**UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL**

**FACULDADE DE AGRONOMIA**

**PROGRAMA DE PÓS-GRADUAÇÃO EM ZOOTECNIA**

**NAYARA OLIVEIRA DA CRUZ**

# BIOTECNOLOGIAS PARA AVALIAÇÃO DA VIABILIDADEE CRIOPRESERVAÇÃO DE GAMETAS DO CORAL ENDÊMICO *Mussismilia harttii* (VERRILL, 1868)

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Tese apresentada como requisito para obtenção do Grau de Doutor em Zootecnia. Área de concentração: Produção Animal, na Faculdade de Agronomia da Universidade Federal do Rio Grande do Sul.

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**BIOTECNOLOGIAS PARA AVALIAÇÃO DA VIABILIDADE**

**ECRIOPRESERVAÇÃO DE GAMETAS DO CORAL ENDÊMICO** *Mussismilia harttii* **(VERRILL, 1868)[1]**

Autor: Nayara Oliveira da Cruz
Orientadora: Vivian Fischer

**Resumo:** Os recifes de coral constituem um dos ecossistemas mais diversos do planeta, exercendo um papel fundamental na manutenção dos recursos pesqueiros que mantem diversas comunidades humanas em todo o mundo. No entanto, os fatores antrópicos e mudanças ambientais vem ocasionando severos danos a este ecossistema. Dessa forma, o desenvolvimento de técnicas que visem sua conservação é fundamental, entre elas, o uso da criopreservação seria uma ferramenta alternativa para a manutenção de novos corais. O objetivo desse trabalho foi desenvolver biotecnologias para avaliar a viabilidade dos gametas do coral endêmico *M. harttii*, e desenvolver um protocolo de criopreservação para armazenamento do gameta masculino por tempo indeterminado, criando assim o primeiro banco de gametas de coral do Atlântico Sul. Para a avaliação da viabilidade oocitária, foi utilizado o teste de brometo de azul de tiazolil tetrazólio (MTT) e o teste de azul de tripano (TB). Os testes de TB foram realizados em duas condições. Inicialmente os oócitos foram expostos a 1, 3, 5, 7 e 9 $mol^{-1}$ de dimetilsulfóxido (DMSO) por 20 min, depois incubados em 0,4% de TB e tiveram sua integridade de membrana avaliada. Posteriormente, os oócitos mortos foram incubados em TB diluído em água do mar filtrada ou dodecil sulfato de sódio (SDS). Para experimentos de MTT, um ensaio de diluição em série foi realizado usando DMSO como composto citotóxico. O TB conseguiu atravessar a membrana do oócito, mas devido ao seu alto teor de lipídios, a observação da coloração azul não foi possível, inviabilizando este teste para oócitos de *M. harttii*. Conseguimos padronizar o ensaio MTT para determinar a atividade mitocondrial em oócitos maduros do coral, e consequentemente avaliar sua viabilidade. Através do ensaio MTT descobrimos a diluição de DMSO que nos permitiu definir uma concentração segura (1,4 M) para trabalhar com oócitos de coral. O protocolo para a criopreservação das células espermáticas foi desenvolvido o melhor crioprotetor, concentração e técnica de criopreservação. Os espermatozoides foram expostos ao crioprotetor dimetilsulfóxido e metanol em concentrações de 10, 15 e 20%. Os espermatozoides foram criopreservados com sucesso em DMSO 20% usando congelamento lento. De modo geral, como resultados desse trabalho alcançamos um método confiável para avaliação da viabilidade de oócitos e um protocolo eficiente para congelamento do sêmen de *M. harttii*. Essas ferramentas biotecnológicas poderão ajudar na preservação da diversidade genética, e assegurar a existência dos recifes de coral.

**Palavras-chave:** Toxicity; cryobiology; MTT; coral brasileiro; citometria; conservação.

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[1]Tese de Doutorado em Zootecnia – Produção Animal, Faculdade de Agronomia, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brasil. (168 p.) Março, 2022.

**BIOTECHNOLOGIES FOR ASSESSMENT OF VIABILITY AND CRYOPRESERVATION OF GAMETES FROM THE ENDEMIC CORAL** *Mussismilia harttii* **(VERRILL, 1868)**[2]

Author: Nayara Oliveira da Cruz
Advisor: Vivian Fischer

**Abstract:** Coral reefs are one of the most diverse ecosystems on the planet, playing a fundamental role in maintaining fishing resources that support diverse human communities around the world. However, anthropic factors and environmental changes have caused severe damage to this ecosystem. Thus, the development of techniques aimed at their conservation is fundamental, among them, the use of cryopreservation would be an alternative tool for the maintenance of new corals. The objective of this work was to develop biotechnologies to evaluate the viability of gametes from the endemic coral *M. harttii*, and to develop a cryopreservation protocol for indefinitely storing the male gamete, thus creating the first coral gamete bank in the South Atlantic. To assess oocyte viability, the thiazolyl blue tetrazolium bromide (MTT) test and the trypan blue (TB) test were used. TB tests were performed under two conditions. Initially the oocytes were exposed to 1, 3, 5, 7 and 9 $mol^{-1}$ of dimethylsulfoxide (DMSO) for 20 min, then incubated in 0.4% TB and their membrane integrity was evaluated. Subsequently, the dead oocytes were incubated in TB diluted in filtered seawater or sodium dodecyl sulfate (SDS). For MTT experiments, a serial dilution assay was performed using DMSO as the cytotoxic compound. TB was able to cross the oocyte membrane, but due to its high lipid content, the observation of blue staining was not possible, making this test unfeasible for *M. harttii* oocytes. We were able to standardize the MTT assay to determine mitochondrial activity in mature coral oocytes, and consequently assess their viability. Through the MTT assay we discovered the DMSO dilution that allowed us to define a safe concentration (1.4 M) to work with coral oocytes. The protocol for cryopreservation of sperm cells was developed using the best cryoprotectant, concentration and cryopreservation technique. Spermatozoa were exposed to DMSO and methanol at concentrations of 10, 15 and 20%. Sperm was successfully cryopreserved in 20% DMSO using slow freezing. In general, as a result of this work, we achieved a reliable method for evaluating oocyte viability and an efficient protocol for freezing *M. harttii* sperm. These biotechnological tools could help preserving genetic diversity, and ensure the existence of coral reefs.

**Keywords:** *Mussismilia harttii*; cryopreservation; MTT; Brazilian coral; cytometry; conservation.

[2]Doctoral thesis in Animal Science, Faculdade de Agronomia, Universidade Federaldo Rio Grande do Sul, Porto Alegre, RS, Brazil. (168 p.) Março, 2022.

## RELAÇÃO DE FIGURAS

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## RELAÇÃO DE TABELAS

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### CAPÍTULO III

## RELAÇÃO DE ABREVIATURAS E SÍMBOLOS

AMF – Água marinha filtrada
ATP–Trifosfato de adenosina
°C – Graus Celsius
CASA – Computer Assisted Sperm Analysis
Cm – Centímetro
CPA – Agente crioprotetor
DMSO – Dimetilsulfóxido
DNA – Ácido desoxirribonucleico
FDA – Diacetato de fluoresceína
FIV – Fertilização in vitro
FL1 – Filtro passa-banda 585/30
FL3 – Filtro passa-banda 585/40
FL4 – Filtro passa-banda 675/25
GBA – Grande barreira de corais
ICSI – Injeção intracitoplasmática de espermatozoides
L – Litro
M – Molar
mL – Mililitro
Min – Minuto
nm – Nanômetro
MTT – (3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide)
PBS – Tampão fosfato-salino
pH – Potencial hidrogeniônico
PI – Iodeto de propídio
RNs –Espécie reativa de nitrogênio
ROs – Espécie reativa de oxigênio
SDS – Dodecil sulfato de sódio
TB – Azul de Tripano
TE – transferência de embriões
TN – Transferência nuclear
µL – Microlitro
µm – Micrometro

**SUMÁRIO**

# CAPÍTULO I

## 1. INTRODUÇÃO GERAL

Os recifes de coral estão entre os ecossistemas mais diversos do planeta. Apesar da área total dos recifes de coral ser muito inferior a 1% de todo o ambiente marinho, algumas estimativas colocam a diversidade total de vida associada em até 2 milhões de espécies, cerca de 25% da vida marinha conhecida (Mulhall, 2007; Wilkinson, 2008). Além disso, facilitam a fixação de nitrogênio, a regulação do carbono e funcionam como barreiras naturais que protegem as áreas costeiras.

Portanto, os recifes de coral desempenham um papel fundamental na renovação dos estoques pesqueiros que sustentam milhares de comunidades humanas em todo o mundo. Os corais são organismos eucarióticos chave que possuem relações intrincadas com um conjunto de micro-organismos, incluindo dinoflagelados endossimbióticos (zooxantelas), fungos, bactérias e archaea. Nos últimos 20 anos, os impactos antrópicos locais e globais têm sido de grande preocupação, sendo este último particularmente preocupante devido aos eventos de mudanças climáticas (Baker et al., 2008; Hughes, 2016; Beyer, et al., 2018). O aumento da temperatura da superfície do oceano (NOAA, 2016) rompe a simbiose entre as microalgas (zooxantelas) que vivem no tecido dos corais e que dão ao coral seu alimento, tornando-os brancos – o chamado branqueamento. Dependendo da intensidade do branqueamento o quadro se torna irreversível, causando a morte dos corais. As previsões indicam que esses eventos se tornarão ainda mais frequentes (Heron et al., 2016), diminuindo a resiliência dos corais e ameaçando os ecossistemas recifais em todo o planeta.

Os únicos recifes de águas rasas verdadeiros do Oceano Atlântico Sul estão ao longo da costa brasileira e abrigam 16 espécies de corais pétreos, com cinco delas endêmicas do Brasil (Castro e Zilberberg, 2016). O coral-cérebro, *Mussismilia harttii* conhecido popularmente como coral couve-flor, está entre os principais construtores dos recifes brasileiros, e já foi afetado por eventos de branqueamento e doenças (Castro e Pires, 1999; Leão et al., 2016) e encontra-se ameaçado de extinção (Pires et al., 2018).

*M. harttii* é uma espécie hermafrodita de reprodução anual, no entanto, pouco se conhece sobre seus gametas. Na tentativa de conhecer a biologia reprodutiva do

gênero Mussismilia, Pires et al., (1997) avaliaram histologicamente três espécies de corais brasileiros. As avaliações ocorreram em períodos diferentes do ano e conseguiram detectar os estágios de desenvolvimento do oócito e do espermatozoide, porém trabalhos que avaliem os gametas vivos ainda não foram desenvolvidos.

Enquanto práticas de conservação *in situ* como as áreas marinhas protegidas podem ajudar a diminuir os impactos locais, os principais impactos antrópicos que afetam os recifes de coral em todo o mundo incluem despejo de esgoto, sedimentação desencadeada pelo desmatamento, aquecimento e acidificação do oceano (Harper et al. 2007 ; Harris et al. 2010 ) continuarão causando declínios populacionais em taxas ainda mais rápidas. Grandes mortalidades sem recuperação levam a uma diminuição na diversidade genética das populações de corais e a possibilidade de extinção de espécies é alta, uma vez que a persistência de uma população ou espécie é amplamente depende da sua diversidade genética (Laikre et al., 2010). Uma maneira segura de conservar a existência de corais é através da preservação de seus gametas (espermatozoide e oócito) em baixas temperaturas (criopreservação), permitindo o uso futuro dessas células para a formação de novos corais. Embora seja um método comprovado para manutenção de células a longo prazo (Mazur, 1984) e conservação de espécies, estudos sobre criopreservação de gametas de coral são recentes e limitados a poucos relatos na literatura. Os espermatozoides de três espécies de corais australianos (*Acropora tenuis*, *Acropora millepora* e *Acropora digitifera*) e um caribenho (*Fungia scutaria*) foram congelados com sucesso (Hagedorn et al., 2006; 2012; Ohki et al., 2014), sendo eficientes na fecundação dos oócitos e gerando larvas saudáveis capazes de assentar e se desenvolver (Hagedorn et al., 2018).

Dessa forma, a criobiologia como ferramenta para a conservação de recifes de coral ainda há muito para expandir. No entanto, criopreservação é uma metodologia bem estabelecida para diferentes tipos de células, tanto em espécies de animais quanto em humanos (He et al., 2008; Santos et al., 2010; Cheuiche et al., 2011). Existem crioprotetores estabelecidos que podem ser testados em curvas de temperaturas diferentes. Nesse sentido, o desenvolvimento de biotécnicas que auxiliem na preservação de espécies ameaçadas de extinção são de grande importância. A criopreservação de bolas de tecidos, fragmentos de tecido, estruturas ápices, (Hagedorn et al., 2013; Feuillassier et al., 2014a; Feuillassier et al., 2014b;

Feuillassier et al., 2015) e gametas de corais pode ser uma ferramenta eficiente e capaz de suprir essa demanda.

Esse projeto traz uma proposta inédita de pesquisa no Brasil, buscando aplicar ferramentas tecnológicas em prol da conservação do coral *M. harttii*. O estabelecimento dessas biotecnologias permitirá a criação do primeiro banco de gametas de corais do Atlântico Sul, permitindo a estocagem dos espermatozoides por tempo indeterminado, sendo descongelados quando desejado, promovendo a fecundação e o crescimento de novos recrutas de corais, que poderão ser utilizados na restauração de recifes degradados.

## 2. REVISÃO BIBLIOGRÁFICA

### 2.1. Recifes de Coral

O recife é um ecossistema muito valioso, pois o ambiente coralíneo é considerado o berçário da vida marinha, suportando uma rica e complexa fauna e flora. Os recifes de coral ocupam no planeta apenas 1% dos oceanos e um quarto das espécies marinhas dependem dele. Com aproximadamente 2 milhões de espécies vivendo dentro e ao redor dos recifes, oferecendo-lhes alimento e abrigo a mais de 50 milhões de anos. Outra função muito importante que os recifes apresentam é a proteção contra impactos das ondas através da barreira que eles exercem evitando a erosão da costa continental. Também é fonte de proteínas para a dieta alimentar da população costeira, durante séculos proporciona empregos através da pesca e da indústria do turismo sendo as maiores e mais espetaculares estruturas da terra criada a partir da atividade biológica (Veron et al., 2000, 2009).

### 2.2. Recifes de corais: Geomorfologia e biologia

Os recifes de coral são caracterizados por serem estruturas rochosas, rígidas, resistentes à ação mecânica das ondas e correntes marinhas, construídos por organismos marinhos (animais e vegetais) portadores de esqueleto calcário (Buddemeier et al., 2004). Do ponto de vista biológico, os recifes coralíneos são

comunidades de organismos com estrutura tridimensional biogênica, em geral, formada pelo acúmulo dos seus próprios esqueletos, são bioconstruídas por corais que secretam carbonato de cálcio (CaCO3) e por algas coralíneas que se caracterizam pela presença de carbonato de cálcio em suas paredes celulares. Sua constituição é fundamentada na atuação conjunta de uma infinidade de seres, formando uma complexa teia de associações e de eventos em sucessão (Castro e Zilberberg, 2016).

Os corais são encontrados em todo o mundo, e estão entre os animais de maior longevidade e crescimento. São animais cnidários pertencentes a mesma classe das anêmonas e podendo ser solitários ou viver em colônia, sendo alguns formados por pólipos e por esqueleto calcário, caracterizados como hermatípicos, que são corais da ordem Scleractinia responsáveis por depositar material de calcário duro em seu esqueleto, formando uma estrutura pedregosa que é responsável pela construção do recife (Kikuchi e leão, 1997).

Outra característica importante é que todos os corais são seres simbiônticos, associação essa que surgiu no era mesozóica depois de uma quase extinção, quando os corais voltaram a ser desenvolver em águas rasas associando-se à microalgas (Stanley Jr, 1988). Fato curioso foi que após o período triássico, as microalgas se diversificaram, justamente quando surgem os corais com simbiose (Frankowiak et al*.,* 2016; (Marcelino e Verbruggen 2016). A descoberta, além de indicar que os microbiomas já coexistiam dentro de corais, também sugere que algumas linhagens de algas são até mais antigas do que os próprios corais e já existiam 250 milhões de anos antes de começarem a habitar esses organismos. Tal associação ocorre com uma microalga unicelular chamada zooxantela. Essas são como órgãos, responsáveis pela produção fotossintética e transferindo grande parte da energia para a nutrição e o crescimento dos corais. (Garrido et al., 2016; Fonseca e Marangoni, 2017). No entanto, a associação zooxantelas e corais está gravemente ameaçada por conta das alterações ambientais no planeta.

## 2.3. Corais Brasileiros

O Brasil é portador dos únicos recifes de coral verdadeiros do Oceano Atlântico Sul. A fauna de corais brasileira é composta predominantemente por formas massivas e os principais construtores de recifes são espécies endêmicas (Leão et al., 2019). Porém, os recifes brasileiros contém uma baixa diversidade, contando com 23 espécies de corais pétreos zooxantelados (Ordem *Scleractinia*), sendo 4 endêmicas (Leão et al., 2016; Zilberberg et al., 2016). A renovação e reprodução dos recifes de coral é o processo fundamental para que as populações de coral sejam mantidas. Informações da biologia e estratégias reprodutivas são fundamentais para a conservação desses ambientes, para o aperfeiçoamento de práticas de manejo e para a recuperação de áreas degradadas (Castro e Zilberberg, 2016).

Dentre essas espécies construtoras está a *M. harttii*, pertencente à família mussidae do gênero mussismilia, essa espécie apresenta os cálices separados de forma dicotômica, sem formar ramos laterais. Três diferentes variações morfológicas são descritas por Laborel (1969), na forma laxa, apresentam os cálices bastantes separados e encontram-se em ambientes de águas mais calmas. A variedade confertifolia apresenta características de cálices poucos separados e é comumente encontrada em águas mais agitadas e a variação intermédia, engloba todas as formas que não apresentam as características extremas das outras variedades. A colônia viva apresenta coloração variada em tons de cinza, amarelo, verde e marrom, também apresenta características primitivas presente no período terciário da bacia sedimentar do mediterrâneo.


**Figura 1.** Descrição de três diferentes variações morfológicas (Laborel, 1969): A- Forma laxa Foto: Carlos Secchin, B- Forma confertifolia Foto: Luiz Cassino e Roberta Decnop, C- Forma com variação intermédia Foto: Magris et al., (2018).

A *M. hattii* é facilmente encontrada no ambiente marinho, desde o estado do Rio Grande do Norte até o Espírito Santo, também havendo incidência em Pernambuco. Ocorre em águas rasas de 2 a 3 m, resistindo a turbidez moderada e também, registrada em águas mais profundas de 15 a 30 m e, ocasionalmente, a 80 m (Laborel, 1969). É construtor primário nos recifes do estado de Pernambuco. Sua reprodução ocorre todos os anos ditada pelas fases da lua, especificamente na lua nova, sendo assim bem previsível o período de desova (Castro e Zilberberg, 2016).

Segundo Pires et al., (1999) ambos gametas masculinos e femininos desenvolvem-se no mesmo mesentério, divisória vertical dos antozoários, porém seu desenvolvimento ocorre em períodos diferentes. Os cistos espermáticos, que abrigam espermatozoides, surgem aproximadamente no oitavo mês do desenvolvimento dos oócitos e, portanto, seu desenvolvimento tem duração de cerca de três meses (Pires et al., 2016). Dessa forma os dois alcançam a maturidade ao mesmo tempo, podendo ocorrer a desova. Ao atingir a maturidade, as paredes dos mesentérios se rompem e os gametas são liberados para dentro da cavidade gastrovascular dos pólipos, onde ficam envoltos por uma camada de muco. Nos dias de desova, pacotes de ovócitos com espermatozoides são expelidos pelos pólipos, migram para a superfície da água e flutuam. Oócitos e espermatozoides se separam com o movimento da água e, após um tempo, é iniciada a fecundação cruzada, onde o ovócito de uma colônia é fecundado pelo espermatozoide de outra colônia (Godoy et al., 2019).

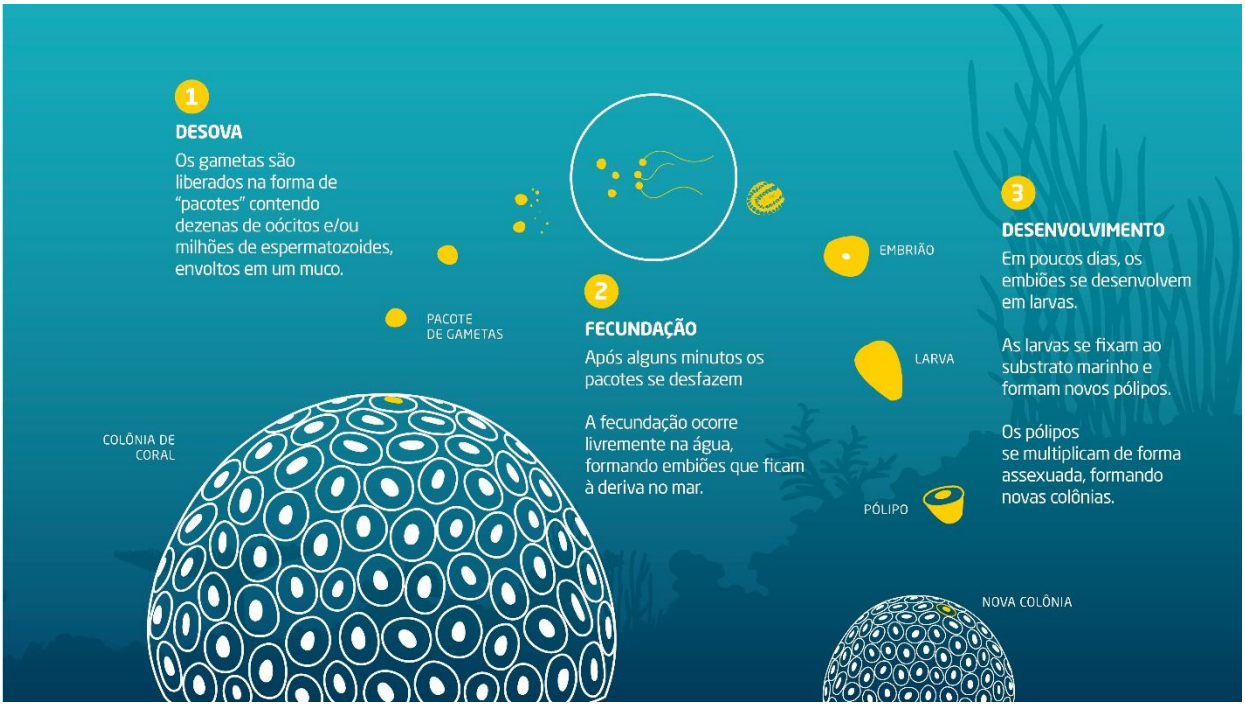


**Figura 2.** Ciclo de vida dos corais liberadores de gametas (Godoy et al. 2019).

## 2.4. Impactos Antrópicos Nos Recifes De Coral

Estima-se que 29% dos corais de água rasa do planeta foram perdidos somente na Grande barreira de corais na Austrália GBA (GBRMPA, 2017; Hughes et al., 2017). Em 2017 ocorreu o branqueamento de 91% dos corais do planeta (Hughes et al., 2019).

Por consequência dos eventos de branqueamento, 59 espécies de peixes recifais já desapareceram da costa das Filipinas e o mesmo vem acontecendo no Quênia (Chabanet et al., 2016; Pratchett et al., 2011). Nos recifes do Leste do mediterrâneo, 64% das espécies de moluscos não são mais encontradas, causados pelo aumento da temperatura da água (Rilov, 2016). É importante ressaltar que aproximadamente 60% das espécies nativas de corais do mediterrâneo oriental não atingem o tamanho reprodutivo, agravando à medida que o clima esquentar, esse colapso da biodiversidade nativa que se intensifica cada vez mais (Albano e Paolo, 2021). Em 2015 o efeito da ação antrópica chamou atenção na região do Caribe, ocorrendo mudanças nas assembleias de peixes, causada pela degradação das

estruturas dos recifes de coral (Alvarez-Filip *et*. al., 2015). E como consequência poderá haver extinção de muitas de espécies marinhas, quem vão dos corais, a espécies de peixes até mamíferos de grande porte (Alvarez-Filip et al., 2015).

