazilian coastal forest. Crustaceana, 70(1), 118-123.

Freire, C. A., Onken, H., & McNamara, J. C. (2008). A structure–function analysis of ion transport in crustacean gills and excretory organs. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 151(3), 272-304.

Giomi, F., Fusi, M., Barausse, A., Mostert, B., Pörtner, H. O., & Cannicci, S. (2014). Improved heat tolerance in air drives the recurrent evolution of air-

breathing. Proceedings of the Royal Society of London B: Biological Sciences, 281(1782), 20132927.

Henry, R. P. (1991). Branchial and branchiostegite carbonic anhydrase in decapod crustaceans: the aquatic to terrestrial transition. Journal of Experimental Zoology, 259(3), 294-303.

Henry, R. P. (1988). Multiple functions of carbonic anhydrase in the crustacean gill. Journal of Experimental Zoology, 248(1), 19-24.

Henry, R. P. (1996). Multiple roles of carbonic anhydrase in cellular transport and metabolism. Annual Review of Physiology, 58(1), 523-538.

Henry, R. P. (1984). The role of carbonic anhydrase in blood ion and acid-base regulation. American Zoologist, 24(1), 241-251.

Henry, R. P., & Cameron, J. N. (1982). The distribution and partial characterization of carbonic anhydrase in selected aquatic and terrestrial decapod crustaceans. Journal of Experimental Zoology, 221(3), 309-321.

Henry, R. P., & Cameron, J. N. (1983). The Role of Carbonic Anhydrase in Respiration, Ion Regulation and Acid-Base Balance in the Aquatic Crab *Callinectes sapidus* and the Terrestrial Crab *Gecarcinus lateralis*. Journal of Experimental Biology, 103(1), 205-223.

Henry, R. P., Gehnrich, S., Weihrauch, D., & Towle, D. W. (2003). Salinity-mediated carbonic anhydrase induction in the gills of the euryhaline green crab, *Carcinus maenas*. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 136(2), 243-258.

Henry, R. P., Lucu, C., Onken, H., & Weihrauch, D. (2012). Multiple functions of the crustacean gill: osmotic/ionic regulation, acid-base balance, ammonia excretion, and bioaccumulation of toxic metals. Frontiers in physiology, 3.

Henry, R. P., & Saintsing, D. G. (1983). Carbonic anhydrase activity and ion regulation in three species of osmoregulating bivalve molluscs. Physiological Zoology, 274-280.

Herreid, C. F. (1969). Integument permeability of crabs and adaptation to land. Comparative Biochemistry and Physiology, 29(1), 423-429.

Hill, R. W., Wyse, G. A. & Anderson, M. (2008). Animal Physiology. Sinauer Associates Inc.

Ivleva, I. V. (1980). The dependence of crustacean respiration rate on body mass and habitat temperature. Internationale Revue der gesamten Hydrobiologie und Hydrographie, 65(1), 1-47.

Kowalczuk, V. G., & Masunari, S. (2000). Estrutura populacional de *Armases angustipes* (Dana)(Decapoda, Brachyura, Grapsidae) na Ilha do Farol, Matinhos, Paraná. Revista Brasileira de Zoologia, 17(1), 1-16.

Lee, C. E., & Bell, M. A. (1999). Causes and consequences of recent freshwater invasions by saltwater animals. Trends in Ecology & Evolution,14(7), 284-288.

Lima, G. V., Soares, M. R., & Oshiro, L. M. (2006). Reproductive biology of the sesarmid crab *Armases rubripes* (Decapoda, Brachyura) from an estuarine area of the Sahy River, Sepetiba Bay, Rio de Janeiro, Brazil. Iheringia. Série Zoologia, 96(1), 47-52.

Lucu, Č. (1990). Ionic regulatory mechanisms in crustacean gill epithelia. Comparative Biochemistry and Physiology Part A: Physiology, 97(3), 297-306.

Luppi, T. A., Spivak, E. D., & Bas, C. C. (2003). The effects of temperature and salinity on larval development of *Armases rubripes* Rathbun, 1897 (Brachyura, Grapsoidea, Sesarmidae), and the southern limit of its geographical distribution. Estuarine, Coastal and Shelf Science, 58(3), 575-585.

Mangum, C. P. (1994). Multiple sites of gas exchange. American Zoologist, 34(2), 184-193.

Maraschi, A. C., Freire, C. A., & Prodocimo, V. (2015). Immunocytochemical localization of V-H+-ATPase, Na+/K+-ATPase, and carbonic anhydrase in gill lamellae of adult freshwater euryhaline shrimp *Macrobrachium acanthurus* (Decapoda, Palaemonidae). Journal of Experimental Zoology Part A: Ecological Genetics and Physiology, 323(7), 414-421.

Masunari, S. (2006). Distribution and abundance of fiddler crabs *Uca* Leach (Crustacea Decapoda Ocypodidae) in Guaratuba Bay, Parana State, southern Brazil. Revista Brasileira de Zoologia, 23(4), 901-914.

Montú, M., Anger, K., & Bakker, C. D. (1990). Variability in the larval development of *Metasesarma rubripes* (Decapoda, Grapsidae) reared in the laboratory. Neritica, 5, 113-118.

Péqueux, A. (1995). Osmotic regulation in crustaceans. Journal of Crustacean Biology, 15(1): 1-60

Ribeiro, F. B., Matthews-Cascon, H., & Bezerra, L. E. A. (2012). Population structure and reproductive biology of the crab *Sesarma rectum* Randall, 1840 (Brachyura, Sesarmidae) in an impacted tropical mangrove in northeast Brazil. Crustaceana, 85(2), 173.

Riisgård, H. U., Zalacáin, D., Jeune, N., Wiersma, J. B., Lüskow, F., & Pleissner, D. (2015). Adaptation of the brine shrimp *Artemia salina* (Branchiopoda: Anostraca) to filter-feeding: effects of body size and temperature on filtration and respiration rates. Journal of Crustacean Biology, 35(5), 650-658.

Rivera-Ingraham, G. A., Barri, K., Boël, M., Farcy, E., Charles, A. L., Geny, B., & Lignot, J. H. (2016). Osmoregulation and salinity-induced oxidative stress: is oxidative adaptation determined by gill function?. Journal of Experimental Biology, 219(1), 80-89.

Schubart, C. D., Cuesta, J. A., Diesel, R., & Felder, D. L. (2000). Molecular phylogeny, taxonomy, and evolution of nonmarine lineages within the American grapsoid crabs (Crustacea: Brachyura). Molecular Phylogenetics and Evolution, 15(2), 179-190.

Schwamborn, R., Neumann-Leitão, S., Silva, T. A., Silva, A. P., Ekau, W., & Saint-Paul, U. (2001). Distribution and dispersal of decapod crustacean larvae and other zooplankton in the Itamaracá estuarine system, Brazil. Tropical Oceanography, 29(1), 1-18.

Smith, W. K., & Miller, P. C. (1973). The thermal ecology of two south Florida fiddler crabs: *Uca rapax* Smith and *U. pugilator* Bosc. Physiological Zoology, 46(3), 186-207.

Souza-Bastos, L. R., & Freire, C. A. (2009). The handling of salt by the neotropical cultured freshwater catfish *Rhamdia quelen*. Aquaculture, 289(1), 167-174.

Thiercelin, N. (2016). *Impact of life history and ecology on rate of diversification and speciation, as exemplified by thoracotreme crabs along the western tropical Atlantic and on both sides of the Isthmus of Panama* (Doctoral dissertation).

Van Horn, J., & Tolley, S. G. (2009). Acute response of the estuarine crab *Eurypanopeus depressus* to salinity and desiccation stress. Journal of Crustacean Biology, 29(4), 556-561.

Vitale, A. M., Monserrat, J. M., Castilho, P., & Rodriguez, E. M. (1999). Inhibitory effects of cadmium on carbonic anhydrase activity and ionic regulation of the estuarine crab *Chasmagnathus granulata* (Decapoda, Grapsidae). Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology, 122(1), 121-129.

Warner, G. F. (1967). The life history of the mangrove tree crab, *Aratus pisoni*. Journal of Zoology, 153(3), 321-335.

Warren, J. H. (1990). Role of burrows as refuges from subtidal predators of temperate mangrove crabs. Marine ecology progress series. Oldendorf, 67(3), 295-299.

Weihrauch, D., & O'Donnell, M. J. (2015). Links between osmoregulation and nitrogen-excretion in insects and crustaceans. Integrative and Comparative Biology, 55(5), 816-829.

Wheatly, M. G., & Henry, R. P. (1992). Extracellular and intracellular acid-base regulation in crustaceans. Journal of Experimental Zoology, 263(2), 127-142.

Willmer, P., Stone, G., & Johnston, I. (2009). Environmental physiology of animals. John Wiley & Sons.

Wilson, K. A. (1989). Ecology of mangrove crabs: predation, physical factors and refuges. Bulletin of Marine Science, 44(1), 263-273.

Wolcott, T. G. (1992). Water and solute balance in the transition to land. American Zoologist, 32(3), 428-437.

CAPÍTULO 3

Carbonic anhydrase activity and cell volume regulation in polychaetes of different environments

Abstract

Polychaetes occur in aquatic environments, mainly marine and estuarine. The abundance of these animals in estuaries and the fluctuations in abiotic factors present in this habitat demand physiological adjustment of internal homeostasis. Carbonic anhydrase exerts functions of osmoregulation, acid-base balance and respiration. Polychaetes are osmoconformers, and, then, under salinity fluctuations, their internal medium changes and expose their tissues and cells to osmotic challenges. The aim of this study was to relate physiological mechanisms of osmoregulation and cell volume regulation, to the environment and euryhalinity of polychaetes. Three species of polychaetes, *Scolelepis goodbody* (marine intertidal), *Laeonereis culveri* (estuarine euryhaline), and *Nephtys fluviatilis* (estuarine oligohaline) were studied. The constitutive osmolality and carbonic anhydrase activity (CAA) were analyzed, and the cell volume regulation capacity was tested under hypo and hyperosmotic shocks of 50% intensity in relation to the isosmotic control. The most euryhaline species, *S. goodbody* and *L. culveri*, showed higher CAA and capacity of cell volume regulation than *N. fluviatilis*, which inhabits the oligohaline region of estuaries. Cell volume regulation capacity tested in isolated cells, and CAA represent a great novelty in literature of polychaetes. Moreover, the comparative approach of physiological data related to ecological aspects contributes to better understand tolerance of species.

1 Introduction

Annelids occur in a great variety of habitats, including sea, estuaries, freshwater, and land (Preston 2008; Sket and Trontelj 2008; Grosberg et al. 2012). Polychaetes, specifically, dwell aquatic environments, mainly marine and estuarine (Preston 2008; Filho and Aviz 2013), and few (~2%) freshwater environments (Glasby and Timm 2008; Preston 2008). Polychaetes are abundant organisms in estuaries, which demonstrate their tolerance to fluctuations in abiotic factors, such as salinity and pH (Bastida et al. 2004; Rosa and Bemvenuti 2006a, b; Poersch et al. 2007; Evans et al. 2010; Silva et al. 2011). These fluctuations demand adjusting mechanisms, which are partially exerted by the enzyme carbonic anhydrase (CA), which catalyzes the reversible reaction of CO_2

hydration (Henry and Saintsing 1983; Henry 1988, 1996). The resulting products of this reaction, ions proton (H^+) and bicarbonate (HCO_3^-), are eliminated from cells through the ionic exchangers Na^+/H^+ and Cl^-/HCO_3^-. Thus, H^+ elimination is coupled with Na^+ uptake, and HCO_3^- elimination is coupled with Cl^- uptake (Henry 1996; Henry et al. 2012; Weihrauch and O'Donnell 2015). The movements of H^+ and HCO_3^- into and out the cells result in internal pH balance, being related to acid-base equilibrium. When H^+ is eliminated pH increases, and the opposite occurs for HCO_3^- (Henry 1996; Henry et al. 2012). The uptake of Na^+ and Cl^- results in rise of internal animal concentration, which represent an osmoregulatory function (Henry 1984; Lucu 1990; Henry 1996; Henry et al. 2012). The reversal reaction, of HCO_3^- dehydration, is also catalyzed by CA and results in CO_2 and water. Membranes show low permeability to HCO_3^-, thus it must be eliminated from cells in its non-hydrated molecular form, as CO_2 (Bundy 1977; Farrelly and Greenaway 1994; Henry 1996; Henry et al. 2012).

