UNIVERSIDADE FEDERAL DE MINAS GERAIS
INSTITUTO DE CIÊNCIAS BIOLÓGICAS
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA CELULAR

Wanderson Valente dos Santos

ESTUDO MORFOFUNCIONAL DA GAMETOGÊNESE DO CORAL *MUSSISMILIA HARTTII* (VERRILL, 1868): desenvolvimento interdisciplinar de ferramentas aplicadas à conservação da espécie endêmica do Brasil e ameaçada de extinção

Belo Horizonte

2025

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Tese apresentada ao Programa de Pós-Graduação em Biologia Celular da Universidade Federal de Minas Gerais, como requisito parcial para obtenção do título de Doutor em Ciências.

Área de concentração: Biologia Celular

Orientadora: Profª. Dra. Gleide Fernandes de Avelar
Coorientador: Prof. Dr. Leandro César de Godoy

Belo Horizonte

2025

043 Santos, Wanderson Valente dos.
Estudo morfofuncional da gametogênese do coral Mussismilia Harttii (Verrill, 1868): desenvolvimento interdisciplinar de ferramentas aplicadas à conservação da espécie endêmica do Brasil e ameaçada de extinção [manuscrito] / Wanderson Valente dos Santos. – 2025.
129 f. : il. ; 29,5 cm.

Orientadora: Profª. Dra. Gleide Fernandes de Avelar. Coorientador: Prof. Dr. Leandro César de Godoy.
Tese (doutorado) – Universidade Federal de Minas Gerais, Instituto de Ciências Biológicas. Programa de Pós-Graduação em Biologia Celular.

1. Biologia Celular. 2. Coral. 3. Reprodução. 4. Gametogênese. I. Avelar, Gleide Fernandes de. II. Godoy, Leandro César de. III. Universidade Federal de Minas Gerais. Instituto de Ciências Biológicas. IV. Título.

CDU: 576

Ficha catalográfica elaborada pela bibliotecária Fabiane C M Reis – CRB 6 – 2680

UNIVERSIDADE FEDERAL DE MINAS GERAIS
ICB - COLEGIADO DE PÓS-GRADUAÇÃO EM BIOLOGIA CELULAR - SECRETARIA

ATA DE DEFESA DE TESE

WANDERSON VALENTE DOS SANTOS

À s **quatorze horas** do dia **21 de fevereiro de 2025**, reuniu-se, no Instituto de Ciências Biológicas da UFMG, a Comissão Examinadora da Tese, indicada pelo Colegiado do Programa, para julgar, em exame final, o trabalho final intitulado: **"ESTUDO MORFOFUNCIONAL DA GAMETOGÊNESE DO CORAL MUSSISMILIA HARTTII (VERRILL, 1868): DESENVOLVIMENTO INTERDISCIPLINAR DE FERRAMENTAS APLICADAS À CONSERVAÇÃO DA ESPÉCIE ENDÊMICA DO BRASIL E AMEAÇADA DE EXTINÇÃO"** , requisito final para obtenção do grau de Doutor em Biologia Celular. Abrindo a sessão, a Presidente da Comissão, **Gleide Fernandes de Avelar** , após dar a conhecer aos presentes o teor das Normas Regulamentares do Trabalho Final, passou a palavra ao candidato, para apresentação de seu trabalho. Seguiu-se a arguição pelos examinadores, com a respectiva defesa do candidato. Logo após, a Comissão se reuniu, sem a presença do candidato e do público, para julgamento e expedição de resultado final. Foram atribuídas as seguintes indicações:

Prof./Pesq.	**Instituição**	**Indicação**
Gleide Fernandes de Avelar	UFMG	Aprovado
Leandro Cesar de Godoy	UFRGS	Aprovado
Cristiano Macedo Pereira	Fundação Renova	Aprovado
Nilo Bazzoli	PUC- MINAS	Aprovado
Daniela Chemim de Melo Hoyos	UFMG	Aprovado
Talita de Oliveira Farias	UFMG	Aprovado

Pelas indicações, o candidato foi considerado: **APROVADO**

O resultado final foi comunicado publicamente ao candidato pela Presidente da Comissão. Nada mais havendo a tratar, a Presidente encerrou a reunião e lavrou a presente ATA, que será assinada por todos os membros participantes da Comissão Examinadora. **Belo Horizonte, 21 de fevereiro de 2025.**

Gleide Fernandes de Avelar_____

Leandro Cesar de Godoy_____

Nilo Bazzoli_____

Cristiano Macedo Pereira_____

Daniela Chemim de Melo Hoyos_____

Talita de Oliveira Farias_____

Belo Horizonte, 25 de fevereiro de 2025.

Assinatura dos membros da banca examinadora:

Documento assinado eletronicamente por **Cristiano Macedo Pereira**, **Usuário Externo**, em 25/02/2025, às 11:04, conforme horário oficial de Brasília, com fundamento no art. 5º do Decreto nº 10.543, de 13 de novembro de 2020.

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Dedico este trabalho a minha mãe, Ana Elisabete Valente dos Santos, por todo o amor, pelas palavras de encorajamento nos momentos difíceis e pelo exemplo de resiliência e coragem que sempre me inspirara.

AGRADECIMENTOS

A **Deus**, pela força, sabedoria e fé que me sustentaram em cada passo dessa jornada. Pela luz nos momentos de incerteza e pela serenidade diante dos desafios.

A minha **família**, em especial aos meus pais **Ana Elisabete Valente dos Santos** e **Sinval dos Santos**, pelo amor, apoio e compreensão ao longo de toda essa jornada. Aos meus irmãos **Micheli Valente dos Santos** e **Wesley Valente dos Santos** pela paciência nos momentos de ausência e pelas palavras de encorajamento nos dias difíceis. A minha sobrinha **Ana Clara Valente dos Santos Bonifácio**, mesmo que em sua inocência celebrava cada conquista. Em especial, ao **Renan de Lima Coutinho**, por ser meu porto seguro, meu alicerce e minha maior motivação para seguir em frente. Dedico esta conquista com todo meu amor e gratidão a vocês.

A minha orientadora **Profª. Dra. Gleide Fernandes de Avelar,** pela disponibilidade em me orientar, pelos ensinamentos, confiança, paciência e todo esforço depositado na realização desta pesquisa. Tenho muito respeito e admiração não só pelo seu profissionalismo, mas pela sua busca incansável de fazer sempre o melhor, sem ofender ou diminuir ninguém, pelo desejo de formar pessoas e, principalmente, pela sua honestidade e humildade. Seu comprometimento e sua paixão pela pesquisa servem de inspiração, não apenas para minha formação acadêmica, mas também para meu crescimento pessoal.

Ao meu coorientador **Prof. Dr. Leandro Cesar de Godoy**, pela luta em prol da conservação dos recifes de coral e pelo suporte financeiro para realização desta pesquisa. Além disso, agradeço por todos os ensinamentos e oportunidades oferecidas durante este tempo de convivência. Pode ter certeza que contribuíram muito para minha formação profissional e pessoal.

Aos **professores** que tive durante o doutorado, em especial a **Profª. Dra. Samyra Maria dos Santos Nassif Lacerda** e **Prof. Dr. Guilherme Mattos Jardim Costa**, por todo conhecimento compartilhado durante esses anos de convívio.

Aos amigos do **Laboratório de Biologia Celular**, em especial a **Bárbara Ramalho Ladeira Cardoso,** pela dedicação, proatividade e ajuda em todas as etapas deste trabalho e a **Iara Nogueira Pastor**, pelas risadas e companhia. Foi um privilégio e um aprendizado trabalhar com vocês durante estes anos. Sem o apoio e a amizade de cada um a caminhada teria seria mais difícil.

A **Universidade Federal de Minas Gerais** (UFMG) e ao **Programa de Pós-graduação em Biologia Celular**, pela oportunidade de realização este curso e pelo suporte técnico concedido.

A **Coordenação de Aperfeiçoamento de Pessoal de Nível Superior** (CAPES), pela concessão da bolsa de estudos.

Ao **Fundo Brasileiro para a Biodiversidade** (FUNBIO), pela concessão da bolsa de auxílio a pesquisa.

Aos **membros da banca** pela disponibilidade e valiosas contribuições.

Para finalizar agradeço a **todos** que contribuíram de forma direta ou indireta e que torceram para que este sonho se tornasse realidade.

Muito obrigado!

O objetivo da Ciência não é abrir a porta para a sabedoria infinita, mas estabelecer um limite para o erro infinito

Mario Sergio Cortella

RESUMO

Os recifes de coral estão entre os ecossistemas de maior biodiversidade e produtividade do planeta. Ainda que ocupe uma área inferior a 1% do oceano, exercem um importante papel na renovação dos estoques pesqueiros. Além disso, apresentam grande potencial farmacológico e servem como barreiras de proteção para as regiões costeiras. Os corais são metazoários e possuem uma estreita relação simbiótica com organismos unicelulares chamados de zooxantelas. Apresentam um ciclo de vida simples que compreende duas fases: a fase larval (vida livre) e a de pólipo, que se fixa ao substrato, e sua reprodução pode ocorrer de forma assexuada ou sexuada. A forma mais comum de reprodução assexuada ocorre através do brotamento de um pólipo ou através da fragmentação da colônia. Já a reprodução sexuada consiste na produção de gametas. A *Mussismilia harttii* está entre as principais espécies construtoras dos recifes brasileiros, porém já sofre com eventos constantes de branqueamento, decorrente do aumento da temperatura do oceano, e encontra-se sob ameaça de extinção. Algumas técnicas seguras para preservar a existência dos corais estão relacionadas ao conhecimento básico da sua biologia reprodutiva. A imuno-histoquímica, imunofluorescência e a avalição ultraestrutural aplicada à biologia reprodutiva dos corais do Brasil se limita à poucos relatos na literatura. Atualmente, apenas dois estudos analisaram a organização morfológica e ultraestrutura do pacote de gametas do coral *M. harttii*. Porém, estudos imuno-histoquímicos, imunofluorescência e ultraestruturais da gametogênese nunca foram realizados para qualquer espécie de coral brasileiro. O presente trabalho empregou essas técnicas para caracterizar as fases de formação dos gametas do coral endêmico *M. harttii*. A caracterização da espermatogênese e da oogênese permitirá conhecer em detalhes sobre a formação dos gametas e dará subsídios para melhor compreender as estruturas que podem estar relacionadas com a formação dos pacotes de gametas, a desova e fecundação na espécie.

Palavras-chave: Reprodução sexuada, biologia reprodutiva, gametogênese, ultraestrutura e conservação.

ABSTRACT

Coral reefs are among the most biodiverse and productive ecosystems on the planet. Although they occupy an area of less than 1% of the ocean, they play an important role in renewing fish stocks. In addition, they have great pharmacological potential and serve as protective barriers for coastal regions. Corals are metazoans and have a close symbiotic relationship with unicellular organisms called zooxanthellae. They have a simple life cycle that comprises two phases: the larval phase (free life) and the polyp phase, which attaches to the substrate, and its reproduction can occur asexually or sexually. The most common form of asexual reproduction occurs through budding from a polyp or through colony fragmentation while sexual reproduction is based on the production of gametes. *Mussismilia harttii* is among the main species that build Brazilian reefs, but it already suffers from constant bleaching events, due to the increase of the ocean temperature, and is under threat of extinction. Some safe techniques to preserve the existence of corals are related to basic knowledge of their reproductive biology. Immunohistochemistry and ultrastructural evaluation applied to the reproductive biology of Brazilian corals is limited to a few reports in the literature. Only two studies have analyzed the morphological organization and ultrastructure of the *M. harttii* coral package and mature gametes. However, studies of gametogenesis based on immunohistochemistry and ultrastructure have never been performed for any Brazilian coral species. The present work used these techniques to characterize the phases of gamete formation of the endemic coral *M. harttii*. The characterization of spermatogenesis and oogenesis will allow us to know in detail about the formation of gametes and will provide subsidies to better understand the structures that may be related to the formation of gamete packages, with spawning and fertilization in the species.

Keywords: Sexual reproduction, reproductive biology, gametogenesis, ultrastructure and conservation.

LISTAS DE FIGURAS

CAPÍTULO 1

CAPÍTULO 3

LISTAS DE ABREVIATURAS E SIGLAS

APACC - Área de Proteção Ambiental Costa dos Corais

ax - Axonemas

CMM - Migração das Células Germinativas

ct - Centríolo

cv - Vesículas Corticais

Ed - Endoderme

er - Reticulo Endoplasmático

FG - Gônada Feminina

fg - Flagelo

fl - Flagelo

fm - Matriz flagelar

fn - Nucléolo Fibrilar

GC - Complexo de Golgi

gn - Nucléolo Granular

h - Horas

HMV - Vesícula Membranosa Heterogênea

IM - Mesentério Imperfeito

L - Litro

lg - Grânulos de Lipídeos

μm - Micrómetro

M - Molar

mc - Muco

MET - Microscopia Eletrônica de Transmissão

MEV - Microscopia Eletrônica de Varredura

MG - Gônada Masculina

Mg - Mesogléia

MGC - Células Germinativas Masculinas

mic - Microvilosidades

min - Minutos

mit - Mitocôndrias

ML - Microscopia de Luz

mL - Mililiitro

mm - Milímetro

MPA - Área de Proteção Marinha

Mt - Mesentério Germinativo

Nc - Nucléolo

nm - Nanomêtro

Nu –Núcleo

OC I - Oócitos em Estágio I

OC II - Oócitos em Estágio II

OC III - Oócitos em Estágio III

OCT I - Oócitos em Estágio I

OCT II - Oócitos em Estágio II

OCT III - Oócitos em Estágio III

OsO4 - Tetróxido de ósmio

PGCs - Células Germinativas Primordiais

PM – Mesentério Perfeito

RM – Músculo Retrator

s - Segundos

SC I - Cistos Espermático em Estágio I

SC II - Cistos Espermático em Estágio II

SC III - Cistos Espermático em Estágio III

SPZ - Espermatozoides

sv - Vesículas Menores

sym – Celulas *Symbiodinum-Like*

um - Mitocôndria unida

v - Vesícula elétron-densa

v/v - Volume/Volume

yb - Corpos de Vitelo

SUMÁRIO

INTRODUÇÃO

Os corais são animais cnidários da classe Anthozoa da ordem Scleractinia, construtores de recifes e estão entre os ecossistemas de maior biodiversidade e produtividade do planeta (Vilaça, 2002). Ainda que os recifes de coral ocupem uma área inferior a 1% do oceano, estima-se que pelo menos um quarto de toda a vida marinha depende diretamente deste ambiente para sobrevivência (Mulhall, 2007; Wilkinson, 2008). Estes organismos exercem um importante papel na renovação dos estoques pesqueiros, gerando alimento e fonte de renda para milhares de comunidades em todo o mundo. Além disso, os corais têm grande potencial farmacológico e são resistentes a choques provocados pela ação mecânica das ondas e correntes marítimas (Hoegh-Guldberg, 2007). São compostos por organismos marinhos (vegetais e animais), os quais formam uma rede de associações e eventos em constante progressão (Castro & Zilberberg 2016).

