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COMPARAÇÃO DAS RESPOSTAS FISIOLÓGICAS AO ESTRESSE HIPOSMÓTICO E INFLUÊNCIA EM BIOMARCADORES DE ESTRESSE OXIDATIVO NO MEXILHÃO *Perna perna* COM DIFERENTES HISTÓRICOS AMBIENTAIS.

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Sumário

RESUMO

A salinidade é um fator abiótico chave em ambientes aquáticos e pode possuir uma ampla variação natural, tendo uma grande influência em atividades fisiológicas e processos adaptativos dos organismos. Além disso, animais aquáticos também são submetidos à exposição a diversos poluentes, os quais podem causar efeitos adversos aos organismos. Com base neste panorama, o objetivo desta tese é comparar as respostas fisiológicas e a influência na análise de biomarcadores no mexilhão *Perna perna* da costa sul do Rio Grande do Sul frente a uma condição de estresse hiposmótico. Cabe salientar que os dois pontos de coleta selecionados para o estudo, um situado nos Molhes da Barra ("Molhes" – ambiente estuarino) e outro no Farol Conceição ("Farol" – ambiente marinho), apresentam um histórico distinto tanto de exposição a poluentes quanto a variações de fatores ambientais naturais. Mexilhões coletados no "Farol" apresentaram respostas clássicas de organismos osmoconformadores com relação ao consumo de oxigênio e aos processos de osmorregulação avaliados através das análises de osmolalidade, concentração de íons e aminoácidos livres na hemolinfa. Entretanto, os mexilhões coletados nos "Molhes" apresentaram respostas similares a organismos osmorreguladores, como regulação da osmolalidade na hemolinfa e aumento da atividade da Na^+K^+-ATPase nas brânquias, além de uma resposta inversa com relação ao consumo de oxigênio. Com relação aos biomarcadores de estresse oxidativo analisados, podemos relatar que suas respostas foram tanto afetadas pelo estresse hiposmótico quanto diferente entre os pontos estudados. Neste sentido, estes resultados reforçam a necessidade da validação dos biomarcadores frente a influência de fatores abióticos para sua utilização em programas de monitoramento ambiental.

Palavras-chave: osmorregulação, estresse hiposmótico, biomarcadores, *Perna perna*, histórico ambiental.

INTRODUÇÃO GERAL

Adaptações fisiológicas ao ambiente

A plasticidade fenotípica é uma propriedade comum de um genótipo, para uma característica, dentro de uma certa gama de condições ambientais. O tipo e magnitude desta plasticidade é específico às características individuais e condições ambientais as quais os organismos são submetidos, podendo ser expressa nos níveis comportamentais, bioquímicos, fisiológicos ou de desenvolvimento. Dentro deste contexto, nem toda plasticidade fenotípica é adaptativa (no sentido evolutivo de melhorar a sobrevivência ou reprodução), mas algumas características são plásticas por causa de restrições inevitáveis impostas pela bioquímica, fisiologia ou biologia do desenvolvimento do organismo (para revisão, ver Pigliucci et al., 2006).

Organismos podem responder a mudanças ambientais de diferentes formas, de acordo com o tempo de exposição a essas alterações e com a magnitude deste estresse, levando assim a diferentes alterações nas suas estruturas e mecanismos fisiológicos. Neste sentido, condições ambientais naturais podem levar a um processo denominado aclimatização nos organismos, definida como as adaptações fisiológicas de um animal às mudanças no clima ou ambiente. De acordo com seu histórico ambiental, estes organismos possuem limites de tolerância aos estresses aos quais estão submetidos. Porém, estes limites, ou seja, esta faixa de tolerância pode ser alterada através do processo de aclimatação (Leake, 1964), sendo que condições ambientais em regimes mais instáveis, ou seja, que sofrem mais flutuações, podem causar uma extensa faixa de respostas celulares (Schroth et al., 2005).

Ecossistemas aquáticos costeiros, e especialmente os estuários, são tipicamente submetidos a uma variedade de estressores, tanto naturais quanto antropogênicos (Adams, 2005). Estes ambientes estuarinos são caracterizados por uma intensa variação

nos parâmetros físico-químicos da água, como salinidade, pH e temperatura. Contrariamente, ambientes oceânicos são mais estáveis, mantendo suas condições dentro de limites mais estreitos de variação. Além disso, ambientes estuarinos frequentemente apresentam misturas complexas de xenobióticos derivadas de efluentes industriais e domésticos (Falciani et al., 2008), os quais podem interagir com o ambiente ou organismos (Poynton e Vulpe, 2009), sendo estes expostos não a apenas um composto, mas a uma mistura de contaminantes. Também, os parâmetros físico-químicos da água podem alterar a biodisponibilidade e, por consequência, a toxicidade dos poluentes (Witters, 1998).

Consequentemente, organismos aquáticos viventes próximo à região estuarina e costeira adjacente são constantemente submetidos a estas alterações ambientais. Neste sentido, organismos estuarinos são normalmente mais tolerantes a estressores ambientais do que organismos estritamente marinhos, como resultado de seleção evolutiva, e parece razoável predizer que habitantes de áreas com fatores abióticos mais variáveis podem ter maior plasticidade fenotípica (Fokina et al., 2014). Por exemplo, em um estudo comparando a tolerância de duas espécies de copépodes, foi observado que a espécie marinha possui maior tolerância a salinidades mais altas enquanto a espécie estuarina foi mais tolerante a salinidades médias e baixas (Svetlichny e Hubareva, 2014). Também, peixes viventes em ambientes com ampla variação térmica, seja ela diária ou sazonal, são mais tolerantes a flutuações na temperatura do que peixes antárticos, os quais sobrevivem apenas à elevação de poucos graus (Hochachka e Somero, 2002).

Dentre diversos fatores abióticos que afetam processos fisiológicos, podemos destacar a salinidade, a qual é um fator determinante na distribuição de espécies em sistemas marinhos, influenciando a sobrevivência, crescimento e processos metabólicos

dos organismos (Brito et al., 2000; Gruffydd et al., 1984). Apesar de ser relativamente constante em mares abertos, em zonas estuarinas e costeiras adjacentes a salinidade pode mudar drasticamente em escalas espaciais e temporais (Kirkpatrich e Jones, 1985; Rowe, 2002), sendo os organismos viventes nestes locais expostos a estas flutuações de salinidade e sua habilidade de existir nestes locais dependente de diferentes adaptações (Berger e Kharazova, 1997). Desta forma, as áreas costeiras são consideradas ambientes estressantes, e organismos habitantes dessas áreas são expostos a mudanças de curta e longa escala na salinidade, causadas por alterações de maré e períodos de chuva, respectivamente (Bussell et al., 2008).

Processos osmorregulatórios

Em geral, dois mecanismos podem estar envolvidos na adaptação a ambientes onde ocorre variação na salinidade. Alguns organismos possuem mecanismos que mantém a concentração do fluído extracelular constante (ou quase) independentemente das variações ambientais, permitindo manter os gradientes osmóticos e iônicos. Estes organismos são denominados osmorreguladores. Porém, outros organismos não possuem a habilidade de manter uma concentração constante do fluído extracelular quando ocorrem variações nas concentrações do meio externo, sendo estes organismos classificados como osmoconformadores. Entretanto, organismos osmoconformadores demonstram mecanismos celulares que respondem às mudanças na concentração do fluído extracelular, fazendo a regulação do volume celular (Davenport e Fletcher, 1978; Lee et al., 2010).

Organismos osmorreguladores podem apresentar mecanismos de hiperregulação ou hiporregulação. Os mecanismos de hiperosmorregulação compreendem uma baixa permeabilidade do tegumento, devido à tendência a entrada de água por osmose nas

células, além de uma captação iônica ativa, já que estes animais perdem íons para o ambiente por difusão. Dentre os mecanismos atuantes para essa captação de íons, destacam-se as bombas e trocadores iônicos, como os trocadores Cl^-/HCO_3^-, Na^+/H^+, Na^+/NH_4^+ e Na^+-K^+-$2Cl^-$, presentes na membrana apical das células, e a Na^+K^+-ATPase, presente na membrana basolateral (Kirschner, 2004). Peixes e invertebrados de água doce apresentam mecanismos hiperosmorreguladores, como a produção de urina diluída, para compensar a entrada de água por osmose, além da obtenção de sais pela dieta concomitante à tomada ativa de íons.

Os mecanismos responsáveis pela hipoosmorregulação compreendem, de maneira geral, uma baixa permeabilidade tegumentar à água, para diminuir perda de água para o ambiente, e a eliminação de íons dos líquidos corporais, os quais são obtidos tanto pela ingestão de água salgada quanto através da alimentação, sendo os principais mecanismos responsáveis pela eliminação de íons a excreção de Cl^-, através de bombas na membrana apical das células, e a excreção passiva ou ativa de Na^+, pelos canais de Na^+ ou ação da Na^+K^+-ATPase, através das brânquias e outros órgãos excretores dos animais (Kirschner, 1993; Wilmer et al., 2005). A hiporregulação pode ocorrer em teleósteos e poucos crustáceos marinhos, como o camarão de água salgada *Artemia salina* (Wilmer et al., 2005). Estes animais habitantes de ambientes hipersalinos possuem reduzida necessidade de ingestão de água, visando reduzir a elevada tomada de íons por esta via, além de possuir mecanismos que minimizam a perda d'água para o ambiente, como a presença de uma cutícula impermeável (Pallarés et al., 2005).

Caranguejos estuarinos, como o *Neohelice granulata*, estão habituados em ambientes com amplas variações na osmolaridade do meio externo, possuindo diversos mecanismos adaptativos para sobreviver em ambientes alterados. Neste sentido, o

caranguejo *Neohelice granulata* pode apresentar mecanismos tanto de hiperregulação, quando expostos a salinidades baixas ou intermediárias, quanto de hiporregulação, apresentando uma variação dos mecanismos atuantes dependendo da situação ambiental (para revisão, ver Bianchini et al., 2008). Durante a hiperosmorregulação, tanto em salinidades intermediárias ou baixas, a Na^+K^+-ATPase tem importante atuação na membrana basolateral. Porém, de forma dependente da situação ambiental, os mecanismos atuantes na membrana apical diferem: sob ação de salinidades intermediárias, o cotransportador Na^+-K^+-$2Cl^-$ parece ser o principal atuante, enquanto durante salinidades baixas, os mecanismos responsáveis pela regulação são principalmente os trocadores Na^+/H^+ e Cl^-/HCO_3^- (para revisão, ver Bianchini et al. 2008). Durante a hiporregulação, em altas salinidades, é levantada a hipótese de que a secreção de íons para o ambiente pode acontecer de forma ativa, com a ação da Na^+K^+-ATPase, ou passiva, através de canais de cloreto, ambos na região paracelular dos ionócitos. Cotransportadores Na^+-K^+-$2Cl^-$ parecem estar relacionados à tomada de íons da hemolinfa pelas células pela membrana basolateral (para revisão, ver Bianchini et al. 2008). Portanto, podemos observar mecanismos contrastantes atuando nos animais dependendo da situação ambiental.

Moluscos bivalves marinhos são classicamente organismos osmoconformadores (Pierce, 1971), ou seja, a concentração osmótica da hemolinfa é semelhante à do ambiente em que vivem, apresentando variações osmóticas da hemolinfa em função das variações do meio externo (Gainey, 1994). Porém, estes organismos apresentam mecanismos comportamentais e fisiológicos para a situação de estresse hiposmótico. Neste sentido, o fechamento de valvas e dos sifões inalantes e exalantes (Davenport, 1979) podem atuar, evitando a circulação de água e, consequentemente, as alterações na concentração osmótica da hemolinfa. Porém, esta condição pode ser desvantajosa em

períodos prolongados. Assim, os organismos possuem outros mecanismos para que, mesmo que a osmolaridade da hemolinfa seja alterada, a turgescência das células seja prevenida durante estresse hiposmótico.

Fisiologicamente, organismos osmoconformadores são capazes de responder a alterações na osmolaridade do ambiente exercendo uma regulação nas concentrações osmóticas de seus fluídos intracelulares, para desta forma evitar alterações de volume celular (Figura 1). Em moluscos bivalves, o volume celular é mais efetivamente regulado pela concentração de aminoácidos livres, proveniente da quebra de proteínas celulares (Navarro e Gonzalez, 1998). Neste sentido, muitos estudos mostram que o conteúdo de aminoácidos livres nos tecidos de bivalves é diretamente proporcional às alterações na salinidade, em uma relação quase linear (Deaton, 2001; Lange, 1963). Assim, em um ambiente hiposmótico, a resposta celular envolve a redução intracelular nos níveis de aminoácidos livres, através do catabolismo destes aminoácidos, anabolismo de proteínas celulares ou excreção de aminoácidos para hemolinfa. Consequentemente, esta relação entre conteúdo de aminoácidos livres e osmolaridade do meio externo parece ser inversa para a hemolinfa (Sadok et al., 1997). Esta relação ocorre pois quando ocorre o estresse hiposmótico, como mencionado, aminoácidos livres são liberados pelas células para a hemolinfa para regulação do volume celular. Quando na hemolinfa, estes aminoácidos podem então ser excretados (Navarro e Gonzalez, 1998) ou constituir novas proteínas (Resgalla Jr., 2008).

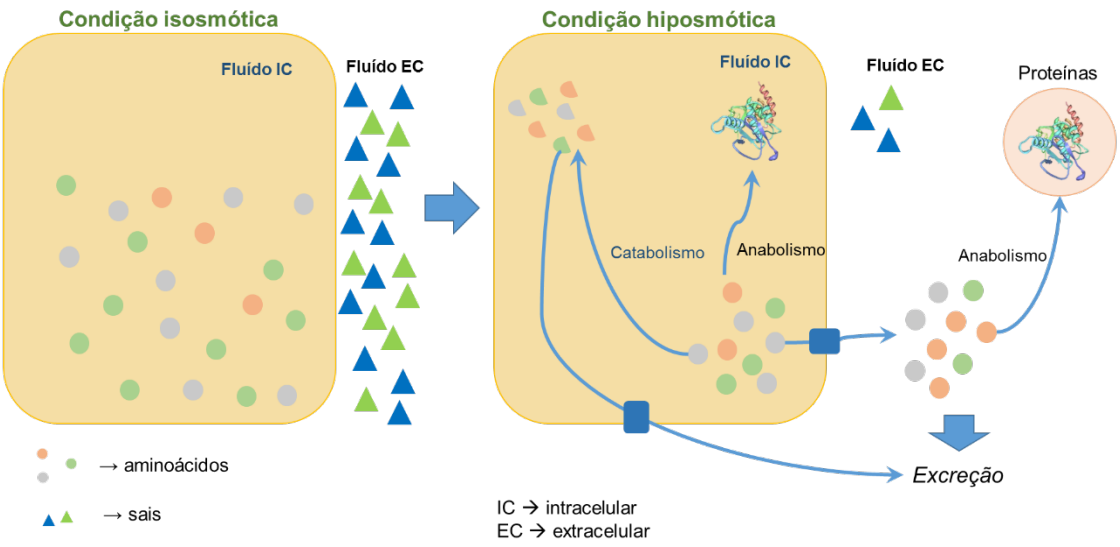

Figura 1. Processo de regulação do volume celular durante estresse hiposmótico em organismos osmoconformadores. Os aminoácidos livres exercem pressão osmótica no fluído intracelular (IC), igualando a pressão osmótica exercida pelos sais no fluído extracelular (EC), como demonstrado na condição isosmótica (salinidade alta). Durante uma condição hiposmótica, devido à redução da concentração de sais no fluído EC, a célula regula seu volume através da eliminação destes aminoácidos livres através de diversos processos, como catabolismo, excreção ou anabolismo de proteínas.

Além da regulação do volume celular, organismos osmoconformadores são capazes de exercer a regulação da concentração de íons específicos em sua hemolinfa, processo este chamado de ionorregulação (Pierce, 1982; Resgalla Jr., 2008; Smith e Pierce, 1987). De uma maneira geral, esta regulação pode ocorrer no processo de entrada e saída dos íons, através de alterações em quantidades e atividades de canais e bombas iônicas, desta forma, alterando a permeabilidade de suas membranas a determinados íons, ou pelo processo de eliminação destes íons através de órgãos excretores. A estabilização da composição e a geração de gradientes iônicos ótimos é

necessária para o efetivo funcionamento celular, particularmente durante flutuações na salinidade ambiental.

Respiração e estresse oxidativo

Organismos osmoconformadores podem apresentar adaptações fisiológicas nas taxas respiratórias relacionadas ao estresse osmótico. Estas variações no consumo de oxigênio em resposta a variações na osmolaridade do meio externo podem estar relacionadas aos mecanismos de osmorregulação citados anteriormente. Além das alterações relacionadas aos processos osmorregulatórios, estudos em bivalves do gênero *Perna* demonstram adaptações destes animais com relação a alterações em faixas de salinidade relacionadas ao consumo de oxigênio (Wang et al., 2012). Quando a alteração na osmolaridade do meio externo é repentina, a resposta inicial de moluscos bivalves é o fechamento de valvas, como já mencionado. Porém, se o estresse hiposmótico ocorre de forma gradual e estes organismo permanecem com suas conchas abertas, o consumo de oxigênio tende a aumentar. Este aumento no consumo de oxigênio pode dever-se em parte a demandas metabólicas necessárias para, por exemplo, o transporte de aminoácidos livres do interior das células para a hemolinfa, como forma de evitar a turgescência, e o anabolismo destes aminoácidos em proteínas (Navarro e Gonzalez, 1998; Resgalla Jr., 2008). Estas alterações nas taxas respiratórias podem levar ao aumento na formação de espécies reativas de oxigênio (ERO), como produtos secundários do metabolismo celular (por exemplo, peróxido de hidrogênio - H_2O_2, radical hidroxila - $^{\cdot}OH$, e radical ânion superóxido - $O_2^{\cdot -}$).

Contrabalanceando os efeitos deletérios da produção das ERO, os organismos possuem um complexo sistema de defesas antioxidantes. Dentre as enzimas participantes deste sistema, podemos destacar a superóxido dismutase (SOD),

responsável por dismutar o $O_2^{\cdot -}$ em H_2O_2, sendo uma família de enzimas com isoformas citosólicas e mitocondriais (Cu,Zn-SOD ou SOD1, e Mn-SOD ou SOD2, respectivamente). O aumento na atividade da SOD sugere um consequente aumento na taxa de formação H_2O_2 intracelular, o qual pode ser decomposto pela enzima catalase (CAT), devendo existir então um balanço entre a atividade destas duas enzimas. Quando a atividade da SOD está aumentada e isto não é acompanhado pela CAT, pode ocorrer um acúmulo de H_2O_2 nas células, o que pode acarretar em danos oxidativos. Outra enzima relacionada ao sistema de defesas antioxidante é a glutationa *S*-transferase (GST), responsável por conjugar o tripeptídeo glutationa (GSH) com moléculas eletrofílicas e outros xenobióticos, tornando mais hidrossolúveis para facilitar sua retirada da célula. Sabe-se que hidroperóxidos orgânicos resultantes de metabolismo oxidativo podem ser considerados como substratos "naturais" de GSTs (Pickett e Lu, 1989) e, desta forma, é estabelecido que as enzimas GSTs também possuem atividades de peroxidase e isomerase (Sheehan et al., 2001), que podem então auxiliar na resposta ao estresse oxidativo (Dondero et al., 2006; Kim et al, 2009).

