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Patologias que afetam o marisco branco *Mesodesma mactroides*

(Bivalvia: Mesodesmatidae)

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médico de homens e outros animais

e aos meus pais Carlos e Sandra.

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Urge escribir este poema

No puede más dentro de mi

Explota, arrasa

Como un niño

Quiere nacer

Amanecí en la playa

Desperté con el sol

Inmenso sol

Debatiéndose

Entre el cielo

Y la tierra

Debatiéndose

Entre la vida

O la muerte

Urge escribir lo que siento

Me siento leve

Como esa gaviota

Que vuela y se pierde dentro del sol

Me siento libre

Como el aire

Como el viento

Donde estas

Donde estuviste

En todo este largo camino

De mi vida

Encendida vida

Sufrida vida

Vivida vida

Maravillosa vida

Donde estuviste

Sera que el tiempo me arrastro

Me arrastro hasta el sur

Solo para encontrarte

Soy del sur

Quiero al sur

Nací en el sur

Te vi en el sur

Moriré en el sur

El sol ya se despego del mar

Escogió el cielo

Un frio viento sopla del sur

Regreso liberado

La escritura me libera

Regreso con tu recuerdo

Con el sol a mis espaldas

Luis Alberto Romano

SUMÁRIO

RESUMO GERAL

O presente trabalho analisou o estado de saúde do molusco de areia *Mesodesma mactroides* do litoral do Estado do Rio Grande do Sul, Brasil. Foram feitas análises histopatológicas de moluscos adultos coletados durante as quatro estações do ano de 2012 na praia do Cassino (Capítulo 1). No Capítulo 2 foram analisados amostras teciduais, com a utilização de microscopia óptica e microscopia eletrônica de transmissão, de juvenis de *M. mactroides* moribundos durante um evento de mortalidade massiva ocorrido no ano de 2011. No Capítulo 3 foi feito um "*screening*" de doenças de notificação obrigatória pela Organização Mundial de Sanidade Animal (OIE) (marteiliose, perkinsose, bonamiose, mikrocitose e infecção pelo vírus herpes de ostreídeos - OsHV µ-var) com a utilização de análise molecular utilizando a técnica de reação em cadeia da polimerase (PCR) em *M. mactroides* coletados desde Capão da Canoa (litoral norte do Rio Grande do Sul) até o Chuí (fronteira sul do Estado). No Capítulo 4 foi analisado o efeito de baixas salinidades na sobrevivência de juvenis e adultos de *M. mactroides* e o quadro patológico associado. Por fim, no Capítulo 5, foi realizado um experimento avaliando a capacidade do marisco branco de reagir a presença de um corpo estranho (partículas de carbono) injetado no pé muscular. Os parasitos mais comuns foram os ciliados *Trichodina* sp. localizados na brânquia e turbelários *Paravortex mesodesma* na luz do intestino. Apesar de nenhum registro de dano aos hospedeiros, tricodinos quando em elevada densidade podem obstruir as brânquias e os turbelários podem causar a oclusão da luz do tubo intestinal. Parasitos de importância intermediária, como coccídeos semelhantes à *Pseudoklossia* e gregarinas *Nematopsis* sp. foram encontrados raramente e em baixa intensidade, causando baixa resposta inflamatória nos hospedeiros. Metacercárias indeterminadas de Digenea localizadas na ponta dos sifões causando a ruptura das fibras musculares e leve infiltração hemocitária foram observados em baixa prevalência e intensidade. Esporocistos indeterminados de Digenea foram registrados raramente entre os túbulos digestivos causando resposta hemocitária severa. Procariontes do gênero *Rickettsia* foram observados raramente nos mariscos adultos, entretanto estes organismos foram registrados em 100% dos juvenis moribundos, logo sendo associado ao evento de mortalidade. Através das analises de PCR, não foi registrada nenhuma doença de notificação obrigatória pela OIE em *M. mactroides*. A salinidade média letal para 48h de exposição foi de 6,5 e 5,7 para adultos e juvenis, respectivamente. Já para 96h de

exposição, a salinidade média letal foi de 10,5 e 8,8 para adultos e juvenis, respectivamente. Os mariscos expostos a salinidades abaixo de 15 apresentaram edema intracelular e focos necróticos no epitélio dos túbulos digestivos e oclusão da luz dos túbulos digestivos. Através do experimento realizado no Capítulo 5, foi possível demonstrar importantes mudanças morfológicas relacionadas a inflamação e morte celular resultante da injeção de tinta nanquim no marisco branco, que foram eficientes em eliminar o material estranho através de fagocitose pelos hemócitos, diapedese pelo epitélio intestinal e também através das brânquias e rim. Analise de microscopia eletrônica evidenciou apoptose e estresse do retículo endoplasmático em células nas quais se acumularam as partículas de carbono injetadas.

Palavras chave: apoptose, bivalves, doenças, estresse do reticulo endoplasmático rugoso, salinidade.

GENERAL ABSTRACT

This work reports on the health state of the yellow clam *Mesodesma mactroides* from the coast of the Rio Grande State, southern Brazil. Histopathological analyses were held using adult clams sampled in Cassino beach in four seasons in 2012 (Chapter 1). In the Chapter 2 it was analyzed, through light microscopy and transmission electronic microscopy, moribund juveniles *M. mactroides* during a mortality episode occurred in 2011. In the Chapter 3, it was realized a screening of notifiable diseases by the World Organization of Animal Health (OIE) (marteiliosis, perkinsosis, bonamiosis, mikrocitosis and the infection of the micro variant of the Ostreid herpes virus - OsHV µ-var) through molecular tools using the polimerase chain reaction (PCR) in yellow clams collected since Capão da Canoa (north shore of the Rio Grande do Sul State) to Chuí (most meridional point of Brazil). In the Chapter 4, it was analyzed the effect of low salinities in the survival of juveniles and adults yellow clams, and the associated pathological alterations. Finally, in the Chapter 5, it was realized an experiment to evaluate the yellow clam capacity to react to presence of a foreign particle (India ink = carbon particles) artificially injected in the muscular foot. The most common parasites recorded were ciliates *Trichodina* sp. in the gills and turbellarians *Paravortex mesodesma* in the intestinal lumen. Despite no evidence of damage to the hosts, trichodines when in high intensity can obtrude the gill and the turbellarians can obtrude the intestinal lumen. Parasites with intermediate importance, resembling the coccidian *Pseudoklossia* and gregarines *Nematopsis* sp. were rarely found, causing low inflammatory response to the hosts. Undeterminate metacercariae (Digenea) located in the siphons causing disruption of the muscle and light infiltration of hemocytes were found in low prevalence and intensity. Undeterminated sporocysts (Digenea) were rarely recorded in the hemocoele of the digestive gland causing severe hemocyte response. Procaryotes of the *Rickettsia* genus were rarelly found in the adult clams, however, these prokaryotes were found in 100% of moribund juveniles, and therefore associated to the mortality event. Through the PCR analyses, it was not recorded any notifiable diseases by OIE in the yellow clam. The lethal salinity for 48h of exposition were 6.5 and 5.7 to adults and juveniles, respectively. For 96h of exposition, the mean lethal salinity were 10.5 e 8.8 to adults and juveniles, respectively. The clams exposed to salinities < 15 presented intracellular edema and necrotic focus in the epithelium of digestive tubules and the occlusion of the digestive tubules. Through the experiment

realized in the Chapter 5, it was possible to demonstrate important morphological alterations related to inflammation and cell death resulting from the India ink injections in the yellow clam, which were efficient in eliminate the ink particles through phagocytosis by the hemocytes, diapedesis across the epithelia of the intestine, gills and kidney. Electron microscopy analyses revealed apoptosis and endoplasmic reticulum stress in the cells where the ink particles accumulated.

Key-words: apoptosis, shellfish, diseases, salinity, endoplasmic reticulum stress.

INTRODUÇÃO GERAL

Na aquicultura, de forma geral é necessário disponibilizar alguma forma de alimento (geralmente uma ração contendo farinha de peixe) no sistema de produção para criar os organismos de interesse. Em contrapartida, para a produção de moluscos bivalves não há necessidade de fornecimento de ração, já que os bivalves se alimentam, principalmente, de microalgas naturalmente presentes no fitoplâncton dos oceanos e corpos de água. Esta é uma das principais características que tornam tão atrativo a criação de bivalves, pois a ração está entre os insumos que mais encarecem a produção de organismos aquáticos (FAO 2012).

Os moluscos bivalves estão entre os organismos aquáticos mais produzidos pela aquicultura, representando em torno de 23% da produção aquícola total do ano de 2013, perdendo apenas para os peixes ósseos, que representaram 66% da produção total aquícola. Já quando se trata de maricultura, o cultivo de moluscos bivalves é citado como a principal atividade, representando 75% do total produzido. Em contrapartida, apesar dos moluscos bivalves serem mais produzidos que os crustáceos, que representam apenas 10% da produção aquícola total, o lucro gerado pelo comércio de crustáceos foi muito maior que o gerado pelos bivalves. Isto se deve ao fato de que os camarões marinhos são considerados como a principal *commodity* no mercado internacional (FAO 2014).

Além disso, a grande maioria dos moluscos bivalves produzidos para alimentação humana possui baixo valor comercial, com exceção de alguns moluscos considerados iguarias, como as vieiras e os gastrópodes pertencentes ao gênero *Haliotis* (abalones). O baixo valor comercial dos moluscos bivalves, associado com a capacidade deles de aproveitarem a produtividade natural do ecossistema, faz com que a criação de bivalves seja uma importante alternativa para aumentar a disponibilidade de alimento saudável e acessível para uma população carente que cresce em um ritmo alarmante (FAO 2014).

Os bivalves com importância para alimentação humana (neste caso não incluindo a produção de ostras perlíferas da família Pteriidiae) pertencem basicamente a quatro grupos: (1) os moluscos de areia (grande parte pertencente a ordem Veneroida) representam o grupo de moluscos bivalves mais produzido mundialmente (35% do total

dos moluscos produzidos), (2) as ostras (família Ostreidae) que representam por volta de 33% da produção de moluscos bivalves, (3) os mexilhões da família Mytilidae (por volta de 15%), e (4) as vieiras (família Pectinidae) (ao redor de 15%) (FAO 2012).

Uma parte considerável da produção mundial de bivalves marinhos, particularmente na Europa e América, está baseada na utilização de espécies amplamente introduzidas em diversas regiões do mundo, como é o caso do venerídeo *Ruditapes phillipinarum* e da ostra do Pacífico *Crassostrea gigas*, ambos originários da Ásia (FAO 2012).

Segundo dados da FAO (2012), a produção de moluscos de areia cresce muito mais rapidamente que a produção de outros bivalves. Em 1990, a produção de moluscos de areia era somente a metade da produção de ostras, entretanto a partir de 2008, a produção de moluscos de areia superou a das ostras, tornando-se o grupo de espécies de moluscos mais produzidos. Segundo Cáceres-Martínez & Vásquez-Yeomans (2008), entre os grandes gargalos que impedem a expansão da malacocultura podemos citar: (1) a falta de laboratórios de produção de sementes e (2) o problema de enfermidades, sendo este último o tema da presente tese.

Mecanismos de defesa dos moluscos bivalves

Os moluscos bivalves, assim como todos os demais invertebrados, possuem um sistema imunológico inato, sem a presença de anticorpos, receptores específicos e nem células de memória como ocorre nos vertebrados. Este sistema imune está essencialmente ligado ao seu sistema circulatório de hemolinfa (Galloway & Depledge 2001). Em moluscos bivalves, o sistema imunológico, possui mecanismos de defesas que envolvem reações celulares, cujo ponto central da resposta imune é a fagocitose. Esta fagocitose é desempenhada por células circulantes na hemolinfa, denominadas hemócitos (Cheng 1996, Betti *et al.* 2006). Os hemócitos dos moluscos estão envolvidos em diversas funções vitais, incluindo digestão, transporte de nutrientes, cicatrização e defesa interna (Cheng 1996). Relata-se comumente a ocorrência de dois grupos básicos de hemócitos em bivalves; os ricos em grânulos, denominados hemócitos granulares (granulócitos), e os com pouco ou nenhum grânulo, conhecidos como hemócitos hialinos (Vargas-Albores & Barraco 2001).

Quando um corpo estranho é muito grande para ser fagocitado, como por exemplo um parasito metazoário ou um grão de areia, ocorre o processo de encapsulação, na qual os hemocitos vão isolar o corpo estranho. Tal processo pode inclusive gerar a produção de pérolas (Cheng, 1996).

Apoptose

A apoptose, também denominada morte celular programada (Kerr *et al.* 1972) é um processo natural do ciclo celular animal podendo ser caracterizada como um fenômeno biológico, não pertencendo ao sistema imune propriamente dito, mas auxiliando biologicamente o sistema em várias circunstancias, por exemplo, no controle de infecções virais (Roulston *et al.* 1999). A apoptose também pode ser induzida somente através da fagocitose. Se o material a fogocitar for de grande tamanho ou em grande quantidade, pode se gerar um estresse do retículo endoplaspático (RE) através do acúmulo de material fagocitado nos túbulos e cisternas do RE (Bergmann *et al*. 1998). Este mecanismo no qual o estresse do RE induz a apoptose foi recentemente descrito (Lee & Lee 2006).

Doenças na aquicultura

A patologia (derivado do grego *pathos*, sofrimento, doença; e *logia*, ciência, estudo) é a ciência que estudo das doenças. Ela envolve tanto a ciência básica quanto a prática clínica, e é devotada ao estudo das alterações estruturais e funcionais das células, dos tecidos e dos órgãos que estão ou podem estar sujeitos a doenças (Robbins & Cotran 2014).

A doença pode ser definida como um conjunto de fenômenos que se produzem em um organismo vivo que sofre uma alteração mórbida e reage contra a mesma. Existem distintos tipos de enfermidades (hereditárias, carência nutricional, metabólicas, degenerativas e infecciosas) (Mohan 2015).

O impacto econômico causado pelas doenças na aquicultura pode ser devido a redução na taxa de crescimento, diminuição no valor de mercado do produto e também devido a mortalidade generalizada dos organismos cultivados (Cáceres-Martínez & Vásquez-Yeomans 2008, Lightner 2005).

Destaca-se que nas últimas décadas os surtos de doenças epizoóticas têm afetado a produção do salmonídeos no Chile, de ostras na Europa (a ostra portuguesa *Crassostrea angulata* e a ostra plana européia *Ostrea edulis*) e de camarões marinhos (*Litopenaeus vannameii*) na Ásia, América do Sul e África, resultando nos casos mais drásticos na perda total da produção (Bondad-Reantaso *et al.* 2001, FAO 2012).

Somente para citar alguns dados, em 2010, a aquicultura na China sofreu perdas de 1,7 milhões de toneladas de organismos aquáticos causadas por doenças, catástrofes naturais e poluição. Surtos de doenças praticamente acabaram com a produção de camarões em Moçambique em 2011 (Bondad-Reantaso *et al*. 2001, FAO 2012, OIE 2012). Mais recentemente, um evento de mortalidade catastrófico dizimou os estoques de um dos bivalves mais importantes da Espanha, o "berberecho" *Cerastoderma edule*, sendo apontado como causador desta catastrofe uma doença causada pelo protozoário *Marteilia conchilla* (Villalba *et al*. 2014).

Organização Mundial da Sanidade Animal

A Organização Mundial da Sanidade Animal, antigamente denominada Oficina Internacional de Epizotias OIE (sigla em francês OIE = "Office International des Epizooties") é uma organização intergovernamental que se encarrega de melhorar a sanidade animal no mundo e garantir a seguridade sanitária do comércio internacional de animais e de seus produtos derivados, graças a uma descrição detalhada das medidas sanitárias que as autoridades veterinárias dos países importadores e exportadores devem aplicar para evitar a transmissão de agentes patógenos aos animais e também às pessoas, além de impedir a instauração de barreiras sanitárias injustificadas. Para tal, foi estabelecido um código zoosanitário para animais aquáticos no qual se encontra a lista das doenças de notificação obrigatória por grupo de animais produzidos na aquicultura.

No manual de diagnóstico para doenças de organismos aquáticos da OIE, é possível relatar uma série de métodos padronizados para realizar o diagnóstico de doenças em peixes, moluscos e crustáceos. Basicamente, a técnica rotineira empregada nos laboratórios de diagnóstico de enfermidades de moluscos bivalves é a histológica clássica, que permite observar a interação entre o patógeno e hospedeiro

Além disto, este manual lista uma série de técnicas moleculares fundamentalmente de reação em cadeia da polimerase (PCR) e hibridação *in situ*, indicados como métodos confirmatórios em o caso de suspeita de uma doença de notificação obrigatória (Walker e Subasinghe 2010, Berthe et al. 1999). Doenças de notificação obrigatória pela OIE são definidas no Código Internacional de Saúde de Animais Aquáticos. Elas são consideradas por ter importância sócio-econômica dentro de países e também para o comércio internacional de animais aquáticos e seus produtos derivados, e necessitam ser relatadas para a OIE como especificada no Código.

Doenças de moluscos bivalves

Segundo a OIE, a produção mundial de moluscos bivalves foi severamente afetada por diversas doenças infecciosas durante as últimas décadas. De fato, as doenças se tornaram um dos fatores limitantes para o crescimento da aqüicultura no mundo. Os agentes etiológicos causadores de enfermidades em molusco bivalves compreendem: os vírus, as bactérias, os protozoários e os metazoários (Lauckner 1983).

De acordo com Cáceres-Martínez & Vásquez-Yeomans (2008), a transferência de agentes infecciosos via o transporte de bivalves vivos, tem sido a principal causa de surtos epizoóticos. A dinâmica do livre comércio e a do desejo legítimo dos países em buscar novas alternativas para aumentar a produção de alimento e para a geração de desenvolvimento socioeconômico, em um prazo curto, fazem com que se passe despercebido por fatores sanitários essenciais que, se não considerados no seu justo contexto, podem fracassar a indústria produtiva de moluscos bivalves.

Entre os fatores sanitários que são necessários conhecer antes de se iniciar a atividade aquícola está: (1) Quais são os problemas de doenças que afetam os bivalves que desejamos cultivar; (2) Quais são os riscos que estas doenças afetem os bivalves nativos da zona receptora; (3) Quais são as doenças que os moluscos da zona receptora podem transmitir aos bivalves transferidos. O conhecimento destas informações traz uma maior garantia do sucesso de um empreendimento aquícola, ajuda a proteger a biodiversidade de moluscos e outras espécies nativas e também protege o público consumidor (Cáceres-Martínez & Vásquez-Yeomans 2008).

A maioria da informação científica existente sobre os agentes patógenos de bivalves se refere a espécies valiosas cultivadas e introduzidas ao redor do mundo como é o caso de *C. gigas e R. philippinarum*, ou espécies de bivalves nativas de países desenvolvidos do hemisfério norte, como por exemplo, a ostra americana *Crassostrea virginica*, a ostra portuguesa *Crassostrea angulata*, a ostra européia *Ostrea edulis*, o mexilhão azul *Mytilus edulis*, o mexilhão do mediterrâneo *Mytilus galloprovincialis*, a "*hard clam" Mercenaria mercenaria* nos Estados Unidos da América e *Ruditapes decussatus*, nativa da Europa. Estas informações permitiram caracterizar as doenças mais importantes de moluscos bivalves que conhecemos até o momento. Algumas destas espécies de parasitos que causam estas enfermidades foram listadas pela OIE como "doenças de notificação obrigatória". No caso dos patógenos destaca-se *Bonamia ostreae*, *B. exitiosa*, *Marteilia refringens*, *Mikrocytos mackini*, *Perkinsus marinus*, *P. olseni*, e a microvariente do vírus herpes de ostreídeos OsHV µvar.

Doenças de moluscos bivalves no Brasil

A pesquisa de doenças em moluscos bivalves no Brasil é recente, comparada aos países do Hemisfério Norte. A maioria dos trabalhos sobre patologia de bivalves foram realizados no sul (Santa Catarina) e no nordeste (Bahia e Ceará) utilizando espécies economicamente importantes oriundas de cultivo e também de bancos naturais, como as ostras *C. gigas*, *Crassostrea rizophorae* e *Crassostrea brasiliana*, os mitilídeos *Perna perna* e *Mytela falcata*, os moluscos de areia *Tagelus plebeius* e *Anomalocardia brasiliana* (da Silva *et al.* 2009, 2011; Boehs *et al.* 2010, Sabry *et al.* 2011).

Produção de moluscos no Brasil

No Brasil existe uma enorme riqueza de espécies de bivalves nativos que habitam uma ampla variedade de ecossistemas (manguezais, praias arenosas, costões rochosos, planícies de maré, água salobra ou marinha, clima tropical e subtropical), os quais suportam atividade extrativista de pequena/média escala (Rios 2009).

Apesar da enorme biodiversidade de moluscos bivalves, poucas espécies nativas são produzidas, como é o caso das ostras nativas *C. rizophorae* e *C. brasiliana*, criadas principalmente nos manguezais do Paraná, São Paulo e nos Estados do nordeste e norte do Brasil, da vieira *Nodipecten nodosus* produzida em Santa Catarina e Rio de Janeiro e

do mexilhão *P. perna* (Santa Catarina, Paraná, São Paulo, Rio de Janeiro e nos Estados do nordeste e norte do Brasil). Além das espécies nativas, a ostra exótica *C. gigas* é uma das principais espécies produzidas em Santa Catarina, que representa o principal Estado produtor de moluscos do Brasil (Ferreira & Oliveira Neto 2007).

Com a finalidade de diversificar a produção de moluscos no Brasil, algumas espécies nativas estão sendo cultivadas em escala de pesquisa, como é o caso dos moluscos de areia *Cyrtopleura costata* (Albuquerque 2010) e *Anomalocardia brasiliana* (Lagreze 2014) e da ostra perlífera *Pteria hirundo* (Albuquerque 2010). Neste contexto, o marisco branco *Mesodesma mactroides* é um candidato a aquicultura, especialmente no Estado do Rio Grande do Sul, devido a importância sócio-cultural que este molusco possui na região.