Os corais apresentam uma relação simbiótica com algas dinoflageladas do gênero *Symbiodinium*, associação essa que surgiu na era Mesozoica, logo após uma quase extinção (Stanley Jr, 1988). Assim, após o período triássico, microalgas denominadas zooxantelas se diferenciaram próximo aos corais simbiônticos (Frankowiak et al., 2016; Marcelino & Verbruggen, 2016). As zooxantelas são responsáveis pela fotossíntese possibilitando o desenvolvimento dos recifes. Além de dar coloração ao animal, as zooxantelas vivem no interior de células específicas chamadas de simbiossoma, onde estão protegidas e recebem suprimentos e nutrientes fundamentais para a realização da fotossíntese. Em contrapartida, elas fornecem ao seu hospedeiro substratos provenientes da fotossíntese que são utilizados pelos corais para nutrição e crescimento (Garrido et al., 2016; Fonseca et al., 2017).

Os corais apresentam um ciclo de vida simples que compreende duas fases: a fase larval (vida livre) e a de pólipo, que se fixa ao substrato (Ventura & Pires 2009), e sua reprodução

pode ocorrer de forma assexuada ou sexuada. A forma mais comum de reprodução assexuada ocorre através do brotamento de um pólipo que se desprende do organismo parental ou através da fragmentação da colônia (Veron, 2000; Twan et al., 2006). Esse processo continua durante toda a vida do animal, sendo que os novos corais são clones da colônia parental. A reprodução sexuada consiste na produção de gametas (espermatozoides e oócitos), promovendo a diversidade genética por meio da fecundação cruzada entre indivíduos (Harrison & Wallace, 1990; Richmond, 1997; Harrison, 2011). Os corais podem desenvolver colônias ou pólipos de um único sexo (masculino ou feminino), ou hermafroditas. Nos hermafroditas, o animal desenvolve ambos os sexos, podendo ocorrer colônias de pólipos hermafroditas, ou colônias de pólipos machos e fêmeas (Harrison, 2011; Pires et al., 2016). Os gametas masculinos e femininos são formados no mesmo pólipo, em diferentes regiões do mesentério. Desse modo, quando maduros, a parede do mesentério se rompe, liberando oócitos e espermatozoides para o interior da cavidade gastrovascular, onde são envoltos por uma camada de muco formando um pacote compacto (Pires et al., 1999; Valente et al., 2023). No período da desova os pacotes com os gametas são liberados pelas bocas dos pólipos (Kinzie, 1996). Quando eliminados na água, os pacotes flutuam até atingir a superfície do mar e se rompem liberando os espermatozoides e oócitos na água para a fecundação (Pires et al., 2016).

O Oceano Atlântico Sul abriga 16 espécies de corais pétreos (verdadeiros) de águas rasas, sendo cinco delas endêmicas do Brasil (Castro & Zilberberg 2016). O coral-couve-flor (*Mussismilia harttii*) pertencente a família Mussidae, apresenta cálices separados com aspecto dicotômico, sem disposição de ramos laterais, embora três padrões morfológicos distintos possam ser observados, conforme descritos por Laborel, (1969). Assim, as formas laxas apresentam cálices muito separados e são encontradas em águas lentas, enquanto cálices densamente concentrados são encontrados em águas mais agitadas e caracterizam a forma confertifolia. Os corais com morfologia intermediária reúnem todas as outras formas que não

apresentam características semelhantes às dos outros dois tipos. A colônia exibe coloração heterogênea em tons de marrom, cinza, amarelo e verde e apresentam traços primitivos que remetem ao período terciário da bacia sedimentar do Atlântico (Laborel, 1969). A *M. harttii* é encontrada naturalmente no ambiente marinho, desde o Norte do Espírito Santo, Pernambuco e até o Rio Grande do Norte. Encontra-se em maior abundância em águas rasas, de 2 a 3 metros de profundidade e turbidez intermediária. Contudo, sua ocorrência já foi registrada em águas mais profundas, entre 15 e 30 metros e eventualmente, a 80 metros de profundidade (Laborel, 1969). Está entre as principais espécies responsáveis pela construção dos recifes brasileiros, porém encontra-se sob ameaça de extinção (LVFBAE, 2018) pois já vem sofrendo com eventos constantes de branqueamento, decorrentes de doenças e do aumento da temperatura do oceano nos últimos anos (Castro & Pires 1999; Leão et al., 2016). Nesse sentido, anomalias térmicas potencializadas pelo El Niño ocorreram no primeiro semestre de 2019 e causaram o branqueamento de cerca de 80% das colônias de *M. harttii* no Parque Marinho do Recife de Fora (Porto Seguro, BA). Porém, a extensão desse dano ainda é desconhecida (Godoy et al., 2021).

Assim como para vertebrados, o conhecimento detalhado da biologia reprodutiva dos diversos grupos de animais invertebrados é fundamental para o desenvolvimento de estratégias de preservação. Particularmente, são raros os trabalhos que abordam a gametogênese nas espécies de corais de ocorrência exclusiva na costa brasileira (Pires et al., 1999; Neves & Pires 2002). Estudos sobre a gametogênese em corais escleractíneos ao redor do mundo revelam características importantes para o sucesso na reprodução sexuada (Harrison, 2011). De acordo com estudo realizado por Shikina et al., (2015), anticorpos anti-vasa específicos para coral *Euphyllia ancora* (Eavas) marcou positivamente espermatogônias não agrupadas ao lado da mesogleia (estágio 0), espermatogônias agrupadas (estágio I) e espermatócitos primários (estágio II) dos mesentérios masculinos. A marcação para Eavas foi sutil e quase indetectável

nos espermatócitos secundários, espermátides e espermatozoides (estágio III-V). Os estudos apresentados por Shikina et al., (2015; 2016; 2020) não revelam apenas características celulares únicas sobre o tecido mesentérico do coral *Euphyllia ancora*, mas também estabelece técnicas básicas para futuros estudos sobre os estágios de desenvolvimento das células germinativas na espermatogênese.

Adicionalmente, o conhecimento acerca da ultraestrutura de corais além de escasso, não contempla espécies originárias da costa brasileira. A análise ultraestrutural da gametogênese de corais tem sido utilizada como ferramenta para investigar relações filogenéticas entre espécies de corais, como demonstrado na comparação filogenética em 12 espécies de Antozoários do Oceano Atlântico – Mediterrâneo (Schimidt et al., 1979). Ainda, a abordagem ultraestrutural em duas espécies de corais, *Pocillopora damicornis* e *Pocillopora elegans*, pertencentes a família Pocilloporidae possibilitou identificar inúmeras características semelhantes na disposição estrutural das células durante a gametogênese (Steiner & Cortés, 1996). Ademais, os pacotes formados por oócitos-espermatozoides liberados durante o período de reprodução também foram objetos de estudos ultraestruturais (Richmond et al., 1997). Assim, foi possível determinar o tempo necessário para abertura dos pacotes, refletindo no êxito durante a atividade reprodutiva (Wolstenholme et al., 2004; Padilla-Gaminõ et al., 2011).

Grupos de pesquisa têm desenvolvido alguns estudos voltados à conservação do coral *M. harttii* no Brasil, no entanto, existem lacunas no conhecimento básico acerca da sua biologia reprodutiva. A avaliação ultraestrutural aplicada à biologia reprodutiva dos corais brasileiros se limita a dois estudos realizados por Valente et al., (2023; 2024). Nestes trabalhos investigou-se a organização morfológica e ultraestrutural dos pacotes e gametas maduros do coral *M. harttii*. No entanto, avaliações imuno-histoquímicas e de imunofluorescência, assim com a avaliação ultraestrutural da formação dos gametas (gametogênese) nunca foram realizadas para quaisquer espécies de corais brasileiros.

Nesse contexto, o presente trabalho utilizou, de forma inédita, técnicas de imuno marcação (peroxidase e fluorescência) e microscopia eletrônica para caracterizar as fases de formação dos gametas, além de contribuir com subsídios para a compreensão das estruturas envolvidas na formação dos pacotes de espermatozoides-oócitos. Adicionalmente, a aplicação das técnicas mencionadas possibilitou conhecer a formação dos gametas masculinos e femininos de forma detalhada contribuindo para a compreensão da formação dos pacotes, desova e manutenção da viabilidade dos gametas. Portanto, essa tese gerou um conhecimento básico, sólido e crucial que servirá de referência para futuros estudos relacionados na área. A associação desses conhecimentos somará esforços que poderão ser utilizados para a conservação dessa espécie endêmica da costa brasileira.

JUSTIFICATIVA

O coral couve-flor (*Mussismilia harttii*), uma das principais espécies construtoras dos recifes brasileiros, está ameaçada de extinção devido a eventos constantes de branqueamento decorrentes do aumento da temperatura do oceano e poluição. Por essa razão, é fundamental compreender como a biologia reprodutiva pode ser afetada por um ambiente em constante mudança, bem como a sua capacidade de adaptação a essas condições, além de suportar o desenvolvimento de estratégias de conservação. Nesse sentido, poucos relatos na literatura utilizaram técnicas de imuno-histoquímica, imunofluorescência e avaliação ultraestrutural como ferramentas para estudar a biologia reprodutiva de corais. Essas técnicas nunca foram utilizadas para avaliar a gametogênese de nenhuma espécie de coral brasileiro, até o momento. Neste contexto, o presente trabalho visa caracterizar a gametogênese do coral endêmico *M. harttii* utilizando, pela primeira vez, as técnicas de imuno marcação e microscopia eletrônica. A caracterização ultraestrutural e histomolecular da espermatogênese e oogênese nos permitirá compreender melhor os mecanismos envolvidos na formação dos gametas, desova, formação dos pacotes de gametas e na fecundação. Assim, com os achados deste estudo esperamos

agregar conhecimento consistente e que possibilitará compreender a biologia reprodutiva da *M. harttii*, com aplicação prática em biotécnicas de preservação e conservação desta espécie endêmica da costa brasileira.

OBJETIVOS

Objetivo Geral

Investigar a gametogênese do coral *Mussismilia harttii* ao longo do ciclo anual, visando compreender o desenvolvimento dos gametas, a dinâmica de formação dos pacotes de espermatozoides-oócitos e como a morfologia dos gametas maduros auxiliam no sucesso reprodutivo.

Objetivos específicos

ARTIGO 1: Morphofunctional evaluation of gametogenesis in the endemic South Atlantic reef-builder *Mussismilia harttii*

- Avaliar a oogenêse e espermatogênese por meio da microscopia de luz, ensaios imuno histoquímico e de imunofluorescência.
- Avaliar as características morfofuncionais da oogenêse e espermatogênse;
- Investigar a organização das gônadas e a disposição dos gametas em seu interior.
- Avaliar as principais características reprodutivas para a espécie;

ARTIGO 2: Evaluating the maturation of male and female gametes in the South Atlantic endemic coral *Mussismilia harttii* using ultrastructural analysis

- Avaliar a oogenêse e a espermatogênese por meio da microscopia eletrônica de varredura e transmissão.

- Avaliar as caracteristicas morfoultraestuturais durante a oogenêse e espermatogênse;
- Identificar e mapear as organelas presentes nas células germinativas masculinas e femininas, bem como inferir suas principais interações.

ARTIGO 3: Ultrastructural evaluation of the oocytes and spermatozoa of the scleractinian coral *Mussismilia harttii*

- Caracterizar a ultraestrutura dos espermatozoides utilizando microscopia eletrônica de varredura (MEV) e microscopia eletrônica de transmissão (MET);
- Caracterizar a ultraestrutura dos oócitos utilizando MEV e MET;
- Identificar e localizar as organelas presentes nos oócitos e espermatozoides e inferir suas principais funções.

RESULTADOS

CAPÍTULO 1

ARTIGO 1 – "Morphofunctional evaluation of gametogenesis in the endemic South Atlantic reef-builder *Mussismilia harttii*"

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Morphofunctional evaluation of gametogenesis in the endemic South Atlantic reef-builder *Mussismilia harttii*

Journal:	*Reproduction*
Manuscript ID	Draft
mstype:	Research paper
Date Submitted by the Author:	n/a
Complete List of Authors:	Valente, Wanderson; Federal University of Minas Gerais, Department of Morphology Cardoso, Bárbara Ramalho Ladeira; Federal University of Minas Gerais, Department of Morphology Cotrim, Francisco de Assis Pinheiro; Federal University of Minas Gerais, Department of Morphology Pastrana , Yugo Moraes ; Federal University of Pernambuco, Biochemistry Amaral, Amanda Pereira ; Federal University of Pernambuco, Biochemistry Pereira, Pedro Henrique Cipresso ; Federal University of Pernambuco, Oceanography França, Luís Guilherme ; Federal University of Pernambuco, Oceanography Lima, Gislaine ; Federal University of Sao Paulo, Health and Sea Science Gradutate Program Costa, Guilherme; Federal University of Minas Gerais Institute of Biological Sciences, Morphology Lacerda, Samyra; Federal University of Minas Gerais, Morphology Godoy, Leandro ; Federal University of Rio Grande do Sul, Animal Biology Graduate Program Avelar, Gleide; Federal University of Minas Gerais Institute of Biological Sciences, Morphology
Keywords:	Coral reproduction, Histological Analysis, In Vitro Fertilization, Hermaphrodite Coral, Coral Conservation

Morphofunctional evaluation of gametogenesis in the endemic South Atlantic reef-builder *Mussismilia harttii*

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Abstract

Decline of coral reefs worldwide emphasizes the need for biological information to subside species conservation and management strategies. Herein, we thoroughly explored gametogenesis of the endemic and endangered Brazilian coral *Mussismilia harttii*. A distinct distribution of male (proximal) and female (distal) regions in the same mesentery confirms the hermaphroditism of *M. harttii*. Primordial germ cells (PGCs), originated from endoderm, expressed VASA/DDX4 and found the mesoglea as physical support and source of nutrition during gametogenesis. Based on the monthly follow-up study, we found three stages of gamete maturation. In spermatogenesis, the germ cell maturation inside the mesogleal compartment, which forms the stage I cysts, coincides when co-inhabiting with stage III oocytes. The stage II cysts incorporate new migrating germ cells and hold germ cell differentiation as well. In stage III, spermatozoa are organized in rows and exhibit mature characteristics, such as round to oval heads and long flagella. Oogenesis, on the other hand, begins with the differentiation of PGCs into oogonia, which migrate to the mesoglea and give rise to primary oocytes. These oocytes undergo growth and differentiation processes, becoming secondary oocytes. In stage III, mature oocytes exhibit a high concentration of lipid granules, yolk bodies and disassemble of the nuclear membrane. *M. harttii* populations have been dramatically reduced in Brazilian waters with high extinction risk. In this regard, our findings shed light into the current knowledge of endemic scleractinian coral reproduction, with potential application to develop biotechnologies, such as *in vitro* fertilization, aiming to safeguard this endangered coral species.