Marine and estuarine species are generally osmoconformers, and thus have internal medium (e.g. blood) nearly isosmotic to environmental water, but slightly hyperosmotic in relation to it (Oglesby 1965a; Clauss 2001; Preston 2008). Thus, under an external (i.e. environmental) change in salinity, animal internal medium (i.e. blood, coelomic fluid) follows the fluctuation. When internal animal concentration fluctuates (i.e. in osmoconformers), cells and tissues are osmotically challenged (Pierce 1982). When a decrease in environmental salinity occurs, the concentration (i.e. osmolality) of extracellular fluid of osmoconformers is reduced, and then cells swell due to water entry. Conversely, when environmental salinity increases, extracellular fluid follows the same tendency and, then, cells lose water and shrink (Häussinger 1996; Sardini et al. 2003; Wehner et al. 2003; Hoffmann et al. 2009). In the first case, a regulatory volume decrease (RVD) can be performed, and in the second condition, the response is a regulatory volume increase (RVI) (Häussinger 1996; Sardini et al. 2003; Hoffmann et al. 2009). These regulatory responses, both called isosmotic intracellular regulation (IIR), occur through fluxes of ions (inorganic osmolytes) and aminoacids (organic osmolytes), both followed by compensatory water fluxes (Pierce 1982; Pierce and Politis 1990; Hoffmann and Dunham 1995; Häussinger 1996; Wehner et al. 2003). Ions and aminoacids are eliminated from cells in RVD, followed by water, resulting in reduction of cell volume. In RVI, intracellular

proteins are hydrolyzed and salts are absorbed by cells, also followed by water, causing increase in cell volume. In both cases, a partial or total recover of volume can occur (Häussinger 1996; Sardini et al. 2003; Hoffmann et al. 2009). Polychaetes use aminoacids in this process (Clark 1968a, b; Freel et al. 1973; Fletcher 1974b; Jorgensen 1979; Costa et al. 1980; Koenig et al. 1981; Costa and Pierce 1983; Schöttler et al. 1990; Blank et al. 2004; Hoeger and Abe 2004).

The aim of this study was to relate physiological mechanisms of osmoregulation and cell volume regulation, to the environment and euryhalinity of polychaetes. Three species were studied: one marine intertidal, one estuarine euryhaline, and one estuarine stenohaline and oligohaline. We hypothesized that the osmotic gradient in relation to water, the carbonic anhydrase activity, and the capacity of cell volume regulation would be directly proportional to the degree of euryhalinity of species (*L. culveri*, *S. goodbody* > *N. fluviatilis*).

2 Material and methods

2.1 Species, collection and acclimation

Three species of polychaetes were studied, one marine, *Scolelepis goodbody* (Jones, 1962), and two estuarine, *Laeonereis culveri* (Webster, 1879), and *Nephtys fluviatilis* (Monro, 1937). *Scolelepis goodbody* was collected in Pontal do Sul Beach, Pontal do Paraná – PR (25°34’39”S 48°20’54”W), *L. culveri* in Cotinga Island, Paranaguá Bay-PR (25°30’12”S 48°28’24”W), and *N. fluviatilis* in Antonina Harbor, Antonina-PR (25°25’42”S 48°42’28”W). All species were collected through the same method. Sediment, in which animals were buried, was collected with shovels and transported for ~2 h, in plastic containers, to the Laboratório de Fisiologia Comparativa da Osmorregulação, of Physiology Department of Federal University of Paraná, Curitiba-PR. Animals were acclimated for 4-5 days in the same plastic containers in which they were transported, with water (35 psu for *S. goodbody*, and 15 psu for both estuarine species), constant aeration, natural photoperiod (~12 h light: 12 h dark).

2.2 Sampling for osmolality and CAA assays

After acclimation period, animals were directly removed from containers (sifted from sediment), gently dried in filter paper, placed in Eppendorf tubes, and

frozen for osmolality and carbonic anhydrase activity (CAA) assays. Each sample for osmolality was composed of 2-3 individuals of *L. culveri* (n= 10 samples), and 3-4 of *N. fluviatilis* (n=8 samples). For CAA, samples were composed of 8-15 individuals of *S. goodbody*, 1 of *L. culveri*, and 2 of *N. fluviatilis* (n=10 samples of each species). After frozen, samples for osmolality were macerated with a plastic pistil, and then centrifuged at 21380Xg for 5 min (Hettich Zentrifugen Mikro 200R). The supernatant is the total body fluids (TBF) of animals, used for osmolality dosage. Even with 50 individuals of *S. goodbody* for each sample, TBF could not be extracted from samples, and then, osmolality was not measured in this species.

2.3 Osmolality

Osmolality of TBF was measured in a vapour-pressure micro-osmometer (Wescor, VAPRO 5520) in undiluted samples.

2.4 Carbonic anhydrase enzymatic activity (CAA)

Samples of whole animals were weighed and sonicated (20 s, at 1 pulse/s, in 50% amplitude - Fisher Scientific, Model FB120) in buffer at a ratio of ~10% tissue weight/ buffer volume. Then the homogenate was centrifuged at 2000xg for 5 min at 4°C (Hettich Zentrifugen Mikro 200R), and the supernatant was reserved for analysis. CAA was determined through the protocol established by Vitale et al (1999), and described in Souza-Bastos and Freire (2009). As a negative control of the technique, the same assay was also performed, but with samples preincubated for 10 min in buffer with acetazolamide addition (final concentrations of 100 μM in the sample - concentration used by Henry et al., 2012), the inhibitor of CA (n = 10 of each species). The composition of homogenization buffer was of 225 mM mannitol, 75 mM sucrose, 10 mM Tris-phosphate, with pH adjusted to 7.4. The total protein concentration of each homogenate, required to calculate CAA, was obtained through the method of Bradford (1976).

2.5 Cell volume regulation assay

This experiment was conducted according to Amado et al. (2012), and adapted from Capó-Aponte et al. (2006) and Hamann et al. (2002). For this assay, animals were sifted from sediment after acclimation, placed in Petri plates with ~10 ml of PBS (Phosphate Buffer Solution) containing EDTA (5 mM) isosmotic to the environmental water of each species (see composition in Table 1). Three to five individuals of *S. goodbody* were placed in each plate (n=5 plates), 1-2 of *L. culveri* (n=8 plates), and 1-2 of *N. fluviatilis* (n=5 plates). Then, using a scalpel, whole animals were minced into small pieces, and the resultant cell suspension was pumped up and down with a Pasteur pipette and filtered through a 30 µm-mesh, to eliminate tissue debris and obtain isolated cells. These isolated cells were counted using a Neubauer chamber, and each sample consisted of ~10^4 cells in 200 µl of control isosmotic saline (also isosmotic to environmental water of each species – Table 1), placed in a well of a black 96-wells microplate (Optiplate 96-well, Black). The measurement of the autofluorescence of each cell sample was proceeded, and then samples were incubated in 10 µM calcein (Calcein-AM from Sigma-Aldrich), in isosmotic saline for 1 h. Calcein uptake by cells was monitored, every 3 min, through the increasing fluorescence intensity. After this incubation, cells were washed with isosmotic saline, and, as an internal control, a curve of cells fluorescence in isosmotic saline was plotted, with readings every 30 s, until 5 min, to verify cell response under isosmotic condition. Lastly, each cell sample was submitted to a saline, isosmotic (control), 50% hyposmotic in relation to control (hypo 50), or 50% hyperosmotic in relation to control (1 well per saline was used for each initial cell suspension) (Table 1), and the fluorescence intensity was again measured every 30 s, but for a total of 20 min. Osmolality of salines and PBS was measured through the same method described for TBF. Fluorescence measurements were conducted in spectrofluorimeter (Tecan InfiniteM200, Austria). Immediately before each solution change, plates were centrifuged (Eppendorf Centrifuge 5810 R, Germany) for 5 min at 290 ×g (20 °C), in order to prevent cell loss. Fluorophores have the property of “fluorescence self-quenching” (Hamann et al. 2002; Capó-Aponte et al. 2006), according to which an increase in fluorescence intensity reflects increase in cell volume, and a decrease in fluorescence intensity reflects decrease in cell volume. We measured the fluorescence intensity at initial

time (0 min) and until 20 min of exposure to saline, and a relative scale was used. The fluorescence intensity at time 0 was considered a reference measure of each sample / well (100%), and the following fluorescence measures along time were relative to it (e.g., according to Hoffmann and Dunham 1995; Wehner et al. 2003; Hoffmann et al. 2009).

Table 1. Composition, concentration and pH of salines (hypo 50, control, hyper 50) and PBS used in cell volume regulation assay of *S. goodbody, L. culveri*, and *N. fluviatilis*.

	S. goodbody	***L. culveri***	***N. fluviatilis***
Hypo 50			
NaCl (mM)	235	100.5	100.5
KCl (mM)	5	2.1	2.1
$MgCl_2$ (mM)	27	11.6	11.6
$CaCl_2$ (mM)	5	2.1	2.1
Osmolality (mOsm/kg.H_2O)	489.5	234.5	234.5
pH	8.2	7.6	7.6
% reduction in relation to control	51.8	48.1	48.1
Isosmotic control			
NaCl (mM)	470	201	201
KCl (mM)	10	4.3	4.3
$MgCl_2$ (mM)	54	23.1	23.1
$CaCl_2$ (mM)	10	4.3	4.3
Osmolality (mOsm/kg.H_2O)	1015	452	452
pH	8.2	7.6	7.6
Hyper 50			
NaCl (mM)	705	301.5	301.5
KCl (mM)	15	6.4	6.4
$MgCl_2$ (mM)	81	34.7	34.7

$CaCl_2$ (mM)	15	6.4	6.4
Osmolality (mOsm/kg.H_2O)	1502	676	676
pH	8.2	7.6	7.6
% increase in relation to control	48.0	49.6	49.6
PBS			
NaCl (mM)	400	171	171
Na2HPO4 (mM)	25	11	11
KH2PO4 (mM)	3.5	1.5	1.5
KCl (mM)	20	8.6	8.6
Osmolality (mOsm/kg.H_2O)	1054	455	455
pH	8.2	7.6	7.6

For all salines the same concentration of the following reagents was used: D-glucose (5 mM), glycine (5 mM), HEPES acid (5 mM), and NaHCO3 (2mM).

An index of cell volume regulation capacity was calculated for each species and experimental saline, according to Foster et al. (2010). The greatest variation of cell volume, in any time of exposure to a saline, is calculated as a percentage of the control, at the same time of experiment. Then, the absolute variation in osmolality of the experimental saline is calculated in relation to the control saline. The index consists on the ratio of the cell volume variation by the osmolality variation, multiplied for 1000. This multiplication was performed in order to result in a number value, which would be easy to handle. A low index reflects low variation of cell volume under osmotic shocks, and, thus, a high capacity for cell volume regulation of the species.

2.6 Statistics

Osmolality of TBF was compared between species through t-test, as data achieved normality requirements. TBF osmolality was compared to expected values for water of 35 (for *S. goodbody*) and 15 psu (for *L. culveri*, and *N. fluviatilis*) (according to Prosser 1973) through 95% confidence interval of

osmolality of each species (as conducted in Santos et al. 2013). CAA was compared between species through 1-way ANOVA with Holm-Sidak *post hoc* (as normality requirements were met). Cell volume (i.e. fluorescence) was analyzed through 2-way repeated measures (RM) ANOVAs, with 3 levels of time (1.5, 5, 20 min), and 3 levels of salines (hypo 50, control, hyper 50), followed by the Holm-Sidak *post hoc* test (for both data, which met or not normality requirements). The significance limit of all analyses was always set at 0.05.

3 Results

Osmolality of total body fluids was lower in *L. culveri* than in *N. fluviatilis*. In addition, *L. culveri* is isosmotic to water, while *N. fluviatilis* is hyperosmotic. This last species is hyperosmotic in relation to water (Fig. 1A). Carbonic anhydrase activity was higher in *S. goodbody* and *L. culveri* than in *N. fluviatilis* (Fig. 1B). Carbonic anhydrase activity was 48.15% inhibited in *S. goodbody*, 92.39% in *L. culveri*, and 92.28% in *N. fluviatilis*, under acetazolamide 100 µM.