A espécie alvo do trabalho: *Mesodesma mactroides*

Seguindo a classificação taxonômica proposta por Rios (2009), *Mesodesma mactroides* pertence:

- Filo MOLLUSCA

- Classe BIVALVIA

- Subclasse HETERODONTA

- Ordem VENEROIDA

- Superfamília MACTROIDEA

- Família MESODESMATIDAE

- Gênero *Mesodesma*

- Espécie *M. mactroides* (Deshayes, 1854)

O termo molusco de areia (tradução do termo inglês *clam* e do espanhol *almeja*) é comumente aplicado, sem caráter taxonômico, aos bivalves marinhos pertencentes, em sua grande maioria, à ordem Veneroida (Manzi & Castagna 1989). Possuem como característica marcante a presença de sifões e pés para escavar no sedimento.

De acordo com Rios (2009), na costa brasileira existem diversas espécies de moluscos de areia comestíveis (para citar algumas: *Amiantis purpurata, Iphigenia brasiliana, Lucina pectinata, Mactra isabelleana, Tagelus plebeius, Tivela mactroides*) que são coletados diretamente de bancos naturais. Estudos recentes foram realizados sobre a produção de larvas do berbigão *Anomalocardia brasiliana* (Lagreze 2014) e também da asa-de-anjo *Cyrtopleura costata* (Albuquerque 2010) visando à diversificação do cultivo de espécies de bivalves nativos do Brasil.

A família Mesodesmatidae está representada por moluscos de areia que apresentam importância socioeconômica em diversas regiões de clima temperado do hemisfério sul, sendo espécies apontadas como promissoras para aquicultura, como é o caso da "toheroa" *Paphies ventricosa* e "pipi" *Paphies australis* na Nova Zelândia e da "macha" *Mesodesma donacium* no Chile e Peru (Olivier *et al.* 1971).

No litoral Atlântico do sul do Brasil, no Uruguai e na Argentina o bivalve infaunal *M. mactroides* ocorre de maneira endêmica. Conhecido popularmente no litoral do Rio Grande do Sul como "marisco branco" e como "almeja amarilla" pelos uruguaios e argentinos, *M. mactroides* se destaca como um dos bivalves mais importantes na região.

A distribuição do marisco branco vai desde o sudeste do Brasil (23ºS), na Ilha Grande (RJ) até o sul da Província de Buenos Aires (41ºS), Argentina (Rios 1994). Segundo pesquisa biogeográfica realizada por Fiori & Defeo (2006), o centro de distribuição geográfica desta espécie está localizado entre a praia do Cassino (extremo sul do Brasil) e a Barra do Chuí (fronteira entre Brasil e Uruguai), onde o crescimento e abundância de *M. mactroides* são maiores que nas populações localizadas tanto ao sul como ao norte.

Segundo Gianuca (1985), organismos dessa espécie vivem enterrados a uma profundidade de 5 a 30 cm (quanto maiores, mais fundo se enterram) na zona do entre-marés de praias arenosas expostas dissipativas. Ocorrem de forma agregada e não uniforme, sendo fácil encontrar os bancos de *M. mactroides* durante a maré-baixa, pois é possível observar os pequenos orifícios na areia característicos de sua presença.

O marisco branco possui rápido crescimento e uma expectativa de vida de cerca de três anos (Defeo *et al.* 1992). Indivíduos adultos medem em torno de 7-8 cm de comprimento de concha, sendo que o recorde de maior espécime (comprimento de concha = 9,8 cm) foi coletado na praia de Mostardas, litoral central do Rio Grande do Sul (Gianuca 1975).

Com relação a reprodução, os indivíduos com comprimento de concha de 4 cm já estão sexualmente ativos. Christiansen (1971) observou com a utilização de análise histológica do tecido gonadal, que os mariscos brancos habitantes da região de Buenos Aires, Argentina, maturam sexualmente durante o inverno - de junho a setembro - e realizam liberação total dos gametas, com o aumento da temperatura, durante a primavera - de outubro a novembro. Mesmo resultado foi obtido recentemente por Herrman *et al.* (2009), analisando o ciclo reprodutivo de *M. mactroides* também da Argentina.

Com respeito ao período de recrutamento de *M. mactroides*, este é muito imprevisível. Geralmente, os recrutas são observados entre os meses de julho e fevereiro, com picos no final do inverno (agosto) e inicio da primavera no Estado do Rio Grande do Sul (Bergonci & Thomé 2008)

Estudos relatam que salinidades reduzidas são desfavoráveis ao desenvolvimento de *M. mactroides* (Olivier *et al.* 1971, Defeo *et al.* 1992, Marins & Levy 2000). Marins & Levy (2000) observaram que a desembocadura da Laguna dos Patos forma uma barreira entre as populações de *M. mactroides* que vivem ao norte (praia do Mar Grosso, cidade de São José do Norte) e ao sul (praia do Cassino, cidade de Rio Grande) da desembocadura, evidenciado através de diferenças genéticas entre estas duas populações. Segundo estes autores, as variações de salinidade na proximidade da desembocadura poderiam ser responsáveis pelo estresse ambiental que o animal sofre, uma vez que os indivíduos habitantes desta região não se desenvolvem até a maturidade.

Defeo *et al.* (1992) afirmam que a distribuição especial de recrutas *Mesodesma mactroides* é afetada significativamente pelo gradiente de salinidade que ocorre na saída do canal Andreoni e da Barra do Chuí. Entretanto, não foi encontrado nenhum trabalho determinando os níveis mínimos de salinidade que o marisco branco é capaz de tolerar.

No passado, o marisco branco foi considerado um dos bivalves mais comuns e abundantes das praias oceânicas dissipativas ao longo do litoral extremo sul do Brasil (Gianuca 1985), Uruguai (Defeo 1989) e Argentina (Coscarón 1959). Somente para citar alguns dados, Gianuca (1985) registrou em janeiro de 1980 na praia do Cassino uma densidade máxima de 8450 ind/m^2, apenas de recrutas e juvenis. Com relação à biomassa, Gianuca (1985) registrou um máximo de 232,47 g/m^2 e mínimo de 108,78 g/m^2, sendo determinado principalmente pela porcentagem de indivíduos grandes.

Aliado a exploração descontrolada dos principais estoques de *M. mactroides* que ocorreram durante as décadas de 1950 até 1980, que contribuíram com a redução dos estoques populacionais, a partir da década de 1990, foram registrados surtos de mortalidades que ocorreram no sul do Brasil, Uruguai e Argentina que culminaram no quase desaparecimento do outrora tão abundante *M. mactroides* (Odebrecht *et al*. 1995, Mendes 1995, Fiori & Cazzaniga 1999, Cremonte & Figueras 2004). Devido a estes fatores, atualmente, os estoques de *M. mactroides* se encontram impossibilitados de serem explorados comercialmente (Fiori & Cazzaniga 1999, Hermann *et al*. 2011).

A portaria do Ibama número 100-N, de 1° fevereiro de 1990 resolve: "Proibir a extração do marisco branco (*Mesodesma mactroides*), nas áreas sob jurisdição nacional compreendidas entre a Barra do Rio Grande e o Arroio Chuí, no Estado do Rio Grande do Sul. Sendo aos infratores destas disposições aplicadas às penalidades previstas..."

Apesar da proibição, ainda é possível observar "marisqueiros" coletando ilegalmente, com auxílio de pás de corte, mariscos brancos ao sul do Balneário do Cassino (na região entre o navio encalhado "Altair" e o Farol do Albardão, aonde ainda é possível encontrar remanescentes de populações de mariscos adultos). Os mariscos, além de serem vendidos aos restaurantes, são vendidos para pescadores amadores que utilizam *M. mactroides* como isca para a pesca do papa terra *Menticirrhus americanus*.

Diversos estudos foram realizados para tentar explicar os surtos de mortalidade que ocorreram a partir da década de 1990. Agentes biológicos (parasitos, floração de microalgas tóxicas) ou abióticos (metais pesados, mudanças climáticas) foram apontados como os causadores destas mortalidades. Apesar do enorme esforço para determinar as causas destes eventos de mortalidade massiva, até o momento nenhuma explicação satisfatória foi formulada.

Segundo Villalba (comunicação pessoal) o primeiro passo para conseguir dados úteis para a gestão e para a exploração de um recurso pesqueiro é conhecer as alterações patológicas que afetam o organismo-alvo de estudo, para em seguida aprofundar o estudo das alterações patológicas das espécies mais patogênicas. Somente assim será possível concluir o último passo, que é desenvolver ferramentas efetivas para enfrentar os problemas patológicos para que estes se mitiguem e/ou desapareçam.

Através do exposto até aqui, se reconhece a importância de realizar estudos acerca das patologias que afetam o marisco branco do Estado do Rio Grande do Sul, como um pré-requisito para posteriores linhas de pesquisa relacionadas ao seu cultivo e restabelecimento dos estoques naturais.

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OBJETIVO GERAL

O objetivo geral deste trabalho é avaliar o estado de saúde do principal bivalve explorado artesanalmente no Estado do Rio Grande do Sul, o marisco branco *Mesodesma mactroides*, a partir da descrição das enfermidades que o afetam e a expressão patológica da sua presença.

Objetivos específicos

Os objetivos específicos são:

1) Identificar os agentes etiológicos que afetam as populações naturais do marisco branco *Mesodesma mactroides* na praia do Cassino;
2) Descrever microscopicamente as patologias (reações tissulares e celulares) observadas sobre os tecidos do marisco branco;
3) Procurar, mediante PCR (reação em cadeia da polimerase), por doenças de notificação obrigatória listadas pela Organização Mundial de Sanidade Animal (OIE) no marisco branco do Estado do Rio Grande do Sul;
4) Determinar a salinidade mínima letal (CL_{50}) para 96 horas de mariscos juvenis e adultos e as alterações patológicas que as salinidades reduzidas causam;
5) Avaliar a capacidade dos mariscos brancos oriundos do meio ambiente de reagir frente a um agente estranho (partículas de carbono).

1. Avaliação histopatológica no marisco branco *Mesodesma mactroides* do sul do Brasil.

Histopathological survey of the yellow clam *Mesodesma mactroides* from southern Brazil.

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Resumo

O objetivo deste estudo foi analisar a condição histopatológica do marisco branco *Mesodesma mactroides* (Mesodesmatidae) do sul do Brasil. Uma amostra de 30 mariscos foi coletada no entremarés da praia do Cassino (32˚24'S - 52˚20'W, Estado do Rio Grande do Sul, Brasil) em janeiro de 2012, e amostras de tecido, incluindo sifões, brânquias, gônadas, glândula digestiva e pé foram processados para exame histológico. O exame microscópico revelou a presença de três táxons de parasitos ou comensais: ciliados cf. *Trichodina*, turbelários cf. *Paravortex mesodesma* e trematódeos digenéticos (esporocistos e metacercária). Os ciliados foram identificados nas brânquias dos mariscos tanto em alta prevalência (100%) como em densidade (média = 39,4 ciliados por corte histológico), sem nenhum dano celular aparente. Os turbelários foram registrados na luz do intestino com uma prevalência de 26,7%, sem dano celular aparente. Metacercárias não identificadas foram registradas no topo do sifão inalante com prevalência de 16,6% e densidade de um parasito por corte histológico, causando a ruptura das fibras musculares e infiltração de hemócitos. Esporocístos de um Digenea indeterminado foram registrados na glândula digestiva de 6,6% dos mariscos examinados, e infiltração massiva de hemócitos e necrose da glândula digestiva foram observados. Este foi o primeiro registro de trematódeos digenéticos em *M. mactroides*, e também o primeiro estudo histopatológico de mariscos brancos do Brasil.

Abstract

The aim of this study was to analyse the histopathological condition of the yellow clam *Mesodesma mactroides* (Mesodesmatidae) from southern Brazil. A sample of 30 clams was collected in the tidal zone of Cassino beach (32˚24'S - 52˚20'W, Rio Grande do Sul State, Brazil) in January 2012, and tissue samples, including siphons, gills, gonads, digestive glands and foot, were processed for histological examination. Microscopical examination revealed the presence of three parasitic or commensal taxa: ciliates cf. *Trichodina*, turbellaria cf. *Paravortex mesodesma* and digenean trematodes (sporocysts and metacercariae). The ciliates were identified on the gills of the clams with both high prevalence (100%) and density (mean density = 39.4 ciliates per histological section), without apparent cell damage. Turbellaria specimens were found in the lumen of the intestine with a prevalence of 26.7%, without apparent cell damage. Metacercariae of unidentified Digenea were found in the tip of the inhalant siphon with a prevalence of

16.6% and a density of one parasite per histological slide, in addition to disruption of the muscle and the presence of infiltrated hemocytes. Sporocysts, of undetermined Digenea, were found in the digestive gland of 6.6% of clams that were examined, and hemocyte infiltration and massive necrosis of digestive tissue were observed. This is the first report on the identification of digenean trematodes in *M. mactroides*, in addition to the first histopathological report in yellow clams from Brazil.

Introduction

The yellow clam *Mesodesma mactroides* (Mesodesmatidae) was an important economic resource from dissipative sandy beaches in southern Brazil, Uruguay and Argentina between 1940 and 1950 (Coscarón, 1959). However, a drastic reduction of the yellow clam populations occurred as a result of overfishing and successive mass mortality events that covered almost the entire geographical range: 32ºS (Rio Grande do Sul State, Brazil) to 40ºS (Buenos Aires Province, Argentina) (Odebrecht et al., 1995; Fiori & Cazzaniga, 1999).

Several factors have been reported as possible causes of these mortality events, such as heavy metals (Thompson & Sanches de Bock, 2007), harmful algal bloom (Odebrecht et al., 1995), and parasites (Fiori & Cazzaniga, 1999; Cremonte & Figueras, 2004). However, it has not been verified that these were the actual causes of these events.

Few histopathological studies have been conducted on *M. mactroides*. Cremonte & Figueras (2004) analysed yellow clams from Argentina and found Turbellaria in the lumen of the digestive tract, coccidia in the nephridia, gregarines inside digestive epithelial cells, and ciliates similar to *Trichodina* sp. in the gills of clams, while Brusa et al. (2006) described the turbellaria *Paravortex mesodesma* as inhabiting the digestive tract of yellow clams from Uruguay.

However, there is a lack of histopathological studies on *M. mactroide*s populations from the Brazilian coast. According to Fiori & Defeo (2006), the region between Cassino beach (Brazil) and Barra do Chuí (the border between Brazil and Uruguay) is the central distribution range of the yellow clam, as it is where the species is located in the highest densities.

Therefore, in the present study, we performed a histopathological survey of *M. mactroides* from Cassino beach, Brazil, to assess healthy bivalves in the centre of their distribution range.

Material and Methods

In January 2012, a sample of 30 yellow clams (mean shell height = 75 ± 10 mm) was collected manually in the tidal zone of Cassino beach (32˚24'S, 52˚20'W), Rio Grande do Sul State, southern Brazil. The temperature and salinity of the surface water at the sampling time were 27 °C and 36 ppt, respectively. The yellow clams were transported alive to the laboratory and shucked from their shells. The soft tissues of the clams were fixed in Davidson's fixative, stored in 70% ethanol, dehydrated, embedded in Paraplast® and then sectioned (5 μm), following standard histological protocols. Sections of the siphons, gills, gonads, digestive gland and foot were stained with haematoxylin and eosin and then examined under light microscopy for the presence of parasites and pathological alterations. The prevalence and density values were calculated according to the method of Bush et al. (1997).

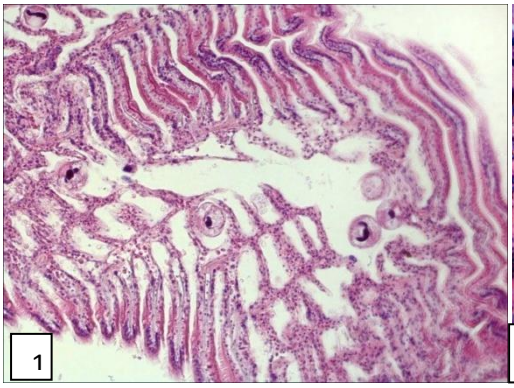
Results

Microscopical examination of the histological sections revealed the presence of three parasitic taxa: ciliates similar to *Trichodina*, Digenea (sporocysts and metacercaria), and Turbellaria. Ciliates similar to *Trichodina* (Figure 1) infected the gill filaments of 100% of the yellow clams that were analysed, and the density was 39.4 ± 66.7 (mean ± standard deviation) ciliates per histological slide. The highest density value that was recorded was 325 ciliates per histological slide. Damage to the gill epithelium was not found to be associated with the ciliate infection. Turbellarians were found in the digestive tract of the yellow clams, with a prevalence of 30%, and the density was one Turbellaria per histological section, except in one case that was recorded two turbelarians (Figure 2). Metacercariae of digenean trematodes were found to be encysted in the tip of the inhalant siphon, with a prevalence of 15% and with one cyst per histological slide, in addition to disruption of muscle and the presence of infiltrated hemocytes (Figure 3). Sporocysts-like, similar to digenean trematodes, were recorded in 6.6% of the clams that were examined, in the digestive gland, and hemocyte infiltration and massive necrosis were observed (Figure 4).

Discussion

The yellow clam was an abundant resource along the Atlantic Coast of South America in past decades (Coscarón, 1959); currently, they are a scarce resource, and since 1994, *M. mactroides* have been considered a threatened species (Fiori & Cazzaniga, 1999). According to Cremonte & Figueras (2004), histopathological research along the entire distribution range of *M. mactroides* would augment the understanding of the causes that underlie the mass mortality that this species has suffered.

According to Lauckner (1983), some *Trichodina* have been identified worldwide, in several bivalve species, and most of these appear to be bacterivorous commensals, although some have been labelled parasitic. Trichodines are recognised by a discoid shape (approximately 30 to 50 µm in diameter), which is characterised by a disk of eosinophil adhesion, ciliature in the shape of a plume and a C-shaped macronucleus (Lauckner, 1983). Heavy infestations of trichodines can cause erosion of gill filaments and can interfere with the respiratory system as a result of excessive mucus production (Bower et al., 1994). In France, the oyster *Crassostrea gigas*, when heavily infested with trichodines, was shown to exhibit an inflammatory response in the gill and a deformed superficial epithelium (Boussaid et al., 1999).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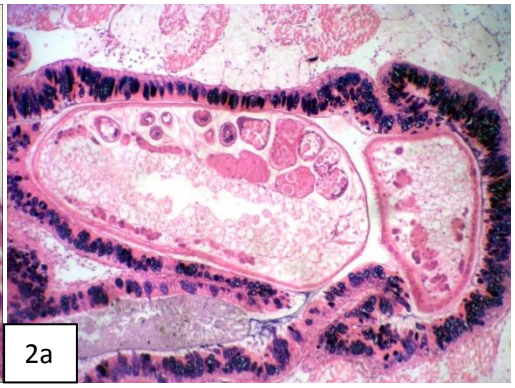
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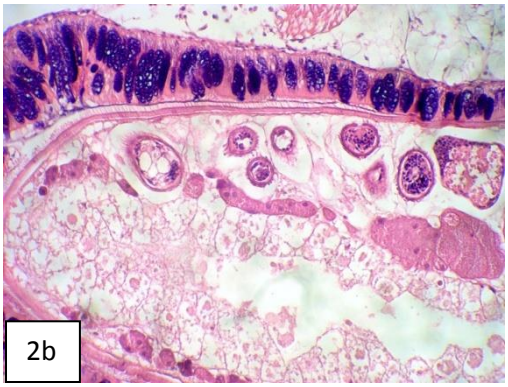
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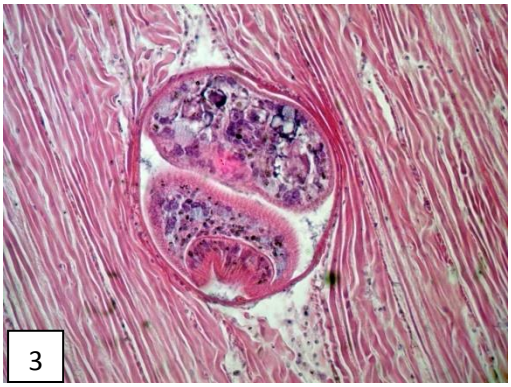
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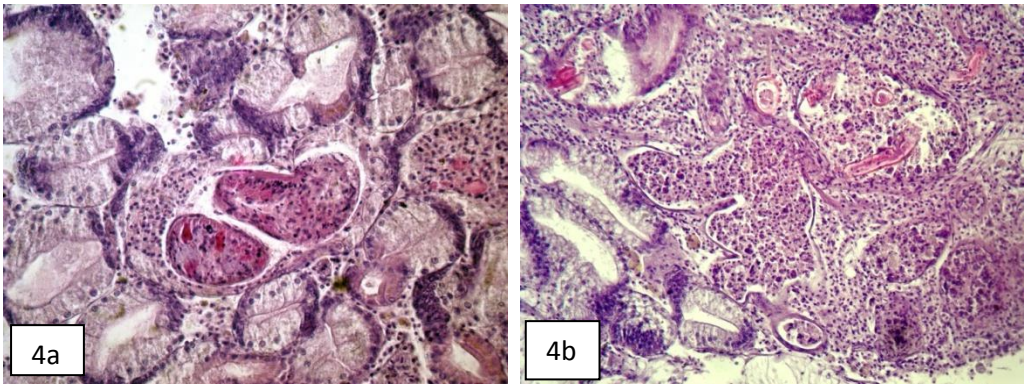

Histological sections (haematoxylin and eosin stained) of the yellow clam *Mesodesma mactroides* from Cassino beach, Rio Grande do Sul State, Brazil. Figure: **1**. *Trichodina*-like ciliates between the gill filaments (arrow). H&E-staining, Bar = 20 µm. Figure: **2.a.** Two Turbellaria cf. *Paravortex mesodesma* obtruding the intestinal lumen (LU) of the host. H&E-staining, Bar = 20 µm. Figure: **2.b.** Detail of the ciliated epithelia of the turbellaria in contact with undamaged intestinal epithelia of the host (arrow). H&E-staining, Bar = 50 µm. Figure: **3**. Metacercariae of unidentified Digenea in the tip of the inhalant siphon with disruption of the muscle (large arrow) and infiltrated hemocytes (short arrow). H&E-staining, Bar = 50 µm. Figure: **4.a.** Sporocysts-like of unidentified Digenea (arrow), with infiltrating hemocytes (HI) in the digestive gland. PAS-staining, Bar = 50 µm. Figure: **4.b**. Digestive gland with PAS-positive cuticle fragments that appear to be sporocysts (arrow) and massive necrosis with hemocyte infiltration (*). PAS-staining, Bar = 50 µm.