Keywords: Coral reproduction, Histological Analysis, In Vitro Fertilization, Hermaphrodite Coral, Coral Conservation.

Introduction

Coral reefs are recognized as one of the most biodiverse ecosystems in the world and, due to the intense anthropogenic activities, these organisms are currently severely threatened (Hughes 2019; Hoegh-Guldberg 2023). Among the multiple consequences of the human-made environmental imbalance, bleaching events, due to rising ocean temperatures, have become more frequent in the recent years (Hughes *et al*. 2018, 2019; Godoy *et al.* 2021, Hoegh-Guldberg 2023; Pereira *et al.* 2024). To protect this ecosystem, it is crucial to understand the reproductive biology of corals to establish conservation strategies.

Scleractinian corals, the most frequent reef's founder, demonstrate two primary modes of reproduction: asexual reproduction, which occurs through mechanisms such as fragmentation or the sprouting of the polyp (Wallace 1985, Ayre & Resing 1986), and sexual reproduction, characterized by the release of oocytes and sperm into the surrounding seawater (Babcock et al. 1986). According to studies published to date, more than 86% of coral species release their gametes into the water for fertilization (Baird *et al*. 2009; Harrison 2011).

Regarding sexual reproductive strategy, some coral species are gonochoric, in which colonies or polyps are from a single sex (male or female), while others are hermaphroditic (Orejas, 2023). In the case of hermaphroditic corals, a single colony

contains both male and female sexes, which can manifest as hermaphroditic polyps or with the occurrence of both male and female polyps in the same colony (Richmond 1997, Harrison & Wallace 1990, Harrison 2011). So far, studies published indicate that approximately 65% of known scleractinian coral species are predominantly hermaphroditic (Guest *et al.* 2008, Baird *et al.* 2009).

In spite of the existence of more than 840 known species of scleractinian corals in the world (Veron 2000, Madin *et al.* 2016, Veron *et al.* 2016), studies approaching the reproductive biology of corals are still scarce (Baird *et al.* 2009). In this scenario, the number of scleractinian inhabiting the Brazilian coast corresponds to approximately 2% of these known species, of which only 5 are recorded as Brazil's endemic (Castro & Zilberberg 2016). Although the relevance of these organisms as reef-builders and also for positioning Brazil as the only country with biogenic reefs in the South Atlantic Ocean, little is known regarding their reproductive strategies (Pires *et al.* 1999, Neves & Pires 2002, Valente *et al.* 2023). This gap of knowledge has a particular meaning to the development of conservation programs, since these endemic scleractinian species are under extinction threatening (ICMbio 2018), victims of bleaching phenomena (Castro & Pires 2001, Leão et al. 2016, Godoy et al. 2021, Pereira et al. 2022; 2024).

Among the scleractinian Brazilian's endemic corals, the *Mussismilia harttii,* also known as cauliflower coral, is distributed from the south of Rio Grande do Norte State to the north of Espirito Santo State, which represents an area of occupancy of approximately 2,000 m^2 (IUCN, 2021). Indeed, the remarkable prevalence of *M. harttii* at the Maragogi's Protection Zone for Marine Wildlife in comparison to the others

environmental protection areas along the Brazilian coast, means the important role this species plays in the reef's ecosystem (ICMBio, 2015)

Nevertheless, the recognized role of *M. harttii* contrasts with the absence of studies approaching its reproductive biology. In this regard, studying gametogenesis is crucial to better comprehend the mechanisms involved in gamete formation as well as the adaptive strategies for the ever-changing environment (Peixoto et al. 2024). So that, revealing the steps of fertilization, for instance, it is an important overcoming to establish well-successful conservation programs (Cameron & Harrison 2020).

In our research, we thoroughly evaluated gametogenesis, describing the organization of the gonads, the arrangement of the gametes, and the main reproductive characteristics for *M. harttii*. To reach these goals, we performed histological analyses, which allowed us to obtain valuable information regarding the reproductive biology characteristics of this endemic coral species.

Material and Methods

Location and permits

The samples were collected at Marine Protected Area (MPA) Costa dos Corais (8º42'16" south latitude and 35º04'40" west longitude), in the municipalities of Tamandaré (PE), São José da Coroa Grande (PE), Maragogi (AL) and Japaratinga (AL) (Fig. 1) (see study area definition at Pereira et al. 2024). The analyses were performed at the Laboratory of Cellular Biology, in the Department of Morphology of the Institute of Biological Sciences of the Federal University of Minas Gerais, located in Belo Horizonte,

Minas Gerais (MG). This research was approved by the Chico Mendes Institute for Biodiversity Conservation - ICMBio (SISBIO No. 78827).

Polyps field collection

Six polyps from different colonies of the coral *Mussismilia harttii* were collected at APACC monthly from October 2021 to December 2022. After collection, the polyps were identified and transferred to fixative solutions and then prepared for light microscopy (n=30), immunohistochemistry and immunofluorescence (n=15).

Light Microscopy

For the histological analysis, polyps (n=30) were fixed in 4% v/v glutaraldehyde with 0.05 M phosphate buffer solution, following the methodology described by Graham & Orenstein (2007), and remained in this solution until the histological processing. The fixed material was decalcified in a solution of 10% v/v formic acid + 5% v/v formalin for 48 hours (Banu *et al.* 2024). After decalcification, the samples were washed in running water (24 hours) to remove excess decalcifying solution. Dehydration was carried out in an increasing series of alcohol concentrations (70% to 100% v/v). After dehydration, fifteen polyps were embedded in glycol methacrylate (Historesin, Leica, Germany), and sections with 4 µm thickness were obtained with a rotative microtome (Leica, RM2255, Germany) using glass knives. Slides were stained with hematoxylin/eosin (HE). Another set of polyps (n=15) were embedded in Paraplast®, and the 5 µm sections were stained

with Masson's trichrome. All samples were mounted with Entellan (Merck, Frankfurt, Germany) and photo documented using an Olympus BX60 photomicroscope (Tokyo, Japan) with an Olympus DP73 camera attached.

Immunohistochemistry

The polyps (n=15) were fixed in a 20% v/v zinc-formalin solution following the methodology described by Layton *et al.* (2018). After fixation, the samples were transferred to 70% v/v alcohol and remained until histological processing. Decalcification took place in a solution of 10% v/v formic acid + 5% v/v formalin, following the methodology described by Banu *et al. (*2024) and embedded in Paraplast®. Sections 5 µm thick were obtained, and after hydration, they were incubated for 30 minutes in a 3% v/v solution of H_2O_2 (hydrogen peroxide) (Sigma, St. Louis, USA) in methanol to block endogenous peroxidase. After blocking, antigen retrieval was carried out for 30 minutes in a proteinase K solution at a concentration of 10ng/mL diluted in PBS. The sections were then incubated with the primary antibody anti-DDX4 (Vasa) (1:100 dilution; produced in rabbits; Abcam, ab13840) for 48 hours at 4ºC. After washing the slides in PBS, the sections were incubated with the goat anti-rabbit biotinylated secondary antibody for 60 min (1:100 dilution; produced in goat; Abcam, ab6720) at room temperature. The sections were then incubated for 30 minutes in streptavidin solution (avidin-biotin-peroxidase), followed by exposure of the samples to the substrate diaminobenzidine (DAB) (Sigma) and counterstained with hematoxylin (Merck) for

153 visualization. The sections were observed and evaluated using an Olympus BX60
154 microscope (Tokyo, Japan) with an Olympus DP73 camera attached.

155

156 ***Immunofluorescence***

157 The fifteen polyps used for the immunohistochemical assay were also used for
158 immunofluorescence. The fixation, decalcification, dehydration, embedding, and
159 microtomy steps were previously described. After deparaffinization and hydration, the
160 samples were immersed in citrate buffer solution (pH 6.0) and heated in a water bath for
161 40 min at 98ºC for antigen recovery.

162 The histological sections were then incubated at 4°C for 48 hours with the primary
163 antibody anti-DDX4 (Vasa) (dilution 1:100; produced in rabbits; Abcam, ab13840). After
164 washing the slides in PBS, they were incubated with the secondary antibodies anti-rabbit
165 – FITC (1:500; produced in goat; Sigma-Aldrich, F0257) for 1 hour in the dark and at
166 room temperature. Finally, the slides were mounted with Fluoromount G mounting
167 medium (EMS, Hatfield, USA) and evaluated under a fluorescence microscope (Olympus
168 BX53F, Japan).

169

170 ***Percentual of germinative components***

171 The evaluation of germinative components distribution along the mesentery was
172 performed using a reticle with 441 intersections (points) per field, at 5x magnification.

For an individual polyp (n=15) from each month of collection, 30 random fields were analyzed, giving a total of 13,230 points

The evaluation focused on male regions, including the male mesentery, spermatozoa, and sperm cysts, as well as female regions, which encompassed the female mesentery and oocytes. After obtaining the percentage of the different reproductive components evaluated, it was possible to determine the frequency (%) between male and female regions.

Histomorphometric evaluation

The images of oocytes and sperm cysts obtained by light microscopy were qualitative and morphometric assessed to gather information regarding the components of gametogenesis. Morphometric analyses were performed using ImageJ software (Microsoft Java 1.1.4.), with quantitative data presented as mean ± standard deviation. The following equation was used to determine the diameter of oocytes and sperm cysts (D):

$$D = \frac{larger\ diameter + smaller\ diameter}{2}$$

Statistical analysis

The data obtained was tested for normality using the Kolmorogov-Smirnov test and for homoscedasticity using Fisher's test. The means of the variables that showed a normal distribution were analyzed using the ANOVA test, and when they did not follow

194 the Gaussian distribution, the Kruskal-Wallis's test was used. For the correlation,
195 Pearson's test was used to examine the relationship between the variables. The data was
196 statistically processed using GraphPad Prism 6.0 for Windows, version 6.01. The
197 significance level was set at $p < 0.05$, and the values represented as mean ± standard error
198 of the mean (SEM).

199 **Results**

200 ***Organization and arrangement of the gonads***

201 Mesenteries were observed from the oral to the aboral body ends and, developing
202 into the extracellular compartment, between the endoderm cells layers, male and female
203 gonads were present (Fig. 2A and B). The male gonads were located substantially close
204 to the oral (upper) region, while the female gonads were positioned in the aboral (lower)
205 region of the polyp and between the retractor muscles (Fig. 2B)

206

207 ***Reproductive strategy of Mussismilia harttii***

208 From the histological analyses, we confirmed that *Mussismilia harttii i*s a
209 hermaphrodite species, displaying female and male gonads in the same individual (Fig.
210 2). Although the gametes occupancy rate during the period of maximum cellular
211 maturation reached approximately 90% of the mesenteries, the distribution between male
212 and female gonads was quite similar, with no differences being reported (Fig. 3A). Based
213 on the monthly follow-up study, we found three stages of gamete maturation (Fig. 3B).
214 Immature oocytes (I) were observed for the first time in November/2021, when the sea
215 surface temperature was around 28º C (Fig. 3A-B). This cell type predominated until

March/2022, when the water temperature reached 29.5º C. So that, in April/2022 an intermediate stage of oocyte maturation was observed (II). However, in late April the temperature started to fall, and in June/2022, the most mature oocyte (III) was seen for the first time. Interestingly, early forms of spermatogenic cysts were identified at this period and were located proximally in the same mesenteries in which mature oocytes were present. Thus, all steps of spermatic maturation evolved from June to August, a narrow window of time when compared with the period demanded for the formation of female's gametes. After reaching the lowest temperature in July, around 25º C, sea water exhibited an increase, which preceded the spawning (Fig. 3A-B). Since female and male gametes reached maturity synchronously in August/2022, the last few weeks represented a final preparation for the spawning that took place between September and October/2022 and is the event that encloses the annual reproductive cycle.

Spreading behavior of female and male gametogenic areas in the mesenteries and fluctuation of sea water temperature were significatively correlated. Overall, gametogenic tissue occupancy was enlarged because of the decreasing temperature of water (female region, r= -0.53, 95%CI -0.82-0.02, P= 0.042; male region, r= -0.76, 95%CI -0.91-0.41, P= 0.0009).

General aspects of gametogenesis

Cells presenting a positive labeling for the VASA undifferentiated cell marker (Fig. 4A and B) and alkaline phosphatase (Supl. Fig. 1) were observed in the endoderm of the mesenteries. The initial differentiation of the $VASA^{+}$ cells, which resulted in oogonia (distal end) or spermatogonia (proximal end), took place in the endoderm.

238 Migration towards the mesoglea defines the beginning of the maturation process. The
239 final maturation of the gametes resulted in the disruption of the mesentery wall, releasing
240 oocytes and spermatozoa into the gastrovascular cavity. Thus, male and female gametes
241 were surrounded by a mucous layer that organizes compact bundles, which are released
242 through the polyps' mouths at spawning time.

243

244 ***Spermatogenesis***

245 As aforementioned, the germ cell maturation process was histologically classified
246 in three steps. Male germ cells migrate from the endoderm (Ed) towards the mesoglea
247 (Mg) (Fig. 5A). Once in the extracellular compartment of the mesentery, male germ cells
248 were arranged into a cystic structure, which was outlined by the mesoglea. At that point,
249 cells from the germinative endoderm were added to the cyst, it was called stage I sperm
250 cyst. During this step, the cyst diameter was approximately 78.1 ± 0.73 μm (n = 422)
251 (Fig. 5D) and cells showed an oval shape and evident nuclei.

252 At stage II, cysts presented a diameter of 100.4 ± 1.39 μm (n = 174), since germ
253 cells were still migrating from the endoderm (Fig. 5B and 5D). However, once inside the
254 cyst, cells showed different nuclear morphologies, which suggests an asymmetric timing
255 of germ cell maturation. Indeed, a densely stained and compacted nucleus was the most
256 frequent type observed, although nuclei with loose arrangement of the genetic material
257 were also distinguished.

