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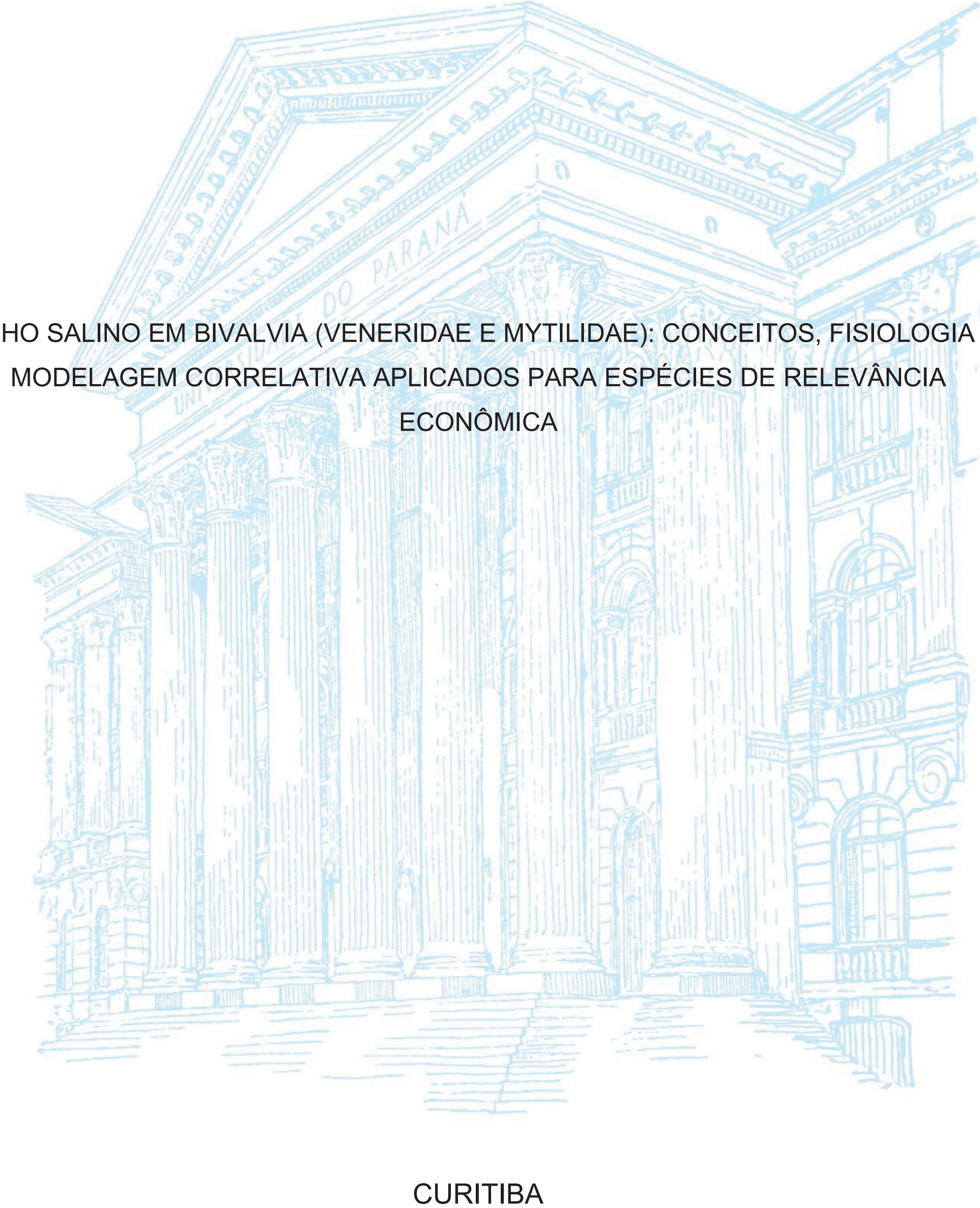
NICOLE STAKOWIAN



NICHO SALINO EM BIVALVIA (VENERIDAE E MYTILIDAE): CONCEITOS, FISIOLOGIA E MODELAGEM CORRELATIVA APLICADOS PARA ESPÉCIES DE RELEVÂNCIA ECONÔMICA

CURITIBA
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Tese apresentada como requisito parcial à obtenção do grau de Doutora em Ciências Biológicas - Zoologia, no Curso de Pós- graduação em Zoologia, Setor de Ciências Biológicas, da Universidade Federal do Paraná.

Orientadora: Carolina Arruda de Oliveira Freire

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TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação ZOOLOGIA da Universidade Federal do Paraná foram convocados para realizar a arguição da tese de Doutorado de **NICOLE STAKOWIAN**, intitulada: **NICHO SALINO EM BIVALVIA (VENERIDAE E MYTILIDAE): CONCEITOS, FISIOLOGIA E MODELAGEM CORRELATIVA APLICADOS PARA ESPÉCIES DE RELEVÂNCIA ECONÔMICA"**, sob orientação da Profa. Dra. CAROLINA ARRUDA DE OLIVEIRA FREIRE, que após terem inquirido a aluna e realizada a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.
A outorga do título de doutora está sujeita à homologação pelo colegiado, ao atendimento de todas as indicações e correções solicitadas pela banca e ao pleno atendimento das demandas regimentais do Programa de Pós-Graduação.

CURITIBA, 28 de Fevereiro de 2025.

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À Vontade do Fogo,
energia de vida que arde na alma daquelas que ousam ir além,
transformar e seguir em frente

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Um doutorado é a realização e um projeto longo e feito a muitas mãos, não caberia citar todos que de alguma forma estão presentes nesse processo. Gratidão!

"O ponto está riscado: há que se ler o encanto para se entender a ciência"
Luiz Antônio Simas e Luiz Rufino (Fogo no Mato, 2018)

RESUMO

Esta Tese de Doutorado apresenta investigações sobre respostas comportamentais, fisiológicas e ecológicas de espécies de bivalves bentônicos epifaunais (*Mytilus galloprovincialis*) e infaunais (*Anomalocardia flexuosa*) frente à variação de salinidade, considerando seus habitats naturais e cenários de mudanças climáticas. No **Capítulo 1**, o nicho salino de *A. flexuosa* foi delimitado experimentalmente: salinidades entre 22 e 34 permitiram a manutenção da osmorregulação e do comportamento de escavação, enquanto valores inferiores a 22 resultaram em redução da atividade da LDH e da abertura das valvas, sugerindo supressão metabólica. A modelagem de nicho ecológico indicou uma tendência de aumento da adequabilidade de hábitat no Norte e diminuição no Sul e Sudeste do Brasil até o final do século XXI, devido à intensificação das chuvas e consequente redução de salinidade em estuários. No **Capítulo 2**, foi projetada a distribuição potencial de *M. galloprovincialis* no Oceano Atlântico utilizando dados ambientais (temperatura, pH e salinidade), com destaque para a perda de hábitats adequados no Atlântico Norte sob cenários futuros e possível diminuição do potencial invasor em regiões tropicais. No **Capítulo 3**, foram avaliadas as respostas teciduais de *M. galloprovincialis* a diferentes salinidades (20 a 40). As brânquias e a glândula digestiva mostraram-se os tecidos mais sensíveis, com destaque para a atividade de SDH, SOD e CBEs como principais biomarcadores. No conjunto, esta tese contribui para a compreensão das estratégias adaptativas de bivalves a variações de salinidade, integrando experimentos fisiológicos e modelagem de nicho correlativa. Os resultados reforçam o valor preditivo de abordagens integradas para a conservação de espécies costeiras frente às mudanças climáticas, evidenciando a importância de dados específicos de ambientes entre-marés para a calibração de modelos ecológicos robustos.

Palavras-chave: ecofisiologia, bivalves, salinidade, estuário, mudanças climáticas.

ABSTRACT

This PhD Thesis presents investigations on behavioral, physiological and ecological responses of epifaunal (*Mytilus galloprovincialis*) and infaunal (*Anomalocardia flexuosa*) benthic bivalve species to salinity variations, considering their natural habitats and climate change scenarios. In **Chapter 1**, the saline niche of *A. flexuosa* was experimentally delimited: salinities between 22 and 34 allowed the maintenance of osmoregulation and burrowing behavior, while values below 22 resulted in reduced LDH activity and valve opening, suggesting metabolic suppression. Ecological niche modeling indicated a trend of increasing habitat suitability in the North and decreasing in the South and Southeast of Brazil until the end of the 21st century, due to the intensification of rainfall and consequent reduction of salinity in estuaries. In **Chapter 2**, the potential distribution of *M. galloprovincialis* in the Atlantic Ocean was projected using environmental data (temperature, pH and salinity), highlighting the loss of suitable habitats in the North Atlantic under future scenarios and possible decline in invasiveness in tropical regions. In **Chapter 3**, the tissue responses of *M. galloprovincialis* to different salinities (20 to 40) were evaluated. The gills and digestive gland were the most sensitive tissues, with emphasis on the activity of SDH, SOD and CBEs as the main biomarkers. Overall, this thesis contributes to the understanding of the adaptive strategies of bivalves to salinity variations, integrating physiological experiments and correlative niche modeling. The results reinforce the predictive value of integrated approaches for the conservation of coastal species in the face of climate change, highlighting the importance of specific data from intertidal environments for the calibration of robust ecological models.

Keywords: ecophysiology, bivalves, salinity, estuary, climate change.

SUMÁRIO

1 INTRODUÇÃO GERAL

Nas últimas décadas, as mudanças climáticas têm se intensificado, com impactos diretos sobre ecossistemas costeiros e os organismos que os habitam (He & Silliman, 2019). Entre os efeitos mais críticos estão o aumento da frequência de eventos extremos — como chuvas intensas e períodos de estiagem —, além da elevação do nível do mar e alterações no regime de salinidade em estuários e regiões entre-marés (Rato et al., 2022). Essas flutuações impõem desafios significativos à fauna bentônica, especialmente aos bivalves, cuja fisiologia está estreitamente ligada ao ambiente aquático e à osmorregulação (Vázquez et al., 2021; Rato et al., 2022).

3.1 Bivalves como modelos no estudo de ecofisiologia

Os bivalves constituem a segunda maior classe do filo Mollusca, com representantes em ambientes marinhos, estuarinos e de água doce (Han et al., 2024; Thorp e Rogers, 2011). Esses organismos são frequentemente descritos como "engenheiros ecossistêmicos" devido ao seu impacto nos ambientes dos ecossistemas em que habitam, como por exemplo filtrando a água, processando nutrientes no sedimento (Ysebaert et al., 2018). Seus representantes podem apresentar modo de vida natante, mas em sua maioria são espécies bentônicas epifaunais ou infaunais (escavadores e perfurantes) tanto de substratos consolidados (rochas, madeira, embarcações e píeres) como inconsolidados (areia, lodo, argila) (Dame, 1993; Hendricks et al., 2015; Ysebaert et al., 2018; Fig. 1). O hábito filtrador os torna filtros naturais, enquanto densos bancos de mexilhões criam micro-hábitats para diversas espécies (Lassen et al., 2006). Além disso, os bivalves infaunais oxigenam o sedimento e promovem a ciclagem de nutrientes por meio da escavação (Wiesebron et al., 2021). Por isso, a presença e saúde de suas populações são amplamente utilizadas como bioindicadores da qualidade ambiental (Yap et al., 2021; Martins et al., 2024).

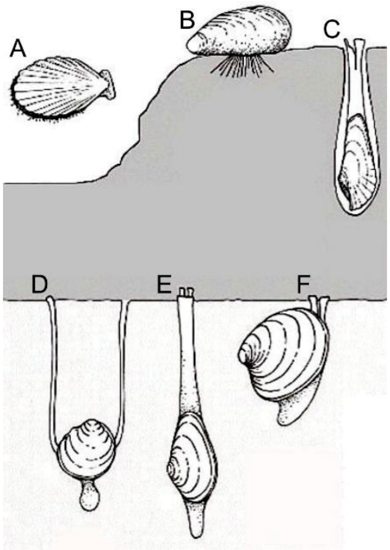

Figura 1. Ilustração de diferentes modos de vida de bivalves: (A) espécies nadadoras, (B) espécies bentônicas epifaunais, (C) espécies bentônicas infaunais de substrato consolidado e (D–F) espécies bentônicas infaunais de substrato inconsolidado. Adaptado de Hendricks et al. (2015).

Mariscos, como são conhecidos os bivalves de forma geral, são fonte de alimento para populações humanas há milhares de anos, como evidenciado pelos sambaquis – depósitos arqueológicos formados por conchas que testemunham a exploração dessas espécies em tempos antigos (Parellada & Gottardi Neto, 1993; Wagner et al., 2011). Como recurso pesqueiro, várias espécies de bivalves sustentam pescarias comerciais e de subsistência em diversas regiões ao redor do mundo (Ferreira et al., 2014; Wijsman et al., 2018). Além de fornecerem proteína de alta qualidade a preços acessíveis, a extração e o cultivo de bivalves geram empregos e fomentam economias locais em áreas costeiras (Oliveira et al., 2013; Ferreira et al., 2014; Willer & Aldridge, 2020). Além da carne, suas conchas também têm múltiplos usos, como em artesanato, construção e rituais religiosos (Ktalav, 2015; Summa et al., 2022; Caroscio et al., 2024). Em muitos países, a aquicultura de bivalves representa uma indústria em crescimento, com impacto ambiental menor em comparação a outras formas de produção de proteína animal (Gawel et al., 2023). A segurança alimentar futura, um dos Objetivos de Desenvolvimento Sustentável (https://brasil.un.org/pt-br/sdgs), está diretamente ligada à conservação e manejo sustentável das populações de bivalves, que há milênios são um recurso vital para as comunidades costeiras (Cox et al., 2024).

No campo de estudo da fisiologia animal, o grupo chama atenção por habitar ambientes altamente variáveis e inóspitos para muitos animais, como os costões rochosos e estuários, sob forte influência das marés (Kon et al., 2020), apesar de seu modo de vida filtrador, que expõe seus tecidos a contaminantes presentes na água, e de sua natureza predominantemente séssil, que limita sua capacidade de migração em resposta a condições ambientais desfavoráveis. Para prosperar nesses ambientes, esses organismos desenvolveram estratégias comportamentais e fisiológicas que permitem manter a homeostase diante de condições adversas. O fechamento das valvas e a escavação no sedimento são respostas rápidas e de baixo custo energético, geralmente acionadas em fases iniciais do estresse, enquanto mecanismos bioquímicos e celulares são ativados em situações mais prolongadas e intensas (Lent, 1969; Deaton, 2009; Pourmozaffar et al., 2020; Leeuwis e Gamperl, 2022).

Para o compreender os efeitos da salinidade na fisiologia dos bivalves, podem ser utilizados biomarcadores bioquímicos (Carregosa et al., 2014; Gonçalves et al., 2017; Cunha et al., 2024). Esses biomarcadores permitem avaliar com precisão como diferentes órgãos — como brânquias, glândula digestiva, músculo e manto — respondem a desafios osmóticos, revelando adaptações fisiológicas que favorecem a tolerância à variação de salinidade. Compreender a fisiologia desses animais é fundamental não apenas para entender os mecanismos que garantem sua sobrevivência em ambientes extremos como estuários e regiões entre-marés, mas também fornece subsídios para o manejo sustentável de populações naturais, permitindo prever o impacto de fatores de estresse, como as mudanças climáticas.

Nesta tese, para quantificar o metabolismo energético, foram avaliadas as atividades das enzimas succinato desidrogenase (SDH, indicadora do metabolismo aeróbico), lactato desidrogenase (LDH, indicadora de metabolismo anaeróbico). Foram também utilizadas quantificações de glicogênio e proteína, para representar as reservas energéticas. Para quantificar a atividade do sistema antioxidante, foram avaliadas as enzimas: superóxido dismutase (SOD) e glutationa peroxidase (GPx), que protegem as células contra espécies reativas de oxigênio (ROS); glutationa redutase (GR) e glutationa-S-transferases (GSTs), que estão envolvidas na regeneração de glutationa e na detoxificação celular; e a capacidade antioxidante total (TAC), que reflete a capacidade geral do tecido de neutralizar as ROS. Para quantificar possíveis danos celulares e neurotoxicidade, foram avaliadas as enzimas carboxilesterases (CbEs, indicadoras de alterações nas membranas celulares e exposição a estressores ambientais) e acetilcolinesterase (AChE, associada à integridade do sistema nervoso e sensível a neurotoxinas), e o nível de peroxidação lipídica (LPO, um indicador clássico de dano oxidativo às membranas celulares).

Além das respostas bioquímicas, a capacidade de manter o equilíbrio interno frente a estressores ambientais pode ser compreendida a partir de dois conceitos fisiológicos: homeostase e alostase (Schulte, 2014; Freire et al., 2020). Homeostase é a manutenção da estabilidade interna por meio de mecanismos compensatórios. É a condição na qual os organismos operam com gasto energético mínimo, favorecendo o crescimento e a reprodução. Alostase, por outro lado, refere-se à estabilidade através da mudança: são os ajustes fisiológicos que o organismo realiza para manter sua função vital frente a condições extremas ou prolongadas. Em bivalves, a ativação do metabolismo anaeróbico, o aumento da atividade antioxidante e a mobilização de reservas energéticas são exemplos de estados alostáticos. Compreender como os bivalves podem alternar entre esses estados nos ajuda a avaliar a real vulnerabilidade dessas populações às mudanças climáticas globais.

3.2 Espécies utilizadas

Dada a diversidade de modos de vida dos representantes desse grupo, para esse projeto foram selecionadas espécies das famílias Veneridae e Mytilidae (*Anomalocardia flexuosa* e *Mytilus galloprovincialis*, respectivamente) que ocorrem em regiões entre-marés de estuários, apresentam hábito epifaunal e infaunal, e que são utilizadas como recurso pesqueiro.

A família Veneridae é uma das mais diversas dentro de Bivalvia, com espécies amplamente distribuídas em ambientes marinhos e estuarinos (Chen et al., 2011). Seus representantes são geralmente infaunais, vivendo enterrados em sedimentos inconsolidados com granulometria que varia entre areia e argila, onde utilizam sifões para filtrar a água da superfície. A espécie *Anomalocardia flexuosa* (Fig. 2) é uma das mais conhecidas desse grupos, conhecida também como berbigão, vôngole ou papa-fumo, dependendo da região (Teixeira e Campos, 2019). Essa é uma espécie infaunal que se enterra cerca de 2cm em substratos que variam entre areia e silte e se distribui desde as índias Ocidentais até o Uruguai, sendo um dos principais recursos extraídos para venda e consumo humano ao longo de toda a sua distribuição (Silva-Cavalcanti *et al.*, 2018). Apesar da escassez dos dados relacionados à pesca, no ano de 2018 foi registrada a captura de 292,92t da espécie apenas no litoral paranaense, advinda principalmente do extrativismo concentrado no município de Paranaguá (FUNDEPAG, 2019).

Figura 2. *Anomalocardia flexuosa* (Linnaeus, 1767), maior diâmetro da concha = 34mm. Foto: Malacopic (www.malacopics.nl).

Além de sua importância econômica em pescarias artesanais, essa espécie se destaca por sua capacidade de tolerar variações significativas de salinidade e temperatura, características que a tornam particularmente adaptada a regiões estuarinas e de entre-marés (Boehs et al., 2008; Maia et al., 2017). No entanto, mortalidades em massa vêm sendo registradas, sugerindo que as populações naturais podem estar em ameaça ou próximas do seu limite de tolerância, evidenciado especialmente sob as mudanças climáticas ambientais, que intensificam o estresse ambiental de ambientes naturalmente variáveis e dinâmicos (Oliveira et al., 2013; Mottola et al., 2020; Lima et al., 2022).

Os mexilhões da família Mytilidae são conhecidos por sua capacidade de colonizar uma ampla gama de substratos duros, frequentemente formando densos bancos em substratos naturais (rochosos) e artificiais (Breeding and Seed 1969; Carranza et al., 2009). Em suas distribuições nativas, os mitilídeos geralmente são utilizados como fonte de alimento e recurso pesqueiro que movimenta economias locais (Lopez et al., 2022). No Brasil, há o exemplo do mitilídeo *Perna perna* no Estado de Santa Catarina, que veio a ser o principal produtor de mexilhão do país (Marenzi & Branco, 2005; Resgalla et al., 2007; INFOAGRO, 2022). Por outro lado, fora de sua distribuição nativa, os representantes desse grupo podem se tornar uma ameaça para espécies nativas, comprometendo a biodiversidade e a saúde do ecossistema (Garci et al., 2007; Lim et al., 2018; Gracia & Rangel-Buitrago, 2020; Han & Dong 2020).