Cremonte & Figueras (2004) identified trichodines in 100% of moribund *M. mactroides* that were analysed during a mortality episode in Monte Hermoso (Argentina) and reported on the possibility that *Trichodina,* as a bacterivorous filter-feeder, would be more prevalent in dying clams that contain high levels of bacteria in the gills.

Fish trichodinids are essentially commensals, as they ingest bacteria associated with their hosts. However, events that increase the availability of this source of bacteria can occur, and an increase in these ciliates can cause damage to their hosts (Lom & Dyková, 1992). It is possible that a similar situation occurs among trichodinids and bivalve hosts.

In the present study, the yellow clams that were evaluated were macroscopically healthy (i.e., burrowing actively in the intertidal zone of Cassino Beach) and the high prevalence and intensity of the ciliates cf. *Trichodina* that were recorded appeared to be

harmless to *M. mactroides* from southern Brazil. In our study, we found no gill damage caused by *Trichodina* sp. and it was not apparent that this parasite was related to the mass mortalities.

Turbellaria members of Graffillidae (*Paravortex* sp.) and Urastomidae (*Urastoma cyprinae*) are intimately associated with marine bivalves (Lauckner, 1983); while urastomids inhabit the mantle cavity, graffillids live in the alimentary tract of these hosts (Lauckner, 1983). Cremonte & Figueras (2004) recorded turbellarians inhabiting the intestine of yellow clams from Argentina with a similar prevalence with that recorded in the present study, without apparent deleterious effect to the hosts. On the other hand, Brusa et al. (2006) found the turbellarian *Paravortex mesodesm*a in the intestine of *M. mactroides* from Uruguay at a high prevalence (91%) and with a maximum of 14 turbellarians per host. The turbellarians found in the present study likely belong to the same species as described by Brusa et al. (2006). No evident deleterious effect related to the presence of turbellarians in the digestive tract of *M. mactroides* was observed in the present study, regardless of their feeding upon their hosts, as previously stated by Lauckner (1983). We found turbellaria obtruding the digestive tract lumen, suggesting that there was a possibility of impaired nutrient absorption.

Larval Digenea are considered the main metazoan parasites of marine bivalves (Lauckner, 1983; Cremonte, 2011). The digenetic trematode described in the present study likely belongs to Monorchidae because members of this family mainly use the same bivalve species as primary (sporocysts) and secondary (metacercariae) intermediate hosts, as stated previously by Lauckner (1983) and Cremonte (2011). It seems that the strategy of parasitising bivalve siphons was widely adopted amongst the monorchids that successfully transmitted to fish as their definitive hosts (Gilardoni et al, 2012). In the present study, unidentified metacercariae of the inhalant siphon caused disruption of the muscle and the presence infiltrated hemocytes. The presence of metacercariae in the inhalant siphon of *M. mactroides* could disrupt their ability to retract the siphons, through a mechanical effect. The accumulation of metacercariae in the siphons may have ecological implications, as parasitised siphons lose their ability to retract and they become more vulnerable to predators that serve as the definitive hosts.

Sporocysts can cause castration in their bivalve hosts (Cremonte et al., 2001); however, this effect was not observed in parasitized yellow clams, as the sporocysts were recorded in the digestive gland of the hosts. The present study is the first report of digenean trematodes that parasitise the yellow clam *M. mactroides*. We did not find sporocysts in the gonads, only in the digestive gland, which presented with necrosis and infiltration of hemocytes.

This study is the first histopathological survey of yellow clams from Cassino beach. In conclusion, with the exception of *Trichodina*, the parasites that were identified caused tissue damage and functional responses in their hosts. Turbellaria almost filled the lumen, and while it was not observed, epithelial lesions could disrupt nutrient absorption. The metacercariae found in the inhalant siphon produced muscular disruption and possibly caused functional impairment of the inhalant siphon. Finally, the sporocysts that were found in the digestive gland generated significant necrosis and infiltration of hemocytes, which can compromise digestive function. In contrast, these injuries only affect the physiology of some individuals; they do not compromise the survival of an entire population. In the meantime, frequent monitoring of yellow clam stocks in Cassino beach would be of importance.

Acknowledgements

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2. Mortalidade do marisco branco *Mesodesma mactroides* (Bivalvia: Mesodesmatidae) associada à *Rickettsia* no sul do Brasil

Rickettsia-associated mortality of the yellow clam *Mesodesma mactroides* (Bivalvia: Mesodesmatidae) in southern Brazil

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KEYWORDS*:* Bivalve mortality, Cassino Beach, *Mesodesma mactroides*, *Rickettsia*, southern Brazil

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Resumo

O marisco branco *Mesodesma mactroides* (Deshayes, 1854) foi um importante recurso pesqueiro no litoral do Rio Grande do Sul. Entretanto, mortalidades massivas e sucessivas (sem causa conhecida) reduziram significativamente os estoques atuais deste bivalve que outrora eram abundantes, impossibilitando a extração desta espécie como uma atividade rentável. No dia 29 de outubro de 2011, durante uma pesquisa histopatológica de rotina acerca de *M. mactroides*, foi registrada uma grande quantidade de juvenis (comprimento de concha entre 20 e 30 mm) moribundos sem capacidade de se enterrar na zona do entremarés na praia do Cassino (32°12'S, 52°11'O) que morreram logo no dia posterior. A fim de investigar a causa da mortalidade destes bivalves, 30 indivíduos ainda vivos foram coletados, imediatamente desconchados e seus tecidos fixados em solução de Davidson para a análise histológica. Para análise em microscopia óptica, 20 indivíduos foram processados no equipamento automático LUPE PT05 17, emblocados em Paraplast®. Cortes de 3 μm foram feitos no micrótomo LUPE MRP03 e corados com hematoxilina-eosina. Para a microscopia eletrônica de transmissão, 10 pequenos fragmentos de tecido do tubo digestivo foram fixados em glutaraldeído 2,5% pH 7,4, logo desidratadas com etanol e embebidos com resina Epon 812. Secções ultrafinas foram coradas com acetato de uranilo e citrato de chumbo, observadas em microscópio JEOL 100S. Através da microscopia óptica, foram evidenciadas inclusões basofílicas por todo epitélio intestinal assim como no epitélio branquial que aparentavam ser organismos semelhantes a rickettsias em elevada prevalência (100% dos indivíduos analisados). Através de microscopia eletrônica confirmou-se que estas inclusões eram colônias de rickettsias infectando as células epiteliais do intestino e brânquias dos juvenis de *M. mactroides*. As rickettsias são pequenas bactérias parasitas intracelulares obrigatórias que ocorrem em diversos invertebrados marinhos e estão associados à mortalidade dos hospedeiros quando em elevada intensidade de infestação. Mortalidades massivas de bivalves ocorrem ao longo de todo litoral brasileiro e pouco se sabe sobre as causas, já que programas de monitoramento do estado de saúde dos bivalves só existem em poucos lugares onde a malacocultura é uma atividade economicamente importante. Este parece ser o primeiro registro de *M. mactroides* com rickettsiosis. Apesar de não podermos afirmar que as rickettsias tenham causado a mortalidade dos juvenis analisados, podemos afirmar que

estes procariotos contribuíram para a morte da população de mariscos amostrada neste estudo.

Palavras-chave: Mortalidade de bivalves, praia do Cassino, *Mesodesma mactroides*, *Rickettsia*, sul do Brasil

INTRODUCTION

The yellow clam *Mesodesma mactroides* (Deshayes, 1854) is an intertidal sandy beach bivalve that is distributed from the southeast of Brazil (Ilha Grande, Rio de Janeiro State, 23°S) to Argentina (Isla del Jabalí, Buenos Aires Province, 41°S) (Rios, 2009). Historically, the yellow clam had been considered an important economic resource (Coscarón, 1959). However, yellow clam populations collapsed as a result of overfishing that was associated with repeated mass mortalities in Brazil, Uruguay and Argentina in the mid-1990s. (Odebrecht et al., 1995; Fiori & Cazzaniga, 1999; Cremonte & Figueiras, 2004). Presently, *M. mactroides* is identified as a threatened species with a critically endangered status (Fiori & Cazzaniga, 1999).

As more knowledge about the factors that can influence the population sizes of sea animals have become available, it has become clear that disease can drastically affect abundance (Sindermann, 1990). Major disease-causing agents of marine bivalves include viruses, prokaryotes, fungi, protists, and invertebrates, such as Digenea, Annelida and Copepoda (Kinne, 1983). According to Lauckner (1983), prokaryotes belonging to Rickettsiales are capable of causing disease and possibly death in marine bivalves from natural beds around the world and, both juveniles and larvae of bivalves are more susceptible to diseases caused by prokaryotes infections (Elston, 1999). Rickettsiae are small, pleomorphic coccobacilli considered to occupy a special taxonomic niche between bacteria and viruses (Lauckner, 1983). The first rickettsia-like organisms detected in molluscs have been found in *Mya arenaria* from Chesapeake Bay (Harshbarger et al., 1977). According to Harshbarger et al. (1977), the ribosome-rich, undulating rods, measuring 300 × 2,000 nm occurred in roundish inclusions, up to 100 µm in diameter and usually located singly in the epithelial-cell cytoplasm and lumen of digestive diverticula.

Although the yellow clam has important economic value, few histopathological studies of this species have been completed. For example, Cremonte & Figueras (2004) reported *Trichodina* sp., gregarins, coccidians and Turbellaria in yellow clams from the Argentinean coast, and Brusa et al. (2006) discovered a new species of Turbellaria, *Paravortex mesodesma*, in the intestinal lumen of *M. mactroides* from the Uruguayan coast. However, the parasites identified in these studies could not be responsible for yellow clam mortality, and there is little known about the pathological conditions of *M. mactroides* populations in Brazil.

Despite the increasing concern about diseases in edible bivalves in the Brazilian coast, particularly in Santa Catarina State in southern Brazil (da Silva et al., 2002; da Silva et al., 2011; Sabry et al., 2011) and the northeastern states of Brazil (Azevedo et al., 2005; Sabry et al., 2007; Boehs et al., 2010), the occurrence and impact of bivalve diseases remain poorly studied. Furthermore, mortalities can occur without notice because a systematic surveillance programme is not currently in place (da Silva et al., 2011). Prior to this study, no histopathological research on the diseases of bivalves had been completed in the State of Rio Grande do Sul.

The programme to monitor the health status of *M. mactroides* began in 2011. The study area included Cassino beach, which lies between Rio Grande City and Barra do Chuí on the south shore of Rio Grande do Sul State in southern Brazil. In October 2011 (austral spring), the local recruitment period of *M. mactroides* (Bergonci & Thomé, 2008), a large number of empty juvenile shells and moribund juveniles unable to burrow in the sand were observed at Cassino beach. Initially it was thought that temperature or salinity could be responsible for the mortalities. However, abnormal fluctuations of these parameters were not observed (the temperature and salinity at the sampling time were 20°C and 30‰, respectively).

The objective of the present study was to determine the cause of the mortality event that affected juveniles of the yellow clam *M. mactroides* using histological assays for the detection of potential pathogens.

MATERIAL AND METHODS

Thirty moribund yellow clam juveniles (shell height ranging from 25 to 35 mm, mean = 29.35 ± 2.94 mm) were manually collected in the intertidal zone of Cassino beach in southern Brazil (32°12'S, 52°11'W) (Fig. 1) in October 2011. The samples were transported to the laboratory as quickly as possible, and the soft parts of the moribund clams were carefully removed from their shells. They were fixed in Davidson's solution (Shaw & Battle, 1957) for one day and then stored in 70% ethanol.

Twenty whole individuals were processed with automated equipment (Lupe Tec, model PT 05, Brazil) and embedded in Paraplast®. Sections (3μm) were cut on a microtome (Lupe Tec, model MRP03, Brazil) and stained with haematoxylin and eosin (HE).

For transmission electron microscopy (TEM), small fragments of the intestines of 10 clams were post-fixed in 2.5% glutaraldehyde and 1% OsO_4 in cacodylate buffer (0.05 M) (pH 7.4). The epithelial cells of the intestine were then dehydrated in ethanol and embedded in Epon 812 resin. Ultrathin sections were stained with uranyl acetate and lead citrate. Finally, the samples were examined with a microscope (JEOL, model 100S, Japan).

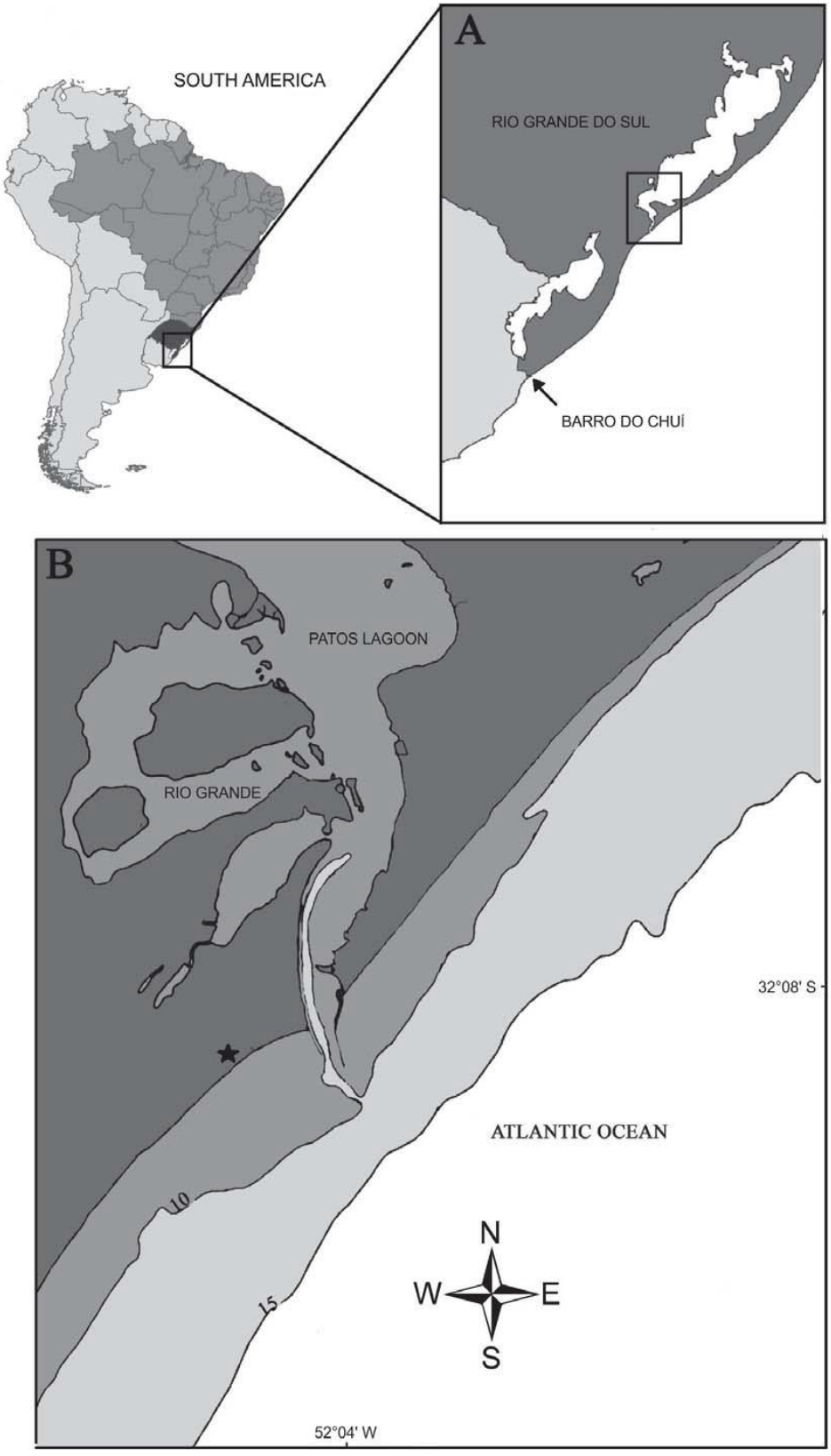


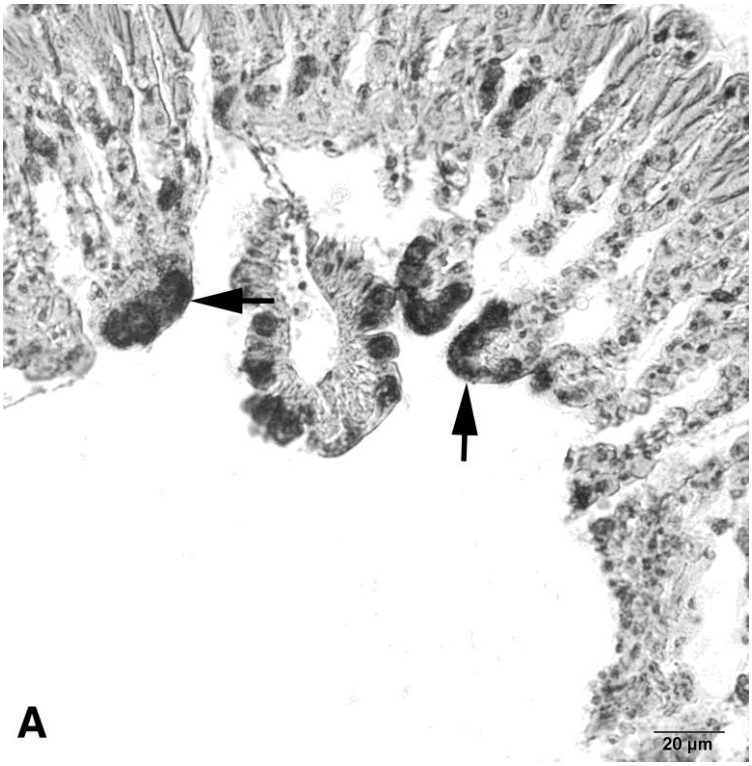
FIG. 1. Map of South America depicting the state of Rio Grande do Sul in southern Brazil. The star indicates the sampling location of moribund *Mesodesma mactroides* juveniles in Cassino beach.

RESULTS

In 100% of the clams analysed, light microscopy revealed the accumulation of basophilic material infecting the epithelial cells of the intestine and the gills that corresponded to *Rickettsia* colonies (Fig. 2). No other parasites were recorded in the studied specimens.

Transmission electron microscopy of the infected midgut revealed varying numbers of rickettsiae in the lumen. Some were free in the lumen while others were intimately associated with the plasma membrane of the intestinal epithelial

cells. In thin sections, the microorganisms were observed as circular shapes, approximately 0.2 to 0.6 µm wide and up to 1.6 µm in length. The limiting boundary was formed by two clearly defined structures: the rickettsial cell wall, which surrounds the whole microorganism, and the underlying plasma membrane, which limits the cytoplasm. Sections that were taken perpendicular to the cell wall and plasmalemma revealed the total thickness of the rickettsial interspace to be approximately 200 to 250 Å. The cell wall was approximately 90 Å thick, and the plasma membrane thickness was approximately 75 Å (Fig. 3B).