258 At the last step of maturation, male germ cells were assembled with the needed
259 tools to successfully perform the fertilization. Thus, at stage III germ cells acquired a

round to oval head and developed a long flagellum, characterizing the mature spermatozoa. A polarized arrangement of the spermatozoa resulted in a bouquet-like cyst, since the flagella converged to the cyst extremity and heads were concentrated in the opposite rim (Fig. 5C). Stage III cysts reached the widest diameter observed during maturation (171.2 ± 1.44 μm; n = 448), indicating a progressive increase in the cysts size (Fig. 5D).

Oogenesis

Early events of oogenesis took place in the endoderm of female germinative mesentery. Thus, oogenesis was launched when the $VASA^{+}$ cells differentiated into more advanced germ cells. After their migration to the mesoglea, these cells developed a homogeneous cytoplasm with low density granules of lipids and yolk bodies (Fig. 6A). Their slightly stained nuclei, suggesting an euchromatic pattern and a transcriptional activity of these cells, contrasted with their remarkable nucleoli. Based on the morphological characteristics, these germ cells were called oocyte I. The nuclear and nucleolar diameters were obtained and corresponded to 26.95 ± 0.43 μm (n = 47), and 16.3 ± 0.52 μm (n = 47), respectively.

In the following phase of female germ cell maturation, the cytoplasm of oocytes at stage II was filled with lipid granules and vitelline bodies (VB). Although VBs were distributed throughout the cytoplasm, they were concentrated in a particular pole of the cell and arranged as an inner layer juxtaposed to the plasma membrane (Fig. 6B). The nucleus and nucleolus were spherical and, despite the uneven VB distribution, centrally

situated in the cell. At this stage, the nucleus was homogeneously stained, and its diameter was 63.4 ± 1.41 µm (n = 122), while the nucleolus diameter was 17.8 ± 0.41 µm (n = 97).

At the last step of maturation, the lipid granules and yolk bodies were abundant and widely dispersed in the cytoplasm of oocytes at stage III (Fig. 6C). Also, these female germ cells were featured by the consistent absence of an evident nuclear apparatus.

In association with the qualitative evidence, measurements of the cellular diameter showed that oocytes III were, approximately, 130% and 65% larger ($p<0.05$) than oocytes II and I, respectively (Fig. 6D).

Discussion

This paper presents unique insights regarding the morphofunctional features of gametogenesis in the endemic, and endangered, Brazilian coral *Mussismilia harttii*. We evaluated the gonadal organization of the mesentery, as well as the gradual development of female and male gametes based on the histological assessment acrossing one year of the reproductive cycle. Also, we found a correlation between germ cell differentiation and sea water temperature. Thus, our data shed some light over the main aspects that address the sexual reproduction of scleractinian corals (Harrison 2011, Shikina et al. 2015) and ensure population variability through cross-fertilization (Richmond 1997, Harrison & Wallace 1990).

Organization and arrangement of the gonads

303 Mesenteries are radial extensions found inside the gastrovascular cavity of polyps
304 that are responsible for coral digestion and reproduction (Bocharova & Kozevich 2011).
305 In *M. harttii*, the male gonads are observed near to the oral region, while the female
306 gonads are situated closely to the aboral end of the polyp. According to Burgess &
307 Babcock (2005), the preferential positioning of male and female gonads along the
308 germinative mesentery has functional implications. Thus, male gametes developing close
309 to the oral region would have the releasing of sperm into the water column facilitated,
310 while the aboral region offers a protected environment for the oocytes, since these cells
311 demand a wider window to proper growth and maturation. This spatial separation of
312 gonads in different regions of the polyps could represent adaptations for sexual
313 reproduction, reduce the risks of self-fertilization and vary according to the species
314 (Kaposi et al. 2023).

315 ***Reproductive strategy of Mussismilia harttii***

316 Hermaphroditism is quite common among scleractinian corals, with many species
317 classified as simultaneous hermaphrodites (Harrison 2011). The organization of the
318 gonads and the presence of both oocytes and sperm in a single individual, or the same
319 polyp, are key characteristics that differentiate hermaphroditic corals from those with
320 separate sexes (Harrison 2011). The observed reproductive behavior, involving the
321 organization of the gonads and the presence of oocytes and sperm in the same individual,
322 provides robust evidence that the coral *M. harttii* is a hermaphroditic species,
323 corroborating the findings of Pires et al. 1999 and Neves & Pires 2002.

In *M. harttii,* oocytes require eight months to mature from stage I to III while male gametes need one third of this period to reach the matured status. Oocyte maturation is associated with vitellogenesis, a process in which the energetic substrate for the new individual development is produced. According to Shikina & Chang (2016), the female germinative mesenteries play an important role in the process of vitellogenesis, which corroborates our hypothesis that *M. harttii* allocates more energy to the development of the female mesenteries to ensure the yolk synthesis and an optimal number of oocytes for successful reproduction (Waller *et al.* 2005).

Synchrony during gametogenesis in scleractinian corals can vary depending on the species (Guest *et al.* 2005, Harrison 2011, Bouwmeester *et al.* 2015 Shlesinger & Loya 2019a). The synchronous process ensures that gametes are released at the most opportune moment for successful reproduction (Shlesinger & Loya 2019b). It is a reproductive strategy to avoid the risk of predation during spawning and fertilization (Jones & Negri 2015). However, because of the climate crisis, some coral species in the Mediterranean Sea are showing reproductive asynchrony (Rossy et al. 2019).

According to our findings, *M. harttii* sperm was identified from the eighth month of oocyte development, corroborating the findings by Pires et al. 1999; Neves & Pires 2002. For the coral *Montipora capitata*, it has been observed that sperm development occurs after the appearance of oocytes (Padilla-Gamiño *et al.* 2011). This phenomenon, known as protogyny, is a common reproductive strategy among marine invertebrates (Petraitis 1990, Levitan & Sewell 1998), including scleractinian corals (Fadlallah 1983).

345 This study found that *M. harttii* spawns between September and October 2022,
346 during the spring. It is already known that spring provides ideal environmental conditions
347 for spawning and fertilization in corals (Gilmour et al. 2016, Osman et al. 2024).
348 Furthermore, the months of September and October coincide with periods of increased
349 sea currents, facilitating the dispersal of larvae to new habitats and increasing their
350 settlement potential (Buck-Wiese et al. 2018). Since the APACC (studied area) shows a
351 calm current cycle between the months of September and October, we suggest that the
352 reproductive success of *M. harttii* would be dependent on variations mainly based on
353 local environmental conditions and species-specific adaptations that may influence
354 spawning.

355 The association between germ cell maturation and water temperature was already
356 mentioned in the literature. Ding and colleagues (2022) found that germinative
357 mesenteries show the best patterns of development when temperature ranges from 25 to
358 29ºC, although in *Goniopora columna,* the suitable temperature for growing and
359 expansion of the mesenteries was defined at 25°C. Additionally, a study by Higuchi *et al.*
360 (2017) on the coral *Acropora solitaryensis* indicated that temperatures between 25-27ºC
361 can trigger the initial stages of sperm development. These findings corroborate our results
362 for *M. harttii* and are consistent with observations from others (Brooke & Järnegren 2013,
363 Okubo *et al.* 2016; Osman *et al*. 2024), suggesting that temperatures between 25-29°C
364 create the optimal environment for the development of key structures, such as germinative
365 mesenteries and the onset of spermatogenesis.

366 ***General aspects of gametogenesis***

Vasa-like proteins are present in the germ cell lines in a range of invertebrates and vertebrates animals, which indicates a well conserved molecular mechanism addressing the formation and maintenance of the germ cells (Isaeva et al. 2009). Similarly, alkaline phosphatase (APH) activity is noticeable in embryonic and initial germ cells in mammals, birds and fish species. Typical APH brick-red staining, observed in cultured mouse embryonic stem cells, was also referred to colonial cnidarians, arthropods, and chordates species already investigated (reviewed in Isaeva et al. 2009). Accordingly, our findings demonstrate for the first time the labeling of PGCs using the DDX4 antibody (Vasa) for an endemic Brazilian coast species of coral. The PGC identity was corroborated through the high APH activity observed on these cells. PGCs are initially observed in the endoderm of the germinative mesenteries, which provides a safe environment to protect them against physical damage and exposure to harmful substances present in the water (Sawall and Al-Sofyani, 2015). For the sea anemone *Nematostella vectensis*, the endoderm assists the differentiation of PGCs and acts as a signal for gonadal development (Perez 2014).

Although it does not participate directly in the differentiation of gametes, the mesoglea acts as a physical and nutritional support during reproductive processes (Lommel *et al.* 2018), maybe mimicking the somatic cells' supporting role. Thus, mesoglea provides structural support for the gametes and maintains the shape of the reproductive tissues (including gonads) (Shikina 2016). In addition, mesoglea directs the traffic of nutrients during gametogenesis. This transport is crucial for the growth and development of germ cells (Yong 2021). Since we found that in *M. harttii,* mesoglea holds the most advanced phases of germ cell maturation, it is reasonable to suggest that

its functions as a provider of physical support and energy supplier throughout gametogenesis as observed for other species, are preserved for *M. harttii*.

Spermatogenesis

After migration, rounded male germ cells form an aggregate and are surrounded with a thin envelope of mesogleal origin. This process, called cystogenesis, results in organized arrangement of germ cells as well as yields a protective environment for maturation of gametes (Milazzo *et al.* 2016, Koutsouveli *et al.* 2020, Bertho *et al.* 2021).

In overall, spermatogenesis begins with the development of spermatogonia, which through mitotic divisions produce spermatocytes that after meiosis, result in haploid cells called spermatids. Spermatids undergo spermiogenesis, transforming into a cell with fertilization ability and then, after release, these cells become mature spermatozoa (Schulz et al. 2010). All these steps were characterized by Chiu and colleagues (2020a, 2020b) through elegant studies of transcriptome assembly from the gonads of *Euphyllia ancora*. Based on our histological findings, spermiogenesis of *M. harttii* is occurring in the cysts and all process is concluded in three months, by the time oocytes are ready to form gamete bundles into the gastrovascular cavity of the polyp, before the spawning event (Valente et al. 2023). Indeed, we suggest that the spermatogonial and spermatocitary phases of spermatogenesis are taking place inside the endoderm of the germinative mesentery. To further investigate this aspect of male gametogenesis of *M. harttii,* ongoing studies of our group are analysing these gonads under transmission electron microscopy.

411 It is not still clear if the early development of oocytes at the bottom of mesentery
412 would contribute with a suitable environment for male germ cell differentiation upwards.
413 However, despite the lack of studies focused on this issue, the synchronic interaction
414 between these two different segments, since spermatic cysts I are observed only after
415 oocytes III, corroborates this hypothesis.

416 It is already known that gametes' morphology is narrowly associated with its
417 function. In this regard, sperm are smaller than oocytes due to their distinct roles in
418 reproduction. The former is motile and prepared to swim through the water column to
419 reach and fertilize oocytes (Levitan 2006, Henley *et al.* 2021). Oocytes, on the other hand,
420 are larger and immotile because they contain the cellular machinery necessary for the
421 initial development of embryos (Voronina 2003). Considering the sperms, not only their
422 morphology plays a role on the reproduction, but also, the manner how these cells are
423 organized inside the cyst. At stage III, sperms are arranged in rows, with heads pointing
424 all to the same side of the cyst and flagella converging to the opposite. This orientation
425 may have functional significance, potentially aiding in the motility and movement of the
426 sperm after the dissociation of the bundles (see Valente *et al.* 2023), since by positioning
427 the flagella in this way, sperms are prepared for coordinated and efficient swimming,
428 increasing their ability to reach and fertilize oocytes efficiently (Kholodnyy *et al.* 2020).

429 ***Oogenesis***

430 Oogenesis is divided into several stages, including the first step in which PGCs
431 differentiate to oogonia (Eckelbarger & Hodgson 2021). These cells pass through mitotic
432 divisions that result in primary oocytes (Shikina *et al.* 2012). Besides the remarkable

433 change in size, as a result of energy reserves accumulation, oocytes I reside inside
434 mesoglea (Eckelbarger & Hodgson 2021). Once these cells colonize the extracellular
435 compartment of mesentery, they advance towards the maturation process. Thus, the
436 upcoming stages are characterized by the presence of large amounts of lipidic granules,
437 as already observed in *Junceella juncea* oocytes (Tsai *et al.* 2014), yolk bodies and
438 acquisition of cortical vesicles (Pires *et al.* 1999). It is worth mentioning that the
439 regulation of lipid granules in oocytes may involve a combination of cellular processes
440 and regulatory factors (Lin *et al.* 2018). Kramarsky-Winter (2015) found that
441 environmental factors such as water temperature and pH influence the reduction of lipid
442 granules in oocytes in anthozoans, particularly scleractinian corals. In the sea anemone
443 *Nematostella vectensis* was demonstrated that high temperatures affect the enzymes
444 responsible for lipid synthesis, breakdown, and transport, leading to a decrease in the size
445 and number of lipid granules within the oocytes (Lebouvier 2021).

446 In our conditions, yolk bodies were first observed at stage II oocytes, surrounding
447 the cytoplasmic layer of the cellular membrane. Yolk bodies are specialized structures
448 rich in lipids and proteins (Eckelbarger & Hodgson, 2021), and their location, as we
449 recorded, may suggest a functional meaning for transporting and distribution of nutrients
450 within the oocyte. A strikingly morphofunctional feature of *M. harttii* stage III oocytes
451 was the absence of nuclei and nucleoli. The loss of the nuclear membrane and the
452 dispersion of genetic material in the cytoplasm, known as nuclear collapse or nuclear
453 reorganization, occurs during the final stages of oocyte maturation (De Souza & Osmani
454 2007). This process is part of oocyte activation, allowing the genetic material to become
455 more accessible to spermatozoa during fertilization (Santella et al. 2020).

456 In summary, this study describes the gametogenesis of *Mussismilia harttii*, a
457 scleractinian coral endemic to the South Atlantic Ocean. The results presented here will
458 support future studies aiming to better understand the reproductive biology of
459 scleractinian corals, as well as the development of biotechnologies such as gamete
460 cryopreservation and *in vitro* fertilization, as important tools applied to restore and
461 conservative programs, approaching severely threatened species. Also, knowing the
462 normal parameters that drive the gametogenesis of *M. harttii* is a meaningful step forward
463 needed to recognize any disruptive effects of biotic or abiotic interferences on this rich
464 ecosystem.

465

466 **Declaration of interest**

467 The corresponding author declares, on behalf of all authors, that there is no conflict of
468 interest that could be perceived as prejudicing the impartiality of the research reported

469

470 **Funding**

471 This work was supported by the Brazilian Biodiversity Fund (Funbio), which granted a
472 field research scholarship and Humanize Institute (No. 121/2019), which contributed to
473 the acquisition of equipment and reagents used during the collection and processing of
474 samples.