O mexilhão azul, *Mytilus galloprovincialis* (Fig. 3), é um exemplo de espécie que é considerada recurso pesqueiro no Atlântico leste (Europa e Mediterrâneo), mas invasor recém-chegado na costa brasileira (Santa Catarina). Essa espécie é amplamente consumida na Europa devido ao sabor, potencial nutritivo e elevada taxa de crescimento em ambientes costeiros de entre-marés (Lopez et al., 2022), mas invasora em várias regiões do mundo (África do Sul, costa leste dos Estados Unidos, e América Latina, incluindo o Oceano Pacífico) onde compete com espécies nativas por espaço e recursos. Se por um lado essa espécie é foco de estudos para garantir sua conservação e produção onde ela é nativa, há o interesse de saber o potencial invasor e quais são as áreas mais prováveis que essa espécie ocupe em outros continentes, sob o risco de ameaçar as espécies nativas (Bownes and McQuaid, 2006; Lowe et al., 2000; Lins et al., 2021).

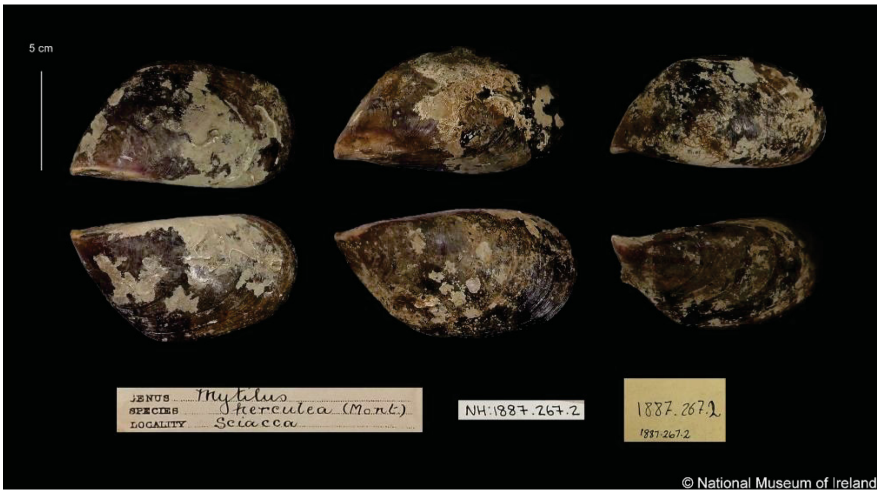

Figura 3. *Mytilus galloprovincialis* (Lamarck, 1819). Foto: Mollusca Types in Britain & Ireland (https://gbmolluscatypes.ac.uk/specimens/8745).

3.3 Ameaças às populações naturais

Atualmente, as populações naturais de bivalves enfrentam ameaças estreitamente relacionadas às atividades humanas (Apte et al., 2019; Tan et al., 2020; Tan et al., 2023). A poluição dos ecossistemas costeiros, por meio de metais pesados, esgoto e resíduos industriais, afeta esses organismos principalmente através da filtração, enquanto a urbanização crescente e as práticas agrícolas reduzem as áreas adequadas para o assentamento e desenvolvimento dos bancos naturais (Thushari et al., 2017; Puccinelli et al., 2022; Momota & Hosokawa, 2021). Além disso, as mudanças climáticas globais, refletidas na intensificação de eventos climáticos extremos, como aumento na precipitação, enchentes e secas, alteram drasticamente as condições ambientais às quais os bivalves estão adaptados, especialmente a salinidade (Hu, 2021; Yuan et al., 2023).

Diante dessas ameaças, temos pouca ou nenhuma previsibilidade sobre o futuro das populações naturais de bivalves e uma abordagem eficiente para avaliar a adequabilidade de hábitat sob cenários de mudança climática é a modelagem de nicho ecológico (Coro et al., 2015; Tytar et al., 2015; Martínez-López et al., 2021). O conceito de nicho, conforme definido por Hutchinson (1957), considera o conjunto de variáveis abióticas que permitem a sobrevivência indefinida de uma espécie. Feng and Papes (2017) expandem esse conceito ao qualificar o tipo de presença: se a espécie (1) apenas sobrevive, (2) sobrevive e cresce, ou (3) sobrevive, cresce e se reproduz.

3.4 O conceito de Nicho salino

Nesse contexto, Zimmer et al. (2021) introduziram o conceito de "nicho químico fundamental", definido como a faixa tolerável de condições químicas para uma espécie, e o "nicho químico realizado", que abrange as condições químicas sob as quais a espécie ocorre na natureza. As respostas fisiológicas às variações de pH e salinidade refletem a função íon-regulatória do organismo, tornando essas variáveis adequadas para estimar o nicho químico. Para que os dados de fisiologia osmorregulatória possam ser usados como ferramentas de manejo e conservação, os autores propõem uma estrutura metodológica que inclui: (1) o estabelecimento do nicho químico espécie-específico como ferramenta preditiva e (2) a identificação de características íon-regulatórias que permitam prever a resposta das espécies às mudanças nas condições químicas.

Para animais osmoconformadores, como os bivalves, que ajustam sua composição interna de solutos conforme a salinidade do ambiente, a salinidade é uma das principais variáveis que determinam a presença ou ausência da espécie (Mcleod & Wing, 2008; Carregosa et al., 2014; Pourmozaffar et al., 2020). Por isso, o conceito de “nicho salino” permitiria Identificar a amplitude de salinidade na qual a espécie mantém a homeostase sem mobilizar recursos energéticos para proteção e reparo celular favorece o crescimento e a reprodução (Sokolova et al., 2012), aproximando-se do que seria o nicho salino fundamental ou a zona de conforto salino (Freire, 2025).

Delimitar o nicho salino fundamental de osmoconformadores pode ser desafiador, já que os limites não são bem definidos e estão intimamente relacionados com o tempo de exposição (Freire, 2025). É necessário compreender os mecanismos pelos quais os bivalves enfrentam as flutuações de salinidade habitualmente para então poder identificar quando há um distúrbio da homeostase. Para bivalves em geral, a primeira resposta ao estresse é o comportamento de escape, uma solução rápida e de baixo custo energético, sendo suficiente para lidar com estresses de curta duração, como os regimes de marés (Woodin et al., 2020; Andrade et al., 2018; Haider et al., 2018). No entanto, sob estresses prolongados (com duração de dias), outros mecanismos fisiológicos são acionados, como a troca para metabolismo anaeróbico, a redução da taxa metabólica e a regulação do volume celular (Zwaan and Wijsman, 1976; Vooys, 1979; Stakowian and Freire, 2024; Freire, 2025). Além disso, enzimas antioxidantes e canais de transporte de membrana são ativados para lidar com as espécies reativas de oxigênio (ROS) e transportar íons orgânicos e inorgânicos envolvidos na regulação do volume celular (Deaton, 2009; Pourmozaffar et al., 2020).

3.5 Perguntas e objetivos

Com base no conceito de nicho de Hutchinson (1957) e nos estudos de Feng and Papes (2017), Zimmer et al. (2021), e no comentário de Freire (2025), a proposta deste estudo é desenvolver o conceito de nicho salino fundamental e realizado, e identificar os processos biológicos que contribuem para a performance osmorregulatória dos bivalves, integrando esses conhecimentos com a modelagem de nicho ecológico para prever a vulnerabilidade das populações naturais e o potencial invasor de espécies exóticas. Essa abordagem integrada de fisiologia e modelagem de nicho pode fornecer, pela primeira vez, uma previsibilidade sobre o futuro das populações naturais de bivalves, seja apontando vulnerabilidades ou demonstrando o potencial invasor de espécies que possam ameaçar a biodiversidade nativa.

Nesse sentido, o primeiro capítulo investiga uma população de *A. flexuosa* do Complexo Estuarino de Paranaguá, exposta a diferentes salinidades por 24 horas, avaliando comportamentos como a abertura das valvas e a escavação, bem como respostas fisiológicas (osmolalidade, atividade enzimática e regulação do volume celular). O segundo capítulo avalia a disponibilidade de hábitat adequado para o mexilhão azul *M. galloprovincialis* no clima presente e no clima previsto para o final deste século ao longo do Atlântico, considerando populações nativas e invasoras, por meio de modelagem de nicho correlativo. Por fim, o terceiro capítulo investiga as respostas fisiológicas de diferentes tecidos (brânquias, glândula digestiva, músculo e manto) de *M. galloprovincialis* à diferentes salinidades (20, 25, 30, 35 e 40) com o objetivo de identificar biomarcadores e tecidos que melhor refletem os desafios à homeostase causados por diferentes salinidades.

2 CAPÍTULO 1

Evidence of *Anomalocardia flexuosa* sensitivity to hyposaline stress through experiments, saline niche characterization and correlative niche modeling

Nicole Stakowian[1], Carolina A. Freire[2]

[1]Departament of Zoology, Federal University of Paraná, Centro Politécnico, Curitiba, PR CEP 81531-980, Brazil.
[2]Department of Physiology, Federal University of Paraná (UFPR), Centro Politécnico, Curitiba, PR CEP 81531-980, Brazil.

Correspondence: stakowian.zoo@gmail.com

2.1 ABSTRACT

The niche modelling approach has become increasingly relevant for estimating how vulnerable species and populations can be to environmental change, particularly when considering native species that are used as fishing resources by human populations, such as *Anomalocardia flexuosa*. The purpose of this study is to delimit the fundamental (laboratory experiments) and realized (occurrence points) saline niche, which is the salinity range in which the species maintains homeostasis without mobilizing energy resources for cellular protection and repair, and use ecological niche modeling to project the suitable area for this species in the climate projected for the end of this century. For this, individuals were exposed individually to one experimental salinity in 500mL recipients. To identify the limits of the saline niche of the population, the salinities used were: 13, 16, 19, 22 (hypoosmotic), 25, 28 (control), and 31, 34, 37 (hyperosmotic), with independent specimens exposed to the different salinities, until a N of X was reached. In the first 4 hours of exposure, behavioral data were obtained (valve opening and excavation). After 24 hours, the behaviors were again recorded, and samples were obtained: hemolymph (for osmolality evaluation), a foot section (to estimate water content), and adductor muscle (for LDH and protein assessment). Our results suggest in the salinities between 22 and 34 clams are able to burrow completely, while maintaining their metabolism, osmotic balance, and tissue hydration. At lower salinities (<22), the bivalves failed to open their valves and burrow completely, despite being able to maintain tissue hydration through valve closure. Projections of Maxent algorithm for the climate of the end of this century indicate a significant loss of habitat suitability for populations in the southeast and south of Brazil, as these regions are already close to the salinity tolerance limits of *A. flexuosa*. The findings of this study highlight the interplay between behavioral and physiological strategies employed by infaunal bivalves to tolerate dynamic salinity conditions in estuarine habitats. For the first time the concept of saline niche was used for bivalves, globally, and highlights the importance of salinity in the distribution of this group, and of osmoconformers in general.

Key words: osmoregulation, clam, fundamental and realized niche, salinity, estuarine invertebrate.

2.2 INTRODUCTION

Bivalves are remarkably tolerant of dynamic environments, allowing them to inhabit estuarine and intertidal zones where salinity can fluctuate rapidly due to tidal cycles, freshwater input, and evaporation (Deaton, 2009; Pourmozaffar et al., 2020). To survive in such conditions, bivalves employ several behavioral and physiological strategies. Escape behavior, such as burrowing in the sediment, helps individuals avoid prolonged exposure to unfavorable conditions (Pourmozaffar et al., 2020; Haider et al., 2018; Woodin et al., 2020). Cell volume regulation, achieved by the balance of intra- and extracellular osmolytes, allows bivalves to maintain controlled tissue hydration and cellular function even under osmotic stress (Pierce, 1982; Deaton, 2009; Stakowian and Freire, 2024). Furthermore, bivalves can facultatively switch to anaerobic metabolism, increasing survival during periods of escape (closed valves) and burrowing in poorly oxygenated sediment (Vooys, 1979; Sokolova et al., 2019). These are strategies that have allowed bivalves to occupy the most diverse habitats throughout their evolutionary history (Bieler et al., 2013).

Anomalocardia flexuosa is an infaunal bivalve species inhabiting intertidal zones which habitually experiences salinity fluctuations, freshwater inflow (rain) and hypoxia. Existing research indicates that the species demonstrates a salinity tolerance (with larval survival and growth optimized) between salinities 25 and 30 (Lima et al., 2024). Salinity also influences its reproductive physiology, as germinal vesicle breakdown and post-fertilization events occur most effectively within a salinity range of 30-35 (Lopes et al., 2023). Furthermore, mass mortality events recorded for *A. flexuosa* in Brazilian coast have been strongly associated with episodes of decreased salinity following extreme rainfall (Oliveira et al., 2014; Vaquero et al., 2022). Adult individuals, typically measuring around 2cm and burrow only 2 to 4cm into poorly oxygenated substrate due to their short siphons (Silva-Cavalcanti et al., 2018; Lee, 1996). This species exhibits limited vertical and horizontal mobility, allowing it to reestablish in the sediment following disturbances (Netto & Lana, 1994). Populations of *A. flexuosa* tend to form aggregations in fine to medium sandbanks characterized by low-medium hydrodynamic energy (Boehs et al., 2008; El-Deir et al., 2009; Corte et al., 2017).

Native to the Brazilian coast, this clam has a longstanding association with human populations, with archaeological evidence found in shell mounds (sambaquis) (Wagner et al., 2011). Its shells are used in souvenirs, religious ornaments, and construction materials, though the primary use remains as a food resource, both for subsistence and commercial purposes (Tavares et al., 2021; Teixeira and Campos, 2019). Harvesting and consumption of *A. flexuosa* occur across the entire Brazilian coastline (Boehs et al., 2008;

Pezzuto et al., 2010; Teixeira and Campos, 2019; Mourão et al., 2021; Nascimento et al., 2022). In 2018, 292 tons of *A. flexuosa* were harvested only in Paranaguá, Paraná (Fundepag, 2019). In order to balance the exploitation of natural banks with the livelihoods of local communities, extractive reserves as Reserva Extrativista Marinha do Pirajubaé (RESEX) in Brazil have implemented management practices, including minimum size limits and rotational harvesting (Araujo, 2001; Pezzuto and Silva, 2015). However, in the last years have seen an increasing concern over mass mortality events in natural banks, likely related to periods of excessive rainfall (Oliveira et al., 2013; Mottola et al., 2020; Lima et al., 2022).

In addition to fishing and capture, global climate change is already exerting significant impacts on coastal ecosystems, and projections indicate that extreme weather events will become more frequent (Kundzewicz, 2016; Des et al., 2021; Houston, 2024). In Brazil, trends suggest increased droughts in the North and Northeast regions, alongside more intense rainfall in the Southeast and South, influenced by atmospheric circulation patterns affected by high- and low-pressure systems (Cunha et al., 2018; Escobar and Rosa, 2024). Ecological niche models, which correlate species occurrence with environmental conditions that support survival, reproduction, and persistence, are commonly used to assess species vulnerability to environmental changes (Peterson and Soberón, 2012; Zhao et al., 2022).

In general, these models are calibrated using occurrence points and climatic variables for both current and future conditions, aligned with IPCC scenarios. The 2–4.5 emission scenario represents a more optimistic outlook, while the 5–8.5 scenario is more pessimistic (IPCC, 2021; Asghar et al., 2019). Given recent climatic trends, the pessimistic scenario appears increasingly probable, with the optimistic pathway becoming less probable (Garnaut et al., 2008; IPCC, 2021). Although occurrence data provide a useful framework for identifying suitable environmental conditions, they do not fully capture the physiological capacities of species.

Incorporating physiological data into correlative niche models enhances the robustness of assessments regarding population vulnerability. The mechanistic model requires specific and predetermined data which are difficult to obtain (Feng and Papes, 2017; Sillero et al., 2021). For osmocomforming species like bivalves, that accommodate their internal composition of solutes according to the salinity of the environment, salinity is a key factor in determining habitat suitability. Understanding the mechanisms by which these organisms deal with salinity variations, such as escape strategies, metabolic adjustments and regulation of tissue hydration (Deaton, 2009; Medeiros et al., 2020; Pourmozaffar et al., 2020; Stakowian and Freire 2024), is fundamental to delineating the non-lethal limits of salinity tolerance. These limits allow the organism to invest energy in growing, reproducing and establishing themselves in new habitats (Sokolova et al., 2012; Feng and Papes, 2017): it has been here referred to as the "fundamental saline niche”.

The “realized saline niche,” by contrast, reflects the salinity range currently experienced by populations *in situ*. Comparing the fundamental and realized saline niches can provide insights into species vulnerability to climate change (Mirón-Gatón et al., 2022; Smith & Barton, 2024). A realized niche narrower than the fundamental niche suggests potential adaptive capacity and range expansion, while a realized niche that closely approaches the limits of the fundamental niche implies a greater risk of local population decline in response to environmental shifts (Zimmer et al., 2021).

Within this context, the present study aims to delineate the fundamental and realized saline niches of *A. flexuosa* by integrating physiological, behavioral, and ecological approaches. Controlled laboratory experiments were conducted to determine non-lethal salinity tolerance limits and behavioral responses to hypoosmotic and hyperosmotic conditions. By incorporating physiological data, occurrence records, and climate data, we characterized the realized saline niche and projected potential shifts in habitat suitability under future climate scenarios.

We hypothesize that (1) *A. flexuosa* employs behavioral and anaerobic mechanisms to tolerate low salinity conditions, (2) clams from median portion of estuary exhibit broader tolerance to salinity fluctuations due to greater freshwater influence, (3) due to mass mortality events, habitat suitability for *A. flexuosa* may change under future climate conditions, as populations are close to their tolerance limit. This study provides novel insights into the resilience and physiological plasticity of an ecologically and economically important bivalve, offering valuable data to support conservation and management strategies in the face of climate change.

2.3 MATERIAL AND METHODS

2.3.1 Field characterization and salinity

The Complexo Estuarino de Paranaguá (CEP), located in Paraná, southeastern Brazil, is a subtropical estuarine system comprising interconnected bays and diverse habitats, including tidal flats, sand banks, rocky shores, and mangroves (Mantovanelli, 1999; Lana et al., 2001). Its main water bodies are Baía de Paranaguá (East-West) and Baía das Laranjeiras (North-South) - strongly influenced by tidal circulation and freshwater discharge (Lana et al., 2001; Marone et al., 2005). The main connection with the Atlantic Ocean is through the channels that surround Ilha do Mel, which protect the CEP from high-energy waves (Lessa et al., 2018; Angulo, 1999). The salinity in the CEP exhibits a horizontal gradient from the inner part to the mouth of the estuary. In its middle portion, the salinity can vary from 22 to 28, while at the mouth of the estuary it varies between 29 and 30; throughout the water column, the salinity remains homogeneous, with minimal stratification (Falkenberg, 2009).

All clams for the saline niche experiment were captured in five collections in Baía de Paranaguá (median portion of the CEP, 25°47’S, 48°45’W) between August 2022 and August 2023. To assess the impact of burrowing on salinity fluctuations during rainy days, which affects the infaunal habit, salinity was measured at three levels at collection site: (1) in the water column, (2) in the water film above the sediment (primarily rainwater), and (3) in the interstitial water (of sediment).

2.3.2 Clam sample, acclimation and experiments

For five days the clams remained with water and sediment from the collection site (salinity 25), constant aeration and natural photoperiod. After acclimatization, for the saline niche experiment, each clam was individually placed in a 500mL container with sediment, positioning the umbo upwards. They were exposed to increasing or decreasing salinity levels across three steps, starting from the average salinity of 25, recorded at the collection site. The salinities tested were as follows: hypoosmotic range (13, 16, 19), control range (22, 25, 28 - dominant at the collection site), and hyperosmotic range (31, 34, 37).

2.3.3 Behavioral and physiological biomarkers for saline niche

For the first four hours of exposure, the behavior (burrowing and valve opening) was observed every 30 minutes. After 24 hours, all clams, regardless of whether they had burrowed or not, were repositioned in the sediment with the umbo upwards and observed for an additional four (4) hours, with behavior recorded every 30 minutes.

After 28 hours of exposure, hemolymph, foot, and adductor muscle samples were collected to assess osmolality, tissue hydration, lactate dehydrogenase (LDH) activity, and protein levels, respectively. Osmolality was measured using a Vapor Pressure Osmometer (Wescor® 5520 VAPRO, USA) with undiluted hemolymph samples. Foot tissue slices were weighed, dehydrated at 60°C for 72 hours, and reweighed to determine tissue water content (Stakowian & Freire, 2024). Adductor muscle samples were homogenized in phosphate buffer (0.1 M, pH 7.0) at a 1:5 ratio and centrifuged at 15,000g for 30 minutes at 4°C (Guiloski et al., 2015). The supernatant was collected for LDH activity and protein content. Commercial kits from Labtest® (Brazil) were used for LDH assays, with absorbances read at 340nm on a spectrophotometer (Amersham Pharmacia Biotech® - Ultrospec 2100 PRO, Sweden). The Bradford (1976) method was used to quantify protein levels, allowing enzyme activity to be expressed per mg of protein.