Impactos antrópicos locais e globais têm se tornado preocupantes (Baker et al., 2008; Hughes, 2016;). Dentre os estressores globais destaca-se a crise climática, provocando o aquecimento e acidificação (Hughes et al., 2017, 2018, 2019). Como estressores locais são identificados os filtros químicos presentes nos protetores solares, a poluição por esgoto e metais pesados, derramamento de petróleo, lixiviação de sedimentos e produtos químicos, sobrepesca, turismo desordenado, espécies bioinvasoras, entre outros fatores (Jones, 1997; Guldberg et al., 2007; Hughes et al., 2017, 2018; Raffa et al., 2019; Mantellato et al., 2018). O estresse térmico, causado pelo aumento da temperatura da superfície do oceano (NOAA, 2016) desfaz a simbiose entre as microalgas (zooxantelas) que vivem no tecido dos corais e que dão ao coral seu alimento (glicose, glicerol, aminoácidos, etc.). Quando essas microalgas têm contato com um agente causador de estresse, como o aumento da temperatura da água, elas passam a produzir elevadas concentrações de espécies reativas de oxigênio (ROS) e espécies reativas de nitrogênio (RNS), as quais causam diversos danos celulares (Lesser et al., 1990; Lesser, 2006). Em resposta, o coral expulsa as zooxantelas de seus tecidos, deixando seu esqueleto calcário visível sob o tecido mole, tornando-se branqueado e perdendo sua principal fonte de alimento (Glynn, 1993; Guldberg, 1999; Hughes et al., 2003, 2017; Weis, 2008; Lesser, 2011). Dependendo da intensidade do estresse, os corais não se recuperam e morrem.

O primeiro evento global de branqueamento durou de 1998 a 1999 (Nzali et al., 1998). No ano de 1998, a (GBA), sofreu branqueamento principalmente na região costeira e severamente nas regiões Centro e Sul (Hughes et al., 2017), onde 45% dos corais branquearam parcialmente e 18% foram seriamente branqueados, e o branqueamento de quase todas as espécies de corais moles (Wilkinson, 1998). O segundo evento global de branqueamento ocorreu nos anos de 2009 e 2010, nesse evento o arquipélago de Los Roques, na Venezuela, teve a temperatura da água em 5 m de profundidade atingindo 30,85°C, os registros de aquecimento da água foram de mais de 16°C de agosto a novembro de 2010. Onde 72% das 563 colônias de

corais escleractíneos estudadas ficaram parcialmente ou totalmente branqueadas e a cobertura de corais teve uma redução de 34% nos transectos estudados (Bastidas et al., 2012).

Em 2016, a temperatura recorde dos oceanos levou a um branqueamento massivo nos recifes de corais australianos. Dados de monitoramentos subaquáticos mostraram que a mortalidade média foi de 22% para toda a Grande Barreira de Corais, chegando a 67% em algumas regiões (AIMS, 2016; Hughes et al., 2016). No Brasil, já existem registros de ocorrência de branqueamento em espécies de corais e zoantídeos em várias áreas recifais ao longo do litoral, observadas desde o primeiro registro na década de 90 (Castro e Pires, 1999) durante o período do verão de 2010, relacionando com possíveis anomalias térmicas das águas superficiais do oceano e outros fatores climático-oceanográficos (Soares e Rabelo, 2014).

Mies et al. (2020) observaram dados copilados entre 1980 à 2018 que os episódios de branqueamento estão associados com mortalidade dos corais em 48% no Indo-Pacífico, 38% no Caribe e 20% no Atlântico Sul. Ficou evidenciado que os recifes do Atlântico Sul passaram por menos episódios de branqueamento, com taxas de mortalidade inferiores quando comparado com o Indo-Pacífico e o Caribe. Apesar das taxas de mortalidade serem variáveis entre as regiões, estima-se que entre 50 e 75% da cobertura global de recifes de coral tenha diminuído nas últimas quatro décadas (BRUNO et al., 2019).

O *El Niño* de 2019 desencadeou o quarto evento de branqueamento, estendendo-se até 2020 (GBRMPA, 2020). No ano de 2019, a costa brasileira sofreu o mais intenso e duradouro evento de branqueamento já registrado no país. Entre março e maio de 2019, os recifes marginais do banco de Abrolhos foram afetados por uma onda de calor, a mais forte desde 1985. Atingindo o grau de aquecimento histórico (DHW) 19,65, ocasionando o branqueamento de 100% das colônias de *Millepora alcicornis* e 80% das colônias de *Mussismilia braziliensis.* O branqueamento de *Millepora alcicornis* foi rapidamente coberto por cianobactérias, seguidas por *tufos* de algas e algas calcárias, ocasionando a mudança de fases e levando a mortalidade de 90% das *Millepora alcicornis*. Já as *Mussismilia braziliensis* conseguiram se recuperar para um estado saudável após ter atingindo branqueamento de 90%

(Ferreira et al., 2021). Os recifes de coral do Atlântico Sul são mais resistente que os recifes do Indo-Pacífico e Caribe (Mies et al., 2020). Apresentam mais resistência às mudanças climáticas, possuindo uma maior resiliência e tolerância ao estresse térmico (Leão et al., 2010; Mies et al., 2020), evidenciado no episódio relatado por Godoy et al . (2021) onde colônias totalmente branqueadas de *M. hispida* e *M. harttii* desovaram gametas ainda viáveis.

Os eventos de branqueamento estão se tornando cada vez mais frequentes (Hughes et al., 2019). Se as mudanças no ambiente marinho continuarem acontecendo nesse ritmo, os corais não terão tempo para se adaptar e estaremos diante do colapso desse ecossistema, já que possuem crescimento lento e sua dinâmica é frequentemente associada a uma estreita flutuação ambiental (Wilson et al., 2006; Alvarez-Filip et al., 2011).

## 2.5. Efeitos Antrópicos Nos Corais Brasileiros

Os recifes de corais sofrem os impactos das ações antrópicas próximos com o litoral, principalmente as relacionadas ao turismo, como por exemplo os recifes de coral de Pirangi no estado do Rio Grande do Norte que são particularmente vulneráveis aos efeitos antropogênicos. Uma delas é o atropelamento da cobertura biológica pelas centenas de visitantes que frequentam o recife a cada ano (Azevedo et al., 2011). Outro fator antrópico e o aumento da carga sedimentar e a entrada de detritos orgânicos na área recifal. Em novembro de 2015, o rompimento da barragem de rejeitos da mineração do Fundão resultou na liberação de aproximadamente 50 milhões de $m^3$ de óxido de ferro e lama rica em quartzo no Rio Doce, tal aporte sedimentar alcançou o banco de Abrolhos através de sedimentos suspensos encontrados no recife de coral (Evangelista et al., 2022).

Segundo Dutra et al., (2006), em locais onde a taxa de sedimentação é superior a acumulação de sedimentos de 10 mg $cm^{-2}$ $dia^{-1}$, há um declínio considerável nos indicadores de saúde da comunidade de corais avaliados em colônias de *Millepora alcicornis*, número total de recrutas e de recrutas de *Siderastrea stellata*. As variações morfológicas e de distribuição das estruturas dos recifes são, muito provavelmente,

os principais fatores que expõem os corais aos efeitos do influxo de sedimentos. Os pontos do recife de estudos mais próximos do continente apresentam os maiores valores para a taxa de acumulação de sedimentos e a maior porcentagem de sedimentos siliciclásticos, o que também parece influenciar a vitalidade dos recifes.

A poluição provocada pelo homem promove efeitos da eutrofização no ecossistema recifal. A concentração de silicato, juntamente com os valores de salinidade, pode ser usada como um marcador de descarga de águas subterrâneas (Montagionni et al., 1993). Indicando que as águas residuais domésticas podem ser fonte de contaminação, acarretando uma quantidade significativa de nutrientes terrestres para o recife. Este modelo de enriquecimento de nutrientes via infiltração de águas subterrâneas é um mecanismo plausível que pode explicar a eutrofização que ocorre em sistemas de recifes de coral, como ocorre no litoral norte da Bahia. Esses dados sugerem que a alta disponibilidade de nutrientes afeta a estrutura trófica, com o aumento do crescimento rápido de turfa e macroalgas que tendem a colonizar a maior parte dos substratos abertos reduzindo a penetração de luz nas colônias de coral, competindo espaço e inibindo o assentamento de novas larvas de coral (Costa Jr. et al., 2000).

Efeitos antrópicos estão afetando as espécies de corais brasileiras, em especial *a M. hartti* que é endêmica e já se encontra ameaçada de extinção por habitar áreas de recifes costeiros rasos, na qual as ações do turismo marinho, a exploração de recursos naturais e a poluição costeira estão entre os principais agentes de impacto humano, apresentando um declínio de 50% de sua população em um período de 30 anos (Pires et al., 2018).

Enquanto práticas de conservação *in situ* como áreas marinhas protegidas podem ajudar a diminuir os impactos locais, os impactos antrópicos como o aquecimento global continuarão causando declínios populacionais em taxas ainda mais rápidas. Altas taxas de mortalidade sem recuperação elevam-se a níveis alarmantes, levando à queda na diversidade genética das populações de corais e a possibilidade de extinção, uma vez que a persistência de uma população ou espécie é amplamente depende da sua diversidade genética (Laikre et al., 2010).

## 2.6. Efeito do estresse térmico na reprodução

O branqueamento dos corais e o estresse térmico podem afetar de forma negativa o seu sucesso reprodutivo (Mendes e Woodley, 2002; Suggett e Smith, 2011; Hagedorn et al., 2016), prejudicando a gametogênese, reduzindo o tamanho dos ovos, afetando a fecundidade e levando a perda de desovas sincrônicas (Mendes e Woodley, 2002; Shlesinger e Loya, 2019). Outro fator que afeta a reprodução dos corais é a substituição temporal de corais por organismos não construtores, como as algas, gerando menor complexidade estrutural no recife e diminuindo a diversidade na comunidade, além de interferir no assentamento dos recrutas (Hughes, 1994; Speare et al., 2019).

Estudos mostraram que 80 a 99% dos corais *Acropora palmata* e *A. cervicornis* do Caribe já foram perdidos (Precht et al., 2002; Aronson et al., 2001). Quando ocorre reprodução sexuada dessas espécies, as larvas são produzidas uma vez por ano, geralmente não se autofecundam e o sucesso reprodutivo pode ser esporádico e sobrecarregado nas pequenas populações isoladas do Caribe (Hagedorn et al., 2012). Em algumas regiões, como em Florida Keys, a diversidade genotípica pode ser tão baixa que a reprodução sexuada é quase impossível (Hagedorn et al., 2012). Além disso, os jovens recrutas estão sendo adversamente afetados pelo aumento da cobertura de macroalgas nos recifes (Hagedorn et al., 2012). Segundo Hagedorn et al., (2012), juntos, esses fatores aumentaram a probabilidade de extinção dos corais *A. palmata* e *A. cervicornis* nos recifes do Caribe nos próximos 5 a 20 anos.

Afim de protege as populações de corais, a criopreservação de gametas, embriões e larvas são excelentes ferramentas que permite a preservação temporária de células em baixas temperaturas. (Lin e Tsai, 2012; Tsai e Lin, 2012).

## 2.7. Criopreservação

A criopreservação tem muitas aplicações biotecnológicas em diferentes áreas. Destacando-se na preservação de células que têm muitas aplicações em medicina humana e veterinária, agricultura e aquicultura. Além disso, esta biotecnologia tem muitas aplicações em pesquisas biomédicas, especificamente nas áreas de imunologia, virologia, neurobiologia, toxicologia e indústria farmacêutica (Chao et al., 2001; Rall, et al., 2001). Isso levou a um

aumento na importância da criobiologia como ciência tornando-se o principal método de preservação de espécimes biológicos, buscando aumentar a importância, examinando o efeito da temperaturas ultrabaixas para manter a viabilidade em células, tecidos, órgãos e organismos. (Bozkurt, 2017).

A tecnologia é crucial para o armazenamento e disponibilidade de recursos genéticos para a área biomédica (Matthews et al., 2018). Sendo uma biotecnologia promissora na criopreservação de gametas é na na preservação de material biológico de coral (Hangedorn et al., 2006; Lin eTsai 2012; Tsai e Lin 2012; Li et al., 2012).

A criobiologia envolve diversos setores da pesquisa tornando-se um campo multidisciplinar, integrando áreas como a biologia molecular, engenharia, matemática, clínicos em medicina humana e veterinária, entre outros (Benson et al., 2012). A criopreservação baseia-se no armazenamento das células ou dos tecidos em baixas temperaturas (criogênicas) (Karlsson& Toner, 1996) garantindo sua viabilidade e manutenção dos níveis de energia cinética, mantendo-os baixos, impedindo o movimento molecular (Grout et al., 1990), induzindo à parada das reações enzimáticas e assim possibilitando que após o aquecimento deste material a atividade metabólica possa ser restaurada normalmente (Mazur et al., 1984). Apesar disso, o próprio processo de criopreservação é associado às injúrias celulares que podem ocorrer durante o resfriamento ou aquecimento (retorno à temperatura fisiológica original).

As injúrias provocadas pelo resfriamento e choque frio ocorrem quando há formação de gelo intracelular. A produção de gelo inicia quando a estrutura das moléculas de água e das pontes de hidrogênio tornam-se mais ordenadas pela exposição à baixa temperatura. Acredita-se que as ligações hidrofóbicas entre as cadeias proteicas laterais são afrouxadas e, consequentemente, a conformação das moléculas da proteína mudam resultando em desnaturação (Tajima e Shimizu, 1973). A exposição ao resfriamento pode estimular o estresse oxidativo, promovendo a formação de espécies reativas de oxigênio (McKersie et al., 1990; Tsang et al., 1991; Scandalios, 1993), causando apoptose celular, desnaturação de proteínas e peroxidação de lipídios (Wood e Youle, 1995; Prasad, 1996). Os danos

causados às células em condições hipotérmicas podem ser divididos em dois tipos principais: injúrias causadas pelo frio durante o processo de resfriamento lento e por altas taxas de resfriamento (choque frio) (Belous e Grischenko, 1994).

Para minimizar as injúrias pelo resfriamento, são utilizadas soluções crioprotetoras que apresentam como funções principais a substituição de água, existente no meio intracelular, realizando a desidratação parcial da célula com o mecanismo de gradiente osmótico, reduzindo o limiar de congelamento e evitando a formação de cristais de gelo no interior das células (Rosato e Laffaldano, 2013). Crioprotetores e solutos, de forma geral, aumentam a viscosidade do meio e diminuem a temperatura de congelamento (Carmichael et al., 2009).

Os crioprotetores são utilizados com a finalidade de proteger as células e tecidos durante a redução da temperatura; contudo, sabe-se que eles podem causar danos osmóticos e toxicidade (Guan et al., 2010). Os efeitos tóxicos da solução crioprotetora devem ser evitados, com taxas rápidas de resfriamento ideal, mas lenta o suficiente para que as células possam desidratar e evitar a formação de gelo intracelular (Mazur et al., 1972).

Existem dois grupos de agentes crioprotetores (CPA) que diferem de acordo com a sua capacidade de passar através das membranas celulares: os permeáveis (intracelulares ou internos) e não permeáveis (extracelulares ouexternos). Os CPA permeáveis são em teoria substâncias com alta solubilidade em água e de baixo peso molecular capazes de penetrar a membrana celular e desempenhar sua ação crioprotetora por todo o citoplasma e organelas, diminuindo a temperatura de congelamento intracelular, suprimindo a formação de cristais de gelo (Hubálek, 2003). As características de um CPA permeável devem apresentar baixo peso molecular, baixa toxicidade e alta capacidade de atravessar a membrana celular (Fahy, 2010). Segundo Fahy, 2007 os CPA não permeáveis são aquelas substâncias que possuem alto peso molecular, portanto não conseguem penetrar no interior da célula, mas promovem a ação protetora externamente. Por serem compostos hidrofílicos, eles se ligam as moléculas de água, aumentando a viscosidade da solução e diminuindo a formação de cristais de gelo (Fahy, 2007).

A criopreservação dos oócitos e dos espermatozoides é um desafio para

diversas áreas do conhecimento. Durante a última metade do século 20 houve avanços consideráveis nas tecnologias reprodutivas de mamíferos , incluindo a produção in vitro de embriões pré-implantação e sexagem de embriões, e até mesmo clonagem em algumas espécies (woods et al., 2004). No entanto, a dificuldade para os espermatozoides ocorre devido a morfologia, ao seu citoesqueleto complexo e aos mecanismos de ativação (Benson et al., 2012), principalmente por conta da toxicidade do CPA que deve ser compreendida e para entender a toxicidade do CPA, é necessário entender quais macromoléculas ou organelas estão sendo quimicamente danificadas e como elas são sendo danificado. (Best, 2015). Para os oócitos de coral o desafio é devido ao tamanho aumentado da célula e a baixa permeabilidade causada pela quantidade de lipídios (Arai et al., 1993; Lin et al., 2013; Tsai et al., 2016). Além de que os protocolos de criopreservação devem ser acessíveis não apenas para grandes laboratórios equipados com alta tecnologia, mas também de uso em campo (Benson et al., 2012).

A criopreservação do material biológico geralmente é realizada através das seguintes técnicas de congelamento: vitrificação e congelamento lento controlado.

Segundo Rall e Fahy, 1985, a vitrificação caracteriza-se pela solidificação de um líquido provocada não pela cristalização, mas por uma elevação extrema da sua viscosidade usando altas concentrações de CPA e taxa de resfriamento ultra-rápida. Dessa forma, quando a solução é rapidamente resfriada e atinge o estado vítreo, toda a solução fica livre de cristais de gelo (Fahy, 1986; Chong et al., 2016). Na qual diminui o tempo no procedimento de criopreservação, que é muito mais simples e rápido. Não exige equipamentos especializados ou caros, o que a torna uma técnica barata. É uma boa alternativa para o campo (Saragusty e Arav, 2011).

Congelamento lento controlado consiste na colocação das amostras em gelo seco, suspensão em vapor de nitrogênio e refrigeração com freezer de taxa controlada (Martínez-Páramo et al., 2017), sendo as duas primeiras opções mais baratas e práticas, podendo serem utilizadas em coletas a campo e em laboratórios menos estruturados. Tal técnica deve atingir uma taxa ótima de resfriamento e alcançar um congelamento equilibrado para um dado tipo celular, ocorrendo lenta

o bastante para permitir o grau de desidratação celular necessário durante o resfriamento (o qual previne a formação de cristais de gelo intracelular), e rápida o suficiente para evitar a superexposição das células aos efeitos tóxicos dos CPA (Muldrew e McGann, 1997). No entanto, existem muitas variáveis a serem estudadas e aprimoradas no processo de todas as técnicas de criopreservação.

O descongelamento é uma etapa muito importante em um protocolo de criopreservação, em vista os muitos processos que podem acontecer, afetando negativamente a viabilidade das células preservadas. O descongelamento lento é geralmente realizado em um congelador programável ou com menos frequência, apenas expondo a amostra temperatura ambiente. O descongelamento rápido é conduzido através da imersão da amostra em banho-maria temperatura controlada (Gordienko e Pushkar, 1994).

## 2.8. Criopreservação De Gametas Para Conservação De Corais

A criopreservação em material biológico de coral é aplicada há 16 anos, no entanto encontra-se ainda, em fases iniciais (Lin e Tsai 2020). Diferentes tipos de técnicas de criopreservação são usados atualmente para criopreservação de gametas de corais. O mais comum é o congelamento lento, que foram aplicados para oócitos de corais *Echinopora sp.* (Tsai e Lin,2012) e *Ellisella robusta* (Lin et al., 2014; Goh, 2019). Para os espermatozoides, os primeiros relatos foram de corais australianos (*A. tenuis*, *A. millepora* e *A. digitifera*) e um caribenho (*Fungia scutaria*) que foram congelados com sucesso (Hagedorn et al., 2006; 2012; Tsai et al., 2010; Ohki et al., 2014), contando com boas taxas de fecundação dos oócitos e produzindo larvas saudáveis capazes de assentar e desenvolver-se (Hagedorn et al., 2017). Outros experimentos com corais foram realizados utilizando o congelamento em duas etapas, com *Symbiodiniaceae* (Chong et al., 2016; Lin et al., 2019; Di Genio et al., 2020) e espermatozoide *A. humilis* (Viyakarn et al., 2017).

Para oócitos a criopreservação foi realizada pela primeira vez em 2010 por Tsai et al. Permanecendo ainda um desafio devido à sua sensibilidade a CPAs, baixa permeabilidade e resfriamento (Li net al., 2011, Tsai et al., 2014), sendo o metanol o

que melhor protege os oócitos reduzindo a formação de cristais de gelo (Tsai et al 2014). Em trabalhos de 2015 e 2016 Tsai et al., observaram que o metanol possui uma baixa ação de vitrificação e elencaram o etilenoglicol como melhores resultados de vitrificação para oócitos de coral.

Um grande desafio para a criopreservação dos oócitos é o alto teor lipídico, resultando em baixas proporções de área de superfície para o grande volume da célula, associados essa característica a dificuldades de congelamento e descongelamento (Li net al., 2013). A elevada quantidade de Fosfatidiletanolaminas e ácidos graxos estão ligadas à alta fluidez da membrana, que protege as células do estresse por frio (Lina t al., 2012, 2013). Tsai et al., (2012) descobriram que os oócitos de coral toleram temperaturas de 0 e 5°C, mas não a -5°C por isso o estudo recomenda a vitrificação, pois observarão menos lesões por frio nesta técnica.

A vitrificação proporcionou bons resultados na criopreservação de bolas de tecido de *Pocillopora damicornis* (Feuillassier et al., 2015), em oócitos juncea (Tsai et al., 2015, 2016), *Symbiodiniaceae* (Hagedorn e Cárter, 2015; Zhao, 2017) e larvas de coral (Daly et al., 2018). O trabalho realizado por Cirino et al. (2019) vitrificou larvas de *Seriatopora caliendrum* em simbiose com dinoflagelados e realizou o descongelamento a laser com nano-ouro, gerando sucesso no assentamento e desenvolvimento das larvas.

A criopreservação bem-sucedida para corais requer um protocolo de congelamento adequado que limita danos biomoleculares, resultando amostras viáveis após o descongelamento. As taxas de resfriamento, composição de CPA e concentração, tempo de equilíbrio e o tempo de descongelamento podem ser otimizados para melhorar a viabilidade celular. A Criobiologia mostra-se uma importante ferramenta para conservação do coral *M.harttii*, pois através dela pode-se estudar e produzir alternativas que irão viabilizar a criopreservação do gametas através de um protocolo e assim desenvolver o primeiro banco de germoplasma de corais brasileiros.

Os criobancos preservam material genético de diferentes espécies em distintas formas  e para diversos fins, incluindo estudos científicos e esforços de conservação. Eles são particularmente importantes para a aquicultura e biopreservação (Clarke,

2009). Mayfield et al., 2019 especula que os corais podem ser criopreservados até um ponto no tempo, no futuro, em que a propagação de corais poderá ser realizada de forma segura e saudável. Ainda segundo Mayfield et al., 2019 os corais descongelados poderão ser criados em cativeiro, nos quais diferentes genótipos provavelmente terão capacidade de serem cruzados para aumentar a diversidade genética e potencialmente produzir populações mais resilientes. Atualmente, encontram-se criobancos de coral no Museu Nacional de Biologia marinha e Aquario (NMMBA) de Taiwan (Mayfield et al., 2019; Di Genio et al., 2020), no Instituto do Havaí de Biologia Marinha (EUA) e em Taronga Western Plains Zoo (Austrália; Hagedorn e Spindler, 2014).