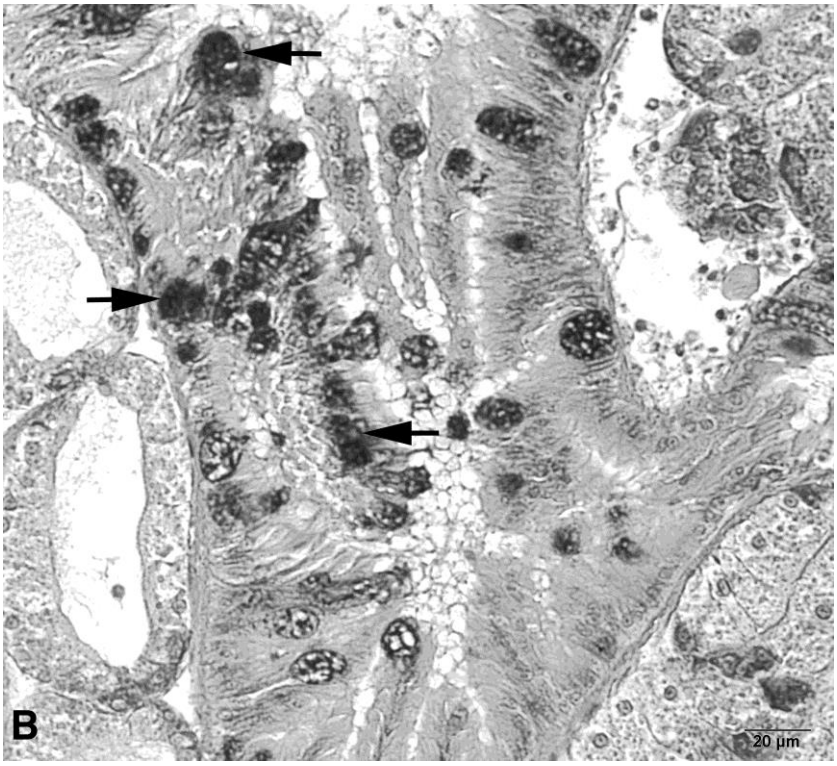
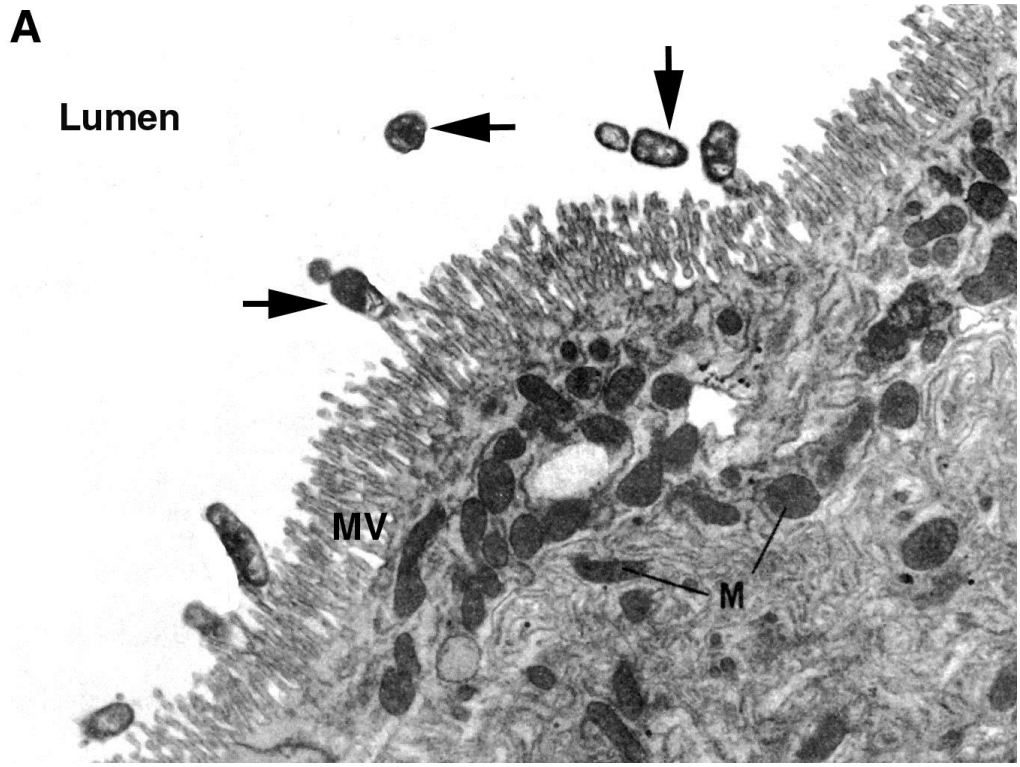


FIG. 2A: FIG. 2A. Gill of *Mesodesma mactroides* showing basophilic inclusions (arrows). Hematoxilin-Eosin staining. FIG. 2B. Intestinal epithelium of *Mesodesma mactroides* with basophilic inclusions. Hematoxilin-Eosin staining.



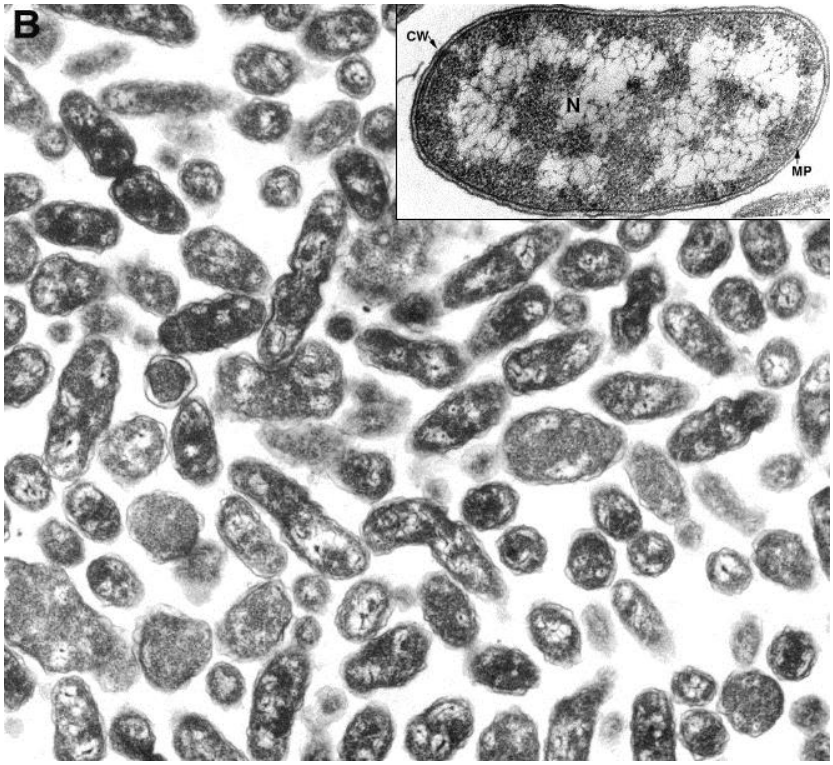


FIG. 3A. Electron micrograph through the intestinal epithelium of *Mesodesma mactroides*. The lumen contains several rickettsiae (arrows); some are in close association with the microvilli (MV). The dense mitochondria in the epithelial cells have a different internal structure than the extracellular microorganisms (M). X 9,500. FIG. 3B. Electron micrograph through the central portion of a colony of *Rickettsia*.

Circular profiles represent transverse sections, and elongate forms are sectioned longitudinally. Note the constriction furrows across the centres of several rickettsiae. With the exception of several pleomorphic forms, the colony is remarkably homogeneous. X 24,000. Insert: *Rickettsia* cells from the intestine of an infected *Mesodesma mactroides.* Both cell wall (CW) and plasma membrane (MP) are trilaminar when viewed on edge. The nuclear component (N) is represented as areas with fine filaments. X 125,000.

DISCUSSION

The present work investigated the cause of juveniles yellow clam (*Mesodesma mactroides*) mortality event that occurred in October of 2011 at an intertidal bed in Cassino beach, State of Rio Grande do Sul, in southern Brazil. Herein, no adults of yellow clam were recorded in the sampling area. According to Sindermann (1990), infectious diseases are common suspects in shellfish mortalities. In this study, we provide the first report of prokaryotes of the genus *Rickettsia* in the gill and intestinal epithelial cells of *M. mactroide*s.

The order of Rickettsiales pertains to the Alphaproteobacteria class and is composed of microorganisms with an obligate intracellular and parasitic lifestyle (Dumler & Walker, 2005). This group of prokaryotes is best known for its medically important genus *Rickettsia*, whose species can cause severe and occasionally fatal diseases in man (Parola & Raoult, 2001).The utrastructural characteristics of rickettsiae were clearly recorded in the present study. In thin sections the microorganisms are seen as circular or rod-shaped profiles. The limiting boundary is formed by two clearly defined structures: the rickettsial cell wall, which surrounds the whole microorganism, and the underlying plasma membrane, which limits the cytoplasm. The contours of the rickettsiae are generally smooth, with few irregularities. Some specimens show wrinkled or bulbous outpocketings of the cell wall. The plasmalemma has occasional vesicular inpocketings, these invaginations resemble the pinocytotic vesicles or vacuoles seen in animal cells. Their contents are of low electron density and are continuous with the structure less clear space between the plasma membrane and the cell wall. At the site of these invaginations, the cell wall is uninterrupted and does not parallel the infolded plasmalemma (Robertson, 1959; Cheville, 2009)

The first record of rickettsiae in bivalves was in 1977 in the soft shell clam, *Mya arenaria* from Chesapeake Bay, USA (Harshbarger et al., 1977). Since then, rickettsiae have been reported in several bivalve species, and they predominantly inhabit in the epithelia of the host's intestines and gills (Buchanan, 1978; Elston & Peacock, 1984; Mialhe et al., 1987; Fries & Grant, 1991). Members of the order Rickettsiales have previously been associated with mortalities of natural populations of different invertebrate groups (Sparks, 1985).

Some studies claim that *Rickettsia* infections cause little harm to bivalves as too few cells become infected (Harshbarger et al., 1977; Bower et al., 1994; Cremonte et al., 2005; Boehs et al., 2010). Indeed, when *Rickettsia* infection occurs in high intensity, it has been associated with significant mortalities in bivalve populations (Buchanan, 1978; Lauckner, 1983; Elston, 1986; Le Gall et al., 1988; Norton et al., 1993; Villalba et al., 1999). Bivalve larvae and juveniles are more vulnerable to prokaryote infections than adults as stated by Kinne (1983) and Elston (1999). In other invertebrates and vertebrates rickettsiae that invade the intestinal epithelial cells and produce an alteration in nutrient obsorption (Walker et al., 2003). It may also happen in *Mesodesma mactroides*.

The first description of a Rickettsiales-like organism (RLO) in the Brazilian aquatic fauna was recorded by Azevedo et al. (2005). They found RLOs infecting the gill epithelium of the mangrove oyster, *Crassostrea rhizophorae*, in the State of Piauí in northeastern Brazil. They suggested that RLOs could be associated with mortality of the parasitised oyster due to the disappearance of the apical cilia with concomitant lysis of the parasitised epithelial cells of the gill. da Silva et al. (2011) and Sabry et al. (2011) found RLOs in very low prevalence in epithelial cells of the digestive glands of oysters (*Crassostrea gigas* and *C. rhizophorae*) from Santa Catarina State in southern Brazil, and those cells did not sustain serious damage.

This is the first report of *Rickettsia* infection among *Mesodesma mactroides* and the first histopathological survey of diseases of marine bivalves from Rio Grande do Sul State. We observed that 100% of the bivalve specimens analysed were infected with *Rickettsia*. Furthermore, the specimens were moribund at the time of collection, and upon returning to the sampling site for observations, it was noted that all juveniles were

dead. Therefore, our data suggests that *Rickettsia* contributed to the mortality event of *M. mactroides* reported in Brazil in 2011.

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3. USO DA REAÇÃO EM CADEIA DA POLIMERASE PARA DETECÇÃO DE PATOGENOS NO MARISCO BRANCO *Mesodesma mactroides*

USE OF POLYMERASE CHAIN REACTION FOR BIVALVE PATHOGENS SURVEILLANCE IN THE YELLOW CLAM *Mesodesma mactroides*

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Resumo

O marisco branco *Mesodesma mactroides* é um bivalve valioso que ocorre do sudeste do Brasil ao norte da Argentina. Populações de *M. mactroides* estão desaparecendo de toda a sua distribuição geográfica e a causa continua desconhecida. O objetivo deste artigo foi procurar por doenças de notificação listadas pela Organização Mundial de Sanidade Animal (OIE), que inclui *Marteilia* sp., *Perkinsus* sp., *Bonamia* sp., *Mickrocytos mackini*, e a microvariante 1 do vírus herpes dos ostreideos (OsHV 1µvar) em populações de marisco branco do sul do Brasil, utilizando protocolos de histologia clássica e técnicas moleculares. Um total de 180 mariscos foram coletados utilizando uma pá na zona intertidal da praia em 6 pontos amostrais cobrindo a costa inteira do Estado do Rio Grande do Sul (extensão = 622 km) em 2013. Amostras de tecidos foram testados utilizando ensaios de reação em cadeia da polimerase convencional (cPCR). As análises não apresentaram nenhuma evidência de sequências específicas de protistas parasitos e patógenos virais em nenhum dos 6 pontos amostrais. Recomenda-se o contínuo monitoramento dos moluscos da região.

Abstract

The yellow clam *Mesodesma mactroides* is a valuable shellfish occurring from southeastern coast of Brazil to the northern coast of Argentina. Populations of *M. mactroides* are disappearing from the entire distribution range and the cause still unknown. The objective of this paper was to search for notifiable diseases listed by the World Organization of Animal Health (OIE), which include *Marteilia* sp., *Perkinsus* sp., *Bonamia* sp., *Mickrocytos mackini*, and the ostreid herpesvirus 1 µ-var in the yellow clam population in southern Brazil, using molecular techniques and classic histology protocols. A total of 180 clams were collected using a shovel in the intertidal region of the beach in six sample points covering the entire coast of the Rio Grande do Sul State (extension = 622 km) in 2013. Tissue samples were tested by OIE recommended single-step conventional polymerase chain reaction assays (cPCR). The screening showed no evidence of the specific sequences of the protistan parasites and viral pathogens at any site of the six zones under study. We recommend continuous monitoring of the molluscs of the region.

Introduction

Epizootic diseases have been claimed to cause catastrophic mortalities of marine bivalves, which can severely impact the fisheries and aquaculture activities around the world (Fisher 1988). Some of these diseases have become a serious primary constraint for the development and sustainability of shellfish farming (Villalba et al. 2014).

Currently, it is considered that the main cause associated with epizootic outbreaks is the transfer of infectious agents through the transport of live shellfish (Figueras and Novoa 2004). The dynamics of free trade and the desire of countries to seek new alternatives to food production and economic development may overlook essential health factors, which if not considered in its proper context, can cause the collapse of the entire shellfish culture (Cáceres-Martínez and Vásquez-Yeomans 2008).

According to the World Organization for the Animal Health (OIE), notifiable diseases are considered to be of socio-economic and/or public health importance within countries and also significant in the international trade of aquatic animals and their products. The OIE establishes a series of diseases that can drastically affect the survival and growth of bivalve mollusks with economical importance. The OIE (2014) list of notifiable diseases of bivalves and the pathogens causing them comprises: marteiliosis (*Marteilia refringens*), bonamiosis (*Bonamia ostreae*, *Bonamia exitiosa*), perkinsosis (*Perkinsus marinus*, *Perkinsus olseni*) and the infection with ostreid herpesvirus-1 microvariant (OsHV-1 µVar).

There are some reports of notifiable diseases in bivalves from the Atlantic coast of the South America. For instance, *Perkinsus marinus* and *Perkinsus* sp. were recently recorded infecting natural stocks of the mangrove oyster *Crassostrea rizophorae* in the northeastern of Brazil (Brandão et al. 2013; da Silva *et al*. 20013) while *Perkinsus olseni* was recorded infecting the venerid clam *Pitar rostrata* in the Uruguayan coast (Cremonte et al. 2005). Another notifiable disease detected in southwestern Atlantic coast was bonamiosis (*Bonamia exitiosa*) infecting the flat oyster *Ostrea puelchana*, in the Argentinean coast, where it caused severely mortality in cultured flat oysters (Kroeck and Montes 2005).

The yellow clam *Mesodesma mactroides* was an abundant shellfisheries resource in the dissipative oceanic beaches from Argentina, Uruguay and southern coast of Brazil, especially in the Rio Grande do Sul State (Coscarón 1959; Defeo 1989; Gianuca 1985). However, a drastic decline of the population collapsed the fishery industry. These reductions in the yellow clam stocks were attributed to the over-exploitation (Brazeiro and Defeo 1999) and mass mortalities events that were firstly reported in Brazil (Odebrecht et al. 1995), and then in Uruguay and Argentina (Fiori and Cazzanniga 1999; Cremonte and Figueras 2004). Several studies were carried out for search the cause of these mortalities events (red tide, heavy metals, pollution, parasites etc.). However no definitive conclusion was obtained.

Molecular technologies, such as the polymerase chain reaction (PCR) have provided a very sensitive and specific tool for the detection of pathogens in aquatic animals. Diagnostic by conventional PCR (cPCR) methodology is now widely available and increasingly being used as a helpful molecular technique for the diagnosis of the most important mollusc diseases (Figueras and Novoa 2004). The aim of the present study was to search for notification diseases in the yellow clam using molecular techniques (cPCR) and histological analysis.

Methods

Clams sampling

Adult yellow clams (shell height between 5 to 8 cm) were sampled during winter 2013 (seawater temperature = 14ºC, salinity = 30‰). The clams were collected using shovels along the intertidal region of dissipative sand beaches. The study area comprises six sampling points distributed along the entire coast of the Rio Grande do Sul State (extension = 622 km). The sampling points were: “Capão da Canoa”, 29º44' S, 50º00' W; “Farol de Mostardas”, 31°14’ S, 50°54’ W; “Estação Marinha de Aquacultura”, 32º12’ S, 52º10’ W; “Farol da Sarita”, 32°37’ S, 52°25’ W; “Farol do Albardão”, 33°12’ S, 52°42’ W; “Farol do Chuí”, 33°44’ S, 53°22’ W. Thirty adult clams were collected per sample point, totalizing 180 clams analyzed.

PCR analysis

DNA extraction

For cPCR assays, cross sections including mantle, gills and labial palps were cut and preserved in 96% ethanol. Samples of the preserved tissues were dissected from each individual and rinsed twice with sterilized double-distilled water to remove residual ethanol. Total DNA was extracted from the samples using the commercial DNeasy kit from Qiagen following the spin column protocol with the addition of two more washing steps with buffer AW1 and AW2. To avoid false negative results total DNA isolated from each sample was first screened using the eucariotic universal primer set, 18S Eu F 5´-TCT-GCC-CTA-TCA-ACT-TTC-GAT-GG-3´ and 18S Eu R 5´-TAA-TTT-GCG-CGC-CTG-CTG-3´ (140 bp) (Fajardo et al. 2008).

This primer set amplifies eucariotic 18S rRNA DNA to verify that the sample does not contain contaminants that may inhibit the PCR reaction. Amplifications were performed in an Eppendorf Mastercycler PCR thermocycler according to the following conditions: Initial denaturation 10 min at 94°C, thirty-five reaction cycles (94°C for 1 min, 61°C for 1 min, 72°C for 1 min) followed by a 4 min extension at 72°C.

cPCR based assays

A series of diagnostic OIE-recommended single-step conventional cPCR assays were set up and applied for the detection of notifiable bivalve mollusc pathogens (OIE 2009, 2010, 2011). cPCR assays were carried out in a total volume of 25 µl containing each diagnostic primer at 2 ng/µl and template genomic DNA at 50-200 ng/µl; Go Taq PCR green buffer at 1 X final concentration including MgCl2 at 1.5 mM; deoxynucleotides (dNTPs) at 0.2 mM; and Go *Taq* polymerase (Promega) at 0.625 U/µl. Amplified products were electrophoresed on a 2% agarose (in 1 X Tris-borate-EDTA) gel containing 0.1 µg/ml ethidium bromide and visualized under UV transillumination.

Single-step cPCR assay was performed for the *Perkinsus* Genus diagnosis using the primer set PerkITS85, 5' CCG-CTT-TGT-TTG-GAT-CCC-3' and Perk ITS-750 5'-ACA-TCA-GGC-CTT-CTA-ATG-ATG-3', to amplify a DNA region of the internal transcribed spacer (ITS) of the small subunit ribosomal RNA (SSU rRNA, 703 bp) (OIE 2009, 2010, 2011). The temperature profile included an initial denaturation at 94°C for 4 min; 40 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min; and a final extension at 72°C for 10 min.

For *Mikrocytos mackini* detection we used primers, Mikrocytos F 5'-AGA-TGG-TTA-ATG-AGC-CTC-C-3' and Mikrocytos R 5'-GCG-AGG-TGC-CAC-AAG-GC-3', that target the SSU rRNA region, (546 bp) (OIE 2009, 2010, 2011). Amplification conditions: initial denaturation at 94°C for 10 min; 40 cycles of 94°C for 1 min, 60.5°C for 1 min, and 72°C for 1 min; and a final extension at 72°C for 10 min.

To detect DNA from the ostreid herpesvirus (OsHV 1-µVar), we used two different primers combinations C2 F 5'-CTC-TTT-ACC-ATG-AAG-ATA-CCC-ACC-3', C6 R 5'-GTG-CAC-GGC-TTA-CCA-TTT-TT-3' (709 bp), and C13 F 5'-CCT-CGA-GGT-AGC-TTT-TGT-CAA-G-3', C5 R , 5'-CCG-TGA-CTT-CTA-TGG-GTA-TGT-CAG-3'(765 bp) from the ORF4 C region of the OsHV-1 genome (Batista et al. 2007). Reaction mixtures were heated to 94°C for 5 min and cycled 35 times at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min for each cycle, with a final extension at 72°C for 7 min.

Additionally to screen *Bonamia ostrae* and *Bonamia exitiosa, M. mactroides* DNA samples were analyzed with primer set F Bo 5'-CAT-TTA-ATT-GGT-CGG-GCC-GC-3', and R Boas 5'-CTG-ATC-GTC-TTC-GAT-CCC-CC-3' targeting the small subunit ribosomal RNA gene (SSU rRNA, 304 bp) (OIE 2009, 2010, 2011). Amplification profile: initial denaturation at 94°C for 5 min; 30 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min; and a final extension at 72°C for 10 min.

If a sample was found positive each individual of the pool was re-tested and checked using a different cPCR protocol and confirmed by sequencing. Negative controls, no template controls, and positive controls obtained from the OIE reference laboratories were included in each PCR run.

Histological analysis

A piece (5 mm thick) of tissue including visceral mass, gills, siphons and foot was collected and fixed in Davidson´s solution for 24 hours and then stored at ethanol 70% until processing. The tissues were processed, embedded in Paraplast. Sections (5 µm thick) were stained with hematoxylin and eosin (H&E) and were examined with light microscopy for disease diagnosis.

Results

PCR analyses

The screening showed no evidence of the specific sequences of the protistan parasites and viral pathogens at any site of the six zones under study. Total DNA integrity of all the samples was confirmed by the presence of eucariotic the 18S rRNA gene amplicon (140 bp, band).