2.3.4 Realized saline niche

Occurrence points were sourced from GBIF (DOI: 10.15468/dl.w5tpwj) and filtered using the spThin package (Aiello-Lammens et al., 2015) to avoid clustering within a 10 km radius. Since niche assess cannot be performed only based on salinity, additional variables - air temperature (due to the species' shallow intertidal habitat) and chlorophyll concentration (which limits filter-feeding species) were obtained from Bio-Oracle (https://bio-oracle.org).

To evaluate the realized saline niche of *A. flexuosa* and identify the salinity range in which natural populations occur, the k-means algorithm divided the data (occurrence points and environmental data) into 'k' groups based on their characteristics. The algorithm was run 25 times with different initial centers to minimize within-cluster variance and maximize the variance between clusters. The Silhouette Method was used to determine the optimal number of clusters and maximize the average Silhouette Coefficient (Maechler et al., 2023).

2.3.5 Niche Modeling for the current and end-of-century climate

The modeling area was defined using the provinces of the North Brazil Shelf, Tropical Southwestern Atlantic, Warm Temperate Southwestern Atlantic, and Magellanic, as outlined by the Marine Ecoregions of the World (MEOW, Spalding et al., 2007), based on the known distribution of *A. flexuosa*. Salinity, air temperature and chlorophyll concentration for climate 2090s, under the SSP5-8.5 high-emission scenario (IPCC, 2021) were obtained also from Bio-Oracle.

Model calibration used both training and testing datasets (presence and background points), and the algorithms Bioclim and Maxent were applied, commonly used for habitat suitability and climate change projections (Booth, 2018; Wei et al., 2018). Model performance was assessed using the AUC (which measures the model's ability to distinguish between presence and absence) and TSS (the sum of sensitivity and specificity - 1) (McPherson et al., 2004; Allouche et al., 2006). AUC values for Bioclim and Maxent were 0.87 and 0.93, respectively, and TSS values were 0.75 and 0.83, respectively. Therefore, Maxent was chosen for habitat suitability projections for both the current climate and for the climate projected for the end of this century.

2.3.6 Statistical analysis

For the analysis of behavioral and physiological responses, salinities were grouped into visually similar groups (13-19, 22, 25-28, 31, 34-37), to increase the performance of statistical tests due to the limited sample size and very close salinity values. To compare hemolymph osmolality, foot water content and LDH activity across different salinity ranges (13-19, 22, 25-28, 31, 34-37), a one-way ANOVA on ranks with Dunn's post-hoc test was used. Chi-square contingency tables assessed whether burrowing behavior at 4 and 24 hours varied with salinity. All statistical analyses had their prerequisites met, assumed a significance level of 95% and were conducted using R, RStudio (R Core Team, 2024), packages: dismo (Hijmans et al., 2023), ggplot2 (Wickham, 2016), rgdal (Bivand et al., 2023), raster (Hijmans, 2023), sf (Pebesma and Bivand, 2023), sp (Bivand et al., 2013), tidyverse (Wickham et al., 2019) and terra (Hijmans, 2024). Figures were formatted using InkScape (Harrington et al., 2004-2005).

2.4 RESULTS

The salinity of the water column, the water film above the sediment (primarily from rainwater), and the interstitial water (of sediment) were recorded as 22, 19, and 26, respectively, for all sampled points. A total of 102 adult clams (average shell length = 2.6 ± 0.3 cm, average total weight = 8.5 ± 2.7mg) were used in the experiment of saline niche (19-21 clams/salinity group). No mortality was recorded throughout the study.

2.4.1 Behavioral responses

Burrowing activity varied with salinity after 24 hours (chi-squared = 14.029, df = 4, p = 0.0072), but no difference was observed after 4 hours of exposure (chi-squared = 3.77, df = 4, p = 0.4373). During the first 4 hours, most clams did not burrow (>50%), with the highest percentage of burrowed clams (38%) occurring at salinities of 25-31, followed by salinities 22 and 34-37. At 13-19, fewer than 20% of clams burrowed (Fig. 1).

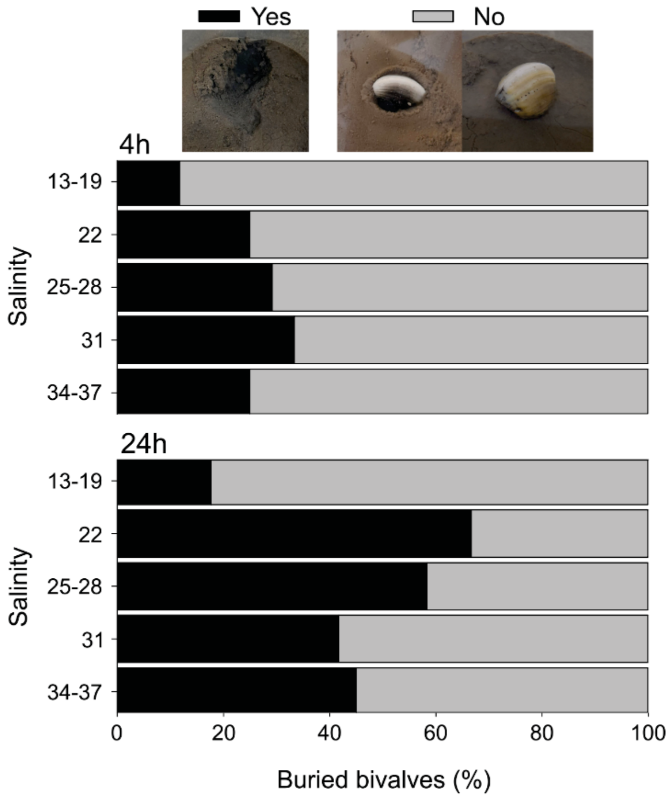

Figure 1. Burrowing ability evaluated in the laboratory. Histograms of frequency of clams *Anomalocardia flexuosa* that burrowed and those that did not burrow after 4 and 24 hours of exposure across different salinity ranges. Photos above illustrate examples of a burrowed clam and a non-burrowed clam.

Valve activity (open or closed) also varied with salinity. At salinities 13-19, clams kept their valves closed when not burrowed, and after 24 hours, the valves opened and closed intermittently. At a salinity of 22, valves remained mostly open but occasionally closed during the initial exposure or permanently closed after 24 hours. In the control (25-28), valves remained open during the first 4 hours, with some clams occasionally closing them. At salinity 31, clams burrowed within the first few hours or kept their valves closed permanently. At salinities 34-37, valves stayed mostly open or opening intermittently at similar rates to those observed at 25-28 (Fig. 2).

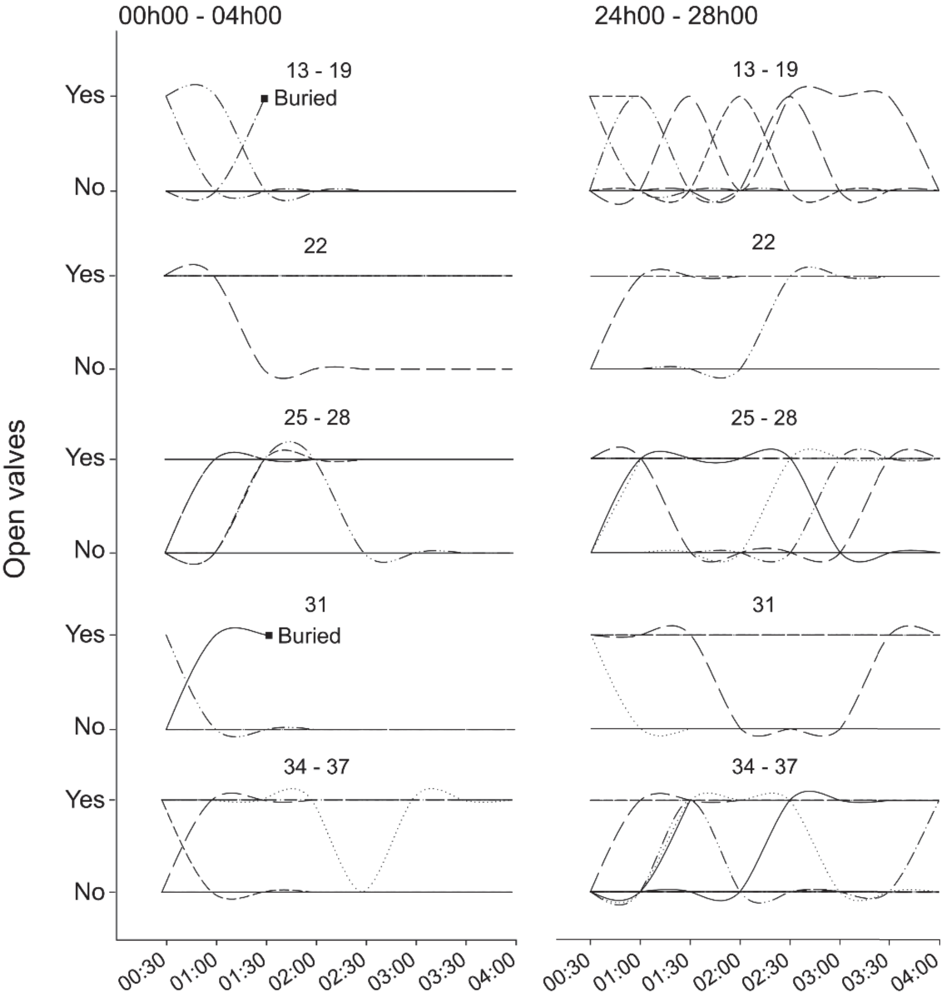

Figure 2. Valve-opening behavior along time and across salinities. Frequency of clams opening or closing their valves during the first 4 hours of exposure and after 24 hours in each salinity range/value. Dashed lines indicate a smaller number of observations. As the line becomes more solid, it indicates that the number of observations has increased.

2.4.2 Physiological responses

Hemolymph osmolality showed typical osmoconformer behavior and varied with salinity (H = 57, df = 4, $p<0.001$). In salinities between 13-19, osmolality was lower than the control, even with clams keeping their shells closed. At 22, osmolality was not different from the control, as observed in salinities 31-37. Tissue hydration of the foot showed minimal variation across salinities, with a difference only between the hypoosmotic challenge (13-19) and the hyperosmotic challenge (31-37), though neither was different from the control. LDH activity in 13-19 and 22 was not different from the control (25-28) or from salinity 31. However, the control exhibited lower LDH activity compared to these groups. The highest LDH activity was observed in the hyperosmotic challenge (34-37), showing differences from all other salinities (Fig. 3). Based on these results, salinities >22, up to the highest tested value of 37 (which exceeds the salinity found in the collection area), were deemed suitable for this *A. flexuosa* population.

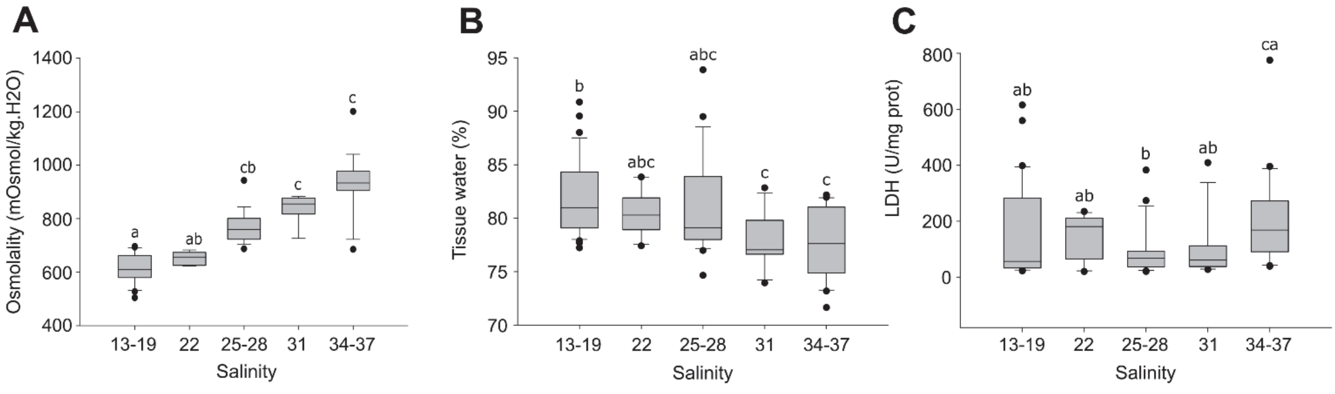

Figure 3. Physiological responses of *Anomalocardia flexuosa* after 24 hours of exposure to different salinity levels. **(A)** Hemolymph osmolality (mOsmol/kg.H_2O). **(B)** Foot tissue water content (%). **(C)** Lactate dehydrogenase (LDH) specific activity (U/mg protein). Dots indicate outliers.

2.4.3 Realized saline niche

For the saline niche characterization, boxplot of occurrence points showed that most individuals of *A. flexuosa* were found in salinities >30. When analyzed by clusters, the boxplots suggested a preference for specific salinity ranges. Clusters 1 and 2 had the highest number of observations (59 and 32, respectively) and were in regions with higher salinities, while clusters 3 and 4 had fewer observations (11 and 3, respectively) and were found in regions with lower salinities (Fig. 4).

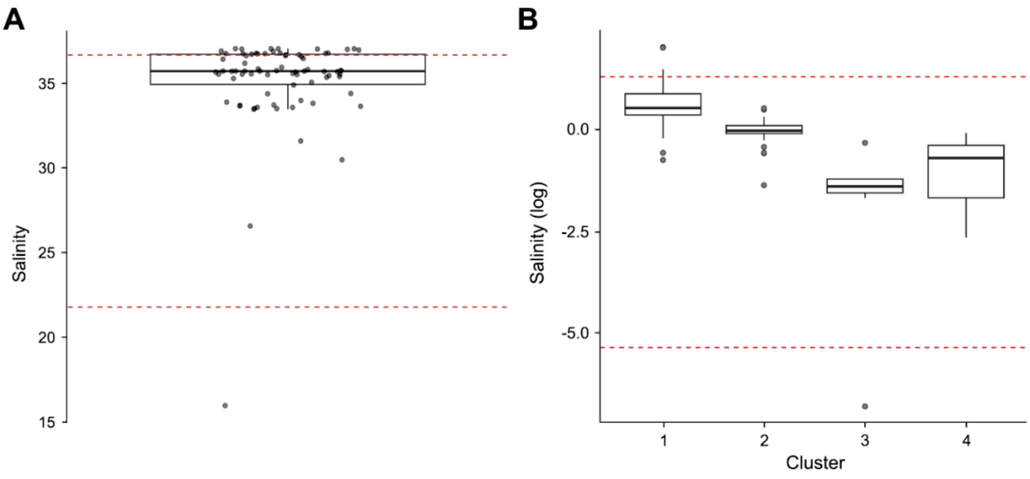

Figure 4. Realized salinity niche characterization of *Anomalocardia flexuosa*. **(A)** Occurrence points (GBIF) distribution across natural environment salinities (Bio-Oracle). **(B)** Distribution of occurrence points by cluster across the log-transformed salinity of the natural environment. Red lines indicate the lower (22) and upper (37) salinity limits of the saline niche recorded in laboratory conditions for the Paranaguá population.

2.4.4 Niche modeling for the current and end-of-century climate

The habitat suitability scale ranges from 0.0 to 1.0, with 0.0 representing minimally suitable habitats and 1.0 representing highly suitable habitats. The Maxent model, which performed the best (AUC = 0.93, TSS = 0.83), identified that 58% of the study area is suitable (>0.0) for *A. flexuosa* under both current and future climate conditions (2090s). However, for future climate projections, there was a 113% increase in areas with suitability >0.6, accompanied by a pronounced shift towards the North (Fig. 8).

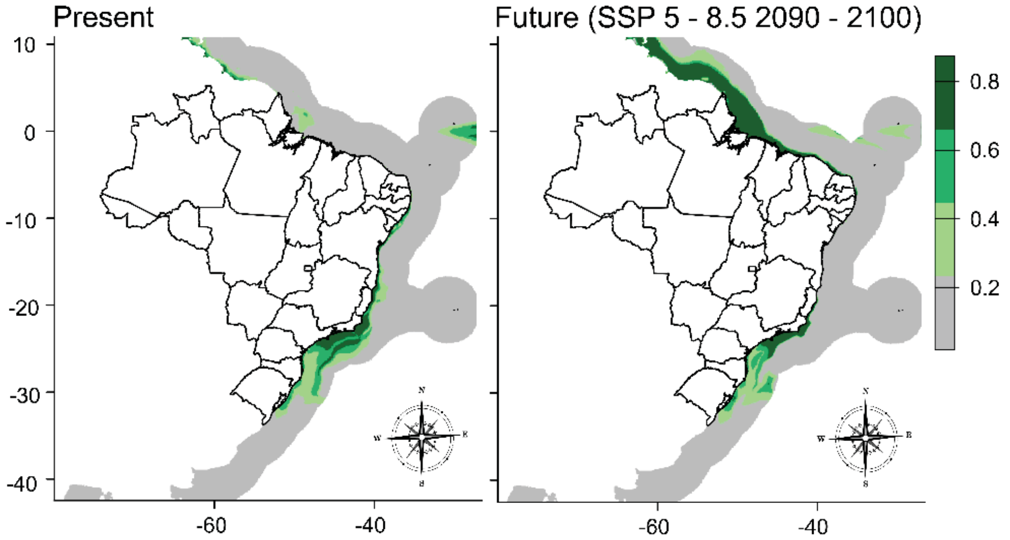

Figure 8. Habitat suitability maps of Maxent model for *Anomalocardia flexuosa* under present and future (2090–2100, SSP 5–8.5 scenario) climate conditions. Suitability values range from 0.0 (minimally suitable) to 1.0 (highly suitable). Climatic variables used in the model include salinity, air temperature, and chlorophyll concentration.

2.5 DISCUSSION

The findings of this study highlight the interplay between behavioral and physiological strategies employed by infaunal bivalves to tolerate dynamic salinity conditions in estuarine habitats. Laboratory experiments revealed that salinities <22 impair key behaviors, such as burrowing and valve opening, while salinities between 25 and 31 represent the (optimal) saline comfort zone. The population used in the fundamental saline niche experiments appears to be already exposed to salinity levels near the lower tolerance limits, particularly during rainy periods. Mass mortality events recorded for *A. flexuosa*, in Santa Catarina (south region of Brazil, Vaquero et al., 2022) and Pernambuco (northeast region of Brazil, Oliveira et al., 2014) have been strongly associated with episodes of decreased salinity following extreme rainfall. If these hyposaline conditions become more frequent and prolonged due to climate change, they could significantly reduce population densities in the southeastern and southern regions of Brazil over time.

2.5.1 Field salinity and behavioral responses

Burrowing in the sediment allows bivalves to create a microenvironment that protects them from rapid changes in salinity (Marsden, 2004; Nestler, 1977). The different salinity values for each water compartments in the field (water column, water film above the sediment and interstitial water) illustrate the potential of burrowing to protect bivalve tissues from low salinities (e.g., rainy season, freshwater inflow). *A. flexuosa* inhabits shallow and intertidal sandbanks and its populations typically experience: (1) when the tide is high, the clams are in the salinity of the water column, (2) when the tide is low, the clams are subjected to higher salinities by evaporation or to lower salinities by rainwater, and (3) burrowing, clams can access a salinity that protects them from the low salinity caused by rainfall.

By understanding the stratification of salinity between the water column, the sediment surface and within the sediment, we can understand the importance of escape behavior in isolating the tissues that allow these animals (which do not have the ability to move) to inhabit intertidal estuaries, places that are completely stressful for most animals. The evolutionary history of valve closure in bivalves is a crucial adaptation that played a significant role in their survival and diversification (Wei et al., 2020; Stanley 1975). The adductor muscle shell attachment is one of the strongest anchoring structures among invertebrates, allowing bivalves to tightly seal their shells (Castro-Claros et al., 2020; Liu et al., 2018).

Like other animals that exhibit escape behavior, this is a strategy limited by time and by ability to burrow even in bad environmental conditions. During the burrowing movement, bivalves must necessarily open the valve to generate thrust with their feet and push water from the mantle cavity through the siphons (Trueman, 1966; Stanley, 1975). Therefore, if external conditions are too stressful, causing not homeostatic overload but homeostatic disruption, exposing the tissues to excavation is not the best option.