## 3. HIPÓTESES E OBJETIVOS

### 3.1. Hipóteses

As hipóteses propostas pelo presente trabalho são de que será possível avaliar a toxicidade dos agentes crioprotetores aos quais os oócitos de *M. harttii* serão submetidos. Além disso, acredita-se que os espermatozoides de *M. harttii*, serão resistentes quando expostos ao resfriamento. Sendo assim possível desenvolver um protocolo de congelamento para o espermatozoide, possibilitando a criação do primeiro banco de gametas de coral do Atlântico Sul utilizando como modelo a espécie *M. harttii.*

### 3.2. Objetivos

#### 3.2.1. Objetivo Geral

Desenvolver biotecnologias para avaliar a viabilidade dos gametas do coral *M. harttii* pós exposição a crioprotetores e criopreservação e desenvolver um protocolo

de criopreservação para armazenamento do gameta masculino por tempo indeterminado, criando o primeiro banco de gametas de coral do Atlântico Sul.

### 3.2.2. Objetivos específicos

- Fornecer uma nova metodologia de analise da viabilidade dos oócitos de *M. harttii*;
- Testar duas diferentes técnicas de criopreservação associadas a dois diferentes crioprotetores e concentrações para o sêmen do coral-cérebro;
- Avaliar a viabilidade dos espermatozoides pós-descongelamento;criopreservacao
- Criar o primeiro banco de gametas de coral do Atlântico Sul utilizando como modelo a espécie *M. harttii.*

## CAPÍTULO II*

# Assessment of viability in coral oocytes: a biochemical approach to achieve reliable assays

*Artigo elaborado seguindo as normas do periódico *Marine Biology*.

# Assessment of viability in coral oocytes: a biochemical approach to achieve reliable assays

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**Abstract**

Given the current threatening scenario faced by coral reefs, reproductive biotechnologies can be a helping hand in the fight against extinction and therefore, safe methods to assess viability of coral gametes are essential. In this study, we aimed to develop a reliable method to assess oocyte viability for scleractinian corals using *Mussismilia harttii* as A model of coral population\community. For this aim, We chose the thiazolyl blue tetrazolium bromide (MTT) assay and trypan blue (TB) test. TB tests were performed in two conditions. First, oocytes were exposed to 1, 3, 5, 7 and 9 $mol^{-1}$ dimethyl sulfoxide (DMSO) for 20 min, then incubated in 0.4% TB and had their membrane integrity assessed. Second, in order to identify if TB was able to stain efficiently, dead oocytes were incubated in TB diluted in filtered seawater or sodium dodecyl sulfate (SDS). For MTT experiments, a serial dilution assay was performed using DMSO as cytotoxic compound. TB was able to cross the oocyte membrane, but because of THE their high lipid content, the observation of blue color was not easily visible, was not clear possible, making this test unfeasible for oocytes of *M. harttii*. We were able to standardize the MTT assay for determining mitochondrial activity in mature coral oocytes, and consequently

assessing their viability. Equally, the positive linear correlation between MTT concentration and DMSO dilution allowed us to set a safe DMSO concentration (1.4 M) to work with coral oocytes. These results provide a new technique to be applied for assessing coral oocyte viability, which can add efforts in reef monitoring programs and conservation strategies.

**Keywords: Triplan blue test,**MTT assay; *Mussismilia harttii*; Brazilian reefs; Toxicity test; Mitochondria; Coral gamete.

## Introduction

Coral reefs are highly biodiverse ecosystems providing important services to shoreline stability (Elliff and Silva 2017), ecotourism, fishing, and are also a source of food and new medicines (El-Naggar 2019). Multiple anthropogenic impacts have led coral reefs to decline worldwide at an accelerating rate (Hughes et al. 2018, 2019; Hoegh-Guldberg et al. 2019) and degradation will have far-reaching implications for other marine ecosystems (Hoegh-Guldberg et al. 2017; UN Environment ISU ICRI and Trucost 2018). Meanwhile, new researches with applications in reef biotechnology and conservation are emerging. Technology alone cannot save reefs, but it can potentially help scientists and conservation practitioners to study, mitigate, and even solve key challenges faced by coral reefs (Madin et al. 2019).

Reproductive biotechnologies find a large field of applications since approximately 90% of shallow water scleractinian corals are broadcast (Harrison, P. L and Wallace 1990; Baird et al. 2009; Harrison 2011), which means both male e female gametes are released in the water column. Although this strategy provides genetic variability and enables dispersal (Harrison and Wallace 1990; Miller and Ayre 2004; Foster et al. 2007), external fertilization makes coral gametes more susceptible to environmental pollution and embryonic growth require an enormous amount of energy to sustain such development outside the parents' body (Yoshida and Asturiano 2020). Therefore, the oocytes are responsible to maintain the initial development of the coral progeny, until they are able to feed. Given the current scenario, the need to create reliable protocols to determine viability of coral oocytes is becoming increasingly important. Whether to evaluate the reproductive performance of individuals, the effects of stressors and environmental contaminants on reproduction and progeny or developing cryopreservation

protocols, basic knowledge of the gamete structure and ways of assessing its viability are crucial.

Although there has been significant advance in the field of coral reproduction, basic physiological aspects of coral gametes are still largely unavailable, especially in the case of oocyte. At the present time, protocol to assess oocyte viability in scleractinian corals at physiological level has been applied only to a single species (*Echinopora spp*. – see Lin and Tsai, 2012; Tsai et al., 2010) using ATP concentration via bioluminescence assay and fluorescein diacetate (FDA) probe. In addition to all limitation, once FDA diffuses into cells, nonspecific esterases in the cytoplasm generate free fluorescein, an as a result, the rate at which fluorescein diffuses out of cells varies greatly (Lindhagen et al. 2008; Johnson et al. 2013).

The criteria of viability is surely the most fundamental of all indicators and, therefore, evaluation of oocytes viability should yield information at metabolic level, making possible to number live, dead and dying cells (Beninger et al. 2021). In fact, it is possible to find several viability tests, considering the integrity of different cell functions, but practically all of them have been developed for mammals. The Trypan blue (TB) and the MTT (3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assays are two of those tests (Didion et al. 1990; Marks et al. 1992; Guan et al. 2010; Beninger et al. 2021). While TB is a vital dye used to detect dead cells in mammals and fish (Wales 1959; Didion et al. 1990; Guan et al. 2010) based on the integrity of the cytoplasmic membrane, the MTT assay is a colorimetric test that allows to assess cellular metabolic activity. Applications include cell proliferation, cytotoxicity and apoptosis (Marks et al. 1992; Nasr-Esfahani et al. 2002). Despite the mentioned applications**,** neither TB nor MTT have been tested for oocytes of scleractinian corals before. Therefore, we aimed to develop a reliable protocol to assess coral oocyte viability with low per-test cost, simplicity for routine use, rapid execution and to obtain efficient and effective results, with a low sample size required.

## Materials and methods

### *Coral collection and permits*

Colonies of *Mussismilia harttii* (*n* = 37) were collected at the Funil site at the Recife de Fora Marine Park (16°24’31″S; 038°58’39″W - Bahia State, Brazil) under SISBIO license Nº 63368-1. Collection was performed two weeks prior to the predicted spawning period of the

species (Pires et al. 1999; Zilberberg et al. 2016). Colonies were placed in 1000-L semi-closed tanks connected to the ocean at the Coral Vivo Research Base, whose team monitors coral spawning episodes since 2004. Local surface seawater temperature was 25.4 ± 0.84°C.

***Oocyte-sperm bundles collection and separation***

Experiments were performed over three nights during the spawning window period. To avoid gamete contamination, each broodstock colony was isolated in a plastic bowl and monitored during spawning (Godoy et al. 2021). Oocyte-sperm bundles were collected from the water surface and immediately transferred to 50 mL tubes (three bundles per tube) containing 10 mL of filtered (0.2 μm) seawater (FSW). Oocytes floating on top of the tube were separated using a Pasteur pipette and transferred to a 2 mL microtube containing FSW for further tests (Fig. 1).

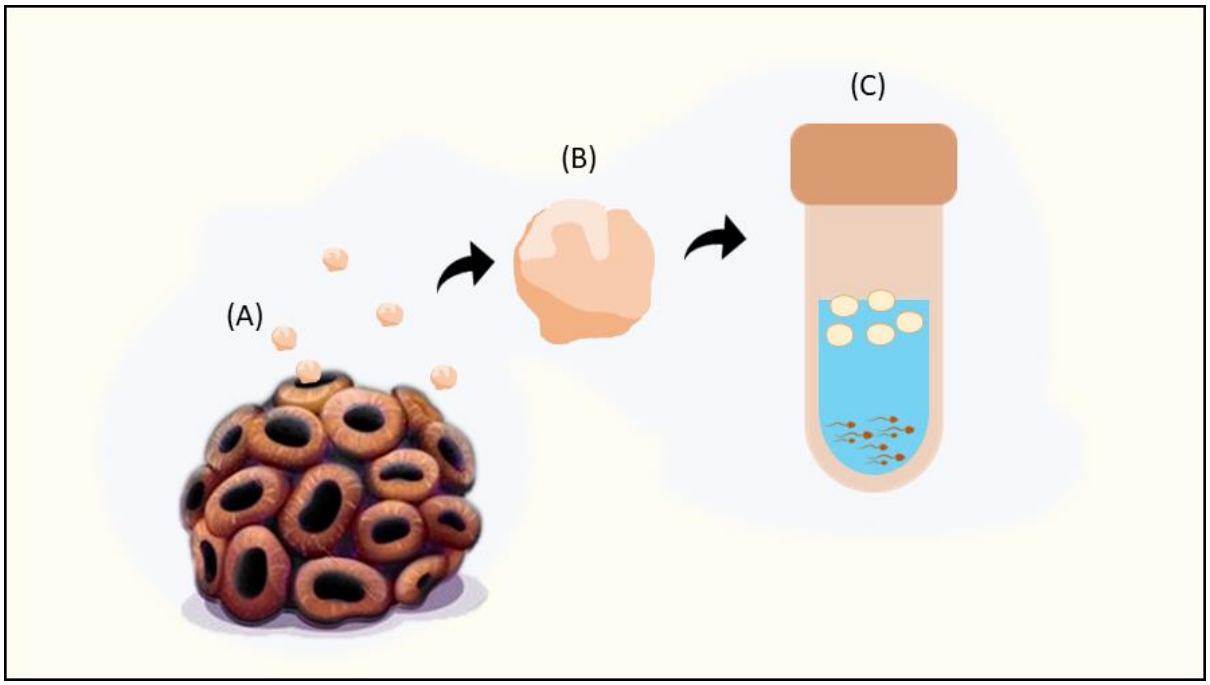



**Fig. 1**(A) Spawning of the hermaphroditic South Atlantic coral *M. harttii*. (B) Oocyte-sperm bundle collected. (C) After dissociation of oocyte-sperm bundles, the oocytes were separated from sperm for the viability tests.

***Choice of oocyte viability tests***

We selected the trypan blue test and the thiazolyl blue tetrazolium bromide (MTT) assay

to carry out the experiments. Trypan blue is a dye exclusion test based on the cell membrane integrity. When the cell membrane is intact, the trypan blue dye is not able to enter the cytoplasm and the cell remain unstained, so it is considered viable (Strober 2001). However, if the cell membrane is damaged trypan blue enters the cytoplasm and the cell becomes blue, which is considered dead. It is a simple and low cost test widely used for quantification of viable cells (Strober 2001; Avelar-Freitas et al. 2014; Chan et al. 2020). The MTT assay is a colorimetric test widely used to assess mammal cells viability, cytotoxicity, proliferation and cell activation determination based on the mitochondrial metabolism (Mosmann 1983; Kumar et al. 2018).The MTT is metabolized in the mitochondria by mitochondrial reductases, which are present only in metabolically viable cells. The mitochondrial reductases reduces the tetrazolium salt, changing its color by turning it into purple formazan crystals (E, Z- 1- (4,5-dimethylthiazol-2-yl) -1,3-diphenylformazan), which can be solubilized in dimethyl sulfoxide (DMSO). The solubilization process generates a purple solution (Fig. 2). The greater the mitochondrial activity the greater the cell viability and therefore a darker purple is the final solution, presenting higher concentration of formazan (Mosmann 1983).It is possible to measure by spectrophotometry, so making possible to quantify which sample presents more cells that are viable.

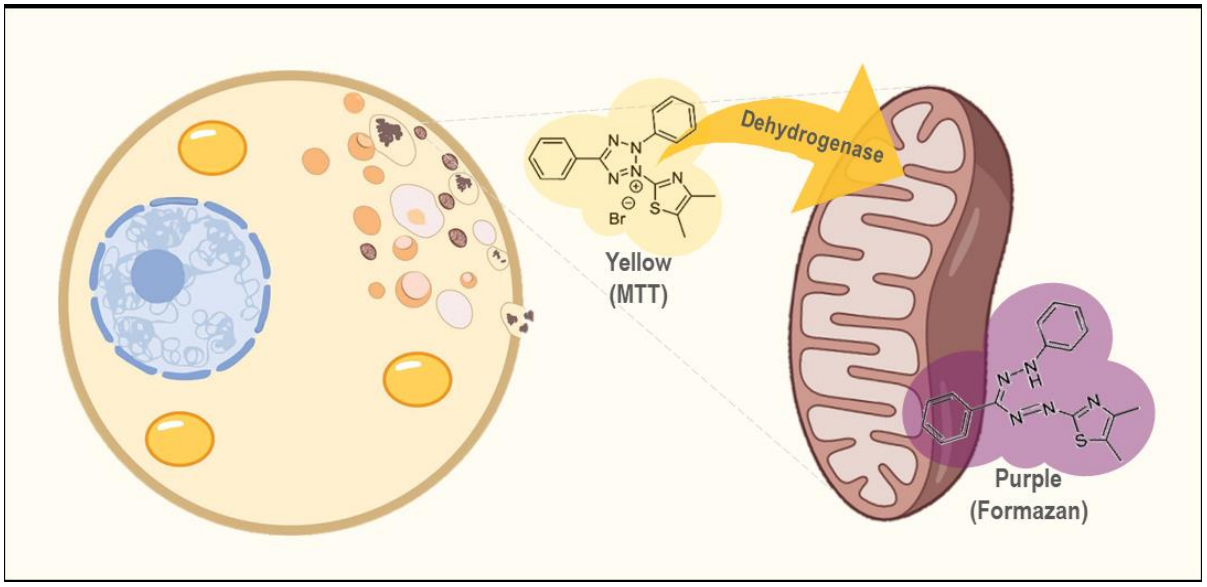



**Fig. 2** MTT viability assay for coral oocytes. The reaction is catalyzed in the oocyte's mitochondria where succinate dehydrogenase reduces thiazolyl blue tetrazolium bromide (MTT) into purple formazan crystals.

*Trypan blue tests*

Trypan blue tests were performed in two different conditions. Firstly, the oocytes were exposed to a cytotoxic compound and then submitted to trypan blue test. Secondly, the trypan blue was used in oocytes that were naturally dead, without the use of any toxic compounds. Therefore, it was possible to identify if the trypan blue was able to stain efficiently the oocytes.

The experiments were started based on a serial dilution assay in order to quantify the viability of coral oocytes after exposed to the cytotoxic compound. We choose DMSO as cytotoxic compound because it is widely used in cytotoxic assays (Huang et al. 2018; Awan et al. 2020) and it is one of the best cryoprotectants patterned for cell cryopreservation (Hagedorn et al. 2012; Xiong et al. 2017; Awan et al. 2020). Since there is no successful protocol for cryopreservation of coral oocytes, it was also important to determine how cytotoxic DMSO can be, and to identify which concentration would be safer for its use in future cryopreservation protocols. We collected a pool of fresh oocytes ($n$ = 242) from three colonies of *M. harttii*, which were randomly assigned to six treatments: control (only oocytes, $n$ = 30)where were they placed?; 1 mol $mL^{-1}$ DMSO ($n$ = 22); 3 mol $mL^{-1}$ DMSO ($n$ = 22); 5 mol $mL^{-1}$ DMSO ($n$ = 100), 7 mol $mL^{-1}$ DMSO ($n$ = 24) and 9 mol $mL^{-1}$ DMSO ($n$ = 44).DMSO diluted in what? The oocytes were exposed to the DMSO solutions for 20 min at room temperature (23°C). Immediately, oocytes were incubated in a trypan blue (0.4%) solution in FSW for 3 min at room temperature, and then washed three times in FSW. Oocytes were assessed under light microscope (Opton TNI-51-IMU) (X 40), with those unstained being classified as intact membrane and the blue stained oocytes considered having a damaged membrane.

To carry out the second trypan blue test we used oocytes that had been collected on the previous spawning night and kept for 24 h in FSW at room temperature. In this case, we needed to have samples of dead cells to use as a control test, since the dye would be expected to penetrate into the dead oocytes. Each microtube received 50 µL of oocytes plus 200 µL of two different trypan blue solutions: (1) 0.4% trypan blue in FSW; and (2) 0.4% trypan blue + 0.01% sodium dodecyl sulfate (SDS) in FSW. The addition of SDS - an anionic detergent suitable to denature proteins - in the trypan blue solution came from the idea of minimizing the possible blockage from the oocyte mucus layer on trypan blue penetration, even in the dead ones, which could generate not accurate viability results. Samples were incubated for 3 min at room temperature and then washed three times in FSW. The oocytes were observed under light microscope (X 40). The unstained oocytes were classified with intact membrane, whereas the blue stained oocytes were considered with damaged membrane. For each solution, three replicates were used and the experiments were repeated three times.

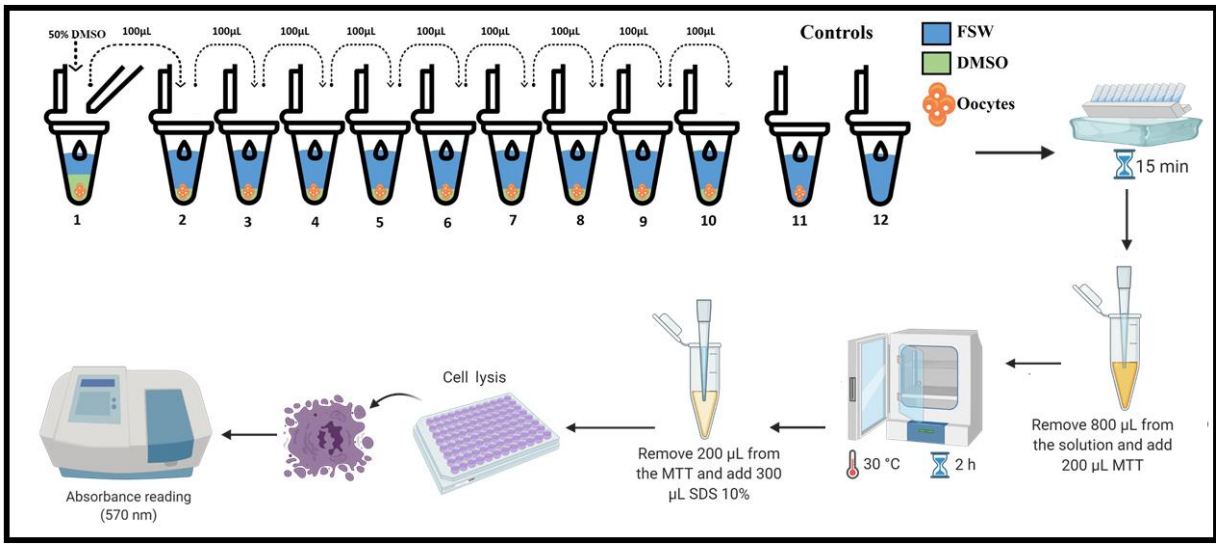
***MTT assays***

To test whether the coral oocytes would be able to metabolize the MTT, we exposed fresh oocytes to two different MTT solutions: solution 1 composed by 5 mg/mL MTT (5%) in FSW (MTT-FSW); and solution 2 composed by 5 mg $mL^{-1}$ MTT (5%) in PBS (MTT-PBS). For each experimental group were selected approximately 50 oocytes and 400 µL of the respective solution. Oocytes were then incubated in the dark for 2 h at 30°C. We adjusted the temperature from 37°C at the original protocol Vybrant® MTT Cell Proliferation Assy Kit (V-13154) of Molecular Probes Inc. (ThermoFisher Scientific) to the ideal 30°C for coral cells. After the incubation period, the MTT solution was removed and 400 µL of DMSO was added to each sample to solubilize the formazan crystals present inside the oocytes. The presence of a final purple solution was considered a positive result. For each solution, three replicates were used and the experiments were repeated three times.

After identifying that coral oocytes were able to metabolize the MTT, a serial dilution assay was performed again with DMSO used as cytotoxic compound. The samples preparation is detailed in Table 1 and the serial dilution procedure is described in (Fig. 3).

**Table 1.** Samples organization prior to the serial dilution for MTT assay.

| Tube | Oocytes suspension (µL) | DMSO (µL) | FSW (µL) | Final volume (µL) |
|---|---|---|---|---|
| 1 | 40 | 500 | 460 | 1000 |
| 2 to 10 | 40 | 0 | 860 | 900 |
| 11 (cell control) | 40 | 0 | 960 | 1000 |
| 12 (solution control) | 0 | 0 | 1000 | 1000 |




Once oocytes were added, the tubes were weighted (0.0388 ± 0.0072 g) before adding the MTT to the samples. Since no statistical differences were found among samples, we considered that it was possible to use the sample weight to equalize the amount of oocytes present in each sample (Fig. 4).

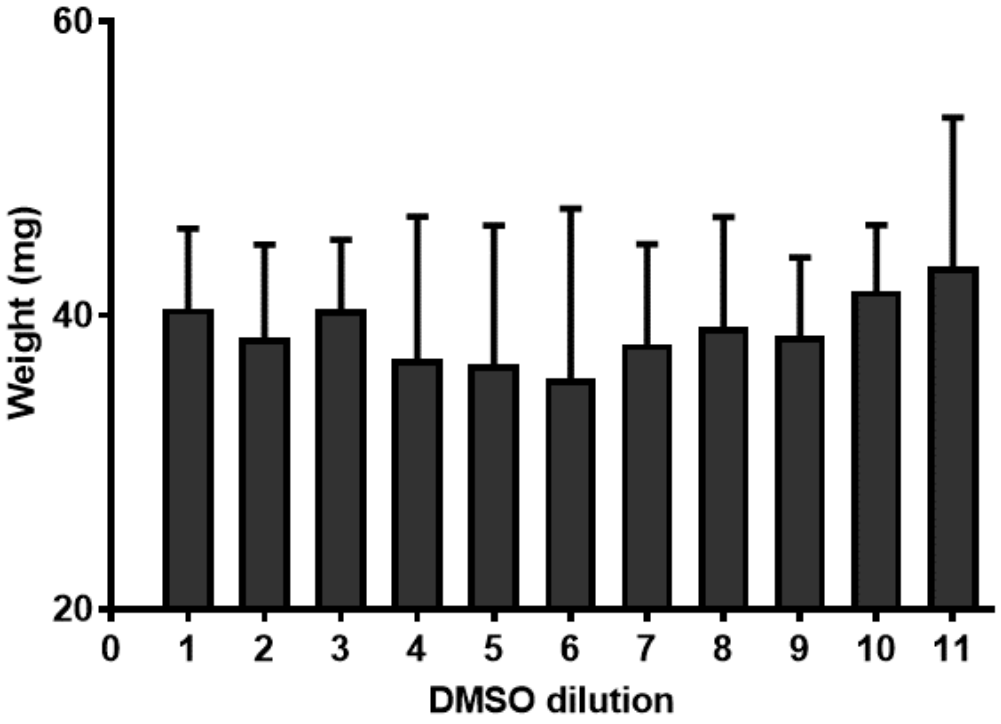



**Fig. 3** Samples showing that the same volume of *M. harttii* oocytes suspension (40 µL) presented similar weights ($P = 0.9677$). Refer to Table 1 for serial dilution details. Data (mean ± SD) are presented for each dilution individually and were analyzed by Shapiro Wilk normality test followed by one-way ANOVA and Tukey's test.