475

476 **Authors contributions**

WV, GFA and LG conceived the study; YMP, APA, PHCP, LGF and GVL collected the biological material; WV, BRLC, FAPC prepared the samples; WV and BRLC carried out the immunohistochemistry and immunofluorescence assays; WV and GFA carried out the morphometric analyses; WV, GFA, GMJC, SMSNL and LG drafted the manuscript; all the authors contributed to revising the manuscript.

Acknowledgements

We thank the Reef Conservation Project (PCR) for the collection and logistical support.

Data availability

Data is available on request.

Ethical approval

The authors declare that all applicable international, national and/or institutional guidelines for sampling, care and experimental use of animals for the study have been followed, all and the necessary approvals from the Chico Mendes Institute for Biodiversity Conservation - ICMBio (SISBIO).

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Figure legends

Fig. 1. Map of the study. Costa dos Corais Environmental Protection Area (APACC), highlighting the municipalities where the collections were conducted during this study on the gametogenesis of the coral *Mussismilia harttii*.

Fig. 2. **Arrangement of the gonads of the coral *M. harttii.*** (A) Low magnification view of a polyp, highlighting the oral (black arrowhead) and aboral (yellow arrowhead)

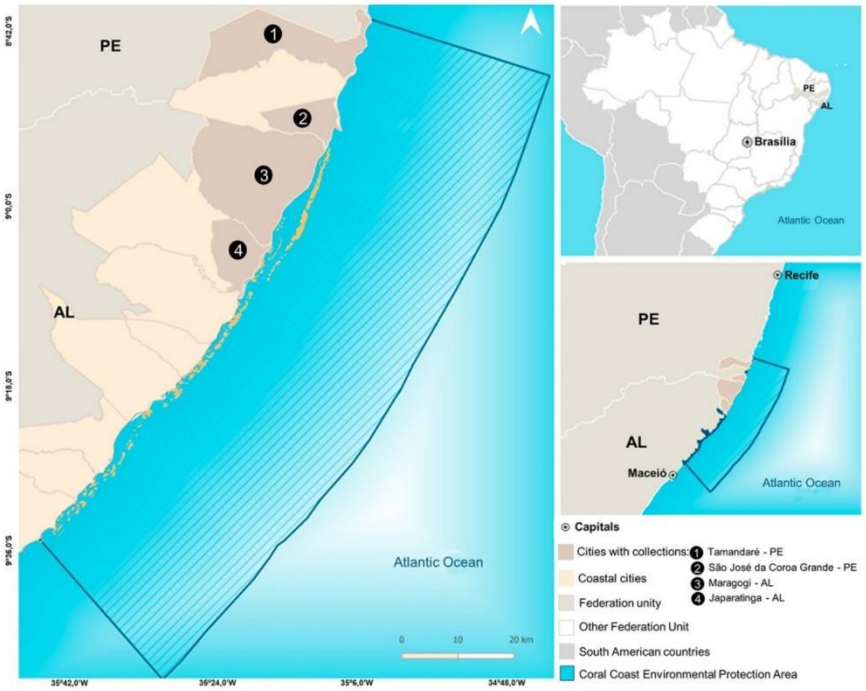
763 regions. Scale bar: 1000 µm. (B) Detailed female gonad (FG), male gonad (MG) and
764 retractor muscle (RM). Scale bar: 500 µm.

765 **Fig. 3. Fig. 3. Gametogenesis of the coral *M. harttii*** (A) Distribution (%) of male and
766 female regions in the germinative mesenteries, sea surface temperatures, period of
767 development of germ cells and spawning time-point (black arrows indicate the spawning
768 months). (B) Timeline depicting male and female gametogenic processes for the species.

769 **Fig. 4 - Primordial germ cells (PGCs) in *M. harttii.*** (A) Immunofluorescence and
770 (B) immunoperoxidase assay to localize $VASA^{+}$ cells (yellow arrowhead) in the
771 endoderm of germinative mesentery. In A' and B', primary antibodies were suppressed
772 (negative control). A and A', scale bar: 100 µm. B scale bar: 25 µm. B' scale bar: 20
773 µm.

774 **Fig. 5. Spermatogenesis of the coral *M. harttii.*** (A) Germinative mesentery (Mt) with
775 stage I spermatic cysts (SC I) adhered to the mesoglea (Mg). Detail of the migration of
776 the male germ cells (CMM) from the endoderm (Ed) to the mesoglea (Mg) (black
777 arrowhead). Scale bar: 10 µm. (B) Male cells within the cysts with oval shape and nucleus
778 intensely stained (yellow arrowheads). Details of secondary cysts (SC II) incorporating
779 new male germ cells (black arrows). Scale bar: 10 µm. (C) Sperm in stage III (SC III),
780 organized in rows and their long flagella converging into a single end of the cyst, giving
781 them a bouquet-like shape (black arrow). After being released from the cysts, mature
782 germ cells are seen into the gastrovascular cavity (Gc). Scale bar: 10 µm. (D) Significant
783 increase in cyst diameter is related to the maturity phase. Statistical differences are
784 represented by different letters, $p < 0.05$.

785 **Fig. 6. Oogenesis of the coral *M. harttii.*** (A) Stage I oocyte (Oc I) adhered to the
786 mesoglea (Mg) with a spherical nucleus (Nu) and a clearly visible nucleolus (Nc). It is
787 also possible to see the presence of a homogeneous cytoplasm with some lipid granules
788 (black arrowhead) and small cortical vesicles (yellow arrowheads). Scale bar: 10 μm. (B)
789 Stage II oocyte (Oc II) near to the endoderm (Ed) of the germinative mesenteries, (Mt)
790 with evident nucleus (Nu) and nucleolus (Nc), many lipid granules (black arrows) and
791 vitelline bodies concentrated at one extremity of the cell and close to the plasma
792 membrane (yellow arrowheads). Scale bar: 10 μm. (C) Stage III oocytes (Oc III) near to
793 the mesoglea (Mg) with a high concentration of lipid granules (black arrow heads),
794 without visible nuclei and nucleoli. Enlargement of the cortical layer in the peripheral
795 region close to the plasma membrane (yellow arrowhead). Scale bar: 10 μm. (D)
796 Significant increase of the oocyte diameter associated with the degree of maturity
797 observed. Statistical differences are represented by different letters, $p < 0.05$.



Map of the study. Costa dos Corais Environmental Protection Area (APACC), highlighting the municipalities where the collections were conducted during this study on the gametogenesis of the coral Mussismilia harttii.

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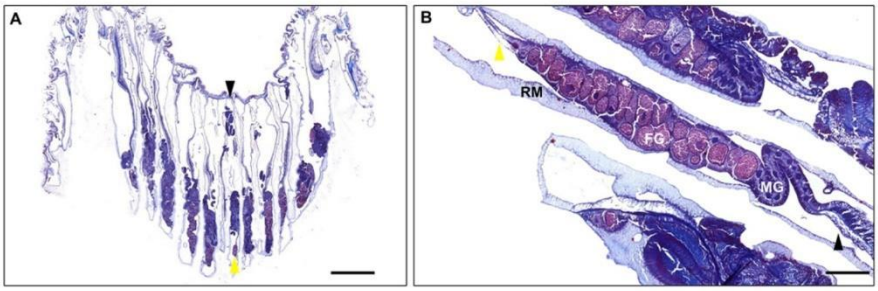

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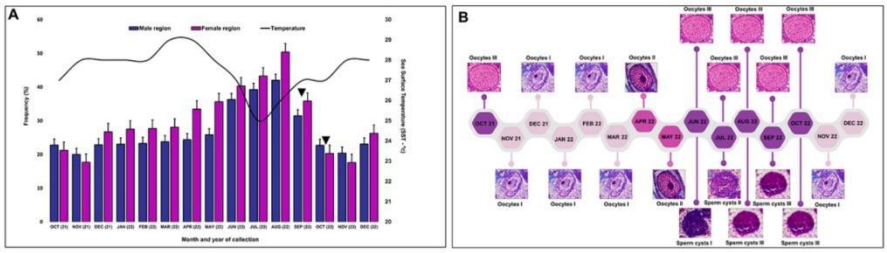

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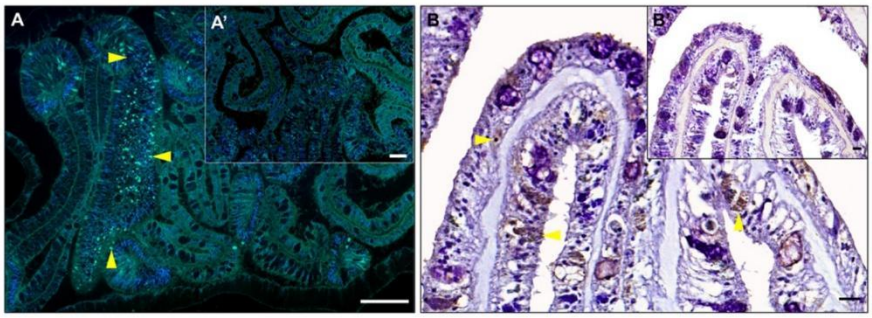

Primordial germ cells (PGCs) in M. harttii. (A) Immunofluorescence and (B) immunoperoxidase assay to localize VASA+ cells (yellow arrowhead) in the endoderm of germinative mesentery. In A' and B', primary antibodies were suppressed (negative control). A and A', scale bar: 100 µm. B scale bar: 25 µm. B' scale bar: 20 µm.

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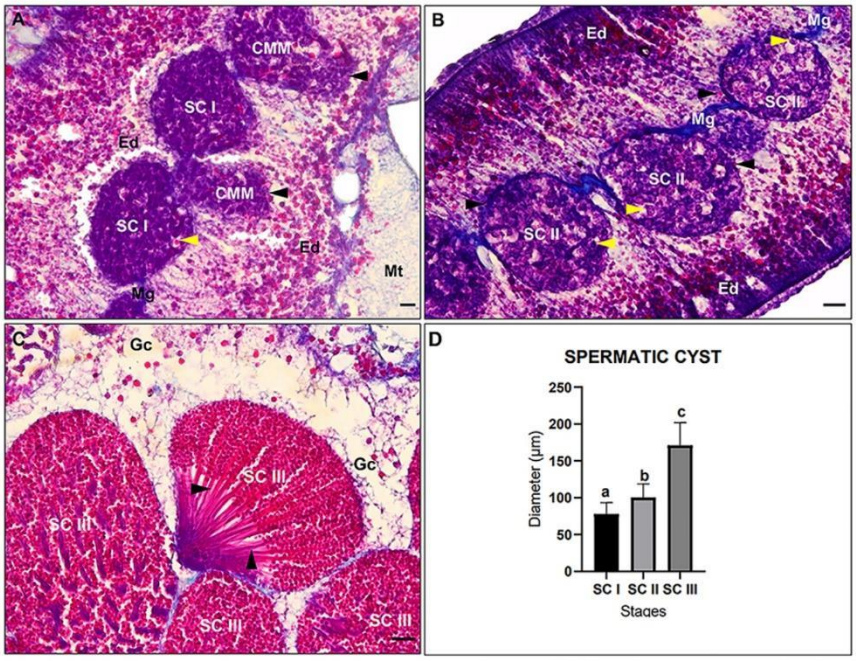

Spermatogenesis of the coral M. harttii. (A) Germinative mesentery (Mt) with stage I spermatic cysts (SC I) adhered to the mesoglea (Mg). Detail of the migration of the male germ cells (CMM) from the endoderm (Ed) to the mesoglea (Mg) (black arrowhead). Scale bar: 10 µm. (B) Male cells within the cysts with oval shape and nucleus intensely stained (yellow arrowheads). Details of secondary cysts (SC II) incorporating new male germ cells (black arrows). Scale bar: 10 µm. (C) Sperm in stage III (SC III), organized in rows and their long flagella converging into a single end of the cyst, giving them a bouquet-like shape (black arrow). After being released from the cysts, mature germ cells are seen into the gastrovascular cavity (Gc). Scale bar: 10 µm. (D) Significant increase in cyst diameter is related to the maturity phase. Statistical differences are represented by different letters, $p < 0.05$.

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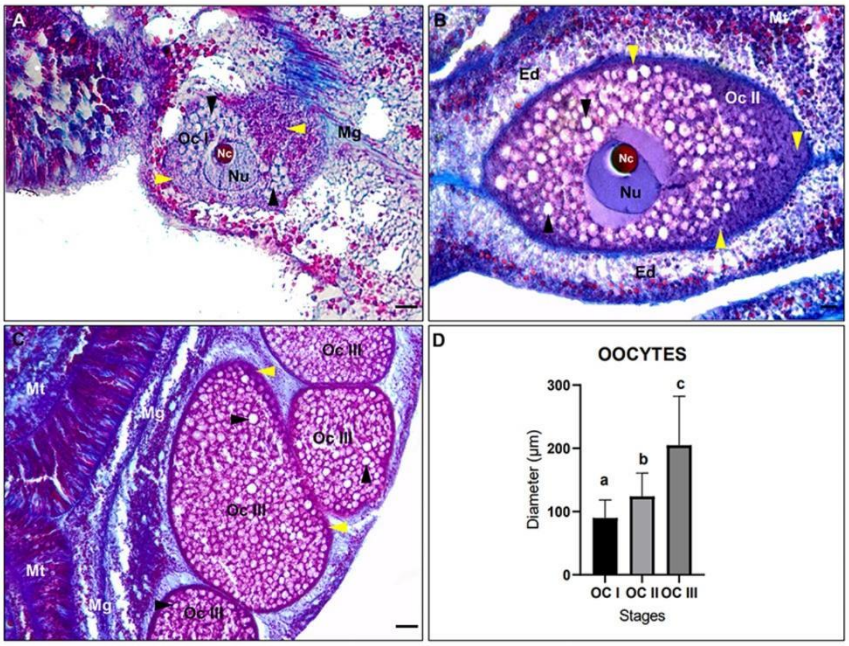

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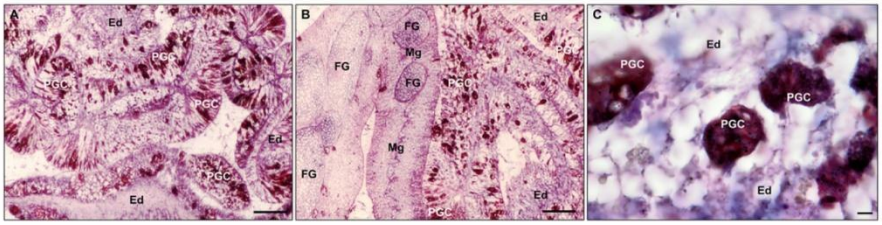

Supplementary figure. Alkaline phosphatase labeling technique for primordial germ cells (PGCs) in tissue. (A) germinative mesentery during proliferative phase with germ cells attached (PGCs) to the endoderm region (Ed) of the polyp. Scale bar: 10 µm. (B). Primordial germ cells (PGC) in the endoderm (Ed) of the germinal mesentery near developing female (FG) gametes attached to the mesoglea (Mg). Scale bar: 10 µm. (C) Details of the cluster of alkaline phosphatase-reactive PGCs in the endoderm (Ed) region. Scale bar: 100 µm.