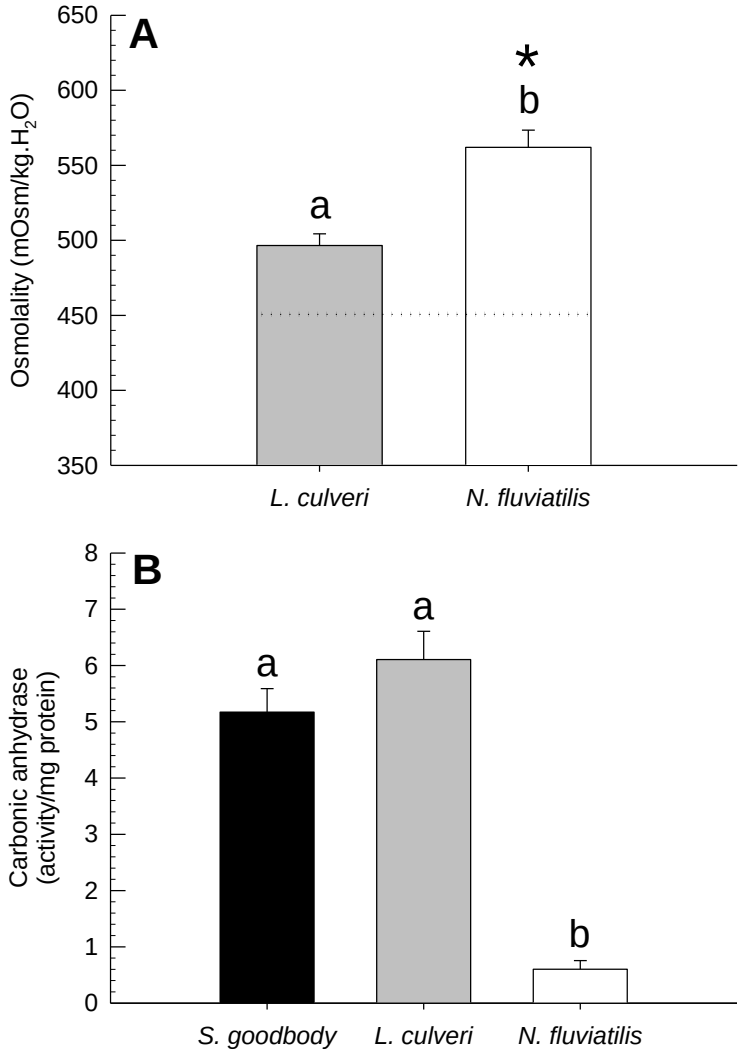

Figure 1. Osmolality of total body fluids of *Laeonereis culveri* (n=10) and *Nephtys fluviatilis* (n=8). Dotted line represents water expected osmolality (A). Body carbonic anhydrase activity of *Scolelepis goodbody, Laeonereis culveri*, and *Nephtys fluviatilis* (n=10 for each species) (B). Lower case letters represent differences between species. * represents difference between total body fluids and water. Error bars=standard error.

Cell volume of *S. goodbody* after 1.5 min was higher in hypo 50 than in the control, and volume return to control level after 5 min of exposure (Fig. 2A). The same pattern was observed in *L. culveri* (Fig. 2B). In *N. fluviatilis* cell volume was lower than control after 20 min in hypo 50 (Fig. 2C). The highest index of cell volume capacity for the hypo 50 saline occurred for *N. fluviatilis*, then for *S. goodbody*, and finally for *L. culveri*. For hyper 50 saline, the highest index was observed for *N. fluviatilis* and *L. culveri*, and the lowest for *S. goodbody* (Table 2).

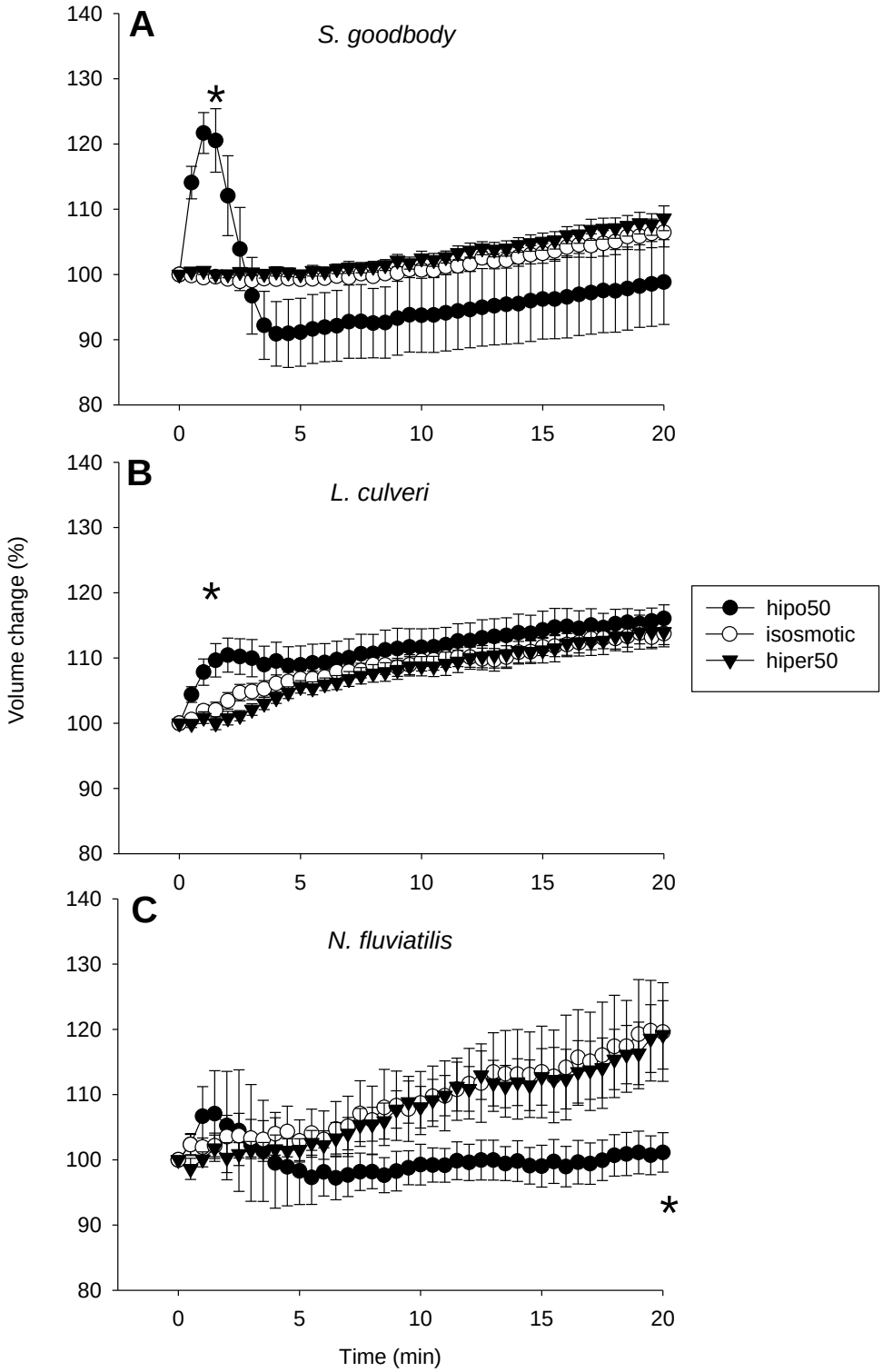

Figure 2. Volume change of cells of *S. goodbody, L. culveri*, and *N. fluviatilis* along 20 min of exposure to hypo 50 (black circles), isosmotic (control – white circles), and hyper 50 (black down triangles) salines. * represent difference in relation to control. Error bars=standard error.

Table2. Index of cell volume regulation capacity of *S. goodbody, L. culveri*, and *N. fluviatilis* face to the hypo and hyper 50 salines. ∆ CV = cell volume variation in relation to control; ∆ Osm = difference of osmolality between experimental and control salines; index = (∆ CV/∆ Osm)*1000.

		S. goodbody	***L. culveri***	***N. fluviatilis***
Hypo 50	∆ CV (%)	22.2	7.6	19.1
	∆ Osm (mOsm/kg H_2O)	525.5	217.5	217.5
	Index	42.2	35.1	87.8
Hyper 50	∆ CV (%)	2.4	3.5	3.7
	∆ Osm (mOsm/kg H_2O)	487.0	224.0	224.0
	Index	5.0	15.8	16.6

4 Discussion

The osmolality of TBF of *L. culveri* was isosmotic to water, which is often observed in the coelomic fluid of estuarine polychaetes in brackish water, as *Nereis vexillosa*, *N. succinea*, and *N. diversicolor* (Oglesby 1965a, b, 1970; Fletcher 1974b). A similar response was also observed for the marine species *Cirriformia spirabrancha* under salinities from 0-225% seawater (Dice 1969). Oppositely, *N. fluviatilis* is ~24% hyperosmotic in relation to water. This species occupies oligohaline regions of estuaries (Lana et al. 1997; Giménez et al. 2006; Rosa and Bemvenuti 2006b; Silva et al. 2011; Melo et al. 2013; Muniz et al. 2000), and probably absorbs salts from water, maintaining osmotic gradients above water values, similarly to *N. limnicola*, which is also an oligohaline species (Oglesby 1965a). Some studies pointed to active salt uptake by polychaetes exposed to low salinities, such as *Onuphis magna*, *Nereis diversiccolor* and *Perinereis cultrifera*, and reduction of this active transport in higher salinities (Fretter 1955; Ebbs and Straiger 1965). Thus, one can think that the acclimation of *N. fluviatilis* to 15 psu, which represent the upper limit of salinity to the species (Lana et al. 1997; Passadore et al. 2007; Braga et al. 2011), would shut down mechanisms of solute absorption. However, if it occurred (see discussion of CAA for this species below) it was as a partial response, and then salt continued to be absorbed, and resulted in a hyperosmotic hemolymph with respect to water. In addition, a hyperosmotic hemolymph in relation to water can be a result of low

permeability to water and chloride flows and of production of a diluted urine (Oglesby 1965b; Larsen et al. 2014). Though, this is not a probable hypothesis, because permeability is directly proportional to salinity, and diluted urine is produced in low salinities (Oglesby 1968; Doneen and Clark 1974; Fletcher 1974a; Oglesby 1981; Larsen et al. 2014). Then, as 15 psu represent a high salinity for *N. fluviatilis* (Lana et al. 1997; Giménez et al. 2006; Rosa and Bemvenuti 2006b; Silva et al. 2011; Melo et al. 2013; Muniz et al. 2000), a high permeability to water and salts, and the production of an isosmotic urine is expected.

CAA was higher in *S. goodbody* and *L. culveri* than in *N. fluviatilis*. This pattern of constitutive physiological parameter is consistent with the degree of euryhalinity of species, as previous studies postulated that euryhaline species have higher CAA than the stenohaline ones (Nielsen and Frieden 1972; Henry and Cameron 1982; Henry et al. 2012). Both, *S. goodbody* and *L. culveri* are euryhaline species. *Scolelepis goodbody* is an intertidal dweller (habitat of high fluctuation in abiotic factors) (MacCord and Amaral 2007), it occurs buried in the sand, and can be exposed to fluctuations of salinity. High salinities can occur due to a high sunny incidence and consequent water evaporation. Oppositely, heavy rains or even the influence of the freshwater from beach groundwater can result in low salinities (Horn 2002, 2006). These aspects denote the euryhalinity of *S. goodbody*. *Laeonereis culveri* dwells freshwater spillways (Palmer et al. 2011), and can face a wide range of salinity, from 0.5 to 30 psu (Mazurkiewicz 1975). Extreme low tides, syzygy, must also be considered as a source of osmotic stress for this species. Finally, *N. fluviatilis* occurs in the oligohaline portion of estuaries, usually in salinities between 4 and 15 psu (Lana et al. 1997; Giménez et al. 2006; Rosa and Bemvenuti 2006b; Silva et al. 2011; Melo et al. 2013; Muniz et al. 2000). Thus *N. fluviatilis* is stenohaline in relation to *S. goodbody* and *L. culveri*, which is coherent to its lowest CAA. In addition, *N. fluviatilis* inhabits oligohaline sites, and the acclimation salinity applied to the estuarine species here tested (15 psu) is near to the highest limit of salinity registered for this species (Lana et al. 1997; Passadore et al. 2007; Braga et al. 2011). As CAA is reduced under salinity increase (e.g. Nielsen and Frieden 1972; Henry et al. 2012), this enzyme was, probably, partially shut down in *N. fluviatilis* acclimated to 15 psu, which represents a high salinity for the species. Finally, *N. fluviatilis* occupies mudy

bottom, which are less permeable to water than sandy substrata in which *L. culveri* and *S. goodbody* occur (Santos and Simon 1974; Kneib 1985; Colling et al. 2007). Then, while remaining buried in the mud, *N. fluviatilis* is exposed to a relatively narrow saline variation, and it “buffers” the environmental fluctuations. Polychaetes burry themselves in the substrate and, therefore, minimize osmotic, thermal, and oxygen stress (Preston 2008). This represent an avoidance behavior (Sanders et al. 1965; Oglesby 1969; Preston 2008).

A RVD signal was observed in the cells of *S. goodbody* and *L. culveri* under hypo 50 saline, characterized by the increase in volume at ~ 1.5 min, followed by the reduction at ~ 5 min of exposure to the osmotic shock (saline). Although the slight increase in volume detected in *N. fluviatilis* at ~ 1.5 min, was not statistically different from the control, at the end of the experiment (20 min), cells of *N. fluviatilis* exposed to hypo 50 saline showed a lower volume than in the control. When exposed to hypo and hypersaline challenges with intensity of 50%, the whole body volume of *L. culveri* showed a ~20% increase and a ~30% decrease, respectively (Oglesby 1965b). These volume variations were lower than the proportional salinity change, and then show some capacity of IIR. This capacity can represent for a species the possibility to dwell in a fluctuating environment. However, our data showed, for a similar osmotic challenge (i.e. hypo and hyper 50 salines), lower changes in volume, also for *L. culveri*. Our time course was of 20 min, and that applied for Oglesby (1965b) was of hours. In addition, we used isolated cells, while Oglesby (1965b) used whole animals. These aspects can be the cause of the differences in results. Volume regulation capacity was also tested in *Glycera dibranchiata*, in red coelomocytes. These cells were challenged with 50% hyposmotic saline for 20 min, and with 40% hyposmotic shock for 120 min. A RVD signal was observed, however it was incomplete, as volume was not totally recovered (Costa et al. 1980; Costa and Pierce 1983). A RVD signal was also verified for larvae and adults of *Diopatra variabilis* (Krishnamoorthi 1963) and in adults of *Glycera embranchiata*, *Onuphis eremita*, *Loimia medusa* and *Clymene insecta* (Krishnamoorthi 1962). These data demonstrate that the three studied species really have high capacity to regulate cell volume under wide osmotic challenges (i.e. 50% intensity). In addition, RVD seems to be a common response, even when isolated cells or whole body are tested.