Histological analyses

The histological examination revealed the occurrence of basophilic inclusion corresponding to *Rickettsia*-like organisms in the digestive gland epithelium, *Trichodina* sp. in the gills, *Nematopsis*-like in the muscular tissue, *Pseudoklossia*-like in the nephridia tubule, turbellarians *Paravortex mesodesma* in the intestinal lumen and metacercariae encysted in the siphons. None of the parasites recorded in the histological slides were included in the OIE-list of diseases of molluscs, confirming the PCR analyses.

Discussion

This is the first study using molecular tools to search for OIE notifiable diseases in the yellow clam *Mesodesma mactroides.* Until the present time, notifiable diseases were not recorded in this species.

The health state surveillance of the of yellow clam population from the coast of the Rio Grande do Sul State, southern Brazil, started in 2011, and since this year, mass mortalities events were recorded in the springtime affecting small juveniles recently settled. The cause of this mass mortality was associated with *Rickettsia* (Carvalho et al. 2013). Nevertheless, a myriad of factors could be associated with the mortality event reported by Carvalho et al. (2013). As stated by Ford (2001), even when clams parasites are associated with mortality, it is not always clear whether they are the only causative agent or simple microbes that proliferate in hosts already compromised by some other factor, such as oxygen depletion, overcrowding and anthropogenic activities.

According to Figueiras and Novoa (2004), bivalve diseases have traditionally been studied using histological techniques, which have proved to be very useful to detect pathogens, mainly protozoan parasites, associated to mortalities or quality losses of cultured stocks but also to determine the lesions and the interaction of the pathogens with the host immune defense mechanisms. In the 90's, molecular biology techniques started to be applied to bivalve diseases. Sensitive techniques, such as PCR, sequencing mainly of the 18s ribosomal RNA and ITS were used for the diagnosis of the main diseases of bivalves (Figueras and Novoa 2004).

Sindermann and Rosenfield (1967) commented on the scarcity of mass mortalities in clam, compared to mussels and oysters. Bower et al. (1994) indicates that clams do have fewer diseases than oysters, although scallops and mussels also have relatively few.

In the present study, the parasites that were recorded in high prevalence in all sample points, ciliates *Trichodina* sp. and the turbellarians cf. *Paravortex mesodesma*, are considered harmless (Lauckner 1983). In the present study, no evidence of damage was associated to these parasites. Others parasites, as coccidians and gregarines are considered of moderate pathogenicity, depending on the intensity of infection (Lauckner 1983; Carballal et al. 2001). Mechanical interference by heavy infections of *Nematopsis* sp. has been suggested to have some harmful effects on the host physiology (Sindermann 1990) and heavy infections of *Pseudoklossia* sp. can cause renal dysfunction (Carballal et al. 2001). However, these two parasites were rarely recorded.

Through the histopathological observations and the molecular analyses, it can be stated that the yellow clam population from the Rio Grande do Sul State are in good health conditions. Despite the absence of positives to the OIE diseases, we recommend continuous monitoring of the diseases affecting the yellow clam *M. mactroides* from Rio Grande do Sul State and other regions were exploitable banks occurs.

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4. Efeito da Salinidade Reduzida no Marisco Branco Mesodesma mactroides

Effect of Low Salinity on the Yellow Clam Mesodesma mactroides

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Resumo

O objetivo deste estudo foi determinar a salinidade letal (CL_{50}) para o marisco branco Mesodesma mactroides (Bivalvia: Mesodesmatidae) e as alterações histopatológicas que poderiam ser úteis para o diagnóstico de mudanças estruturais no tecido dos bivalves. Mariscos de duas classes etárias de tamanho (juvenis e adultos) foram colocados em recipientes de 10 L e expostos à salinidades de 35, 30, 25, 20, 15, 10 e 5 g/L. Os tratamentos foram realizados em triplicata com sete bivalves em cada recipiente, A CL_{50} para 48 h de exposição foi 6,5 g/L e 5,7 g/L para adultos e juvenis, respectivamente. Para 96 h de exposição, a CL_{50} foi 10,5 g/L para adultos e 8,8 g/L para juvenis. O exame histológico dos mariscos expostos à salinidade de 10 g/L por 96 h revelou edema intracelular e focos necróticos no epitélio da glândula digestiva e oclusão da luz da glândula digestiva. Em conclusão, M. mactroides pode ser considera uma espécie eurialina moderada, tolerando salinidades de 35 até 15 g/L.

Palavras-chave*:* marisco branco, salinidade letal, CL_{50}, histologia, extremo sul do Brasil.

Abstract

The aim of this study was to determine the lethal salinity (LC_{50}) for the yellow clam Mesodesma mactroides (Bivalvia: Mesodesmatidae) and identify histopathological alterations that could be used to diagnose structural changes in clam tissue. Clams in two size classes (adults and juveniles) were placed in 10 L chambers and exposed to salinities of 35, 30, 25, 20, 15, 10, and 5 g/L. There were triplicate chambers with seven clams each for each salinity. The LC_{50} values for a 48 h exposure were 6.5 g/L and 5.7 g/L for adults and juveniles, respectively. For a 96 h exposure, the LC_{50} values were 10.5 g/L for adults and 8.8 g/L for juveniles. The histological examination of yellow clams exposed to 10 g/L for 96 h showed intercellular oedema and necrotic foci in the epithelium of the digestive gland and occlusion of the lumen of the digestive gland. In conclusion, M. mactroides can be characterised as a moderately euryhaline species, tolerating salinities from 35 to 15 g/L.

Keywords*:* yellow clam, lethal salinity, LC_{50}, histology, extreme southern Brazil.

Introduction

The yellow clam Mesodesma mactroides (Deshayes, 1854) is an intertidal sandy beach bivalve that is distributed along the Atlantic coast of South America from Brazil to Argentina (Rios, 2009). Historically, M. mactroides had been considered an important economic resource that is commercially exploited by fishermen using shovels in Brazil, Uruguay, and Argentina (Coscarón, 1959; Gianuca, 1985; Bergonci and Thomé, 2008). However, yellow clam populations collapsed as a result of overfishing, which was associated with cyclic mass mortalities due to unknown causes (Odebrecht et al., 1995; Fiori and Cazzaniga, 1999; Cremonte and Figueiras, 2004). Some of the largest numbers of mortalities occurred near the influence of the Patos Lagoon and River Plate, which can affect the salinity of the coastal zone in extreme situations. M. mactroides has been identified as a threatened species with a critically endangered status (Herrmann et al., 2011).

According to Manzi and Castagna (1989), the salinity tolerance range of the species is one of the most fundamental biological information required for assessing its environmental suitability for culture purposes. Studies claim that reduced salinities in regions near freshwater streams or rivers are unfavourable environments for the development of M. mactroides (Olivier et al., 1971; Defeo et al., 1992; Marins and Levy, 2000). However, these studies did not determine the minimum tolerable levels of salinity for the yellow clam. Therefore, the objective of this study was to investigate the influence of salinity on M. mactroides survival. The lethal effects of salinity were examined in juvenile and adult clams in the laboratory through short-term exposure bioassays. In addition, histological changes that could be used to diagnose the exposure of yellow clams to low salinities were analysed.

Materials and Methods

Experiments to determine the lethal effects of salinity were carried out with different-sized M. mactroides clams at the Marine Aquaculture Station of the Federal University of Rio Grande-FURG, southern Brazil. The experiments were carried out during summer–early autumn 2012, and the clams were obtained from Cassino Beach (Figure 1) (32°24’S; 52°20’W). Experimental procedures were based on standard

methods for static acute bioassays with aquatic invertebrates (Rand and Petrocelli, 1985).

The clams were acclimated to laboratory conditions for one week. Juveniles (with no functional gonads, mean shell length = 29.4 ± 2.9 mm) and adults (with functional gonads, mean shell length = 62.2 ± 2.8 mm) were maintained at ambient temperature (25°C) in 200 L tanks with aerated seawater (35 g/L) without sand. The clams were fed daily with 1 L of Nannochloropsis oculata. Only clams showing healthy signs and normal behaviour (normal shell gape and protrusion of siphons and foot) were used in the bioassays.

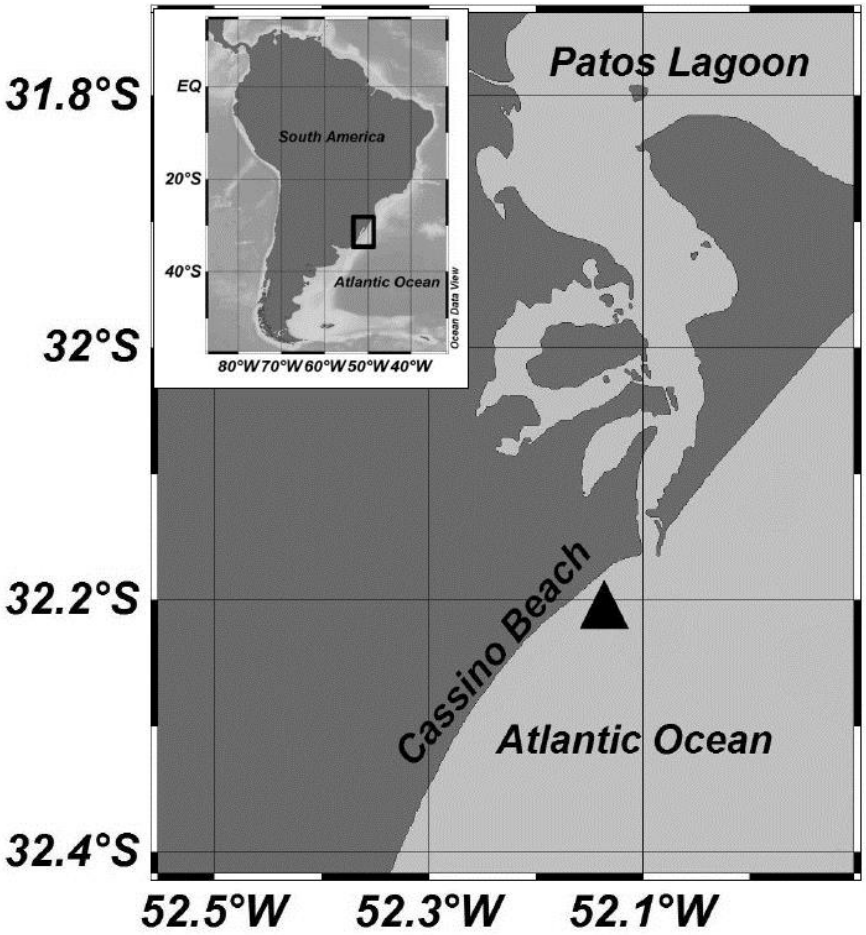

Figure 1. Map of South America depicting Rio Grande do Sul State in southern Brazil. The star indicates the sampling location of Mesodesma mactroides in Cassino beach.

The clams of two size classes were placed in 10 L tanks at 25°C. For each treatment, 21 juvenile clams (7 for each replica) were exposed to salinities of 35, 30, 25, 20, 15, 10, and 5 g/L. The same procedure was performed for adult clams. The different salinities were obtained by diluting seawater (35 g/L) with fresh water. The clams were not fed during the experiment. Mortality was recorded at 12, 24, 36, 48, 72, and 96 h. Test subjects with a permanent wide valve gape with extended siphons and a foot that was not responsive to touch were considered dead.

For each treatment, the percentage survival of clams was plotted against the exposure period. The acute lethal effects of low salinities on different-sized clams were analysed by determining the median lethal concentration (LC_{50}), which represents the salinity estimated to cause 50% mortality of a test population over a specific period (Rand and Petrocelli, 1985). A trimmed Spearman Karber was used to calculate the LC_{50} for each exposure time.

To analyse possible histological changes resulting from osmotic stress, clams that survived at the end of the experiment were fixed in Davidson's solution (Shaw and Battle, 1957). Tissue samples (especially the digestive gland, which appeared sensitive to low salinity exposure in our preliminary experiments) were embedded in Paraplast®, and 5 μm sections were stained with haematoxylin and eosin.

Results

M. mactroides clams of all size classes were tolerant to low salinities. Mortality was recorded at salinities ≤ 10 g/L (Figure 2). All clams succumbed within 96 h of exposure to 5 g/L salinity. The survival after 96 h of exposure to a salinity of 10 g/L was 60% and 27% in juveniles and adults, respectively. At salinities ≥ 15 g/L, all animals tested survived.

The median lethal salinity (LC_{50}) of a 48 h exposure was 6.5 g/L and 5.7 g/L, respectively, for adults and juveniles. For a 96 h exposure, the LC_{50} was 10.5 g/L for adults and 8.8 g/L for juveniles.

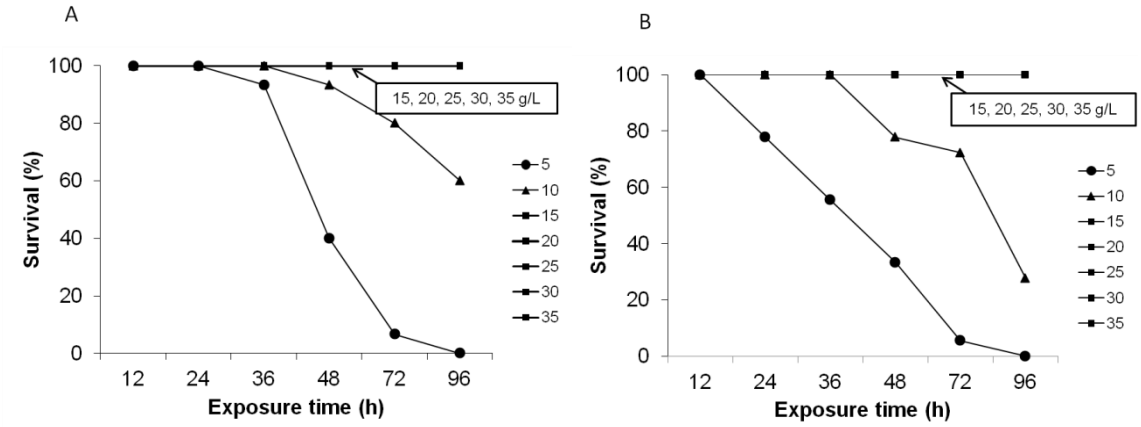

Figure 2. Percentage survival of Mesodesma mactroides exposed to different salinities (g/L) at an ambient temperature of 25°C. (A) juveniles, (B) adults.

The histological evaluation revealed clear trends that could be useful in the presumptive diagnosis of low salinity exposure. Figure 3 shows that in yellow clams exposed to salinities ≥ 15 g/L, the structure of the digestive gland remained normal, whereas structural changes were detected in the digestive gland of clams exposed to a salinity of 10 g/L for 96 h. The pathological signs observed were occlusion of the digestive tubular lumina, necrotic foci, and intracellular oedema in the epithelium of the digestive glands (Figure 4).

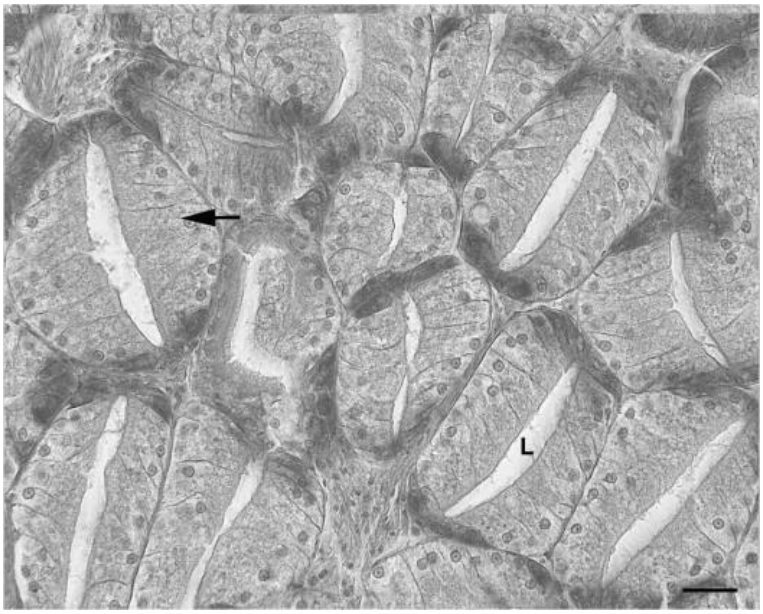

Figure 3. Histological section of a normal Mesodesma mactroides digestive gland showing high absorptive epithelial cell height (arrow) and the open lumen of the digestive gland (L). Haematoxylin and eosin staining. Bar, 20 µm.

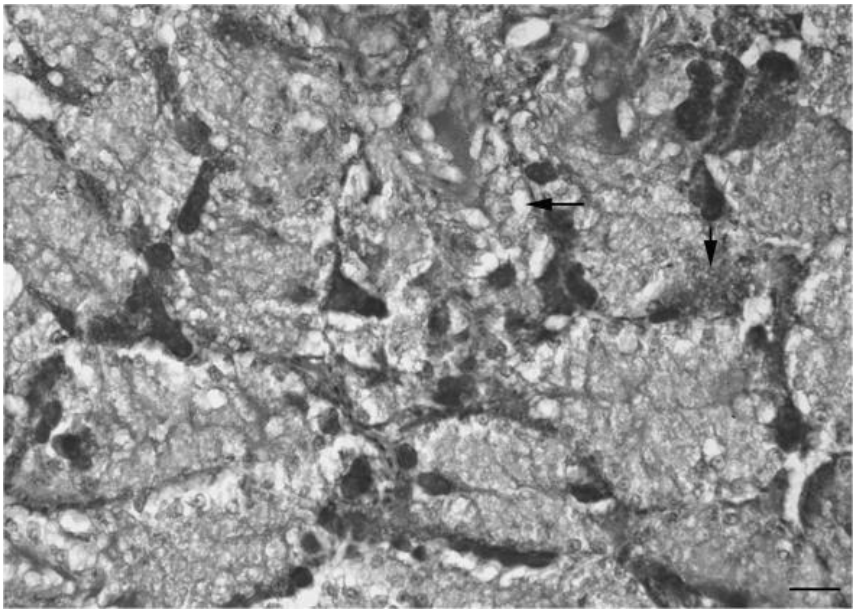

Figure 4. Histological section of a digestive gland from Mesodesma mactroides exposed to 10 g/L salinity for 96 h showing intercellular oedema (long arrow) and necrotic foci (short arrow). The absorptive cells appear to be massively swollen, occluding the lumen of the tubules. Haematoxylin and eosin staining. Bar, 20 µm.

Discussion

Knowledge of the minimum salinity tolerance of commercially important bivalves, such as the yellow clam M. mactroides, will be of prime importance to determine causes of mortality in the environment. According to Kinne (1970), the greater tolerance of juveniles to low salinities than adults could be explained by the additional metabolic demand due to the onset of gonad maturation. Marins and Levy (2000) reported that only juvenile yellow clams lived near the Patos Lagoon outflow because adult clams were not able to survive the low salinities characteristic of this environment.

The coastal marine realms are affected by continental runoff during severe rain, resulting in periods of decreased salinity. Animals inhabiting such habitats adopt different mechanisms for survival (Kinne, 1970). Intertidal and estuarine bivalves are

generally tolerant to sudden and large changes in salinity (Shumway et al., 1977). Sediment burial is a major mechanism used by clams to isolate themselves from unfavourable conditions in the water column. However, the yellow clams were maintained in an aquarium without sand in this study. If the clams were given the opportunity to burrow in the sand, they may have survived for a longer time in a low salinity environment.

On Cassino beach on the coast of Rio Grande do Sul where the yellow clam M. mactroides is frequently found, the salinity ranges from 14 g/L to 38 g/L (mean = 28 g/L), with the minimum values related to El Niño events (Odebrecht et al., 2010). Thus, the lethal low salinities for M. mactroides were not far from the extreme values recorded by Odebrecht et al. (2010), and mortality might occur due to low salinities in some areas.

Most bivalves respond immediately to changes in the environmental salinity by closing their valves to isolate their soft body from the external environment (Dame, 1996). At the salinity of 5 g/L, the valves of M. mactroides were tightly closed until they died. In clams maintained in a salinity of 10 g/L, their valves were closed from the beginning of the experiment to 72 h of exposure.

After 72 h of exposure to 10 g/L salinity, siphons and the foot protruded out slightly and responded to external stimuli at a slow rate. No production of faeces or pseudo-faeces was observed, indicating that these clams were physiologically stressed.

At salinities $\geq$ 15 g/L, yellow clams were active from the beginning of the exposure to the end of the experiment, with the production of faeces and pseudo-faeces. The siphons and foot were withdrawn into the shell cavity at the slightest disturbance. Therefore, low salinities $\geq$ 15 g/L can be considered suitable for the yellow clam, at least for 96 h of exposure.

Histological changes observed in the digestive gland confirm that 10 g/L salinity is unsuitable for M. mactroides. Lesions and structural changes of the gastrointestinal epithelium are important indicators of bivalve health, and a significant loss of digestive gland absorptive cells is a pathological sign associated with mortality in bivalves

(Elston, 1999). Syndromes that involve the digestive gland may result from changes in the environment, such as temperature and salinity (Elston, 1999)

The present study provided data that can be used for the diagnosis or forensic evaluation of yellow clams that are suspected of exposure to lethal or marginal low salinities. Similar results were obtained by Elston et al. (2003) in an experiment analysing the salinity tolerance of the Manila clam Venerupis phillippinarum (Adams and Reeve, 1850). They concluded that the swelling of absorptive cells of the digestive glands of clams exposed to a salinity of 10 g/L might be due the absorption of hypoosmotic seawater, followed by the sloughing of these cells into the lumen of the digestive gland.

In conclusion, the yellow clam M. mactroides can be considered a moderate euryhaline species that is able to tolerate salinities from 35 to 15 g/L, and populations, particularly the adults, that inhabit areas near the mouth of great rivers, such as River Plate or Patos Lagoon, can suffer mortality after several days of rainstorms that occur during strong El Niño events and are accompanied by an elevated discharge of fresh water into the coast.