After addition of DMSO, the samples were kept for 15 min at 4 °C (equilibrium). Then, the DMSO solution was removed (800 µL) from each sample using a syringe with needle (40 x 1.20 18 G1 1/2). We did use the needle in order to prevent the adhesion of oocytes and consequently loss of cells. Hereafter, the MTT solution was added and the samples were incubated for 2 h at 30°C in the dark. After incubation, we carefully removed 200 µL of the MTT solution from each tube, leaving only the cells, and a 300 µL-aliquot of 10% SDS was added to solubilize the formazan crystals. For that assay, we chose to solubilize the formazan crystals with SDS instead of DMSO because we observed that the solubilization process would be more efficient for cell lysis (Fig. 5). Then, the solution was transferred to 96 well plates (Fig. 3) and the absorbance was measured using a spectrophotometer (Multiskan GO, Thermo Scientific) with microplate reader at 570 nm.

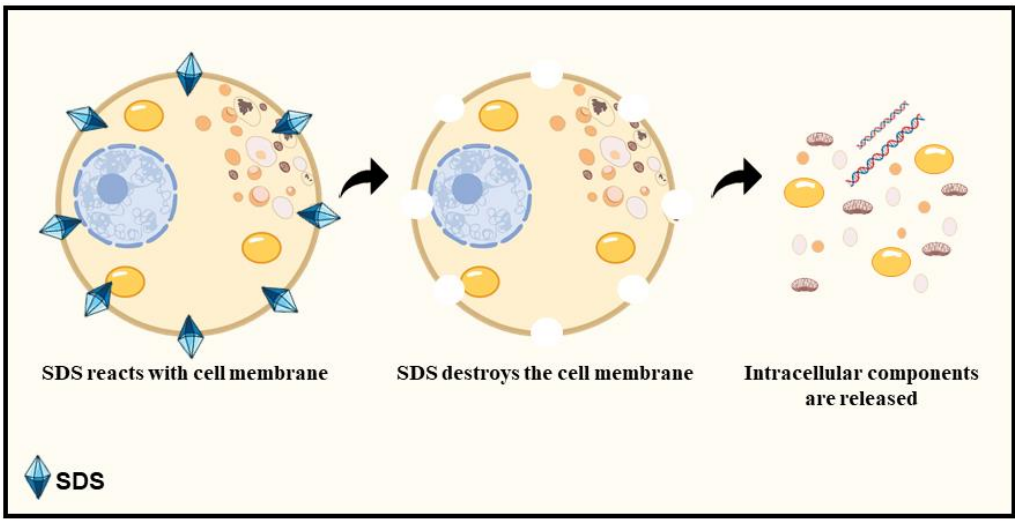



**Fig. 4** Sodium dodecyl sulfate (SDS) action promoting membrane lysis in order to release the intracellular components of coral oocytes.

**Statistical analyses**

All experiments were performed in triplicate and repeated three times. Statistical analyses were carried out using normality test (Kolmogorov-Smirnov, Shapiro Wilk or D'Agostino & Pearson), and homogeneity test (Levene) were applied to assess statistical assumptions. Data that did not present a normal distribution were transformed by using the square root of the plus (k) constant value $\sqrt{\boldsymbol{y+1}}$ and analyzed using Kruskal-Wallis followed by Dunn's multiple comparisons test and considered significantly different at $P < 0.05$. Data from the cytotoxicity assay using MTT were box plotted with median and maximum-minimum values. Analyses were performed using GraphPad Prism 7.0 software.

***Trypan blue tests***

The first test showed that it was not possible to distinguish the viable coral oocytes from the damaged ones by trypan blue solution alone (Fig. 6A). Despite the oocytes had been collected on the previous spawning night (24 h earlier), all of them when exposed to 0.4% trypan blue in FSW remained unstained On the other hand, when SDS was added to the trypan blue solution it was possible to observe in all oocytes the occurrence of membrane rupture with cytoplasm overflow (Fig. 6B). That membrane rupture probably was caused by the SDS, but it

was interesting to observe that the overflowed cytoplasm presented a blue dye, expected in the cytoplasm of dead oocytes (blue droplets in Fig. 6B).

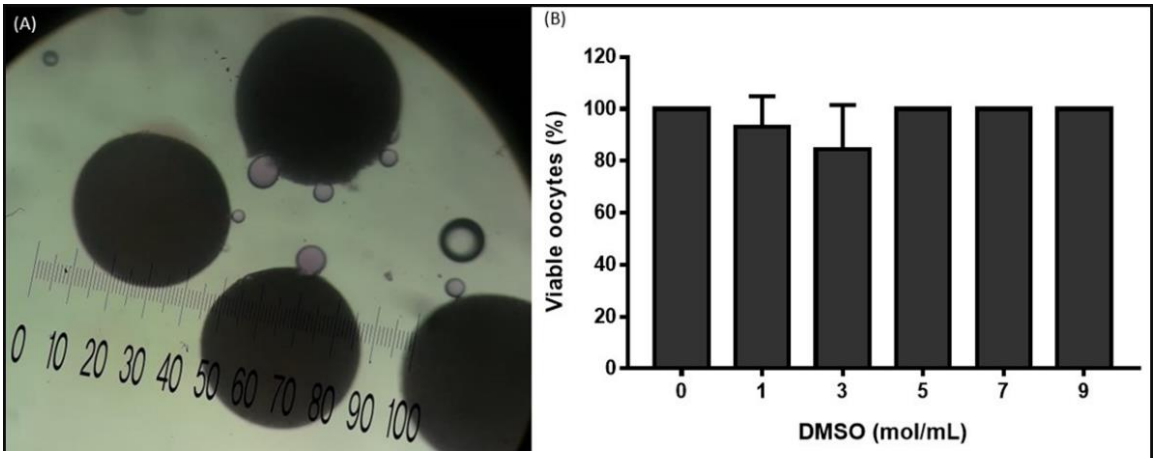



**Fig. 5** Trypan blue (TB) exclusion test for oocytes of the South Atlantic coral *M. harttii*. (A) Oocytes showing membrane rupture after exposure to TB solution supplemented with sodium dodecyl sulfate (SDS). Note the blue cytoplasm droplets. Me parece muito o cell blebbing observado em cells que entram em apoptose. Não é lise celular,pois a membrana plasmática esta presente nos blebs. (B) Quantification of viable oocytes using TB after exposure to dimethyl sulfoxide (DMSO) at different concentrations. Data were analyzed by two-way ANOVA and are expressed as mean ± SE.

Results from the trypan blue test associated with DMSO corroborated the initial test results, showing that it was not possible to distinguish viable oocytes from the dead ones in the sample. Data from the control group (DMSO-free) was the same (one-way ANOVA: $F$ (2,10) = 2.242, $P$ = 0.1569) of those oocytes exposed to DMSO solutions, even at the highest concentration (9 mol $mL^{-1}$), which is known to be lethal (Fig. 6C).

***MTT assays***

The MTT pre-test showed that coral oocytes were able to metabolize the MTT producing formazan crystals when MTT was solubilized in FSW. It was possible to observe that oocytes were purple after the incubation time (Fig. 7A). We could observe in the MTT serial dilution assay that the viability of coral oocytes increased as dilution increased (Fig. 7B). In fact, the higher the concentration of DMSO the greater the damage to coral oocytes, affecting directly

their viability. That negative effect could be observed in the MTT absorbance results (Fig.7B).

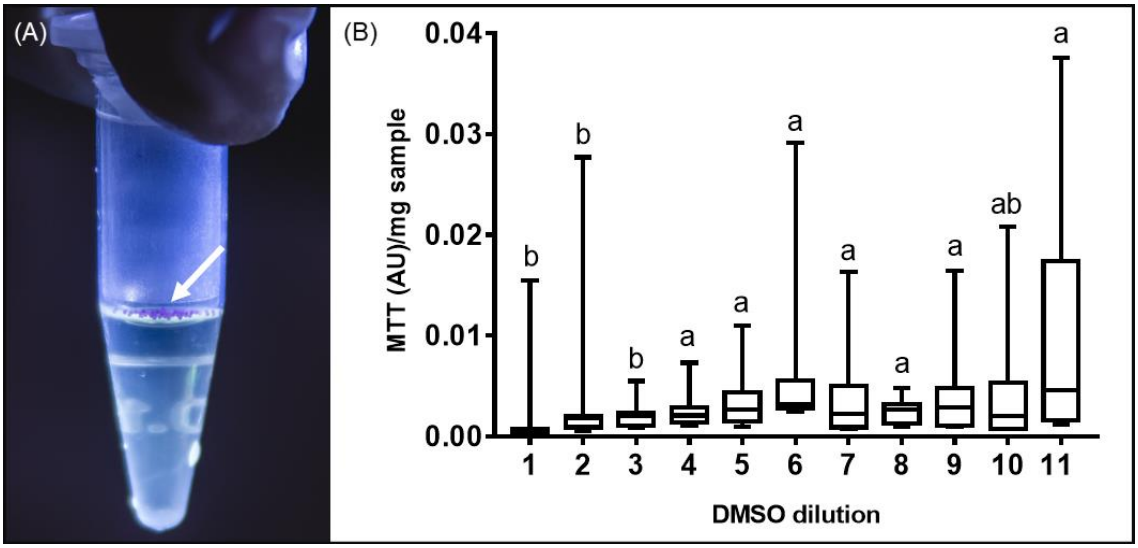



**Fig. 6** MTT assay to assess viability of coral oocytes. (A) Purple oocytes (withe arrow) with intracytoplasmic formazan crystals. (B) Serial dilution test to assess the cytotoxic effect of dimethyl sulfoxide (DMSO) on oocytes. Refer to Table 1 for serial dilute details. Significant difference by Kruskal-Wallis test (H11 = 34.38, *P* = 0.0002). Chi-Square test = 35.42857, *P* = 0.0001. Different letters indicate difference by the Dunn test.

After the MTT assay, the three first dilutions presented the lowest absorbance results and therefore proving the harmful effect of DMSO on the oocytes at such concentrations (Fig. 7B). However, from the fourth dilution (1.4 mol $mL^{-1}$) DMSO seems to be less detrimental to the maintenance of oocytes viability. There was no significant difference (Chi-Square test = 35.42857, *P* = 0.0001) from the fourth to the tenth dilution when compared to the control group (DMSO-free oocytes) (Fig. 7B). A $R^2$ = 0.7273 (Spearman rank correlation, $r^2$ = 0.72, N = 11, *P* = 0.0144) was found after analyzing the absorbance results, showing a positive linear correlation between the MTT concentration and DMSO dilution (Fig. 8).

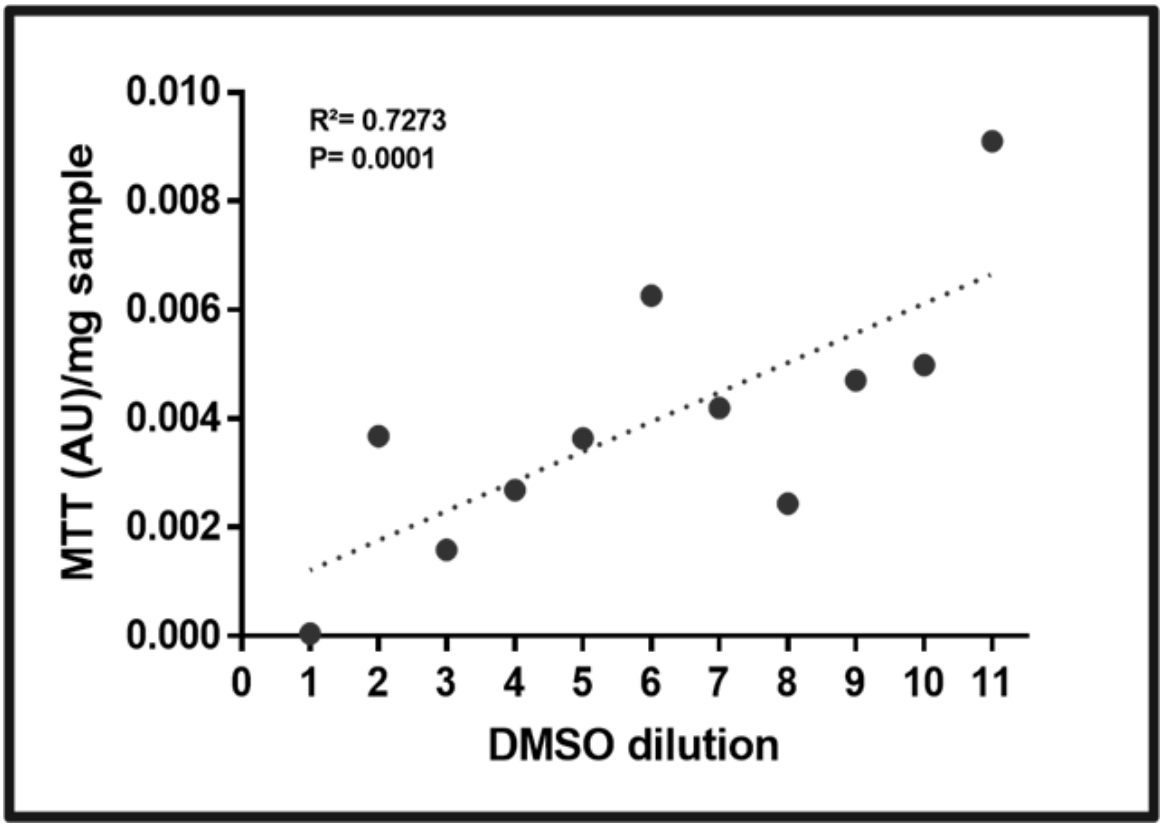



**Fig. 7** Simple linear regression between MTT concentration and dimethyl sulfoxide (DMSO) dilution in oocytes of the South Atlantic coral *M. harttii*. Refer to Table 1 for serial dilution details. Significant difference by Kruskal-Wallis test (H11 = 34.38, *P* = 0. .0002). Chi-Square test = 35.42857, *P* = 0.0001. Determination coefficient for transformed values.

**Discussion**

The global climate crisis places increasing pressure on coral reefs survival. In the current scenario, reproductive biotechnologies can be a helping hand in the fight against species extinction. In this context, the development of reliable methods to assess the quality of coral gametes are essential. The biological features of coral oocytes limiting efficient viability assessment techniques may explain the single test so far available in literature (Lin et al., 2011, 2012, 2014; Tsai et al., 2014).. In order to contribute to this research field, we selected the trypan blue test and the thiazolyl blue tetrazolium bromide (MTT) assay seeking to develop a reliable protocol to assess the viability of scleractinian coral oocyte as well as adding efforts in

reef monitoring programs and biotech conservation strategies.

A high lipid content has been found in the oocytes of scleractinian corals, which occupies approximately 55%-80% of the inner space of the oocytes (Arai et al. 1993; Lin et al. 2013; Tsai et al. 2016). The high content of lipids in oocytes of reef-building corals seems to be an important source of energy for growth of the future larvae (Battey and Patton 1984). Notably, the benefits of this high lipid content go further. The coral offspring uses this stored energy source mainly during the initial larval stages for dispersion. The positive buoyancy conferred by the esters wax during the first days of larval stage increases passive dispersion by current (Arai et al. 1993). However, despite playing these crucial roles in coral reproduction, the large amount of cytoplasmic lipids in the oocytes is a major obstacle, limiting efficient viability assessment techniques and making them very sensitive to low temperature conditions (Jafari et al. 2018). These obstacles are not restricted to aquatic broadcast spawners since some mammalian species like pig also have lipid-rich oocytes (Battey and Patton 1984; Amstislavsky et al. 2019; Yoshida and Asturiano 2020), and scientists have been trying to find ways to overcome such detrimental effects.

Given this point, the initial tests with trypan blue showed that it was not possible to use it to evaluate viability in coral oocytes. Previous knowledge from our research team (Godoy, L. *personal communication*) showed that *M. harttii* oocytes are dead 24 hours after spawning. Therefore, it was expected that all oocytes presented a blue cytoplasm after being exposed to TB. However, when SDS was added to the TB solution it promoted the rupture of the cytoplasmic membrane. It probably occurs because SDS is an ionic detergent, with cytolytic properties (Jafari et al. 2018).The SDS monomers are bound to proteins by predominantly hydrophobic interactions, causing unfolding of the tertiary structure, and at concentrations higher than critical micellar concentration (CMC, see (Bhuyan 2010)) the micelles nucleate on the hydrophobic patches of the protein chain driving it to expand, which is the hallmark of SDS action on proteins (Bhuyan 2010; Tsai et al. 2016). Thus, SDS even at low concentration promoted cell lysis. However, the membrane rupture enabled the visualization of the overflowed cytoplasm presenting a blue dye, which was in fact expected in the cytoplasm of dead oocytes. In brief, that fact demonstrated that the trypan blue was able to enter the cells, but probably because of the high lipid content of the oocytes, the observation of the blue color was not possible by optical microscopy. In other words, the dye crosses the membrane, but we could not see it, which makes the use of this test unfeasible for oocytes of *M. harttii*.

In an attempt to solve the problem of high lipid content impairing visualization of the

cytoplasm, we decide to work with a metabolic assay to evaluate the viability of coral oocytes. To do so, we standardize the MTT assay for determining mitochondrial activity in mature oocytes of *M. harttii*, and consequently assessing their viability. Equally, the positive linear correlation between MTT concentration and DMSO dilution allowed us to set a safe DMSO concentration (1.4 M, fourth dilution) to work with coral oocytes cryopreservation. This concentration is close to the ones that were also less toxic for larvae of the coral *Seriatopora caliendrum*, which showed an ATP content reported to be viable at 0.5-1 M of DMSO (Cirino et al., 2019) 2010). In the same way, DMSO was the second least toxic cryoprotectant to oocytes of the scleractinian coral *Echinopora spp.*, with viability of 72% after exposure to 1 M (Tsai et al. 2010).

The need to create safe and solid protocols to determine viability of coral gametes is urgent given the range of local and global drivers that have been imposed to them, and reproductive biotechnologies can in fact make a difference in the fight against species extinction. Majority of the broadcast-spawning corals reproduce only once a year during a narrow time window. As a result, they need techniques that are easy to carry out, since sample processing is a step that limits several protocols. In this study, we aimed to develop a reliable protocol to assess the viability of scleractinian coral oocytes using TB and MTT assay for the first time. Even though TB test showed as unfeasible due to the biological features of coral oocytes, we found the MTT assay to be a reliable protocol for determining physiological viability via the mitochondrial activity of mature *M. harttii* oocytes. These results provide a new technique to be applied for assessing coral oocyte viability, which can add efforts in conservation programs for coral reefs.

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thank Wanderson Valente for designing the graphical illustration of coral oocyte.

## Conflict of interests

The authors declare that there are no conflict of interests.

## Author contributions

NOC, AGG and LG designed the study; NOC, AGG, AGS and LG performed the experiments; AGS, LSL and DPSJ contributed to infrastructure/material/technical support; NOC and AGG analyzed the data and wrote the first draft of the manuscript; NOC and LG prepared the manuscript for submission. All authors have revised and agreed to the published version of the manuscript.

## Data availability

The data generated and analyzed during this study are available from the corresponding author upon request.

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## CAPÍTULO III*

# Primeiro relato da criopreservação do espermatozoide de um coral endêmico do Atlântico Sudoeste

*Artigo elaborado seguindo modelo de estrutura e formatação do periódico *Cryobiology*

**Primeiro relato da criopreservação do espermatozoide de um coral endêmico do Atlântico Sudoeste**

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**Resumo**

Os recifes de coral são de grande importância para manutenção da vida marinha, no entanto espécies de corais em todo o mundo estão enfrentando severas pressões ambientais. Dessa forma, o desenvolvimento de técnicas que visem sua conservação é fundamental, dentre elas, a criopreservação é uma alternativa. Esse trabalho objetivou desenvolver um protocolo de criopreservação para a espécie de coral brasileiro *Mussismilia harttii*, a fim de compreender a sensibilidade das células espermáticas aos agentes crioprotetores e ao frio. Determinamos o melhor crioprotetor, concentração e técnica de criopreservação. Os espermatozoides foram expostos aos crioprotetores dimetilsulfóxido e metanol em concentrações de 10, 15 e 20% e

criopreservados por vitrificação e congelamento lento controlado. Os espermatozoides foram criopreservados com sucesso quando DMSO 20% foi associado com congelamento lento. Essas descobertas devem aumentar/aprimorar os resultados dos os esforços na conservação de espécies de coral ameaçadas de extinção, proporcionando a construção de novas ferramentas para proteger a biodiversidade de corais brasileiros.

**Palavras-chave:** Coral Brasileiro; Espermatozoide; Crioprotetores; criopreservação; Banco genético;

## Introdução

Os recifes de coral são ecossistemas com uma grande biodiversidade, e verdadeiros berçários para mais de 25% de todas as espécies marinhas conhecidas [1]. Os recifes de coral definem-se por seus componentes biológicos (corais) e geológicos (recife) [2]. São conhecidas em torno de 900 espécies de corais escleractíneos de águas rasas [3,4] com estruturas rígidas, resistentes à ação mecânica das ondas e correntes marítimas. Sua estrutura é construída por carbonato de cálcio, pois têm a capacidade de extrair grandes quantidades de íons cálcio e íons carbonato da água do mar para sintetizarem seu esqueleto [5].

Os ecossistemas recifais são valiosos não apenas para uma infinidade de organismos marinhos que chamam os recifes de lar, mas também para os seres humanos, pois são berçários para peixes comercialmente importantes [6–8]. Junto com a fauna marinha associada que é rica e provê alimento e sustento para as comunidades humanas em seu entorno [2,9]. Os compostos bioativos extraídos dos corais são utilizados na elaboração de produtos da indústria farmacêutica [10]. Além disso, os recifes geram renda para as atividades associadas ao ecoturismo [9].

Infelizmente os corais de todo o mundo estão em risco, pois a maioria são espécies muito frágeis e suscetíveis aos danos causados pelo aquecimento a e acidificação do oceano decorrente do aumento das concentrações de dióxido de carbono, e que tem levado ao branqueamento dos corais [11–13]. O branqueamento é definido pela perda de dinoflagelados simbióticos (*Symbiodiniaceae*) que é sua principal fonte de alimento, induzindo o coral muitas vezes à morte [14]. Ao longo das últimas décadas, os eventos de branqueamento têm ocorrido com mais frequência e maior intensidade, provocando episódios graves de mortalidade em massa de corais [15] e contribuindo para uma redução drástica na cobertura de corais, na complexidade do habitat e na biodiversidade associada [15–19].

O estresse causado pelo branqueamento pode afetar o sucesso reprodutivo dos corais [20]. O aumento da temperatura e distúrbios antropogênicos induziram a perda da sincronia da desova em cinco das espécies de corais mais abundantes no Mar Vermelho, *Acropora eurystoma*, *Galaxea fascicularis*, *Platygyra lamellina*, *Dipsastraea favus* e *Acanthastrea echinata* [21], além de graves efeitos negativos que prejudicam a gametogênese, reduzindo o tamanho dos ovos e fecundidade [20,22–25].

Devido a crescente perda da cobertura de corais, os esforços para desenvolver estratégias para sua conservação devem ser imediatos, principalmente quando se trata de uma espécie endêmica e que possui tanta importância na construção de um recife, como a espécie *Mussismilia harttii* (coral couve-flor), foco do presente estudo e uma das principais construtoras de recifes do Brasil [26]. Uma das técnicas mais promissoras de conservação para os invertebrados marinhos é a criopreservação. Este processo envolve a preservação de gametas, embriões, larvas, tecidos ou fragmentos por congelamento rápido na presença de crioprotetores seguido de armazenamento em temperatura muito baixa para manter viabilidade por um longo período de tempo [27–29].