In the hyperosmotic shock, a different pattern of response was observed. Cell volume did not change in relation to control. Thus, instead of allow fluctuation in cell volume followed by a posterior return (RVD observed in hypo 50 saline), maintenance of volume/hydration was the strategy observed for the three species. Indeed RVD is more often observed than RVI (Oglesby 1981). A higher change in volume under hypo than under hyperosmotic shock was already observed in polychaetes, nematodes, sipunculans, molluscs, crustaceans (Péqueux and Gilles 1979; Oglesby 1981; Dragolovich and Pierce 1992; Amado et al. 2006). Differences in responses of volume change under hypo and hyperosmotic challenges were also observed in the marine polychaete *Cirriformia spirabrancha*. In this species, the increase of whole body volume under 50% hyposmotic shock was more intense than the decrease under 50% hyperosmotic shock (Dice 1969). It seems that, in the hyposmotic shock, the response is a result of water movement into the cells/animals. However, in the hyperosmotic shock, salt gain is the main factor responsible for the osmotic equilibrium between the internal (cell or animal) and the external (saline or experimental water) media (Dice 1969). Thus, under hyperosmotic shock the volume change is relatively narrow.

IIR capacity is also related to the euryhalinity of the species (Florkin and Schoffeniels 1969 apud Pierce 1971; Pierce 1982). Coherently, *L. culveri*, an euryhaline species, has some capacity of volume regulation, while *Nereis succinea*, a stenohaline species, functions nearly as a perfect osmometer, with volume changes similar to the salinity changes (Oglesby 1965b). The IIR capacity of the three studied species is also coherent with their salinity range of occurrence, mentioned above. In addition, an interspecific comparison can be performed, through the index of cell volume regulation capacity. A low index reflects low variation of cell volume under osmotic shocks, and, thus, a high capacity for cell volume regulation of the species. For the hypo 50 saline, the highest index was observed for *N. fluviatilis*, followed by *S. goodbody*, and then by *L. culveri*. For the hyper 50 saline, *L. culveri* and *N. fluviatilis* showed the highest indexes (similar values), followed by *S. goodbody*. The general pattern of these indexes pointed to the lowest capacity of volume regulation for *N. fluviatilis*, which is the less euryhaline, as referred above. In contrast, *L. culveri* was prominent in volume regulation under hyposmotic challenge, while *S. goodbody*

did for the hyperosmotic shock. It is coherent with the occurrence of *L. culveri* in freshwater spillways (Palmer et al. 2011). Moreover, Nereididae, the family to which *L. culveri* belongs, is the most representative family of freshwater polychaetes (Glasby and Timm 2008), which brings an evolutive issue. The indexes are also consistent with the sandy intertidal habitat of *S. goodbody* (MacCord and Amaral 2007). The salinity into the substrate of the marine intertidal polychaete *C. spirabrancha* is more intensely increased under exposure to the sun than decreased when exposed to diluted seawater (Dice 1969). This habitat is similar to that of *S. goodbody* and, then we can expect that this species will face salinity increase in a higher frequency than a salinity decrease. Thus, the index of cell volume regulation capacity is related to the euryhalinity and habitat of species. In addition, the indexes observed for hyposmotic saline were higher than the ones for hyperosmotic saline. It is coherent with the higher variation in volume verified in cells exposed to hypo 50 saline than in the ones exposed to hyper 50 saline.

Our data on CAA and on cell volume regulation represent great novelty in the literature. Concerning to carbonic anhydrase, few studies were conducted in polychaetes. Its expression was detected in *Osedax*, with function of providing H^+ for dissolution of bones (e.g. of whales) for *Osedax* nutrition (Tresguerres et al. 2013). Its gene expression was investigated in *Platynereis dumerilii* under acidifying conditions (Wäge et al. 2015). The enzymatic concentration was studied in *Sabella spallanzanii* exposed to high PCO_2 concentration (Turner et al. 2015). The CAA was studied in the polychaetes *Riftia pachyptila*, *Chaetopterus variopedatus*, and a non-described species, and related to the absorption of inorganic carbon in animals with or without symbiont algae (Kochevar and Childress 1996; Goffredi et al. 1997). Thus, our comparative focus for the three studied species, associated to aspects of habitat and salinity tolerance is novel. In relation to volume regulation, few studies were conducted with isolated cells, most of those on eggs and red coelomocytes (Krishnamoorthi 1963; Costa et al. 1980; Costa and Pierce 1983). Some studies investigated whole body volume or tissue volume under osmotic challenges (Krishnamoorthi 1962, 1963; Ebbs and Straiger 1965; Freel et al. 1973; Oglesby et al. 1982; Schöttler et al. 1990; Van Gaest et al. 2007), others studied aminoacids related to salinity changes (Clark 1968a, b; Freel et al. 1973; Fletcher 1974b; Jorgensen 1979; Costa et al 1980;

Koenig et al. 1981; Costa and Pierce 1983; Schöttler et al. 1990; Blank et al. 2004; Hoeger and Abe 2004). No previous studies were conducted before with isolated cells from a tissue or body. Thus, our data are relevant to the knowledge of polychaetes physiology.

5 Conclusion

The initial hypothesis was essentially confirmed, as CAA and cell volume regulation capacity in polychaetes are directly proportional to euryhalinity of species. However, an osmotic gradient was detected only in the most stenohaline species, *N. fluviatilis*. Cell volume regulation capacity tested in isolated cells represents a great novelty in literature of polychaetes. CA is also very understudied in these invertebrates, and thus represent novel focus for the group. Finally, the comparative approach of physiological data related to ecological aspects contributes to better understand tolerances of species.

Literature cited

Amado, E. M., Freire, C. A., & Souza, M. M. (2006). Osmoregulation and tissue water regulation in the freshwater red crab *Dilocarcinus pagei* (Crustacea, Decapoda), and the effect of waterborne inorganic lead. Aquatic toxicology, 79(1), 1-8.

Amado, E. M., Freire, C. A., Grassi, M. T., & Souza, M. M. (2012). Lead hampers gill cell volume regulation in marine crabs: stronger effect in a weak osmoregulator than in an osmoconformer. Aquatic toxicology, 106, 95-103.

Bastida, R., Trassens, M., & Martin, J. P. (2004). Polychaete assemblages of intertidal mixohaline flats of Bahía Samborombón (La Plata River estuary-Argentina). Thalassas: An international journal of marine sciences, 20(2), 39-53.

Blank, M., Bastrop, R., Röhner, M., & Jürss, K. (2004). Effect of salinity on spatial distribution and cell volume regulation in two sibling species of *Marenzelleria* (Polychaeta: Spionidae). Marine Ecology Progress Series, 271, 193-205.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical biochemistry, 72(1-2), 248-254.

Braga, C. F., Monteiro, V. F., Rosa-Filho, J. S., & Beasley, C. R. (2011). Benthic macroinfaunal assemblages associated with Amazonian saltmarshes. Wetlands Ecology and Management, 19(3), 257-272.

Bundy, H. F. (1977). Carbonic anhydrase. Comparative Biochemistry and Physiology Part B: Comparative Biochemistry, 57(1), 1-7.

Capó-Aponte, J. E., Iserovich, P., & Reinach, P. S. (2005). Characterization of regulatory volume behavior by fluorescence quenching in human corneal epithelial cells. The Journal of membrane biology, 207(1), 11-22.

Clark, M. E. (1968)a. A survey of the effect of osmotic dilution on free amino acids of various polychaetes. The Biological Bulletin, 134(2), 252-260.

Clark, M. E. (1968)b. Free amino-acid levels in the coelomic fluid and body wall of polychaetes. The Biological Bulletin, 134(1), 35-47.

Clauss, W. G. (2001). Epithelial transport and osmoregulation in annelids. Canadian journal of zoology, 79(2), 192-203.

Colling, L. A., Bemvenuti, C. E., & Gandra, M. S. (2007). Seasonal variability on the structure of sublittoral macrozoobenthic association in the Patos Lagoon estuary, southern Brazil. Iheringia. Série Zoologia, 97(3), 257-262.

Costa, C. J., & Pierce, S. K. (1983). Volume regulation in the red coelomocytes of *Glycera dibranchiata*: An interaction of amino acid and K+ effluxes. Journal of comparative physiology, 151(2), 133-144.

Costa, C. J., Pierce, S. K., & Warren, M. K. (1980). The intracellular mechanism of salinity tolerance in polychaetes: volume regulation by isolated Glycera dibranchiata red coelomocytes. The Biological Bulletin, 159(3), 626-638.

Dice, J. F. (1969). Osmoregulation and salinity tolerance in the polychaete annelid *Cirriformia spirabrancha* (Moore, 1904). Comparative Biochemistry and Physiology, 28(3), 1331-1343.

Doneen, B. A., & Clark, M. E. (1974). Sodium fluxes in isolated body walls of the euryhaline polychaete, *Nereis (Neanthes) succinea*. Comparative Biochemistry and Physiology Part A: Physiology, 48(2), 221-228.

Dragolovich, J., & Pierce, S. K. (1992). Comparative time courses of inorganic and organic osmolyte accumulation as horseshoe crabs (*Limulus*

polyphemus) adapt to high salinity. Comparative Biochemistry and Physiology Part A: Physiology, 102(1), 79-84.

Ebbs Jr, N. K., & Staiger, J. C. (1965). Some osmotic adaptations of *Onuphis magna* (Polychaeta: Onuphidae). Bulletin of Marine Science, 15(4), 835-849.

Evans, D. L., Strom, D. G., & Mosura-Bliss, E. L. (2010). Spatial Distribution of Benthic Macroinvertebrates in the Crystal River/Kings Bay System with Emphasis on Relationships with Salinity.

Farrelly, C., & Greenaway, P. E. T. E. R. (1994). Gas exchange through the lungs and gills in air-breathing crabs. Journal of Experimental Biology, 187(1), 113-130.

Filho, J. S. R., & Aviz, D. (2013). Macrobenthic communities of an Amazonian estuary (Guajará Bay, Brazil): temporal and spatial changes. Journal of Coastal Research, 65(sp1), 123-128.

Fletcher, C. R. (1974)a. Volume regulation in *Nereis diversicolor*—I. The steady state. Comparative Biochemistry and Physiology Part A: Physiology, 47(4), 1199-1214.

Fletcher, C. R. (1974)b. Volume regulation in *Nereis diversicolor*—III. Adaptation to a reduced salinity. Comparative Biochemistry and Physiology Part A: Physiology, 47(4), 1221-1234.

Freel, R. W., Medler, S. G., & Clark, M. E. (1973). Solute adjustments in the coelomic fluid and muscle fibers of a euryhaline polychaete, *Neanthes succinea*, adapted to various salinities. The Biological Bulletin, 144(2), 289-303.

Fretter, V. (1955). Uptake of radioactive sodium (24 Na) by *Nereis diversicolor* Mueller and Perinereis cultrifera (Grube). Journal of the Marine Biological Association of the United Kingdom, 34(01), 151-160.

Giménez, L., Dimitriadis, C., Carranza, A., Borthagaray, A. I., & Rodríguez, M. (2006). Unravelling the complex structure of a benthic community: A multiscale-multianalytical approach to an estuarine sandflat. Estuarine, Coastal and Shelf Science, 68(3), 462-472.

Glasby, C. J., & Timm, T. (2008). Global diversity of polychaetes (Polychaeta; Annelida) in freshwater. Hydrobiologia, 595(1), 107-115.

Goffredi, S., Childress, J., Desaulniers, N., Lee, R., Lallier, F., & Hammond, D. O. U. G. (1997). Inorganic carbon acquisition by the hydrothermal vent

tubeworm *Riftia pachyptila* depends upon high external PCO_2 and upon proton-equivalent ion transport by the worm. Journal of Experimental Biology, 200(5), 883-896.

Grosberg, R. K., Vermeij, G. J., & Wainwright, P. C. (2012). Biodiversity in water and on land. Current Biology, 22(21), R900-R903.

Hamann, S., Kiilgaard, J. F., Litman, T., Alvarez-Leefmans, F. J., Winther, B. R., & Zeuthen, T. (2002). Measurement of cell volume changes by fluorescence self-quenching. Journal of fluorescence, 12(2), 139-145.

Häussinger, D. (1996). The role of cellular hydration in the regulation of cell function. Biochemical Journal, 313(Pt 3), 697.