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5. India ink induces apoptosis in the yellow clam *Mesodesma mactroides* (Deshayes, 1854). Optical and ultrastructural study.

Tinta nanquim induz apoptose no marisco branco *Mesodesma mactroides* (Deshayes, 1854). Estudo óptico e ultraestrutural.

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Este artigo foi aceito para publicação na revista *Annals of Brazilian Academy of Science* (AABC-2014-0600)

Resumo

Este artigo reporta o processo inflamatório agudo e celular no marisco branco *Mesodesma mactroides*, induzido através de injeção de tinta nanquim no pé muscular. Obervações histológicas com microscopia óptica e microscopia eletrônica foram realizadas 24 e 48h após a injeção. A resposta inflamatória induzida consistiu de infiltração hemocitária geral sem ocorrência de necrose e atividade apoptótica. Migração de fagócitos carregados de tinta nanquim através do epitélio intestinal foi registrado. Parece que o marisco branco excretou partículas de nanquim pelas brânquias e rim. A tinção positiva para apoptose foi observada na glândula digestiva. Microscopia eletrônica da glândula digestiva revelou mudanças ultraestruturais de estresse do retículo endoplasmático rugoso e corpos apoptóticos. O mecanismos pelo qual as partículas de nanquim induzem apoptose continuam desconhecidos, possivelmente associados ao estresse do reticulo endoplasmático. Este trabalho destacou características que exigem discussões na área restrita da resposta inflamatória de moluscos.

*Palavras-chave***:** tinta nanquim; processo inflamatório; estresse do retículo endoplasmático; apoptose; *Mesodesma mactroides*

Abstract

This paper reports on the acute inflammatory and celular process in the yellow clam, *Mesodesma mactroides*, induced by injection of India ink into the muscular foot. Histological observations with optical and electronic microscopy were made at 24 and 48 hr after injection. The induced cellular inflammatory response consisted of a general hemocyte infiltration without necrosis and apoptotic activity. Migration of ink-laden phagocytes across the intestinal epithelium was recorded. It appeared that the yellow clam “excreted” ink particles through the gill and kidney. The positive staining for apoptosis was observed in digestive gland. Electronic microscopy revealed in digestive gland ultrastructural changes of endoplasmic reticulum stress and apoptotic bodies. The mechanism by which the India ink particles induce apoptosis remains unknown, possibly associated with the endoplasmic reticulum stress. This work has highlighted

features that require further discussion and thought in the restricted field the inflammatory responses of mollusks.

Key words: India ink; Inflammation process; endoplasmic reticulum stress; apoptosis; *Mesodesma mactroides*.

INTRODUCTION

Interest in the immunity of bivalves has been increasing continuously in the last decades due to the catastrophic mortalitiy in aquaculture-produced species and to the decline of natural stocks of economic value (Song et al. 2010).

For decades, the yellow clam *Mesodesma mactroides* (Deshayes 1854) (Mesodesmatidae) was the major shellfish resource from the sandy beaches of the Atlantic coast of Argentina, Uruguay and the southern part of Brazil (Castilla and Defeo 2001). However, a combination of overexploitation and high mortality rates along the entire distribution range of the species culminated in the collapse of these natural stocks (Fiori and Cazzaniga 1999).

In recent years, there has been great emphasis on the study of parasites of the yellow clam and the resulting pathologies (Cremonte and Figueras 2004, Carvalho et al. 2013a, 2013b). However, basic pathological processes, such as inflammation, have been generally overlooked.

Classic studies using injected dyes have greatly helped investigators describe the phagocytic cells and their role in local and general inflammatory processes in oysters (Pauley and Sparks 1966).

Essentially all animal cells have the ability to undergo apoptosis by activating an intrinsic cell suicide program when they are no longer needed or have become seriously damaged. The execution of this program leads to a morphologically distinct form of cell death, termed apoptosis (Kerr et al. 1972, Wyllie et al. 1980). It is now generally accepted that apoptosis is of central importance for the development and homeostasis of metazoan animals (Bergmann et al. 1998).

Apoptosis in mollusks is involved in the larval development process (Gifondorwa and Leise 2006) and most likely constitutes an important immune response that can be initiated by different inducers (Terahara and Takahashi 2008).

The endoplasmic reticulum (ER) is a centrally located, multifunctional, and multiprocess intracellular organelle supporting many mechanisms required by virtually every cell (Groenendyk et al. 2010). The membrane performs a remarkable number of diverse functions, including protein synthesis, translocation across the membrane, integration into the membrane, folding, posttranslational modification including *N*-linked glycosylation, and synthesis of phospholipids and steroids on the cytoplasmic side of the ER membrane, and regulation of Ca^{2+} homeostasis. Development and maintenance of optimally functioning ER membrane is essential for virtually all cellular activities, from intracellular signaling to control of transcriptional pathways; ion fluxes to control of energy metabolism; protein synthesis to multisubunit assembly; and lipid synthesis to transcriptional regulation of steroid metabolism. One of the major advantages of the centrally located ER network for the cell is the ability to control the composition and the dynamics of the ER luminal environment in an extracellular environment- independent way. Furthermore, the ER is not an isolated organelle because it has developed sophisticated mechanisms of communication with many other

cellular compartments, especially mitochondria, the plasma membrane, and the nucleus (Anelli and Sitia 2008, Frischauf et al. 2008).

ER stress conditions in mammals and humans have been observed in numerous diseases including Alzheimer disease, Creutzfeldt–Jakob disease, Huntington disease as well as cardiovascular diseases, indicating that ER stress- induced apoptosis is an important factor in pathophysiological conditions (Boya et al. 2002).

The purpose of the present study was to describe the inflammatory and celular responses experimentally induced after injection of a foreign body (India ink) into the muscular foot of the yellow clam *M. mactroides.*

MATERIALS AND METHODS

All experimental protocols were approved by the Federal University of Rio Grande (FURG) Animal Care Committee.

Clams husbandry

Specimens of yellow clam (n = 60) were collected in December 2012 at the intertidal zone of the Cassino Beach, (32˚19’54.2” S 052˚17’20.4” W), Brazil, by excavating the sand with a shovel. Specimens measured 40-60 mm in shell height (mean = 47 mm). Once in the laboratory, the clams were maintained for one day in tanks filled with 50 L of seawater, which was continuously aerated (temperature = 23 °C, salinity = 33 ppt), and were fed with the microalgae *Nannochloropsis occulata*.

Experimental Design

Prior to injection, the clams were anesthetized with a benzocaine solution (250 mg/L). Using a 25 gauge needle fitted to a syringe, approximately 0.05 ml of a suspension of

Indian ink (Royal Talens, Holland) or filtered seawater (30 clams for each group) was injected into the muscular foot of the *M. mactroides*.

Following the injections, the clams of each group were placed back in separeted tanks with 50 L of aerated seawater until the appropriate period of time had elapsed. Water exchange, tank cleaning and microalgae were provided daily. Ten clams were analyzed at 24 and 48 h after the injection.

Sample processing

Optical microscopy

Clams were shucked and the meat fixed in 20% buffered formalin for 24 hours. Sections that were approximately 5 mm thick, including the mantle, gills, gonad, digestive gland, kidney, and foot were then taken from each specimen. Tissue samples were embedded in Paraplast ® (Sigma, St. Louis, MO, USA) and 5 μm sections were stained with hematoxylin and eosin (H&E).

Ultraestructural analysis

For electron microscopy small fragments of the mantle, gills, gonad, digestive gland, kidney, and foot were cut into 1 mm blocks and immediately fixed in phosphate buffered glutaraldehyde (pH 6.9 at 4 °C), washed in Millonig's solution and post-fixed in 1% osmium tetroxide; the tissue blocks were then dehydrated in a graded series of ethanol-acetone, immersed in propylene oxide and embedded in Durcupan ACNI (Fluka Chemie A.G., Switzerland). Ultrathin sections were cut with an LKH ultramicrotome and doublestained with uranyl acetate and lead citrate before examination in a Jeol JEM-8T electron microscope (Jeol, 32, Tokyo, Japan).

Apoptosis analysis

Detection of apoptosis was performed in several tissues of both animals injected with Indian ink and control group animals injected with physiological solution. These samples were fixed in 20% buffered formalin and embedded in Paraplast®. For apoptosis detection, terminal deoxynucleotidyl transferase-mediated deoxyuridinetriphosphate nick end-labelling (TUNEL) was performed according to the manufacturer's recommendations by using the ApogTag plus Peroxidase in Situ Apoptosis Detection Kit (S7101; Chemicon, International). Anti-cleaved caspase-3 immunostaining was assessed using a rabbit anti-cleaved caspase-3 polyclonal antibody (Asp175; Cell Signalling Technology, Danvers, MA) as described (Schoner et al. 2010)

RESULTS

All of the experimental clams survived until the end of the trial.

Optical microscopy

Within 24 hours after the India ink injection, a massive migration of hemocytes to the location of the injection at the muscular foot was observed. Clumps of India ink granules were seen to be encapsulated, and small granules were phagocytosed and surrounded by inflammatory infiltrate (Figure 1). Within 48 hours after the India ink injection, gross examination of the experimental clams revealed that the India ink had spread to all parts of the body. Clumps of India ink accumulated in the gills, where they caused severe inflammation with massive infiltration of hemocytes, obstruction of the vessels and hemorrhage (Figure 2). Other organs in which the ink particles accumulated were the kidney and the digestive tract (Figure 3).

Evaluation of the apoptotic process by optical microscopy

Forty-eight hours after injection of Indian ink into the muscular foot of *M. mactroides*, in digestive gland tubules and muscle cells was observed the presence of apoptotic cells in all the clams after the India ink injection. Apoptosis was not observed in animals that were not injected with India ink. Tissues were counterstained with hematoxylin and eosin to aid in the morphologic evaluation of normal and apoptotic cells (Figure 4).

Electronic microscopy

Within 24 hours after the injection of the India ink, gill and kidney cells, but more in digestive gland, displayed various abnormalities. The shape of the nucleus was often irregular. In some cells the mitochondria were swollen, these cells were empty or contained osmiophilic lamellar structures that were suggestive of india ink with a variable density. Forty-eight hours after the India ink injection, similar condensed cell fragments containing fragmented nuclear masses were found in the voluminous membrane bound vacuoles in the cytoplasm of cells. The basement membrane appeared fragmented and had disappeared in some areas. When present, it was not closely apposed to the cytoplasmic membrane of the kidney cells, which is the normal configuration. Granular hemocytes were most frequently detected in contact with epithelial cells. They had a rather regular, round or oval nucleus. Their cytoplasm was usually pale and contained scarce isolated ribosomes and a few mitochondria. Furthermore, these cells usually lacked a well developed endoplasmic reticulum and a Golgi apparatus, and one or several lysosomal vesicles of variable size were frequently observed. The abundance of inflammatory cells in the gill and kidney tissue, which was represented by osmiophilic lamellar or granular electron dense material, was often associated with india ink. Fibroblasts predominated in the fibrotic areas, and a basement membrane-like substance was frequently in contact with their cell surfaces. Some fibroblasts showed morphologic signs of great synthetic activity, as evidenced by

cytoplasm containing numerous cisternae of rough ER that was dilated and filled with material of medium electron density. Within 24 hours after the India ink injection, apoptotic bodies were found in the muscle cells (Figures 5, 6, 7, 8, 9)

DISCUSSION

Bivalve hemocytes are primarily responsible for the defense against pathogens and foreign particles (Ottaviani et al. 2010). Phagocytosis and encapsulation are two major mechanisms used by hemocytes to eliminate nonself substances and dead cells (De Vico and Carela 2012). Phagocytosis is a process by which nonself molecules and cell debris are recognized and ingested, while encapsulation is the cellular immune defense reaction against foreign bodies that are too large to be phagocytosed (De Vico and Carela 2012).

The inflammatory response that occurs after pathogen invasion and/or cellular injury is a local defense reaction from the host tissue (Cone 2001). The functional basis of the inflammatory response has not been substantially modified during evolution, and the basic pattern is quite similar, regardless of the nature of the injurious agent, the site of its occurrence, or the injured organism (Ottaviani et al. 2010).

According to the manufacturer, Royal Talens, the carbon black pigment in Indian Ink is extremely fine, less than 2 μm. These particles are in constant motion in the ink, continually bumping into one another. The special preparation method of the ink ensures that they do not form lumps. If a large amount of water is added too quickly, however, the binder (shellac) that surrounds the particles dissolves. The particles then clot to form larger particles and sink to the bottom.

The strong hemocyte infiltration around the site of India ink was probably due to chemotaxis on the part of these cells in response to a release of chemicals from the necrotic muscle and connective tissue cells in the area. Phagocytosis of India ink by individual phagocytic cells in *M. mactroides* is similar to the response observed in other mollusks (Pauley and Krassner 1972).

Soluble substances are eliminated primarily through the gill, kidney, and intestine. In vertebrates, insoluble particulate material, such as carbon, is sequestered in the liver, spleen, lungs, and lymph nodes, all of which are part of the mononuclear phagocyte system (MPS) (Hirsch and Fedorko 1970). Disposal of particulate materials is very slow, except in the lung MPS cells, and may persist for many years. The main pathway of elimination in invertebrates is either the intestine or the renal-pericardial complex. According to Pauley and Krassner (1972), the intestine and the kidney play an important role in the elimination of ink particles from the sea hare *Aplysia californica*. The fact that ink was injected intramuscularly in the foot muscles may account for this result.

The electron microscopic study focused on the abnormalities in the gill, kidney and muscle tissue. A constant feature was the presence of conspicuous necrosis of gill cells and several electrolucent areas of irregular size that did not have apparent osmiophilic material and were not limited by a membrane, which seem to correspond to India ink in the gill, kidney, muscle, and intestinal cells.

Abnormalities in the gill and in the kidney epitelial basement membrane were also observed. These included the presence of areas of rarefaction containing osmiophilic inclusions in a basement membrane that was otherwise thickened or multilayered. The significance of these basement membrane abnormalities is unknown. However, the

suggestion has been made that they result from the deposition of India ink (Cotran 1965).

This is the first electron microscopy study of invertebrates treated with India ink. In vertebrates, however, a few studies have been performed that were based on electron microscopy. Cotran (1965) studied endothelial cells of rats and mice "overloaded" with India ink, and the vascular endothelium was examined by electron microscopy for evidence of phagocytosis. Phagocytosis of carbon was demonstrated in the endothelium of small myocardial vessels, the endocardium, the pulmonary capillary endothelium, the aorta, and the glomerular and peritubular capillary endothelia. Some carbon remained in the endothelium of the heart vessels for at least 7 days after overloading. Carbon particles were also present in circulating mononuclear cells and in perivascular phagocytes.

Apoptosis is a process with typical morphological signatures, including plasma membrane blebbing, cell shrinkage, chromatin condensation and fragmentation (Kerr et al. 1972, Wyllie et al. 1980). A family of cystein-dependent aspartate-directed proteases, called caspases, is responsible for the proteolytic cleavage of cellular proteins that contribute to the characteristic apoptotic features, such as the cleavage of caspase-activated DNase that results in internucleosomal DNA fragmentation (Kerr et al. 1972, Wyllie et al. 1980). Two pathways for activating caspases have been studied in detail. One starts with ligation of a death ligand to its transmembrane death receptor, followed by recruitment and activation of caspases in the death-inducing signalling complex. The other pathway involves the participation of mitochondria, which release caspase-activating proteins into the cytosol, thereby forming the apoptosome, where caspases will bind and become activated.

It is well established that prolonged ER stress can lead to cell apoptosis. Several novel

pathways have been identified that can offer explanations on how cells trigger programmed cell death when faced with irreparable damages that cannot be rescued by the unfolded protein response. Despite these important discoveries, the *in vivo* molecular mechanisms underlying ER stress induced apoptosis are just emerging. Furthermore, it is unclear whether observations derived from specialized cell lines reflect tissue-specific or general mechanisms and whether results from *in vitro* reconstitution assay systems apply to endogenous cellular mechanisms (Li et al. 2006)

The efficient functioning of the ER is essential for most cellular activities and survival. Conditions that interfere with ER function lead to the accumulation and aggregation of unfolded proteins. If the stress is prolonged, or the adaptive response fails, apoptotic cell death ensues. In mammals and humans, many studies have focused on how this failure initiates apoptosis, as ER stress-induced apoptosis is implicated in the pathophysiology of several neurodegenerative and cardiovascular diseases (Szegezdi et al. 2006). In this study, mollusks treated with India ink showed apoptosis, whereas those treated with a physiological solution did not. The cause of apoptosis in this context is still unknown; however, India ink accumulation within the cells may be related to ER stress.

Bordem (2012) showed ultrastructural changes of ER stress, these are thickening of the membrane of the endoplasmic reticulum, dilated cisterns, ribosome dissociation and electron-subjects grouping the outside of the membrane. Some of these findings were observed in cells studied with electron microscopy in this work.

Previous studies have shown that bivalve hemocytes synthesize and secrete catecholamines including noradrenaline and dopamine (Ottaviani & Franceschi, 1996). On the other hand, noradrenaline has the capacity to induce the apoptosis of oyster

hemocytes (Lacoste et al., 2002) and norepinephrine induces endoplasmic ER stress in PC12 cells. These three factors (apoptosis, norepinephrine and ER stress) could be related and be associated with a foreign body in mollusks.

CONCLUSION

In conclusion, India ink proved to be a useful experimental model for inflammation in mollusks. Finally, note that India ink iduces apoptosis, and the cause of this phenomenon is not known, the association of apoptosis and ER in mollusks is unknown requires further studies.

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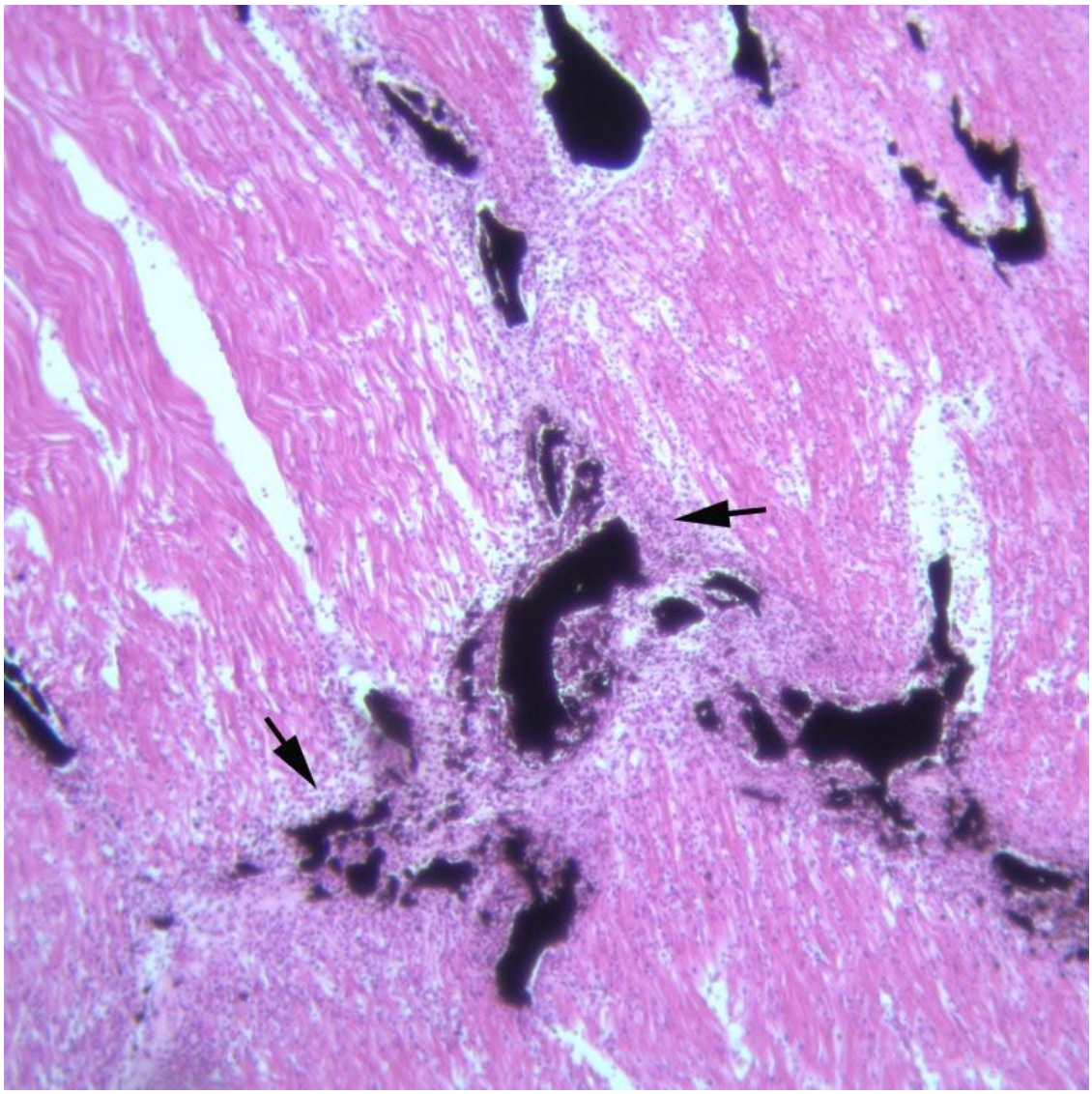

Figure 1: Muscle tissue, 24 hours after the ink injection, with clumps of India ink granules surrounded by massive infiltration of hemocytes (arrow). H&E staining.