Several studies have shown that unfavorable salinities can impair the burrowing behavior of several bivalve species: *Mya arenaria* showed reduced burrowing performance when acclimated to low (5) or variable (5–15) salinity compared to control conditions (15) (Haider et al., 2018). One study found that burrowing of *Venerupis corrugata*, *Ruditapes decussatus*, and *Ruditapes philippinarum* was reduced in all three species at salinities equal to or below 15 (Woodin et al., 2020). This strategy is evident in *A. flexuosa* by the low frequency of clams that burrowed at the lowest salinities tested. Furthermore, clams that did not burrow also did not remain open, but after 24 hours they opened and closed frequently. This behavior probably reflects the need for respiration, circulation and elimination of excreta after a period of unfavorable salinity greater than normal (which would be the tidal time) (Roden, 2018; Woodin et al., 2020).

2.5.1 Physiological responses

Among the physiological parameters evaluated, the osmolality of *A. flexuosa* was typical of a euryhaline osmoconformer, which allows and can tolerate a wide variation in extracellular fluid concentration for a given period of time (Deaton, 2009; Santos et al., 2013). After 24h of exposure, with some individuals gaining or losing more or less water (intraspecific variability), the stability of tissue hydration demonstrates the efficiency of both the escape behavior in protecting tissues and the ability to regulate tissue hydration in the tested salinity range (13-37). Some individuals can regulate and fully recover cell volume, while others recover partially, but enough to ensure cellular integrity and function (Pierce, 1982, Torre et al., 2013; Stakowian and Freire, 2024).

Although LDH is a valuable indicator of anaerobic metabolism (Livingstone, 1983; Nie et al., 2018), it is underutilized in assessing the metabolism of infaunal bivalves. This enzyme has a well-preserved structure across species, making it a suitable target for studying anaerobic metabolism in different organisms, including bivalves (Boldina-Cosqueric et al. 2010; Strahl et al., 2011). Carneiro et al. (2021) investigated how different levels of contamination affect the tolerance of *A. flexuosa* to different intensities and durations of marine heatwaves and found no differences in LDH activity. This may be because the activity of this enzyme in *A. flexuosa* is more related to burrowing in the thin and poorly oxygenated substrate (which cannot be identified in experiments without sediment) than to valve closure alone, which can occur with or without sediment.

Considering the mean of LDH activity, salinities between 25 and 31 (more frequent from the median region to the mouth of the CEP) seem to represent the “saline/osmotic comfort zone” of *A. flexuosa*, which is the salinity range in which the animal does not need to expend energy to protect itself (escape) or repair potential damage (Falkenberg, 2009; Sokolova et al., 2012). A study evaluating the larviculture of *A. flexuosa* under different salinities identified that a salinity range of 25-30 is optimal for survival and growth, confirming the physiological comfort zone (Lima et al., 2024).

Salinities <19 can lead to a decrease in the metabolism of clams as a whole (aerobic or anaerobic), hence the lower LDH activity in this condition. Due to the need to remain closed (and potentially exposed, without burrow), it is prudent that the metabolism slows down to require less oxygen (and using anaerobic metabolism) and produce less excreta that can intoxicate the water in the mantle cavity (Zhang et al., 2020). This decrease in metabolism at low salinities has already been observed in other bivalves that use this adaptive strategy to avoid contact and exposure of tissues to unfavorable salinities (especially hyposaline stress). The oyster *Crassostrea virginica* demonstrated zero oxygen consumption during periods of valve closure (Hammen, 1969). Other study analyzed *A. flexuosa* shells from Brazil and revealed that salinity <16 significantly influences growth patterns. These findings suggest that lower salinities (deviations from the optimal salinity range) may require the organism to expend more energy on stress responses and repair than growing and reproducing (Sokolova et al., 2012; Colonese et al., 2017).

Salinities 22 and ≥34 appear to be more challenging than the control salinity, represented by increased mean LDH activity in this condition. Salinities ≥34 are rare in the CEP, but salinities ≤22 are commonly observed, especially during rainy periods (Falkenberg, 2009). If these salinities (≤22 and ≥34) become more frequent, they may represent a burden on homeostasis and potentially affect the energy balance of the organism in the long term (Sokolova et al., 2012). With increased precipitation in the South and Southeast regions indicated by climate change (Lyra et al., 2017; Zilli et al., 2017; Alves et al., 2021), salinities ≤22 will be more frequent and may last longer than usual, potentially increasing mass mortality events of *A. flexuosa*, as already suggested by previous studies (Maia et al., 2018; Lima et al., 2022; Oliveira et al., 2013; Mottola et al., 2020).

It is questionable why populations of *A. flexuosa* can thrive in the CEP and other estuaries where salinity levels are 22 or less. This is due to the efficiency of escape behavior of bivalves. The valves can remain closed long enough for the salinity of the surrounding water to restore a suitable condition for the tissues, and the sediment microhabitat potentially buffers this salinity variation (Nestler, 1977; Haider et al., 2018; Carvalho et al., 2015). Estuaries are rich in biodiversity, with abundant food, protection from wave energy (which can displace clams), and sediment that allows *A. flexuosa* to burrow (Lana et al., 2001; McLusky and Elliott, 2004; Kennish, 2002). The behavioral and physiological strategies of the bivalves, plus the dynamics of the estuary (conditions to change constantly) allow these populations to persist and thrive. Climate change disrupts this ‘stability in chaos’, causing unfavorable conditions to become more frequent and last longer, threatening locally adapted biodiversity (Des et al., 2021; Worm and Lotze, 2016; Reyer et al., 2017).

2.5.3 Projections for the climate at the end of this century in Brazil

In addition to the fundamental saline niche experiments and the realized saline niche, the Maxent algorithm projections indicate an increase in areas with greater suitability for *A. flexuosa* in the Northern coast of Brazil, or areas of lower latitude South. This may be due to the fact that the southeastern and southern regions of Brazil are projected to experience increases in extreme precipitation events due to global climate change (Lyra et al., 2017; Zilli et al., 2017; Alves et al., 2021), which could lower salinity beyond the usual, making the clams' adaptive strategies insufficient. On the other hand, future projections indicate an increased frequency and intensity of droughts in lower latitude regions (Duffy et al., 2015; Parsons, 2020). This pattern may have caused the result of greater habitat suitability in the north observed in the future projection.

Regions with intense freshwater inflows during extreme rainfall events may experience prolonged hyposaline conditions, which could compromise homeostasis, energy balance, and population viability (Sokolova et al., 2012). Mass mortality after periods of heavy rainfall has already been observed for *A. flexuosa* and other bivalves. During a study in northeastern Brazil, significant declines in *A. flexuosa* densities were observed in months of high rainfall and low salinity (<5) (Maia et al., 2018). In European estuarine environments, extreme rainfall events reduced salinity and caused significant mortality in bivalves like *Cerastoderma edule* and *R. philippinarum*. The tropical bivalve *Cardita affinis* also has increased mortality rates linked to reduced salinity because heavy rainfall during the La Niña event in Colombia (Riascos et al., 2008). In Kingston Harbor, Jamaica, mass mortalities among marine animals, including bivalves, were also documented following heavy rainstorms (Goodbody, 1961). These parallels suggest that *A. flexuosa* populations in Brazil may face similar outcomes if extreme rainfall events intensify, as predicted by climate models.

The extent to which northern habitats can support large and stable populations depends on local environmental conditions, resource availability, and connectivity with existing populations. This raises questions about the potential for gene flow and local adaptation, which are essential for the long-term resilience of A. flexuosa in the face of climate change (Bible & Sanford, 2016; Nikula et al., 2008).

2.5.4 Conclusions

The concept of saline niche was used for bivalves for the first time and highlights the importance of salinity in the distribution of this group, and of osmoconformers in general (Gainey & Greenberg, 1977; Podbielski et al., 2022). Our results confirm the evidence that mass mortalities of *A. flexuosa* may be caused by intense rainfall and, considering the projections, this should intensify, and mortality episodes become more frequent in the southeast and south regions of Brazil. Communities that depend on this clam for subsistence will not be left without the resource, because the region remains suitable, but *A. flexuosa* populations in the southeast and south may decrease in density in the long term (Oliveira et al., 2013; Mottola et al., 2020; Lima et al., 2022).

This study, for the first time, searched for physiological and behavioral markers to delimit the fundamental saline niche of *A. flexuosa* and delimited the realized saline niche through a clustering algorithm (occurrence points) and related it to correlative niche modeling to assess the species' vulnerability to climate projections in the present and at the end of this century. Niche modeling projections under future climate scenarios reveal shifts in habitat suitability, probably related to increased precipitation events and consequently, decrease in salinity. These findings highlight the resilience and physiological plasticity of *A. flexuosa* while underscoring its vulnerability to salinity decrease, providing data for conservation and management, and a framework that can be used to assess the vulnerability of osmoconformer populations under climate change.

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3 CAPÍTULO 2

Ecological Niche Modeling reveals projections of suitable areas for blue mussel (*Mytilus galloprovincialis*, Lamarck 1819) across the Atlantic Ocean

Nicole Stakowian[1], Carolina A. Freire[2]

[1]Graduate Program in Zoology, Biological Sciences Sector, Federal University of Paraná, Centro Politécnico, Curitiba, PR CEP 81531-980, Brazil.

Correspondence: stakowian.zoo@gmail.com

3.1 ABSTRACT

Mytilus galloprovincialis, considered native to the Mediterranean, Black Sea, and eastern Atlantic coast, is an economically significant species and a globally recognized invasive mussel. Its broad physiological tolerance has facilitated its establishment in non-native regions, including South Africa, South America, and North America, where it poses ecological and economic risks by displacing native species such as *Perna perna* in South America and South Africa. This study applied ecological niche modeling (ENM) to assess the realized niche of *M. galloprovincialis* and project habitat suitability under current and predicted future (2090s) climate scenarios, in the Atlantic Ocean. Using occurrence data and key environmental variables (pH, salinity, and temperature), k-means clustering identified three climate groups that reflect the environmental adaptability of the species. ENM projections under the SSP2-4.5 climate scenario revealed a global reduction in suitable habitat, particularly in the Temperate North Atlantic ecoregion, with predicted declines from 264.92 km² to 108.93 km². In subtropical regions, habitat suitability has remained relatively stable. These findings highlight the role of temperature in constraining the global distribution of *M. galloprovincialis*, with warmer climates threatening native populations and potentially mitigating invasive pressure. Our approach provides valuable insights not only for managing *M. galloprovincialis* but also for assessing the distributional dynamics of other bivalves or marine species introduced through ballast water. Our results support proactive monitoring that can provide early detection of trends in habitat growth or loss and apply targeted interventions. Such efforts will be essential to mitigate the ecological and economic impacts of Anthropocene pressures.

Keywords: Biochemical biomarkers; Bioinvasion; Climate change; Euryhalinity; Osmoconformer.

3.2 INTRODUCTION

Mytilus galloprovincialis, commonly known as the Mediterranean mussel or blue mussel, is native to the Mediterranean Sea, the Black Sea, and the eastern Atlantic coast, including regions of Portugal, France, and parts of North Africa (Zbawicka et al., 2018; Belz et al., 2020; Ouagajjou et al., 2023). Within its native range, this species holds significant economic importance, particularly in aquaculture, where it is valued for its fast growth, adaptability, and high market demand (Lopez et al., 2022; Ouagajjou et al., 2023). The combination of its nutritional profile and commercial value has established *M. galloprovincialis* as a key species of regional seafood industries.

The remarkable adaptability of *M. galloprovincialis*, including tolerance to variations in salinity and temperature, has facilitated its success in non-native regions, establishing populations on other continents such as South Africa, South America, and North America (Belz et al., 2020; Lins et al., 2021; del Rio-Lavín et al., 2022). *M. galloprovincialis* is listed among the top 100 invasive species worldwide (Lowe et al., 2000) and poses a serious threat to local ecosystems and economically important native species, such as *Perna perna* in Brazil and South Africa (Bownes and McQuaid, 2006; Zardi et al., 2007; Hanekom, 2008). The dispersal of *M. galloprovincialis* has largely been facilitated by human activities, including maritime transport and trade, underscoring the global scale of its invasive potential (Ouagajjou et al., 2023; Westfall et al., 2010).

The dual role of *M. galloprovincialis*—as a key fisheries resource in its native range and a disruptive invasive species in non-native regions—presents complex challenges for management under changing environmental conditions. In native regions, its ecological and economic contributions are indispensable, whereas in invaded regions, its proliferation undermines biodiversity and displaces native species (Lins et al., 2021; Bownes and McQuaid, 2006). Addressing these challenges requires tools capable of predicting how environmental changes influence species distributions. Ecological Niche Modeling (ENM) has emerged as a robust approach to assess species vulnerabilities and potential bioinvasion risks, as it relates species presence to environmental variables and projects changes in habitat suitability under future climate scenarios (Peterson, 2003; Peterson and Soberón, 2012; Feng and Papes, 2017).

Although ENM has been extensively applied to assess invasion risks for species such as snails, flatworms, reptiles, disease-carrying insects (Larson et al., 2010; Tytar et al., 2015; Yu et al., 2024) and freshwater bivalves (Feng and Papeş, 2017), this study represents its first application to *M. galloprovincialis*. Guided by Hutchinson's duality, which distinguishes between environmental space—the range of abiotic conditions under which a species can survive and reproduce—and geographic space, or the physical locations where these conditions occur (Colwell and Rangel, 2009), this study evaluates the occurrence of M. galloprovincialis within environmental space to identify suitable habitats across the Atlantic coast. Subsequently, an ENM framework was employed to project habitat suitability in both native and non-native ranges under a moderate climate change scenario, anticipating a global temperature increase of 2.4°C to 2.6°C by the end of century.

The primary objective of this study is to test this method for a very relevant commercial species and assess future habitat availability for native populations along the eastern Atlantic coast and to evaluate potential shifts in suitable habitats in non-native regions, where reductions in bioinvasion pressure may occur. By providing critical data on habitat suitability and distribution dynamics, this study aims to inform resource managers and policymakers, supporting the development of targeted strategies for managing *M. galloprovincialis* under the climatic pressures of the Anthropocene.

3.3 MATERIAL AND METHODS

To analyze the realized niche of *M. galloprovincialis* in environmental space, the main variables that delimit the occurrence of marine and estuarine species were used: pH, temperature and salinity. These variables were extracted for each occurrence point to characterize the range of environmental conditions the species occupies. Then k-means clustering was used to group occurrence points based on their environmental profiles, allowing to identify distinct "climatic groups" or clusters of conditions that populations of *M. galloprovincialis* may tolerate. Following the cluster analysis, these clusters were mapped geographically to examine spatial patterns in the distribution of environmental conditions suitable for *M. galloprovincialis*.

To perform the ENM on the Atlantic coast, the following regions defined in Marine Ecoregions of the World (MEOW) (Spalding et al., 2007) were selected: Temperate North Atlantic, Tropical Atlantic, Temperate South America and Temperate Southern Africa (Fig. 1). Occurrence data of native and invasive populations of *Mytilus galloprovincialis* were obtained by searching for the species name in the Global Biodiversity Information Facility database (GBIF: https://www.gbif.org/, DOI 10.15468/dl.83qwfq). Data on the main drivers of distribution and persistence of marine invertebrates (pH, salinity, and temperature) for the current and predictions of future (2090s) climate were obtained from the Bio-Oracle database (https://www.bioracle.com.br/), and cropped to the region of interest; these data were based on the SSP2-4.5 climate scenario of the Intergovernmental Panel on Climate Change (IPCC, 2021). This scenario leads to a global temperature increase of approximately 2.4°C to 2.6°C by the end of the 21st century, as compared to pre-industrial levels. This is the moderate scenario, as it includes the effective establishment of policies and measures to reduce emissions, globally.

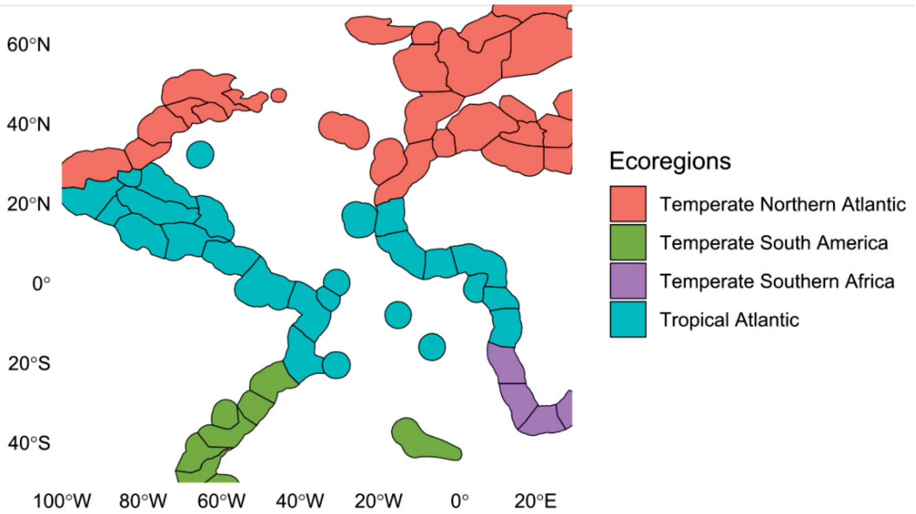

Figure 1. Selected modeling regions based on "Marine Ecoregions of the World" (Spalding et al., 2007), showing the four ecoregions included in the Ecological Niche Modeling of *Mytilus galloprovincialis*. These regions encompass both native and non-native ranges of the species, providing the spatial framework for modeling current and future habitat suitability.

Occurrence points from GBIF were filtered to remove records without Longitude and Latitude data, and the SpThin package was used to remove overly aggregated occurrence points (within a 1km radius) to avoid model overfitting and ensure greater representativity of population distribution. To evaluate the realized niche of *M. galloprovincialis* and identify regions with distinct climates based on the distribution of occurrence points, the k-means algorithm was used, dividing the data into 'k' distinct groups based on their characteristics, aiming to minimize within-cluster variance and maximize between-cluster variance. The Silhouette Method was used to determine the optimal number of clusters and to maximize the average Silhouette Coefficient.

To fit the models, environmental variables of the modelling area were normalized, and the Bioclim, Maxent, and GLM algorithms were used (Busby, 1991; Phillips et al., 2006; MacCullagh and Nelder 1989; Qiao et al., 2015). The models were trained using two datasets: training and testing, including occurrence data, background and environmental variables (pH, salinity, and temperature) for the current and end-of-century climate (IPCC, 2021). After being trained in the 3 algorithms (Bioclim, Maxent and GLM), the models were evaluated using test data to verify their accuracy and generalization capability. The three models were evaluated using Area Under the Curve (AUC), which quantifies the model's ability to distinguish between classes (presence and absence) (McPherson et al., 2004). The packages used for niche modelling were spThin (Phillips and Williams, 2016), raster (Hijmans, 2021), dismo (Hijmans et al., 2021), and sdm (Czarnecki and Cazelles 2020). All analyses were performed using RStudio, assuming a significance level of 95%, and the graphs were edited using the Inkscape software.

3.4 RESULTS

The realized niche of *M. galloprovincialis* spans a broad range of environmental conditions, as indicated by the distribution of occurrence points. This suggests that the species tolerates multiple distinct "climatic groups" within its habitat; the k-means algorithm identified three climatic clusters (Fig. 2A). Cluster 1 is characterized by high pH, indicating a more alkaline environment, with salinity consistent with seawater, suggesting more open and marine-influenced environments. Cluster 2 is slightly alkaline, with typical marine salinity, and temperatures corresponding to subtropical regions. Cluster 3 has a lower pH, approaching more acidic conditions, with seawater salinity similar to Clusters 1 and 2, and cooler temperatures, suggesting temperate climate regions (Fig. 2C). Geographically, when we analyze the global distribution of climatic clusters, Cluster 1 is predominantly found in the Northern Hemisphere, especially in the Mediterranean region. Cluster 2 is prevalent along the Atlantic Ocean, extending from north to south, while Cluster 3 is more frequent in the eastern coast of the Atlantic in the Southern Hemisphere (Fig. 2B).