Henry, R. P. (1988). Multiple functions of carbonic anhydrase in the crustacean gill. Journal of Experimental Zoology, 248(1), 19-24.

Henry, R. P. (1996). Multiple roles of carbonic anhydrase in cellular transport and metabolism. Annual Review of Physiology, 58(1), 523-538.

Henry, R. P. (1984). The role of carbonic anhydrase in blood ion and acid-base regulation. American Zoologist, 24(1), 241-251.

Henry, R. P., & Cameron, J. N. (1982). The distribution and partial characterization of carbonic anhydrase in selected aquatic and terrestrial decapod crustaceans. Journal of Experimental Zoology, 221(3), 309-321.

Henry, R. P., & Saintsing, D. G. (1983). Carbonic anhydrase activity and ion regulation in three species of osmoregulating bivalve molluscs. Physiological Zoology, 274-280.

Henry, R. P., Lucu, C., Onken, H., & Weihrauch, D. (2012). Multiple functions of the crustacean gill: osmotic/ionic regulation, acid-base balance, ammonia excretion, and bioaccumulation of toxic metals. Frontiers in physiology, 3.

Hoeger, U., & Abe, H. (2004). β-Alanine and other free amino acids during salinity adaptation of the polychaete *Nereis japonica*. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 137(1), 161-171.

Hoffmann, E. K., & Dunham, P. B. (1995). Membrane mechanisms and intracellular signalling in cell volume regulation. International review of cytology, 161, 173-262.

Hoffmann, E. K., Lambert, I. H., & Pedersen, S. F. (2009). Physiology of cell volume regulation in vertebrates. Physiological reviews, 89(1), 193-277.

Horn, D. P. (2002). Beach groundwater dynamics. Geomorphology, 48(1), 121-146.

Horn, D. P. (2006). Measurements and modelling of beach groundwater flow in the swash-zone: a review. Continental Shelf Research, 26(5), 622-652.

Jørgensen, N. O. G. (1979). Uptake of L-valine and other amino acids by the polychaete *Nereis virens*. Marine Biology, 52(1), 45-52.

Kochevar, R. E., & Childress, J. J. (1996). Carbonic anhydrase in deep-sea chemoautotrophic symbioses. Marine Biology, 125(2), 375-383.

Koenig, M. L., Powell, E. N., & Kasschau, M. R. (1981). The effects of salinity change on the free amino acid pools of two nereid polychaetes, *Neanthes succinea* and *Leonereis culveri*. Comparative Biochemistry and Physiology Part A: Physiology, 70(4), 631-637.

Krishnamoorthi, B. (1962, December). Salinity tolerance and volume regulation in four species of polychaetes. In Proceedings of the Indian Academy of Sciences-Section B (Vol. 56, No. 6, pp. 363-371). Springer India.

Krishnamoorthi, B. (1963). Volume regulation in eggs, larvae and adults of a brackish-water polychaete, *Diopatra variabilis* (Southern). Proceedings: Plant Sciences, 57(5), 275-289.

Lana, P. C., Couto, E. C., & Almeida, M. V. (1997). Polychaete distribution and abundance in intertidal flats of Paranaguá Bay (SE Brazil). Bulletin of Marine Science, 60(2), 433-442.

Larsen, E. H., Deaton, L. E., Onken, H., O'Donnell, M., Grosell, M., Dantzler, W. H., & Weihrauch, D. (2014). Osmoregulation and excretion. Comprehensive Physiology.

Lucu, Č. (1990). Ionic regulatory mechanisms in crustacean gill epithelia. Comparative Biochemistry and Physiology Part A: Physiology, 97(3), 297-306.

Kneib, R. T. (1985). Predation and disturbance by grass shrimp, Palaemonetespugio Holthuis, in soft-substratum benthic invertebrate assemblages. Journal of Experimental Marine Biology and Ecology, 93(1-2), 91-102.

MacCord, F. S., & Amaral, A. C. Z. (2007). The reproductive cycle of *Scolelepis goodbodyi* (Polychaeta, Spionidae). Marine Biology, 151(3), 1009-1020.

Mazurkiewicz, M. (1975). Larval development and habits of *Laeonereis culveri* (Webster)(Polychaeta: Nereidae). The Biological Bulletin, 149(1), 186-204.

Melo, K. D. R., Tagliaro, C. H., & Beasley, C. R. (2013). Seasonal changes in the subtidal benthic macrofauna of a mangrove coast in northern Brazil. Journal of Coastal Research, 65(sp1), 87-92.

Mettam, C. (1981). Survival strategies in estuarine nereids. In Feeding and Survival Srategies of Estuarine Organisms (pp. 65-77). Springer US.

Muniz, P., Venturini, N., & Rodríguez, M. (2000). Macrobenthic communities in a temperate urban estuary of high dominance and low diversity: Montevideo Bay (Uruguay). Mar. Biol.

Nielsen, S. A., & Frieden, E. (1972). Carbonic anhydrase activity in molluscs. Comparative Biochemistry and Physiology Part B: Comparative Biochemistry, 41(3), 461-468.

Oglesby, L. C. (1965)a. Steady-state parameters of water and chloride regulation in estuarine nereid polychaetes. Comparative biochemistry and physiology, 14(4), 621-640.

Oglesby, L. C. (1965)b. Water and chloride fluxes in estuarine nereid polychaetes. Comparative biochemistry and physiology, 16(4), 437-455.

Oglesby, L. C. (1969). Salinity—stress and desiccation in intertidal worms. American Zoologist, 9(2), 319-331.

Oglesby, L. C. (1970). Studies on the salt and water balance of *Nereis diversicolor*—I. Steady-state parameters. Comparative biochemistry and physiology, 36(3), 449-466.

Oglesby, L. C. (1981). Volume regulation in aquatic invertebrates. Journal of Experimental Zoology, 215(3), 289-301.

Oglesby, L. C., Mangum, C. P., Heacox, A. E., & Ready, N. E. (1982). Salt and water balance in the polychaete *Nereis virens*. Comparative Biochemistry and Physiology Part A: Physiology, 73(1), 15-19.

Palmer, T. A., Montagna, P. A., Pollack, J. B., Kalke, R. D., & DeYoe, H. R. (2011). The role of freshwater inflow in lagoons, rivers, and bays. Hydrobiologia, 667(1), 49-67.

Passadore, C., Giménez, L., & Acuña, A. (2007). Composition and intra-annual variation of the macroinfauna in the estuarine zone of the Pando Stream (Uruguay). Brazilian Journal of Biology, 67(2), 197-202.

Péqueux, A., & Gilles, R. (1979). Effects of hypo-and hyperosmotic shocks on the volume and ions content of *Carcinus maenas* isolated axons. Comparative Biochemistry and Physiology Part A: Physiology, 64(3), 427-431.

Pierce, S. K. (1982). Invertebrate cell volume control mechanisms: a coordinated use of intracellular amino acids and inorganic ions as osmotic solute. The Biological Bulletin, 163(3), 405-419.

Pierce, S. K. (1971). Volume regulation and valve movements by marine mussels. Comparative Biochemistry and Physiology Part A: Physiology, 39(1), 103-117.

Pierce, S. K., & Politis, A. D. (1990). Ca2+-activated cell volume recovery mechanisms. Annual review of physiology, 52(1), 27-42.

Poersch, L., Castello, J. P., Wasielesky Jr, W., & Cavalli, R. O. (2007). The challenge of sustainable aquaculture: effects on the environment of the Patos Lagoon estuary. Journal of Coastal Research, 130-135.

Preston, R. L. (2008). 5 Osmoregulation in Annelids. Osmotic and Ionic Regulation: Cells and Animals, 135.

Rosa, L. C. D., & Bemvenuti, C. E. (2006)a. Seasonal stratification of the estuarine macroinfauna of the Patos Lagoon estuary, southern Brazil. Thalassas, 22 (2), 17-23.

Rosa, L. C. D., & Bemvenuti, C. E. (2006)b. Temporal variability of the estuarine macrofauna of the Patos Lagoon, Brazil. Revista de Biología Marina y Oceanografía, 41(1), 1-9.

Sanders, H. L., Mangelsdorf, P. C., & Hampson, G. R. (1965). Salinity and faunal distribution in the Pocasset River, Massachusetts. Limnology and Oceanography, 10(suppl).

Santos, I. A., Castellano, G. C., & Freire, C. A. (2013). Direct relationship between osmotic and ionic conforming behavior and tissue water regulatory capacity in echinoids. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 164(3), 466-476.

Santos, S. L., & Simon, J. L. (1974). Distribution and abundance of the polychaetous annelids in a south Florida estuary. Bulletin of Marine Science, 24(3), 669-689.

Sardini, A., Amey, J. S., Weylandt, K. H., Nobles, M., Valverde, M. A., & Higgins, C. F. (2003). Cell volume regulation and swelling-activated chloride

channels. Biochimica et Biophysica Acta (BBA)-Biomembranes, 1618(2), 153-162.

Schöttler, U., Daniels, D., & Zapf, K. (1990). Influence of anoxia on adaptation of euryhaline polychaetes to hyposmotic conditions. Marine Biology, 104(3), 443-451.

Silva, R. F., Rosa Filho, J. S., Souza, S. R., & Souza-Filho, P. W. (2011). Spatial and temporal changes in the structure of soft-bottom benthic communities in an Amazon estuary (Caeté estuary, Brazil). Journal of Coastal Research, (64), 440.

Sket, B., & Trontelj, P. (2008). Global diversity of leeches (Hirudinea) in freshwater. Hydrobiologia, 595(1), 129-137.

Souza-Bastos, L. R., & Freire, C. A. (2009). The handling of salt by the neotropical cultured freshwater catfish *Rhamdia quelen*. Aquaculture, 289(1), 167-174.

Tresguerres, M., Katz, S., & Rouse, G. W. (2013). How to get into bones: proton pump and carbonic anhydrase in *Osedax* boneworms. Proceedings of the Royal Society of London B: Biological Sciences, 280(1761), 20130625.

Turner, L. M., Ricevuto, E., Massa-Gallucci, A., Gambi, M. C., & Calosi, P. (2015). Energy metabolism and cellular homeostasis trade-offs provide the basis for a new type of sensitivity to ocean acidification in a marine polychaete at a high-CO2 vent: adenylate and phosphagen energy pools versus carbonic anhydrase. Journal of Experimental Biology, 218(14), 2148-2151.

Van Gaest, A. L., Young, C. M., Young, J. J., Helms, A. R., & Arellano, S. M. (2007). Physiological and behavioral responses of Bathynerita naticoidea (Gastropoda: Neritidae) and Methanoaricia dendrobranchiata (Polychaeta: Orbiniidae) to hypersaline conditions at a brine pool cold seep. Marine Ecology, 28(1), 199-207.

Vitale, A. M., Monserrat, J. M., Castilho, P., & Rodriguez, E. M. (1999). Inhibitory effects of cadmium on carbonic anhydrase activity and ionic regulation of the estuarine crab *Chasmagnathus granulata* (Decapoda, Grapsidae). Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology, 122(1), 121-129.

Wäge, J., Hardege, J. D., Larsson, T. A., Simakov, O., Chapman, E. C., Arendt, D., & Rotchell, J. M. (2015). Effects of low seawater pH on the marine polychaete *Platynereis dumerilii*. Marine pollution bulletin, 95(1), 166-172.

Wehner, F., Olsen, H., Tinel, H., Kinne-Saffran, E., & Kinne, R. K. (2003). Cell volume regulation: osmolytes, osmolyte transport, and signal transduction. In Reviews of physiology, biochemistry and pharmacology (pp. 1-80). Springer Berlin Heidelberg.

Weihrauch, D., & O'Donnell, M. J. (2015). Links between osmoregulation and nitrogen-excretion in insects and crustaceans. Integrative and comparative biology, 55(5), 816-829.

CONCLUSÃO

A novidade deste trabalho foi a abordagem comparativa, relacionando AAC a diferentes grupos zoológicos de invertebrados, e ao seu grau de conquista de ambientes de água doce e terrestres. Além disso, AAC é muito pouco estudada nos grupos dos equinodermos (cerca de 5 trabalhos) e dos poliquetas (apenas dois trabalhos encontrados). Então, sob essa visão altamente comparativa, foi possível detectar os padrões fisiológicos que seguem: 1) AAC se relaciona ao ambiente, e tende a ser mais alta em habitantes marinhos, dulcícolas e de água muito diluídas do que em espécies estuarinas, o que não havia sido constatado anteriormente; 2) AAC e os gradientes osmóticos / iônicos são diretamente proporcionais à eurihalinidade e ao sucesso na conquista de novos ambientes por uma espécie, e por um grupo como um todo, e ao controle de permeabilidade e capacidade de transporte vetorial de sal pelos epitélios do grupo zoológico; 3) a capacidade de manutenção de volume celular / tecidual contribui para a tolerância à salinidade de osmoconformadores e para o grau de eurihalinidade de osmorreguladores. Os presentes dados podem ser utilizados futuramente, em conjunto com dados coletados futuramente, como ferramentas para realização de inferências sobre os efeitos do aquecimento global sobre as espécies.