Neste sentido, além da influência direta do consumo de oxigênio na produção de ERO, muitos poluentes, como metais, pesticidas e hidrocarbonetos, podem modificar direta ou indiretamente o balanço entre a concentração de pró-oxidantes e antioxidantes. Neste contexto, caracteriza-se uma situação de "estresse oxidativo" quando a taxa de produção de compostos pró-oxidantes, como as ERO, excede a sua taxa de degradação pelo sistema antioxidante, levando a um aumento nos danos oxidativos a diferentes alvos celulares, como proteínas, DNA e ácidos graxos, além de desregular as vias de sinalização redox (Jones, 2006).

Assim, a determinação de danos oxidativos em biomoléculas (dano de DNA, oxidação de proteínas, peroxidação lipídica) e/ou respostas antioxidantes em espécies

aquáticas é comumente empregado em programas de monitoramento ambiental (Bainy et al., 1996; Geracitano et al., 2004). Mais do que isso, a atividade e quantidade destas enzimas pode ser alterada pela exposição a poluentes, sendo estas enzimas uma barreira de defesa celular. Desta forma, estas mudanças na atividade e quantidade podem ser medidas e são um indicativo de exposição a poluentes.

Monitoramento ambiental e biomarcadores

O desenvolvimento de métodos para a identificação, estimação, avaliação comparativa e manejo dos riscos associados a descargas de poluentes químicos ao ambiente e recursos naturais é de extrema relevância. Durante décadas, os programas de monitoramento ambiental concentraram-se na medida de variáveis físicas e químicas do ambiente. De fato, estes são importantes componentes dos programas de avaliação de qualidade de água (Galloway et al., 2004), porém, a confiança no critério químico sozinho pode prover uma avaliação inadequada das condições biológicas e ecológicas dos sistemas aquáticos (Adams, 2005). Também, a avaliação deste critério isoladamente provém pouquíssima informação da real ou potencial atividade biológica de contaminantes (Hahn, 2002). Assim, a avaliação dos riscos não pode ser somente baseada em análises químicas de amostras ambientais, pois esta abordagem não fornece nenhuma indicação dos efeitos deletérios dos contaminantes na biota (Cajaraville et al., 2000) e se, de fato, estes poluentes encontram-se biodisponíveis para exercerem seus efeitos nos organismos.

Sendo assim, o uso de organismos para avaliar mudanças no ambiente, ou seja, o biomonitoramento, torna-se relevante para a avaliação da saúde ambiental. Esta abordagem pode envolver desde a dosagem de determinados contaminantes nos tecidos dos organismos, importante para avaliar a biodisponibilidade dos contaminantes no

ambiente, até a avaliação dos efeitos adversos destes compostos nos sistemas biológicos (Clark, 2001), permitindo inferir sobre a contaminação ambiental. É importante ressaltar que o biomonitoramento permite a inferência sobre a ação dos contaminantes mesmo em concentrações abaixo do limite de detecção química, ou ainda após o término da exposição ao agente tóxico, além de permitir a análise das possíveis interações entre os contaminantes e fatores do ambiente.

Neste sentido, os estressores podem exercer seus efeitos inicialmente no nível molecular ou submolecular, por exemplo, através de mudanças em componentes ou processos moleculares, bioquímicos e fisiológicos, como expressão de genes e proteínas, integridade do DNA, atividade enzimática, metabolismo e respiração (Adams, 2005). Por último, estes efeitos podem refletir em mudanças nos níveis superiores de organização biológica, como os níveis de organismo, população e comunidade. Assim, medidas nos níveis bioquímicos ou fisiológicos detectam rápida e especificamente a presença de diversos compostos tóxicos, permitindo a identificação precoce de mudança, antes dos efeitos deletérios alcançarem estes níveis superiores de organização biológica (Monserrat et al., 2003).

Neste contexto, biomarcadores têm sido definidos como variações moleculares, bioquímicas, celulares, fisiológicas ou comportamentais que podem ser medidas nas células, tecidos ou fluídos corpóreos e que provém evidência de exposição e/ou efeitos de um ou mais poluentes químicos (Depledge et al., 1995). Como mencionado, muitos poluentes são conhecidos por influenciarem o estado oxidativo dos organismos, então medidas na atividade enzimática de participantes das defesas antioxidantes e reações de biotransformação têm sido frequentemente propostos como biomarcadores em programas de monitoramento ambiental (Capela et al., 2016; Capó et al., 2015; Geret et al., 2002; Hoarau et al., 2006). Cravo e colaboradores (2009) utilizaram uma abordagem de

múltiplos biomarcadores para acessar a qualidade ambiental em um mexilhão da família Mytilidae (*Mytilus galloprovincialis*), dentre os quais estavam as enzimas antioxidantes (SOD, CAT e GST). Estes autores reportaram que o conjunto de biomarcadores utilizado é adequado para identificar a saúde ambiental de forma ampla, porém relatam que alguns dos biomarcadores escolhidos são fortemente influenciados pelas condições ambientais. Outro estudo, também utilizando a abordagem de biomarcadores múltiplos em outra espécie de mitilídeo (*Mytilus trossolus*) também utilizou, dentre outros, as enzimas SOD, CAT e GST (Turja et al., 2014). Estes autores destacam que o uso destes biomarcadores em combinação com outras análises leva a um entendimento global da integridade ambiental.

Invertebrados aquáticos são organismos amplamente utilizados e importantes para investigações de efeitos subletais de poluentes (Rickwood e Galloway, 2004) e especificamente bivalves marinhos, como os mexilhões, os quais são constantemente submetidos a poluentes, devido a sua condição séssil. Devido ao hábito de alimentação por filtração, tem sido demonstrado que esses organismos são capazes de acumular contaminantes da água do mar circundante e resistem a uma larga série de concentrações de contaminantes (Baumard et al., 1998; Baumard et al., 1999; Connor, 1996; Resgalla et al., 2007), sendo capazes de acumular tanto poluentes orgânicos quanto inorgânicos (Cajaraville et al., 2000). Assim, uma série de testes têm sidos realizados em organismos pertencentes a este grupo, dentre eles, o bivalve marinho *Perna perna* (Abessa et al., 2005; Bainy et al., 2000; Gregory et al., 1999; Gregory et al., 2002).

Problemática do monitoramento ambiental: validação de biomarcadores

O principal problema na utilização de biomarcadores está relacionado com a variabilidade encontrada nas respostas dos organismos, podendo estas serem

influenciadas tanto por fatores abióticos (como temperatura, salinidade, oxigênio dissolvido, etc.), quanto por fatores bióticos (como genótipo, plasticidade fenotípica, idade, sexo, etc.), até mesmo independente da presença de contaminantes no ambiente (Handy et al., 2003). Neste sentido, é muito importante a realização de estudos prévios para a validação da utilização dos biomarcadores na avaliação da qualidade do ambiente.

Claramente, não podemos identificar o efeito das alterações de um fator específico para o metabolismo de um organismo num ambiente natural, pois os sistemas vivos estão sob influência aditiva, sinergística ou antagonística de um amplo espectro de fatores ambientais (como abióticos, bióticos e antropogênicos) (Fokina et al., 2014). Parâmetros ambientais não relacionados à poluição, como temperatura, salinidade e outros, podem causar mudanças importantes em alguns sistemas bioquímicos que têm sido propostos como biomarcadores, além de alterações no estado fisiológico dos organismos. Assim, é essencial caracterizar as variações naturais nas respostas de biomarcadores de organismos sentinela para diferenciar os efeitos induzidos pela poluição dos efeitos de flutuações ambientais naturais (Cravo et al., 2009; Hamer et al., 2008; Tedengren et al., 1988; Zanette et al., 2011).

Esta problemática é aumentada em programas de monitoramento de larga escala, onde os organismos são submetidos a um amplo espectro de condições ambientais. Quanto maior a área coberta por um programa de monitoramento, maior a variabilidade natural esperada em fatores ambientais como disponibilidade de comida, temperatura ou salinidade, e também em fatores intrínsecos como idade, estado de condição ou reprodutivo (Fokina et al, 2014).

Para validar o uso de biomarcadores e sua aplicabilidade em monitoramentos de poluição marinha é indispensável o entendimento da importância das variáveis

ambientais nas respostas biológicas estudadas e a capacidade de discriminar entre respostas causadas por poluentes e a influência da variabilidade em processos naturais (Tankoua et al., 2012; Thain et al., 2008).

Caracterização ambiental

Sob uma perspectiva regional, podemos destacar o complexo lagunar Patos-Mirim. A Laguna dos Patos (Figura 2) recebe água de uma bacia de drenagem de 201.626 km² (Toldo Jr., 1994). A grande industrialização e ocupação desordenada das margens da Laguna dos Patos contribui para a geração de impactos ambientais devido à liberação de esgotos domésticos e atividades ligadas aos setores industrial, portuário e agrícola (Seeliger e Costa, 1998).

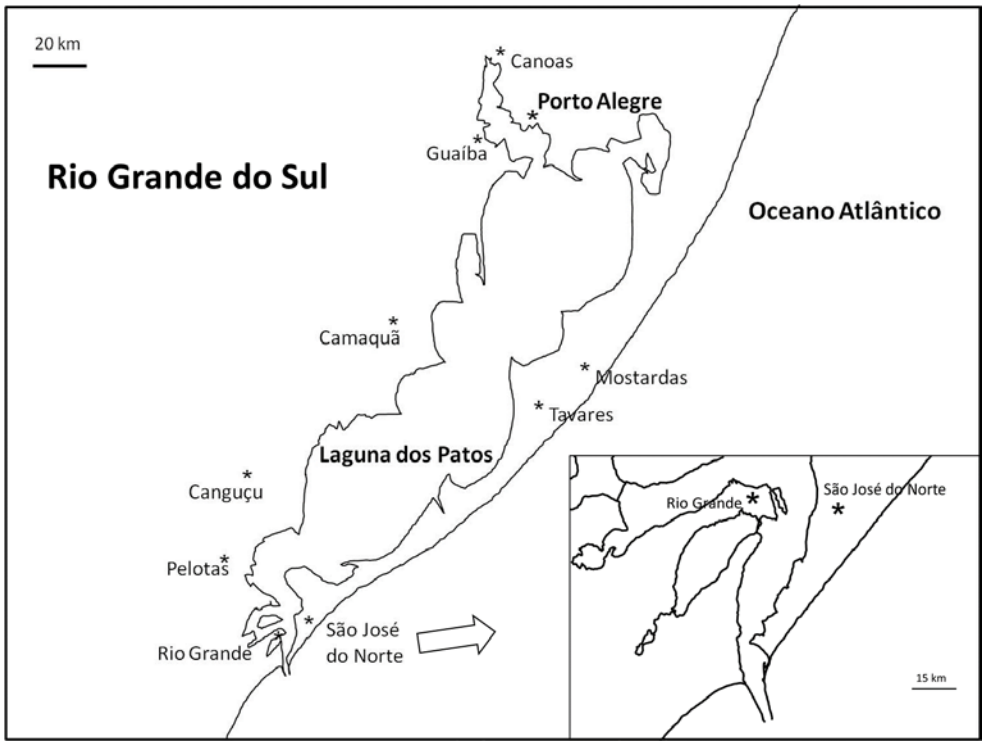

Figura 2. Laguna dos Patos. Localização da Laguna no estado do Rio Grande do Sul e sua desembocadura, na cidade de Rio Grande e visão aproximada da desembocadura (Rola et al., 2012). Asteriscos (*) representam as principais cidades no entorno da Laguna dos Patos.

Estudos utilizando o monitoramento físico e químico em conjunto com o biomonitoramento, inclusive utilizando o enfoque de biomarcadores, têm sido realizados na região, mostrando que algumas espécies encontradas na região são capazes de acumular elevadas concentrações de contaminantes ambientais. Além disso, em algumas destas espécies já foram observados efeitos adversos da exposição a estes contaminantes (Amado et al., 2006; Baumgarten e Niencheski, 1990; Amado et al., 2006). Também, a análise de alguns biomarcadores moleculares e bioquímicos para as condições do estuário da Laguna dos Patos para o mexilhão *Mytilus edulis* foi efetuada anteriormente pelo nosso grupo de estudo (Rola et al., 2012), onde foram analisados alguns biomarcadores relacionados ao estresse oxidativo e evidenciaram efeitos decorrentes possivelmente de poluentes presentes nas águas da desembocadura da Laguna dos Patos.

A região estuarina existente na desembocadura da Laguna dos Patos é influenciada pelo escoamento de suas águas e, neste sentido, além de apresentar variações em escala temporal e espacial em fatores abióticos, como a salinidade, também recebe o aporte de diversas fontes de contaminação. Segundo Burrage e colaboradores (2008) e Marques e colaboradores (2009), a ação dos ventos é o fator dominante no padrão de deslocamento da água que sai pela desembocadura da Laguna, sendo essa desviada predominantemente para o sul, devido à predominância de ventos do quadrante norte durante a maior parte do ano. Em alguns períodos do ano, esta água da Laguna pode chegar a atingir regiões ao norte da desembocadura, porém não ultrapassando 35 km de extensão ao norte (Fernandes et al., 2002; Marques et al., 2009; Figura 3). Desta forma, ocorrem processos de mistura destas águas na zona de plataforma continental próxima à desembocadura da Laguna, estando esta região então

submetida a grandes variações de salinidade, temperatura e exposição a poluentes diversos.

Neste sentido, é importante ressaltar que todo este aporte de contaminantes que está afetando a Laguna dos Patos e seu estuário pode atingir sua desembocadura, chegando até a costa adjacente e os organismos lá existentes. Estes dados são de extrema importância para este estudo, tendo em vista que o ponto “Farol” (Farol da Conceição) a ser utilizado neste estudo encontra-se a uma distância de 75 km ao norte do ponto “Molhes” (Molhes da Barra), estando então em um local sem a influência da variação de salinidade regida pelo deságue da Laguna dos Patos, e longe também da influência dos contaminantes presentes nessa água de mistura.

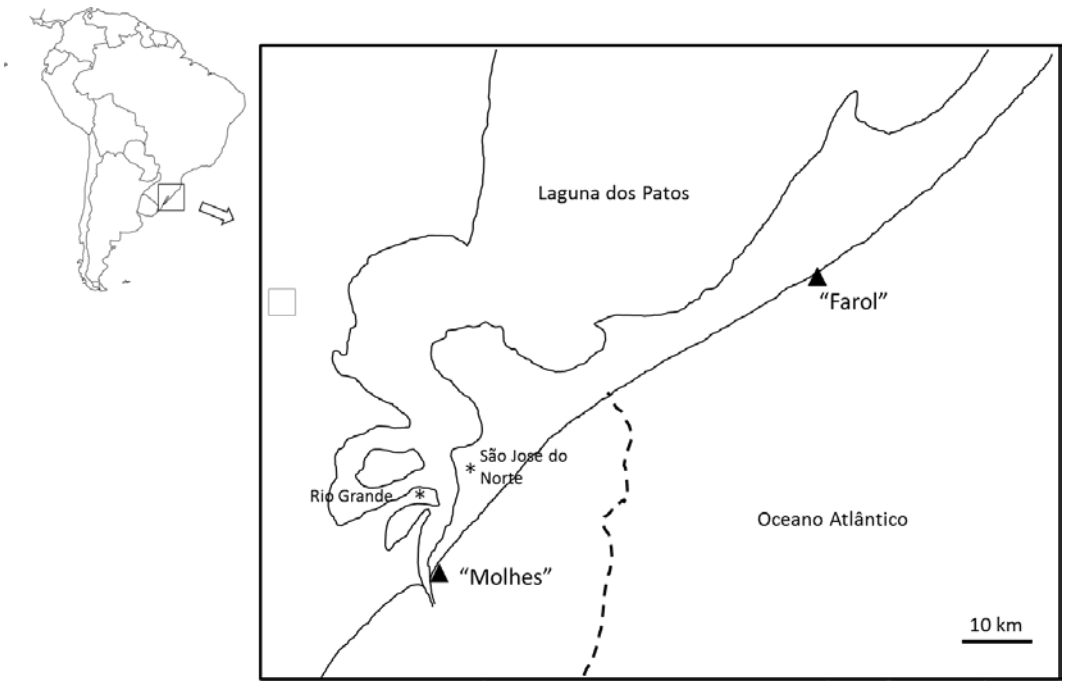

Figura 3. Localização dos pontos de coleta. Os triângulos (▲) representam os pontos de coleta, os asteriscos (*) as cidades de Rio Grande e São José do Norte e a linha pontilhada indica o alcance máximo da pluma da Laguna dos Patos, delimitando a área com flutuações de salinidade frequentes. Ponto de coleta nos Molhes da Barra ("Molhes"), na desembocadura da Laguna dos Patos, considerado um ponto estuarino; Ponto de coleta no Farol da Conceição ("Farol"), 75 km ao norte, considerado ponto estritamente marinho.

Organismo modelo: mexilhão *Perna perna*

Moluscos bivalves da família Mytilidae são organismos com baixa mobilidade e que se alimentam através de filtração, sendo expostos ao estresse ambiental de forma contínua e cíclica, enquanto permanecerem vivos e fixos ao local em questão. Para lidar com estressores, estes organismos possuem uma série de adaptações que permitem a eles sobreviverem sob condições adversas, incluindo mudanças em taxas respiratória e

metabólica totais, ativação de vias alternativas de produção de energia e a indução de sistemas de defesas bioquímicas e mecanismos de reparo (Almeida et al., 2007).

Estudos em bivalves do gênero *Perna* demonstram adaptações destes animais com relação a alterações em faixas de salinidade, sendo estas adaptações em processos fisiológicos, como mudanças de potenciais de membrana e no consumo de oxigênio (Stucchi-Zucchi e Salomão, 1998; Wang et al., 2012). Além disso, estes organismos possuem uma ampla faixa de tolerância a salinidade, sendo sua faixa de tolerância situada entre 15 e 40‰ (Resgalla Jr. et al., 2007). Também, esse organismo já é utilizado em um programa de monitoramento efetuado pelo Porto do Rio Grande na região. Neste sentido, escolhemos o mexilhão *Perna perna* como organismo modelo para esse estudo, visto que este organismo encontra-se presente em ambos locais, marinho e estuarino, e, assim, pode possuir adaptações distintas em função das flutuações ambientais.