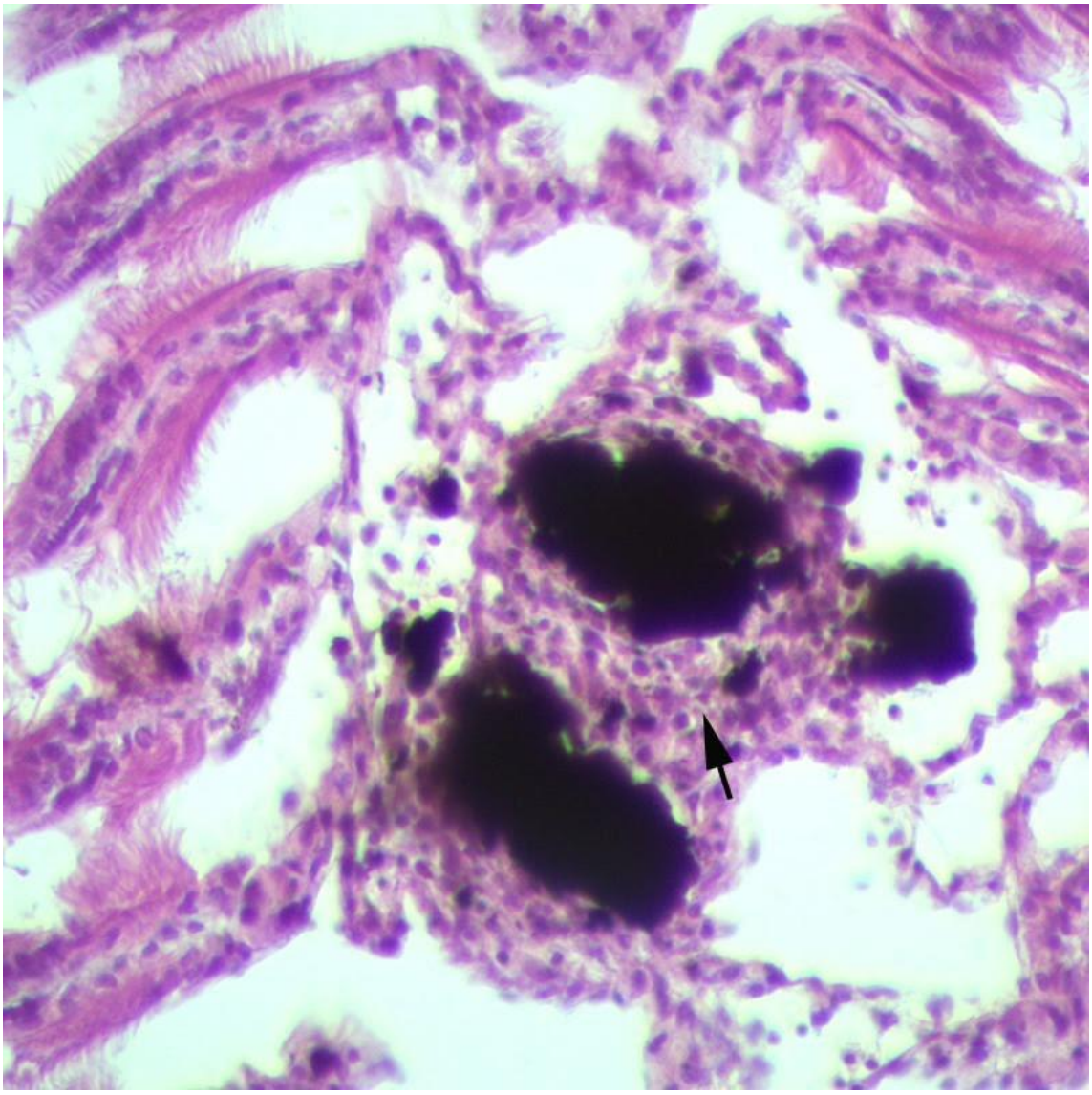

Figure 2: Gill, 48 hours after the ink injection, with India ink encapsulated and surrounded by inflammatory infiltrate (arrow). H&E staining.

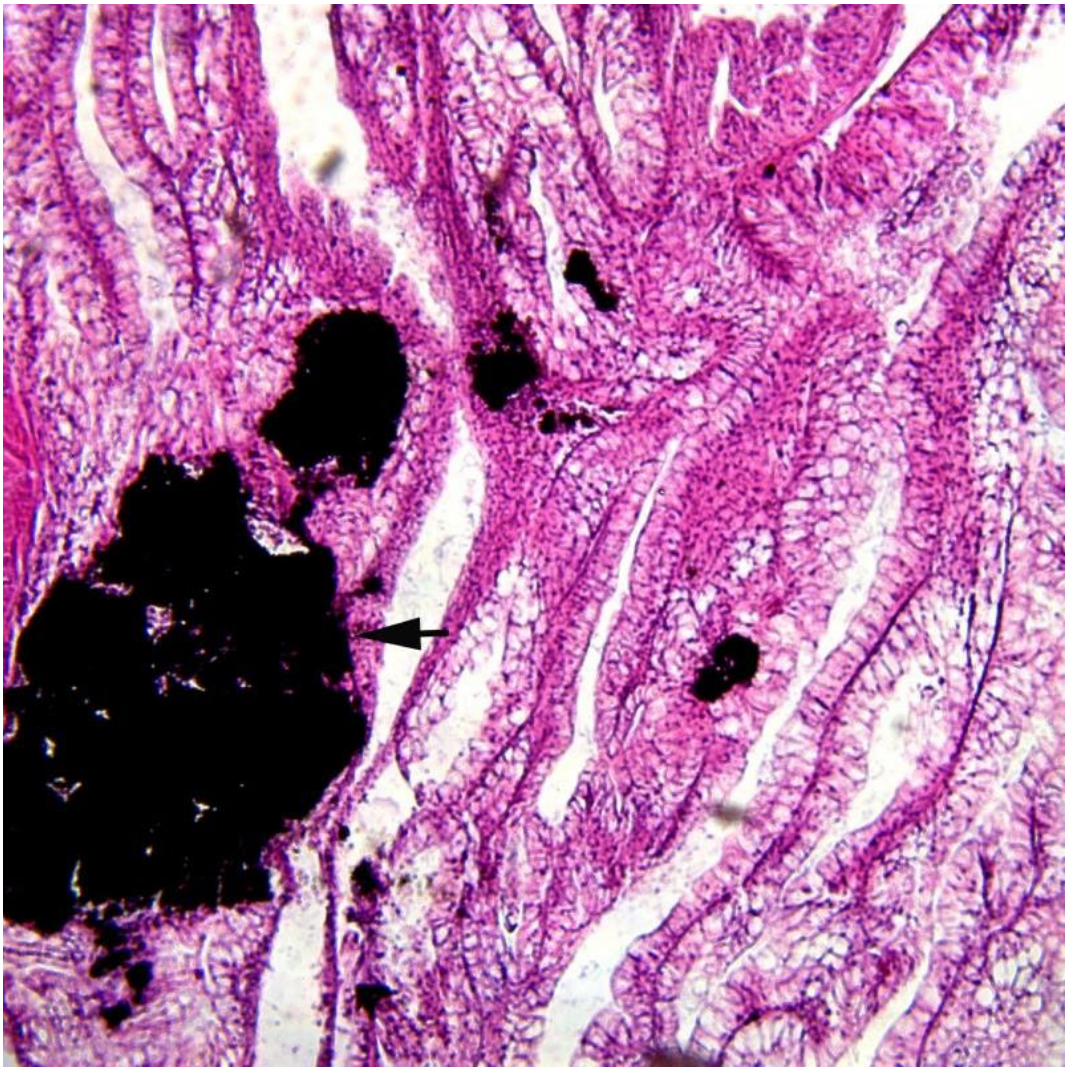

Figure 3: Kidney, 48 hours after the ink injection, with India ink between the tubular structures (arrow). H&E staining.

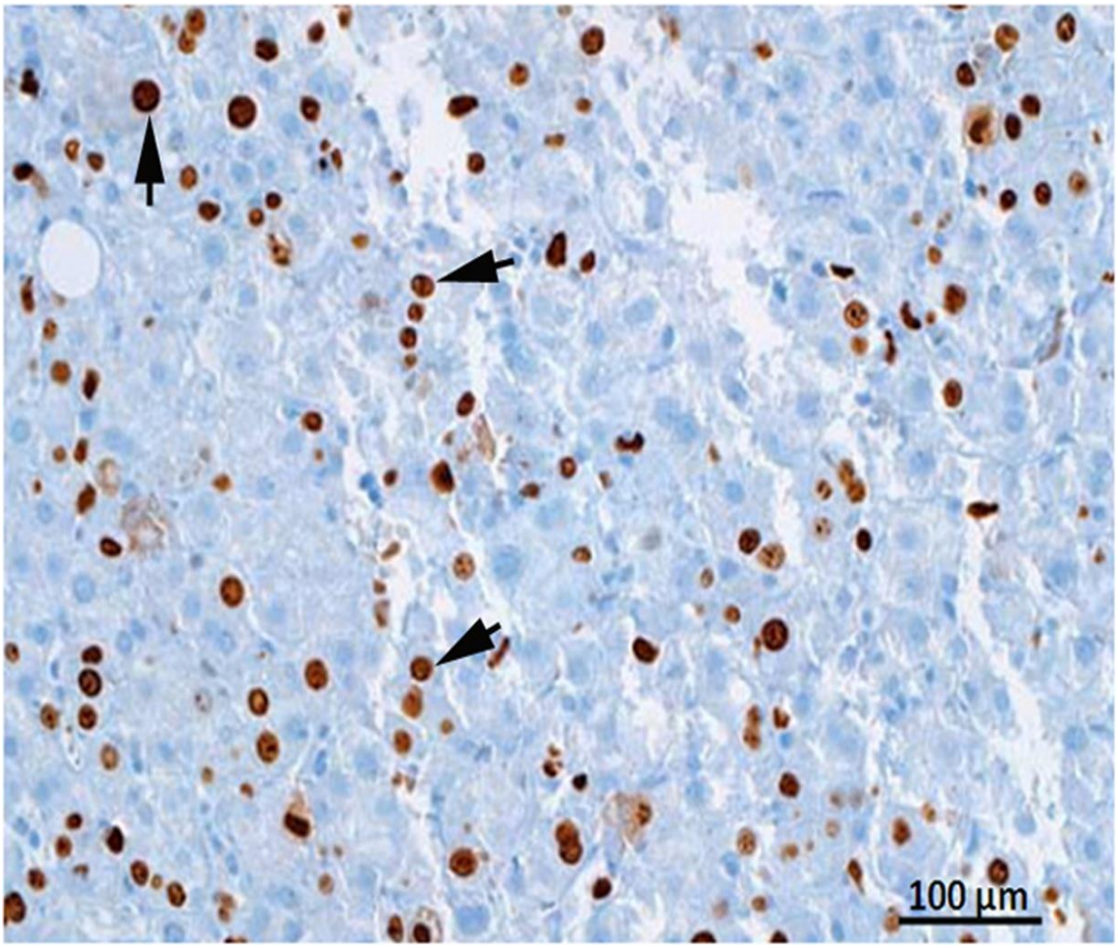

Figure 4: Presence of apoptotic cells (arrow) in digestive gland tubules, 24 hours after the ink injection.

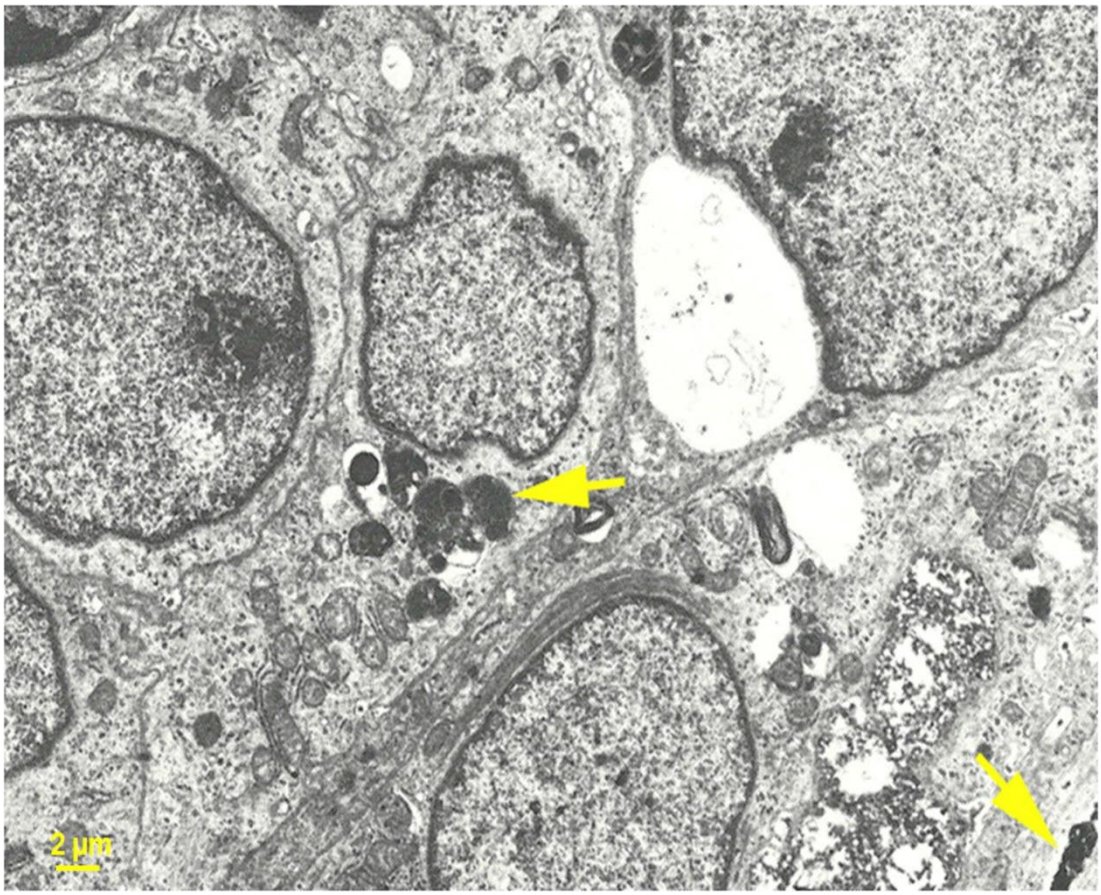

Figure 5: Low magnification of kidney tissue, 24 hours after the ink injection. Cells display numerous cytoplasmic osmiophilic lamellar structures. (arrows).

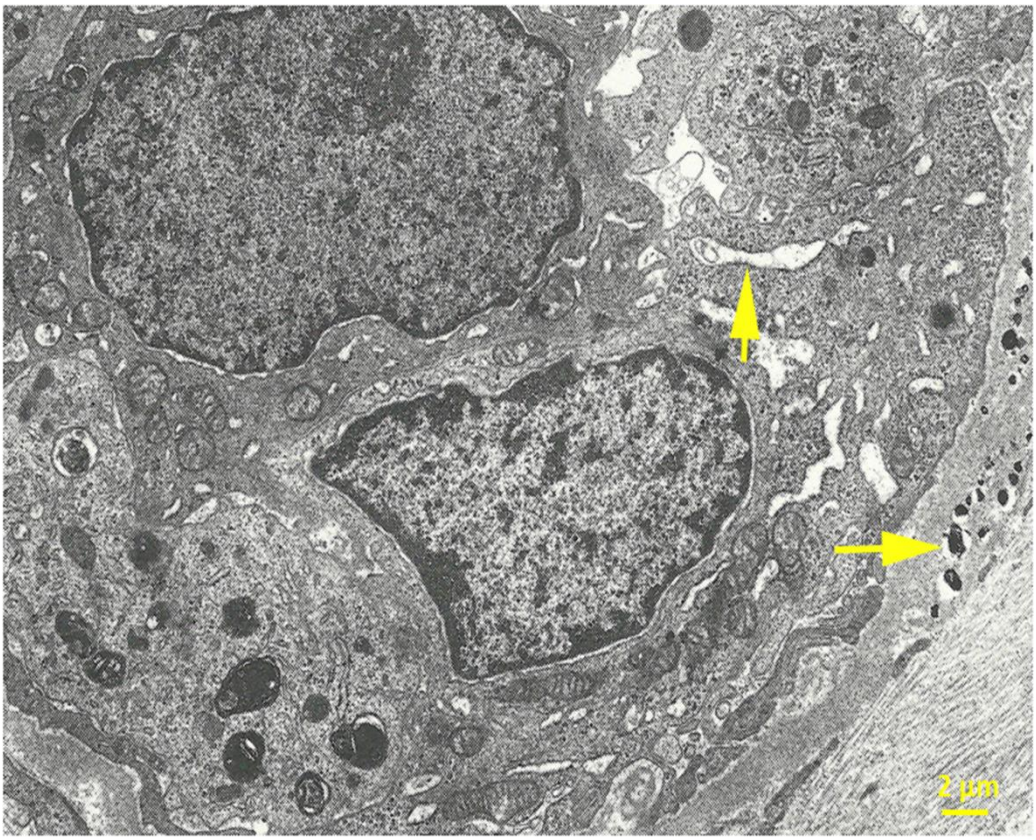

Figure 6: Gill cell, 48 hours after the ink injection, showing dilated endoplasmic reticulum (ER) (short arrow) and osmiophilic and electrodense lamellar structures (long arrow), which may correspond to India ink. M: mitochondria.

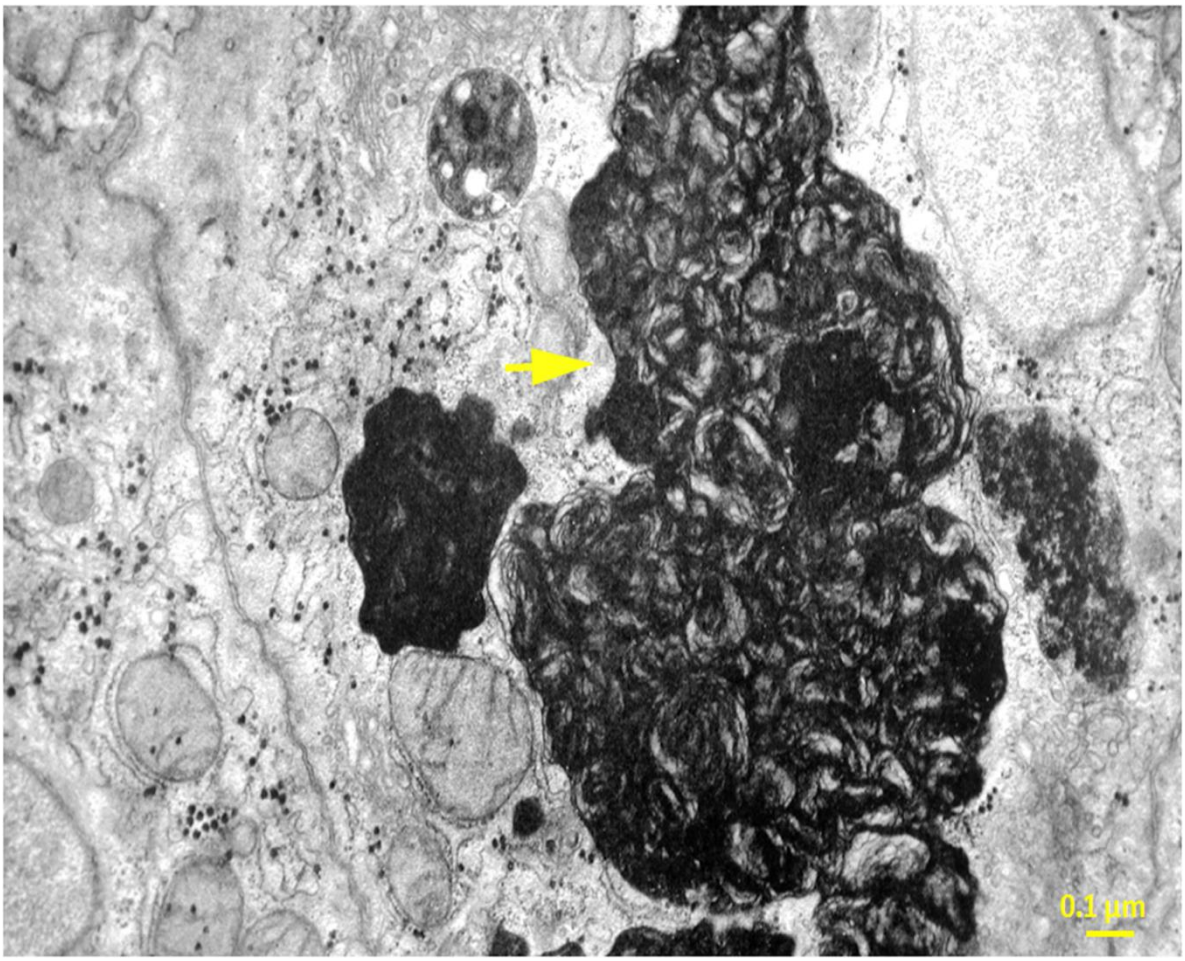

Figure 7: Higher magnification of Figure 5, osmiophilic and electrodense lamellar structures (arrows), which may correspond to India ink.