REFERÊNCIAS GERAIS

Abele, L. G. (1992). A review of the grapsid crab genus *Sesarma* (Crustacea: Decapoda: Grapsidae) in America, with the description of a new genus (No. 527). Smithsonian Institution Press.

Abessa, D. M. D. S., Zaroni, L. P., Sousa, E. C. P. M. D., Gasparro, M. R., Pereira, C. D. S., Rachid, B. R. D. F., ... & King, R. S. (2005). Physiological and cellular responses in two populations of the mussel *Perna perna* collected at different sites from the coast of São Paulo, Brazil. Brazilian Archives of Biology and Technology, 48(2), 217-225.

Amado, E. M., Freire, C. A., & Souza, M. M. (2006). Osmoregulation and tissue water regulation in the freshwater red crab *Dilocarcinus pagei* (Crustacea, Decapoda), and the effect of waterborne inorganic lead. Aquatic toxicology, 79(1), 1-8.

Amado, E. M., Freire, C. A., Grassi, M. T., & Souza, M. M. (2012). Lead hampers gill cell volume regulation in marine crabs: stronger effect in a weak osmoregulator than in an osmoconformer. Aquatic toxicology, 106, 95-103.

Anger, K. (1995). The conquest of freshwater and land by marine crabs: adaptations in life-history patterns and larval bioenergetics. Journal of Experimental Marine Biology and Ecology, 193(1), 119-145.

Anger, K. (1996). Salinity tolerance of the larvae and first juveniles of a semiterrestrial grapsid crab, *Armases miersii* (Rathbun). Journal of experimental marine biology and ecology, 202(2), 205-223.

Anger, K. (2001). The biology of decapod crustacean larvae (Vol. 14, pp. 1-420). Lisse: AA Balkema Publishers.

Anger, K., & Charmantier, G. (2000). Ontogeny of osmoregulation and salinity tolerance in a mangrove crab, *Sesarma curacaoense* (Decapoda: Grapsidae). Journal of experimental marine biology and ecology, 251(2), 265-274.

Anger, K., & Moreira, G. S. (2004). Biomass and elemental composition of eggs and larvae of a mangrove crab, *Sesarma rectum* Randall (Decapoda: Sesarmidae) and comparison to a related species with abbreviated larval development. Scientia Marina, 68(1), 117-126.

Augusto, A., Greene, L. J., Laure, H. J., & Mcnamara, J. C. (2007) a. Adaptive shifts in osmoregulatory strategy and the invasion of freshwater by brachyuran crabs: evidence from *Dilocarcinus pagei* (Trichodactylidae). Journal of Experimental Zoology Part A: Ecological Genetics and Physiology, 307(12), 688-698.

Augusto, A., Greene, L. J., Laure, H. J., & McNamara, J. C. (2007) b. The ontogeny of isosmotic intracellular regulation in the diadromous, freshwater palaemonid shrimps, *Macrobrachium amazonicum* and *M. olfersi* (Decapoda).Journal of Crustacean Biology, 27(4), 626-634.

Bastida, R., Trassens, M., & Martin, J. P. (2004). Polychaete assemblages of intertidal mixohaline flats of Bahía Samborombón (La Plata River estuary-Argentina). Thalassas: An international journal of marine sciences, 20(2), 39-53.

Berger, V. J., & Kharazova, A. D. (1997). Mechanisms of salinity adaptations in marine molluscs. In Interactions and Adaptation Strategies of Marine Organisms (pp. 115-126). Springer Netherlands.

Blank, M., Bastrop, R., Röhner, M., & Jürss, K. (2004). Effect of salinity on spatial distribution and cell volume regulation in two sibling species of *Marenzelleria* (Polychaeta: Spionidae). Marine Ecology Progress Series, 271, 193-205.

Bliss, D. E. (1968). Transition from water to land in decapod crustaceans. American Zoologist, 8(3), 355-392.

Bliss, D. E. (1979). From sea to tree: saga of a land crab. American Zoologist, 19(2), 385-410.

Böttcher, K., & Siebers, D. (1993). Biochemistry, localization, and physiology of carbonic anhydrase in the gills of euryhaline crabs. Journal of Experimental Zoology, 265(4), 397-409.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical biochemistry, 72(1-2), 248-254.

Braga, C. F., Monteiro, V. F., Rosa-Filho, J. S., & Beasley, C. R. (2011). Benthic macroinfaunal assemblages associated with Amazonian saltmarshes. Wetlands Ecology and Management, 19(3), 257-272.

Bundy, H. F. (1977). Carbonic anhydrase. Comparative Biochemistry and Physiology Part B: Comparative Biochemistry, 57(1), 1-7.

Burggren, W. W., & McMahon, B. R. (1988). Biology of the land crabs. Cambridge University Press.

Capó-Aponte, J. E., Iserovich, P., & Reinach, P. S. (2005). Characterization of regulatory volume behavior by fluorescence quenching in human corneal epithelial cells. The Journal of membrane biology, 207(1), 11-22.

Capitoli, R. R., Benvenuti, C. E., & Gianuca, N. M. (1977). Ocorrência e observações bioecológicas do caranguejo *Metasesarma rubripes* (Rathbun) na região estuarina da Lagoa dos Patos. Atlântica, 2(1), 50-62.

Castellano, G. C., Santos, I. A., & Freire, C. A. (2016). Maintenance of ionic gradients and tissue hydration in the intertidal sea cucumber *Holothuria grisea* under hypo-and hyper-salinity challenges. Journal of the Marine Biological Association of the United Kingdom, 1-8.

Clark, M. E. (1968)a. A survey of the effect of osmotic dilution on free amino acids of various polychaetes. The Biological Bulletin, 134(2), 252-260.

Clark, M. E. (1968)b. Free amino-acid levels in the coelomic fluid and body wall of polychaetes. The Biological Bulletin, 134(1), 35-47.

Clauss, W. G. (2001). Epithelial transport and osmoregulation in annelids. Canadian journal of zoology, 79(2), 192-203.

Colling, L. A., Bemvenuti, C. E., & Gandra, M. S. (2007). Seasonal variability on the structure of sublittoral macrozoobenthic association in the Patos Lagoon estuary, southern Brazil. Iheringia. Série Zoologia, 97(3), 257-262.

Conde, J. E., & Díaz, H. (1989). The mangrove tree crab *Aratus pisonii* in a tropical estuarine coastal lagoon. Estuarine, Coastal and Shelf Science, 28(6), 639-650.

Conde, J. E., Tognella, M. M. P., Paes, E. T., Soares, M. L. G., Louro, I. A., & Schaeffer-Novelli, Y. (2000). Population and life history features of the crab *Aratus pisonii* (Decapoda: Grapsidae) in a subtropical estuary. Interciencia-Caracas-, 25(3), 151-158.

Costa, C. J., & Pierce, S. K. (1983). Volume regulation in the red coelomocytes of *Glycera dibranchiata*: An interaction of amino acid and K+ effluxes. Journal of comparative physiology, 151(2), 133-144.

Costa, C. J., Pierce, S. K., & Warren, M. K. (1980). The intracellular mechanism of salinity tolerance in polychaetes: volume regulation by isolated Glycera dibranchiata red coelomocytes. The Biological Bulletin, 159(3), 626-638.

Cumberlidge, N., Fenolio, D. B., Walvoord, M. E., & Stout, J. (2005). Tree-climbing crabs (Potamonautidae and Sesarmidae) from phytotelmic microhabitats in rainforest canopy in Madagascar. Journal of Crustacean Biology, 25(2), 302-308.

Da Silva Castiglioni, D., de Oliveira, P. J. A., da Silva, J. S., & Coelho, P. A. (2011). Population dynamics of *Sesarma rectum* (Crustacea: Brachyura: Grapsidae) in the Ariquindá River mangrove, north-east of Brazil. Journal of the Marine Biological Association of the United Kingdom, 91(07), 1395-1401.

Dall, W. H. B. J., Hill, B. J., Rothlisberg, P. C., & Sharples, D. J. (1990). The biology of the Penaeidae. Advances in marine biology, 27.

Davenport, R. (1995). *Perna perna* enters the bays. Texas Conchologist, 31, 92.

Davenport, J., & Wong, T. M. (1986). Responses of the blood cockle *Anadara granosa* (L.)(Bivalvia: Arcidae) to salinity, hypoxia and aerial exposure. Aquaculture, 56(2), 151-162.

De Arruda Leme, M. H. (2006). Seasonal changes in reproductive traits of the crab *Sesarma rectum* (Grapsoidea: Sesarmidae) on the northern coast of São Paulo State, Brazil. Journal of Crustacean Biology, 26(2), 141-147.

De Melo, G. A. S. (1996). Manual de identificação dos Brachyura (caranguejos e siris) do litoral brasileiro. Editora Plêiade; Fundação de Amparo à Pesquisa do Estado de São Paulo.

De Vooys, C. G. N. (1991). Anaerobic metabolism in sublittoral living *Mytilus galloprovincialis* in the Mediterranean—IV. Role of amino acids in adaptation to low salinities during anaerobiosis and aerobiosis. Comparative Biochemistry and Physiology Part A: Physiology, 100(2), 423-431.

Deaton, L. (2008). 4 Osmotic and Ionic Regulation in Molluscs. Osmotic and Ionic Regulation: Cells and Animals, 107.

Díaz, H., & Conde, J. E. (1988). On the food sources for the mangrove tree crab *Aratus pisonii* (Brachyura: Grapsidae). Biotropica, 20(4), 348-350.

Díaz, H., & Conde, J. E. (1989). Population dynamics and life history of the mangrove crab *Aratus pisonii* (Brachyura, Grapsidae) in a marine environment. Bulletin of Marine Science, 45(1), 148-163.

Diaz, H., & Ewald, J. J. (1968). A comparison of the larval development of *Metasesarma rubripes* (Rathbun) and *Sesarma ricordi* H. Milne Edwards (Brachyura, Grapsidae) reared under similar laboratory conditions. Crustaceana. Supplement, 225-248.

Dice, J. F. (1969). Osmoregulation and salinity tolerance in the polychaete annelid *Cirriformia spirabrancha* (Moore, 1904). Comparative Biochemistry and Physiology, 28(3), 1331-1343.

Diehl, W. J. (1986). Osmoregulation in echinoderms. Comparative Biochemistry and Physiology Part A: Physiology, 84(2), 199-205.

Diehl, W. J., & Lawrence, J. M. (1985). Effect of salinity on the intracellular osmolytes in the pyloric caeca and tube feet of *Luidia clathrata* (Say)(Echinodermata: Asteroidea). Comparative Biochemistry and Physiology Part A: Physiology, 82(3), 559-566.

Doneen, B. A., & Clark, M. E. (1974). Sodium fluxes in isolated body walls of the euryhaline polychaete, *Nereis (Neanthes) succinea*. Comparative Biochemistry and Physiology Part A: Physiology, 48(2), 221-228.

Dragolovich, J., & Pierce, S. K. (1992). Comparative time courses of inorganic and organic osmolyte accumulation as horseshoe crabs (*Limulus polyphemus*) adapt to high salinity. Comparative Biochemistry and Physiology Part A: Physiology, 102(1), 79-84.

Ebbs Jr, N. K., & Staiger, J. C. (1965). Some osmotic adaptations of *Onuphis magna* (Polychaeta: Onuphidae). Bulletin of Marine Science, 15(4), 835-849.

Eshky, A. A., Atkinson, R. J. A., & Taylor, A. C. (1995). Physiological ecology of crabs from Saudi Arabian mangrove. Marine Ecology Progress Series, 126, 83-95.

Evans, D. H. (Ed.). (2008). Osmotic and ionic regulation: cells and animals. CRC Press.

Evans, D. L., Strom, D. G., & Mosura-Bliss, E. L. (2010). Spatial Distribution of Benthic Macroinvertebrates in the Crystal River/Kings Bay System with Emphasis on Relationships with Salinity.

Farrelly, C., & Greenaway, P. E. T. E. R. (1994). Gas exchange through the lungs and gills in air-breathing crabs. Journal of Experimental Biology, 187(1), 113-130.

Filho, J. S. R., & Aviz, D. (2013). Macrobenthic communities of an Amazonian estuary (Guajará Bay, Brazil): temporal and spatial changes. Journal of Coastal Research, 65(sp1), 123-128.

Fischer, E. A., Duarte, L. F., & Araujo, A. C. (1997). Consumption of bromeliad flowers by the crab *Metasesarma rubripes* in a Brazilian coastal forest. Crustaceana, 70(1), 118-123.

Fletcher, C. R. (1974)a. Volume regulation in *Nereis diversicolor*—I. The steady state. Comparative Biochemistry and Physiology Part A: Physiology, 47(4), 1199-1214.

Fletcher, C. R. (1974)b. Volume regulation in *Nereis diversicolor*—III. Adaptation to a reduced salinity. Comparative Biochemistry and Physiology Part A: Physiology, 47(4), 1221-1234.

Florkin, M. (1962). La régulation isosmotique intracellulaire chez les invertébrés marins euryhalins. Académie royale.