Durante a criopreservação, o espermatozoide enfrenta vários estresses que podem levar ao comprometimento funcional ou morte do gameta [30,31]. Abaixo das temperaturas subfisiológicas, as atividades metabólicas podem ser paralisadas por um período indefinido de tempo, preservando a viabilidade celular [32]. No entanto, durante esse processo, as células são suscetíveis a danos, como formação de cristais de gelo intracelular, lesões por congelamento e estresse osmótico [33,34]. Portanto, os produtos químicos referidos como agentes crioprotetores (CPAs) são essenciais para o tratamento celular antes da criopreservação. CPAs, taxas de resfriamento e temperaturas de resfriamentosão adaptados para cada espécie e tipo celular devido a diferentes tolerâncias à baixas temperaturas e toxicidade dos CPAs.

A maioria dos protocolos de criopreservação aplicados a corais estão em estágios iniciais, com os estudos atuais concentrando-se em células reprodutivas como oócitos, espermatozoides e embriões, para futura recuperação populacional e aumento de dados genômicos em bancos de germoplasma [29,35–38,38,39].

Desta forma, o objetivo desse trabalho foi desenvolver um protocolo seguro para criopreservação do espermatozoide de *M. harttii*, determinando as condições ideais de manipulação como: o melhor crioprotetor, sua concentração e a técnica ideal de criopreservação. Esse estudo traz um ineditismo na pesquisa brasileira, buscando aplicar ferramentas tecnológicas em prol da conservação do coral *M. harttii*. O estabelecimento dessas

tecnologias permitirá a criação do primeiro banco de gametas de corais do Atlântico Sul, permitindo a estocagem do espermatozoide por tempo indeterminado.

## Materiais e métodos

### Coleta de corais e licenças ambientais

As colônias de *Mussismilia harttii* (n = 50) foram coletadas por mergulhadores no Funil do Parque Marinho do Recife de Fora (16 ° 24'31 ″ S; 038 ° 58'39 ″ W - Bahia, Brasil) sob a licença SISBIO Nº 63368-1. A coleta foi realizada duas semanas antes do período previsto de desova da espécie [40,41]. As colônias com diâmetro de 11,8 ± 4,0 cm foram alocadas e aclimatadas em tanques de 1000 L com sistema de captação semiaberto conectado ao oceano, localizados na Base de Pesquisa do Projeto Coral Vivo, cuja equipe monitora episódios de desova de corais desde 2004. A temperatura da superfície da água do mar era 25,4 ± 0,84 ° C.

### Coleta dos pacotes e separação dos espermatozoides e oócitos

Os experimentos foram realizados ao longo de três noites durante o período de desova da espécie. Para evitar a contaminação dos gametas, cada colônia foi isolada em um recipiente plástico e monitorada durante a desova [42]. Os pacotes compostos por oócitos e espermatozoides foram coletados da superfície da água e imediatamente transferidos para tubos de 50 mL (dez pacotes por tubo) contendo 10 mL de água marinha filtrada (0,2 μm) (AMF). Ocorrendo a dissociação do pacote, os oócitos flutuantes deslocavam-se para o topo do tubo e logo foram separados usando uma pipeta Pasteur. Os espermatozoides que se depositavam ao fundo foram mantidos no tubo de 15 mL para a realização da centrifugação por 10 minutos em 7000 rpm à 22 °C (temperatura ambiente da sala). Após esta etapa, os espermatozoides foram transferidos para microtubos de 2 mL.

As amostras centrifugadas foram analisadas quanto a motilidade e concentração espermática seguindo a metodologia de Godoy et al. [42]. Para avaliação da integridade de membrana, os espermatozoides foram expostos ao corante supravital eosina que é indicador da integridade da membrana plasmática, corando apenas células com a membrana plasmática lesada, e é associado à nigrosina, que é responsável pelo contraste do fundo da lâmina, aplicando o método de Blom [43].

Após essas avaliações as amostras prosseguiram para o processo de criopreservação,

sendo realizado um pool espermático de três colônias, os quais foram distribuídos nos tratamentos. O desenho experimental constituiu de um esquema fatorial 2 x 3 x 2, sendo composto por dois agentes crioprotetores permeáveis à membrana (CPAs) metanol e dimetilsulfóxido (DMSO); em três concentrações (10, 15 e 20%); utilizando duas técnicas de criopreservação: o congelamento lento e a vitrificação. Os CPAs e suas concentrações foram selecionados com base em estudos anteriores com [27,38,44–46] e ensaios prévios do nosso grupo de pesquisa.

As diferentes concentrações de metanol (Honeywell Riedel-de-Haën™) e DMSO (Sigma-Aldrich) foram preparadas com água milli-q e sacarose 0,9 M e distribuídas em tubos criogênicos de polipropileno (2 mL) com roscado externo. Para o congelamento lento as amostras foram alocadas em um container Bicell®, mantido overnight em ultra freezer (-80°C) e então as amostras em criotubos foram transferidas e armazenadas em nitrogênio líquido. Para a vitrificação, as amostras foram equilibradas por 5 minutos sobre uma placa de gelo (4 °C) e em seguida mergulhadas em nitrogênio líquido. Para cada tratamento, foram utilizados 10 criotubos contendo 500 µL de solução crioprotetora com espermatozoides. O descongelamento foi realizado após 26 meses de armazenamento em nitrogênio líquido. As amostras foram descongeladas em banho-maria a 70°C [47] durante 50 segundos e em seguida foram adicionados 300 µL de AMF para diluir o crioprotetor, seguido de centrifugação por 10 minutos em 7000 rpm à 22 °C. O sobrenadante foi descartado, restando 100 µL de espermatozoides que foram diluídos em 900 µL de AMF (pH 8.2) em temperatura ambiente. A motilidade espermática foi avaliada logo após o descongelamento em (triplicata) de forma subjetiva usando um microscópio óptico (P502- Precision®) com objetiva de 40X e alíquotas de 40 µL.

**Ensaio de MTT**

O teste de Brometo de 3- (4,5-dimetiltiazolil-2) -2,5-difeniltetrazólio (MTT) foi usado para investigar o efeito das técnicas de criopreservação e concentração dos CPAs sobre a viabilidade das células espermáticas do coral *M. harttii*. Para iniciar as análises, as células foram mantidas em pellets à uma concentração $3.58 \times 10^7 \pm 2.43 \times 10^7$ /mL. Em seguida, 200 µL da solução de MTT (0,5 mg / mL concentração final) foram adicionados e as amostras foram incubadas por 2 h à 30°C no escuro. Após a incubação, retiramos cuidadosamente 200 µL da solução de MTT de cada tubo, deixando apenas o pellet espermático, e uma alíquota de 300 µL de dodecil sulfato de sódio (SDS) a 10% foi adicionada para solubilizar os cristais de formazan (Cruz et al. 2022, *under review*). Em seguida, a solução foi transferida para placas de cultura

de 96 poços e a absorbância medida em espectrofotômetro (Multiskan GO, Thermo Scientific) com leitor de microplacas a 570 nm.

**Citometria de fluxo e viabilidade celular**

O kit de viabilidade espermática LIVE / DEAD (molecular Probes, Leiden) foi utilizado seguindo as instruções do fabricante. Os pellets possuíam concentrações avaliadas entre 3, 5 e 7 x $10^7$ células/ mL. Os espermatozoides foram resuspensos em uma solução de AMF com volume final de 1 mL, corados com 5 µL de SYBR-14 (100 nM) e incubados no escuro a 37°C por 10 min. As amostras foram então coradas com 5 µL de iodeto de propídio (PI) (12 nM) e incubadas por mais 10 min antes da análise via citometria de fluxo.

A citometria de fluxo foi conduzida usando um BD Citômetro de fluxo Accuri™ C6 (Becton Dickinson, Franklin Lakes, NJ). Este instrumento foi equipado com um laser azul de estado sólido de 448 nm e um laser diodo vermelho de 640 nm. As emissões da sonda fluorescente SYBR-14 foram avaliadas com FL1 (filtro passa-banda 533/30) e a fluorescência do iodeto de propídio foi avaliada com FL3 (filtro passa-banda 585/40). A fluorescência vermelha profunda do MitoTracker foi medida usando um filtro passa-banda vermelho escuro FL4 675/25. As amostras de controle com coloração única foram usadas para definir quadrantes e compensações, bem como para determinar a fluorescência de fundo. Os arquivos foram exportados e analisados usando FloJo (Software FloJo V 10).

Durante o preparo dos controles para refinamento da avaliação, uma amostra do mesmo tratamento foi dividida em 5 subamostras de 50 µL. A primeira subamostra foi corada apenas com 0.25 µL de SYBr (0,02mM) e a segunda subamostra recebeu 1 µL (1.19mM) de iodeto de propídio. À terceira, que precisava conter apenas células mortas, adicionamos 1 µL de iodeto de propídio e a congelamos e aquecemos por 3 vezes. A quarta subamostra recebeu 0.25 µL SYBr + 1 µL de Iodeto de propídio e foi analisada usando a combinação bem validada, SYBR-14 / PI, enquanto a quinta subamostra era livre de corantes fluorescentes. Cada sonda recebeu um tempo de incubação em temperatura ambiente (22 °C), sendo o SYBr incubado por 4 min e o iodeto de propídio por 1 min.

**Análise estatística**

Todas as análises foram realizadas em triplicata. As análises estatísticas foram realizadas usando o teste de normalidade (Kolmogorov-Smirnov, Shapiro Wilk ou D'Agostino

& Pearson), e o teste de homogeneidade (Levene) foi aplicado para avaliar as suposições estatísticas. Os dados com distribuição normal foram analisados usando o teste ANOVA two-way seguido por Teste de Tukey para comparação post hoc de médias que apresentaram diferença significativa entre as concentrações na técnica de criopreservação. Para averiguar a diferença entre as técnicas de criopreservação na mesma concentração de crioprotetor, os dados foram analisados por meio de ANOVA de medidas repetidas de duas vias, seguido do teste de Bonferroni para comparação post hoc de médias. Os dados que não apresentaram uma distribuição normal foram analisados usando Kruskal-Wallis seguido pelo teste de comparações múltiplas de Dunn, e considerados significativamente diferentes quando $P < 0,05$. Os dados foram plotados com médias brutas ± desvio padrão. As análises foram realizadas usando o software GraphPad Prism 7.0.

**Resultados**

O sêmen fresco de *M. harttii* apresentou excelente qualidade para o congelamento. A concentração espermática foi de 6,59 ± 3,27 x $10^7$ espermatozoides/mL. A motilidade espermática estava em 97 ± 5,06% e a integridade da membrana (avaliada por coloração vital) em 98 ± 3,42%

**Análise subjetiva da motilidade**

As taxas de motilidade do sêmen criopreservado com 10 e 20% de metanol (4,67 ± 0,5% e 11,67 ± 2,8%, respectivamente) diferiram significativamente ($P < 0,0001$), porém essa diferença não foi observada entre as técnicas de congelamento ($F (1,12) = 0,01961$; $P = 0,8910$)). No entanto, a motilidade espermática foi significativamente afetada pelos CPAs ($F (1,2) = 49,35$; $P < 0,001$)). Foi detectada diferença significativa entre as interações das três concentrações de CPAs ($F (2,2) = 570,4$; $P < 0,0001$)), sendo que a combinação de DMSO 20% com congelamento lento resultou na maior taxa de motilidade (29,67 ± 0,58%) observada entre todos os tratamentos (Tabela 1). As amostras submetidas ao metanol 15% e ao DMSO 10 e 15%, para ambas as técnicas de criopreservação, não apresentaram espermatozoides com motilidade progressiva, apenas vibração com movimentos circulares foi observado (Tabela 1).

**Tabela 1.** Motilidade subjetiva dos espermatozoides criopreservados do coral *M. harttii.*

| | METANOL | | DMSO | |
|---|---|---|---|---|
| | Vitrificação | Congelamento lento | Vitrificação | Congelamento lento |
| *10%* | 3,33 ± 1,53%$^{b}$ | 5 ± 0 %$^{b}$ | * | * |
| *15%* | * | * | * | * |
| *20%* | 11,67 ± 2,89%$^{a}$ | 11,67 ± 2,88%$^{a}$ | 23,33 ± 2,88%$^{a}$ | 29,67 ± 0,577%$^{aA}$ |

Letras minúsculas distintas representam diferença significativa entre as concentrações na técnica de criopreservação e letras maiúsculas distintas representam diferença entre as técnicas de criopreservação na mesma concentração de crioprotetor. ($F(2,2) = 570,4$; $P < 0,0001$)). * Indica células sem motilidade, porém apresentando o comportamento vibratório das células espermáticas.

**Citometria de fluxo**

Quando o metanol foi utilizado como crioprotetor, não houve efeito significativo ($P > 0,05$) da técnica de criopreservação na viabilidade espermática (Fig. 1). Da mesma forma, a viabilidade espermática não sofreu alteração ($P > 0,05$) das diferentes concentrações de metanol durante a vitrificação, não ultrapassando 40%. No entanto, quando submetidos ao congelamento lento controlado, a viabilidade dos espermatozoides em 10% de metanol (33,99 ± 1,70%) foi superior àqueles congelados na concentração 20%.

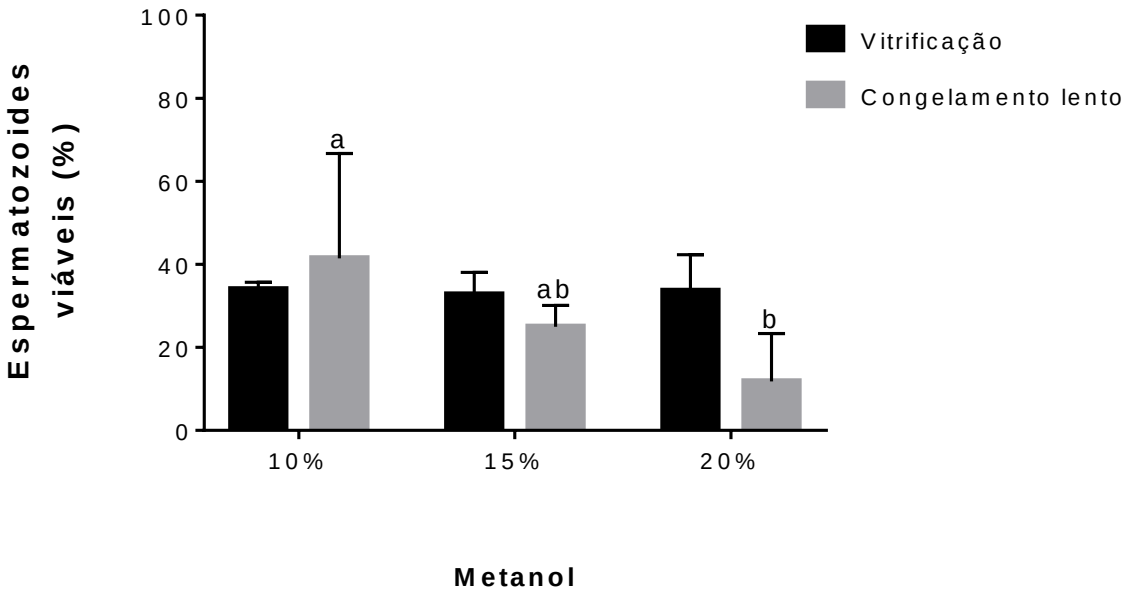


**Fig. 1.** Análise da viabilidade dos espermatozoides de *M. harttii* medida pela técnica de citometria de fluxo após criopreservação com metanol em três distintas concentrações. Letras minúsculas distintas representam diferença entre as concentrações na técnica de criopreservação (F (2 12) = 2,126, P = 0.1620)).

A combinação entre DMSO 20% e a técnica de congelamento lento controlado foi a que proporcionou o melhor resultado, com 83,57 ± 2,22% de espermatozoides (Fig. 2). As viabilidades nas concentrações 10 e 15% de DMSO não diferiram entre si (34,46 ± 6,36% e 42,13 ± 2,50%, respectivamente) no congelamento lento controlado (Fig. 2). Foi possível constatar que a técnica de congelamento lento promoveu taxas de viabilidade espermática significativamente superiores àquelas obtidas pela vitrificação em todas as concentrações de DMSO.

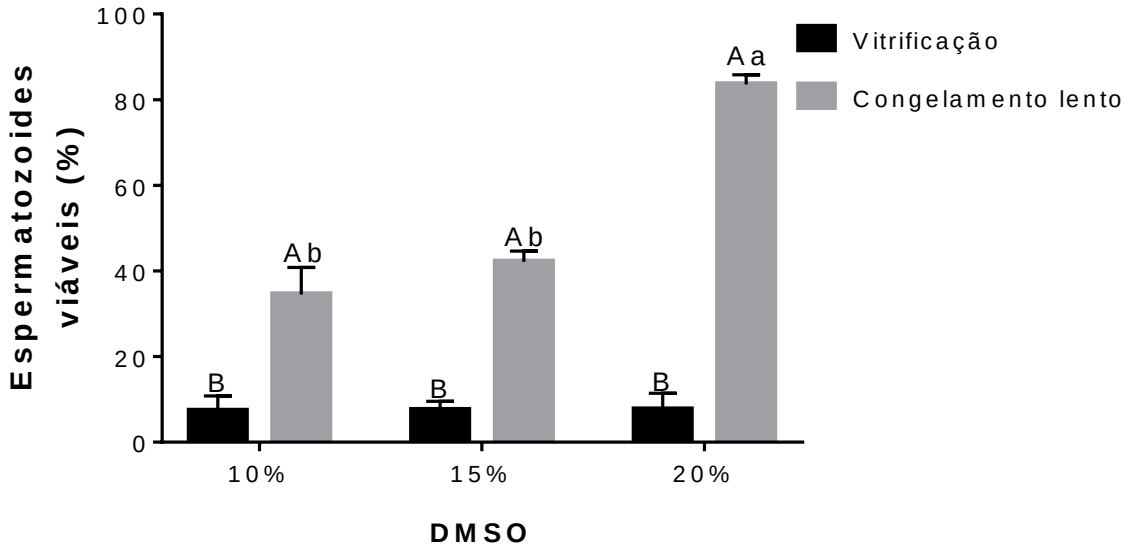


**Fig. 2.** Análise da viabilidade dos espermatozoides de *M. harttii* medida pela técnica de citometria de fluxo após criopreservação usando DMSO em três distintas concentrações. Letras minúsculas distintas representam diferença significativa entre as concentrações na técnica de criopreservação e letras maiúsculas distintas representam diferença entre as técnicas de criopreservação na mesma concentração de crioprotetor. $F(2,12) = 75{,}09$; $P < 0.0001$.

A Fig. 3 apresenta uma visão global de todos os tratamentos e suas interações. De forma geral, os resultados mais baixos de viabilidade espermática foram observados em todas as concentrações do DMSO quando a técnica de vitrificação foi empregada. Por outro lado, quando empregada a técnica de congelamento lento, a concentração de 20% foi significativamente a melhor frente aos demais cenários comparativos.

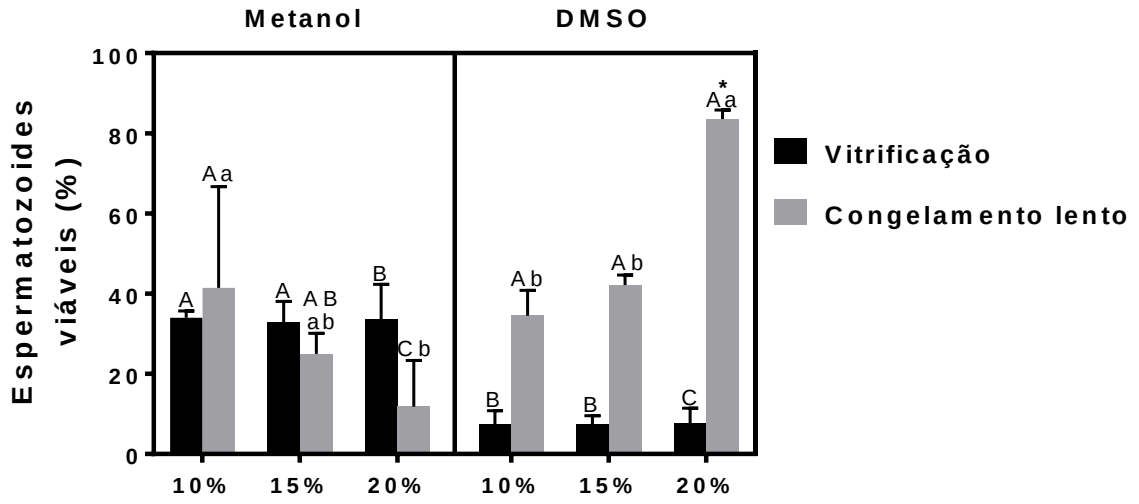


**Fig. 3.** Análise da viabilidade dos espermatozoides de *M. harttii* medida pela técnica de citometria de fluxo após criopreservação usando DMSO e metanol em três diferentes concentrações por meio de vitrificação e congelamento lento. Letras maiúsculas distintas apresentam diferença nos CPAs e técnicas de criopreservação para cada concentração testada. Letras minúsculas distintas representam diferença entre as concentrações dos CPAs na técnica de criopreservação (F (2,2) =14,69; P<0.0001)). Asterisco (*) indica quando há diferença entre vitrificação e congelamento lento, dentro dos mesmos CPAs e da mesma concentração de CPA.

**Ensaio MTT**

Podemos observar no ensaio MTT que a viabilidade dos espermatozoides do coral foi significativamente maior que todos os demais tratamentos quando metanol 15% foi utilizado (F (6,24) = 5,574, P = 0,0010) (Fig. 4). É possível observar que os resultados de maior absorbância foram encontrados na técnica de congelamento lento, independente dos CPAs. Quando utilizado o crioprotetor DMSO os resultados de absorbância foram significativamente maiores na concentração 15%. De fato, a técnica de vitrificação afetou diretamente a viabilidade mitocondrial dos espermatozoides e esse efeito negativo pôde ser observado nos resultados de absorbância do MTT (Fig.4).

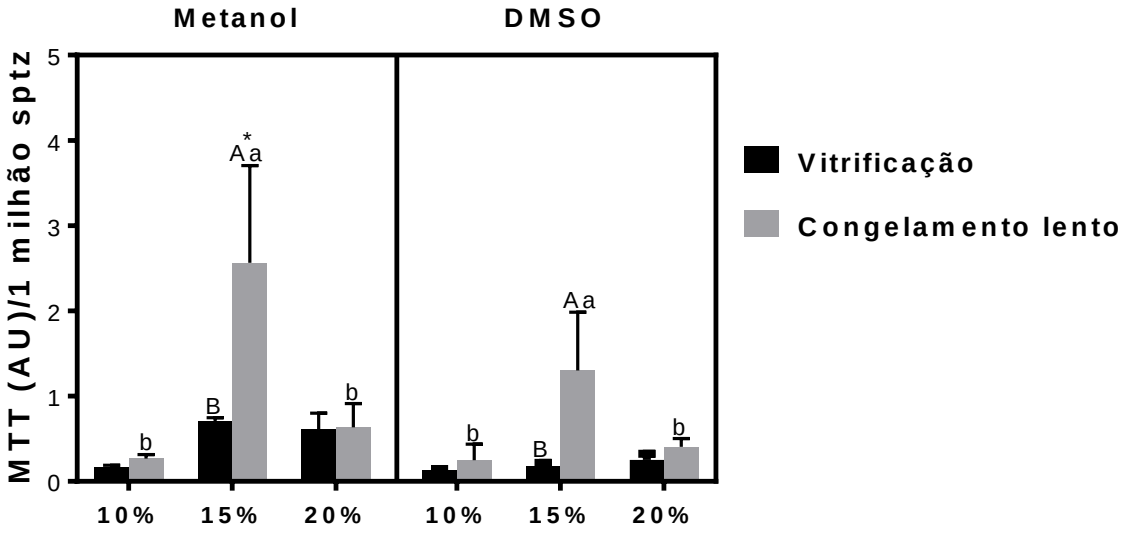


**Fig. 4.** Viabilidade das células espermáticas de M. harttii após criopreservação usando DMSO e metanol em três diferentes concentrações por meio de vitrificação e congelamento lento. O eixo Y indica as unidades de absorbância (AU) a 570 nm no ensaio MTT. A análise de ANOVA de três vias foi realizada para comparar todos os grupos experimentais, levando em consideração os efeitos do metanol e DMSO, a técnica (vitrificação e congelamento lento) e as concentrações dos crioprotetores (CPAs) e suas interações. Quando observado efeito significativo, utilizou-se teste de Tukey e de Bonferroni. Letras maiúsculas distintas indicam diferença entre as técnicas, dentro do mesmo CPA e concentração. Letras minúsculas distintas indicam diferença entre as concentrações dentro do mesmo CPA e da mesma técnica de criopreservação. Asterisco (*) indica quando há diferença entre o metanol e DMSO, dentro da mesma técnica e da mesma concentração de CPA.