Portanto, ambos pontos de amostragem deste estudo possuem um histórico distinto tanto de exposição a poluentes quanto a variações de fatores ambientais, e, assim, os organismos viventes nesses locais são submetidos a diferentes tipos de adaptações de acordo com seus históricos. Desta forma, se faz necessário o estudo da influência dos fatores abióticos na fisiologia destes organismos e sua influência na validação de alguns biomarcadores amplamente utilizados no monitoramento ambiental.

OBJETIVOS

Objetivo Geral

O objetivo geral da presente tese é comparar as respostas fisiológicas e sua influência na análise de biomarcadores em mexilhões *Perna perna*, coletados em dois pontos distintos, expostos em laboratório a diferentes condições de estresse hiposmótico.

Objetivos Específicos

- Avaliar a influência de estresse hiposmótico sobre respostas fisiológicas relacionadas ao processo de osmorregulação no mexilhão *Perna perna*;
- Analisar a resposta em termos de consumo de oxigênio, níveis de espécies reativas de oxigênio e biomarcadores de estresse oxidativo em *P. perna* expostos em condições de laboratório a uma condição hiposmótica;
- Verificar a influência do histórico ambiental nas respostas fisiológicas e análises de biomarcadores frente ao estresse hiposmótico em mexilhões *P. perna*, relacionando com possíveis mecanismos adaptativos de cada grupo de animais.

MANUSCRITO 1

Can marine bivalves display osmoregulatory mechanisms? A study of two populations of the mussel *Perna perna* (Linnaeus, 1758) in southern Brazil

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Can marine bivalves display osmoregulatory mechanisms? A study of two populations of the mussel *Perna perna* (Linnaeus, 1758) in southern Brazil

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Can marine bivalves display osmoregulatory mechanisms? A study of two populations of the mussel *Perna perna* (Linnaeus, 1758) in southern Brazil

Abstract

Salinity is an abiotic factor that can undergo temporal and spatial oscillations in environments such as estuaries, and organisms inhabiting these environments must respond to these variations to survive. Some authors hypothesized that the ecological history of organisms may be related to different responses and adaptations to stressors. This study aims to evaluate whether marine mussels (*Perna perna*) collected from sites with distinct histories of fluctuations in abiotic parameters, including salinity, respond differently to hypoosmotic stress. Mussels collected at the "Farol" site (no history of salinity variation) showed reduced haemolymph osmolality when exposed to hypoosmotic stress (salinities of 25 and 20‰) in the laboratory, as expected for osmoconforming organisms. Moreover, these animals showed reduced concentrations of Na^+, Cl^-, and K^+; increased levels of ninhydrin-positive substances in the haemolymph; and no changes in gill Na^+/K^+-ATPase activity. For animals collected at the "Molhes" site (typical salinity variations), this same pattern was observed on day 1 of hypoosmotic stress. On days 4 and 14, the osmolality and ionic concentration returned to near-baseline values (corresponding to salinity of 35‰), and these mussels showed an increase in gill Na^+/K^+-ATPase activity at day 4. This long-term response observed for "Molhes" mussels is similar to that observed for osmoregulating organisms. These results suggest that the ecological history, evidenced here by differences in organismal origin (sampling sites), can influence the physiological parameters of mussels in response to a stressful situation. Furthermore, "Molhes" mussels seem to be adapted to this condition, showing responses similar to those of osmoregulating organisms.

Keywords: estuary, salinity adaptation, Patos Lagoon, osmoregulation, haemolymph

Introduction

Physiological processes are affected by various abiotic factors, including salinity. Variations in salinity have a direct effect on metabolism, reproduction and osmoregulation and play an important role in species distributions within coastal and estuarine environments (Kinne 1966; Rowe 2002). Estuaries and coastal areas are considered stressful environments, and organisms inhabiting these areas are exposed to short- and long-term changes in salinity caused by tidal alterations and by rainy periods, respectively (Bussell et al. 2008). As such, organisms living in estuarine environments can tolerate fluctuations in the solute concentration of their external media. In general, two mechanisms might be involved in the adaptation to these environments. Some organisms are unable to maintain a stable concentration of extracellular fluid when variations in external media concentration occur, demonstrating cellular mechanisms that respond to these changes in extracellular fluid concentration (osmoconformer organisms). However, other organisms have mechanisms that stabilize the concentration of extracellular fluid independent of environmental variations, allowing them to maintain osmotic and ionic gradients (osmoregulator organisms) (Davenport and Fletcher 1978; Lee et al. 2010).

Marine bivalve mollusks are known osmoconformers (Pierce 1971) that have behavioral and physiological mechanisms to prevent the swelling of cells during hypoosmotic stress (for a review of mollusks' osmoregulatory processes, see Deaton 2009). In this context, behavioral changes consist of the closure of exhalant and inhalant siphons as well as complete valve adduction (Davenport 1979). When valves are closed, mussels have no access to an external supply of oxygen, and the mantle cavity fluid content falls (Davenport and Fletcher 1978), demonstrating that this condition can be disadvantageous over the long term. In relation to physiological mechanisms,

observations show that tissue levels of organic molecules (free amino acids - FAAs) decrease in response to declining environmental salinity and haemolymph osmolality (Lange 1963). In this context, cells of bivalve tissues exposed to hypoosmotic media export these FAAs to haemolymph, from which the FAAs can be excreted or absorbed by other cells, where protein synthesis can occur (Lange 1963).

Despite the osmoconforming pattern found for marine bivalves, differences in the response to salinity fluctuations among individuals have been described in the literature. For example, the osmolality of the retained mantle fluid of closed mussels (*Mytilus edulis*) is higher in organisms from areas characterized by severe salinity changes than in animals from places where environmental changes are less extreme (Davenport 1979). Moreover, the lower limit of lethal salinity for some species of bivalve mollusks can be reduced if the animals are exposed to fluctuating rather than continuously low salinities (Shumway 1977). Thus, we tested whether mussels (*Perna perna*) collected from sites with distinct histories of fluctuation in abiotic parameters responded to hypoosmotic stress by demonstrating different adaptive responses. Mussels collected at a salinity of 35‰ from two different sites were exposed to hypoosmotic conditions in the laboratory (salinities of 25 and 20‰) for up to 14 days, and haemolymph parameters (osmolality, ionic concentration, ninhydrin-positive substances and gill Na^+/K^+- ATPase activity) were regularly monitored.

Materials and methods

Experimental design and sampling

P. perna brown mussels (30–40 mm) were collected at two sites in a southern littoral region of Rio Grande do Sul (Brazil; Fig. 1) during March 2012. From a regional perspective, the outfall of the Patos-Mirim Lagoon complex (southern Brazil) occurs

near the city of Rio Grande, and its output is delimited by breakwaters. Thus, this is an estuarine environment where water mixing occurs at the ledge near the Lagoon outfall. As such, this region is subjected to large variations in abiotic factors, and the organisms living in this habitat and in the adjacent coast are constantly exposed to these variations. Salinity variations at this location range from 12 to 27‰ (Burrage et al., 2008). According to Burrage and collaborators (2008) and Marques and collaborators (2009), wind is the dominant factor that determines the displacement pattern of water coming out of the Lagoon outfall. During some periods of the year, the water of the Lagoon can extend to regions north of the mouth, but no more than 35 km (Fig. 1; Marques et al., 2009). These data are extremely important for this study, given that one of the sampling points ("Farol da Conceição" – Conceição's lighthouse – "Farol" site, 31º43'834"S, 51º28'932"W) is 75 km north of the Lagoon outfall. Thus, this area is not influenced by the variation in salinity governed by the outflow of the Patos Lagoon. In contrast to this location, the other sampling point ("Molhes da Barra" - Barra's breakwaters – "Molhes" site, 32º09'050"S, 52º04'550"W) is located near the Lagoon outfall and is constantly subjected to salinity fluctuations. The two sampling points selected for the present study have distinct histories of abiotic parameter fluctuations, and living organisms may show different adaptive responses according to their ecological history. Our research group (Rola et al. 2012) has previously used both sites in other studies. Mussels from both sites were collected in the intertidal zone at the same salinity (35‰) and immediately transported to the laboratory in seawater. In the laboratory, mussels were maintained for 15 days at 20ºC and 35‰ salinity (Resgalla et al. 2006).

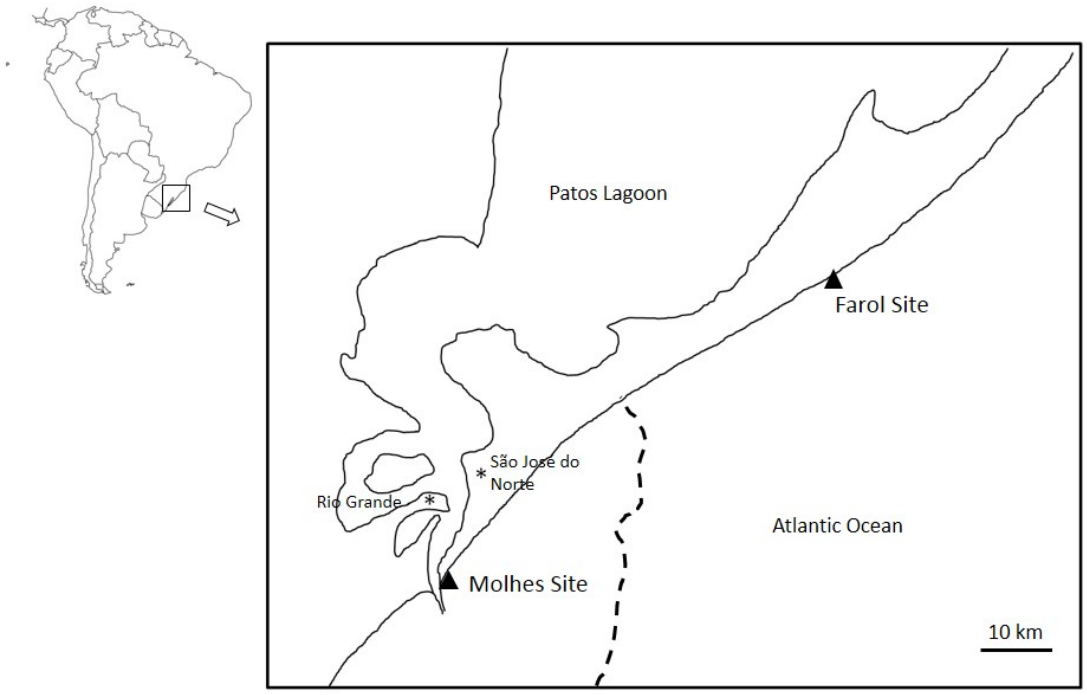

Fig. 1 Map of the sampling points located in southern Brazil - Rio Grande do Sul. Asterisks (*) show the locations of the Rio Grande and of São José do Norte, and triangles (▲) show the locations of the "Farol" and "Molhes" sites. The dotted line indicates the maximum range of the plume of Patos Lagoon, outlining the area with frequent salinity fluctuations.

After this period, a control group was kept in natural seawater (35‰ salinity), and the remaining animals were exposed to diluted seawater (25 and 20‰ salinity, diluted with dechlorinated water) for up to 14 days. The experiment was conducted with three aquaria per treatment, with each aquarium containing 6 L of water and 5 animals per liter. Every 48 h, the water was renewed and mussels were fed with the alga *Conticribra weissflogii*. The initial salinity (35‰) was decreased gradually (according

to Fig. 2) to avoid valve closure. The haemolymph of the adductor muscle and the gills (for the Na^+/K^+-ATPase activity assay) from six mussels per group was sampled immediately and after 1, 4 and 14 days from the beginning of treatment. Samples were immediately centrifuged at 800 g for 10 minutes (at 4ºC), and the supernatant, termed haemolymph for the results and discussion, was immediately stored at -70ºC for further analysis. Analyses were conducted at least in triplicate.

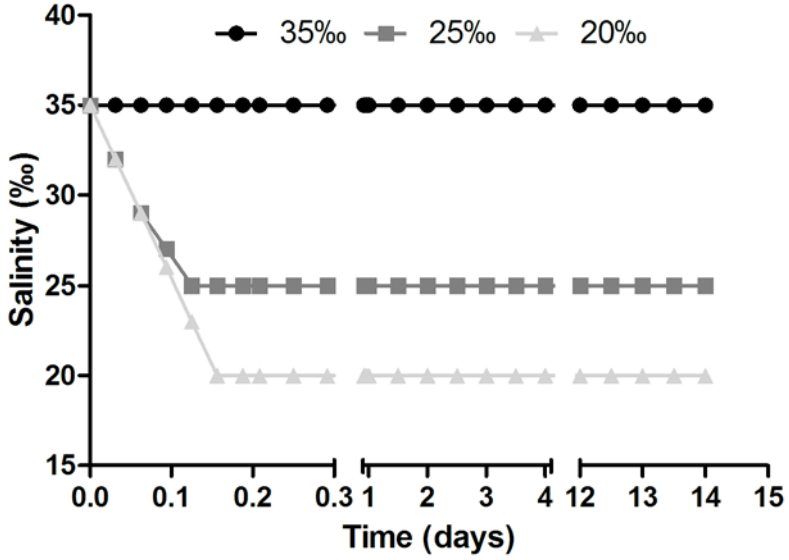

Fig. 2 Diagram showing the gradual decrease in salinity in the experimental media during the exposure period. The control group was kept in seawater (salinity of 35‰), and the remaining animals were exposed to diluted seawater (salinities of 25 and 20‰) for up to 14 days.

Haemolymph analysis

Haemolymph aliquots were used to determine the osmolality in a VaPro® vapor pressure osmometer (model 5600, Wescor Inc., Logan, Utah, USA). Corresponding

tank water was also measured (35‰ – ±980 mOsm; 25‰ – ±650 mOsm; 20‰ – ±520 mOsm).

Concentrations of sodium and potassium in the extracellular fluid were obtained by flame photometry (Digimed NK 2004, São Paulo, BR), and chloride concentrations were obtained using a colorimetric test (chloride colorimetric assay, Doles Reagentes, Goiânia, BR).

Haemolymph aliquots were used for ninhydrin-positive substances (NPS) analysis according to Clark (1964) and read using a microplate reader. This analysis was used as an indicator of free amino acid content. We constructed a standard curve (between 0.625 and 25 μg/ml glycine diluted in 95% ethanol), and samples were diluted in 95% ethanol. The reaction began with the addition of citrate buffer and ninhydrin to the samples (and standards), which were then incubated for 20 minutes at 100°C and then cooled for 5 minutes in Eppendorf tubes. Then, 60% ethanol was added. In 96-well microplates, we added 250 μL of the reaction mixture (containing a sample or standard) per well, and readings were performed by spectrophotometry at 585 nm.

Na^+/K^+-ATPase (NKA) activity assay

Gills were homogenized (1:4, w/v) in buffer (Tris-HCl 100 mM, EDTA 2 mM, $MgCl_2.6H_2O$ 5 mM; pH 7.75) according to Jorge et al. (2013) and centrifuged (20000 g at 4°C for 20 min). The supernatant obtained was stored at -70ºC for analysis. Aliquots were utilized in an NKA activity assay according to McCormick (1993). The protein content was evaluated using a commercial kit (Doles Reagentes) based on the biuret protein assay, and enzyme activity is expressed as μmol ADP produced/mg protein/h.

Statistical analysis

The results are presented as the mean ± standard error. The statistical analysis (n = 6) was performed using two-way ANOVA ($p<0.05$) followed by Tukey's test. Previously, we tested the prerequisites for analysis of variance (normality and homogeneity of variances).

Results

Notably, for animals that were kept at a salinity of 35‰ (control) from both sampling sites, none of the analyzed haemolymph parameters changed during the exposure period (14 days).

Osmolality measurement

Mussels from the two sites differed in their osmotic responses to hypoosmotic stress (Fig. 3). "Farol" mussels (Fig. 3a) exposed to salinities of 25 and 20‰ showed decreases in haemolymph osmolality at days 1, 4 and 14 (salinity of 25‰: reductions of 25, 45 and 21%, respectively; salinity of 20‰: reductions of 27, 56 and 63%, respectively) in relation to the osmolality observed at day 0 ($p<0.05$). In contrast, "Molhes" mussels (Fig. 3b) exposed to salinities of 25 and 20‰ showed decreased osmolality in relation to day 0 ($p<0.05$) only at day 1 (reductions of 22 and 49%, respectively), returning to the initial osmolality at subsequent times.

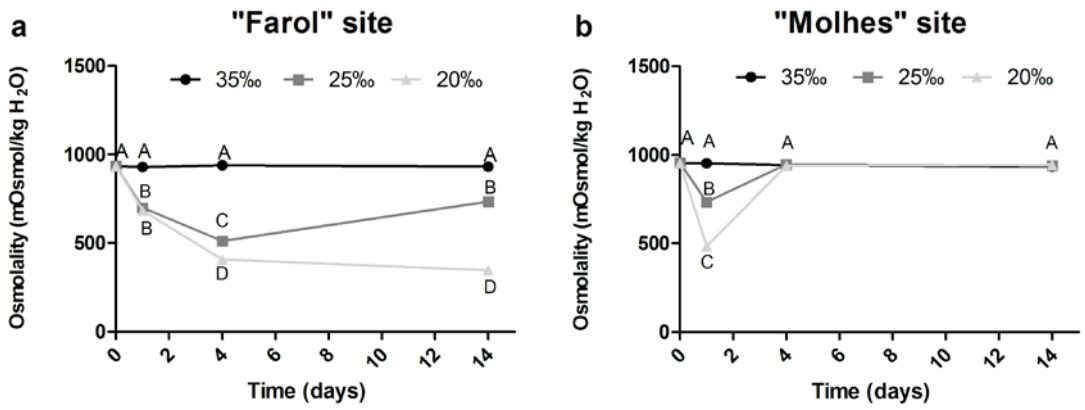

Fig. 3 Effects of hypoosmotic stress on haemolymph osmolality. Haemolymph osmolality of "Farol" mussels (a) and "Molhes" mussels (b) at a salinity of 35‰ (control) and under hypoosmotic conditions (salinities of 25 and 20‰) were measured at days 0, 1, 4 and 14 of exposure. Values are expressed as the mean ± SE (n=6). Significant differences ($p<0.05$) between the initial time (0 days) and subsequent times (1, 4 and 14 days) are indicated by different letters. No significant differences ($p>0.05$) were observed for mussels kept at 35‰ salinity.