Flück, M., Webster, K. A., Graham, J., Giomi, F., Gerlach, F., & Schmitz, A. (2007). Coping with cyclic oxygen availability: evolutionary aspects. Integrative and comparative biology, 47(4), 524-531.

Foster, C., Amado, E. M., Souza, M. M., & Freire, C. A. (2010). Do osmoregulators have lower capacity of muscle water regulation than osmoconformers? A study on decapod crustaceans. Journal of Experimental Zoology Part A: Ecological Genetics and Physiology, 313(2), 80-94.

Freel, R. W., Medler, S. G., & Clark, M. E. (1973). Solute adjustments in the coelomic fluid and muscle fibers of a euryhaline polychaete, *Neanthes succinea*, adapted to various salinities. The Biological Bulletin, 144(2), 289-303.

Freire, C. A., Cavassin, F., Rodrigues, E. N., Torres, A. H., & McNamara, J. C. (2003). Adaptive patterns of osmotic and ionic regulation, and the invasion of fresh water by the palaemonid shrimps. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 136(3), 771-778.

Freire, C. A., Onken, H., & McNamara, J. C. (2008). A structure–function analysis of ion transport in crustacean gills and excretory organs. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 151(3), 272-304.

Freire, C. A., Souza-Bastos, L. R., Amado, E. M., Prodocimo, V., & Souza, M. M. (2013). Regulation of muscle hydration upon hypo-or hyper-osmotic shocks: differences related to invasion of the freshwater habitat by decapod crustaceans. Journal of Experimental Zoology Part A: Ecological Genetics and Physiology, 319(6), 297-309.

Fretter, V. (1955). Uptake of radioactive sodium (24 Na) by *Nereis diversicolor* Mueller and Perinereis cultrifera (Grube). Journal of the Marine Biological Association of the United Kingdom, 34(01), 151-160.

Furlan, M., Castilho, A. L., Fernandes-Goes, L. C., Fransozo, V., Bertini, G., & COSTA, R. C. (2013). Effect of environmental factors on the abundance of decapod crustaceans from soft

bottoms off southeastern Brazil. Anais da Academia Brasileira de Ciências, 85(4), 1345-1356.
Gilmour, K. M., & Perry, S. F. (2009). Carbonic anhydrase and acid–base regulation in fish. Journal of Experimental Biology, 212(11), 1647-1661.
Giménez, L., Dimitriadis, C., Carranza, A., Borthagaray, A. I., & Rodríguez, M. (2006). Unravelling the complex structure of a benthic community: A multiscale-multianalytical approach to an estuarine sandflat. Estuarine, Coastal and Shelf Science, 68(3), 462-472.
Giomi, F., Fusi, M., Barausse, A., Mostert, B., Pörtner, H. O., & Cannicci, S. (2014). Improved heat tolerance in air drives the recurrent evolution of air-breathing. Proceedings of the Royal Society of London B: Biological Sciences, 281(1782), 20132927.
Glasby, C. J., & Timm, T. (2008). Global diversity of polychaetes (Polychaeta; Annelida) in freshwater. Hydrobiologia, 595(1), 107-115.
Goffredi, S., Childress, J., Desaulniers, N., Lee, R., Lallier, F., & Hammond, D. O. U. G. (1997). Inorganic carbon acquisition by the hydrothermal vent tubeworm *Riftia pachyptila* depends upon high external PCO_2 and upon proton-equivalent ion transport by the worm. Journal of Experimental Biology, 200(5), 883-896.
Grosberg, R. K., Vermeij, G. J., & Wainwright, P. C. (2012). Biodiversity in water and on land. Current Biology, 22(21), R900-R903.
Hamann, S., Kiilgaard, J. F., Litman, T., Alvarez-Leefmans, F. J., Winther, B. R., & Zeuthen, T. (2002). Measurement of cell volume changes by fluorescence self-quenching. Journal of fluorescence, 12(2), 139-145.
Häussinger, D. (1996). The role of cellular hydration in the regulation of cell function. Biochemical Journal, 313(Pt 3), 697.
Hayashi, Y., & Motokawa, T. (1986). Effects of ionic environment on viscosity of catch connective tissue in holothurian body wall. Journal of Experimental Biology, 125(1), 71-84.
Henry, R. P. (1984). The role of carbonic anhydrase in blood ion and acid-base regulation. American Zoologist, 24(1), 241-251.
Henry, R. P. (1988). Multiple functions of carbonic anhydrase in the crustacean gill. Journal of Experimental Zoology, 248(1), 19-24.
Henry, R. P. (1991). Branchial and branchiostegite carbonic anhydrase in decapod crustaceans: the aquatic to terrestrial transition. Journal of Experimental Zoology, 259(3), 294-303.
Henry, R. P. (1996). Multiple roles of carbonic anhydrase in cellular transport and metabolism. Annual Review of Physiology, 58(1), 523-538.
Henry, R. P., & Cameron, J. N. (1982). The distribution and partial characterization of carbonic anhydrase in selected aquatic and terrestrial decapod crustaceans. Journal of Experimental Zoology, 221(3), 309-321.
Henry, R. P., & Cameron, J. N. (1983). The Role of Carbonic Anhydrase in Respiration, Ion Regulation and Acid-Base Balance in the Aquatic Crab *Callinectes sapidus* and the Terrestrial Crab *Gecarcinus lateralis*. Journal of Experimental Biology, 103(1), 205-223.
Henry, R. P., Gehnrich, S., Weihrauch, D., & Towle, D. W. (2003). Salinity-mediated carbonic anhydrase induction in the gills of the euryhaline green crab, *Carcinus maenas*. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 136(2), 243-258.
Henry, R. P., Lucu, C., Onken, H., & Weihrauch, D. (2012). Multiple functions of the crustacean gill: osmotic/ionic regulation, acid-base balance, ammonia excretion, and bioaccumulation of toxic metals. Frontiers in physiology, 3.
Henry, R. P., & Saintsing, D. G. (1983). Carbonic anhydrase activity and ion regulation in three species of osmoregulating bivalve molluscs. Physiological Zoology, 274-280.
Herreid, C. F. (1969). Integument permeability of crabs and adaptation to land. Comparative Biochemistry and Physiology, 29(1), 423-429.
Hill, R. W., Wyse, G. A. & Anderson, M. (2008). Animal Physiology. Sinauer Associates Inc.
Hoeger, U., & Abe, H. (2004). β-Alanine and other free amino acids during salinity adaptation of the polychaete *Nereis japonica*. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 137(1), 161-171.
Hoffmann, E. K., & Dunham, P. B. (1995). Membrane mechanisms and intracellular signalling in cell volume regulation. International review of cytology, 161, 173-262.
Hoffmann, E. K., Lambert, I. H., & Pedersen, S. F. (2009). Physiology of cell volume regulation in vertebrates. Physiological reviews, 89(1), 193-277.
Horn, D. P. (2002). Beach groundwater dynamics. Geomorphology, 48(1), 121-146.
Horn, D. P. (2006). Measurements and modelling of beach groundwater flow in the swash-zone: a review. Continental Shelf Research, 26(5), 622-652.

Ivleva, I. V. (1980). The dependence of crustacean respiration rate on body mass and habitat temperature. Internationale Revue der gesamten Hydrobiologie und Hydrographie, 65(1), 1-47.

Jørgensen, N. O. G. (1979). Uptake of L-valine and other amino acids by the polychaete *Nereis virens*. Marine Biology, 52(1), 45-52.

Kirschner, L. B. (1991). 2 Water and Ions. Comparative Animal Physiology, Environmental and Metabolic Animal Physiology, 1, 13.

Kneib, R. T. (1985). Predation and disturbance by grass shrimp, Palaemonetespugio Holthuis, in soft-substratum benthic invertebrate assemblages. Journal of Experimental Marine Biology and Ecology, 93(1-2), 91-102.

Kochevar, R. E., & Childress, J. J. (1996). Carbonic anhydrase in deep-sea chemoautotrophic symbioses. Marine Biology, 125(2), 375-383.

Koenig, M. L., Powell, E. N., & Kasschau, M. R. (1981). The effects of salinity change on the free amino acid pools of two nereid polychaetes, *Neanthes succinea* and *Leonereis culveri*. Comparative Biochemistry and Physiology Part A: Physiology, 70(4), 631-637.

Kowalczuk, V. G., & Masunari, S. (2000). Estrutura populacional de *Armases angustipes* (Dana)(Decapoda, Brachyura, Grapsidae) na Ilha do Farol, Matinhos, Paraná. Revista Brasileira de Zoologia, 17(1), 1-16.

Krishnamoorthi, B. (1962, December). Salinity tolerance and volume regulation in four species of polychaetes. In Proceedings of the Indian Academy of Sciences-Section B (Vol. 56, No. 6, pp. 363-371). Springer India.

Krishnamoorthi, B. (1963). Volume regulation in eggs, larvae and adults of a brackish-water polychaete, *Diopatra variabilis* (Southern). Proceedings: Plant Sciences, 57(5), 275-289.

Kristensen, E., & Kostka, J. E. (2005). Macrofaunal burrows and irrigation in marine sediment: microbiological and biogeochemical interactions. Interactions between macro-and microorganisms in marine sediments, 125-157.

Lana, P. C., Couto, E. C., & Almeida, M. V. (1997). Polychaete distribution and abundance in intertidal flats of Paranaguá Bay (SE Brazil). Bulletin of Marine Science, 60(2), 433-442.

Larsen, E. H., Deaton, L. E., Onken, H., O'Donnell, M., Grosell, M., Dantzler, W. H., & Weihrauch, D. (2014). Osmoregulation and excretion. Comprehensive Physiology.

Lawrence, J. M., & Kafri, J. (1979). Numbers, biomass, and caloric content of the echinoderm fauna of the rocky shores of Barbados. Marine Biology, 52(1), 87-91.

Lee, C. E., & Bell, M. A. (1999). Causes and consequences of recent freshwater invasions by saltwater animals. Trends in Ecology & Evolution,14(7), 284-288.

Lima, G. V., Soares, M. R., & Oshiro, L. M. (2006). Reproductive biology of the sesarmid crab *Armases rubripes* (Decapoda, Brachyura) from an estuarine area of the Sahy River, Sepetiba Bay, Rio de Janeiro, Brazil. Iheringia. Série Zoologia, 96(1), 47-52.

Lucu, Č. (1990). Ionic regulatory mechanisms in crustacean gill epithelia. Comparative Biochemistry and Physiology Part A: Physiology, 97(3), 297-306.

Luppi, T. A., Spivak, E. D., & Bas, C. C. (2003). The effects of temperature and salinity on larval development of *Armases rubripes* Rathbun, 1897 (Brachyura, Grapsoidea, Sesarmidae), and the southern limit of its geographical distribution. Estuarine, Coastal and Shelf Science, 58(3), 575-585.

MacCord, F. S., & Amaral, A. C. Z. (2007). The reproductive cycle of *Scolelepis goodbodyi* (Polychaeta, Spionidae). Marine Biology, 151(3), 1009-1020.

Magalhães, C. A., Taniguchi, S., Cascaes, M. J., & Montone, R. C. (2012). PCBs, PBDEs and organochlorine pesticides in crabs *Hepatus pudibundus* and *Callinectes danae* from Santos Bay, State of Sao Paulo, Brazil. Marine pollution bulletin, 64(3), 662-667.

Mangum, C. P. (1994). Multiple sites of gas exchange. American Zoologist, 34(2), 184-193.

Maraschi, A. C., Freire, C. A., & Prodocimo, V. (2015). Immunocytochemical localization of V-H+-ATPase, Na+/K+-ATPase, and carbonic anhydrase in gill lamellae of adult freshwater euryhaline shrimp *Macrobrachium acanthurus* (Decapoda, Palaemonidae). Journal of Experimental Zoology Part A: Ecological Genetics and Physiology, 323(7), 414-421.

Masunari, S. (2006). Distribution and abundance of fiddler crabs *Uca* Leach (Crustacea Decapoda Ocypodidae) in Guaratuba Bay, Parana State, southern Brazil. Revista Brasileira de Zoologia, 23(4), 901-914.

Mazurkiewicz, M. (1975). Larval development and habits of *Laeonereis culveri* (Webster)(Polychaeta: Nereidae). The Biological Bulletin, 149(1), 186-204.

Melo, K. D. R., Tagliaro, C. H., & Beasley, C. R. (2013). Seasonal changes in the subtidal benthic macrofauna of a mangrove coast in northern Brazil. Journal of Coastal Research, 65(sp1), 87-92.

Mettam, C. (1981). Survival strategies in estuarine nereids. In Feeding and Survival Srategies of Estuarine Organisms (pp. 65-77). Springer US.

Mirjana, H. B. (2006). The basket shell, *Corbula gibba* Olivi, 1792 (Bivalve Mollusks) as a species resistant to environmental disturbances: A review. Acta adriatica, 47(1), 49-64.

Montú, M., Anger, K., & Bakker, C. D. (1990). Variability in the larval development of *Metasesarma rubripes* (Decapoda, Grapsidae) reared in the laboratory. Neritica, 5, 113-118.