**Tabela 2**. Análise da interação entre criopreservação usando DMSO e metanol em três diferentes concentrações por meio das técnicas de vitrificação e congelamento lento.

| Three-way ANOVA (Valor de p<0,05) | Valores de F (DFn, DFd) | Valores de P |
|---|---|---|
| Efeito da concentração | F (2,2) = 19,14 | *P* < 0,0001 |
| Efeito do crioprotetor (CPA) | F (1,2) = 9,142 | *P* = 0,0059 |
| Efeito da técnica de Criopreservação | F (1,2) = 17,77 | *P* = 0,0003 |
| Interação da concentração x CPA | F (2,2) = 3,661 | *P* = 0,0410 |
| Interação da concentração x técnica | F (2,2) = 12,03 | *P* = 0,0002 |
| Interação do CPA x técnica | F (1,2) = 0,05234 | *P* = 0,4764 |
| Interação concentração x CPA x Técnica | F (2,2) = 1,034 | *P* = 0,3709 |

**Discussão**

Em todo o mundo, os recifes de coral estão sendo degradados. O aumento na concentração de gases de efeito estufa está aquecendo e acidificando o oceano, tornando os corais cada vez mais suscetíveis ao estresse, branqueamento, doenças emergentes e assim afetando negativamente seus serviços ecossistêmicos [12,13,48,49]. A criopreservação pode atuar como ferramenta na preservação da biodiversidade e diversidade genética dos recifes [50], podendo conceder segurança no caso de uma catástrofe que afete tanto populações selvagens quanto a *ex situ*. Até o momento houve sucesso na criopreservação de espermatozoides de cerca de 30 espécies de espécies de corais [51]. O conhecimento básico e inédito que tem sido gerado pelo nosso grupo de pesquisa acerca da biologia e fisiologia dos gametas de corais brasileiros foi fundamental para alcançarmos o sucesso descrito nesse estudo. Aqui, nós mostramos pela primeira vez que os espermatozoides do coral endêmico *M. harttii* podem sobreviver à criopreservação. Essa conquista pode representar o primeiro e crucial passo para a conservação da diversidade genética dos corais do brasil.

Nesse estudo as amostras permaneceram estocadas em nitrogênio líquido por 26 meses.

Contudo, a motilidade subjetiva do material pós-criopreservação mostrou-se abaixo de 30%, com uma redução de 68 pontos percentuais em relação às amostras frescas. Uma queda na taxa de motilidade é esperada após o processo de criopreservação. O sêmen criopreservado do coral *D. labyrinthiformis* permaneceu viável para uso em fertilização *in vitro* por pelo menos 13 meses após a estocagem, embora sua motilidade tenha reduzido em média 24% na comparação com sêmen fresco [45]. A taxa de espermatozoides móveis tem sido o critério amplamente utilizado para avaliar a qualidade seminal após a criopreservação na maioria das espécies aquáticas [32,44]. No entanto, tal avaliação quando aplicada aos espermatozoides de corais pode não ser a melhor ferramenta, considerando a grande variação na qualidade dos gametas a cada noite de desova, a limitada janela de tempo para a reprodução sexual a cada ano e que algumas espécies de coral apresentam espermatozoides com padrões de movimento e deslocamento muito distintos [52,53]. Diante dos resultados de queda na motilidade, nós sugerimos utilizar uma elevada concentração ($6,59 \pm 3,27 \times 10^8$ /mL) de espermatozoides para a fertilização. É importante destacar que a fertilização acontecerá *in vitro*, ou seja, em um ambiente totalmente controlado, onde as chances de sucesso são significativamente aumentadas quando comparadas ao oceano. Além disso, cada oócito precisa de apenas um espermatozoide para que a fecundação. A baixa taxa de motilidade subjetiva dos espermatozoides criopreservados (29,67 ± 0,58%) mesmo no melhor tratamento (DMSO 20% + congelamento lento), mostra que tanto o CPA quanto a técnica de criopreservação podem impactar negativamente a motilidade devido à toxicidade e as crioinjúrias. O sêmen dos corais *A. tenuis* e *A. millepora* após criopreservação com DMSO (10%) apresentou taxa de motilidade de 41%, e as larvas produzidas a partir dos espermatozoides criopreservados tiveram a mesma taxa de assentamento que àquelas oriundas de sêmen fresco [37]. De forma semelhante, o sêmen do coral *A. digitifera* com motilidade abaixo de 40% pós-descongelamento foi capaz de promover taxas de fertilização estatisticamente iguais ao controle com espermatozoides frescos [46]. A menor taxa de motilidade pode também ser compensada adicionando mais espermatozoides criopreservados aos oócitos para aumentar a concentração [37,45,52]. O estudo de Zuchowicz et al. [52] sugere um aumento na concentração da dose inseminante com $3,0 \times 10^3$ células/oócito para conseguir a fertilização *in vitro* da espécie *Lobactis scutaria*, que apresentou motilidade espermática de 6% pós-descongelamento.

Fica claro que baixas taxas de motilidade podem promover altas taxas de fertilização *in vitro*, e que isso se torna particularmente importante quando estamos lidando como o material genético de espécies ameaçadas de extinção. No entanto, nós devemos nos atentar para outras

características da célula espermática. No presente estudo, nós utilizamos pela primeira vez a técnica de citometria de fluxo e a coloração combinada de Sybr-14 com PI para avaliar a viabilidade dos espermatozoides de um coral escleractíneo brasileiro. O SYBR-14 e o PI são corantes específicos de DNA, marcando as células vivas de verde (SYBR-14) e as células mortas de vermelho (PI), respectivamente [54]. A citometria de fluxo tem sido aplicada com sucesso na avaliação da viabilidade espermática em invertebrados marinhos como a ascídia *Ciona intestinalis*, o ouriço-do-mar *Paracentrotus lividus*, *Evechinus chloraticus*, mexilhões *Mytilus galloprovincialis* e *Perna canaliculus* e abalone *Huliotis íris* [55,56].

Apesar de o DMSO ser considerado tóxico para várias células animais, a concentração de 20% combinada com o CPA não permeável sacarose (0,9 M) proporcionou proteção para a estrutura do espermatozoide de *M harttii*. O DMSO 2M também ofereceu proteção para os espermatozoides de *A. humilis* de crioinjúrias, garantindo sucesso de fertilização e assegurando melhor proteção que os CPAs propilenoglicol e metanol [29]. O DMSO também foi eficaz na criopreservação do sêmen de *A. humilis* e *A. palmata* no estudo de Hagedorn et al.[53]. Independentemente da toxicidade dos CPAs, concentração e o período de equilíbrio são espécie-dependentes e as membranas celulares podem variar em estrutura e composição e não serem compatíveis com o mesmo CPA ou concentração [29].

Exemplificando, de acordo com o presente estudo o ensaio MTT apontou a combinação de metanol 15% e congelamento lento como os de melhor resultado em termos de atividade e viabilidade mitocondrial. O metanol penetra na célula a uma velocidade comparável à taxa de transporte da água, e portanto, a incubação das células com metanol não leva à tensões osmóticas pronunciadas [57]. O ensaio MTT é um teste colorimétrico originalmente descrito por Mosmann [58] e tem sido utilizado para medir a atividade metabólica em células vivas. O princípio subjacente deste teste é baseado na redução do sal de tetrazólio amarelo transformando em cristais de formazan roxo não solúveis em água através da introdução de células metabolicamente ativas [59], confirmando que a técnica de congelamento lento foi a que melhor assegurou a viabilidade mitocondrial das células espermáticas do coral *M. harttii*. As mitocôndrias são pequenas organelas responsáveis pela funcionalidade dos espermatozoides, suprindo energia através do metabolismo aeróbico, em um processo nomeado fosforilação oxidativa [60]. Este processo faz com que o espermatozoide adquira motilidade e fique apto para se deslocar ao encontro do oócito.

Michelmann [61] sugere que em humanos, a gravidez pode ser alcançada por injeção intracitoplasmática de espermatozoides mesmo que a célula espermática não possua motilidade.

No entanto, tais células precisam apresentar certas características de qualidade como estrutura inteiriça do DNA, não podendo apresentar reação acrossômica, nem espermatozoides imaturos ou com anomalias. Esta observação é algo importante, pois em situações extremas, como em espécies raras ou ameaçadas de extinção, todo o material genético é importante para ser preservado. A exemplo os mamíferos ameaçados das espécies *Damaliscus pygargus phillipsi*, *Connochaetes taurinus* e *Syncerus caffer*, cuja motilidade progressiva das três espécies (22,15; 13,83 e 8,4%; respectivamente) obtiveram sucesso na fertilização [62].

Interessante observar que no tratamento com metanol 15% as células espermáticas não apresentavam motilidade, mas um tipo de movimento vibratório, indicando que as células estavam vivas. No entanto, quando analisadas por citometria de fluxo, essas amostras apresentaram viabilidade de 25%. Nas condições experimentais aqui executadas, o congelamento lento mostrou-se melhor que a vitrificação para os espermatozoides de *M. harttii*. Esse resultado corrobora os achados para as espécies *A. digitifera*, *A. palmata* e *Fungia scutaria*, cujos estudos alcançaram os melhores resultados com o emprego da técnica de congelamento lento controlado [46,53].

Os desafios enfrentados neste trabalho vão desde a coleta do material, que é dificultada pela curta janela reprodutiva da espécie (uma vez ao ano) e a baixa quantidade de amostras para se trabalhar. Sob essas condições, o refinamento dos protocolos foi longo e árduo, como muito bem relatado por Zuchowicz et al. [52], que levaram anos para desenvolver e definir protocolos para avaliação dos espermatozoides de coral no sistema CASA. Durante os testes nos deparamos com a dificuldade em definir um protocolo eficiente de descongelamento das amostras contidas em criotubos, e finalmente determinando a temperatura de 70°C por 50 s, como a encontrada por Gwo et al. [47]. Aplicamos movimentos suaves até o total descongelamento, conforme relatado por Grosso-Becerra et al. [63] para ajudar no descongelamento dentro do tempo proposto. O protocolo de criopreservação desenvolvido aqui para o coral *M. harttii* também servirá de base fundamental para o desenvolvimento de novos protocolos para outras espécies de corais brasileiros. O protocolo usado ao longo deste estudo provou ser eficiente mesmo em campo, onde o acesso a estrutura laboratorial é escasso.

A pressão das mudanças climáticas, a perda de habitat e a exploração excessiva dos recursos naturais estão resultando em riscos cada vez maiores de extinção. Biotecnologias como técnicas avançadas de criopreservação, fertilização in vitro (FIV), injeção intracitoplasmática de espermatozoides (ICSI) e transferência nuclear (TN) para manter a heterogeneidade genética em populações *ex situ* [64], devem ser aplicadas também para conservação de corais. Como o

exemplo bem-sucedido de criopreservação de larvas de um coral do recife do Indo-Pacífico, o *Seriatopora caliendrum*, que contém dinoflagelados simbióticos (família *Symbiodinaceae* ) que são vitais para sua sobrevivência, sugerindo que a abordagem de vitrificação e aquecimento a laser ultrarrápido podem ser aplicáveis a outras espécies marinhas ameaçadas [36].

Sendo necessários mais estudos para continuar desenvolvendo protocolos de criopreservação de gametas de corais, de forma segura e sólida, sua urgência é dada a gama de fatores locais e globais que lhes foram impostos, e as biotecnologias reprodutivas podem de fato fazer a diferença na luta contra a extinção dessas espécies. O uso de técnicas de criopreservação de sêmen, como avaliados neste estudo, podem ajudar a preservar a diversidade genética, evitar o desaparecimento de corais e criar oportunidades para diversificar as populações. Além disso, permite avançar na investigação sobre biologia do desenvolvimento, genética, sistemática e biologia molecular de corais.

Programas de melhoramento para garantir a viabilidade genética e os Bancos de Recursos Biológicos podem contribuir potencialmente para esse desafio, fornecendo uma fonte de genes que podem ser usados para combater os efeitos de pressões de seleção externa, deriva genética e depressão endogâmica em populações pequenas ou fragmentadas. Esses bancos geralmente contêm materiais biológicos criopreservados; como espermatozoides, embriões e culturas de células como recursos genéticos para pesquisas [64,65]. Este trabalho marca a história da criobiologia aplicada à conservação dos corais brasileiros, deixando como fruto o primeiro banco de gametas de coral do atlântico sul.

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# CAPÍTULO V

## 4. CONSIDERAÇÕES FINAIS

Os protocolos de criopreservação de coral começaram a serem desenvolvidos há 16 anos, aplicados em corais australianos e da região do caribe. Consequentemente, os estudos em criobiologia de corais estão em fase inicial, com metodologias que ainda precisam ser refinadas e mais estudadas. A necessidade em produzir protocolos seguros e sólidos para determinar a viabilidade dos gametas de coral é urgente, pois cada espécie de coral, seus tipos de materiais biológicos e os efeitos das mudanças ambientais influenciam no processo. As biotecnologias reprodutivas podem de fato fazer a diferença na luta contra a extinção dessas espécies.

Dessa forma, os desafios em desenvolver tais protocolos começam no momento da coleta do material que é dificultada pela curta janela reprodutiva da espécie, pois a maioria dos corais de desova de transmissão se reproduzem apenas uma vez por ano. A pressão das mudanças climáticas, a perda de habitat e a exploração excessiva dos recursos naturais estão resultando em riscos cada vez maiores, prejudicando a produção de gametas, gerando células de baixa qualidade.

Necessitam-se de técnicas de fácil execução, pois o processamento de amostras *in situ* ou *ex situ* é uma etapa que limita diversos protocolos. Portanto, desenvolvemos o primeiro método confiável para avaliar a viabilidade de oócitos de coral e aplicamos técnicas de criopreservação para sêmen de *M. harttii*. O nosso protocolo provou ser eficiente mesmo em campo, onde o acesso a estrutura laboratorial é escasso e o uso dessas técnicas pode ajudar a preservar a diversidade genética, evitar o desaparecimento de corais e criar oportunidades para diversificar as populações. Agregando esforços em programas de conservação de recifes de corais brasileiros. Além disso, permite avançar ainda mais nas investigações sobre a criobiologia.

O protocolo de criopreservação desenvolvido no presente trabalho para o coral *M. harttii* também servirá de base fundamental para o desenvolvimento de novos protocolos para outras espécies de corais brasileiros. No entanto, mais estudos são necessários para continuar desenvolvendo protocolos de criopreservação de gametas de corais, para contribuir em programas de melhoramento e garantir a viabilidade genética. E assim, criando Bancos de Recursos Biológicos que contribuíram

potencialmente para esse desafio, fornecendo uma fonte de matérias biológicos que podem ser usados para combater os efeitos de pressões de seleção externa, deriva genética e depressão endogâmica em populações pequenas ou fragmentadas. Esta tese marca a história da criobiologia aplicada à conservação dos corais brasileiros, deixando como fruto o primeiro banco de gametas de coral do atlântico sul.

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# APÊNDICES

**Apêndice 1 –** Normas do periódico Under reviews Marine Biology

Marine Biology International Journal on Life in Oceans and Coastal Waters

## Submission guidelines

## Instructions for Authors

General Information

Please note that the journal does not offer pre-evaluation. Therefore please directly submit your manuscript to EditorialManager at the link below. The Editors will then contact you.

When preparing your manuscript, it is important that you consider the points listed below very carefully. We also recommend using a copy of a recent article as an additional guide. For questions please contact the Editors at marinebiology@geomar.de. Manuscripts that do not fit our standard cannot be considered for publication.

For manuscripts on ocean acidification please see also the ‘Guidelines for reporting ocean acidification data’ at the end of this document.

Only articles of interest to readers of Marine Biology, presenting novel and useful information for the scientific community, and contributing to scientific progress in a particular field can be considered for publication. The potential impact and importance of the work should be described in the manuscript to ensure that the article will be read and cited.

Submitted manuscripts are first checked for English language, ethical issues, and

plagiarism. Manuscripts exhibiting problems cannot be considered for publication and may be irrevocably rejected.

The submission template contains questions about the specific contribution of the manuscript to the field. The replies to these questions are of utmost importance, because the initial decision as to whether a manuscript will be sent out for review or will be rejected without review mainly depends upon the title, the abstract, and the replies to these questions. Replies that are vague will be taken as an indication that the authors are unable to condense information on these points, or that they have not considered the relationship of their work to progress in the field.

**Important Notes**

- Formatting as per journal instructions is essential, otherwise the manuscript will be returned without review. Carefully prepared manuscripts which are conform to the regulations of the journal have a higher chance to be considered for publication.
- Authors are advised to use blank Word files when writing their cover letter, as formatting discrepancies may be introduced when special headings/symbols (e.g. of universities) are included on the page.
- **Language**

  Manuscripts should conform to standard rules of English grammar and style. Either British or American spelling and punctuation may be used, but must be consistent throughout the article. Submitted manuscripts will first be checked for language, presentation and style. Manuscripts which are substandard in these respects will be returned without review. Scientists for whom English is a foreign language are strongly recommended to have their manuscript read by a native English-speaking colleague or edited by a professional editing service. Information about editing services is available on the journal web page. It is the collective responsibility of the authors to submit a linguistically correct manuscript.

- **Cover letter**

When submitting a manuscript, a cover letter addressed to the Editor in Chief should be uploaded under the item ‘Cover Letter’. It will be visible for the editors, but it is not included into the manuscript file (PDF) which is made for the reviewers. The cover letter should contain a statement, that all authors have agreed to the submitted version of the manuscript, that the manuscript or parts of it have not been published elsewhere, and that the paper is not under consideration elsewhere. If the research was done in a country where none of the authors is resident, evidence that official permission to conduct the research has been given must be provided.

For questions please contact the Managing Editor at marinebiology@geomar.de

Types of Papers

- Original papers: These are the most important components of Marine Biology. They report on original research in all fields of marine biology and conform to the accepted standards of scientific quality. Interim reports and papers with inconclusive results will usually not be published. In the latter case, exceptions can be made if the inconclusiveness is a robust and important result with relation to widely debated theory.
- Reviews, concepts, and syntheses: Review papers are invited on any topic related to the focus of Marine Biology. These reviews may either summarize recently terminated research areas of wide importance, provide an up-to-date account of the present status of active research areas, or set the perspective for future research. They are not meant to be mere literature surveys. These reviews are meant to be in-depth and comprehensive efforts, and authors should have demonstrated expertise in the topic area. Very high standards of assessment with respect to quality and importance are applied to these reviews.
- Methods: Method articles may describe methods developed by the authors or a compendium of methods from the “grey” literature, if these methods deserve the attention of a wider community. Application examples demonstrating the usefulness of the method are welcome.
- Rapid communications: Rapid communications are reports of important new research results or discoveries which deserve to be published more rapidly than usual articles. The reasons for the special urgency have to be given in the cover

letter. The articles have to conform to the highest priority criteria in respect to originality and importance. They can only be accepted, if no major revision of the original manuscript is needed. Rejected rapid communications cannot be submitted as regular manuscripts.

- Short notesShort notes are brief papers that contain significant observations that do not warrant full-length papers or important experimental results that are not sufficiently elaborated or developed as to justify an original paper. They may also present opinions or novel interpretation of existing ideas. Short Notes must be of considerable potential significance for a wide readership, preliminary work will not be considered. Short notes could combine the results and discussion.
- Comments and replies: Comments relate to articles in Marine Biology not older than one year. Their intention has either to be a substantial critique of the original article or the clarification of a major misunderstanding that could have been caused by the original article. The authors of the criticized articles have the right to write a reply. Comment and reply will be published together. The comment will be reviewed externally, while the reply will only be edited for clarity.
- Highlight articles:Outstanding papers of all categories may be selected as highlight articles. These articles must be exceptional in respect to the originality of the study, the importance to a diverse group of marine biologists and to the robustness of the methods. The specific importance of the article is emphasized by an accompanying comment of the responsible Editor. Highlight articles are promoted in social media.

Manuscript Submission

**Manuscript Submission**

Submission of a manuscript implies: that the work described has not been published before; that it is not under consideration for publication anywhere else; that its publication has been approved by all co-authors, if any, as well as by the responsible authorities – tacitly or explicitly – at the institute where the work has been carried out. The publisher will not be held legally responsible should there be any claims for compensation.

**Permissions**

Authors wishing to include figures, tables, or text passages that have already been published elsewhere are required to obtain permission from the copyright owner(s) for both the print and online format and to include evidence that such permission has been granted when submitting their papers. Any material received without such evidence will be assumed to originate from the authors.

**Online Submission**

Please follow the hyperlink “Submit manuscript” and upload all of your manuscript files following the instructions given on the screen.

**Source Files**

Please ensure you provide all relevant editable source files at every submission and revision. Failing to submit a complete set of editable source files will result in your article not being considered for review. For your manuscript text please always submit in common word processing formats such as .docx or LaTeX.

Title Page

**Title Page**

Please make sure your title page contains the following information.

**Title**

The title should be concise and informative.

**Author information**

- The name(s) of the author(s)
- The affiliation(s) of the author(s), i.e. institution, (department), city, (state), country
- A clear indication and an active e-mail address of the corresponding author
- If available, the 16-digit ORCID of the author(s)

If address information is provided with the affiliation(s) it will also be published.

For authors that are (temporarily) unaffiliated we will only capture their city and country of residence, not their e-mail address unless specifically requested.

**Abstract**

Please provide an abstract of 150 to 250 words. The abstract should not contain any undefined abbreviations or unspecified references.

*For life science journals only (when applicable)*

- Trial registration number and date of registration for prospectively registered trials
- Trial registration number and date of registration, followed by “retrospectively registered” for retrospectively registered trials

**Additional Details on General Structure**

The manuscript should be submitted as a word file or in LaTeX. The manuscript should be organized into Abstract, Introduction, Materials and Methods, Results, Discussion/Conclusion, Compliance with Ethical Standards, Acknowledgments, References, Figures (with captions) and Tables. Marine Biology does not publish footnotes or supplements, but additional data or videos may be submitted as electronic supplementary material which will be available online.

No full justification for the text should be used. Line numbers should run consecutively throughout the text, from the title page through the figure legends. Lines in tables or figures should not be numbered. Abbreviations and acronyms must be defined at first mention in the Abstract, again in the main body of the text, and also in the Figure Legends. A list of abbreviations may be included as a table, but should not appear at the beginning of the manuscript.

The **Title** should be meaningful and signal the importance of the study for the field. It should be descriptive and tell the reader what the paper is about. It should be general rather than restrictive to species and geographic areas. If scientific names of species are used, they must be accompanied by a higher taxonomic classification term and/or

by a common name.

The **Abstract** should summarize the manuscript and attract the reader. It should be short and clear (150-250 words). The abstract should reflect what was done, why it was done, and what major results were obtained. It should not be written in the first person. The abstract should include the date(s) of the study and the latitude and longitude where the samples or experimental organisms were collected. It should not contain descriptions of the state of the art; such information should be limited to the introduction. No undefined abbreviations or unspecified references should be used. The abstract may decide whether a manuscript will be sent out for review; papers may be rejected due to poor or confusing abstracts.