Ionic concentration

In mussels collected at the "Farol" site, we observed significant decreases ($p<0.05$) in the concentrations of all the examined ions in the haemolymph (Fig. 4a, c and e) when compared to day 0 (Na^+ - salinity of 25‰: reductions of 14, 35 and 48%; salinity of 20‰: reductions of 40, 48 and 46% at days 1, 4 and 14, respectively; K^+ - salinity of 25‰: reductions of 30, 46 and 58%; salinity of 20‰: reductions of 65, 72 and 77%, at days 1, 4 and 14, respectively; Cl^- - salinity of 25‰: reductions of 17, 24 and 15%; salinity of 20‰: reductions of 20, 36 and 19%, at days 1, 4 and 14, respectively). For "Molhes" mussels, the concentrations of all examined ions in the haemolymph (Fig. 4b, d and f) initially decreased significantly ($p<0.05$) when compared

to day 0 (Na^+ - reductions of 40 and 42% at day 1, for salinities of 25 and 20‰, respectively; K^+ - reductions of 55 and 74% at day 1 for salinities of 25 and 20‰, respectively; Cl^- - reductions of 23 and 21% at day 1 for salinities of 25 and 20‰, respectively) and returned to the initial values during later periods of hypoosmotic stress.

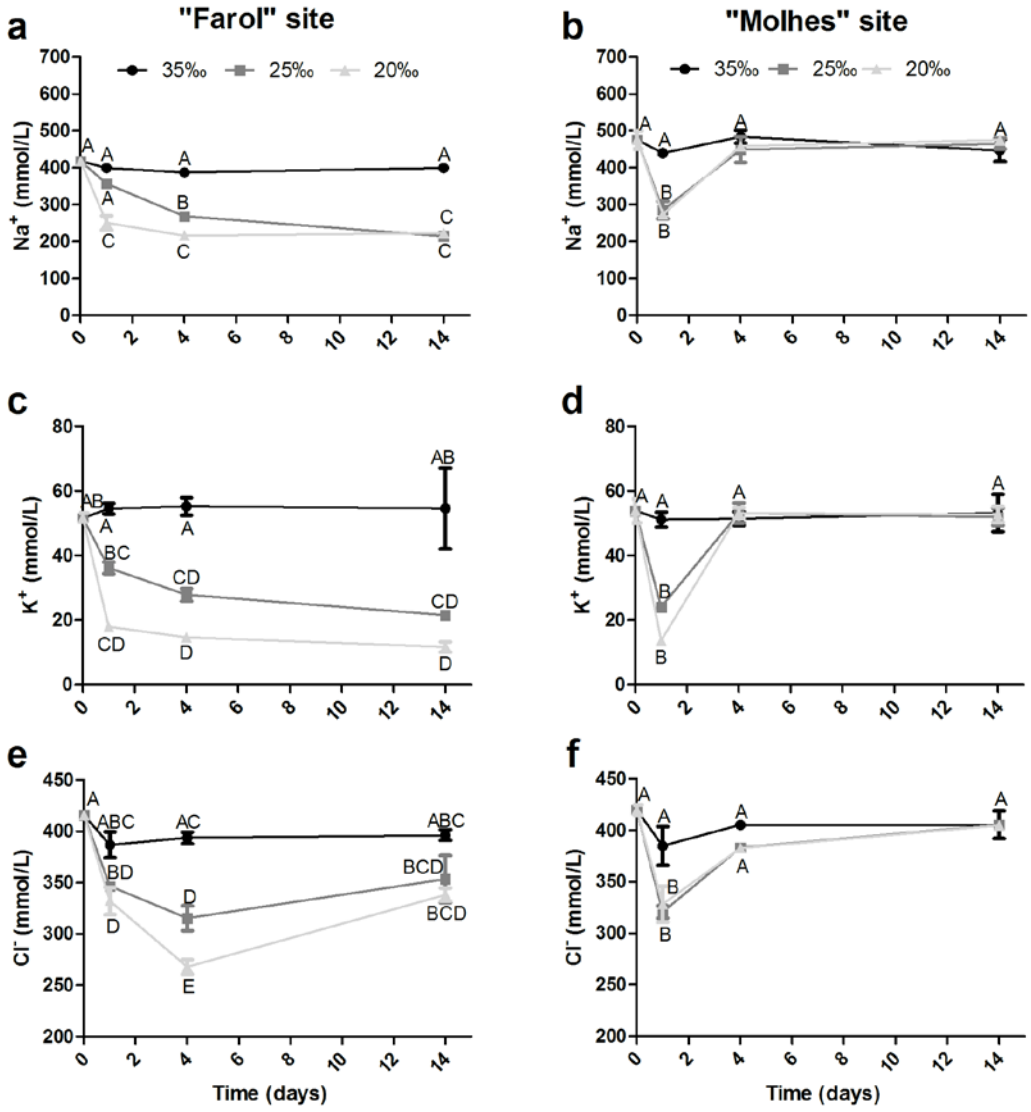

Fig. 4 Effects of hypoosmotic stress on the ionic concentration of the haemolymph. Na^+, K^+, and Cl^- measured in the haemolymph from "Farol" mussels (a, c and e, respectively) and "Molhes" mussels (b, d and f, respectively) at a salinity of 35‰ (control) and under hypoosmotic conditions (salinities of 25 and 20‰) were measured at days 0, 1, 4 and 14 of exposure. Values are expressed as the mean ± SE (n=6). Significant differences ($p<0.05$) between the initial time (0 days) and subsequent times (1, 4 and 14 days) are indicated by different letters. No significant differences ($p>0.05$) were observed for mussels kept at 35‰ salinity.

Ninhydrin-positive substances analysis

Haemolymph ninhydrin-positive substances (NPS) were used as indicators of free amino acid content (Fig. 5). “Farol” mussels (Fig. 5a) transferred to salinities of 25 and 20‰ showed significantly increased NPS levels at 1 day (230 and 243% at salinities of 25 and 20‰, respectively; $p<0.05$), but they returned to initial values (day 0, corresponding to salinity 35‰) after 4 and 14 days of hypoosmotic stress. In contrast, “Molhes” mussels (Fig. 5b) maintained at salinities of 25 and 20‰ showed increased haemolymph NPS levels at day 4 (167 and 190% at salinities of 25 and 20‰, respectively; $p<0.05$) and sustained these higher levels at day 14 (211 and 241% at salinities of 25 and 20‰, respectively; $p<0.05$).

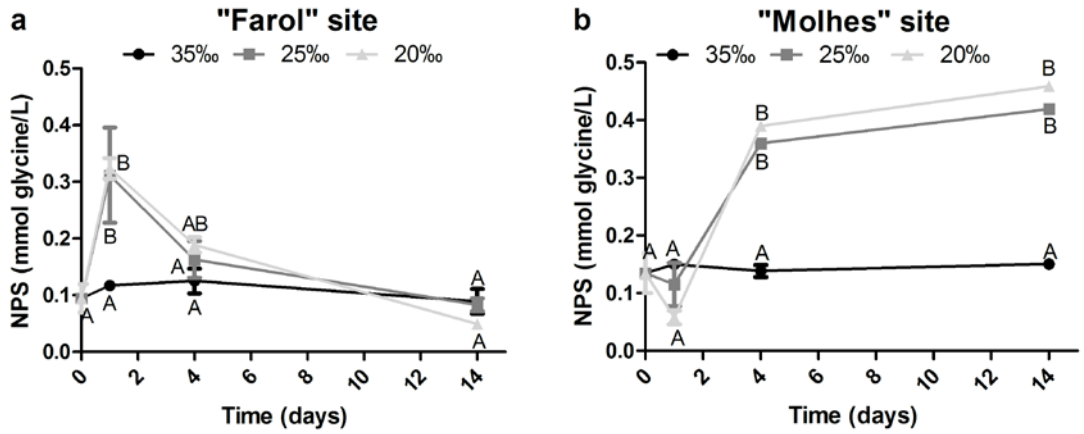

Fig. 5 Ninhydrin-positive substances (NPS) in the haemolymph after hypoosmotic stress. NPS levels in “Farol” mussels (a) and “Molhes” mussels (b) at a salinity of 35‰ (control) and under hypoosmotic conditions (salinities of 25 and 20‰) were measured at days 0, 1, 4 and 14 of exposure. Values are expressed as the mean ± SE (n=6). Significant differences ($p<0.05$) between the initial time (0 days) and subsequent times (1, 4 and 14 days) are indicated by different letters. No significant differences ($p>0.05$) were observed for mussels kept at 35‰ salinity.

NKA activity assay

"Farol" mussels (Fig. 6a) showed no changes in their gill NKA activity during the exposure period. In contrast, "Molhes" mussels (Fig. 6b) exposed to salinities of 25 and 20‰ showed increases in their NKA activity relative to day 0 ($p<0.05$) at day 4 (76 and 80%, respectively), returning to the initial levels at day 14. No difference in basal NKA activity was observed between the two populations (data not shown).

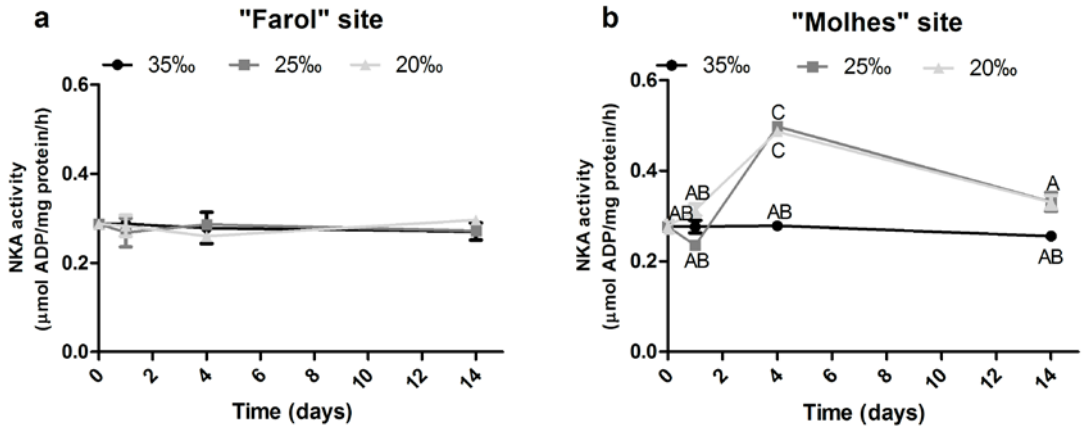

Fig. 6 Na^+/K^+-ATPase (NKA) activity in the gills after hypoosmotic stress. NKA activity levels in "Farol" mussels (a) and "Molhes" mussels (b) at a salinity of 35‰ (control) and under hypoosmotic conditions (salinities 25 and 20‰) were measured at days 0, 1, 4 and 14 of exposure. Values are expressed as the mean ± SE (n=6). Significant differences ($p<0.05$) between the initial time (0 days) and subsequent times (1, 4 and 14 days) are indicated by different letters. No significant differences ($p>0.05$) were observed for mussels kept at 35‰ salinity.

Discussion

In this study, we tested the hypothesis that mussels with distinct ecological histories could respond differently to a situation of stress (hypoosmotic stress) based on

the peculiarities of their environments and, consequently, the adaptations displayed by these organisms. Considering the characteristics of the two sampling sites ("Farol" and "Molhes"), particularly the salinity fluctuations observed at the "Molhes" site and the mussels' responses observed in the laboratory after hypoosmotic stress, our results support this hypothesis.

As mentioned above, salinity is a limiting factor for the distribution of marine invertebrates, specifically mussels, and it influences several physiological parameters that lead to potentially important implications for their health, such as alterations in oxygen consumption and changes in heart rate and total metabolism (Stickle and Sabourin 1979). Wang and collaborators (2012) and Westebom and collaborators (2002) noticed a decline in mussel size and biomass with decreased salinity. Thus, decreased environmental salinity may be disadvantageous for these organisms, and Maar and collaborators (2015) demonstrated that hypoosmotic shock causes changes in energy balance.

A strategy used by some mussels to avoid this disadvantageous effect is valve closure. It is important to emphasize that in our study, we observed that valves remained open during the entire experiment, probably due to the gradual decline in water salinity. This observation is essential because other authors have previously demonstrated that physiological responses differ between mollusks isolated from the external environment (by closing their valves) or maintained in these environments (valves kept open, either voluntarily or by the insertion of catheters) (Davenport 1979). The valve-closure reaction has been suggested as the primary response to short periods of exposure to low salinity among intertidal and estuarine bivalves (Gilles 1972; Hoyaux et al. 1976).

However, the response of mussels exposed to less extreme changes in salinity indicates that isolation by valve closure does not occur until the osmotic concentration

of the medium falls below a critical level between 400 and 500 mOsm/kg H_2O (Costa and Pritchard 1978). Davenport (1979) found that mussels exposed to decreasing salinities passively reduced their haemolymph osmolality based on the medium, consistent with our results for the "Farol" mussels, which responded as classical osmoconformers.

Regarding the physiological adaptations, osmoconforming marine bivalve mollusks have some mechanisms that regulate cellular volume. In this sense, an almost linear relationship between environmental salinity and total FAA in cells has been observed for *Mytilus edulis* tissues (Lange, 1963). Many studies show that the content of FAAs in bivalve tissues as measured by NPS is directly proportional to the alterations in salinity. For example, Deaton (2001) found increased FAA content (total and specific amino acids) at the gills of the mussel *Geukensia demissa* concomitantly with increases in the salinity of the medium. This response might be due to alterations in haemolymph osmolality to attempt to regulate cellular volume (for a review, see Larsen et al. 2014). That is, during exposure to higher salinity, the osmolality of mussel haemolymph increases, and the cell response involves increasing the level of FAAs through cellular protein catabolism or uptake from the haemolymph. However, this relation could be reversed for haemolymph, similar to the results found by Sadok and collaborators (1997), which showed an increase in NPS haemolymph content with reduced salinity. This result could be explained by the release of FAAs by cells to the haemolymph to regulate cell volume. Similarly, the increased NPS levels observed in the haemolymph of "Farol" mussels at day 1 could be caused by cellular FAA excretion. A release of amino acids and other organic molecules was also reported in response to cellular swelling induced by hypoosmotic stress in other Mytilidae mussel

species (Wright et al. 1989; Weber et al. 1992; Silva and Wright 1994; Neufeld and Wright 1996).

The mechanisms responsible for cellular volume regulation are more complex than originally thought, and Pierce (1982) and Smith and Pierce (1987) have shown that both free amino acids and intracellular ions are involved in this process. The stabilization of optimal ionic composition and gradients is necessary for effective cellular function, particularly during fluctuations in environmental salinity. In addition, in their study of *Mytilus edulis*, Hoyaux and collaborators (1976) found reductions in ion concentrations in haemolymph in response to reduced salinity. This is a classic response that corroborates our results for "Farol" mussels. At this point, it is important to note that "Farol" mussels respond to all of the analyzed parameters as classic osmoconformers. It is crucial to remember that "Farol" mussels do not suffer from the influence of salinity changes due to their localization far from the Patos Lagoon mouth (Fig. 1).

It has been suggested that the stress imposed on osmoconforming bivalves by short-term salinity fluctuations triggers behavioral and physiological mechanisms different from those elicited during long-term salinity adaptation, which may explain the differences in the responses of populations with distinct ecological backgrounds. It is known that organisms living in estuaries have a higher tolerance to salinity variations (Shumway 1977) because long-term adaptation has allowed them to develop physiological strategies to manage reduced salinity (Normant et al. 2005). Regarding mussels collected at the "Molhes" site, we observed certain changes in the classical osmoconformer response. These differences may be related to localization of the "Molhes" site at the Patos Lagoon mouth and to factors cited above, such as the fact that

long-term adaptation allows estuarine mussels to develop physiological strategies for managing reduced salinity.

Although we noted that at day 1 the "Molhes" mussels slightly reduced their haemolymph osmolality, these values returned to the initial levels at days 4 and 14, which could be explained by a period of adjustment to the new salinity regime. In this sense, the "Molhes" mussels' response was more similar to that of an osmoregulating organism, which would maintain haemolymph osmolality independent of changes in the medium. Hyperosmotic haemolymph has also been found in an invasive estuarine mussel (*Perna viridis*) when exposed to salinities below 10‰ (McFarland et al. 2013) and in *Mytilus edulis* at salinities below 25.5‰ (Costa and Pritchard 1978). It is important to note that this effect may be related either to valve closure (McFarland et al., 2013) or to ineffective exchange between the mantle fluid and the exterior media (Davenport, 1979). Here, we found that haemolymph from mussels collected at the "Molhes" site was both hyperosmotic with respect to the external medium and similar to baseline values (time 0, salinity 35‰), even in mussels with open valves. It is important to note that this finding is different than the results of McFarland and collaborators (2013) and of Costa and Pritchard (1978) and contrasts with the results observed for "Farol" mussels.

Classic responses of osmoconforming animals with regard to their ion concentration were found in "Farol" mussels, as described above, but not for "Molhes" mussels, which responded like osmoregulating organisms (Normant et al. 2005). These responses were similar to those observed for haemolymph osmolality. Considering the results of total osmolality and the ion concentrations in the haemolymph of "Molhes" mussels after hypoosmotic treatments, it is important to note a biphasic response pattern. Thus, if the salinity regime is altered for a short-term period (approximately 1

day or less), the organisms show reduced osmolality and ion concentration; however, if the stress persists, they invest in regulating their ion concentration. It is important to note that this is the strategy adopted by many animals for the maintenance of haemolymph hyperosmolality (and not the FAA concentration, as already mentioned), which is very similar to the strategy of osmoregulating organisms, which regulate their fluids because NKA activity increases (Pierce 1982; Smith and Pierce 1987). Thus, similar to the observations of Brooks and Mills (2006) for crustacean species, gill Na^+/K^+-ATPase activity in "Molhes" mussels was surprisingly elevated when the mussels were exposed to hypoosmotic medium. This increase was observed on day 4, simultaneous with the return of haemolymph osmolality to near-baseline values (corresponding to a salinity of 35‰). For euryhaline crustacean species, gill Na^+/K^+-ATPase is the driving force for active ion uptake (Péqueux 1995), and this seems to be occurring in "Molhes" mussels. Consequently, we noticed an initial decrease in all analyzed ions (at day 1) followed by a return to baseline conditions after 4 and 14 days. Based on this biphasic response, we hypothesized that "Molhes" mussels could adopt distinct strategies depending on the duration of hypoosmotic stress. For short-term exposure, "Molhes" mussels showed responses similar to those of "Farol" mussels (osmoconformer strategy); however, during long-term exposure, they seemed to adopt an osmoregulator strategy.

In conclusion, we observed that organismal origin (collection point) influences biological responses to environmental stress. "Farol" and "Molhes" mussels respond quite differently to hypoosmotic stress, and this appears to be related to their ecological history. Mussels collected from the "Farol" site (located outside the influence of the Patos Lagoon plume) are not naturally exposed to regular variations in salinity patterns, and they respond to these changes in the laboratory as classic osmoconforming

organisms. However, the “Molhes” mussels (collected from a site located at the Patos Lagoon mouth and under constant influence of the plume) naturally undergo changes in this environmental parameter and somehow adapt to these changes by responding like an osmoregulating organism, including by modulating NKA activity.