Morris, S. T. E. P. H. E. N. (2001). Neuroendocrine regulation of osmoregulation and the evolution of air-breathing in decapod crustaceans. Journal of Experimental Biology, 204(5), 979-989.

Motokawa, T. A. T. S. U. O. (1994). Effects of ionic environment on viscosity of Triton-extracted catch connective tissue of a sea cucumber body wall. Comparative Biochemistry and Physiology Part B: Comparative Biochemistry, 109(4), 613-622.

Muniz, P., Venturini, N., & Rodríguez, M. (2000). Macrobenthic communities in a temperate urban estuary of high dominance and low diversity: Montevideo Bay (Uruguay). Mar. Biol.

Nielsen, S. A., & Frieden, E. (1972). Carbonic anhydrase activity in molluscs. Comparative Biochemistry and Physiology Part B: Comparative Biochemistry, 41(3), 461-468.

Oglesby, L. C. (1965)a. Steady-state parameters of water and chloride regulation in estuarine nereid polychaetes. Comparative biochemistry and physiology, 14(4), 621-640.

Oglesby, L. C. (1965)b. Water and chloride fluxes in estuarine nereid polychaetes. Comparative biochemistry and physiology, 16(4), 437-455.

Oglesby, L. C. (1969). Salinity—stress and desiccation in intertidal worms. American Zoologist, 9(2), 319-331.

Oglesby, L. C. (1970). Studies on the salt and water balance of *Nereis diversicolor*—I. Steady-state parameters. Comparative biochemistry and physiology, 36(3), 449-466.

Oglesby, L. C. (1981). Volume regulation in aquatic invertebrates. Journal of Experimental Zoology, 215(3), 289-301.

Oglesby, L. C., Mangum, C. P., Heacox, A. E., & Ready, N. E. (1982). Salt and water balance in the polychaete *Nereis virens*. Comparative Biochemistry and Physiology Part A: Physiology, 73(1), 15-19.

Palmer, T. A., Montagna, P. A., Pollack, J. B., Kalke, R. D., & DeYoe, H. R. (2011). The role of freshwater inflow in lagoons, rivers, and bays. Hydrobiologia, 667(1), 49-67.

Passadore, C., Giménez, L., & Acuña, A. (2007). Composition and intra-annual variation of the macroinfauna in the estuarine zone of the Pando Stream (Uruguay). Brazilian Journal of Biology, 67(2), 197-202.

Péqueux, A. (1995). Osmotic regulation in crustaceans. Journal of Crustacean Biology, 15(1), 1-60.

Péqueux, A., & Gilles, R. (1979). Effects of hypo-and hyperosmotic shocks on the volume and ions content of *Carcinus maenas* isolated axons. Comparative Biochemistry and Physiology Part A: Physiology, 64(3), 427-431.

Pierce, S. K. (1982). Invertebrate cell volume control mechanisms: a coordinated use of intracellular amino acids and inorganic ions as osmotic solute. The Biological Bulletin, 163(3), 405-419.

Pierce, S. K. (1971). Volume regulation and valve movements by marine mussels. Comparative Biochemistry and Physiology Part A: Physiology, 39(1), 103-117.

Pierce, S. K., & Politis, A. D. (1990). Ca2+-activated cell volume recovery mechanisms. Annual review of physiology, 52(1), 27-42.

Poersch, L., Castello, J. P., Wasielesky Jr, W., & Cavalli, R. O. (2007). The challenge of sustainable aquaculture: effects on the environment of the Patos Lagoon estuary. Journal of Coastal Research, 130-135.

Preston, R. L. (2008). 5 Osmoregulation in Annelids. Osmotic and Ionic Regulation: Cells and Animals, 135.

Pritchard, D. W. (1967). What is an estuary: physical viewpoint. American Association for the Advancement of Science.

Rasmussen, A., & Andersen, O. (1996). Apparent water permeability as a physiological parameter in crustaceans. The Journal of experimental biology, 199(12), 2555-2564.

Ribeiro, F. B., Matthews-Cascon, H., & Bezerra, L. E. A. (2012). Population structure and reproductive biology of the crab *Sesarma rectum* Randall, 1840 (Brachyura, Sesarmidae) in an impacted tropical mangrove in northeast Brazil. Crustaceana, 85(2), 173.

Riisgård, H. U., Zalacáin, D., Jeune, N., Wiersma, J. B., Lüskow, F., & Pleissner, D. (2015). Adaptation of the brine shrimp *Artemia salina* (Branchiopoda: Anostraca) to filter-feeding: effects of body size and temperature on filtration and respiration rates. Journal of Crustacean Biology, 35(5), 650-658.

Rivera-Ingraham, G. A., Barri, K., Boël, M., Farcy, E., Charles, A. L., Geny, B., & Lignot, J. H. (2016). Osmoregulation and salinity-induced oxidative stress: is oxidative adaptation determined by gill function?. Journal of Experimental Biology, 219(1), 80-89.

Robertson, J. D. (1949). Ionic regulation in some marine invertebrates. Journal of Experimental Biology, 26(2), 182-200.

Rosa, L. C. D., & Bemvenuti, C. E. (2006)a. Seasonal stratification of the estuarine macroinfauna of the Patos Lagoon estuary, southern Brazil. Thalassas, 22 (2), 17-23.

Rosa, L. C. D., & Bemvenuti, C. E. (2006)b. Temporal variability of the estuarine macrofauna of the Patos Lagoon, Brazil. Revista de Biología Marina y Oceanografía, 41(1), 1-9.

Ruppert, E. E., Fox, R. S., Barnes, R. D. Zoologia dos Invertebrados – Uma Abordagem Funcional-Evolutiva. São Paulo: Roca, 2005; 1145 pp

Sanders, H. L., Mangelsdorf, P. C., & Hampson, G. R. (1965). Salinity and faunal distribution in the Pocasset River, Massachusetts. Limnology and Oceanography, 10(suppl).

Santos, I. A., Castellano, G. C., & Freire, C. A. (2013). Direct relationship between osmotic and ionic conforming behavior and tissue water regulatory capacity in echinoids. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 164(3), 466-476.

Santos, S. L., & Simon, J. L. (1974). Distribution and abundance of the polychaetous annelids in a south Florida estuary. Bulletin of Marine Science, 24(3), 669-689.

Santos-Gouvea, I. A., & Freire, C. A. (2007). Effects of hypo-and hypersaline seawater on the microanatomy and ultrastructure of epithelial tissues of *Echinometra lucunter* (Echinodermata: Echinoidea) of intertidal and subtidal populations. ZOOLOGICAL STUDIES-TAIPEI-, 46(2), 203.

Sardini, A., Amey, J. S., Weylandt, K. H., Nobles, M., Valverde, M. A., & Higgins, C. F. (2003). Cell volume regulation and swelling-activated chloride channels. Biochimica et Biophysica Acta (BBA)-Biomembranes, 1618(2), 153-162.

Schöttler, U., Daniels, D., & Zapf, K. (1990). Influence of anoxia on adaptation of euryhaline polychaetes to hyposmotic conditions. Marine Biology, 104(3), 443-451.

Schubart, C. D., Cuesta, J. A., Diesel, R., & Felder, D. L. (2000). Molecular phylogeny, taxonomy, and evolution of nonmarine lineages within the American grapsoid crabs (Crustacea: Brachyura). Molecular Phylogenetics and Evolution, 15(2), 179-190.

Schwamborn, R., Neumann-Leitão, S., Silva, T. A., Silva, A. P., Ekau, W., & Saint-Paul, U. (2001). Distribution and dispersal of decapod crustacean larvae and other zooplankton in the Itamaracá estuarine system, Brazil. Tropical Oceanography, 29(1), 1-18.

Sender, S., Böttcher, K., Cetin, Y., & Gros, G. (1999). Carbonic anhydrase in the gills of seawater- and freshwater-acclimated flounders *Platichthys flesus*: purification, characterization, and immunohistochemical localization. Journal of Histochemistry & Cytochemistry, 47(1), 43-50.

Silva, R. F., Rosa Filho, J. S., Souza, S. R., & Souza-Filho, P. W. (2011). Spatial and temporal changes in the structure of soft-bottom benthic communities in an Amazon estuary (Caeté estuary, Brazil). Journal of Coastal Research, (64), 440.

Sket, B., & Trontelj, P. (2008). Global diversity of leeches (Hirudinea) in freshwater. Hydrobiologia, 595(1), 129-137.

Smith, W. K., & Miller, P. C. (1973). The thermal ecology of two south Florida fiddler crabs: *Uca rapax* Smith and *U. pugilator* Bosc. Physiological Zoology, 46(3), 186-207.

Souza-Bastos, L. R., & Freire, C. A. (2009). The handling of salt by the neotropical cultured freshwater catfish *Rhamdia quelen*. Aquaculture, 289(1), 167-174.

Thiercelin, N. (2016). *Impact of life history and ecology on rate of diversification and speciation, as exemplified by thoracotreme crabs along the western tropical Atlantic and on both sides of the Isthmus of Panama* (Doctoral dissertation).

Tresguerres, M., Katz, S., & Rouse, G. W. (2013). How to get into bones: proton pump and carbonic anhydrase in *Osedax* boneworms. Proceedings of the Royal Society of London B: Biological Sciences, 280(1761), 20130625.

Truchot, J. P. (1990). Respiratory and ionic regulation in invertebrates exposed to both water and air. Annual review of physiology, 52(1), 61-74.

Turner, L. M., Ricevuto, E., Massa-Gallucci, A., Gambi, M. C., & Calosi, P. (2015). Energy metabolism and cellular homeostasis trade-offs provide the basis for a new type of sensitivity to ocean acidification in a marine polychaete at a high-CO2 vent: adenylate and phosphagen

energy pools versus carbonic anhydrase. Journal of Experimental Biology, 218(14), 2148-2151.

Van Gaest, A. L., Young, C. M., Young, J. J., Helms, A. R., & Arellano, S. M. (2007). Physiological and behavioral responses of Bathynerita naticoidea (Gastropoda: Neritidae) and Methanoaricia dendrobranchiata (Polychaeta: Orbiniidae) to hypersaline conditions at a brine pool cold seep. Marine Ecology, 28(1), 199-207.

Van Horn, J., & Tolley, S. G. (2009). Acute response of the estuarine crab *Eurypanopeus depressus* to salinity and desiccation stress. Journal of Crustacean Biology, 29(4), 556-561.

Veiga, M. P. T., Gutierre, S. M., Castellano, G. C., & Freire, C. A. (2015). Tolerance of high and low salinity in the intertidal gastropod *Stramonita brasiliensis* (Muricidae): behaviour and maintenance of tissue water content. Journal of Molluscan Studies, eyv044.

Vitale, A. M., Monserrat, J. M., Castilho, P., & Rodriguez, E. M. (1999). Inhibitory effects of cadmium on carbonic anhydrase activity and ionic regulation of the estuarine crab *Chasmagnathus granulata* (Decapoda, Grapsidae). Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology, 122(1), 121-129.

Zardi, G. I., Nicastro, K. R., McQuaid, C. D., Rius, M., & Porri, F. (2006). Hydrodynamic stress and habitat partitioning between indigenous (*Perna perna*) and invasive (*Mytilus galloprovincialis*) mussels: constraints of an evolutionary strategy. Marine Biology, 150(1), 79-88.

Warner, G. F. (1967). The life history of the mangrove tree crab, *Aratus pisoni*. Journal of Zoology, 153(3), 321-335.

Warren, J. H. (1990). Role of burrows as refuges from subtidal predators of temperate mangrove crabs. Marine ecology progress series. Oldendorf, 67(3), 295-299.

Weihrauch, D., & O'Donnell, M. J. (2015). Links between osmoregulation and nitrogen-excretion in insects and crustaceans. Integrative and Comparative Biology, 55(5), 816-829.

Wheatly, M. G., & Henry, R. P. (1992). Extracellular and intracellular acid-base regulation in crustaceans. Journal of Experimental Zoology, 263(2), 127-142.

Willmer, P., Stone, G., & Johnston, I. (2009). Environmental physiology of animals. John Wiley & Sons.

Wilson, K. A. (1989). Ecology of mangrove crabs: predation, physical factors and refuges. Bulletin of Marine Science, 44(1), 263-273.

Wolcott, T. G. (1992). Water and solute balance in the transition to land. American Zoologist, 32(3), 428-437.

Wäge, J., Hardege, J. D., Larsson, T. A., Simakov, O., Chapman, E. C., Arendt, D., & Rotchell, J. M. (2015). Effects of low seawater pH on the marine polychaete *Platynereis dumerilii*. Marine pollution bulletin, 95(1), 166-172.

Wehner, F., Olsen, H., Tinel, H., Kinne-Saffran, E., & Kinne, R. K. (2003). Cell volume regulation: osmolytes, osmolyte transport, and signal transduction. In Reviews of physiology, biochemistry and pharmacology (pp. 1-80). Springer Berlin Heidelberg.