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Supplementary methodology

Alkaline phosphatase

The polyps (n=15) were fixed in a 20% (v/v) zinc-formalin solution, according to the methodology described by Layton et al. (2018). After fixation, the samples were transferred to 70% (v/v) alcohol, where they remained until histological processing. The decalcification process was performed in a 10% (v/v) formic acid solution combined with 5% (v/v) formalin, following the protocol described by Banu et al. (2024), and subsequently, dehydrated and embedded in Paraplast®. Histological sections of 5 µm thickness were deparaffinized and incubated for 60 minutes in 0.1 M Tris-HCl buffer solution at 37°C. They were then incubated for 30 minutes in the alkaline phosphatase solution BCIP (5-bromo-4-chloro-3-indolyl phosphate) and NBT (nitro blue tetrazolium chloride) (Sigma, St. Louis, USA) at 37°C (Koç & Yüce, 2012; Akbulut & Yön, 2019). Subsequently, the slides were washed in the same buffer and then counterstained with hematoxylin (Merck) and mounted for viewing. The sections were evaluated using an Olympus BX60 microscope (Tokyo, Japan) with an Olympus DP73 camera attached.

Supplementary figure. Alkaline phosphatase labeling technique for primordial germ cells (PGCs) in tissue. (A) germinative mesentery during proliferative phase with germ cells attached (PGCs) to the endoderm region (Ed) of the polyp. Scale bar: 10 µm. (B). Primordial germ cells (PGC) in the endoderm (Ed) of the germinal mesentery near developing female (FG) gametes attached to the mesoglea (Mg). Scale bar: 10 µm. (C) Details of the cluster of alkaline phosphatase-reactive PGCs in the endoderm (Ed) region. Scale bar: 100 µm.

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CAPÍTULO 2

ARTIGO 2 – "Evaluating the Maturation of Male and Female Gametes in the South Atlantic Endemic Coral *Mussismilia harttii* Using Ultrastructural Analysis"

Em submissão na revista: **Microscopy and Microanalysis**

Link com as imagens em alta resolução:

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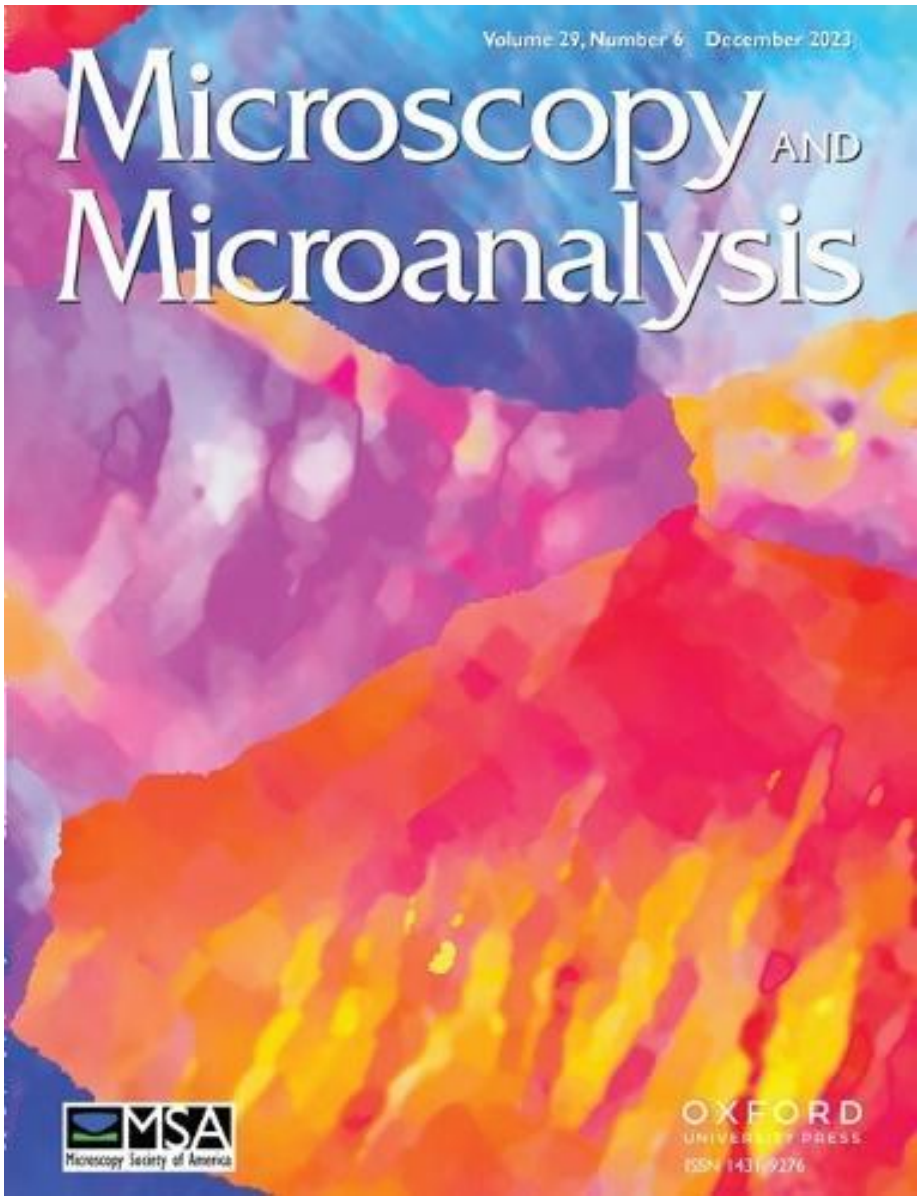

Evaluating the Maturation of Male and Female Gametes in the South Atlantic Endemic Coral *Mussismilia harttii* Using Ultrastructural Analysis

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Abstract

Since the 1950s, the reef ecosystem has shrunk by 50%, with species severely impacted by the climate crisis, putting the future of coral reefs at risk. The decline of coral reefs emphasizes the importance of conserving species around the world. Despite their importance, the reproductive biology of scleractinian corals remains poorly understood. This study investigated the gametogenesis of the coral *Mussismilia harttii*, focusing on gametogenesis through ultrastructural analysis. In stage I spermatogenesis, the cysts are oval, with elongated male germ cells and developed nuclei. There is a Golgi complex and an axoneme with a 9+2 arrangement of microtubules. In stage II, the cysts are ovoid, and the germ cells have a euchromatic nucleus with a central nucleolus, small, oval mitochondria, and a Golgi complex. In stage III, the bouquet-like cysts with germ cells in rows, condensed nuclei, and axoneme, the central mitochondria surround the base of the flagellum and the Golgi complex overlapping the nucleus. In oogenesis, stage I oocytes are oval, presenting microvilli, cortical vesicles, and lipid granules, as well as mitochondria and endoplasmic reticulum. In stage II, the oocytes become rounded, with short microvilli, a centralized euchromatic nucleus, and cortical vesicles close to the membrane. Lipid granules and yolk bodies are also present in the cytoplasm. In stage III, the oocytes maintain their spherical shape, with long, thin microvilli, cortical vesicles in the membrane, and lipid granules surrounded by the yolk bodies, and the mitochondria remain dispersed in the cytoplasm. Symbiodinium-like cells were observed near the membrane or in the cytoplasm, suggesting a symbiotic relationship with oocytes still during oogenesis. These observations provide valuable information on the reproductive process of *M. harttii* and contribute to future related studies involving the reproductive biology and conservation of coral reefs.

Keywords: Sexual reproduction, Ultrastructure, Gametogenesis, Fertilization, Coral conservation.

Introduction

Sexual reproduction is an important strategy to guarantee the genetic variability of the species, and ultimately, the adjustment to environmental changes (Santiago-Valentín et al., 2019; Pratchett et al., 2019). Although some studies have documented sexual reproduction in scleractinian corals worldwide, data regarding gametogenesis are still scarce (Shikina et al., 2020). The lack of information on gametogenesis and gametes also hinders the efforts to manage and develop conservation strategies for coral reefs, which are increasingly threatened by climate change (Goffredo et al., 2012; Fang et al., 2023).

To date, 32 species of scleractinian corals from different reef ecosystems have been described regarding gamete morphology (Wallace, 1985; Steiner, 1991, 1993; Goffredo et al., 2000; Vargas-Ángel et al., 2006; Padilla-Gamiño et al., 2011; Tsai et al., 2016; Lin et al., 2018; Valente et al., 2024), and 16% of those were evaluated at the ultrastructural level (Steiner, 1991, 1993; Goffredo et al., 2000; Padilla-Gamiño et al., 2011; Valente et al., 2024).

The South Atlantic Ocean provides suitable conditions for housing 16 species of shallow-water scleractinian corals, five of which are endemic to the Brazilian coast (Castro & Zilberberg, 2016). The cauliflower coral (*Mussismilia harttii*) is one of the main reef-building endemic species in Brazil, and the knowledge about the gametogenesis of scleractinian corals from the South Atlantic Ocean environment is limited to this genus so far (Pires et al., 1999). However, constant bleaching events, a consequence of ocean temperature elevation (Leão et al., 2016; Teixeira et al., 2019; Pereira et al., 2022), and the occurrence of diseases (Francini-Filho et al., 2008) have led this species to the risk of extinction (LVFBAE, 2018).

Strategies for coral reef conservation increasingly depend on the understanding of reproductive biology, particularly gamete formation and adaptations to the environment (Lin et al., 2013; Toh et al., 2022). In this study, we investigated the ultrastructural morphology of the

gametogenesis of *Mussismilia harttii* to elucidate the role of the organelles involved in the maintenance and viability of gametes. As well as understanding how gamete morphology helps with sexual reproduction and, consequently, reproductive success.

Material and Methods

Location and legal licenses

For this study, samples were collected in the Costa dos Corais Marine Protection Area (MPA), located at 8º42'16“ S and 35º04'40” W, which included the municipalities of Tamandaré (PE), São José da Coroa Grande (PE), Maragogi (AL) and Japaratinga (AL) (see Pereira et al., 2024). Samples in liquid media (fixative solutions) were transported under refrigerated conditions to the Laboratory of Cellular Biology at the Federal University of Minas Gerais, in Belo Horizonte, Minas Gerais (MG), and processed for ultrastructural evaluation. The Chico Mendes Institute for Biodiversity Conservation (ICMBio) authorized the full research steps (SISBIO license number 78827).

Collecting polyps in the field

The inclusion criteria used to choose the colonies of *Mussismilia harttii* considered the size (bigger than 50 polyps) and health, with no bleaching symptoms. Polyps from the center of the colonies were sampled monthly from October 2021 to December 2022, approaching the different stages of gametogenesis. Immediately after collection, polyps were identified and stored in fixative solutions. They were then processed for scanning (n=15) and transmission electron microscopy (n=30).

Scanning Electron Microscopy (SEM)

Scanning electron microscopy samples were fixed in 3% glutaraldehyde and 0.1 M phosphate buffer, following the protocol described by Graham & Orenstein (2007). Following

fixation, the material was decalcified in 10% EDTA solution at 35ºC for 10 days (Savi et al., 2017). After this step, samples were washed in running water (24 hours) to remove the excess decalcifying solution. Dehydration was carried out using an increasing series of acetone (70% to 100%) at 10-minute intervals for each bath. The samples were then dried using a Critical Point Dryer (Leica CPD 030, Germany). Once the critical point was reached, the specimens were carefully mounted on aluminum stubs with the aid of a magnifying glass to ensure proper orientation and optimal visualization of the target area. Conducting was carried out using carbon deposition (Bal-Tec MED 20, Germany), and samples were examined using a scanning electron microscope (Jeol JSM 6360LV, USA).

Transmission Electron Microscopy (TEM)

Samples assigned for the TEM study were fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffered solution, following the method described by Karnovsky (1965). After the initial fixation, samples were decalcified (Savi et al., 2017) and then post-fixed for 45 minutes in 2% OsO_4 0.2 M phosphate buffered solution and rinsed three times in the same buffer (15 minutes each). Dehydration was performed through 10-minute baths, using increasing acetone concentrations (30% to 100%).

Plastic resin (Epoxy Embedding Kit) combined with acetone, or pure, was used for samples pre-infiltration and infiltration steps, respectively. After 24 hours of infiltration, embedding was carried out in silicone molds filled with pure resin, which were kept at 60°C for 72 hours. Ultra-thin sections (60 nm) were obtained and transferred to copper grids, contrasted with 2% uranyl acetate and lead citrate. Sections were evaluated in a transmission electron microscope (Tecnai G2-12 Spirit Biotwin 120kV - Thermo Fisher / FEI, USA).

Image analysis

Scanning and transmission electron photomicrographs were digitized and processed with Adobe Photoshop CS3 (Adobe Systems Inc., 345 Park Avenue, San Jose, California, USA). Adjustments were made to improve focus, contrast, brightness, and grayscale. Sperm cysts (n=48), oocytes (19 cells), and spermatozoa (72 cells) were qualitative and morphometrically analyzed regarding their structural components. Morphometric measurements were taken using ImageJ software (Microsoft Java 1.1.4), with quantitative results expressed as mean ± standard deviation. The diameter of the sperm cysts and oocytes (D) was calculated using the following formula:

$$D = \frac{larger\ diameter + smaller\ diameter}{2}$$

Results

Spermatogenesis

Stage I sperm cysts depicted an ovoid shape and an average diameter of 59.5 ± 5.08 μm (n=21) (Fig. 1A). At this stage, male germ cells presented a rounded nucleus (1.92 ± 0.34 μm; n=16) with non-condensed chromatin predominating in the nucleoplasm. Surrounding the inner nuclear membrane, condensed chromatin was observed, although subjacent to the nuclear pores, it was also disrupted. The nucleolus was evident and centrally positioned (Fig. 1B), and the Golgi complex released its secretory vesicles (Fig. 1C and 1D). Axoneme was observed, and the arrangement of the microtubules was conserved, following the pattern (9+2) (Fig. 1E and 1F).