**Keywords:** The keywords indicated in the submission template should also be included in the manuscript.

The **Introduction** should describe why the study was done and end with some testable hypotheses or clear objectives. Manuscripts which do not present a clear hypothesis are likely to be rejected without review.

**Methods:** All details required to repeat the work must be provided. Usage of publicly accessible data from repositories must be indicated. The respective accession information must be provided in the References.

**Results:** Where specific results are being presented or discussed the past tense should be used. The present tense should only be used for generalizations arising from the study results.

The **Discussion** should highlight the importance or significance of the study for the field and the resulting new insights.

**Compliance with Ethical Standards** must be included as a separate section. The authors should give information about funding and explicitly declare that they have no conflict of interest.

They should also declare that all applicable international, national and/or institutional

guidelines for sampling, care and experimental use of organisms for the study have been followed and all necessary approvals have been obtained. Details about permissions should be provided; documentary evidence must be available on request.

Please do not write "Informed consent was obtained from all individual participants included in the study" if (as usual) no human participants were involved in the study.

In the **Acknowledgement** grants, funds, and contributing people should be mentioned. The reviewers should be acknowledged, but please consider that Marine Biology now allows reviewers to have their names disclosed on the manuscript. You might include the name of a reviewer who has agreed to disclose her/his name into the acknowledgements when you receive the proofs (names are printed at the first page of the paper), but this is not mandatory. Write e.g. "We thank the reviewers" or "We thank X.Y. and an anonymous reviewer"

The **References** must be formatted in MABI style (see more details under "Citations"). Data taken/used from public Databases (e.g. PANGAEA) must be cited by accession numbers.

**Figures:** For ease of reviewing the figures with their captions should be included into the running text. More details are given under 'Illustrations' and 'Figure Captions' (see below). In addition the figure source files without captions must be submitted.

**Tables:** Tables should be numbered using Arabic numerals and have a table caption (title) on top, explaining the components of the table. All abbreviations in the table should be explained in the caption. Tables must not contain vertical lines.

Text
Text Formatting
Manuscripts should be submitted in Word.

- Use a normal, plain font (e.g., 10-point Times Roman) for text.
- Use italics for emphasis.
- Use the automatic page numbering function to number the pages.
- Do not use field functions.

- Use tab stops or other commands for indents, not the space bar.
- Use the table function, not spreadsheets, to make tables.
- Use the equation editor or MathType for equations.
- Save your file in docx format (Word 2007 or higher) or doc format (older Word versions).

Manuscripts with mathematical content can also be submitted in LaTeX. We recommend using Springer Nature's LaTeX template.

**Headings**

Please use no more than three levels of displayed headings.

**Abbreviations**

Abbreviations should be defined at first mention and used consistently thereafter.

**Footnotes**

Footnotes can be used to give additional information, which may include the citation of a reference included in the reference list. They should not consist solely of a reference citation, and they should never include the bibliographic details of a reference. They should also not contain any figures or tables.

Footnotes to the text are numbered consecutively; those to tables should be indicated by superscript lower-case letters (or asterisks for significance values and other statistical data). Footnotes to the title or the authors of the article are not given reference symbols.

Always use footnotes instead of endnotes.

**Acknowledgments**

Acknowledgments of people, grants, funds, etc. should be placed in a separate section on the title page. The names of funding organizations should be written in full.

**Important note:**

**Contrary to the above text, the journal does not encourage the use of footnotes.**

**Additional Details:**

- Use 1.5 or double-space formatting and enable line numbering. No full justification for the text should be used. Superscript must be used to denote the denominator in units, e.g. kg y-1, 24 hr time for time of day, e.g. 0700 hr.
- Use of a recent article as a guideline is recommended. Correct formatting is a prerequisite for acceptance of a manuscript. This concerns especially statistics, units, and citations/references.

Scientific style
Genus and species names should be in italics.

**Statistics**

Describe statistical methods in sufficient detail to allow a knowledgeable reader with access to the original data to verify the reported results. Use the same font for the same mathematical symbol regardless where it appears in the manuscript (text, equations, tables, figures, figure legends).

Give means and standard errors/standard deviations with their associated sample size in the format: X ± SE = 35.09 ± 0.07 km, n = 15. When standard deviation/error is shown in an illustration, n should be given as well.

**Statistical tests** use the following formats: (ANOVA, F (1,25) = 8.56, P= 0.035)

(Kruskal-Wallis test, H25 = 123.7, P= 0.001) (Chi-square test, X22 = 0.23, P = 0.57) (Paired t test, t24 = 2.33, P= 0.09)

(Linear regression, r2 = 0.94, F1,66 = 306.87, P ‹ 0.001) (Spearman rank correlation, rs = 0.60, N = 33, P ‹ 0.01) (Wilcoxon signed-ranks test, T = 7, N = 33, P ‹ 0.05) (Mann-Whitney U test, U = 44, N1 = 7, N2 = 24, P ‹ 0.02)

Please either give the exact P-value of a statistical test, or state P‹0.0xxx, if this is not possible. P=0 is not valid.

**Units**

Use of SI and SI-derived units is preferred. Internationally accepted units can be also be used, e.g. “min” for “minute”. The capital letter “L” must be used for liter.

Please use superscripts instead of “/” or “per …” for ratios. Exponents should also be written as superscripts.

When using a number and a unit of measure to make a qualifying adjective, put a hyphen between them, e.g. 300-µm sieve.

Please refer to the following examples.

Length, Area, Volume: pm, nm, µm, mm, cm, m, km, mm2, cm2, m2, L, mL, µL, mm3, cm3, m3 Mass: pg, ng, µg, mg, g, kg, t, Da, kDa

Time: s, min, h, d, y Temperature: °C,

Absolute quantity: pmol, nmol, µmol, mmol, mol

Concentration: pM, nM, µM, mM, M, N, %, µg L-1,

Work, Energy, Heat quantity: J, erg, cal, kcal Force: dyn, N, gw, kgw

Pressure: Pa, mmHg, atm, bar Electricity: V, W, mA, A, Hz

Photometry: if possible, avoid cd, lx, lm, cd m-2, energy or photon flux density would be preferable

Sound: Hz, kHz, mHz, Abar, dB Speed: cm s-1, m s-1, kn, rad s-1 (some speeds, e.g. sedimentation rates are better expressed per day or even year)

Radioactivity: dpm, cps, cpm, mBq, Bq, kBq, Gy, kGy, mSv, Sv, R, kR Rotation: ×g, cycle Use the symbols ‹ and › to stand for less than and more than.

Also note that salinity has no units and should be presented as: salinity of X or salinity

X.

**Organisms**

Genus and species name must be in italics. It is recommended that the species names appear in full at the beginning of each section of the manuscript and when they appear at the beginning of a sentence. In other places use the contraction e.g. *A. islandica* for *Arctica islandica*. Do not abbreviate genus names if several genera with the same initials can lead to confusion, or when only the genus name is used. *Genus* sp. and *Genus* spp. should only be used when speciation to species level was generally sought, but not completely reached and several species should be treated together, respectively.

The species author may follow the first use of the study species name in either the Abstract or the Materials and Methods. If it is included, the reference to the original description must appear in the References section.

Common names can be used in addition to the scientific names, they are useful especially in the title. Common names such as “water fleas” for cladocerans, or common names that might be misleading must be avoided. E.g.: **Sandfish** is a common name of: *Gonorynchus*, a genus of fish, *Scincus scincus*, a skink, and *Holothuria scabra*, a sea cucumber. It should only be used for the fish.

Only use the words ‘animal’ and ‘plant’ in the most general sense. When referring to the individual organisms used in a study, use the most specific term possible such as the species name (in full or contracted), the common name such as ‘mud shrimp’ for *Upogebia pugettensis*, or ‘individuals’ where appropriate.

When describing the general attributes of a species use a singular verb. When referring to the multiple organisms belonging to the species used in a study, use a plural verb.

**Seasons**

When describing the seasonal timing of events, be aware that fall and winter occur at different times of the year in the northern and southern hemispheres. It is best to

specify the months rather than just the seasons.

**Study Locations**

When writing the names of states in the USA do not use the postal abbreviation but write them in full− thus Virginia not VA.

If a map is used to show study locations, it must have a scale, an arrow indicating due north or a compass rose and a border with the latitude/longitude marked on it. It should show all geographical locations mentioned in the study. The source of the map must be given in the caption of the figure.

**Guidelines for reporting ocean acidification data in scientific journals**

A document prepared in the framework of the data management activity of the Ocean Acidification International Coordination Centre of the International Atomic Energy Agency can be found at the following link:

Guidelines for reporting ocean acidification data in scientific journals

References

**Citation**

Cite references in the text by name and year in parentheses. Some examples:

- Negotiation research spans many disciplines (Thompson 1990).
- This result was later contradicted by Becker and Seligman (1996).
- This effect has been widely studied (Abbott 1991; Barakat et al. 1995a, b; Kelso and Smith 1998; Medvec et al. 1999, 2000).

**Reference list**

The list of references should only include works that are cited in the text and that have been published or accepted for publication. Personal communications and unpublished works should only be mentioned in the text.

Reference list entries should be alphabetized by the last names of the first author of

each work. Please alphabetize according to the following rules: 1) For one author, by name of author, then chronologically; 2) For two authors, by name of author, then name of coauthor, then chronologically; 3) For more than two authors, by name of first author, then chronologically.

If available, please always include DOIs as full DOI links in your reference list (e.g. “https://doi.org/abc”).

- Journal article

  Gamelin FX, Baquet G, Berthoin S, Thevenet D, Nourry C, Nottin S, Bosquet L (2009) Effect of high intensity intermittent training on heart rate variability in prepubescent children. Eur J Appl Physiol 105:731-738. http://dx.doi.org/10.1007/s00421-008-0955-8

  Ideally, the names of all authors should be provided, but the usage of “et al” in long author lists will also be accepted:

  Smith J, Jones M Jr, Houghton L et al (1999) Future of health insurance. N Engl J Med 965:325–329

- Article by DOI

  Slifka MK, Whitton JL (2000) Clinical implications of dysregulated cytokine production. J Mol Med. http://dx.doi.org/10.1007/s001090000086

- Book

  South J, Blass B (2001) The future of modern genomics. Blackwell, London

- Book chapter

  Brown B, Aaron M (2001) The politics of nature. In: Smith J (ed) The rise of modern genomics, 3rd edn. Wiley, New York, pp 230-257

- Online document

Cartwright J (2007) Big stars have weather too. IOP Publishing PhysicsWeb. http://physicsweb.org/articles/news/11/6/16/1. Accessed 26 June 2007

- Dissertation

Trent JW (1975) Experimental acute renal failure. Dissertation, University of California

Always use the standard abbreviation of a journal's name according to the ISSN List of Title Word Abbreviations, see

ISSN LTWA

If you are unsure, please use the full journal title.

**Additional Remarks on Citation**

When citing references in the text, put them in parentheses in chronological order with the earliest first. Separate them with semicolons. Do not put a comma between the author(s) and date.

Examples:

- (Thompson 1990; Abbott et al. 2005; Elliott and Green 2009)
- Same author, multiple years. E.g. (Brown 1997, 2000, 2005)
- Same author, same year. E.g. (Brown 2005a, b)
- Two authors (Brown and Smith 2007; Abbott and Green 2009)
- Multiple authors (Zar et al. 1998; Brown et al. 2008)
- As part of a sentence, e.g. This result was later contradicted by Becker and Seligman (1996)
- Abbreviate Personal Communications to (pers comm
- Abbreviate Unpublished data to (unpubl data)

**Additional Remarks on References**

References should be alphabetized by the last names of the first author of each work.

When there are more than two references with the same first author, the references should be arranged so that the single-authored papers come first in chronological order with the earliest first, then the two-authored papers in alphabetical order by second author, then the multi-authored papers in chronological order with the earliest first.

Journal articles: Journal names must be abbreviated without punctuation. DOIs should be checked with the doi system website, to make sure that the cite is correct.

Online documents: Websites should only be cited if absolutely essential because they will change with time.

Data from a database must be cited in the references by using a Digital Object Identifier (DOI).

Conference Proceedings should not be cited. Every cited printed work should be publicly accessible by ISBN or ISSN number.

When revising your manuscript please examine the validity of your journal references with the 'Automatic Reference Checking' module of the Editorial Manager.

The results of the reference checking are provided by clicking the corresponding link provided in your “Main Menu” in the Editorial Manager, as well as in the PDF file containing your manuscript. If "not validated" is displayed for a reference, it should be checked carefully and corrected where appropriate, as in most cases typos, wrong journals, issues or pages preclude its validation.

Statements & Declarations
The following statements must be included in your submitted manuscript under the heading 'Statements and Declarations'. This should be placed after the References section. Please note that submissions that do not include required statements will be returned as incomplete.

**Funding**

Please describe any sources of funding that have supported the work. The statement

should include details of any grants received (please give the name of the funding agency and grant number).

Example statements:

*“This work was supported by […] (Grant numbers […] and […]). Author A.B. has received research support from Company A.”*

*“The authors declare that no funds, grants, or other support were received during the preparation of this manuscript.”*

**Competing Interests**

Authors are required to disclose financial or non-financial interests that are directly or indirectly related to the work submitted for publication. Interests within the last 3 years of beginning the work (conducting the research and preparing the work for submission) should be reported. Interests outside the 3-year time frame must be disclosed if they could reasonably be perceived as influencing the submitted work.

Example statements:

*“Financial interests: Author A and B declare they have no financial interests. Author C has received speaker and consultant honoraria from Company M. Dr. C has received speaker honorarium and research funding from Company M and Company N. Author D has received travel support from Company O. Non-financial interests: Author D has served on advisory boards for Company M and Company N.”*

*“The authors have no relevant financial or non-financial interests to disclose.”*

Please refer to the “Competing Interests” section below for more information on how to complete these sections.

**Author Contributions**

Authors are encouraged to include a statement that specifies the contribution of every

author to the research and preparation of the manuscript.

Example statement:

*“All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by [full name], [full name] and [full name]. The first draft of the manuscript was written by [full name] and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.”*

Please refer to the “Authorship Principles ” section below for more information on how to complete this section.

**Data Availability**

All original research must include a Data Availability Statement. Data Availability Statements should provide information on where data supporting the results reported in the article can be found. Statements should include, where applicable, hyperlinks to publicly archived datasets analysed or generated during the study. When it is not possible to share research data publicly, for instance when individual privacy could be compromised, data availability should still be stated in the manuscript along with any conditions for access.

Example statements:

*“The datasets generated during and/or analysed during the current study are available in the [NAME] repository, [PERSISTENT LINK TO DATASETS]”*

*“The datasets generated during and/or analysed during the current study are not publicly available due to [REASON(S) WHY DATA ARE NOT PUBLIC] but are available from the corresponding author on reasonable request.].”*

Please refer to the “Research Data Policy and Data Availability” section below for more information on how to complete this section.

In addition to the above, manuscripts that report the results of studies involving humans and/or animals should include the following declarations:

**Ethics approval**

Authors of research involving human or animal subjects should include a statement that confirms that the study was approved (or granted exemption) by the appropriate institutional and/or national research ethics committee (including the name of the ethics committee and reference number, if available). For research involving animals, their data or biological material, authors should supply detailed information on the ethical treatment of their animals in their submission. If a study was granted exemption or did not require ethics approval, this should also be detailed in the manuscript.

*"This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of University B (Date.../No....)."*

*"This is an observational study. The XYZ Research Ethics Committee has confirmed that no ethical approval is required."*

For detailed information on relevant ethical standards and criteria, please refer to the sections on "Research involving human participants, their data or biological material", "Research involving animals, their data or biological material".

**Consent to participate**

For all research involving human subjects, freely-given, informed consent to participate in the study must be obtained from participants (or their parent or legal guardian in the case of children under 16) and a statement to this effect should appear in the manuscript.

Example statement:

*"Informed consent was obtained from all individual participants included in the study."*

*"Written informed consent was obtained from the parents."*

Please refer to the section on “Informed Consent” for additional help with completing this information.

**Consent to publish**

Individuals may consent to participate in a study, but object to having their data published in a journal article. If your manuscript contains any individual person’s data in any form (including any individual details, images or videos), consent for publication must be obtained from that person, or in the case of children, their parent or legal guardian. This is in particular applicable to case studies. A statement confirming that consent to publish has been received from all participants should appear in the manuscript.

Example statement:

*“The authors affirm that human research participants provided informed consent for publication of the images in Figure(s) 1a, 1b and 1c.”*

Please refer to the section on “Informed Consent” for additional help with completing this information.

Tables

- All tables are to be numbered using Arabic numerals.
- Tables should always be cited in text in consecutive numerical order.
- For each table, please supply a table caption (title) explaining the components of the table.
- Identify any previously published material by giving the original source in the form of a reference at the end of the table caption.
- Footnotes to tables should be indicated by superscript lower-case letters (or asterisks for significance values and other statistical data) and included beneath the table body.

Artwork

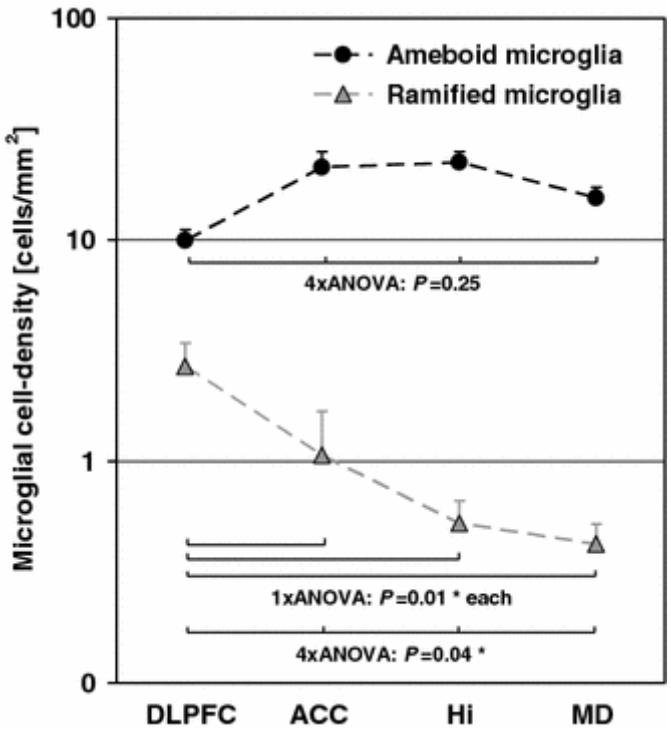
For the best quality final product, it is highly recommended that you submit all of your

artwork – photographs, line drawings, etc. – in an electronic format. Your art will then be produced to the highest standards with the greatest accuracy to detail. The published work will directly reflect the quality of the artwork provided.

**Electronic Figure Submission**

- Supply all figures electronically.
- Indicate what graphics program was used to create the artwork.
- For vector graphics, the preferred format is EPS; for halftones, please use TIFF format. MS Office files are also acceptable.
- Vector graphics containing fonts must have the fonts embedded in the files.
- Name your figure files with "Fig" and the figure number, e.g., Fig1.eps.

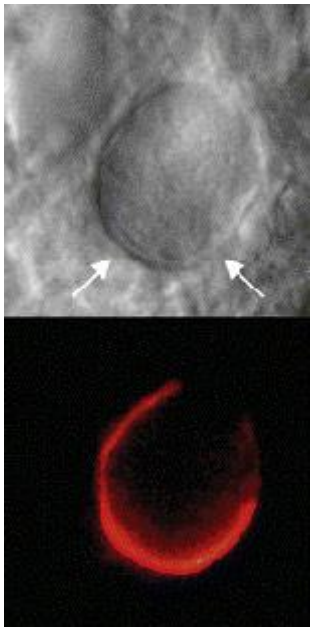
**Line Art**




- Definition: Black and white graphic with no shading.
- Do not use faint lines and/or lettering and check that all lines and lettering within the figures are legible at final size.
- All lines should be at least 0.1 mm (0.3 pt) wide.
- Scanned line drawings and line drawings in bitmap format should have a minimum resolution of 1200 dpi.

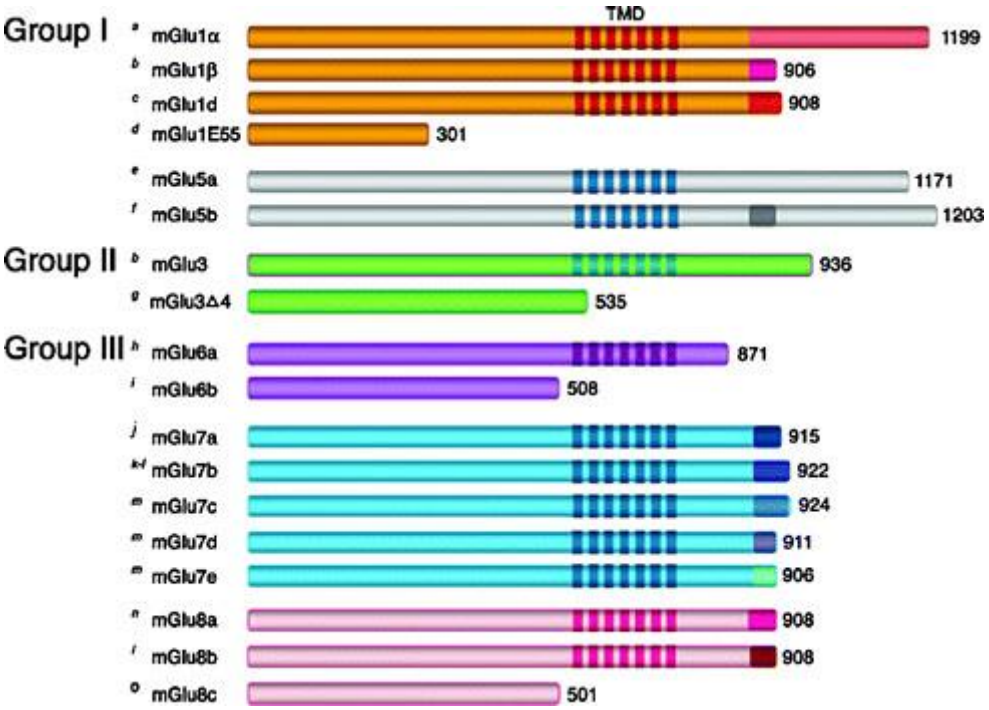
- Vector graphics containing fonts must have the fonts embedded in the files.

**Halftone Art**



- Definition: Photographs, drawings, or paintings with fine shading, etc.
- If any magnification is used in the photographs, indicate this by using scale bars within the figures themselves.
- Halftones should have a minimum resolution of 300 dpi.

**Combination Art**




- Definition: a combination of halftone and line art, e.g., halftones containing line drawing, extensive lettering, color diagrams, etc.
- Combination artwork should have a minimum resolution of 600 dpi.

**Color Art**

- Color art is free of charge for print and online publication.
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- **Financial interests:** Author A and B declare they have no financial interests. Author C has received speaker and consultant honoraria from Company M and Company N. Dr. C has received speaker honorarium and research funding from

Company M and Company O. Author D has received travel support from Company O.

**Non-financial interests:** Author D has served on advisory boards for Company M, Company N and Company O.

**Examples of statements to be used when authors have nothing to declare:**

- The authors have no relevant financial or non-financial interests to disclose.
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- All authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript.
- The authors have no financial or proprietary interests in any material discussed in this article.

Authors are responsible for correctness of the statements provided in the manuscript. See also Authorship Principles. The Editor-in-Chief reserves the right to reject submissions that do not meet the guidelines described in this section.

Research involving human participants, their data or biological material

**Ethics approval**

When reporting a study that involved human participants, their data or biological material, authors should include a statement that confirms that the study was approved (or granted exemption) by the appropriate institutional and/or national research ethics committee (including the name of the ethics committee) and certify that the study was performed in accordance with the ethical standards as laid down in the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards. If doubt exists whether the research was conducted in accordance with the 1964 Helsinki Declaration or comparable standards, the authors must explain the reasons for their approach, and demonstrate that an independent ethics committee or institutional review board explicitly approved the doubtful aspects of the study. If a study was granted exemption from requiring ethics approval, this should also be detailed in the manuscript (including the reasons for the exemption).