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MANUSCRITO 2

Biomarkers of oxidative stress in the marine bivalve *Perna perna*: effects of hypoosmotic stress on different mussel populations

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Biomarkers of oxidative stress in the marine bivalve *Perna perna*: effects of hypoosmotic stress on different mussel populations

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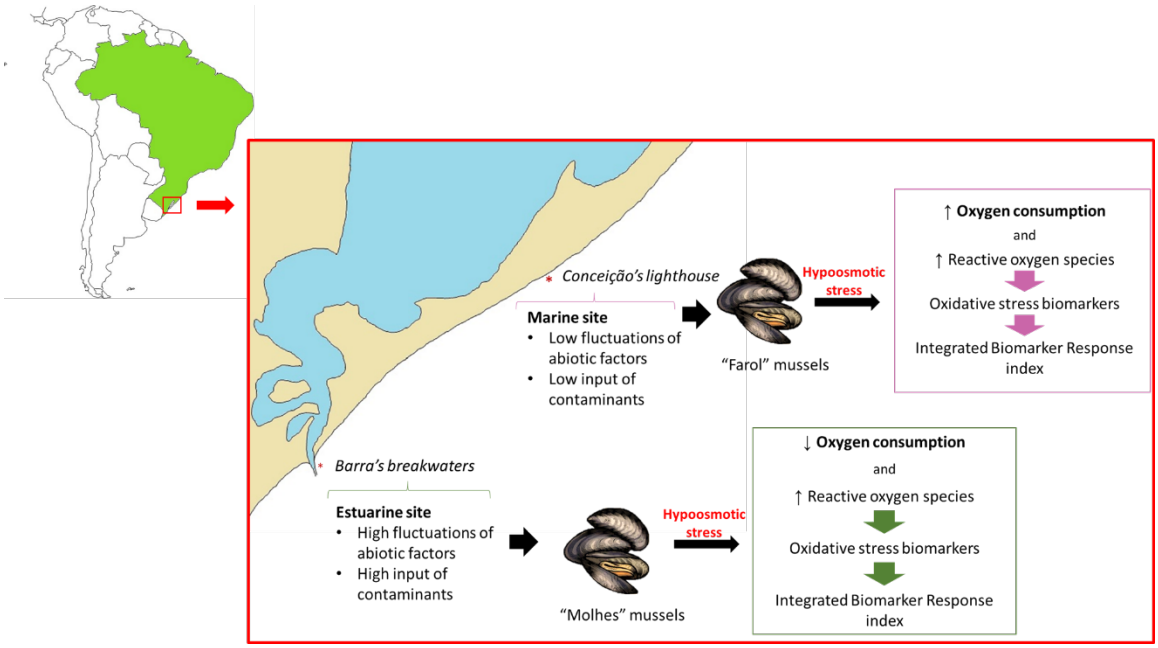
Highlights

1. Influence of abiotic factors, as salinity, could confound biomarker analysis;

2. Oxidative stress biomarkers are altered by hypoosmotic stress in mussel *Perna perna;*

3. Ecological history of organisms could influence differently the analyzed responses.

Graphical abstract



Abstract

Salinity affects physiology and metabolism of aquatic organisms, which may be reflected in oxygen consumption (VO_2) and enhanced reactive oxygen species generation (ROS). In addition, exposure to various pollutant classes may lead to an increase in ROS generation, and consequent changes in components of antioxidant defenses. Therefore, it seems essential to characterize natural variations in these biomarkers in order to differentiate pollution-induced effects from the effects of natural environmental fluctuations. In the present study, we evaluated responses to hypoosmotic stress of two mussel populations in order to determine whether or not such biomarker variation depends on ecological organism history. *Perna perna* mussels were collected at two sites, an estuarine and a marine environment, and mussels were exposed to salinities of 35 and 20‰ for 1, 4 and 14 days. VO_2 and ROS levels, total antioxidant capacity and activities of antioxidant enzymes (superoxide dismutase, catalase and glutathione *S*-transferase) were measured. The integrated biomarker response (IBR) index was calculated. Hypoosmotic stress affected all analyzed biomarker responses and IBR values, and these changes were distinct between populations. These results suggest that the ecological history of organisms, represented here by differences in organism origin (sample sites), and variation in abiotic factors at collecting sites should be taken into account in environmental monitoring programs.

Keywords: biomarkers, hypoosmotic stress, Patos Lagoon, oxidative stress, mussel

1. Introduction

Aquatic organisms are exposed to a wide range of environmental pollutants. In this sense, it is established that exposure to different classes of pollutants would induce variation in reactive oxygen species (ROS) levels (Regoli and Giuliani, 2014). When the rate of ROS production exceeds the rate of its decomposition by antioxidant defenses and repair systems, oxidative stress can occur, leading to the oxidation of key cell components like proteins, DNA and fatty acids (Sies, 1993). Antioxidant defenses include both enzymatic and non-enzymatic components, and the enzymatic system comprises antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione *S*-transferase (GST) (Choi et al., 2008). The activity and amount of these enzymes may be altered as a consequence of exposure to pollutants, which may be a defense barrier against ROS effects. In this sense, measurable changes in antioxidant defenses can indicate levels of pollutant exposure, and are widely investigated as biomarkers in environmental monitoring programs (Geret et al., 2002; Hoarau et al., 2006; Khessiba, 2005). In order to obtain combined chemical and biological data to assess the quality of aquatic environments, pollution monitoring programs have included the analysis of induced biological effects in sentinel species exposed to chemical pollution (Bellas et al., 2014; Thain et al., 2008). Marine bivalves such as mussels are widely used as sentinel species, mainly due to low mobility and filter-feeding characteristics (Baumard et al., 1999; Connor, 1996; Resgalla et al., 2007).

Pollution monitoring programs often involve the collection of organisms from environments such as estuaries and intertidal zones, where salinity and temperature are important factors affecting metabolic and physiological parameters of inhabitants (Bussell et al., 2008; Hamer et al., 2008; Prevodnik et al., 2007; Widdows and Donkin, 1991). Previous literature indicates that enzymatic activities associated with biomarker

responses could change due to factors such as natural physiological or reproductive cycles (Borkovic et al., 2005; Sheehan and Power, 1999), differences in food availability, water temperature (Viarengo et al., 1998), or reductions in salinity (Abele et al., 2011). As such, variation in biomarker responses may be misinterpreted as an effect of contaminant exposure.

Salinity is a key factor influencing the physiology and distribution of organisms in aquatic systems. Variations in salinity impose osmotic challenges, affecting metabolism, growth and survivorship of aquatic organisms (Brito et al., 2000; Gruffydd et al., 1984). Salinity fluctuations may affect animal metabolic rate and has been associated with enhanced ROS generation in aquatic organisms (Liu et al., 2007). Therefore, it is important to characterize natural variations in biomarker responses of aquatic organisms in order to differentiate pollution-induced effects from effects due to natural environmental fluctuations (Cravo et al., 2009; Hamer et al., 2008; Tedengren et al., 1988; Zanette et al., 2011). Previous studies (Albentosa et al., 2012; Bellas et al., 2014) indicate that some physiological and biochemical biomarkers seem to be more affected by biotic parameters such as feeding condition and reproductive status, than by chemical pollution in the mussel *Mytilus galloprovincialis*. To validate the applicability of biomarkers in aquatic pollution monitoring it is fundamental to discriminate the pollutant-induced response of an organism from the response to variability in natural environmental processes (Tankoua et al., 2012; Thain et al., 2008).

When in large scale monitoring programs, these problems are magnified, where organisms are subjected to a wide range of environmental conditions. The larger the area covered by a monitoring program, the greater the expected influence of natural variability (Fokina et al., 2014). Estuarine environments are characterized by their instability, including variations in water temperature, oxygen and salinity. Contrarily,

oceanic environments are more stable. In this sense, estuarine organisms have previously been more tolerant to environmental stress than marine organisms as a result of natural selection. Additionally, it seems reasonable to predict that inhabitants of areas with fluctuating abiotic factors would exhibit higher phenotypic plasticity (Fokina et al., 2014). Therefore, populations that developed in highly variable environments would present distinct strategies do deal with abiotic parameter fluctuations. In order to investigate this hypothesis, we evaluated the biomarker responses of two mussel populations subjected to salinity variations. For this, mussels were collected at two sites with distinct ecological history, an estuarine and a marine environment, which have previously been used in environmental monitoring studies (Rola et al., 2012).

2. Materials and methods

2.1.Experimental design, sampling and acclimation

Brown mussels *Perna perna* (30–40 mm) were collected at two sites in the southern littoral of Rio Grande do Sul (Brazil; Fig. 1) during late summer (March 2012). The first site is located at ‘‘Molhes da Barra’’ (Barra’s breakwaters, 32°09’050’’S, 52°04’550’’W) in Mar Grosso beach (São José do Norte, RS), an estuarine site. The second site is located at “Farol da Conceição” (Conceição’s lighthouse, 31°43’834’’S, 51°28’932’’W), 75 km far from the first site, a fully marine site. These sites have been used in a previous study with a different mussel species from the Mytilidae family, *Mytilus edulis* (Rola et al., 2012). Mussels were collected and immediately transported to the laboratory in water from the sampling site, where they were then acclimated to laboratory conditions (20°C, photoperiod 12D:12L) for 15 days according to Resgalla Jr. et al. (2007) prior to the tests. During the acclimation period,

mussels from both sites were kept in natural seawater at salinity 35‰ and fed with the algae *Conticribra weissflogii*.

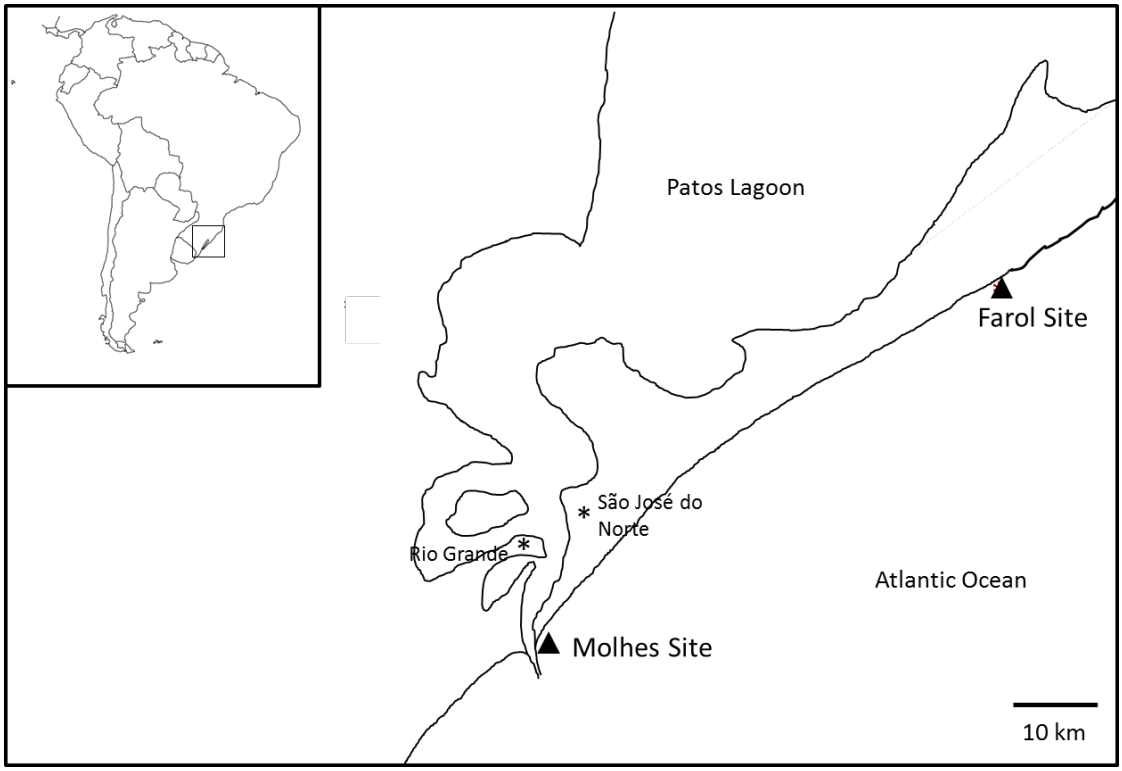


Figure 1. *Map of sampling points (southern Brazil - Rio Grande do Sul).* Triangles (▲) show the location of "Farol" and "Molhes" sites, and asterisks (*) show the location of Rio Grande and São José do Norte cities.

2.2.Salinity treatment assay

Mussels were exposed to salinities of 35 and 20‰ in 6L tanks (5 mussels/1L of water). Firstly, animals were placed in tanks with 35‰ salinity seawater. The water salinity from 20‰ group was gradually decreased 2‰ each 45min. After 1, 4 and 14 days the oxygen consumption (VO_2) of mussels was measured. Also, during the same sampling times gills and digestive gland samples were separated for further analysis. For enzymatic activity measurement, samples of gills and digestive gland were stored at -70°C.

2.3.Biomarker analyses

2.3.1. Oxygen consumption (VO_2)

Mussel VO_2 measurements were performed according to Resgalla Jr. et al. (2007). For this, mussels were individually placed in flasks containing aerated seawater (salinity 35‰ or 20‰), with at least six replicates per experimental treatment. Flasks were closed and held for 30 minutes. The initial and final dissolved oxygen in the water was measured using a digital oxymeter (SoilControl, OMC-900). The values were expressed in terms of mg O_2 consumed/L of water/kg of wet weight.

2.3.2. Reactive oxygen species (ROS) levels

Fresh mussel tissues (gills and digestive gland) were homogenized (1:4, w/v) in phosphate buffer 50mM, pH 7.5, and then centrifuged (20,000g, 4ºC, 20 minutes), from which the supernatant was taken for ROS assays. ROS levels were measured according to the methodology of Ferreira-Cravo et al. (2009) and analyzed in a fluorimeter (485nm excitation and 535nm emission; Victor 2, Perkin Elmer). Total fluorescence production was calculated by integrating the fluorescence units (FU) and standardized per minute and mg of protein. The results were expressed as relative ROS levels: the results of salinity 20‰ relative to the results of salinity 35‰.

2.3.3. Antioxidant capacity against peroxyl radicals

Antioxidant capacity against peroxyl radicals (ACAP) was evaluated according to Amado et al. (2009), by measuring the generation of ROS (mentioned in above item) in samples treated or not treated with a peroxyl radical generator: 2-dihydrochloride 2-azobis-2-metilpropionamidin (ABAP, Aldrich). The test was performed in fluorimeter (485nm excitation and 535nm emission; Victor 2, Perkin Elmer). Total fluorescence

production was calculated by integrating the fluorescence units (FU) and standardized per minute and mg of protein. Relative area was calculated and area of samples with ABAP was subtracted from samples without ABAP. The results were expressed as relative ACAP: the results of salinity 20‰ relative to the results of salinity 35‰.

2.3.4. Enzymatic activity

Mussel tissues were homogenized (1:4 w/v) in cold (4°C) buffer containing 1 mM EDTA, 500 mM sucrose, 20 mM Tris-base, 150 mM KCl with pH adjusted to 7.6. Homogenates were centrifuged (1000g, 4ºC, 10 min; and 12,000g, 4ºC, 30 min) and the resulting supernatant from these two centrifugation steps was collected and stored at -70°C for further enzymatic analyses. Six samples were used in biochemical assays for each experimental group. Catalase (CAT) activity was quantified following Beutler's method (1975), which measures the H_2O_2 (10 mM) decomposition per minute at 240 nm, the results of which were expressed as CAT units. One CAT unit is defined as the amount of enzyme able to decompose 1 µmol of H_2O_2 per minute per milligram of protein. SOD activity was measured according to McCord and Fridovich (1969) in spectrophotometer at 550 nm. One SOD unit is defined as the sample amount that inhibits cytochrome c reduction by 50% under the assay specified conditions. GST activity was measured at 340 nm following the method of Habig et al. (1974), using 1 mM 1-chloro-2,4-dinitrobenzene (CDNB; Sigma) and 1 mM reduced glutathione (GSH), pH 7.0. One GST unit represents the enzyme amount needed to conjugate 1 µmol of CDNB per minute and per milligram of total protein. Protein content was evaluated according to a commercial kit (Doles Reagentes Ltd., Goiânia, Brazil), which is based on the Biuret protein assay. All assays were performed at 25°C and at least in duplicate.

2.4.Integrated biomarker response (IBR) index calculation

The integrated biomarker response index (IBR) was calculated using the method described by Beliaeff and Burgeot (2002) and modified by Marigómez et al. (2013). The biomarkers ROS, ACAP, SOD, CAT and GST were considered for IBR calculation. This analysis was done in order to provide a data overview, since this does not represent a correlation test. The calculation method is based on the relative differences between biomarkers in each given data set. Thus, the IBR index is calculated by summing-up triangular star plot areas (a simple multivariate graphic method) for each two neighboring biomarkers in a given data set. Since the IBR value is directly dependent on the number of biomarkers in the data set, we divided the obtained IBR value by the number of biomarkers used in each analysis (n = 5) to calculate IBR/n, according to Broeg and Lehtonen (2006).

2.5.Statistical analysis

Results are presented as mean ± standard error. The statistical analysis of enzymatic assays (n=6) was performed using Student's t test ($p<0.05$), between different salinities, in the same experimental time.

3. Results

3.1.Oxygen consumption

A significant increase ($p<0.05$) in oxygen consumption (VO_2) was observed for "Farol" mussels after 4 days of hypoosmotic stress. In relation to "Molhes" mussels, a significant decrease ($p<0.05$) was observed at day 14 (Fig. 2). No significant differences ($p>0.05$) were found in others exposure times.