At Stage II, the diameter of spermatic cysts was approximately 85.0 ± 5.61 μm (n=17) (Fig. 2A). Male germ cells maintained the rounded nuclear shape despite an increase of its diameter (4.0 ± 1.25 μm, n=12). Non-condensed chromatin and centralized nucleolus were

observed at this step. The scarce organelle content observed at Stage I contrasted with numerous mitochondria (diameter 0.37 ± 0.04 μm, n=32), besides the axoneme and Golgi complex juxtapositioned to the nucleus in these cells at Stage II (Fig. 2B-2F).

Remarkable modifications featured the Stage III spermatogenic cell cysts. In addition to the diameter increase of approximately 2.5-fold (191.5 ± 5.83 μm, n=15) compared to the previous stage, the bouquet-like cysts (Fig. 3A) indicated cells were uniformly oriented (Fig. 3B). At this point, male gametes developed the cellular tools needed for fertilization, such as nuclear condensation, although the maintenance of its rounded shape; Golgi complex, which produces secretory vesicles that migrates to the cellular boundaries, and a mitochondrial collar around the flagellum insertion. These are the ordinary components of the sperm head (Fig. 3B-3D). In this regard, sperm heads were concentrated at one side of the cyst and the flagella towards the opposite.

Oogenesis

The oocytes in *M. harttii* presented three morphologically distinct stages of development. At stage I, the oocytes were spherical, and the cell diameter was approximately 187.2 ± 2.21 μm (n = 7). Microvilli, cortical vesicles, lipid granules, yolk bodies, rough endoplasmic reticulum (RER), and mitochondria were the organelles and cell specializations that characterized oocytes along the maturation process (Fig. 4A). Cortical vesicles positioned in the cytoplasm close to the cell membrane depicted a low electron-dense content (Fig. 4B), which contrasts with the electron-dense yolk bodies. At this phase, lipids were gathered to form irregular granules (Fig. 4C). Mitochondria (0.42 ± 0.04 μm, n = 36) and ER were distributed throughout the cytoplasm (Fig. 4D and 4E). In addition, stage I oocytes were observed sharing with secondary oocytes the same milieu (Fig. 4F).

The second step of oocyte maturation (Stage II) was marked by the enlargement of the cell (226.7 ± 5.4 μm, n = 12), which maintained the spheric form. Microvilli project toward the intercellular space, and in the subjacent cytoplasm, intense vesicular traffic was observed since heterogeneous electron-dense cortical vesicles vary in size, shape, and electron density (Fig. 5A and 5B). The euchromatic nucleus was located in the central region of the cell, with a noticeable nucleolus (Fig. 5C). The maturing lipid granules vary in shape from oval to irregular (Fig. 5D). Mitochondria (0.63 ± 0.07 μm, n = 21) were widely distributed in the cytoplasm along with the endoplasmic reticulum (Fig. 5E). Yolk bodies have adhered around the lipid granules (Fig. 5F).

Mature oocytes classified at Stage III did not show meaningful differences in comparison to the previous stage. Indeed, the cellular diameter slightly increased (267.3 ± 7.2 μm), and the ultrastructural components were conserved, although following the cell environment, these elements depicted a matured status. Microvilli were found to be longer in stage III oocytes compared to earlier developmental stages, and lipid granules reached the biggest volume at this phase. During oocyte maturation, the diameter of mitochondria did not change significantly (0.54 ± 0.04 μm, n = 12) (Fig. 6D). Different from the earlier stages, Symbiodinium-like cells were observed in the cytoplasmic compartment, either close to the cellular membrane, or dispersed in the cytoplasm of oocyte III (Fig. 6A - 6F).

Discussion

This paper presents new information regarding *Mussismilia harttii* gamete maturation from an ultrastructural view, which enabled a more detailed description of the organelles and cell specialization that develops during male and female gamete maturation inside the scleractinian coral mesenteries. Since gametogenesis is a primary biological process that directly influences the reproductive success of the species, the knowledge produced in this field

will contribute to establishing conservationist programs, which are urging, particularly in the face of climate change and the anthropogenic impacts that are punishing the environment lately (Waller et al., 2014; Ayalon et al., 2021; Sakai et al., 2020).

Spermatogenesis

Spermatogenesis is a complex process that takes place within the gonads and involves the sequential development of male gametes through three main phases: spermatogonial, spermatocyte, and spermiogenesis (Chiu et al., 2020a; Chiu et al., 2020b). During the spermatogonial phase, a proliferative wave of spermatogonia increases their number (Chiu et al., 2020a). Primary spermatocytes result from the last spermatogonia cell type mitosis. These cells enter meiosis to form secondary spermatocytes and, subsequently, spermatids (Chiu et al., 2020b). However, based on our observations, the stages of spermatogonia and spermatocytes in *M. harttii* do not occur inside the coral's gonads, suggesting that spermatogonial and spermatocyte phases would be taking place in the endoderm of the germinal mesenteries, since inside the spermatic cysts (gonads) we only observed the presence of male germ cells in the spermiogenesis (maturation) stage.

Sperm cysts are structures associated with the storage, support, and maturation of male gametes (Hernandez et al., 2005; Hazar et al., 2015). In aquatic organisms, such as sponges, cysts are formed from specialized cell structures that facilitate the development of spermatogonia (Ereskovsky et al., 2012). For the fish *Danio rerio*, the organization of sperm cysts is formed by the presence of Sertoli cells, which provide structural and nutritional support during sperm maturation (Cacialli et al., 2017). Therefore, in *M. harttii* sperm cysts can play important roles in the reproductive process, from nutritional support and storage to the maturation of developing male gametes.

The nucleus plays an important role during the stages of spermatogenesis. Euchromatic regions are associated with active transcription and gene expression, which are essential for

germ cell differentiation and development (Wen et al., 2016). In the crab *Eriocheir sinensis*, nuclear morphology undergoes significant changes during spermatogenesis, with the euchromatic nucleus in the early stages and condensation of the genetic material in the final stages of maturation (Sun et al., 2010). Therefore, the nucleus in *M. hartti* serves as a center for the regulation of cell transcription and morphogenesis, which are essential during spermatogenesis (Sun et al., 2010).

Our observations indicate that the Golgi complex in *M. harttii* exhibits consistently high vesicle production during spermatogenesis. According to Goffredo et al. (2000), the Golgi apparatus plays a vital role in the synthesis and packaging of proteins and vesicles necessary for the development of gametes. The vesicles produced by the Golgi are essential for the transportation of proteins and other molecules necessary for the maturation of male germ cells (Goffredo et al., 2000). Furthermore, Yao et al. (2002) state that the Golgi apparatus is responsible for the formation of acrosomal vesicles. During spermatogenesis, the vesicles produced in the Golgi migrate to the upper region of the nucleus and fuse to form the acrosome (Nozawa et al., 2020). This information corroborates our findings for *M. harttii*, since the male germ cells in the early stages did not have acrosomal vesicles inside, while the male gametes in stage III have small electron-dense vesicles underlining the inner plasma membrane.

The axoneme, the structural core of the flagella, normally displays an arrangement of 9+2 microtubules, which is crucial for its functions during motility (Inaba, 2007). In marine invertebrates, this structure is essential for sperm motility and plays an important role during flagellar beating (Sukhan et al., 2020). It is also a highly conserved structure in different marine invertebrate taxa. According to the ultrastructural studies carried out by Ereskovsky & Tokina (2022) for the marine sponges *Crellomima imparidens* and *Hymedesmia irregularis*, they revealed that the male gametes have axoneme configurations similar to other marine

invertebrates and may provide similarities about the evolutionary relationships between the invertebrate taxa.

Mitochondria are essential organelles involved in energy production and metabolic processes (Jeong et al., 2014). During spermatogenesis, mitochondria undergo significant changes, including the fusion process that contributes to their functionality (Hock & Kralli. 2009). This process ensures the supply of energy to the entire cell (Hock & Kralli, 2009; Jeong et al., 2014). According to our findings for *M. harttii*, in the early stages of spermatogenesis, small mitochondria are distributed throughout the cytoplasm of the cells. As spermatogenesis progresses, these smaller mitochondria aggregate to form a single larger mitochondrion, which is essential for energy supply during flagellar beating and motility (Alevi et al., 2015).

Oogenesis

In many marine invertebrates, oogenesis can involve different stages of development and can be categorized into three main phases: oogonia proliferation, vitellogenesis, and gamete maturation (Goffredo et al., 2012; Zhang et al., 2023). For the octopus *Sepia pharaonis*, oogenesis is divided into the stages of oogonia, cell growth, and vitellogenesis (Basch & Pearse, 2022). For *M. harttii*, oogenesis begins with the formation of oogonia in the endoderm, which migrate towards the mesoglea, located in the central region of the germinal mesenteries. When they adhere to the mesoglea, they undergo successive structural and morphological changes until they become mature oocytes.

The outer plasma membrane plays vital roles during oogenesis, including nutrient absorption and protection of the developing oocytes (Rojas et al., 2021). In the early stages of oocyte development in *M. harttii*, significant morphological changes were identified in the structure of the outer plasma membrane, including the presence of adhered vesicles. According to Goffredo et al. (2010), the presence of lipid vesicles and other membrane components

increases as oogenesis progresses, suggesting that these elements contribute to the structural and functional properties of the membrane.

In the early stages of oogenesis in *M. harttii*, oocytes have short, compact microvilli, which become long and thin in the later stages. Microvilli arise from the cytoplasmic membrane and are supported by a cytoskeleton of actin filaments (Hayakawa et al., 2007). It is recorded that microvilli participate in the transport of yolk protein in the scleractinian coral *Galaxea fascicularis* (Baird et al., 2009). It also allows communication between female gametes, as observed in the corals *Acropora cytherea* and *Acropora tenuis* (Ibrahim, 2021). Microvilli may also be involved in reproduction, preventing polyspermy. According to Marshall & Bolton (2007), after fertilization, changes in the structure of the microvilli, such as retraction or modification, can help prevent additional sperm from attaching to the surface of the oocyte.

Cortical vesicles play important roles in the oogenesis of *M. harttii*. According to Weng et al., (2021), cortical vesicles are closely bonded to the cytoplasmic accumulation and secretion of substances. According to Apparício et al., (2011), the formation of cortical vesicles initially occurs in the cytoplasm and as oogenesis progresses, they end up accumulating under the plasma membrane of mature oocytes. Its functions may also be involved in preventing polyspermy during fertilization (Haley & Wessel, 2004), as well as assisting in the formation of gamete packets, as observed for the coral *Montipora capitata* (Padilla-Gamiño et al., 2011).

During oogenesis in *M. harttii*, we observed a progressive increase in the number and size of lipid granules in stages II and III. Lipid granules serve as energy reserves for oocyte maturation and subsequent survival of embryos and larvae (Lin et al., 2012). During the stages of oocyte development, including pre-vitellogenesis and vitellogenesis, there is a marked increase in organelles responsible for lipid synthesis, such as the smooth endoplasmic reticulum

and the Golgi complex (Lin et al., 2013). This increase correlates with the accumulation of lipid droplets, which will be used as energy reserves for the survival of the embryo (Rey et al., 2015).

Yolk bodies were identified throughout oogenesis in the coral *M. harttii*, but in the more advanced stages, they were abundant and maintained an organization around the lipid granules. Yolk bodies are formed during vitellogenesis, the stage of oogenesis in which oocytes grow and accumulate nutrients from the external environment or are synthesized by the endoplasmic reticulum and Golgi complex (Shikina et al., 2013). In addition, interactions between somatic and germ cells support oocyte development, influencing the development and increase in oocyte size (Coelho & Lasker 2014). Studies have shown that somatic cells in the coral *Euphyllia ancora* produced yolk proteins, including vitellogenin, and these were transported to the oocytes, where they accumulated as yolk bodies (Shikina et al., 2016; Shikina et al., 2013; Shikina et al., 2015). Therefore, the presence of yolk bodies is essential for oocyte maturation, as they provide necessary nutrients that support gamete development and post-fertilization (Shikina et al., 2013).

During oogenesis, mitochondria are involved in energy production and the synthesis of essential biomolecules for the development of the oocyte, as well as contributing to the quality of gametes, which is fundamental for successful reproduction (Kirillova et al., 2021). According to Shikina et al. (2015), the formation of mitochondria is linked to oocyte development, as they provide the necessary energy and metabolic support during oogenesis. In addition, the role of mitochondria extends to energy production; they are also involved in regulating apoptosis during oogenesis. This process ensures that only healthy oocytes are retained, which is essential for reproductive success (Tworzydło et al., 2016).

During oogenesis, in stages I and II, a high concentration of rough endoplasmic reticulum was observed dispersed in the cytoplasm of *M. harttii* oocytes. This organelle is

fundamental in the production of vitellogenin, a precursor of yolk proteins that are essential for the nutrition of developing oocytes (Twan et al., 2006). This process has been shown in the coral *Astroides calycularis*, where the synthesis of vitellogenin and other proteins produced by the rough endoplasmic reticulum directly influences the maturation of oocytes (Twan et al., 2006). On the other hand, smooth endoplasmic reticulum is involved in lipid metabolism, which is crucial for the formation of lipid granules and cell membranes during oocyte maturation (Chen et al., 2019). Overall, the endoplasmic reticulum, in both versions, is a fundamental organelle that supports oogenesis through its roles in protein synthesis and lipid metabolism.

Nuclear activity is essential for oogenesis, regulating and promoting the synthesis necessary for oocyte development (Trounson, 2001). Nuclei were not observed in stage III oocytes during *M. harttii* oogenesis. The loss of the nuclear membrane and the dispersion of genetic material in the cytoplasm is known as nuclear collapse or nuclear reorganization (De Souza & Osmani 2007). This phenomenon occurs during the final stages of oocyte maturation, as they prepare for fertilization (De Souza & Osmani 2007). According to Santella et al., (2020), this process is part of oocyte activation, allowing the genetic material to become accessible to the incoming sperm during fertilization.

Symbiont cells described as Symbiodinium-like, were observed in stage III oocytes in *M. harttii*. According to Hazraty-Kari et al. (2022), the dinoflagellates of the *Symbiodiniaceae* family are acquired through vertical transmission, when the gametes obtain the symbionts directly from the parent coral. This strategy highlights the adaptability of oocytes in establishing symbiotic relationships with photosynthetic organisms, which are crucial for their development (Jung, 2023).

In summary, male and female germ cells of *Mussismilia harttii* were evaluated at the ultrastructural level regarding the morphological modifications during the maturation process

until they reached the status of oocytes and spermatozoa, enabled to follow with fertilization. These findings expand the knowledge regarding the reproductive biology of this endemic scleractinian coral to the South Atlantic Ocean, which supports further research in the field, focusing on the conservation and recovery of degraded reef areas.