**Retrospective ethics approval**

If a study has not been granted ethics committee approval prior to commencing, retrospective ethics approval usually cannot be obtained and it may not be possible to consider the manuscript for peer review. The decision on whether to proceed to peer review in such cases is at the Editor's discretion.

**Ethics approval for retrospective studies**

Although retrospective studies are conducted on already available data or biological material (for which formal consent may not be needed or is difficult to obtain) ethics approval may be required dependent on the law and the national ethical guidelines of a country. Authors should check with their institution to make sure they are complying with the specific requirements of their country.

**Ethics approval for case studies**

Case reports require ethics approval. Most institutions will have specific policies on this subject. Authors should check with their institution to make sure they are complying with the specific requirements of their institution and seek ethics approval where needed. Authors should be aware to secure informed consent from the individual (or parent or guardian if the participant is a minor or incapable) See also section on **Informed Consent**.

**Cell lines**

If human cells are used, authors must declare in the manuscript: what cell lines were used by describing the source of the cell line, including when and from where it was obtained, whether the cell line has recently been authenticated and by what method. If cells were bought from a life science company the following need to be given in the manuscript: name of company (that provided the cells), cell type, number of cell line, and batch of cells.

It is recommended that authors check the NCBI database for misidentification and contamination of human cell lines. This step will alert authors to possible problems with the cell line and may save considerable time and effort.

Further information is available from the International Cell Line Authentication Committee (ICLAC).

Authors should include a statement that confirms that an institutional or independent ethics committee (including the name of the ethics committee) approved the study and that informed consent was obtained from the donor or next of kin.

**Research Resource Identifiers (RRID)**

Research Resource Identifiers (RRID) are persistent unique identifiers (effectively similar to a DOI) for research resources. This journal encourages authors to adopt RRIDs when reporting key biological resources (antibodies, cell lines, model organisms and tools) in their manuscripts.

**Examples:**

**Organism:** *Filip1*$^{tm1a(KOMP)Wtsi}$ **RRID:MMRRC_055641-UCD**

**Cell Line:** RST307 cell line **RRID:CVCL_C321**

**Antibody:** Luciferase antibody DSHB Cat# LUC-3, **RRID:AB_2722109**

**Plasmid:** mRuby3 plasmid **RRID:Addgene_104005**

**Software:** ImageJ Version 1.2.4 **RRID:SCR_003070**

RRIDs are provided by the Resource Identification Portal. Many commonly used research resources already have designated RRIDs. The portal also provides authors links so that they can quickly register a new resource and obtain an RRID.

**Clinical Trial Registration**

The World Health Organization (WHO) definition of a clinical trial is "any research study that prospectively assigns human participants or groups of humans to one or more health-related interventions to evaluate the effects on health outcomes". The WHO defines health interventions as “A health intervention is an act performed for, with or

on behalf of a person or population whose purpose is to assess, improve, maintain, promote or modify health, functioning or health conditions" and a health-related outcome is generally defined as a change in the health of a person or population as a result of an intervention.

To ensure the integrity of the reporting of patient-centered trials, authors must register prospective clinical trials (phase II to IV trials) in suitable publicly available repositories. For example www.clinicaltrials.gov or any of the primary registries that participate in the WHO International Clinical Trials Registry Platform.

The trial registration number (TRN) and date of registration should be included as the last line of the manuscript abstract.

For clinical trials that have not been registered prospectively, authors are encouraged to register retrospectively to ensure the complete publication of all results. The trial registration number (TRN), date of registration and the words 'retrospectively registered' should be included as the last line of the manuscript abstract.

**Standards of reporting**

Springer Nature advocates complete and transparent reporting of biomedical and biological research and research with biological applications. Authors are recommended to adhere to the minimum reporting guidelines hosted by the EQUATOR Network when preparing their manuscript.

Exact requirements may vary depending on the journal; please refer to the journal's Instructions for Authors.

Checklists are available for a number of study designs, including:

Randomised trials (CONSORT) and Study protocols (SPIRIT)

Observational studies (STROBE)

Systematic reviews and meta-analyses (PRISMA) and protocols (Prisma-P)

Diagnostic/prognostic studies (STARD) and (TRIPOD)

Case reports (CARE)

Clinical practice guidelines (AGREE) and (RIGHT)

Qualitative research (SRQR) and (COREQ)

Animal pre-clinical studies (ARRIVE)

Quality improvement studies (SQUIRE)

Economic evaluations (CHEERS)

**Summary of requirements**

The above should be summarized in a statement and placed in a ‘Declarations’ section before the reference list under a heading of ‘Ethics approval’.

Please see the various examples of wording below and revise/customize the sample statements according to your own needs.

Examples of statements to be used when ethics approval has been obtained:

• All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. The study was approved by the Bioethics Committee of the Medical University of A (No. ...).

• This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of University B (Date.../No. ...).

• Approval was obtained from the ethics committee of University C. The procedures used in this study adhere to the tenets of the Declaration of Helsinki.

• The questionnaire and methodology for this study was approved by the Human Research Ethics committee of the University of D (Ethics approval number: ...).

Examples of statements to be used for a retrospective study:

• Ethical approval was waived by the local Ethics Committee of University A in view of the retrospective nature of the study and all the procedures being performed were part of the routine care.

• This research study was conducted retrospectively from data obtained for clinical purposes. We consulted extensively with the IRB of XYZ who determined that our study did not need ethical approval. An IRB official waiver of ethical approval was granted from the IRB of XYZ.

• This retrospective chart review study involving human participants was in accordance with the ethical standards of the institutional and national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. The Human Investigation Committee (IRB) of University B approved this study.

Examples of statements to be used when no ethical approval is required/exemption granted:

• This is an observational study. The XYZ Research Ethics Committee has confirmed that no ethical approval is required.

• The data reproduced from Article X utilized human tissue that was procured via our Biobank AB, which provides de-identified samples. This study was reviewed and deemed exempt by our XYZ Institutional Review Board. The BioBank protocols are in accordance with the ethical standards of our institution and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Authors are responsible for correctness of the statements provided in the manuscript. See also Authorship Principles. The Editor-in-Chief reserves the right to reject submissions that do not meet the guidelines described in this section.

Research involving animals, their data or biological material

The welfare of animals (vertebrate and higher invertebrate) used for research, education and testing must be respected. Authors should supply detailed information on the ethical treatment of their animals in their submission. For that purpose they may use the ARRIVE checklist which is designed to be used when submitting manuscripts describing animal research.

For studies involving client-owned animals, authors must also document informed consent from the client or owner and adherence to a high standard (best practice) of veterinary care.

Authors are recommended to comply with:

• The International Union for Conservation of Nature (IUCN) Policy Statement on Research Involving Species at Risk of Extinction and consult the IUCN red list index of threatened species.

• Convention on the Trade in Endangered Species of Wild Fauna and Flora

When reporting results authors should indicate:

• … that the studies have been approved by a research ethics committee at the institution or practice at which the studies were conducted. Please provide the name of ethics committee and relevant permit number;

• … whether the legal requirements or guidelines in the country and/or state or province for the care and use of animals have been followed.

Researchers from countries without any legal requirements or guidelines voluntarily should refer to the following sites for guidance:

– The Basel Declaration describes fundamental principles of using animals in biomedical research

– The International Council for Laboratory Animal Science (ICLAS) provides ethical

guidelines for researchers as well as editors and reviewers

– The Association for the study of Animal Behaviour describes ethical guidelines for the treatment of animals in research and teaching

– The International Association of Veterinary Editors' Consensus Author Guidelines on Animal Ethics provide guidelines for authors on animal ethics and welfare

Researchers may wish to consult the most recent (ethical) guidelines available from relevant taxon-oriented professional societies.

If a study was granted exemption or did not require ethics approval, this should also be detailed in the manuscript.

**Summary of requirements**

The above should be summarized in a statement and placed in a 'Declarations' section before the reference list under a heading of 'Ethics approval'.

Please see the various examples of wording below and revise/customize the sample statements according to your own needs.

*Examples of statements to be used when ethics approval has been obtained:*

• All procedures involving animals were in compliance with the European Community Council Directive of 24 November 1986, and ethical approval was granted by the Kocaeli University Ethics Committee (No. 29 12 2014, Kocaeli, Turkey).

• All procedures performed in the study were in accordance with the ARVO Statement for Use of Animals in Ophthalmic Vision and Research. The ethical principles established by the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8523, revised 2011) were followed. The research protocol was approved by the Ethics Committee on Animal Use (Protocol No. 06174/14) of FCAV/Unesp, Jaboticabal.

• This study involved a questionnaire-based survey of farmers as well as blood sampling from their animals. The study protocol was assessed and approved by Haramaya University, research and extension office. Participants provided their verbal informed consent for animal blood sampling as well as for the related survey questions. Collection of blood samples was carried out by veterinarians adhering to the regulations and guidelines on animal husbandry and welfare.

• All brown bear captures and handling were approved by the Ethical Committee on Animal Experiments, Uppsala, Sweden (Application C18/15) and the Swedish Environmental Protection Agency in compliance with Swedish laws and regulations.

• The ethics governing the use and conduct of experiments on animals were strictly observed, and the experimental protocol was approved by the University of Maiduguri Senate committee on Medical Research ethics. Proper permit and consent were obtained from the Maiduguri abattoir management, before the faecal samples of the cattle and camels slaughtered in this abattoir were used for this experiment.

*Examples of statements to be used when no ethical approval is required/exemption granted:*

• No approval of research ethics committees was required to accomplish the goals of this study because experimental work was conducted with an unregulated invertebrate species.

• As the trappings of small mammals were conducted as part of regular pest control measures in accordance with the NATO Standardized Agreement 2048 "Deployment Pest and Vector Surveillance and Control ", no approval by an ethics committee was required.

• All experiments have been conducted as per the guidelines of the Institutional Animal Ethics Committee, Department of Zoology, Utkal University, Bhubaneswar, Odisha, India. However, the insect species used in this study is reared for commercial production of raw silk materials, as a part of agro-based industry. Therefore, use of this animal in research does not require ethical clearance. We have obtained permission

from the office of Research officer sericulture, Baripada, Orissa, India for the provision of infrastructure and support for rearing of silkworm both in indoor and outdoor conditions related to our study to promote sericulture practices.

Authors are responsible for correctness of the statements provided in the manuscript. See also Authorship Principles. The Editor-in-Chief reserves the right to reject submissions that do not meet the guidelines described in this section.

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**Apêndice 2 –** Normas do periódico Cryobiology

## Guide for Authors

## Introduction

The Official Journal of the Society for Cryobiology

### *Types of article*

•Regular Papers

•Brief Communications

•Reviews

•Letters to the Editor

Regular papers will describe experimental findings, techniques, or theory. They will consist ofan abstract that summarizes the objective of the study, the methods used, and the conclusionsreached. Abstracts should not exceed 250 words and should be adequate for direct presentation to abstracting services. After the abstract a list of up to 10 keywords that will be useful for indexing or searching must be included. The Introduction will contain a statement ofthe purpose of the work, the problem that stimulated it, and a brief summary of relevant published investigations. The Materials and Methods section must be presented in sufficient detail to enable other investigators to repeat the work. The Results should be concise and should avoid redundant tables and figures illustrating the same data. The Discussion should interpret the results, with minimal recapitulation of findings.

Brief Communications are concise reports of original findings, techniques or theory and includean abstract no longer than 150 words and a list of up to 10 keywords. They are not divided intosections. As a guideline it is suggested that there should be no more than 3 tables and/or figures and a maximum of 10 references. The total length, including references, should not exceed 2500 words.

Reviews should only be submitted after first discussing the article with the Editor or a memberof the Editorial Board. As with regular papers and brief communications, reviews will be subject to peer review.

Letters to the Editor should concern matters of general interest to the readership of the journalor papers recently published in the journal. Authors of papers that are the subject of commentwill be given an opportunity to reply. Letters may not exceed 1 printed page in length and if publication deadlines are pressing, proofs may not be provided. The Editor's decision will be final.

### *Contact details for submission*

Please submit your article via http://ees.elsevier.com/cryo.

For questions on the reviewing process or for proposals for Review Articles, please contactthe Editor-in-Chief:

Prof. David M. Rawson

E-mail: david.rawson@societyforcryobiology.org

## Submission checklist

You can use this list to carry out a final check of your submission before you send it to the journal for review. Please check the relevant section in this Guide for Authors for more details.**Ensure that the following items are present:**

One author has been designated as the corresponding author with contact details:

• E-mail address

• Full postal address

All necessary files have been uploaded:

*Manuscript*:

• Include keywords

• All figures (include relevant captions)

• All tables (including titles, description, footnotes)

• Ensure all figure and table citations in the text match the files provided

• Indicate clearly if color should be used for any figures in print

*Graphical Abstracts / Highlights files* (where applicable)

*Supplemental files* (where applicable)Further considerations

• Manuscript has been 'spell checked' and 'grammar checked'

• All references mentioned in the Reference List are cited in the text, and vice versa

• Permission has been obtained for use of copyrighted material from other sources(including the Internet)

• A competing interests statement is provided, even if the authors have no competing intereststo declare

• Journal policies detailed in this guide have been reviewed

• Referee suggestions and contact details provided, based on journal requirementsFor further information, visit our Support Center.

**Ethics in publishing**

Please see our information pages on Ethics in publishing and Ethical guidelines for journalpublication.

**Studies in humans and animals**

If the work involves the use of human subjects, the author should ensure that the work described has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans. The manuscript should be in line with the Recommendations for the Conduct, Reporting, Editing and Publication of Scholarly Work in Medical Journals and aim for the inclusion of representative human populations (sex, age and ethnicity) as per those recommendations. The terms sex and gender should be used

correctly.

Authors should include a statement in the manuscript that informed consent was obtained for experimentation with human subjects. The privacy rights of human subjects must always be observed.

All animal experiments should comply with the ARRIVE guidelines and should be carried out in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments, or the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978) and the authors should clearly indicate in the manuscript that such guidelines have been followed. The sex of animals must be indicated, and where appropriate, the influence (or association) of sex on the results of the study.

**Declaration of interest**

All authors must disclose any financial and personal relationships with other people or organizations that could inappropriately influence (bias) their work. Examples of potential competing interests include employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications/registrations, and grants or other funding. Authors must disclose any interests in two places: 1. A summary declaration of interest statement in the title page file (if double-blind) or the manuscript file (if single-blind). If there are no interests to declare then please state this: 'Declarations of interest: none'. This summary statement will be ultimately published if the article is accepted. 2. Detailed disclosures as part of a separate Declaration of Interest form, which forms part of the journal's official records. It is important for potential interests to be declared in both places and that the information matches. More information.

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Submission of an article implies that the work described has not been published previously (except in the form of an abstract, a published lecture or academic thesis, see 'Multiple, redundant or concurrent publication' for more information), that it is not under consideration for publication elsewhere, that its publication is approved by all authors and tacitly or explicitly bythe responsible authorities where the work was carried out, and that, if accepted, it will not bepublished elsewhere in the same form, in English or in any other language, including electronically without the written consent of the copyright-holder. To verify originality, your article may be checked by the originality detection service Crossref Similarity Check.

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**Changes to authorship**

Authors are expected to consider carefully the list and order of authors **before** submitting their manuscript and provide the definitive list of authors at the time of the original submission. Any addition, deletion or rearrangement of author names in the authorship list should be made only **before** the manuscript has been accepted and only if approved by the journal Editor. To request such a change, the Editor must receive the following from the **corresponding author**:

(a) the reason for the change in author list and (b) written confirmation (e-mail, letter) from all authors that they agree with the addition, removal or rearrangement. In the case of addition or removal of authors, this includes confirmation from the author being added or removed.

Only in exceptional circumstances will the Editor consider the addition, deletion or rearrangement of authors **after** the manuscript has been accepted. While the Editor considers the request, publication of the manuscript will be suspended. If the manuscript has already been published in an online issue, any requests approved by the Editor will result in a corrigendum.

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### *Language (usage and editing services)*

Please write your text in good English (American or British usage is accepted, but not a mixture of these). Authors who feel their English language manuscript may require editing to eliminate possible grammatical or spelling errors and to conform to correct scientific English may wish to use the English Language Editing service available from Elsevier's Author Services.

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Our online submission system guides you stepwise through the process of entering your articledetails and uploading your files. The system converts your article files to a single PDF file used in the peer-review process. Editable files (e.g., Word, LaTeX) are required to typeset your article for final publication. All correspondence, including notification of the Editor's decision and requests for revision, is sent by e-mail.

### *Submission address*

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### Peer review

This journal operates a single blind review process. All contributions will be initially assessed by the editor for suitability for the journal. Papers deemed suitable are then typically sent to a minimum of two independent expert reviewers to assess the scientific quality of the paper. TheEditor is responsible for the final decision regarding acceptance or rejection of articles. The Editor's decision is final. More information on types of peer review.

### *Use of word processing software*

It is important that the file be saved in the native format of the word processor used. The text should be in single-column format. Keep the layout of the text as simple as possible. Most formatting codes will be removed and replaced on processing the article. In particular, do not use the word processor's options to justify text or to hyphenate words. However, do use bold face, italics, subscripts, superscripts etc. When preparing tables, if you are using a table grid, use only one grid for each individual table and not a grid for each row. If no grid is used, use tabs, not spaces, to align columns. The electronic text should be prepared in a way very similar to that of conventional manuscripts (see also the Guide to Publishing with Elsevier). Note thatsource files of figures, tables and text graphics will be required whether or not you embed your figures in the text. See also the section on Electronic artwork.

To avoid unnecessary errors you are strongly advised to use the 'spell-check' and 'grammar- check' functions of your word processor.

Manuscripts must be prepared in double or triple line spacing and lines must be numbered. Pages should be numbered in consecutive order.

### *LaTeX*

You are recommended to use the latest Elsevier article class to prepare your manuscript and BibTeX to generate your bibliography.

Our Guidelines has full details.

### *Subdivision*

Regular papers will describe experimental findings, techniques, or theory. They will consist ofan abstract that summarizes the objective of the study, the methods used, and the conclusionsreached.

Brief Communications should not be divided into sections.

### *Introduction*

The Introduction will contain a statement of the purpose of the work, the problem that stimulated it, and a brief summary of relevant published investigations.

### *Material and methods*

Provide sufficient details to allow the work to be reproduced by an independent researcher. Methods that are already published should be summarized, and

indicated by a reference. If quoting directly from a previously published method, use quotation marks and also cite the source. Any modifications to existing methods should also be described.

***Results***

Results should be clear and concise.

Avoid redundant tables and figures illustrating the same data.

***Discussion***

This should explore the significance of the results of the work, not repeat them. A combined Results and Discussion section is often appropriate. Avoid extensive citations and discussionof published literature.

**Essential title page information**

***Title.*** Concise and informative. Titles are often used in information-retrieval systems. Avoid abbreviations and formulae where possible.

• ***Author names and affiliations.*** Please clearly indicate the given name(s) and family name(s) of each author and check that all names are accurately spelled. You can add your name between parentheses in your own script behind the English transliteration. Present the authors' affiliation addresses (where the actual work was done) below the names. Indicate allaffiliations with a lower-case superscript letter immediately after the author's name and in frontof the appropriate address. Provide the full postal address of each affiliation, including the country name and, if available, the e-mail address of each author.

• ***Corresponding author.*** Clearly indicate who will handle correspondence at all stages of refereeing and publication, also post-publication. This responsibility includes answering any future queries about Methodology and Materials. **Ensure that the e-mail address is given and that contact details are kept up to date by the corresponding author.**

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**Highlights**

Highlights are optional yet highly encouraged for this journal, as they increase the discoverability of your article via search engines. They consist of a short collection of bullet points that capture the novel results of your research as well as new

methods that were used during the study (if any). Please have a look at the examples here: example Highlights.

Highlights should be submitted in a separate editable file in the online submission system. Please use 'Highlights' in the file name and include 3 to 5 bullet points (maximum 85 characters, including spaces, per bullet point).

**Abstract**

A concise and factual abstract is required. The abstract should state briefly the purpose of the research, the principal results and major conclusions. An abstract is often presented separately from the article, so it must be able to stand alone. For this reason, References should be avoided, but if essential, then cite the author(s) and year(s). Also, non-standard or uncommon abbreviations should be avoided, but if essential they must be defined at their first mention in the abstract itself.

Abstracts of Regular Papers should not exceed 250 words, abstracts of Brief Communications should not exceed 150 words

***Graphical abstract***

Although a graphical abstract is optional, its use is encouraged as it draws more attention to the online article. The graphical abstract should summarize the contents of the article in a concise, pictorial form designed to capture the attention of a wide readership. Graphical abstracts should be submitted as a separate file in the online submission system. Image size: Please provide an image with a minimum of 531 × 1328 pixels (h × w) or proportionally more. The image should be readable at a size of 5 × 13 cm using a regular screen resolution of 96 dpi. Preferred file types: TIFF, EPS, PDF or MS Office files. You can view Example Graphical Abstracts on our information site. Authors can make use of Elsevier's Illustration Services to ensure the best presentation of their images and in accordance with all technical requirements.

***Keywords***

Immediately after the abstract, provide a maximum of 10 keywords, using American spelling and avoiding general and plural terms and multiple concepts (avoid, for example, "and", "of"). Be sparing with abbreviations: only abbreviations firmly established in the field may be eligible. These keywords will be used for indexing purposes.

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# VITA

Nayara Oliveira da Cruz, filha de Eluiza Elena Oliveira da Cruz e José Balbino Lopes da Cruz, nasceu no dia 09 de junho de 1990 na cidade de Belém, no estadodo Pará.

Concluiu o ensino fundamental no Colégio Nossa Senhora da Anunciação em 2005 no município de Ananindeua-PA e o ensino médio na Escola Estadual Agroindustrial Juscelino Kubitschek de Oliveira em 2008, no município de Marituba-PA. Em 2010 ingressou no curso de Tecnologia em Aquicultura, no Instituto Federal de Educação, Ciência e Tecnologia do Pará (Campus Castanhal), finalizando em 2013. Durante a graduação realizou estágio de iniciação científica nas áreas de piscicultura de corte, piscicultura ornamental, sanidade, tecnologia do pescado, carcinicultura, ostreicultura e extensão. Ao final do curso de graduação, realizou o Estágio Curricular Obrigatório no Proex-Aquicultura, do IFPA (Campus Castanhal),onde desenvolveu atividades de pesquisa e extensão na área de piscicultura de corte.

Possuiu vínculo de enquadramento funcional, onde atuou como Técnica responsável de laboratório na empresa Alimento Seguro Consultoria/Serviços entre os anos de 2013-2015. Atuando nas áreas de piscicultura ornamental, conservação, reprodução e nutrição. Em 2015 ingressou no mestrado do Programa de Pós-Graduação em Aquicultura, pela Universidade Nilton Lins em ampla associação com o Instituto Nacional de Pesquisa da Amazônia, sendo Bolsista CAPES. Durante esse período realizou atividades de pesquisa nas áreas de aquicultura, reprodução, fisiologia, comportamento e bem-estar. Em 2017, obteve o título de Mestre em Aquicultura.

Em 2018 ingressou no doutorado do Programa de Pós-Graduação em Zootecnia, pela Universidade Federal do Rio Grande do Sul, na área de concentração de Produção Animal, sendo bolsista CAPES. Contemplada com a Bolsa FUNBIO – Conservando o Futuro em parceria com o Instituto Humanize. Nesse período realizou atividades de pesquisa e ensino no Projeto Reef Bank e no Grupo de Pesquisa AQUAM (Produção e Conservação da Biodiversidade das Espécies Aquáticas), com foco principal no desenvolvimento e aprimoramentos de técnicas relacionadas às metodologias de criopreservação de gametas de coral.