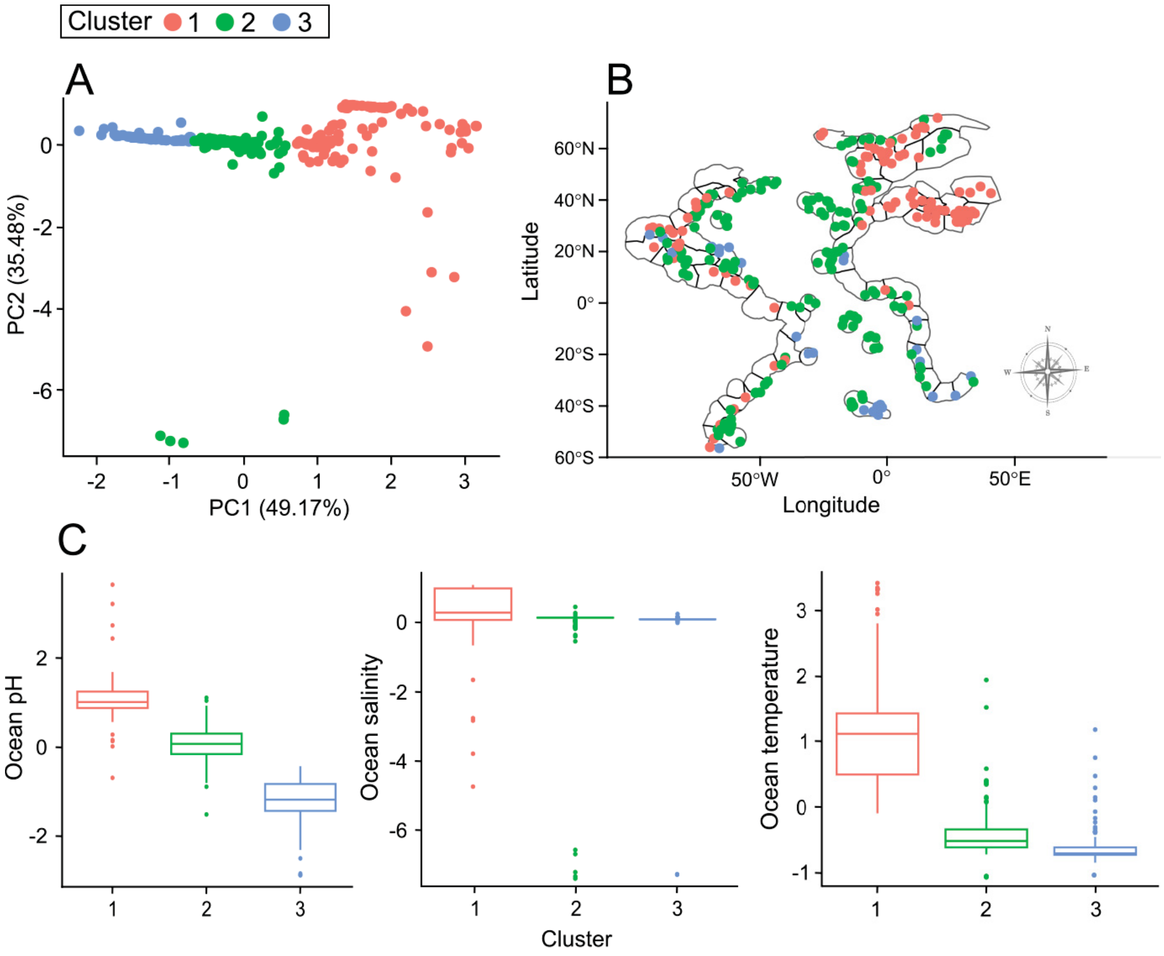

Figure 2. Global distribution of the realized niche of *Mytilus galloprovincialis*, including native and invasive populations in environmental and geographic space. **(A)** Occurrence points plotted in environmental space. Different colors indicate clusters with common environmental characteristics identified through the k-means algorithm. **(B)** Distribution of environmental clusters of the realized niche in geographic space. **(C)** Variations in ocean pH, salinity and temperature within each cluster. The values were scaled by subtracting the meaning and dividing it by the standard deviation.

The AUC values for each model, after fitting for the three algorithms, were 0.89 (BIOCLIM), 0.76 (GLM) and 0.74 (Maxent). Based on this, the BIOCLIM model was identified as the best fit for the dataset, suggesting good discriminatory power. The response curves of the BIOCLIM model indicate that suitability for the species is sensitive to variations in pH, salinity, and temperature (Fig. 3). The model shows that the species' habitat suitability is maximized within certain ranges, suggesting that these variables collectively define the species' preferred ecological niche'.

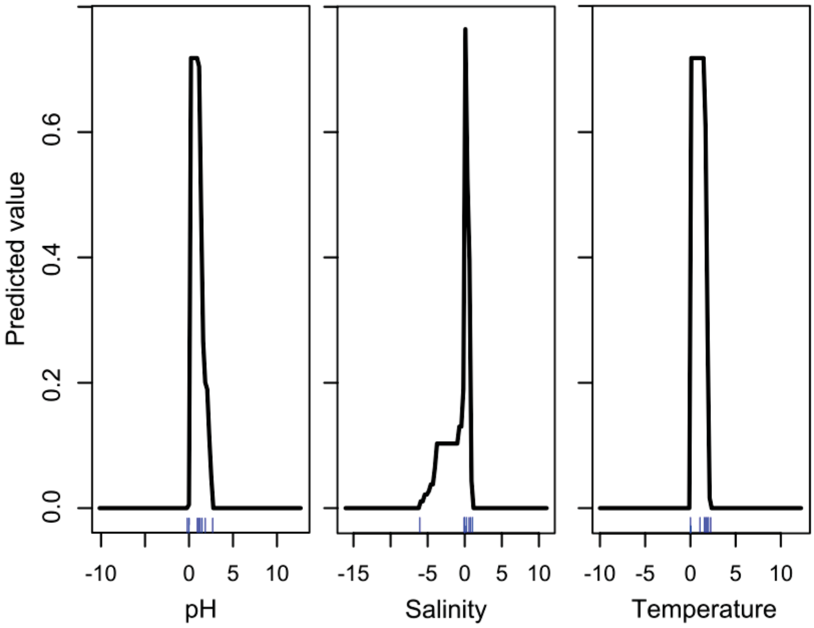

Figure 3. Response curves of the BIOCLIM model showing the predicted habitat suitability of *Mytilus galloprovincialis* as a function of environmental variables. The x-axis represents the standardized values (z-scores) of average pH, salinity, and ocean temperature, with 0 corresponding to the mean value for each variable across the study region. Positive values indicate conditions above the mean, while negative values indicate conditions below the mean. The y-axis indicates the predicted suitability, ranging from 0 (unsuitable) to 1 (highly suitable). Black lines represent the model's response curves, and blue tick marks on the x-axis indicate the distribution of species occurrence records across the range of each variable.

Current and future (2090’s) habitat suitability projections show suitability values ranging from 0.0 to 0.80, with being >0.5 a strong indicator of the presence of the species. The current projection revealing a slightly larger area of suitable habitat (2,857.2 km² for suitability > 0.1) compared to the future projection (2,664.9 km²), reduction of approximately 190km² (Fig. 4).

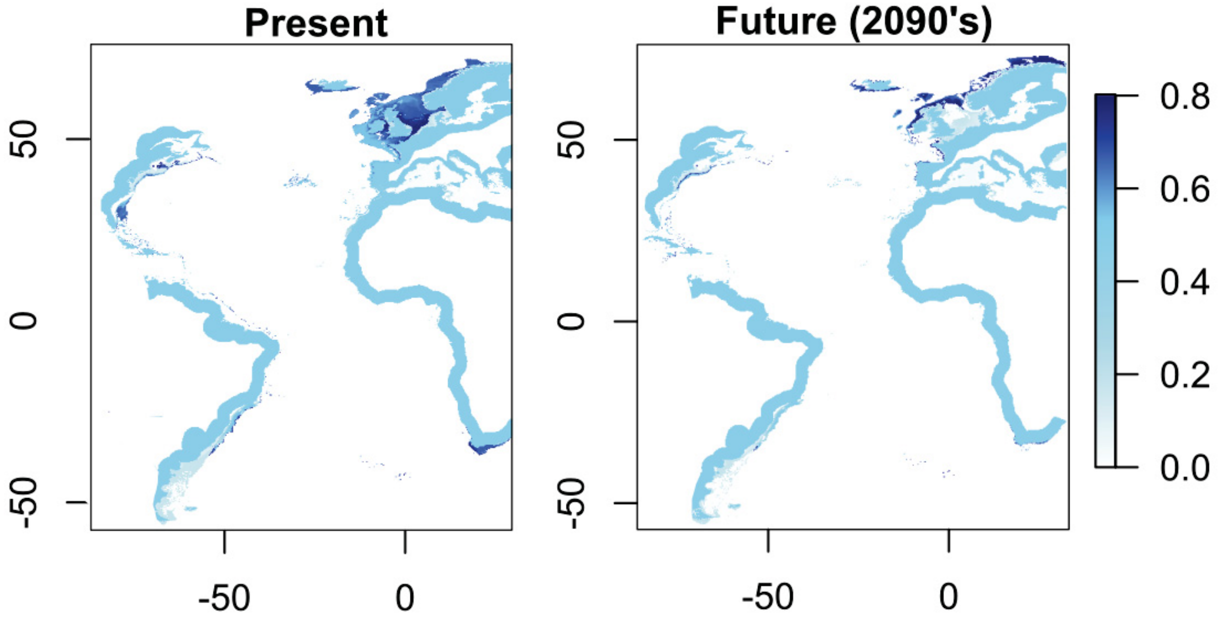

Figure 4. Bioclimatic projections of habitat suitability for *Mytilus galloprovincialis* under present climatic conditions (left) and future climatic conditions (end of century – 2090’s, SSP 2-4.5 scenario; right). The color gradient represents habitat suitability values, ranging from 0 (unsuitable; light blue) to 0.8 (highly suitable; dark blue).

When evaluated by ecoregions, the Temperate Northern Atlantic showed the largest decline, with suitable area decreasing from 264.92 km² in the present to 108.93 km² in the future, a reduction of 155.99 km². The Tropical Atlantic, Temperate South America, and Temperate Southern Africa ecoregions exhibited less pronounced declines, with reductions of 12.40 km², 10.94 km², and 9.99 km², respectively (Fig. 5).

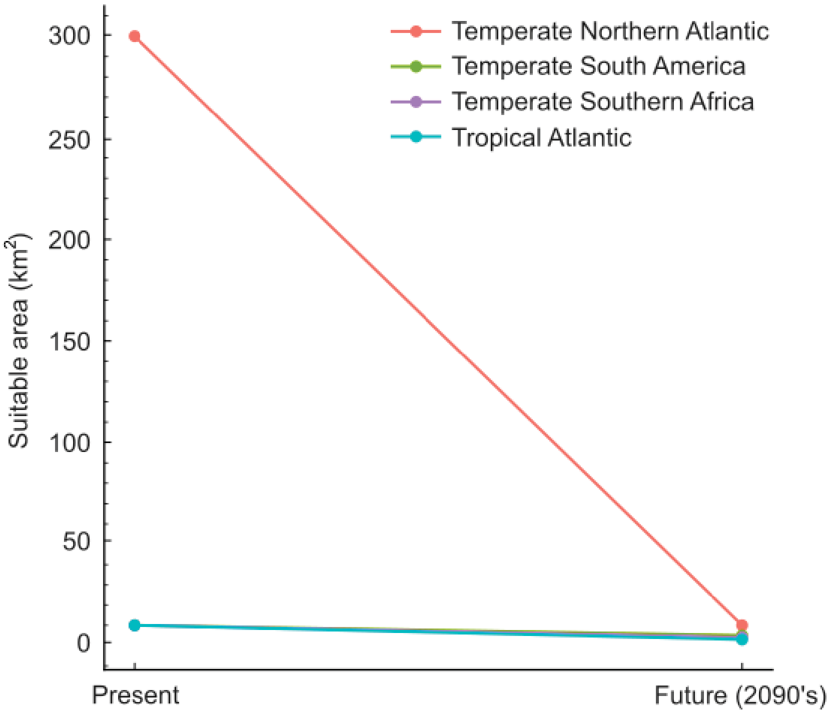

Figure 5. Change in suitable habitat area (suitability > 0.1) for *Mytilus galloprovincialis* across four ecoregions between the present and future climate projections (2090s, SSP 2-4.5 scenario).

3.5 DISCUSSION

This study provides new insights into the ecological distribution and environmental tolerance of *M. galloprovincialis* through climate cluster analysis and ENM. The distinct climate groups defined by variations in pH, salinity, and temperature align with the species' native distribution in the Mediterranean and with its native and invasive distribution in subtropical environments across the Atlantic, highlighting the species' physiological plasticity and adaptability—traits linked to its invasive potential (Zardi et al., 2018). Projections under future climate conditions (2090s) showed an overall reduction in suitable habitat, with the North Atlantic experiencing the largest declines, consistent with studies indicating that rising temperatures threaten temperate marine species (Anestis et al., 2007; Mesas & Tarifeño, 2015). However, the stability of suitable habitats in subtropical regions highlights the species' ability to persist under variable conditions.

3.5.1 Invasiveness of M. galloprovincialis along the Atlantic coast

In North America, the Smithsonian Institution's National Exotic Marine and Estuarine Species Information System (NEMESIS) indicates that *M. galloprovincialis* is introduced and widespread on the Atlantic coast of western North America (www.invasions.si.edu/nemesis), but studies confirming and discussing its presence have not been found. In South America, the presence of *M. galloprovincialis* is relatively recent and has been reported in Argentina, specifically in the Puerto Madryn. According to Zbawicka et al. (2018), 88% of the individuals in this area are the result of hybridization between *M. galloprovincialis* and *M. platensis*. In Brazil, *M. galloprovincialis* has been reported in mussel farms in Santa Catarina (Belz et al., 2020), where it has threatened the native species farm, *Perna perna*, through competition for space to settle in the substrate (Lins et al., 2021; Suplicy, 2022).

In South Africa, *M. galloprovincialis* established itself in Saldanha Bay in 1970 and spread along the west coast at an estimated rate of 115 km/year, eventually colonizing the southern half of Namibia and large portions of the South African coastline (Branch & Steffani, 2004). In Tsitsikamma, there is a spatial segregation between natural populations of *M. galloprovincialis* and *P. perna* has been observed, with *M. galloprovincialis* occupying higher zones and *P. perna* inhabiting lower zones (Bownes and McQuaid, 2006).

3.5.2 Evolutionary context of Mytilus complex distribution

The blue mussel is widely recognized for its robust invasive capabilities, attributed to its broad physiological tolerance and adaptability to novel environments (Han et al., 2024; Vasquez et al., 2022). Other estuarine bivalve species also exhibit remarkable physiological tolerance and environmental adaptability. Oysters occupying estuarine and intertidal habitats have evolved well-established stress tolerance mechanisms to withstand harsh and dynamically changing environments (Zhang et al., 2015). This adaptability is crucial for the survival of species in dynamic estuarine environments and contributes to their success in aquaculture and natural ecosystems. Our environmental clusters derived through the k-means algorithm demonstrate the variety of conditions under which *M. galloprovincialis* is found globally providing direct evidence of its adaptive and invasive nature.

Often considered more invasive than other *Mytilus* species, the evolutionary history of the *Mytilus* genus provides insights into the adaptability and invasiveness of *M. galloprovincialis* (Lockwood et al., 2010; Lockwood and Somero, 2011; Zardi et al., 2011). Originating from *M. edulis* in the North Atlantic, which in turn originated from *M. trossolus* around 3.5 million years ago, *M. galloprovincialis* has undergone adaptations specific to the Mediterranean and has a distribution that extends to overlap with *M. edulis* and *M. trossolus* in temperate and subpolar waters (Evans and Somero, 2010; Williams, 2023).

Our analysis of environmental clusters corroborates previous observations, with Cluster 1 concentrated in the Mediterranean and Cluster 2 extending across the North Atlantic, suggesting potential coexistence with *M. edulis* and *M. trossolus* in temperate zones (Fly and Hilbish, 2013). This represents a novel approach, as applying clustering techniques to identify these environmental patterns offers a method to confirm previously observed geographic distributions or investigate potential areas of occurrence. Integrating environmental data with ENM, this study provides additional evidence to support and refine the understanding of spatial overlap and interactions among *Mytilus* species.

The overlap of Cluster 2 from the Atlantic to southern Brazil points to ongoing differentiation in the South Atlantic and Mediterranean populations (del Rio-Lavín et al., 2022). Genetic studies indicate that *M. galloprovincialis* populations in Brazil are most closely related to Mediterranean populations, which could explain the clustering pattern between southern Brazil and the Mediterranean (Lins et al., 2021). Hybridization in overlapping zones may enhance adaptability, facilitating the spread of *M. galloprovincialis* through genetic introgression (Han and Dong, 2020).

The differentiation between these species is difficult and only possible through genetic analyses (Lins et al., 2021; Jilberto et al., 2023), making it likely that the occurrence points used to characterize the realized niche may not refer exclusively to *M. galloprovincialis*; indeed, our analysis suggest that this species overlaps with congeners in hybridization zones. This also is suggested by the variation in allele frequencies across different environmental conditions reported in other studies (Riginos and Cunningham, 2005; Zbawicka et al., 2018). Share niche characteristics with other *Mytilus* species is an important aspect of its ecological flexibility and expansion potential (Gardner, 1996; Lins et al., 2021; Diz and Skibinski, 2023; Jilberto et al., 2023).

3.5.3 Habitat projections under climate change

The SSP 2-4.5 scenario represents a moderate pathway for greenhouse gas emissions and societal development, balancing mitigation and adaptation measures, and serving as a middle ground for evaluating potential climate impacts and adaptation strategies (IPCC, 2021). Under this scenario, the ENM approach indicated a reduction in overall suitable habitat for *M. galloprovincialis* in the end of century, particularly in the Temperate North Atlantic ecoregion, where a decline of 155.99 km² was observed. The stability of suitable areas in subtropical regions illustrates how temperature can constrain the global distribution of this species (Anestis et al., 2007; Mesas & Tarifeño, 2015). That is, increasing global temperatures should, on the one hand, limit the bioinvasion of *M. galloprovincialis* but, on the other hand, threaten local native populations.

In subtropical regions outside the native range (North and South America and South Africa), efforts to control and mitigate the spread of *M. galloprovincialis* should focus on improving biosecurity measures, such as restricting larval dispersal via ballast water (D'Aloia et al., 2015; Lim et al, 2020) and protecting native species through conservation and restoration of habitats for larval and adult settlement (Rodríguez-Pérez et al., 2019; Wilcox et al., 2020).

3.5.4 Conclusions

This study successfully assessed current and future habitat availability for populations of *M. galloprovincialis* along the Atlantic coast and detected stability in the extent of invaded areas and potential habitat loss in native regions under a moderate climate change scenario. By employing a novel combination of ENM and climatic clustering, this approach provides valuable insights not only for managing *M. galloprovincialis* but also for assessing the distributional dynamics of other bivalves or marine species introduced through ballast water. Our results support proactive monitoring that can provide early detection of trends in habitat growth or loss and apply targeted interventions. Such efforts will be essential to mitigate the ecological and economic impacts of *M. galloprovincialis* under Anthropocene pressures.

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4 CAPÍTULO 3

Evidence of allostasis in an osmoconformer: tissue-specific responses of *Mytilus galloprovincialis* to salinity changes

Nicole Stakowian[1], Marta Cunha[2], Rosa Freitas[2], Carolina A. Freire[3]

[1]Graduate Program in Zoology, Federal University of Paraná (UFPR), Centro Politécnico, Curitiba, PR CEP 81531-980, Brazil.
[2]Department of Biology, University of Aveiro, 3810-193, Aveiro, Portugal.
3Department of Physiology, Federal University of Paraná (UFPR), Centro Politécnico, Curitiba, PR CEP 81531-980, Brazil.
Correspondence: stakowian.zoo@gmail.com

4.1 ABSTRACT

Bivalves exhibit diverse physiological mechanisms to cope with salinity fluctuations, a key environmental factor influencing their distribution and survival. This study investigates tissue-specific biochemical responses in *Mytilus galloprovincialis*, an osmoconforming euryhaline mussel, exposed to five salinity levels (20, 25, 30, 35, and 40) for 14 and 28 days. Metabolic, antioxidant, and cellular damage biomarkers were assessed in four tissues: digestive gland, gills, mantle, and muscle. Principal Coordinate Analysis (PCoA) and PERMANOVA revealed that tissue type was the primary factor driving biochemical variability ($R^2 = 63.0\%$, $p < 0.001$), followed by salinity ($R^2 = 2.3\%$, $p < 0.001$) and exposure time ($R^2 = 1.5\%$, $p < 0.001$). Metabolic biomarkers activities (succinate dehydrogenase, electron transport system, protein and glycogen levels) were most pronounced in the digestive gland and mantle, indicating elevated metabolic activity under changing salinity conditions. Antioxidant responses were strongest in the digestive gland, with elevated superoxide dismutase (SOD) and total antioxidant capacity (TAC) at salinity 35. Cellular damage markers, including lipid peroxidation (LPO) and acetylcholinesterase (AChE), were more pronounced in the gills and digestive gland. Our findings suggest that *M. galloprovincialis* maintains physiological balance through metabolic and enzymatic adjustments, with 'suboptimal' salinities (25 and 35) inducing allostatic adaptations. This study highlights the role of salinity in shaping stress responses and provides insights into the resilience of osmoconforming animals under environmental fluctuations, contributing to our understanding of their adaptability to climate change-induced salinity shifts.