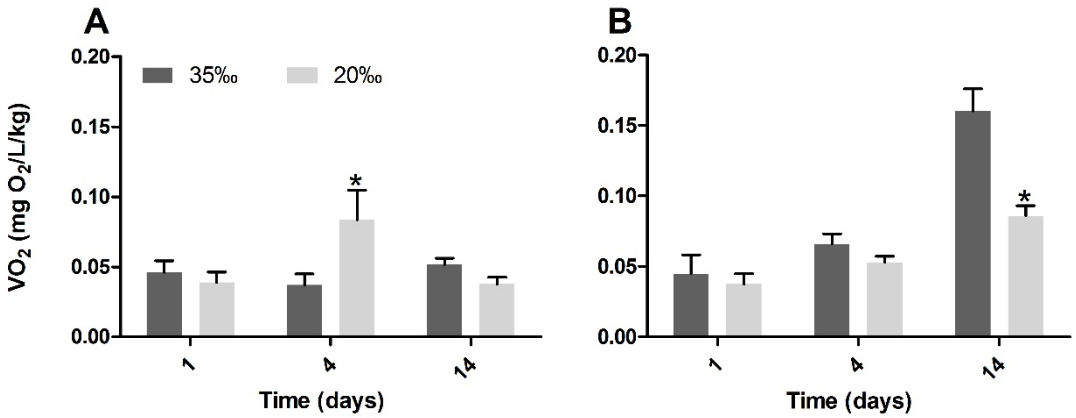


Figure 2. *Effects of hypoosmotic stress on oxygen consumption (VO_2).* Oxygen consumption of "Farol" mussels (A) and "Molhes" mussels (B) at salinities 35‰ (control) and 20‰ (hypoosmotic treatment) were measured at days 1, 4 and 14 of exposure. Values are expressed as mean ± SE (n=6). Significant difference ($p<0.05$) between salinities at the same time is indicated by asterisks (*).

3.2.Reactive oxygen species (ROS) levels

Concerning ROS levels (Fig. 3), a significant increase ($p<0.05$) was observed in the digestive gland of "Farol" mussels exposed for 14 days to hypoosmotic stress. This increase in ROS levels was also observed for gills of "Molhes" mussels at days 1 and 14 of hypoosmotic stress. No significant difference ($p>0.05$) was found in other exposure times.

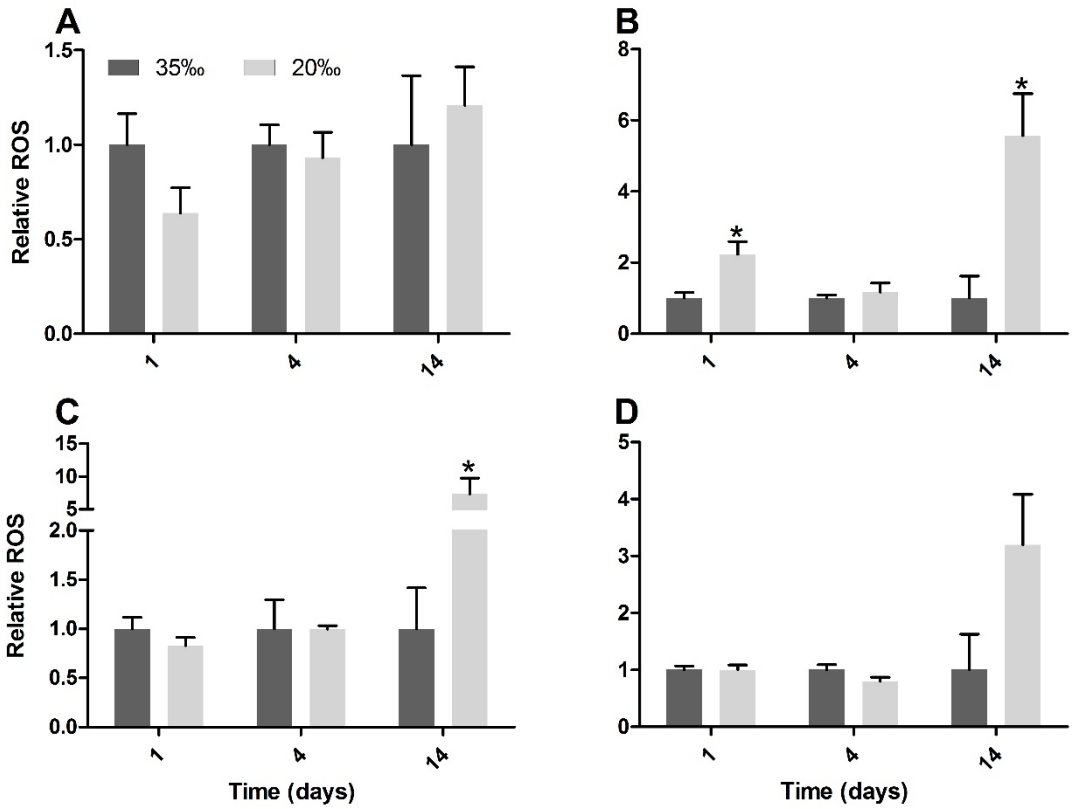


Figure 3. *Effects of hypoosmotic stress on ROS levels.* Relative ROS measurements in gills and digestive gland from "Farol" mussels (A and C respectively) and "Molhes" mussels (B and D respectively) at salinities 35‰ (control) and 20‰ (hypoosmotic treatment) were measured at days 1, 4 and 14 of exposure. Values are expressed as mean ± SE (n=6). Significant difference ($p<0.05$) between salinities at the same time is indicated by asterisks (*).

3.3.Antioxidant Capacity (ACAP) against peroxyl radicals

Regarding ACAP (Fig. 4), a significant increase ($p<0.05$) was observed in gills and digestive gland of "Farol" mussels exposed to hypoosmotic stress for 14 days. For "Molhes" mussels, a significant increase ($p<0.05$) was observed only in gills at day 14

of treatment. Contrarily, a significant decrease ($p<0.05$) was found in digestive gland of "Molhes" mussels at days 1 and 14 of hypoosmotic stress. No significant difference ($p>0.05$) was found in other exposure times.

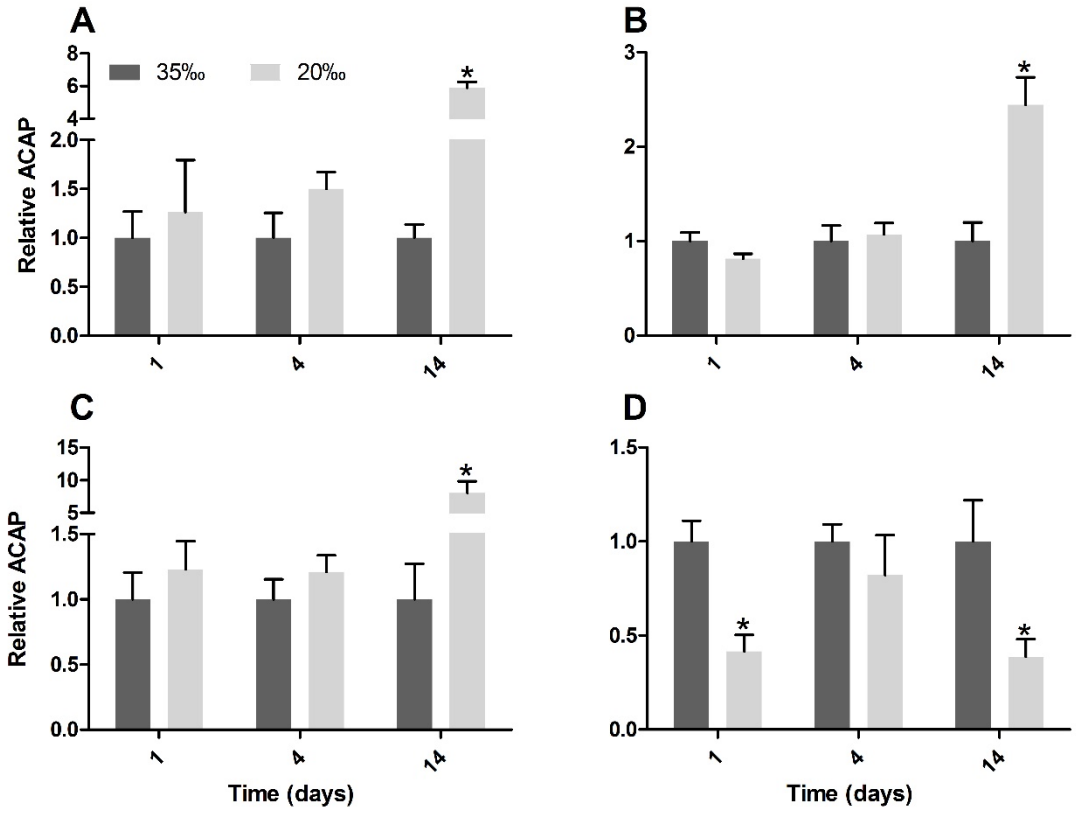


Figure 4. *Effects of hypoosmotic stress on ACAP levels.* Relative ACAP measurements in gills and digestive gland from "Farol" mussels (A and C respectively) and "Molhes" mussels (B and D respectively) at salinities 35‰ (control) and 20‰ (hypoosmotic treatment) were measured at days 1, 4 and 14 of exposure. Values are expressed as mean ± SE (n=6). Significant difference ($p<0.05$) between salinities at the same time is indicated by asterisks (*).

3.4.Enzymatic activity

3.4.1. Catalase (CAT)

The effects of hypoosmotic stress on CAT activity (Fig. 5) were dependent on mussel origin. For “Farol” mussels, a significant decrease ($p<0.05$) was found regarding CAT activity in digestive glands at day 14 of hypoosmotic stress. In relation to “Molhes” mussels, a significant increase ($p<0.05$) was observed in gills at day 1 of treatment and a significant decrease ($p<0.05$) at day 4. For digestive gland of “Molhes” mussels, a significant decrease ($p<0.05$) was found at day 4 of treatment. No significant difference ($p>0.05$) was found in other exposure times.

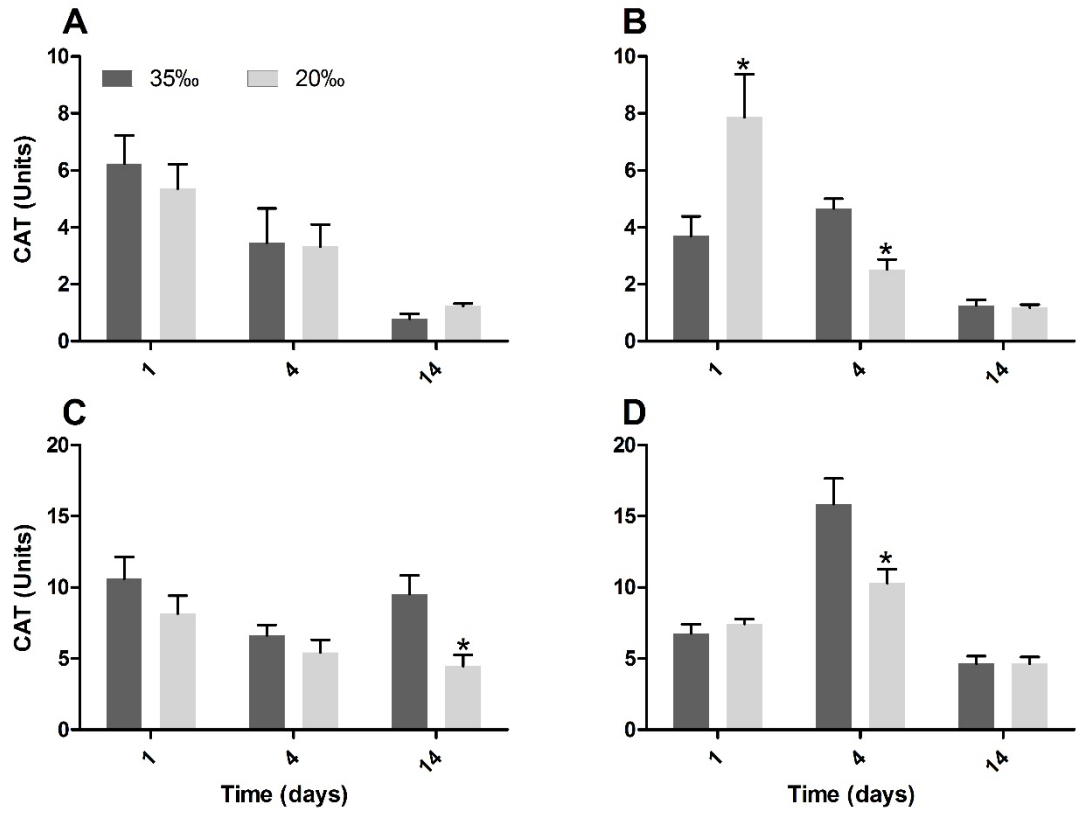


Figure 5. *Effects of hypoosmotic stress on CAT activity.* CAT activity measurements in gills and digestive gland from "Farol" mussels (A and C respectively) and "Molhes" mussels (B and D respectively) at salinities 35‰ (control) and 20‰ (hypoosmotic treatment) were measured at days 1, 4 and 14 of exposure. Values are expressed as mean ± SE (n=6). Significant difference ($p<0.05$) between salinities at the same time is indicated by asterisks (*).

3.4.2. Superoxide dismutase (SOD)

Concerning "Farol" mussels, a significant increase ($p<0.05$) in SOD activity (Fig. 6) was observed at day 14 for gill and at day 1 for digestive gland samples, and a significant decrease ($p<0.05$) was found at day 4 and 14 of treatment in digestive gland. For "Molhes" mussels, a significant decrease ($p<0.05$) was found in gills at day 4 of hypoosmotic stress. In digestive gland, a significant decrease ($p<0.05$) was observed at

day 1 and a significant increase ($p<0.05$) at day 14 of treatment. No significant difference ($p>0.05$) was found in other exposure times.

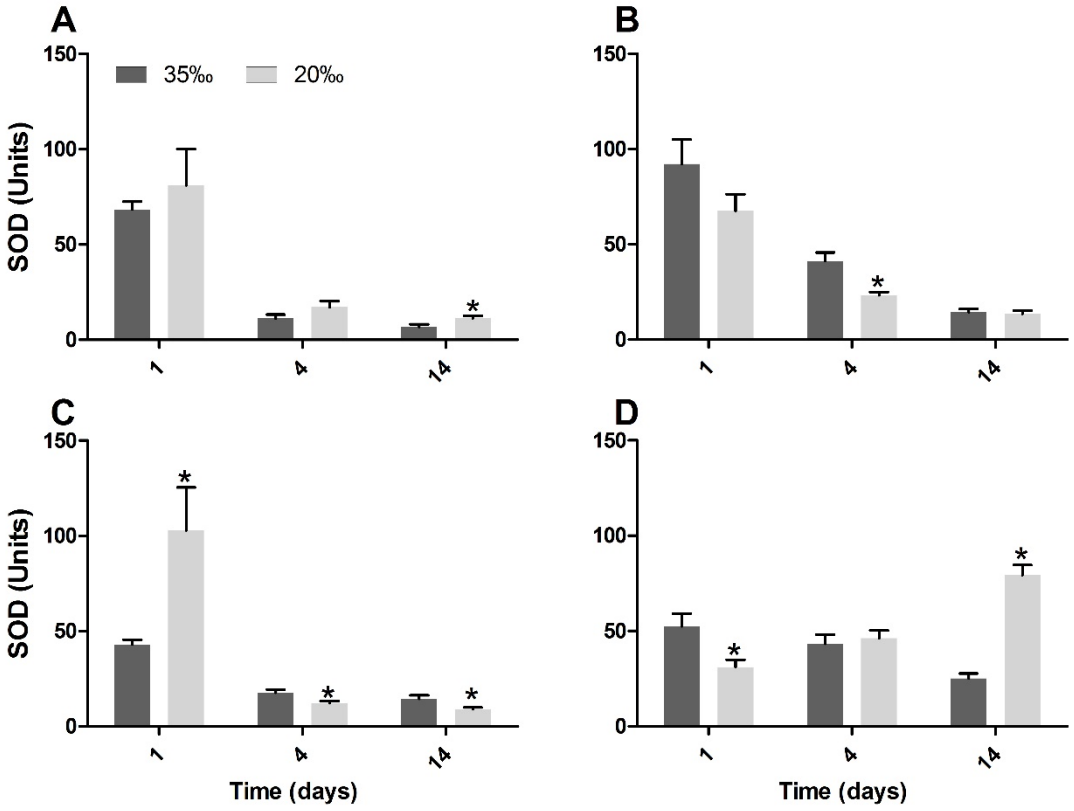


Figure 6. *Effects of hypoosmotic stress on SOD activity.* SOD activity measurements in gills and digestive gland from "Farol" mussels (A and C respectively) and "Molhes" mussels (B and D respectively) at salinities 35‰ (control) and 20‰ (hypoosmotic treatment) were measured at days 1, 4 and 14 of exposure. Values are expressed as mean ± SE (n=6). Significant difference ($p<0.05$) between salinities at the same time is indicated by asterisks (*).

3.4.3. Glutathione S-transferase (GST)

In relation to "Farol" mussels, a significant increase ($p<0.05$) in GST activity (Fig. 7) was observed in gills at day 4 and in digestive gland at day 14 of hypoosmotic stress. For "Molhes" mussels, a significant increase ($p<0.05$) in GST activity was observed in gills at day 1. No significant difference ($p>0.05$) was found in other exposure times.

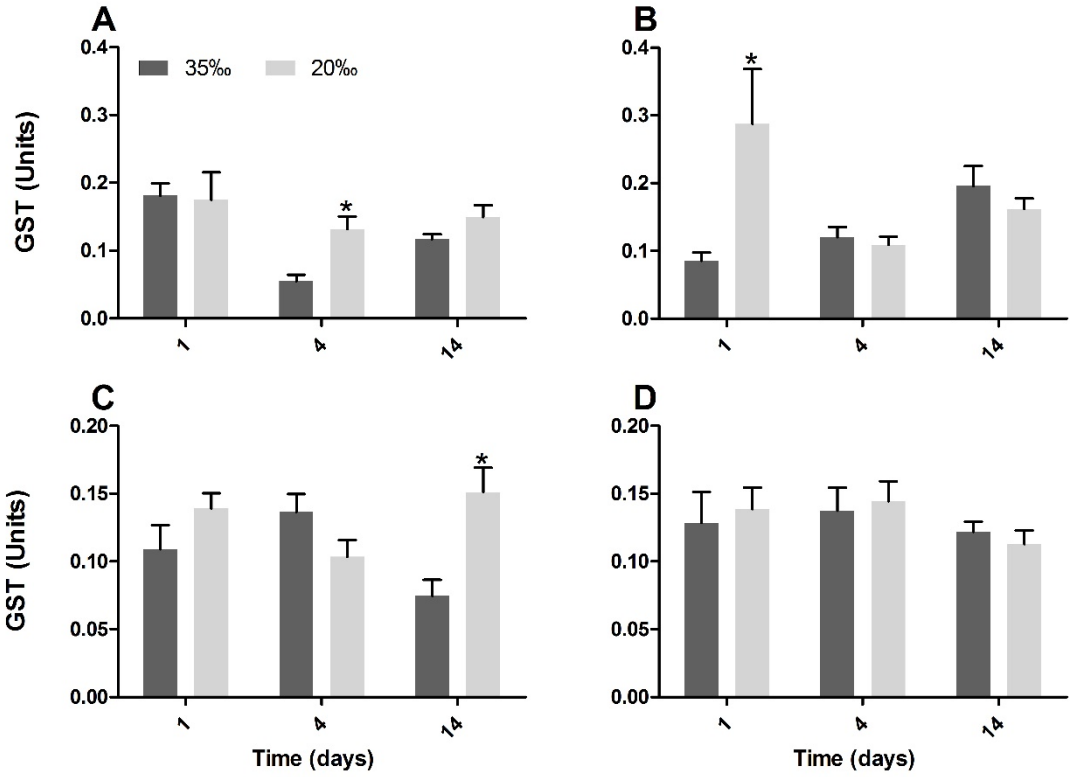


Figure 7. *Effects of hypoosmotic stress on GST activity.* GST activity measurements in gills and digestive gland from "Farol" mussels (A and C respectively) and "Molhes" mussels (B and D respectively) at salinities 35‰ (control) and 25‰ (hypoosmotic treatment) were measured at days 0, 1, 4 and 14 of exposure. Values are expressed as mean ± SE (n=6). Significant difference ($p<0.05$) between salinities at the same time is indicated by asterisks (*).