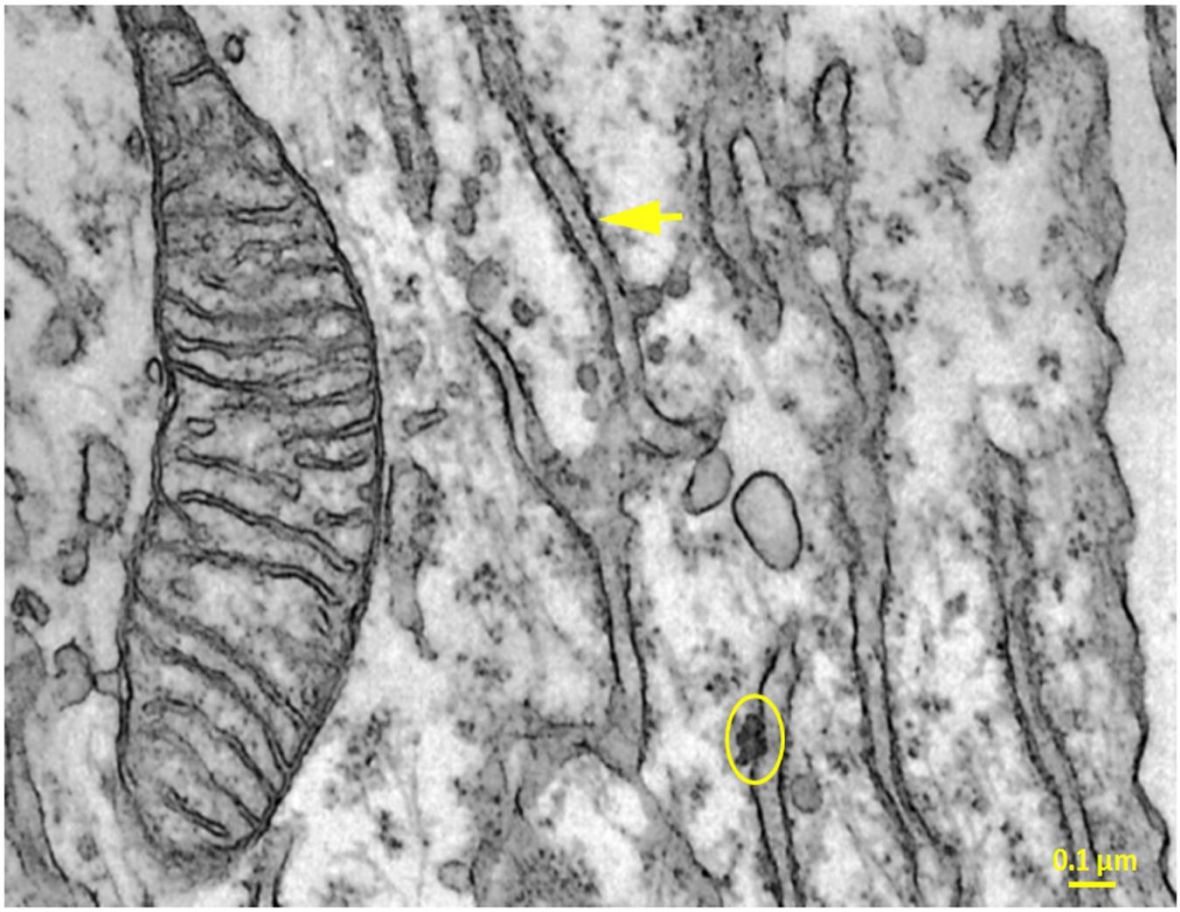

Figure 8: Higher magnification of digestive gland cell which thickening of the membrane of the endoplasmic reticulum (arrow) , dilated cisterns, ribosome dissociation and electron-subjects grouping the outside of the membrane (0).

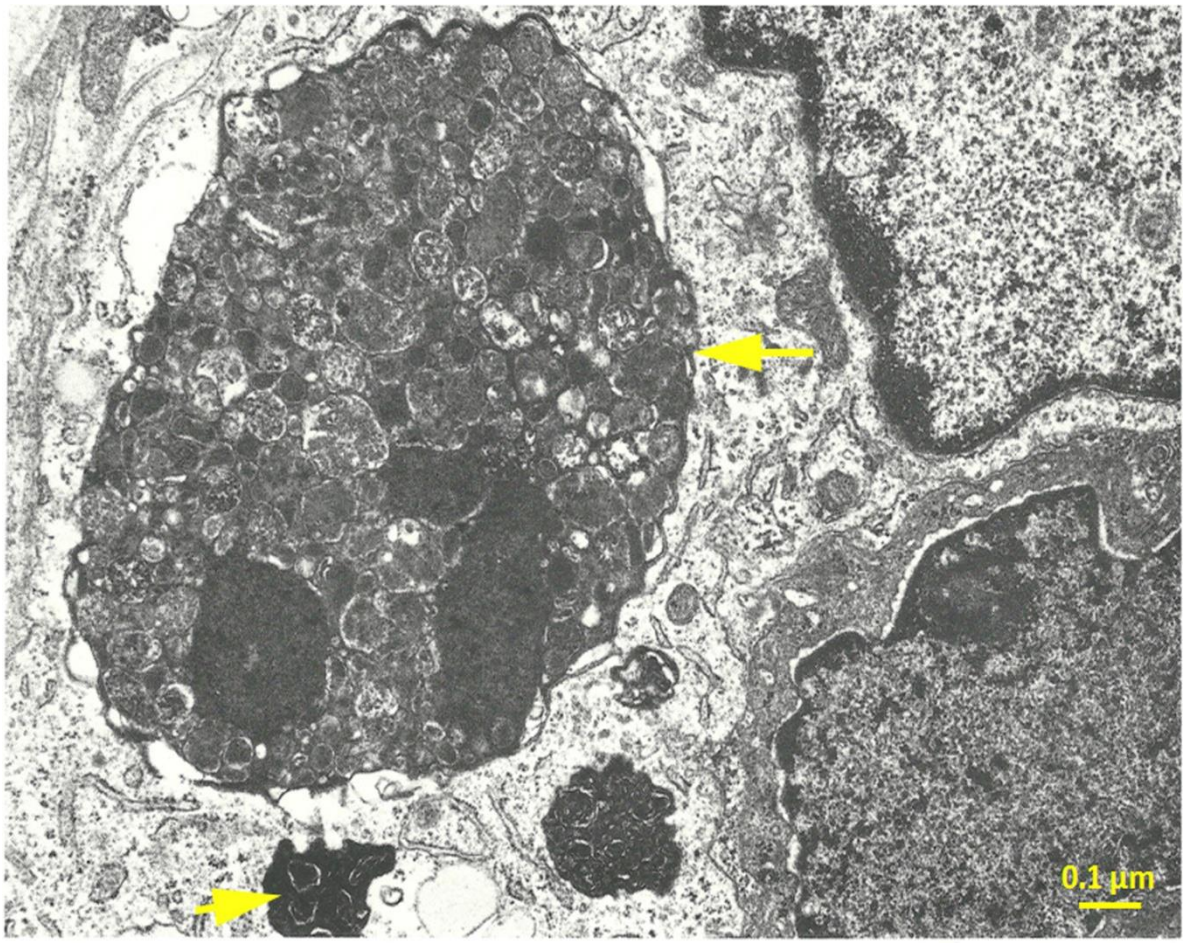

Figure 9: Digestive gland cell, 48 hours after the ink injection, with a voluminous cytoplasmic membrane bound vacuole containing an apoptotic body (long arrow) and electrodense structures (short arrow), which may correspond to India ink.

DISCUSSÃO GERAL

A presente tese produziu os primeiros conhecimentos acerca dos patógenos que afetam o marisco branco *Mesodesma mactroides* do Estado do Rio Grande do Sul, Brasil.

De uma forma resumida, foram registrados procariontes *Rickettsia* sp., protozoários (ciliados *Trichodina* sp., coccídeos *Pseudoklossia*-like e gregarinas *Nematopsis*-like.) e também metazoários (o turbelário c.f. *Paravortex mesodesma* e digeneos não identificados no estágio de esporocisto e no estágio de metacercária)

Apesar de ter sido apresentado no Capítulo 1 apenas os resultados de parasitos relacionados a uma coleta de mariscos realizada no verão, durante esta pesquisa foram realizadas amostragens de marisco branco (n = 30) de tamanho adulto (entre 5 – 7 cm) a cada três meses, cobrindo todas as estações do ano de 2012, totalizando um *n* = 120 mariscos. Os únicos parasitos encontrados em elevada prevalência e em todas as estações do ano foram os tricodinos e os turbelários, que segundo revisão de Lauckner (1983) são considerados parasitos inofensivos aos bivalves hospedeiros. Já os parasitos considerados de importância intermediária, como é o caso dos coccídeos e gregarinas, foram encontrados tão raramente (2 registros de cada parasito) e com ausência de danos aos hospedeiros.

Apesar de estes mesmos parasitos terem sido considerados como possíveis causadores de mortalidade em massa de *M. mactroides* na Argentina (Cremonte & Figueras 2004), não há evidências de que estes sejam uma ameaça a população de *M. mactroides* no sul do Brasil.

Apesar de ter sido registrado rickettsias em poucos casos nos *M. mactroides* adultos analisados, essas mesmas rickettsias foram registradas em 100% dos juvenis moribundos. Durante a primavera (novembro) dos anos de 2011, 2012 e 2013 foram registrados mortalidades massivas de juvenis de *M. mactroides*. A análise das amostras de mariscos moribundos coletadas na primavera de 2011 deu origem ao artigo apresentado no Capítulo 2. Apesar de não haver sido mostrado os resultados referentes as amostras de juvenis moribundos de 2012 e 2013, estas também foram analisadas histologicamente, e foi diagnosticado rickettsiose.

Nos três anos foi possível observar que o fenômeno de mortalidade inicia-se com os mariscos juvenis com comportamento anômalo desenterrados na beira do mar, flutuando com sinais claros de fraqueza (acúmulo de areia nas brânquias e a falta de atividade). Segundo Castinel *et al.* (2004) estabelecer a causa de um surto de mortalidade em uma determinada população de bivalves não é um exercício simples, primeiramente porque o ambiente aquático tem muitos fatores dinâmicos e complexos a se considerar. As rickettsias são agentes infecciosos que afetam a todas as espécies animais e são capazes de gerar mortalidades massivas (Walker 1996, Raoult & Roux 1997, Dumler & Walker 2005, Parola *et al.* 2005).

A detecção do agente patogênico em associação com a mortalidade não necessariamente significa que o agente sozinho foi responsável pelo evento. Outros fatores podem ser necessários para criar a condição estressante e exacerbar a capacidade do agente de causar a doença (Castinel *et al*. 2014).

Portanto, é muito difícil afirmar que a causa da diminuição ou até mesmo o desaparecimento dos estoques do outrora tão abundante *M. mactroides* seja devido so à rickettsiose, já que a uma miríade de fatores (que vai desde o aquecimento global, acidificação dos oceanos, até os impactos antrópicos locais do porto de Rio Grande com a dragagem e aumento de sedimentos lamosos na praia do Cassino, aumento do número de pessoas vivendo neste Balneário e consequente aumento na quantidade de carros circulando na beira do mar) poderiam atuar sinergicamente a fim de causar diminuição das respostas imunes do marisco branco e posteriormente a morte. Sabe-se que os mariscos brancos são muito sensíveis a ações antrópicas e por isso, utilizados como indicadores de poluição ambiental (Fontana *et al.* 2003).

Segundo definição da Organização Mundial de Sanidade Animal (OIE), é considerado como um evento de mortalidade massiva quando mais de 15% dos organismos de uma espécie de um determinado local morrem em curto período de tempo. Entretanto, como questiona Figueras (2011): “Conhecemos realmente as taxas de mortalidade dos moluscos bivalves tanto oriundos de cultivos como de bancos naturais? O que entendemos por uma mortalidade anormal? Os critérios que diferenciam uma mortalidade anormal e uma mortalidade não explicada devem ser definidos urgentemente”

Recomenda-se que estudos devem ser direcionados para o entendimento dos fatores associados a evolução da rickettsiose no marisco branco para futuramente determinar quais estratégias possam ser tomadas com a finalidade de se evitar estes eventos de mortalidade.

Para que o Programa Nacional de Sanidade de Animais Aquáticos possa colocar em prática o programa de diagnóstico, vigilância e comércio de organismos aquáticos, torna-se fundamental que este Programa obtenha resultados de laboratórios válidos. Para tal finalidade, a OIE produz uma série de protocolos de diagnóstico que constituem uma importante ferramenta para que o Programa Nacional de Sanidade de Animais Aquáticos realize as analises de laboratório para a detecção de agentes patogênicos que afetem os animais aquáticos a um nível global (Berthe *et al.* 1999).

As técnicas moleculares normalmente oferecem uma vantagem com respeito a sensibilidade, que frequentemente é neutralizada por problemas técnicos. A PCR é especialmente sensível às condições nas quais se executa, podendo gerar tanto falsos positivos como falsos negativos. Sempre que se utilizem técnicas moleculares, estas devem ser utilizadas com cautela e com especial atenção a inclusão de controles positivos e negativos adequados, com o fim de superar a possível falta de robustez, assim como para manter uma suficiente exatitude. É importante reconhecer que a PCR e as provas baseadas na sequência de DNA detectam apenas o ácido nucléico do agente patogênico e não indicam a presença do parasito vivo, nem de infecção ou enfermidade. Contudo, o uso de uma prova de PCR baseada em RNA pode detectar a presença de um parasito vivo, porém ainda assim não confirmará a presença de infecção ou doença (Diggles *et al.* 2003).

No Capitulo 3 descrevemos os métodos segundo a normativa da OIE para o diagnóstico de doenças de notificação obrigatória em *M. mactroides* do Estado do Rio Grande do Sul, esta pesquisa foi apenas um passo inicial. Propõe-se que a análise molecular das doenças de notificação em bivalves seja realizada de maneira rotineira em mariscos brancos e outras espécies de bivalves de toda a costa do Rio Grande do Sul a fim de certificar a região como "Livre de Doenças de Notificação Obrigatória".

Segundo a OIE, os estudos de patógenos em populações naturais de moluscos bivalves são fundamentais para definir o "status" de doença de um país. Estes métodos

moleculares são capazes de detectar a ocorrência de patógenos que estejam em baixa intensidade de infecção, que através da histologia clássica poderia passar despercebidos. Até o presente momento nenhuma doença de notificação foi encontrada em *M. mactroides.*

A salinidade da água pode ser um fator de causa de mortalidade de populações de bivalves que ocorrem em regiões costeiras próximas a grandes rios e estuários. Localizado no extremo sul do Brasil, a praia do Cassino, considerada uma das maiores praias do mundo (extensão aproximada = 220 km) se inicia ao norte no Molhe Oeste (~8 km de extensão mar adentro), o qual protege a desembocadura da "Laguna" dos Patos e permite a entrada de grandes navios no Porto, e vai até a Barra do Chuí, que é um pequeno arroio que marca a fronteira entre o Brasil e o Uruguai. Durante períodos intensos de chuva na região, associados principalmente com eventos do "El niño", a salinidade da água do mar na região próxima aos molhes pode baixar drasticamente devido principalmente a elevada descarga de água da "Laguna" dos Patos, como foi registrado por Odebrecht *et al.* (2009).

Odebrecht *et al.* (2009) registraram durante um período de 10 anos de monitoramento da salinidade do mar no Balneário Cassino (mais precisamente em frente à Estação Marinha de Aquicultura, EMA-FURG), salinidades variando entre 14 e 38. Portanto, muito próxima da CL_{50} estimada para 96h, que foi 10,5 (IC 95% = 9,36 – 11,81) e 8.8 (IC 95% = 7,67 – 10,12) para adultos e juvenis, respectivamente

A obtenção do dado de salinidade média letal para o *M. mactroides* será útil para prever possíveis surtos de mortalidade devido ao estresse osmótico, e também, para auxíliar estudos futuros que determinarão as condições para o cultivo desta espécie.

A maioria das doenças que afetam os organismos aquáticos está associada a deficiências na resposta imune (Ellis *et al*. 2011). No meio natural, os organismos filtradores como os moluscos bivalves, podem estar em contato com elementos difíceis de fagocitar, seja pela sua quantidade ou pelo seu tamanho (Cheng & Combes 1990). Estabelecer os efeitos que estes materiais exercem na fisiologia do molusco é importante. No Capítulo 5, incorporamos através de injeções no pé do *M. mactroides* tinta nanquim, que na realidade são partículas inertes de carbono de 2 µm, que podem formar aglomerados de mais de 100 µm em contato com a água. O experimento teve a

finalidade de avaliar as consequências de fagocitar um material inerte abundante e possível de ser detectado com as técnicas histológicas clássicas. Observamos neste trabalho que a acumulação de partículas de carbono nas cisternas e túbulos do retículo endoplasmático rugoso produziu um estresse nestas organelas e induziu a apoptose em moluscos bivalves.

Esperamos que o presente trabalho seja uma obra de referência útil sobre a temática "patologia de moluscos" no Brasil e que contribua para a compreensão mais completa sobre os fenômenos de mortalidade que afetam o marisco branco *Mesodesma mactroides* e que estes dados colaborem com o futuro cultivo desta espécie.

CONCLUSÃO

(1) Os parasitos encontrados não são uma ameaça ao *M. mactroides* da região do Estado do Rio Grande do Sul;

(2) Procariontes do gênero *Rickettsia* podem estar associados a eventos de mortalidade de juvenis na praia do Cassino;

(3) Nenhuma doença de notificação obrigatória foi registrada em *M. mactroides* do Estado do Rio Grande do Sul;

(4) *M. mactroides* tolera uma ampla variedade de salinidade (de 15 – 35), abaixo da salinidade 15, tanto os juvenis (comprimento de concha ≤ 3 cm) como adultos (≤ 5 cm) passam a sofrer mortalidade;

(5) Injeção de partículas de carbono (tinta nanquim) causou estresse do retículo endoplasmático rugoso e apoptose em células que acumularam estas partículas.

PERSPECTIVAS

(1) Identificar ao nível molecular os parasitos encontrados na presente tese;

(2) Estudar a epidemiologia das rickettsias em *M. mactroides;*

(3) Continuar o monitoramento de doenças de notificação obrigatória em *M. mactroides* e outros moluscos da região do Rio Grande do Sul;

(4) Determinar as condições para o cultivo de *M. mactroides* e inseri-lo no policultivo com camarões/peixes na Estação Marinha de Aquicultura (EMA-FURG), utilizando-o para o controle de microalgas e sólidos suspensos na água de cultivo dos viveiros.

(5) Aprofundar o conhecimento sobre o estresse do retículo endoplasmático rugoso e a apoptose em moluscos bivalves.

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ANEXO

Figura 1. Exemplar adulto de marisco branco *Mesodesma mactroides* coletado na praia do Cassino, Rio Grande do Sul, Brasil.

Figura 2. Coleta de *Mesodesma mactroides* utilizando pás de corte na praia do Cassino, Rio Grande do Sul, Brasil.

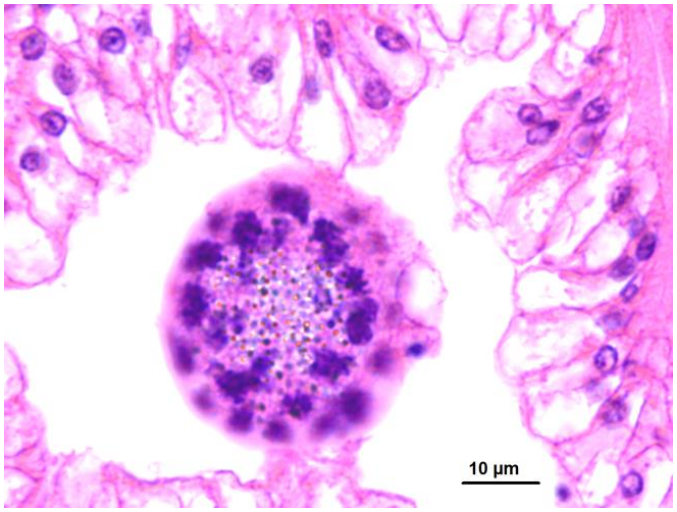

Figura 2. Coccídeo semelhante à *Pseudoklossia* no rim de *Mesodesma mactroides* da praia do Cassino, Rio Grande do Sul, Brasil.

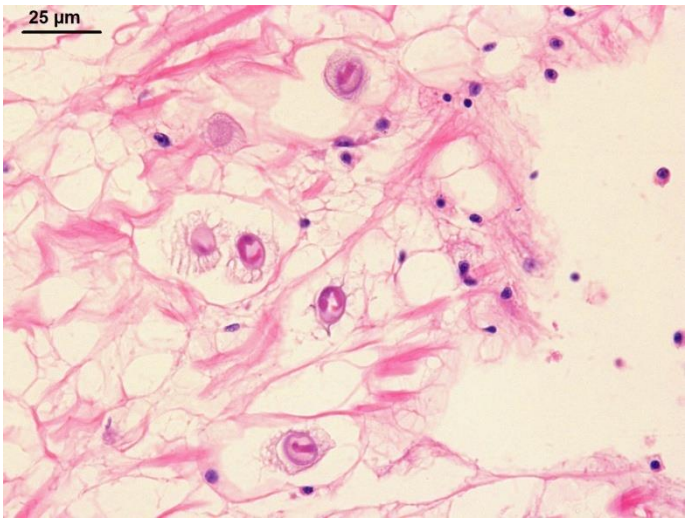

Figura 3. Oocitos de gregarinas semelhantes à *Nematopsis* sp. no tecido conjuntivo de *Mesodesma mactroides* da praia do Cassino, Rio Grande do Sul, Brasil.

Tabela 1. Prevalência das parasitoses registradas em *Mesodesma mactroides* durante diferentes estações do ano de 2012 na praia do Cassino, Rio Grande do Sul, Brasil.

Estação	*RLO*	*Trchd*	*Ntps*	Psdkls	Trbl	Mtcer	Sprct.
Verão	____	100%	_____	_____	30%	13.3%	6.6%
Outono	3.3%	66.6%	_____	_____	23.3%	_____	_____
Inverno	10%	30%	_____	6.6%	3.3%	_____	_____
Primavera	____	40%	6.6%	_____	13.3%	3.3%	_____

RLO = *Rickettsia*-like organisms; Trchd = Trichodina sp.; Ntps = Nematopsis sp.; Psdkls = Pseudoklossia sp.; Trbl = Paravortex mesodesma; Mt. cer = Metacercarias (Digenea); Sprct = Esporocistos (Digenea).