Key words: Osmoregulation; Bivalve; Biochemical biomarkers; Allostasis; Ecophysiology.

4.2 INTRODUCTION

Bivalves have evolved a diversity of physiological and behavioral mechanisms to cope with changes in salinity, a critical environmental factor that directly influences their distribution and survival. Many species, particularly estuarine bivalves (such as *Cerastoderma edule* and *Ruditapes philippinarum*), respond to low salinity by reducing filtration activity, metabolism, and burrowing behavior to conserve energy and minimize osmotic stress (Domínguez et al., 2020). *Trapezium liratum*, for example, tolerates drops in salinity by tightly closing its shells for long periods (Kurihara, 2017).

In osmoconforming bivalves, the antioxidant system plays a crucial role in managing oxidative stress caused by ion and water fluxes during salinity fluctuations. When exposed to hypo- or hyperosmotic conditions, bivalves regulate their cell volume by adjusting intracellular ion concentrations and free amino acid pools, processes that generate reactive oxygen species (ROS) as metabolic byproducts (Zurburg & Zwaan, 1981). Antioxidant enzymes, such as superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione reductase (GR) neutralize ROS and maintain cellular homeostasis (Cunha et al., 2024; Gostyukhina et al., 2023). This is one of the adaptive strategies that allow bivalves to withstand salinity changes caused by tides, rainfall, and climate change, although prolonged exposure to extreme salinity levels can lead to physiological stress, reduced reproductive success, and mortality (Domínguez et al., 2020; Vázquez et al., 2021; Blanco et al., 2022).

When the antioxidant system in bivalves is insufficient to counteract oxidative stress induced by salinity fluctuations, enzymatic activities related to neurotoxicity, such as acetylcholinesterase (AChE) and carboxylesterase (CbEs), can be activated (Mebarki et al., 2015; Cunha et al., 2024). For example, in freshwater mussels (*Unio ravoisieri*), elevated salinity levels suppress oxidative metabolism, leading to increased catalase (CAT) activity and malondialdehyde (MDA) accumulation, both indicators of oxidative damage. This stress also resulted in a significant increase in AChE activity, suggesting that neurotoxic effects may be linked to oxidative imbalance (Lassoued et al., 2023). In *Cerastoderma glaucum*, bivalves exposed to environmental pollutants showed AChE inhibition along with increased glutathione S-transferase (GST) activity, indicative of detoxification efforts (Mebarki et al., 2015). In cases where oxidative stress is prolonged, excessive LPO can impair membrane integrity, leading to cellular dysfunction and apoptosis (Mejdoub et al., 2017).

Tissue-specific responses in bivalves are increasingly recognized as critical for understanding their physiological adaptations to environmental stressors, immunological challenges, and pollutant exposure. Different tissues exhibit distinct responses to metals, reflecting the complexity of detoxification mechanisms and highlighting their adaptive capabilities (Soldatov et al., 2007; Ahmad et al., 2011). The functions of the mantle, in mytilids largely composed of gonadal tissue, and the muscle, responsible for valve movement, also contribute to the overall resilience of the organism (Borković-Mitić et al., 2024). Studies have shown that different tissues exhibit distinct metabolic and immunological responses. For example, in mussels (*Perna canaliculus*), immune responses to infections by *Vibrio* sp. vary by tissue, with hemolymph and gills sharing metabolic changes such as increased itaconic acid, while hepatopancreas tissues show distinct amino acid and fatty acid changes (Nguyen et al., 2019). Furthermore, in bivalves such as *Anadara kagoshimensis*, different tissues display specific antioxidant activities and metabolic profiles, with the hepatopancreas, gills, and foot displaying unique balances of antioxidant enzymes (Golovina et al., 2016).

In order to evaluate how each tissue of an osmoconforming euryhaline animal responds to different salinities, we used the species *Mytilus galloprovincialis* as a model. It is a widely recognized model organism in physiological research due to its adaptability, environmental responsiveness, and experimental accessibility (Miglioli et al., 2024; Lassoued et al., 2023). Its role as a bioindicator is well established, given its ability to bioaccumulate pollutants, reinforcing its importance as a sentinel species (Arrigo et al., 2024). Its immune system and hemocyte function provide insights into comparative immunity and cellular stress responses (Ottaviani et al., 1998). Therefore, this is a good biological model to study metabolism, oxidative stress, and tolerance to environmental variations, contributing to research on the impacts of climate change on marine organisms.

The Mediterranean mussel or blue-mussel, as it is known, is a commercially significant seafood species, particularly in Europe, where it is widely cultivated and consumed (Vendrami et al., 2020). Its ability to efficiently convert low-value food sources into high-quality protein makes it essential in aquaculture and human nutrition (Ayvaz et al., 2023). Spain is the largest producer of M. galloprovincialis in the Mediterranean region and ranks second globally after China (Guéguen et al., 2011; Stankovic and Jovic, 2011). This popularity positions it as one of Europe's most consumed fishery products (Stankovic and Jovic, 2011; Tedde et al., 2019). In other parts of the world, such as South America and South Africa, it is considered invasive and a threat to native species of economic importance (Lowe et al., 2000; Lins et al., 2021; Bownes and McQuaid, 2006).

This study aims to uncover tissue-specific patterns and identify key biomarkers that best reflect the effects of salinity on M. galloprovincialis. We hypothesize that the digestive gland will exhibit the highest metabolic activity and antioxidant action due to its role, followed by the gills and mantle, given their direct exposure to surrounding water (Peters et al., 1999; Soldatov et al., 2007). Muscle, on the other hand, should demonstrate lower activity due to its stable metabolic functions and isolation from external water (Lockwood & Somero, 2011; Castellano et al., 2018; Stakowian and Freire, 2024).

4.3 MATERIAL AND METHODS

Mussels were collected in Ria de Aveiro, Portugal, at the end of January/2024 during low tide. Organisms of similar sizes were selected to minimize the effect of body weight on the study (length: 63.76 ± 3.91 mm; width: 34.04 ± 2.15 mm). After sampling, the mussels were depurated and acclimated in the laboratory for two weeks. During this period, mussels were kept at 17.0 ± 1.0°C and pH 8.0 ± 0.1 in artificial seawater (salinity 30 ± 1) prepared with commercial salt (Tropic Marin® SEA SALT from Tropic Marine Center) and freshwater purified by reverse osmosis. From the fifth day in the laboratory, mussels were fed every other day with 50µL/L of Oceanlife® solution (composed of 1 spoon of Oceanlife Coral Food®, 1mL of Oceanlife Phyto Marine®, and 4mL of deionized water for 100L) until the start of the experiment.

In the second week of acclimation, mussels were divided into tanks with different salinities to prevent abrupt salinity changes during the experiment. Mussels from the stock aquarium (30) were acclimated to various salinities as follows: for those acclimated to salinity 20, seawater of salinity 30 was diluted along 5 days, 2 units per day. Mussels acclimated to salinity 40 were placed in seawater of salinity 30, which was progressively concentrated along 5 days, also 2 units per day.

After 12 days of acclimation to the desired salinity, three mussels were placed in each experimental unit, which consisted of a glass aquarium containing 1.5L of artificial seawater (500mL/mussel). For each salinity level (20, 25, 30, 35 and 40), three aquaria were used, with three mussels each. Mussels in all experimental units were maintained under continuous aeration, and mortality was assessed daily; mussels were considered dead if they failed to close their shells in response to external stimuli. During the 14 or 28-day experimental period, mussels were fed every other day with a mixture of *Isochrysis* sp. (IsoPrime, PROVIRON), *Phaeodactylum* sp., and *Tetraselmis* sp. (Phyto Algae, AQUALGAE) at a 1:1:1 ratio, with a density of 150,000 cells/mussel. The water in each aquarium was fully replaced weekly, maintaining the respective salinity level and ratio of 500mL/mussel.

After 14 days of the experiment, one mussel from each aquarium were sacrificed and used to create four tissue pools: digestive gland, gills, muscle, and mantle (Fig 1). Shells were used to determine the length and width of the mussels. Tissue pools were stored at -80°C until biochemical analyses.

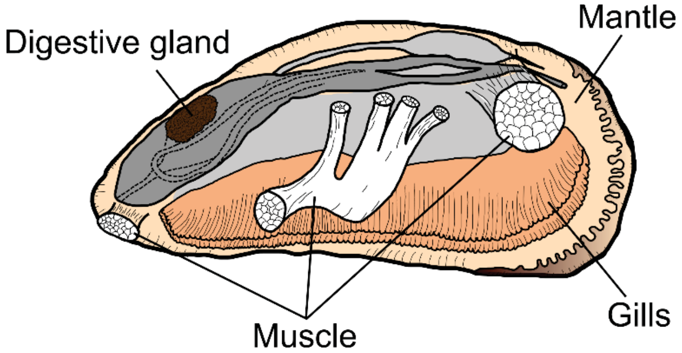

Figure 1. Anatomical diagram of *Mytilus galloprovincialis* illustrating the primary organs analyzed in this study, including the digestive gland, gills, mantle, and adductor muscle. These tissues were selected to evaluate the organism's physiological response as a whole under varying salinity conditions.

4.3.1 Biochemical markers

The biochemical biomarkers evaluated include proxies for metabolic activity: mitochondrial Succinate Dehydrogenase activity (SDH), Electron Transport System (ETS), glycogen (GLY) and protein contents (PROT); for the antioxidant system: Superoxide Dismutase (SOD), Glutathione Peroxidase (GPx), Glutathione Reductase (GR), Glutathione S-transferases activities (GSTs), and Total Antioxidant Capacity (TAC); and for cellular damage: activity of carboxylesterases (CbEs), Lipid Peroxidation (LPO), acetylcholinesterase activity (AChE).

Tissues were weighed and homogenized using a Potter-Elvehjem homogenizer (Sanches et al., 2023). Tissues used for the determination of GLY, PROT, SOD, GPx, GR, GST, TAC, CbEs, LPO and AChE were homogenized using phosphate buffer (50 mM potassium phosphate buffer with 1 mM EDTA, 1% Triton X-100, and 1 mmol/L DTT at pH 7.0) at ratios of 1:3 for gills and muscle, 1:2 for mantle, and 1:5 for digestive gland. Homogenates were then centrifuged at 10,000 *g* for 20 min at 4°C. For the ETS activity samples were homogenized using a TRIS buffer (0.1 mol/L Tris-HCl, 15% (w/v) polyvinylpyrrolidone (PVP), 153µmol/L magnesium sulfate ($MgSO_4$), and 0.2% (v/v) Triton X-100 at pH 8.5) at ratios of 1:3 for gills and muscle, and 1:5 for the digestive gland and mantle. Homogenates were centrifuged at 3,000 *g* for 20 min at 4°C. SDH was analyzed using a phosphate buffer (K_2HPO_4, 0.1 M pH 8.2) in ratios of 1:3 for gills and muscle, and 1:5 for the digestive gland and mantle. Homogenates were centrifuged at 3,000 *g* for 20 min at 4°C. The supernatants were then separated and stored at -80ºC, until used for the biomarker analyses.

SDH activity was assessed based on the ability of the succinate dehydrogenase system of active mitochondria to reduce MTT (3-[4,5-dimethylthiazol-2-y1]-2,5-diphenyltetrazolium bromide) to its water-insoluble form (formazan) using colorimetric analysis. Absorbance was read at 560 nm, and the amount of formazan formed was calculated using a molar extinction coefficient of 51,000 $M^{-1}cm^{-1}$, with results expressed in nmol per g of FW (van Meerloo et al., 2011; Bagchi et al., 2001). ETS activity was measured based on the protocol described by King and Packard (1975), modified by de Coen and Janssen (1997). The absorbance was recorded at 490 nm for 10 min at 25 s. The amount of formazan formed was calculated using $\varepsilon = 15{,}900\ M^{-1}\ cm^{-1}$, and the results were expressed in nmol per min per g fresh weight (FW). GLY was measured using the sulfuric acid method (Dubois et al., 1956). Glucose was used to prepare the standards (0–2 mg/mL). Final reactions were read at 492 nm. PROT was assessed using the Biuret method (Robinson & Hodgen, 1940). Bovine serum albumin was used as standard (0-40 mg/mL). Final reactions were read at 540 nm. GLY and PROT results are expressed in mg/g of FW.

Regarding the antioxidant system, SOD activity was determined using the method described by Beauchamp and Fridovich (1971), with modifications by Carregosa et al. (2014). Standards ranged from 0.25–60 U/mL of SOD. The activity was measured at 560 nm, and the results were expressed in units per mg of PROT. GPx was analyzed following the method described by Paglia and Valentine (1967). Absorbance was measured at 340 nm for 5 min, and readings were recorded every 15 s. The activity was expressed as nmol/min/mg PROT. The GR activity was determined using the method adapted from Carlberg and Mannervik (1985), with the oxidation of NADPH ($\varepsilon = 6.22$ mM^{-1} cm^{-1}) monitored at 340 nm. The results are expressed in nmol/min/mg PROT. GST activity was quantified according to the methodology described by Habig et al. (1974) with modifications by Carregosa et al. (2014). Activity was measured at 340 nm over a 5-min period, with readings taken every 15 s. The results are expressed in nmol/min/mg PROT. The TAC was measured according to the method described by Roma et al. (2024). Absorbance was recorded at 410 nm, and results were expressed as µmol of Trolox equivalent per mg of PROT.

For CbEs analysis, p-nitrophenyl acetate (pNPA) was used as the substrate, and the hydrolysis rate of pNPA was determined using a continuous spectrophotometric enzyme assay (Solé et al., 2018). Activity was measured at 405 nm for 5 min with 15/15 s readings. The results are expressed in nmol/min/mg PROT. LPO levels were measured according to the method of Buege and Aust (1978) by quantifying thiobarbituric acid-reactive substances (TBARS). The samples were incubated for 25 min at 96°C, and the absorbance was read at 532 nm ($\varepsilon = 1.56 \times 105$ M^{-1} cm^{-1}). LPO levels were expressed in nmol of MDA formed per g of FW. AChE activity was determined using acetylthiocholine iodide (ATChI, 5 mM) as the substrate following the method of Ellman et al. (1961) with modifications by Mennillo et al. (2017). AChE activity was measured continuously for 5 min at 412 nm and expressed in nmol per min per mg of PROT, using a molar extinction coefficient of 13,600 M^{-1} cm^{-1} for the yellow dianion of 5-thio-2-nitrobenzoic acid (TNB). Each original tissue homogenate sample was analysed in triplicates, for each biomarker assayed; absorbances were read using a microplate reader (SYNERGY H1 BioTek, United States).

4.3.2 Statistical analysis

To evaluate the differences in the biochemical profiles of *M. galloprovincialis* tissues, a Principal Coordinate Analysis (PCoA) was performed using a distance matrix and considering two main dimensions ($k = 2$). Initially, the biomarker data were standardized (mean = 0, standard deviation = 1) and the distance matrix was calculated using the Euclidean metric. The results were visualized in a two-dimensional graph, in which each point represents a sample and, additionally, confidence ellipses were generated for each tissue, to highlight the distribution of the data in the multidimensional space.

The data of all biomarkers were separated into three functional groups: metabolism biomarkers (SDH, ETS, PROT and GLY), antioxidant system biomarkers (SOD, GPx, GR, GSTs and TAC) and cell damage biomarkers (CbEs, AChE and LPO). The biomarkers of each functional group were analyzed separately following the steps: (1) PCA to evaluate the contribution of each biomarker and the variation of the tissues, (2) PERMANOVA to quantify the influence of the experimental factors (time and salinity) and (3) for the biomarkers with the greatest contribution (loading value) and for the tissues with the greatest dispersion in the PCA, the Student's t-test (for parametric data) and Wilcoxon (for non-parametric data) were used to identify which was and where the effect of time and salinity was observed.

To evaluate the effect of time, it was tested whether the biomarker had different responses between 14 and 28 days at each salinity (20, 25, 30, 35 and 40). To evaluate the effect of salinity, it was tested whether the salinities 20, 25, 35 or 40 were different from the control salinity (30) for 14 and 28 days, separately. The tests and graphs were performed using R language and the RStudio application (R Core Team, 2023). Values of $p<0.05$ were considered significant. For PCoA, the vegan package (Oksanen et al., 2022) was used, and for PCA, the prcomp function of R base was used. To test statistical differences, the Student’s T-test and the Wilcoxon test were used, both implemented in the R stats package. The ggplot2 (Wickham, 2016) package and the InkScape software were used to view and edit the graphs.

4.4 RESULTS

4.4.1 PCoA and PERMANOVA with tissues, salinities and biomarkers

The PCoA based on the Euclidean distance matrix revealed a clear differentiation between the tissues (digestive gland, gills, mantle and muscle). The first axis (PCoA1) explained 41.26% of the variation in the data, while the second axis (PCoA2) explained 16.36%, totaling 57.62% of the variability. The PERMANOVA analysis demonstrated that the variation in the biochemical profiles was explained by all factors analyzed ($p < 0.001$). The tissue factor was the main determinant of the variation in the data ($R^2 = 63.0\%$, $p < 0.001$), followed by salinity ($R^2 = 2.3\%$, $p < 0.001$) and exposure time ($R^2 = 1.5\%$, $p < 0.001$) (Fig. 2).

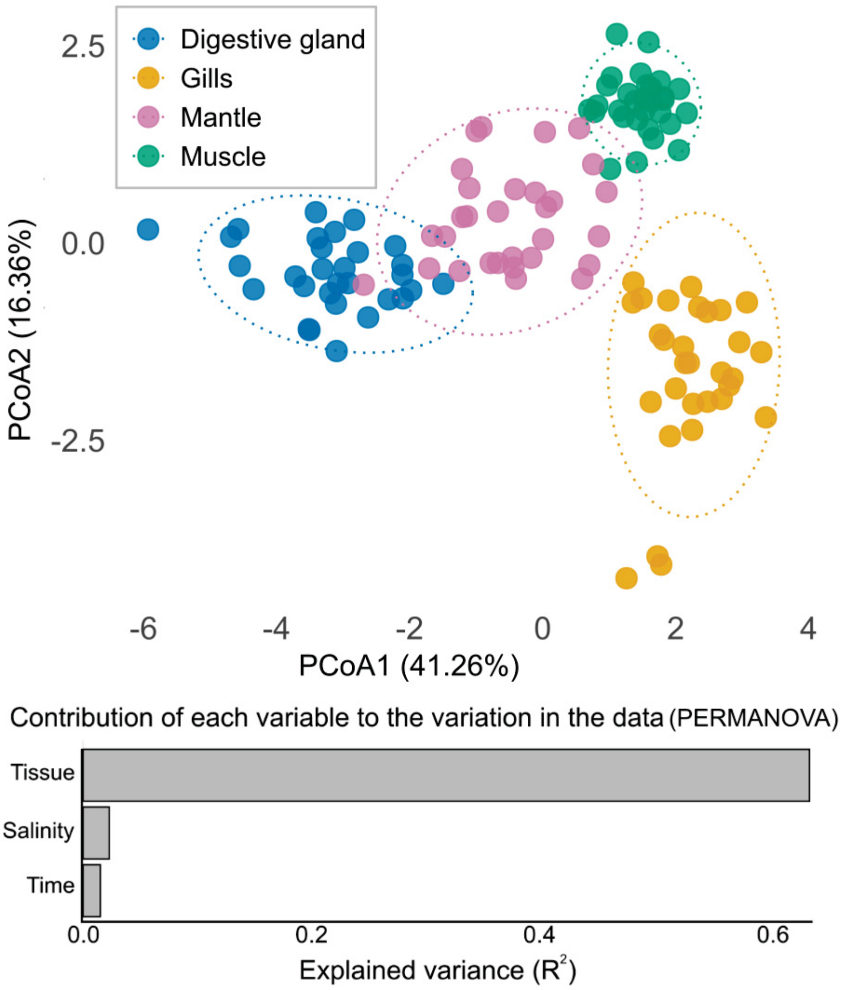

Figure 2. Principal Coordinates Analysis (PCoA) based on the Euclidean distance matrix of the biochemical responses of tissues (digestive gland, gills, mantle and muscle) of Mytilus galloprovincialis exposed to different salinities (20, 25, 30, 35 and 40) for 14 or 28 days. The points represent individual samples, and the ellipses indicate groupings by similarity. The bar graph below the PCoA shows the contribution of the variables tissue, salinity and exposure time to the variation in the data, according to PERMANOVA analysis.