3.5.Integrated biomarker response (IBR)

According to the IBR starplot (Fig. 8), biomarker scores are specific for each population and vary according to the salinity treatment. The analyses of one tissue (gill) and one sampling time (day 4) were displayed. Other graphics may be found in the supplementary data, which displayed the same pattern of response (with different responses between populations). IBR/n index also differed among populations, with IBR/n at "Farol" mussels higher than at "Molhes" mussels (Tab. 1).

Table 1. IBR/n values from "Farol" and "Molhes" mussels at salinities 35‰ (control) and 20‰ (hypoosmotic stress).

	Salinities	
	35‰	*20‰*
"Farol" mussels	0.5607	0.3869
"Molhes" mussels	0.1739	0.2630

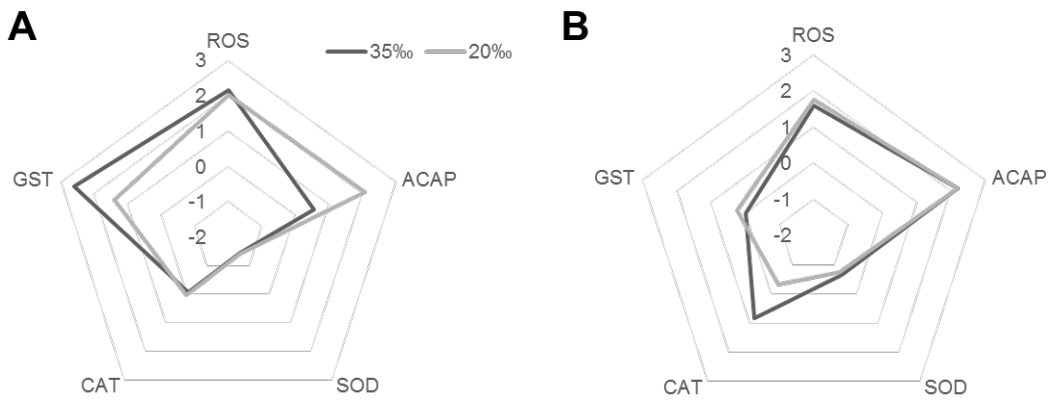


Figure 8. *Effects of hypoosmotic stress as displayed via the IBR index.* Starplots from "Farol" mussels (A) and "Molhes" mussels (B) at salinity 35‰ (control) and hypoosmotic treatment (salinity 20‰) were calculated at day 4 of exposure for gills.

4. Discussion

The present study hypothesized that abiotic factors would affect mussel biomarker responses differently depending on ecological histories of organisms. Overall, all analyzed biomarkers were altered with hypoosmotic stress. Salinity represented a limiting factor for mussel distribution, influencing several physiological parameters such as respiratory rate (oxygen consumption), heart rate and metabolism (Stickle and Sabourin, 1979). The present study observed alterations in oxygen consumption during hypoosmotic stress, reflecting alterations in organism metabolism and osmoregulatory processes. Salinity reduction may cause an increase in metabolic rate due to increased osmoregulatory demands, subsequently increasing ROS production in marine organisms (Abele et al., 2011). It is important to note that hypoosmotic stress indirectly leads to ROS formation and alterations in antioxidant capacity, which can increase the susceptibility of animals to the action of other stressors.

Increases in ROS levels should be intercepted by the action of antioxidants in order to avoid an oxidative stress situation. Superoxide dismutase (SOD) is the enzyme responsible for the removal of $O_2^{\cdot-}$ with formation of hydrogen peroxide (H_2O_2) (Regoli and Giuliani, 2014). SOD therefore represents a source of hydrogen peroxide, which creates a necessity for H_2O_2 reducing enzymes, such as catalase (CAT). Catalase is an extremely active catalyst for reduction of H_2O_2 to H_2O, and is mostly present within peroxisomes. The present study found alterations in SOD and CAT activities as a function of salinity, sampling time and tissue. An and Choi (2010) described increases in SOD and CAT activity in gills of ark shell (*Scapharca broughtonii*) following salinity decreases to 25‰. Carregosa et al. (2014) also stated that at salinity of 14‰ three species of mussel (*Venerupis decussata*, *Venerupis corrugata*, *Venerupis philippinarum*) significantly increased the activity of antioxidant enzymes SOD and CAT. Contrary to this, Verlecar et al. (2008) observed a decrease in SOD activity following slight decreases in salinity for *Perna viridis*. Lau et al. (2004), in a field study with the same species, also observed a decrease in SOD and CAT activities when salinity decreased. These results reinforce the idea that enzymatic activity varies with salinity, depending on species, time and magnitude of hypoosmotic stress.

Glutathione *S*-transferase (GST) was also analyzed in the present study. This enzyme family conjugates electrophilic compounds (from both endogenous and exogenous sources) with the tripeptide glutathione. Here, an increase in GST activity after hypoosmotic stress for both populations with different temporal patterns was observed. Many studies also described an increase in GST activity following salinity decreases, such as within the oyster *Crassostrea gigas* (Damiens et al., 2004), and the mussels *Mytilus galloprovincialis* (Bebianno et al., 2007; Hoarau et al., 2006) and *Perna viridis* (Verlecar et al., 2008). This increase in GST activity is likely related to its

role in detoxification and conjugation of damaged biomolecules, which may have resulted from the observed increase in ROS levels.

All observed results, aside from being distinct between analyzed populations, demonstrate alterations in biomarker responses caused by changes in salinity. Reports from a monitoring program in a European estuary (Water Framework Directive) demonstrated similar results, in an investigation of biomarker responses regarding the effects of a salinity gradient along sampling sites for the fish species *Placiththys flesus* (Capela et al., 2016). It is important to emphasize that alterations of biomarker responses may occur due to variations in natural abiotic factors, and not only due to pollutant exposure. These alterations occur naturally in the environment, and must be taken into account during biomarker validation in monitoring programs, since these biochemical biomarkers are unspecific and may be altered during situations of oxidative stress, and subsequently evaluated in studies (Capela et al., 2016; Capó et al., 2015). Although it is advantageous to assess multiple stressors, oxidative stress biomarkers may be affected by various abiotic factors (Livingstone, 2003). As mentioned above, for monitoring programs acting over a long scale this is a significant issue, since these alterations could mask or overestimate pollutant effects. Thus, it is essential to evaluate the abiotic factors in the study sites, as well as investigate the influence of these factors on biomarkers to be analyzed above monitoring studies itself.

Biomarker responses may present variability in sensitivity, induction and recovery, and this variability may occur at population or tissue levels (Wu et al., 2005). Moreover, this variability could be markedly affected by abiotic factors, and may then eclipse the response to pollutants during monitoring. Thus, as pointed out here and highlighted by Colin et al. (2016), it is extremely important to validate the biomarkers used in monitoring programs, both in laboratory tests in order to elucidate a possible

cause-effect relationship between stressors and biomarkers, and in field studies, testing the strength of these relationships in varying degrees of complexity in natural systems (including influences of biotic and abiotic factors).

Besides variations due hypoosmotic stress, our results demonstrate that the pattern of response is different depending on organism environmental history. The two selected sampling points represent distinct ecological history and likely differing adaptive responses of organisms according to this. The outfall of the Patos-Mirim Lagoon complex (Southern Brazil) occurs near the city of Rio Grande. This output is delimited by breakwaters, which creates an estuarine environment where water-mixing processes occur. As such, this region (Barra's breakwaters – "Molhes" site) is subjected to large variations in abiotic factors, such as salinity. In some periods of the year, the water of the Lagoon can extend to regions north of the mouth, but generally no further than 35 km (Marques et al., 2009). "Farol da Conceição" (Conceição's lighthouse – "Farol" site) is located at a distance of 75 km north of the Lagoon outfall, and therefore not influenced by the salinity variation regimen governed by the outflow of the Patos Lagoon. "Farol" is thus considered a fully marine site.

Considering biomarkers analysis between populations, oxygen consumption increased in "Farol" mussels (fully marine) when salinity decreased at 4 days of treatment. This result is common among osmoconformer invertebrates (Bayne and Newell, 1983), likely reflecting the elevated energetic requirement of salinity "tolerance" during hypoosmotic conditions. Thereby, Hawkins et al. (1987) have shown an increase in oxygen uptake when salinity decreases in *Perna viridis* and *Perna indica* (32 to 20‰ in 24h). Hamer et al. (2008) also demonstrated an increase in oxygen uptake following decreases in salinity in *Mytilus galloprovincialis* (37 to 28 and 18‰ in 14 days). Upon exposure to a prolonged reduction in salinity, bivalves may gradually

acclimate to the new salinity regime, returning to initial oxygen consumption values (Bayne and Newell, 1983), as observed at day 14 in the present experiment for the "Farol" group. In relation to "Molhes" mussels (living in an estuarine site), a contrary response was observed: reduction in oxygen consumption following salinity decreases. In this sense, a considerable depression of *M. edulis* gill movement and *Littorina saxatilis* oxygen consumption occured under a reduction in salinity from 25 to 14‰ (Berger, 2005). It is important to note that these mussels were collected at White Sea, where salinity varies from 0 to 28‰ throughout the year (Filatov et al., 2005). Thus, animals that live in this location have a history of salinity variation, similarly to "Molhes" mussels, which demonstrated similar responses to hypoosmotic stress. The contrasting answer between populations may be due a distinct response regarding osmoregulation processes. In another study, our group demonstrated that these two populations responded differently in relation to osmoregulatory patterns, such that "Farol" mussels displayed a classic osmoconforming response and "Molhes" mussels responding similar to osmoregulating organisms (unpublished data). In this regard, probably because "Molhes" mussels are often exposed to changes in salinity due to the estuarine environment, they have a different strategy to deal with these alterations, which could be reflected in the reduction of oxygen consumption of animals.

For other biomarkers, rather than opposite responses among populations, differences in the time elapsed to onset of a response were observed. For example, hypoosmotic stress increased ROS levels at day 14 in digestive gland of "Farol" mussels and at day 1 and 14 in gills of "Molhes" mussels. These results differ between populations in terms of tissue and exposure time, but hypoosmotic stress induced an increase in ROS levels in each instance. In general, increases in ROS levels were followed by an induction of antioxidant capacity. Contrary to this, only digestive gland

of “Molhes” mussels displayed decreases of antioxidant capacity after 1 and 14 days of hypoosmotic stress. Antioxidant enzymes also responded differently among populations. To exemplify, SOD activity at day 1 of treatment increased when salinity decreased in digestive gland of "Farol” mussels, while no changes were found for CAT activity at the same experimental time. In relation to “Molhes” mussels, SOD activity increases in digestive gland similarly to that found to “Farol” mussels, but only on day 14 of treatment, and this increase was not accompanied by CAT activity. As noted above, biomarkers may differ in their time of induction, and this variability may occur between populations of the same species (Wu et al., 2005). Regarding these results, it is important to emphasize that differences among populations with distinct ecological history have to be evaluated with biomarker validation, since basal levels of biomarkers could be altered due to fluctuations in abiotic factors. This caution is extremely important, especially in monitoring programs covering a large area in which abiotic factors vary among distinct collection points.

In order to provide an integrative approach with respect to biomarkers, the present study obtained the global indices of environmental quality: the Integrated Biological Response (IBR) index (Beliaeff and Burgeot, 2002). The IBR index is not a statistical tool to compare differences between groups, but assists in generating an integration of the biomarker responses from different groups. This may provide a panorama of the animal’s responses due to different treatments with contaminants. However, some authors have observed seasonal variation of IBR in mussels (Bodin et al., 2004; Broeg and Lehtonen, 2006) that could be due to variations in abiotic factors such as temperature, pH and salinity, and others. In this sense, it should be well understood which factors affect the components used in index calculations in order to not confound effects of abiotic factors with pollution effects. Therefore, we used IBR

calculation at distinct salinity, demonstrating that salinity affects IBR values and may depend on organism sampling site and consequently the ecological history of organisms. Salinity affected IBR values and starplot graphs differently, depending on the origin of mussels. Within the same population, the graphs plotted for different salinities resulted in distinct starplots. However, the starplots for "Molhes" mussels in most times were more similar between the two salinities tested when compared with starplots for “Farol” mussels. This result further supports the idea that hypoosmotic stress alters biomarker status of organisms and that these changes are different between the populations studied. Thereby, it was noted that natural stressors, which can mask effects due to pollutant exposure, could alter IBR values. So, this result reinforces the importance of evaluating the influence of abiotic factors when calculating IBR index and reveals that this analysis must be done individually for each population.

5. Conclusions

Primarily, changes in salinity could lead to changes in the analyzed biomarkers, either by generating ROS or by interference in the levels and activity of antioxidants. Furthermore, mussel populations may respond differently to a situation of stress (hypoosmotic stress) based on the pre-exposure to abiotic and biotic conditions in their environments and, consequently, the adaptations displayed by these organisms. The natural variability of organism responses, such as those caused by adaptability to different habitats (as consequences of temperature, salinity, and water quality parameters fluctuations, for example) could confound biomarkers results. In conclusion, it is important to emphasize that variation in abiotic factors (such as salinity) should always be taken into account when assessing biomarkers. Moreover, the difference

among population responses must also be considered, thereby decreasing the probability of erroneous interpretations even in integrated biomarkers analysis.

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Supplementary data

Table S1. IBR/n values from “Farol” and “Molhes” mussels at salinities 35‰ (control) and 20‰ (hypoosmotic stress) at days 1 and 14 in gills.

		Salinities	
		35‰	*20‰*
Day 1	“Farol” mussels	0.7555	0.1634
	“Molhes” mussels	0.2616	0.4474
Day 14	“Farol” mussels	-0.5100	-0.6777
	“Molhes” mussels	-0.4800	-0.3528

Table S2. IBR/n values from “Farol” and “Molhes” mussels at salinities 35‰ (control) and 20‰ (hypoosmotic stress) at days 1, 4 and 14 in digestive gland.

		Salinities	
		35‰	*20‰*
Day 1	“Farol” mussels	-0.0585	0.1831
	“Molhes” mussels	0.0932	0.3682
Day 4	“Farol” mussels	-0.0625	0.1938
	“Molhes” mussels	0.0418	0.2157
Day 14	“Farol” mussels	-0.4678	-0.4371
	“Molhes” mussels	-0.3808	0.0969

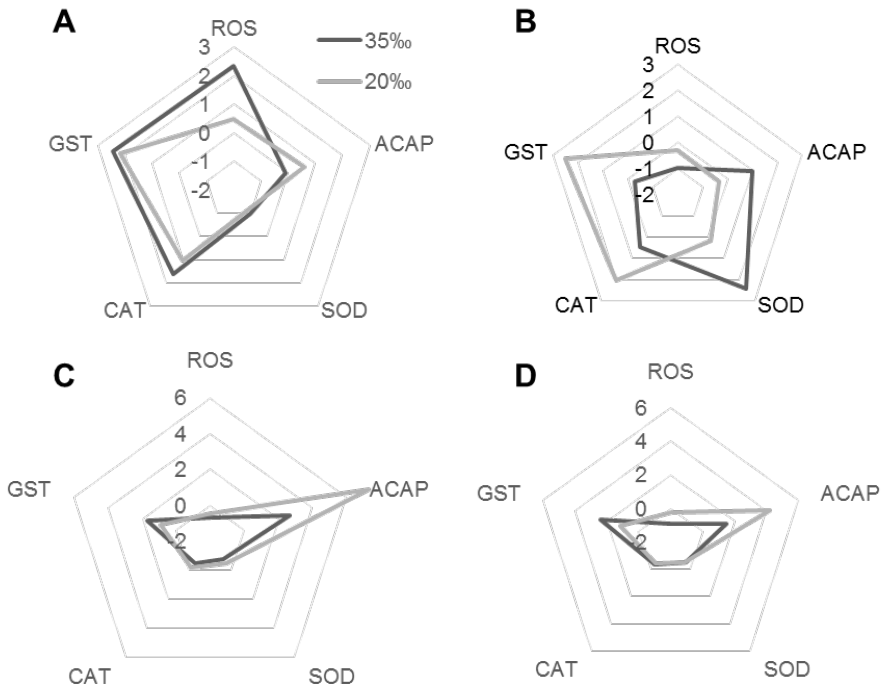

Figure S1. Effects of hypoosmotic stress as displayed via the IBR index. Starplots from "Farol" mussels at day 1 (A) and day 14 (C) and "Molhes" mussels at day 1 (B) and day 14 (D) at salinity 35‰ (control) and hypoosmotic treatment (salinity 20‰) were calculated for gills.