4.4.2 Metabolic biomarkers

The PERMANOVA analysis revealed that metabolic biomarkers were mainly influenced by tissue type ($R^2 = 84.06\%$, $p < 0.001$). In addition, both exposure time ($R^2 = 1.14\%$, $p < 0.001$) and salinity ($R^2 = 1.24\%$, $p = 0.020$) showed significant effects, although to a lesser extent. The interaction between time and salinity was also significant ($R^2 =$

1.70%, p = 0.003), suggesting that the effect of salinity varied depending on the exposure time.

The first principal component (PC1) explained 78.66% of the variation in the data, while the second component (PC2) explained 11.01%, totaling 89.68% of the variance captured by the first two axes (Fig. 3). The mantle and digestive gland presented greater dispersion and are more spread out along PC1, indicating greater variation in these organs. The gills and muscle, on the other hand, appear to be less influenced by these biomarkers. Therefore, only the digestive gland and mantle data were evaluated for the effect of time and salinity. All metabolic biomarkers had similar values of contribution to the data variation (loadings: SDH = -0.526, ETS = -0.49, PROT = -0.52 and GLY = -0.45). Therefore, for all metabolic biomarkers, paired tests were performed to evaluate the effect of time and salinity.

In the digestive gland, mussels exposed to salinities 20 and 30 had higher SDH activity after 28 days of exposure compared to 14 days of exposure (p = 0.0199 for salinity 20 and p = 0.0185 for salinity 30). The opposite was observed at salinity 40, with SDH activity higher after 14 than after 28 days (p = 0.0158). After 14 days of exposure, SDH activity at salinities 20, 25 and 35 was lower than at the control salinity (30) (p = 0.0311, 0.0421 and 0.0213, respectively). ETS activity and PROT levels had no significant effect of either time or salinity (p > 0.05 for all comparisons). GLY had no effect of time (p > 0.05). After 28 days of exposure, mussels exposed to salinity 35 had higher GLY levels than control mussels (30 salinity, p = 0.0107).

In the mantle, SDH and ETS activity were not affected by time or salinity. PROT levels were influenced by time at salinities 25 and 35, being higher after 28 days of exposure compared to 14 days. After 28 days, mussels exposed to salinities 25 (p = 0.042) and 35 (p = 0.014) had higher PROT levels than those in the control (30). GLY levels showed an effect of time at salinities 25 and 30, being higher after 14 days than after 28 days of exposure. Salinity had an effect only in mussels exposed for 14 days, with lower GLY levels at salinities 20 (p = 0.040) and 35 (p = 0.039) than in the control (30).

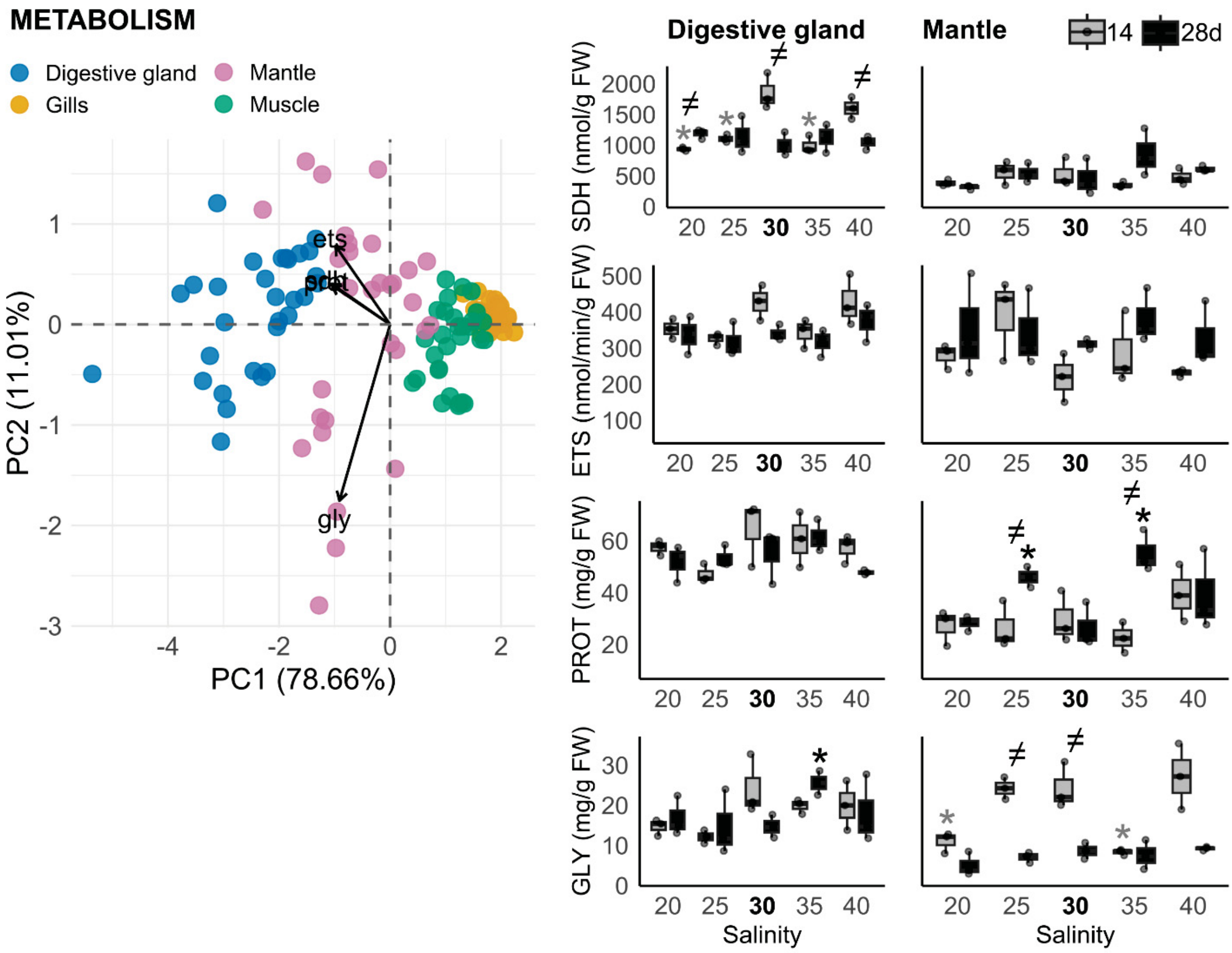

Figure 3. Principal Component Analysis (PCA) of the responses of metabolic biomarkers (ETS, SDH, PROT and GLY) in the tissues (digestive gland, gills, mantle and muscle) of *Mytilus galloprovincialis* exposed to different salinities (20, 25, 30, 35 and 40) for 14 or 28 days. On the left, the PCA shows the distribution of tissues and the variation in metabolic biomarkers. Arrows indicate the variables most correlated with the principal components (PC1 and PC2). On the right, boxplots represent the variation of biomarkers in the digestive gland and mantle for the exposure times of 14 days (gray) and 28 days (black). The symbol # indicates significant differences between 14 and 28 days within the same salinity. The symbol * (gray) indicates significant differences between the given salinity (20, 25, 35 or 40) and the control salinity (30, bold) in the 14-day data, while the symbol * (black) indicates significant differences for the same contrasts in the 28-day data.

4.4.3 Antioxidant system

PERMANOVA analysis revealed that antioxidant biomarkers were mainly influenced by tissue type ($R^2 = 67.78\%$, $p < 0.001$). Both exposure time ($R^2 = 1.19\%$, $p = 0.012$) and salinity ($R^2 = 1.47$ $p = 0.002$) showed significant effects, although to a lesser extent. The interaction between time and salinity was also significant ($R^2 = 1.23\%$, $p = 0.009$), indicating that the effect of salinity may vary depending on the exposure time.

The first principal component (PC1) explained 34.47% of the variation in the data, while the second component (PC2) explained 28.56%, totaling 63.03% of the variance captured by the first two axes (Figure X). Digestive gland and mantle showed greater dispersion along PC1, suggesting greater variation in antioxidant biomarkers in these tissues. Gills and muscle showed less variation in these components. Therefore, only the effects of time and salinity were evaluated for digestive gland and mantle tissues. Among the antioxidant biomarkers, GR showed the greatest contribution to data variation in PC1 (loading = 0.61), followed by SOD (0.56), GPx (0.28), GSTs (0.15) and TAC (-0.44). In PC2, GPx had the greatest influence (loading = 0.63), followed by SOD (-0.37). Based on these results, paired statistical tests were performed to evaluate the effect of time and salinity on GR, SOD, GPx and TAC in the tissues with greater variation (digestive gland and mantle).

In the digestive gland, mussels exposed to salinities 25 and 35 had higher SOD activity after 28 days of exposure compared to 14 days of exposure ($p = 0.037$ for salinity 25 and $p = 0.015$ for salinity 35). After 28 days of exposure, SOD activity was higher at salinity 35 than at the control salinity (30, $p = 0.079$). GPx and GR enzyme activity had no significant effect of time or salinity ($p > 0.05$ for all comparisons). TAC had no effect of time ($p = 0.140$) and, after 28 days of exposure, was higher at salinity 35 than at the control (30, $p = 0.082$).

In the mantle, SOD and GPx activity had no effect of time or salinity ($p > 0.05$). GR had a time effect at all salinities except salinity 35 ($p = 0.108$). At salinities 20 and 25, GR activity was higher after 14 days than after 28 days ($p = 0.005$ and $p = 0.004$, respectively). At salinities 30 and 40, GR activity was higher after 28 days of exposure than after 14 days ($p = 0.012$ and $p = 0.01$, respectively). After 14 days of exposure, GR activity was higher at salinities 20 and 25 than in the control (30) ($p = 0.005$ and $p = 0.004$). After 28 days of exposure, GR activity was lower at all salinities tested (20, 25, 35 and 40) than in the control (30) ($p < 0.05$). TAC was affected by time at salinity 35, being higher after 28 days of exposure than after 14 days of exposure ($p = 0.003$). After 28 days of exposure, TAC was higher at salinity 35 than in the control (30) ($p = 0.03$).

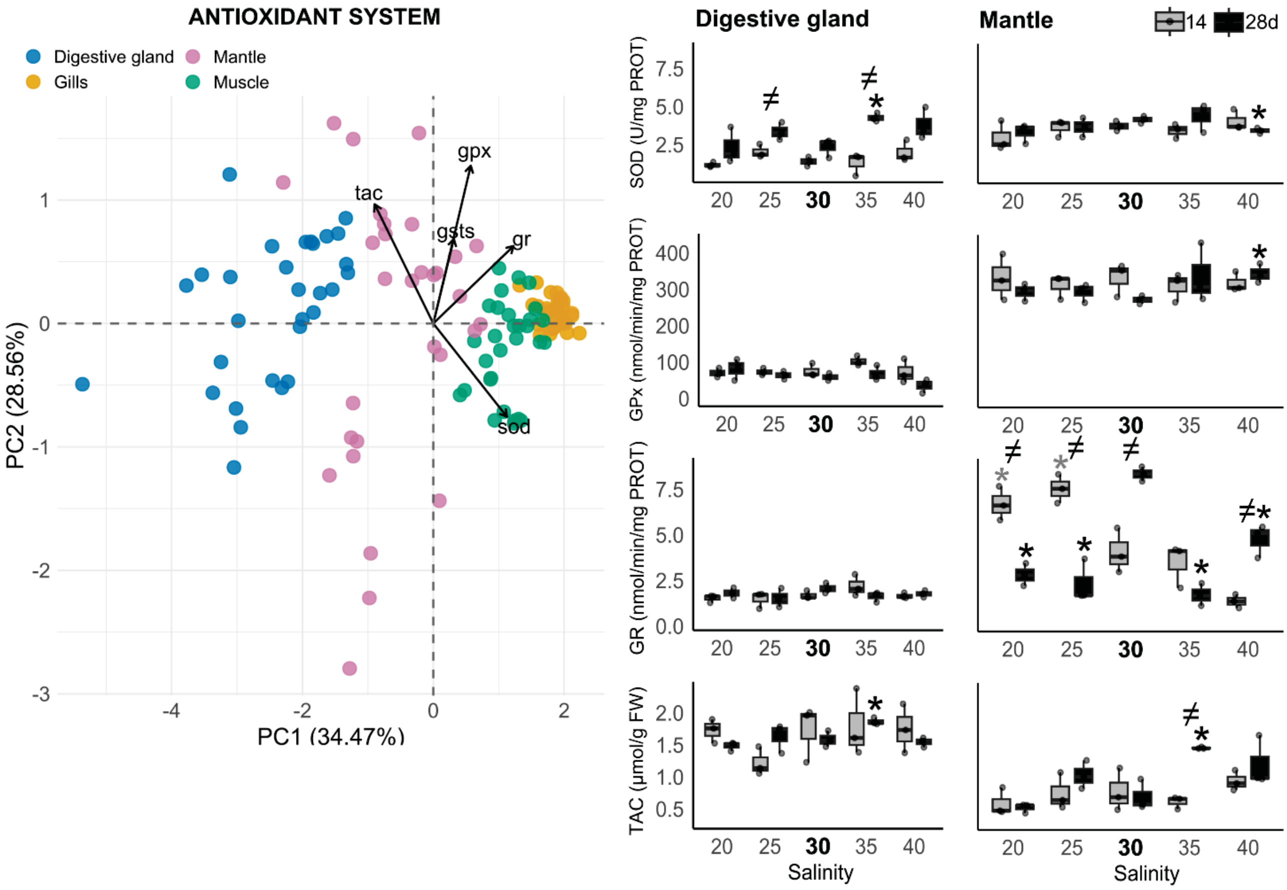

Figure 4. Principal Component Analysis (PCA) and variation of antioxidant biomarkers (SOD, GPx, GR and TAC) in tissues (digestive gland and mantle) of Mytilus galloprovincialis exposed to different salinities (20, 25, 30, 35 and 40) for 14 or 28 days. On the left, the PCA illustrates the separation of tissues based on the variation of antioxidant biomarkers, with arrows indicating the variables most correlated with the principal components (PC1 and PC2). On the right, boxplots show the variation of biomarkers in the digestive gland and mantle tissues for the exposure times of 14 days (gray) and 28 days (black). The symbol # indicates significant differences between 14 and 28 days within the same salinity. The symbol * (gray) indicates significant differences between the given salinity (20, 25, 35 or 40) and the control salinity (30, bold) in the 14-day data, while the symbol * (black) indicates significant differences for the same contrasts in the 28-day data.

4.4.4 Cellular damage biomarkers

The PERMANOVA analysis revealed that cellular damage biomarkers were mainly influenced by tissue type ($R^2 = 71.29\%$, $p < 0.001$). In addition, both exposure time ($R^2 = 1.24\%$, $p = 0.017$) and salinity ($R^2 = 1.31\%$, $p = 0.008$) showed significant effects, although to a lesser extent. However, the interaction between time and salinity was not significant ($R^2 = 0.42\%$, $p = 0.153$), suggesting that the effect of these factors occurs independently.

The first principal component (PC1) explained 50.99% of the variation in the data, while the second component (PC2) explained 33.17%, totaling 84.16% of the variance captured by the first two axes (Fig. 5). The digestive gland and gill tissues showed greater dispersion along PC1, indicating greater variation in the biomarkers of cellular damage in these organs. Muscle and mantle showed less variation in these components. All biomarkers of cellular damage showed a high contribution to the data variation. LPO (-0.70) and CbEs (-0.68) had a greater influence on PC1, while AChE had a more pronounced contribution on PC2 (-0.97). Thus, paired statistical tests were performed for CbEs, AChE and LPO in the tissues with greater variation (digestive gland and gills) to evaluate the effects of time and salinity.

In the digestive gland, CbEs activity was affected by time ($p > 0.05$). After 14 days of exposure, mussels exposed to salinity 25 showed higher levels of this biomarker compared to the control ($p = 0.032$). AChE activity was not influenced by time or salinity ($p > 0.05$). LPO levels were affected by time at salinity 35, with higher values after 28 days of exposure ($p = 0.0003$). In the gills, CbEs activity was affected by time at salinity 20, being higher after 14 days of exposure than after 28 days ($p = 0.028$). After 14 days, CbEs activity was higher at salinities 20 and 40 compared to the control (30, $p = 0.0043$ and $p = 0.0473$, respectively). AChE activity had a significant effect of time at salinity 25, being higher after 14 days than after 28 days of exposure ($p = 0.0017$). After 28 days of exposure, AChE activity was higher at salinity 40 than in the control (30, $p = 0.044$). LPO levels were affected by time at salinity 25, with higher values after 14 days compared to 28 days of exposure ($p = 0.0078$). After 14 days of exposure, LPO levels were higher at salinity 25 than in the control (30, $p = 0.0045$).

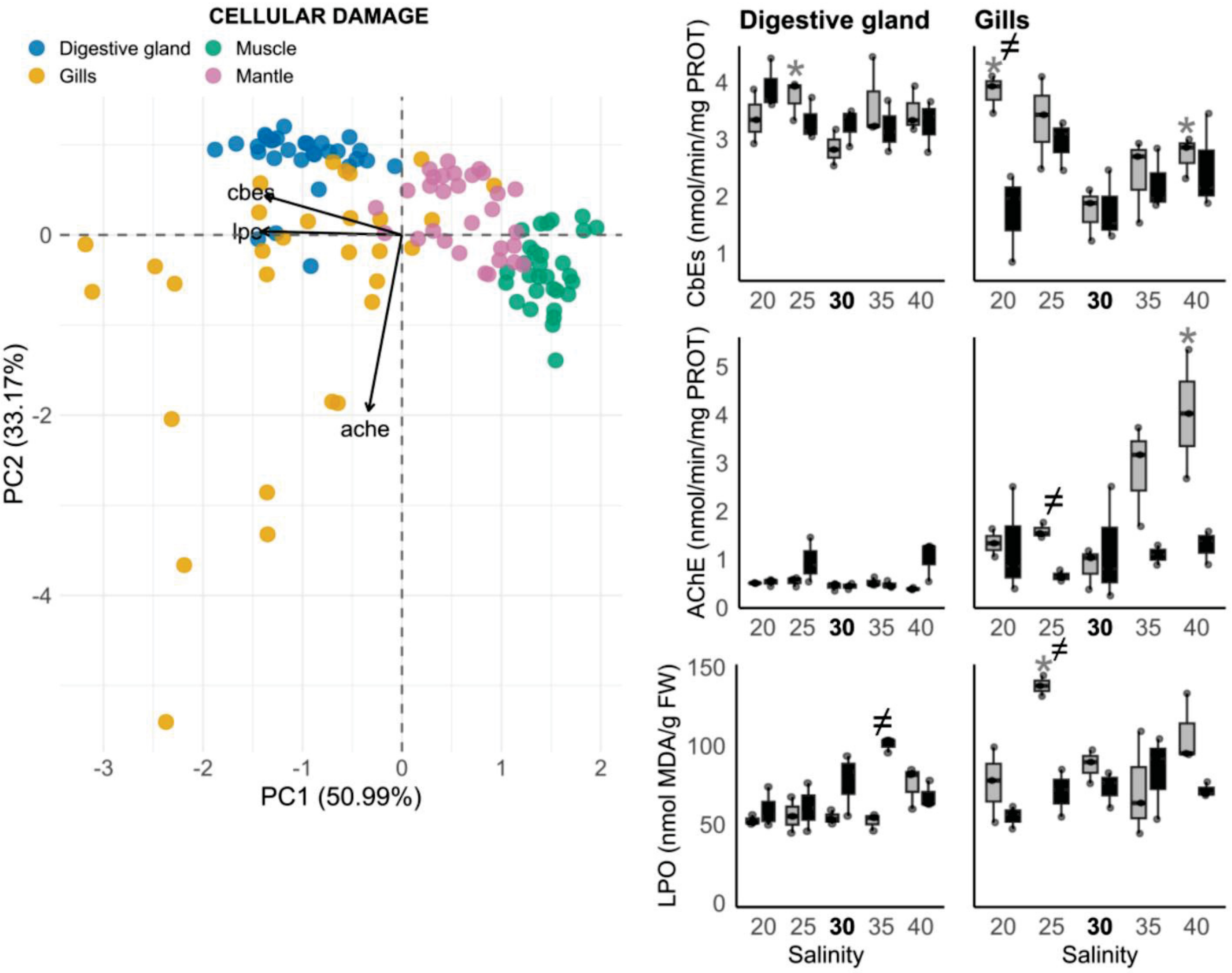

Figure 5. Principal Component Analysis (PCA) and variation of cell damage biomarkers (CbEs, AChE and LPO) in tissues (digestive gland and gills) of Mytilus galloprovincialis exposed to different salinities (20, 25, 30, 35 and 40) for 14 or 28 days. On the left, the PCA shows the separation of tissues and the variation of cell damage biomarkers, with arrows indicating the variables most correlated with the principal components (PC1 and PC2). On the right, boxplots show the variation of biomarkers in digestive gland and gill tissues for the exposure times of 14 days (gray) and 28 days (black). The symbol # indicates significant differences between 14 and 28 days within the same salinity. The symbol * (gray) indicates significant differences between the given salinity (20, 25, 35 or 40) and the control salinity (30, bold) in the 14-day data, while the symbol * (black) indicates significant differences for the same contrasts in the 28-day data.