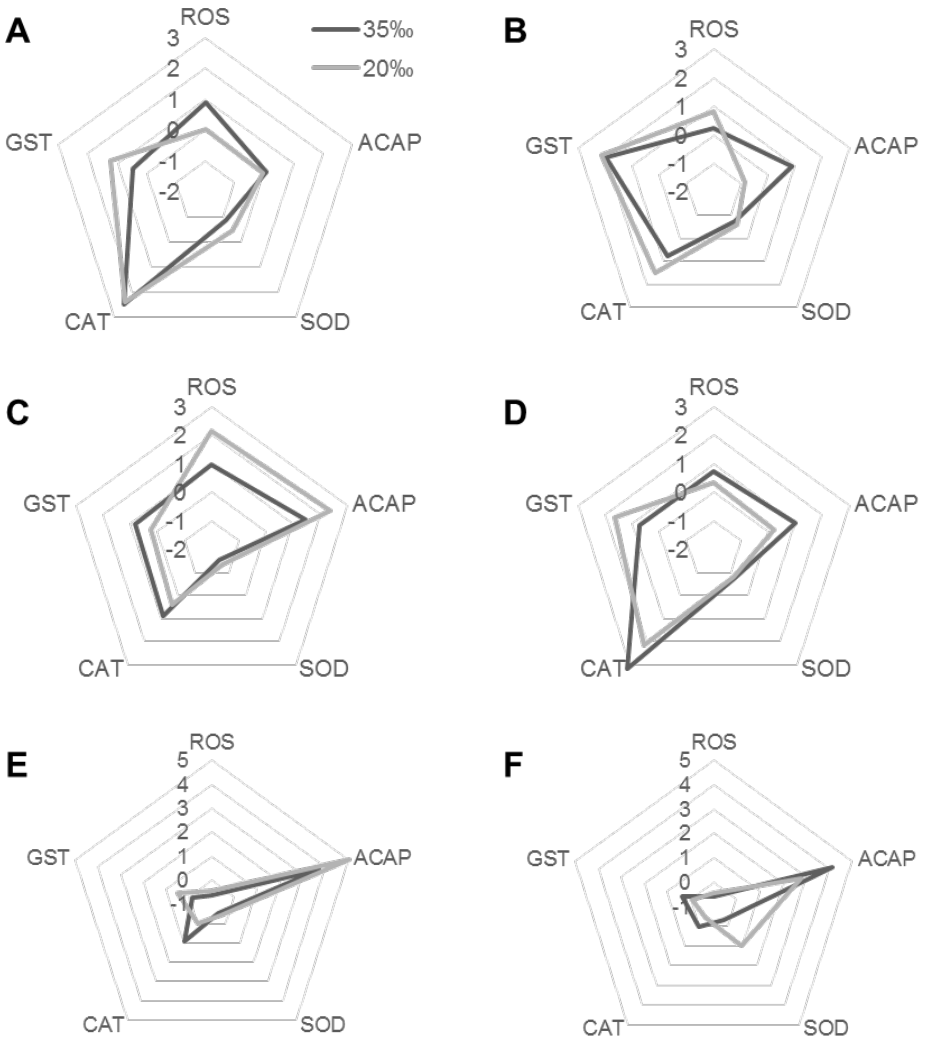

Figure S2. Effects of hypoosmotic stress as displayed via the IBR index. Starplots from "Farol" mussels at day 1 (A), day 4 (C) and day 14 (E) and "Molhes" mussels at day 1 (B), day 4 (D) and day 14 (F) at salinity 35‰ (control) and hypoosmotic treatment (salinity 20‰) were calculated for digestive gland.

DISCUSSÃO GERAL

No presente estudo, testamos a hipótese de que uma situação de estresse hiposmótico afeta as respostas fisiológicas dos mexilhões de forma dependente do histórico ecológico, e que estas alterações afetam a análise de biomarcadores. Estas diferenças nas respostas podem ser baseadas nas peculiaridades do habitat do organismo e, consequentemente, às suas adaptações. Neste sentido, considerando as características dos dois locais de amostragem ("Farol" e "Molhes") e as respostas distintas observadas nos testes em laboratório, nossos resultados suportam esta hipótese.

Como já mencionado, a salinidade é capaz de influenciar diversos parâmetros fisiológicos dos organismos aquáticos, e, neste sentido, em nosso estudo observamos alterações, por exemplo, no consumo de oxigênio durante o estresse hiposmótico. Esta variação pode refletir alterações no metabolismo do organismo, podendo estar ligada ao processo de osmorregulação. Assim, o estresse hiposmótico afetou de forma distinta tanto os processos osmorregulatórios quanto o consumo de oxigênio nos animais dos distintos pontos de coleta.

Os mexilhões coletados no ponto do "Farol", o qual está localizado fora da influência da pluma da desembocadura da Laguna dos Patos e, assim, não estão naturalmente expostos a variações regulares nos padrões de salinidade, respondem ao estresse hiposmótico induzido em laboratório como organismos osmoconformadores clássicos. Neste sentido, foram encontrados níveis aumentados de aminoácidos livres (medidos como substâncias positivas a ninidrina - NPS) na hemolinfa. Este aumento pode ser causado pela liberação de aminoácidos pelos hemócitos, como forma de regulação de volume. Esta excreção de aminoácidos pelas células, juntamente com outros mecanismos compensatórios, como o anabolismo de proteínas a partir destes aminoácidos e a aumentada excreção de amônia (Gainey, 1994; Livingstone et al.,

1979), além da regulação de íons específicos, podem requerer um aumento no gasto energético, o qual se reflete em acréscimos no consumo de oxigênio. De fato, este consumo apresentou-se aumentado nos mexilhões do "Farol" com o estresse hiposmótico, resultado comum entre invertebrados osmoconformadores (Hamer et al., 2008; Hawkins et al., 1987). Também com relação a regulação osmótica, foi observada uma redução na concentração de íons e, consequentemente, uma queda na osmolalidade da hemolinfa, resultados estes dentro do padrão esperado para organismos osmoconformadores e similares aos encontrados por diversos autores (Hoyaux et al., 1976; Neufeld e Wright, 1996; Silva e Wright, 1994; Weber et al., 1992; Wright et al., 1989).

Os mexilhões dos "Molhes", localizado na desembocadura da Laguna dos Patos e sob influência constante da sua pluma e, assim, sob flutuações temporais na salinidade, parecem estar adaptados a estas mudanças respondendo similarmente a organismos osmorreguladores. Com relação a este ponto, foi relatado em nosso estudo uma resposta bifásica para as concentrações iônicas e osmolaridade da hemolinfa. Embora tenhamos notado uma redução inicial na concentração de alguns íons e na osmolalidade da hemolinfa, observado nas análises do dia 1 de exposição ao estresse hiposmótico, estes valores retornaram aos níveis iniciais no dia 4 e permaneceram nestes níveis no dia 14 de exposição ao estresse. Neste ponto, se faz importante ressaltar a atuação da Na^+/K^+-ATPase nas brânquias dos animais dos "Molhes", a qual teve sua atividade aumentada com o estresse hiposmótico simultaneamente ao retorno da osmolalidade da hemolinfa (segunda fase da resposta observada), estratégia similar a adotada por diversas espécies de organismos osmorreguladores (Brooks e Mills, 2006; Péqueux, 1995; Pierce, 1982; Smith e Pierce, 1987).

Neste sentido, a hemolinfa permaneceu hiperosmótica com respeito ao meio externo no dia 4 de tratamento, surpreendentemente mesmo com as valvas abertas. Estes resultados são contrastantes aos encontrados na literatura, mesmo os que relatam um discreto aumento na osmolalidade e concentração de íons na hemolinfa (Costa e Pritchard, 1978; McFarland et al., 2013), já que estes autores relatam o fechamento das valvas, ou seja, uma ausência de trocas com o meio externo. Além disso, os autores demonstraram que estes aumentos nos parâmetros analisados tornaram a hemolinfa hiperosmótica ao meio externo, mas não tendo retornada aos valores iniciais como observado para os mexilhões dos "Molhes". Também, estes resultados são discrepantes quando comparados aos encontrados para os mexilhões do "Farol", os quais embora também tenham permanecido com as valvas abertas, apresentaram a resposta clássica para organismos osmoconformadores.

Por conseguinte, podemos hipotetizar que, apesar de relatarmos a manutenção das valvas abertas durante todo o experimento para os animais dos "Molhes", diferentemente da estratégia clássica de fechamento da concha utilizada por alguns mexilhões (Davenport, 1979; Gilles, 1972; Hoyaux et al., 1976; McFarland et al., 2013), podem estar ocorrendo outros mecanismos que reduzam a circulação de água no interior das valvas e a taxa de filtração de água desses organismos (Riisgard et al., 2013), fazendo com que as trocas entre o fluído do manto e o meio externo sejam menores. Esta menor circulação de água no interior das valvas do organismo pode facilitar o processo de regulação iônica, devido ao menor fluxo de meio hiposmótico para o interior da concha, porém este menor fluxo leva a um menor aporte de oxigênio para as trocas gasosas, e, desta forma, uma consequente redução no consumo de oxigênio pelos animais. De fato, foi relatada uma redução no consumo de oxigênio nos mexilhões dos "Molhes" durante o estresse hiposmótico, resultado similar ao encontrado por autores

utilizando bivalves de locais onde também ocorre esta flutuação no regime salino (Berger, 2005).

Apesar da redução no consumo de oxigênio dos organismo, como outras funções permanecem ativas, como por exemplo a indução da Na^+K^+-ATPase, é provável que estes organismos invistam durante algum tempo no metabolismo anaeróbico para manutenção da homeostasia. Moluscos bivalves são considerados anaeróbios facultativos, e neste sentido possuem diversas estratégias a nível molecular e metabólico para adaptação a baixas concentrações de oxigênio, como, por exemplo, vias de fermentação alternativas que resultam na formação de maiores quantidades de ATP (Hochachka e Somero, 2002). Para confirmar esta hipótese, como perspectivas, pretendemos efetuar outros testes entre estes grupos de animais, tais como medidas na taxa de filtração e produtos do metabolismo anaeróbico destes animais.

Com relação a estas diferentes respostas entre os animais coletados em diferentes pontos, sugere-se que flutuações de curto e longo prazo na salinidade levam a mecanismos adaptativos comportamentais e fisiológicos diferentes. Organismos estuarinos possuem uma maior tolerância a variações na salinidade (Shumway, 1977) devido à adaptação a estas flutuações ter permitido o desenvolvimento de estratégias fisiológicas para administrar a salinidade reduzida (Normant et al., 2005). Os animais dos “Molhes” podem apresentar estas adaptações, devido ao ambiente estuarino, e quanto à resposta bifásica apresentada, também podemos relacionar a diferenças nos mecanismos adaptativos dependentes da duração do estresse hiposmótico. Aparentemente, se o regime de salinidade é alterado por um curto prazo, os organismos mostram reduções na osmolalidade e concentração de íons similares as apresentadas pelos animais do “Farol”, porém se o estresse persiste, ocorre o investimento na regulação da concentração iônica.

As alterações relatadas no consumo de oxigênio dos animais pode resultar no aumento da produção de ERO nos organismos (Abele et al., 2011). É importante notar que o estresse hiposmótico, de maneira geral, leva a formação de ERO, seja pelo aumento da taxa respiratória, como relatado nos animais do "Farol", quando pelos possíveis ciclos de metabolismo anaeróbico e reoxigenação nos animais dos "Molhes". Se faz de extrema importância ressaltar que a alteração em um fator abiótico (salinidade) está alterando a formação de ERO e, neste sentido, estas alterações podem levar a mudanças diretas ou indiretas nas atividades de enzimas antioxidantes classicamente utilizadas como biomarcadores em programas de monitoramento ambiental. Também, da mesma forma que os resultados relatados para os processos osmorregulatórios, os grupos de mexilhões podem responder diferentemente a situações de estresse hiposmótico, baseado no seu histórico ecológico de pré-exposição a este fator abiótico no ambiente e suas adaptações.

De modo geral, todos os biomarcadores analisados foram alterados com o estresse hiposmótico. As atividades das enzimas antioxidantes superóxido dismutase (SOD), catalase (CAT) e glutationa *S*-transferase (GST) foram alteradas em ambos grupos, de forma dependente ao tempo de amostragem e tecido analisado e estes resultados são similares aos encontrados na literatura. Apesar dos resultados para as enzimas SOD e CAT possuírem uma maior variação, tanto sofrendo indução quanto redução quando expostos ao estresse hiposmótico (An e Choi, 2010; Carregosa et al., 2014; Lau et al., 2004; Verlecar et al., 2008), os resultados para a enzima GST revelaram uma indução na atividade enzimática em resposta ao estresse hiposmótico, similar ao encontrado por outros autores (Bebianno et al., 2007; Damiens et al., 2007; Hoarau et al., 2006; Verlecar et al., 2008). Este aumento na atividade da GST está provavelmente relacionado ao seu papel na detoxificação e conjugação de biomoléculas

danificadas, as quais podem resultar do aumento de ERO relatado nos nossos resultados.

Com relação às alterações nas atividades destas enzimas causadas pelo estresse hiposmótico, é importante enfatizar que as alterações nestes biomarcadores podem ocorrer devido a variações em fatores abióticos naturais, e não somente pela exposição a poluentes. Neste sentido, em um estudo de monitoramento no estuário do rio Minho, seguindo a WFD (do inglês Water Framework Directive) foi analisada a variação de biomarcadores com relação a diversos parâmetros, e através de uma análise comparativa relacionando pontos de coleta, biomarcadores, parâmetros abióticos e poluentes, foi encontrada uma relação do gradiente de salinidade com resposta de biomarcadores (Capela et al, 2016).

Os biomarcadores de estresse oxidativo utilizados são avaliados em diversos estudos (Capela et al., 2016; Capó et al, 2015) e, embora sejam vantajosos para acessar múltiplos estressores, podem ser marcadamente afetados por diversos fatores abióticos (Livingstone, 2003), como aqui relatado com relação a alterações na salinidade. Assim, estas alterações podem mascarar ou superestimar os efeitos de poluentes, sendo essencial a avaliação da influência dos fatores abióticos nos biomarcadores, validando a sua utilização em biomonitoramento.

Além da variação direta pela influência do estresse hiposmótico, se faz necessário destacar que biomarcadores podem ter variabilidade na sua sensibilidade, indução e recuperação, e esta variabilidade pode ocorrer entre tecidos e até nos níveis de população (Wu et al., 2005), como relatado em nossos resultados. Os dois grupos utilizados no presente estudo, “Farol” e “Molhes” tiveram a atividade destes biomarcadores alterada pela salinidade, como mencionado, e estas alterações foram diferentes entre os grupos e parecem depender do histórico ecológico enfrentado pelos

organismos. Como descrito acima, grupos de diferentes locais respondem distintamente aos estresse hiposmótico com relação aos processos fisiológicos de osmorregulação e respiração, e estas diferenças parecem afetar também as respostas nas atividades enzimáticas. Estas diferenças nos biomarcadores se deram principalmente quanto ao tempo decorrido para o início da resposta, ou seja, por diferenças no tempo de indução entre os grupos.

Com relação a estas diferenças, diferentes grupos de animais podem apresentar diferentes limiares de indução dos biomarcadores analisados. Esta diferença deve-se, provavelmente, a ação distinta de fatores abióticos e bióticos em seus habitats, ou seja, histórico ambiental e as adaptações fisiológicas apresentadas por estes organismos. Além de limiares de indução distintos, podem ocorrer diferentes tempos de recuperação (retorno aos valores basais após a exposição), e isso pode mascarar de forma distinta o efeito dos contaminantes, podendo sub ou superestimar seus efeitos.

Estas alterações nos biomarcadores, devido às diferenças nas respostas fisiológicas dos animais, por fim, podem alterar índices de qualidade ambiental, utilizados em programas de monitoramento para acessar a saúde do ambiente através de uma abordagem integrativa de biomarcadores. No presente estudo, foi relatada a influência do estresse hiposmótico e do histórico ambiental no IBR (do inglês Integrated Biological Response) (Beliaeff e Burgeot, 2002), e neste sentido, já foi relatada uma variação sazonal no IBR em mexilhões (Bodin et al., 2004; Broeg e Lehtonen, 2006). Neste sentido, para a utilização destes índices em uma abordagem de avaliação da saúde ambiental, deve ser bem entendido quais fatores afetam os componentes usados no cálculo do índice para que os efeitos causados por fatores abióticos não sejam confundidos com os efeitos de poluentes. Este resultado reforça a importância de validação dos biomarcadores frente a variações em fatores abióticos e, mais do que isto,

a diferença nas respostas entre os animais de distintos pontos de coleta deve também ser considerada, assim reduzindo a probabilidade de interpretações errôneas mesmo em analises integradas de biomarcadores.

CONCLUSÃO

A variabilidade natural das respostas dos organismos pode dever-se a diferentes mecanismos fisiológicos responsáveis pela adaptabilidade a diferentes habitats, como consequência da influência diferencial dos parâmetros abióticos e bióticos presentes no ambiente. Neste sentido, os grupos de animais com diferentes históricos ecológicos, “Farol” e “Molhes”, respondem de forma contrastante em processos fisiológicos essenciais a sua sobrevivência (taxas respiratórias e mecanismos de osmorregulação) e estas respostas distintas podem influenciar e estender-se a medidas utilizadas como biomarcadores em programas de monitoramento ambiental (Figura 4).

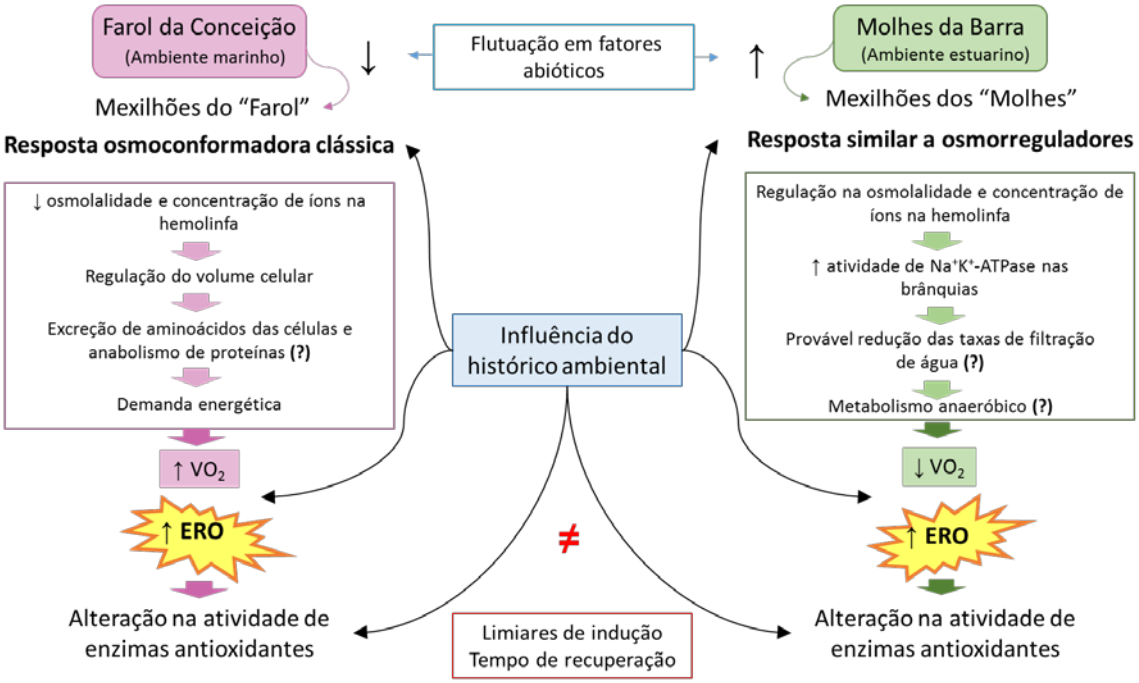

Figura 4. Resumo dos principais resultados encontrados na presente tese, relacionando com os possíveis mecanismos atuantes. VO_2 – consumo de oxigênio; ERO – Espécies reativas de oxigênio; (?) – hipóteses inferidas, não analisadas na tese.

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