4.5 DISCUSSION

This study demonstrates that *M. galloprovincialis* exhibits distinct tissue-specific biochemical responses to salinity variations. This reflects the distinct physiological roles of different organs, aligning with their functional specialization in marine bivalves (Wu et al., 2013; Chen et al., 2018). The observed responses align with previous studies that emphasize the importance of evaluating multiple tissues to capture the complexity of physiological adaptations in marine organisms (Fang et al., 2012; Wu et al., 2013; Chen et al., 2018; Yu et al., 2023).

4.5.1 Metabolic adjustments

Salinity fluctuations influence metabolic processes in marine mussels, affecting energy production, nutrient storage and enzyme activity (Deaton, 2009; McCarthy et al., 2013; Maar et al., 2015; Haider et al., 2018). As part of mitochondrial complex II, SDH catalyzes the conversion of succinate to fumarate, facilitating electron transfer to ubiquinone and directly contributing to ATP generation. The activity of this enzyme is essential for the physiological adaptation of marine organisms to environmental variations, modulating energy production (Orlov et al., 2023). The increase in SDH activity at salinities 20 and 30 after 28 days suggests an adaptive metabolic adjustment, while the decline at salinity 40 indicates possible metabolic suppression due to more extreme salinity conditions. These findings align with previous studies showing metabolic reconfiguration under environmental changes in bivalves (Mejdoub et al., 2017; Cunha et al., 2023; Cruz et al., 2023). Comparable responses have been observed in other marine bivalves, where the digestive system consistently exhibits greater metabolic responsiveness under environmental stressors (Mejdoub et al., 2017; Cunha et al., 2023; Cruz et al., 2023).

The mantle showed low SDH activity and, although not significant, there is a suggestion that the ETS is higher at salinities 25 and 35 than in the control, evidencing a strategy at “suboptimal” salinities (different from the control, but not detrimental). ETS acts in the conversion of chemical energy from respiratory substrates into ATP through oxidative phosphorylation (Missaglia et al., 2021). This process is essential for the generation of the electrochemical proton gradient, which drives the activity of ATP synthase (Complex V) and allows the production of ATP, ensuring energy homeostasis in organisms subjected to dynamic environmental conditions (Sokolova, 2018).

Protein levels play a role in the regulation of energy metabolism, nutrient transport and defense against oxidative stress, and are often modulated by factors such as variations in temperature, salinity and oxygen availability (López-Pedrouso et al., 2020). The observed increase in protein content in the mantle at salinities 25 and 35 suggests intensified protein synthesis, potentially as part of physiological adaptation strategies (Tremblay et al., 1998). GLY, in turn, is the main energy reserve of bivalves. In organisms exposed to environmental stressors, the decrease in glycogen stores is often associated with a higher metabolic demand for maintaining homeostasis, while its accumulation occurs under more favorable conditions, when there is a lower need for energy mobilization (Almeida et al., 2020). The depletion of GLY levels over time observed in this study may reflect the stress of long-term maintenance of mussels in the laboratory.

Muscle tissue exhibited the most stable metabolic responses, consistent with its role in the organism and its indirect exposure to external water. Muscle tissues generally exhibit stable metabolic activity, which likely reflects their role in maintaining basic movements and protective function through escape behavior (valve closure) to avoid direct exposure to the external environment (Stakowian and Freire, 2024; Mejdoub et al., 2017; Lompré et al., 2023).

4.5.2 Antioxidant system responses

Antioxidant enzymes evolved in parallel with the increase in Earth’s atmospheric oxygen around two billion years ago. These enzymes protect their substrates, which are predominantly proteins but also include other macromolecules (e.g., DNA and RNA), from oxidative damage (Kultz, 2020). Active oxidants or free radicals, such as superoxide radicals, hydroxyl radicals, and H2O2, which are derived from oxygen, are collectively called Reactive Oxygen Species (ROS) (Bal et al., 2021). When protection against oxidants fails, cells experience stress, commonly referred to as oxidative stress. SOD, GPX, GST, and GR act cooperatively and synergistically to scavenge ROS (Yan et al., 2008).

The digestive gland exhibited significant variations in SOD activity, with highest levels observed at salinities of 25 and 35 after 28 days. SOD is one of the first lines of defense against oxidative stress. It catalyzes the conversion of superoxide radical (O_2^-) into hydrogen peroxide (H_2O_2) and molecular oxygen (O_2), thereby reducing oxidative damage to biomolecules (Culotta, 2001; Obeme-Nmom et al., 2024). This increase suggests enhanced neutralization of reactive oxygen species (ROS) in the digestive gland under these long-term conditions.

The enzyme GPx acts by removing hydrogen peroxide generated by SOD and reducing lipid hydroperoxides, protecting cell membranes against lipid peroxidation. This enzyme uses reduced glutathione (GSH) as a substrate to neutralize peroxides, forming oxidized glutathione (GSSG) as a product (Cunha et al., 2024). GR, in turn, is responsible for regenerating GSH from its oxidized form (GSSG), using NADPH as an electron donor (Pannala et al., 2013). In the present study, GPx showed no significant changes, although it is more active in the mantle than in the digestive gland, under all conditions. GR also had greater activity in the mantle than in the digestive gland, but with a strong effect of time and salinity, with greater activity at salinities 20 and 25 after 14 days. This suggests an early response to oxidative stress, which declined after prolonged exposure, since after weeks the bivalves had time to reach acclimatization. The enzyme GSTs had minimal activity in this study. Its main function is detoxification, through the conjugation of glutathione to toxic compounds, increasing their solubility and facilitating excretion

(Dourado et al., 2008). Since salinity is not a pollutant or xenobiotic, it makes sense that GSTs were not active in this study. Overall, the significant increase in TAC in both tissues with the highest variation (digestive gland and mantle) at salinity 35 after 28 days indicates a positive regulation of antioxidant defenses.

Mediterranean mussels (*M. galloprovincialis*) exposed to fluctuating salinities (6, 10, 14, 24, and 30) showed increased antioxidant defenses in gill tissues, particularly under hypersaline conditions, suggesting that their antioxidant system is highly adaptive to osmoregulatory stress (Andreyeva et al., 2024). In this study, the gills, a critical tissue directly exposed to seawater through filtration, had minimal antioxidant responses compared to other tissues. This was also the case in muscle, supporting its secondary role in managing oxidative stress observed in marine and estuarine invertebrates (Pati et al., 2023).

4.5.3 Cellular damage biomarkers

Cellular damage biomarkers such as CbEs, AChE, and LPO provide insights into the physiological cost of prolonged salinity adjustments. CbEs are a class of enzymes from the hydrolase superfamily, whose main function is to catalyze the hydrolysis of fatty acid esters, allowing the degradation of endogenous and exogenous substrates (Cunha et al., 2024). Changes in salinity can alter the energetic demands of bivalves, triggering metabolic changes that involve increased lipid metabolism and activation of ester-hydrolyzing enzymes. Although salinity itself is not a toxicant, it can induce physiological stress that leads to increased production of reactive metabolites. In this sense, CbEs play a role in the detoxification of endogenous metabolic byproducts that can accumulate under osmotic stress (Singh et al., 2021). The digestive gland is an important site of energy storage, and the activation of CbEs in this tissue may be linked to the breakdown of energy reserves (e.g., glycogen and lipids) in response to increased energy demands (Thompson et al., 1974).

The enzyme AChE, in turn, regulates neurotransmission in marine organisms. Its main function is to hydrolyze acetylcholine (ACh) in the synaptic cleft, allowing the completion of the nerve signal and the restoration of the neuromuscular system (Behra et al., 2002; Umar and Aisami, 2020). In marine mussels, such as M. galloprovincialis, AChE activity is predominantly localized in the gills, while CbEs show greater activity in the digestive glands (Escartín & Porte, 1997), in agreement with the pattern observed here. In the gills, CbE activity was higher under hyposaline conditions, while AChE was more active under hypersaline conditions, indicating different strategies for hypo- and hypersaline conditions in this tissue (Luchmann et al., 2014; Chen et al., 2018). These enzymatic responses highlight the plasticity of bivalves in dealing with environmental variability and the difference in responses to hypo- and hypersaline challenges (Deaton, 2009; Pourfmozafar et al., 2020).

High levels of LPO at salinity 25 and 35 in the digestive gland and gills, respectively, indicate increased lipid peroxidation, reflecting some level of oxidative damage to cell membranes (Kultz, 2020). LPO is characterized by the degradation of polyunsaturated fatty acids in cell membranes due to the action of reactive oxygen species (ROS), leading to the formation of highly reactive aldehydes, such as 4-hydroxynonenal (4-HNE) and malondialdehyde (MDA) (Alam et al., 2022). These products interact with proteins and DNA, promoting structural and functional modifications that can result in apoptosis. In fish and marine invertebrates, LPO has been associated with mitochondrial dysfunction and impaired cell membrane permeability, affecting essential physiological processes such as cellular respiration and redox signaling (Bal et al., 2021; Shastak & Pelletier, 2023; Cunha et al., 2024). Another marine bivalve, *Anadara broughtonii*, also showed an increase in LPO triggered by osmotic stress, highlighting its role as a biomarker of oxidative damage due to salinity fluctuations (An & Choi, 2009). In mussels (*M. galloprovincialis*) collected from five sites along the coast of Casablanca (Morocco), the highest LPO levels were also observed in the gills and hepatopancreas (functionally analogous to the digestive gland), particularly in the most polluted sites (Mejdoub et al., 2017). These tissues, due to their direct interaction with the environment and their active roles, appear to have a high susceptibility to oxidative damage (Matozzo et al., 2016).

The digestive gland and gills have emerged as highly sensitive to changes in salinity and are a good tissue to be used as an indicator of metabolism and oxidative stress in bivalves. The relatively stable metabolic profile of muscle tissue and the lower direct exposure to environmental water reduce its oxidative stress load, explaining the minimal activation of biomarkers in this tissue. The mantle, as it is composed mainly of gonadal tissue in the Mytilidae family, may have its variation more related to gametogenic processes and gamete release, not being a good tissue indicator of metabolism and oxidative damage caused by variations in salinity.

4.5.4 Osmoconformer and allostasis concept

A key question in the physiology of osmoconforming animals is: what constitutes stress? Unlike osmoregulators, which must actively control ionic balance, osmoconformers such as M. galloprovincialis adjust metabolically and structurally to environmental fluctuations (Deaton, 2009; Larsen et al., 2014; Pourmozaffar et al., 2020), making them excellent models for understanding the concept of allostasis. In this study, no mussels died during the experiment in the 20-40 salinity range. Growth, reproduction, or tissue health were not quantified, but the physiological adjustments were sufficient to maintain the organism's life, which is its primary function (Sokolova et al., 2012). This suggests that the salinity levels tested were not lethal and that these responses represent physiological adjustments.

Coined by Sterling and Eyer (1988), allostasis refers to “stability through change” rather than “stability through constancy” (which is the basis of homeostasis). Unlike homeostasis, which aims to maintain physiological parameters within a fixed range, allostasis recognizes that an organism anticipates and adapts to stressors by adjusting physiological systems accordingly (Schulte, 2014; Freire et al., 2020). This helps to differentiate beneficial adjustments from maladaptive chronic stress, avoiding overgeneralization of responses as “stress” when they may simply be adaptive changes. Adjustments in metabolism, antioxidant defenses, and enzymatic activities allow mussels to function within their salinity tolerance range without entering a detrimental state of stress (Deaton 2009; Schulte, 2014; Pourmozaffar et al., 2020; Stakowian and Freire, 2024). This is a key characteristic for an osmoconformer of estuaries and intertidal waters.

In this study, *M. galloprovincialis* at suboptimal salinities (25 and 35) exhibited metabolic and antioxidant adaptations, reaching a new equilibrium. Even at extreme salinities (20 and 40), metabolic suppression was observed, but these responses do not necessarily indicate stress but may reflect energy conservation strategies. Although a long-term metabolic decrease can be detrimental to the population, the ability to dynamically regulate physiological processes and ensure survival in highly stressful environments highlights the plasticity of osmoconformers and challenges traditional definitions of stress (Schulte, 2014; Freire et al., 2020).

4.5.5 Conclusions

Mytilus galloprovincialis exhibits optimal metabolic performance in the salinity range of 30-35. Exposure to 20 and 40 salinities induced metabolic challenges, evidenced by reductions in glycogen levels, indicating increased energy cost, or depletion of metabolism, leading to similar or lower biochemical responses than the control. Our results reinforce the greater sensitivity of bivalves to hypo- than hypersaline stress, which is in line with the evolutionary history of the group (Deaton, 2009; Pourmozaffar, 2020). At "suboptimal" salinities (specifically 25 and 35), mussels adjusted their metabolism and antioxidant system to reach a new state of equilibrium with the environment (allostasis), leading to physiological adaptations marked by increased biomarkers of CbEs, AChE and LPO.

This paper investigates for the first time the tissue profile of biochemical responses to a salinity gradient in bivalves and relates it to the concept of allostasis. This study reiterates the critical role of salinity fluctuations in shaping stress responses and, beyond the specific case of *M. galloprovincialis*, provides broader insights into the physiological and biochemical adaptations of marine invertebrates to salinity changes. Given the accelerated impact of climate change on coastal ecosystems, the ability of osmoconforming species to dynamically regulate physiological processes should be recognized within the concept of allostasis. Integrating this perspective will improve our understanding of how osmoconforming animals adapt to environmental variability and will inform conservation strategies to preserve biodiversity in rapidly changing habitats.

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5 CONCLUSÃO GERAL

O Capítulo 1 teve como foco o berbigão *Anomalocardia flexuosa*, um bivalve de importância econômica ao longo de toda a costa brasileira. Experimentos de laboratório demonstraram que entre 22 e 34 os indivíduos conseguem realizar atividades essenciais, como escavação, e preservar o equilíbrio osmótico e a hidratação tecidual. Em salinidades inferiores a 22, poucos indivíduos de enterraram e a valvas permaneceram fortemente fechadas, o que limita a respiração, a alimentação e a eliminação de excretas, levando a uma supressão do metabolismo como um todo. O resultado do nicho salino realizado sugere que a maioria das populações naturais de *A. flexuosa* ocorrem em salinidades marinhas (>30), indicando uma adaptação local da população estudada no Complexo Estuarino de Paranaguá (CEP) e, provavelmente, das populações de outros estuários ao longo de sua distribuição. Isso é corroborado pelo experimento de regulação da hidratação tecidual, que revelou que berbigões da parte interna do CEP (expostos a maior variação de salinidade) têm maior capacidade de regular a hidratação tecidual do que populações da desembocadura do CEP (expostos a maior influência marinha).

A modelagem de nicho ecológico projetou para o final deste século (em um cenário de altas emissões) um aumento na adequabilidade do habitat em áreas mais ao norte do país e uma diminuição nas regiões sudeste. Esse resultado pode estar relacionado ao aumento de eventos extremos de precipitação nas regiões sudeste e sul, que reduzem a salinidade em áreas estuarinas, ultrapassando os limites de tolerância da espécie. Esse capítulo alerta que as mudanças climáticas, ao intensificar condições hipossalinas em estuários, podem aumentar os episódios de mortalidade em massa dos berbigões, especialmente no sudeste e sul do Brasil. Esses dados podem ser utilizados como referencial para o manejo sustentável e a mitigação dos impactos climáticos.

O Capítulo 2 utiliza modelagem de nicho ecológico e análise de agrupamentos climáticos para avaliar a distribuição do mexilhão *Mytilus galloprovincialis* no Oceano Atlântico. Os resultados revelaram que a espécie ocupa diferentes "grupos climáticos", caracterizados por variações no pH, salinidade e temperatura. Esses agrupamentos sugerem adaptação local da espécie ao longo da sua ampla distribuição e destacam a plasticidade fisiológica de *M. galloprovincialis*, que faz jus ao seu potencial invasivo. O algoritmo BIOCLIM projetou para o final deste século (em um cenário de emissões moderadas) uma redução global de áreas adequadas para *M. galloprovincialis*, com maior impacto no Atlântico Norte, área nativa da espécie. Esses resultados indicam que as mudanças climáticas globais podem limitar a capacidade de invasão do mexilhão-azul, mas, paradoxalmente, ameaçar populações nativas, onde a espécie é um importante recurso pesqueiro.

O **Capítulo 3** investigou as respostas fisiológicas de *Mytilus galloprovincialis* à variação de salinidade (20, 25, 30, 35 e 40) por meio da análise de biomarcadores bioquímicos em diferentes tecidos (glândula digestiva, brânquias, músculo e manto). Os resultados demonstraram que a glândula digestiva e as brânquias foram os melhores tecidos indicadores das adaptações às mudanças na salinidade, refletindo ajustes no metabolismo energético, no sistema antioxidante e nos danos celulares. Essas respostas variaram de acordo com a salinidade e o tempo de exposição, indicando que os desafios osmóticos podem provocar uma resposta fisiológica tecido-específica para preservar a homeostase. Os achados deste capítulo ressaltam a importância de considerar múltiplos biomarcadores e diferentes tecidos ao avaliar as estratégias adaptativas de bivalves à variação de salinidade, fornecendo informações relevantes para a compreensão dos mecanismos fisiológicos de animais eurialinos e osmoconformadores que habitam estuários e a zona entremarés.

Essa Tese contribui para a compreensão das estratégias adaptativas de osmoconformadores à variação de salinidade ao integrar fisiologia, comportamento e modelagem ecológica para compreender como bivalves epi- e infaunais respondem às variações de salinidade em regiões costeiras sob a influência das mudanças climáticas. Entre as principais contribuições desta pesquisa, destacam-se a definição experimental do nicho salino fundamental para *A. flexuosa*, com evidências de que salinidades abaixo de 22 comprometem o comportamento e a osmorregulação; a validação da plasticidade fisiológica e da sensibilidade tecidual de *M. galloprovincialis* frente a diferentes salinidades, com destaque para brânquias e glândula digestiva como tecidos sentinelas; o uso inédito de biomarcadores bioquímicos em conjunto com a modelagem de nicho correlativa para avaliação da vulnerabilidade de espécies osmoconformadoras às mudanças climáticas; a projeção de áreas adequadas ao longo do Oceano Atlântico para as espécies estudadas, considerando cenários climáticos futuros.

Esta Tese também enfrentou limitações, principalmente quanto a escassa disponibilidade de dados ambientais específicos para zonas costeiras e de entremarés, especialmente em bases globais como Bio-Oracle e WorldClim, que priorizam variáveis marinhas (coluna d’água) ou atmosféricas (temperatura do ar). Tais dados nem sempre refletem com precisão as condições vivenciadas por organismos sésseis-bentônicos no entremarés, como a variação de salinidade em sedimentos ou camadas superficiais após eventos de chuva. Como consequência, a modelagem de nicho pode superestimar ou subestimar a adequabilidade de hábitats para essas espécies, cuja distribuição depende fortemente de micro-hábitats e da capacidade de comportamento de escape.

Portanto, o presente trabalho reforça a necessidade urgente de se desenvolver bases de dados ambientais mais representativas de ambientes costeiros rasos, com alta resolução espacial e temporal, para que modelos ecológicos possam refletir com maior fidelidade os riscos enfrentados por espécies costeiras frente às mudanças climáticas. A integração entre fisiologia e modelagem ecológica demonstrada nesta tese oferece caminhos promissores para pesquisas futuras, manejo adaptativo e conservação de recursos pesqueiros tradicionais ameaçados pela intensificação das pressões ambientais.

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