Acknowledgments

WV received a field research grant from the Brazilian Biodiversity Fund (Funbio) and the Humanize Institute (grant no. 121/2019), which facilitated the acquisition of equipment and reagents used in this work.

Authors contributions

WV, GFA, and LG conceived the study; WV, BRLC, LC, and MLS prepared the samples; WV, BRLC, LC, and MLS conducted the ultrastructural analyses; WV and GFA carried out the morphometric analyses; WV, GFA, and LG drafted the manuscript; all the authors participated in revising the manuscript.

Data availability

Data is available on request.

Statements

Conflict of interest

The corresponding author declares, on behalf of all authors, that there is no conflict of interest.

Ethical approval

The authors declare that all applicable international, national, and institutional guidelines for the sampling, care, and experimental use of animals in this study were followed, along with obtaining the necessary approvals from the Chico Mendes Institute for Biodiversity Conservation (ICMBio/SISBIO).

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Figure legends

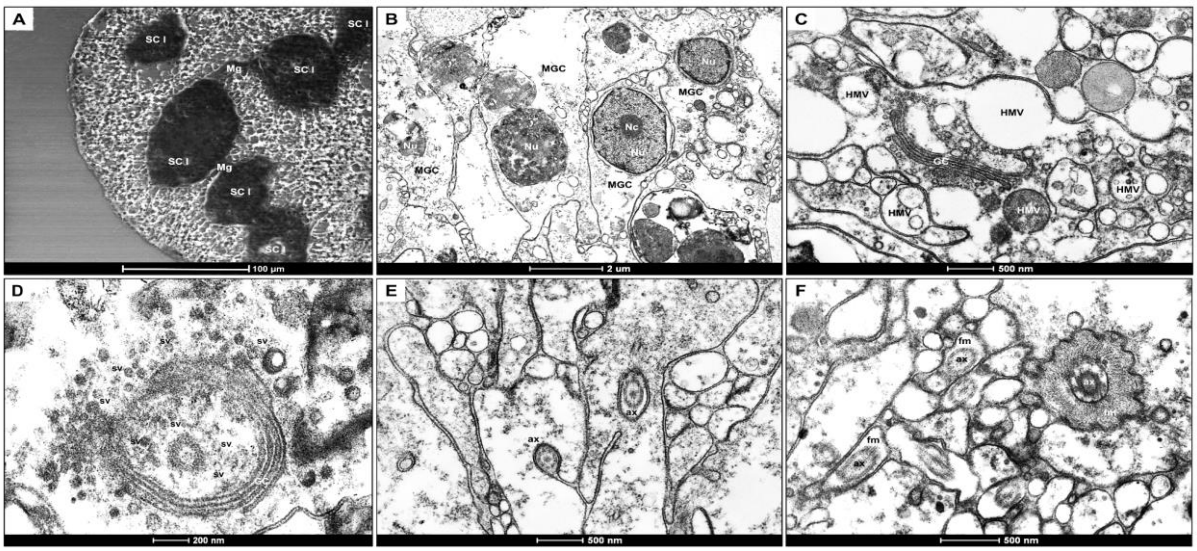

Fig. 1. Ultrastructure of stage I spermatic cysts in the coral *M. harttii.* (A) Scanning photoelectron micrograph (SEM) showing rounded stage I spermatic cysts (SC I) adhered to the mesoglea (Mg). (B) Transmission photoelectron micrograph (TEM) showing individualized male germ cells (MGC), delimited by the plasma membrane, with a nucleus (Nu) and a nucleolus (Nc). (C) TEM photo micrograph highlighting heterogeneous membranous vesicles (HMV) and a lamellar structure, similar to the Golgi complex (GC). (D) TEM detail of the Golgi (GC), surrounding smaller vesicles (sv) and accompanied by smaller electron-dense vesicles (sv). (E) TEM shows a single axoneme (ax) per cell dispersed in the cytoplasm. (F) Detail of the TEM showing the arrangement of the axonemes (ax), with microtubules organized in the 9+2 pattern, surrounded by the cell membrane, forming the flagellar membrane (fm).

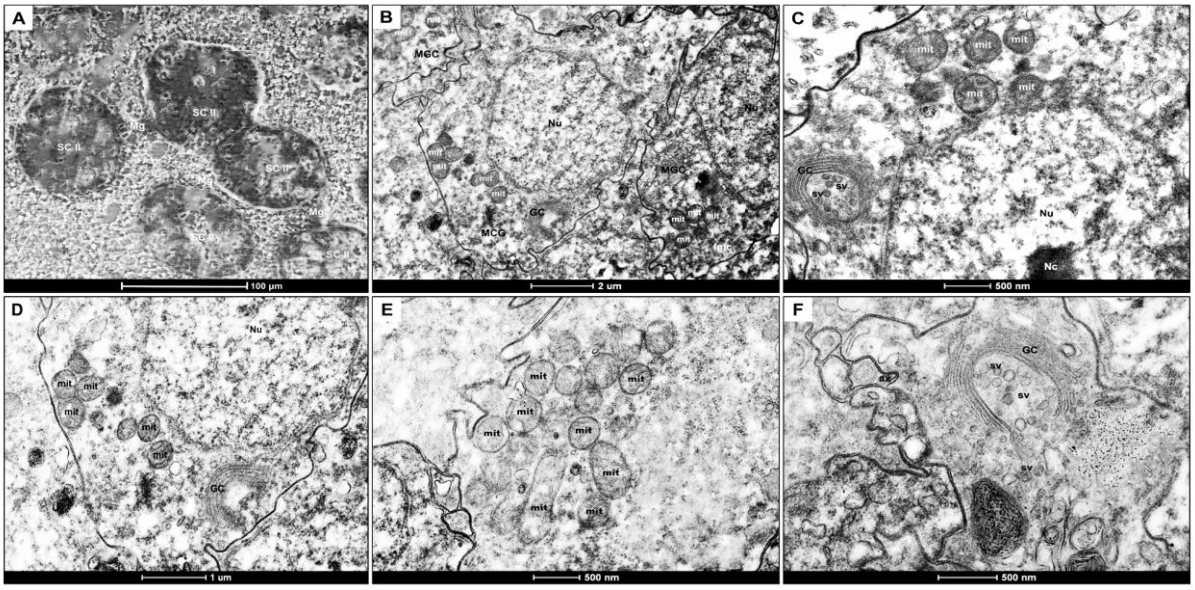

Fig. 2. Ultrastructure of stage II spermatic cysts in the coral *M. harttii*. (A) Scanning photoelectron micrograph (SEM) showing oval-shaped stage II spermatic cysts (SC II) attached to the mesoglea (Mg). (B) Transmission photoelectron micrograph (TEM) showing individualized male germ cells (MGC) with a nucleus (Nu), mitochondria (mit), and a Golgi complex (GC). (C) TEM detail of the completely decondensed nucleus (Nu), with a nucleolus (Nc) evident in the central region of the cell, as well as mitochondria (mit) and the Golgi complex (GC) with its vesicles (vs). (D) TEM of a cell with a rounded euchromatic nucleus (Nu) close to the mitochondria (mit) and Golgi complex (GC). (E) TEM image showing details of the mitochondria (mit) distributed in the cytoplasm, highlighting the internal ridges. (F) TEM detail of the Golgi complex (GC) secreting vesicles (vs) into the cytoplasm and the presence of an axoneme (ax).

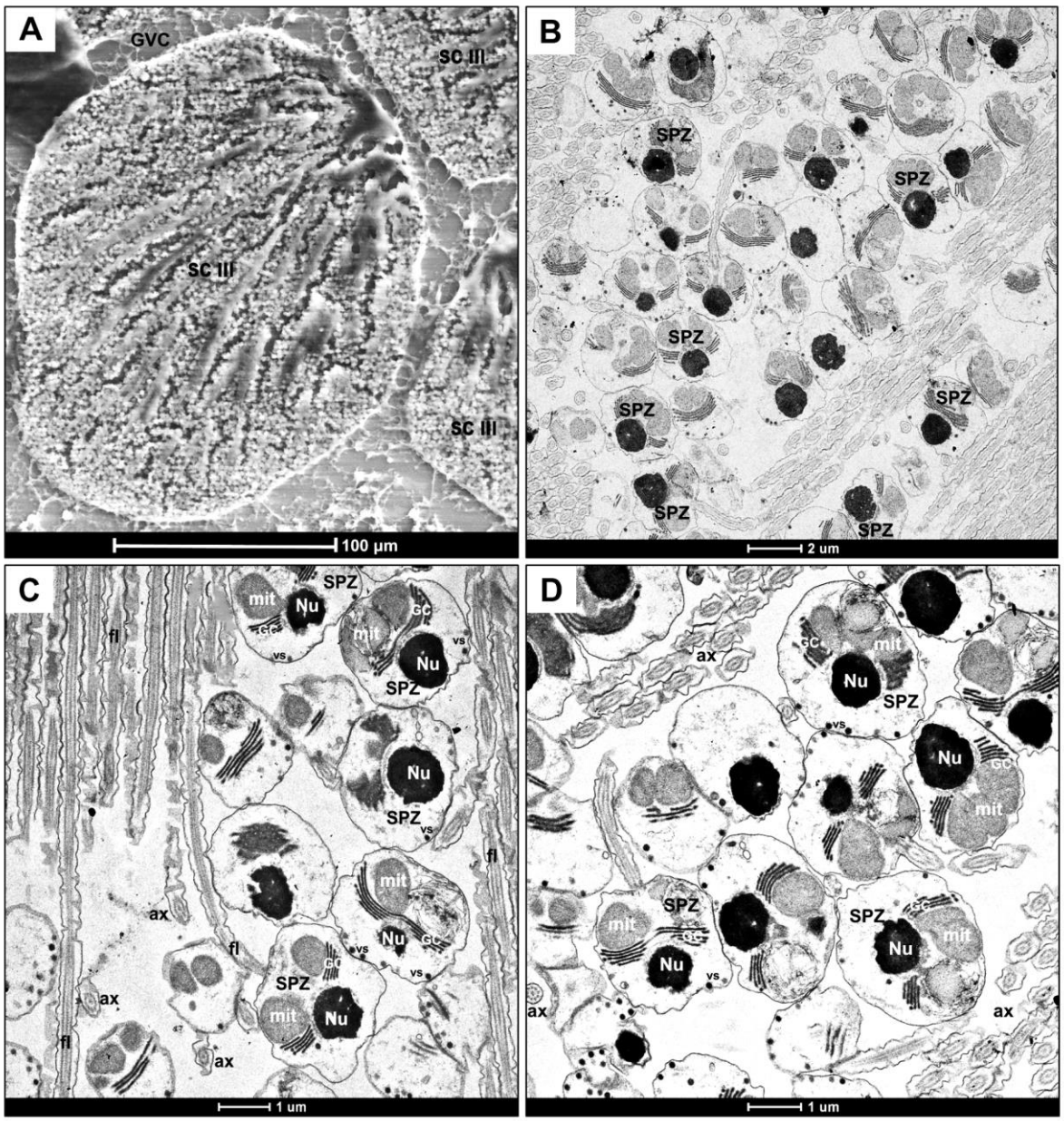

Fig. 3. Ultrastructure of stage III sperm cysts of the coral *M. harttii*. (A) Scanning photoelectron micrograph (SEM) showing the bouquet-like stage III sperm cysts (SC III). (B) Transmission electron microscopy (TEM) shows individualized spermatozoa (SPZ) arranged in rows, demonstrating a pattern of organization within the cyst. (C) TEM image highlighting the spermatozoa with flagella (fl) and the organization of their organelles inside. (D) TEM detail shows spermatozoa with a condensed nucleus (Nu), the presence of a single mitochondrion (mit) surrounding the axoneme, a single Golgi complex (GC), vesicles close to the plasma membrane (vs), and flagella (fl).

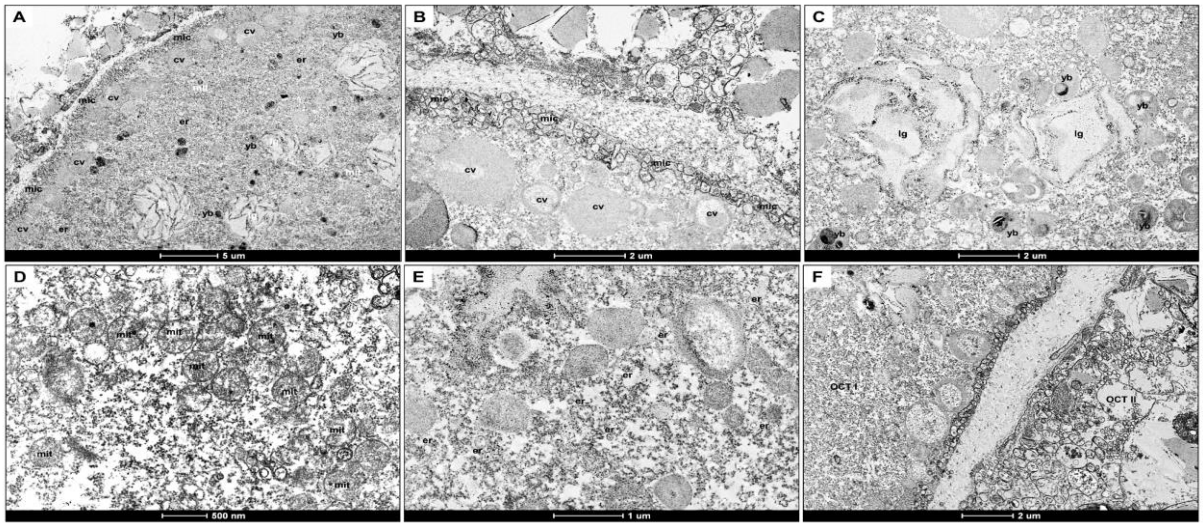

Fig. 4 Transmission photoelectron micrograph showing the internal components of *M. harttii* stage I oocytes. (A) General view: note the presence of evaginations of the microvilli (mic), cortical vesicles (cv), lipid granules (lg), yolk bodies (yb), and mitochondria (mit). (B) Details of the microvilli (mic) with thick evaginations and the presence of cortical vesicles (cv) with an ovoid shape and homogeneous content positioned close to the plasma membrane. (C) Lipid granules in aggregation (lg) and vitelline bodies nearby (yb). (D) Details of the oval mitochondria (mit) distributed throughout the cytoplasm. (E) In addition to the endoplasmic reticulum distributed throughout the cytoplasm (Fig. 1E). (F) Presence of stage I oocytes (OCT I) next to secondary oocytes (OCT II).

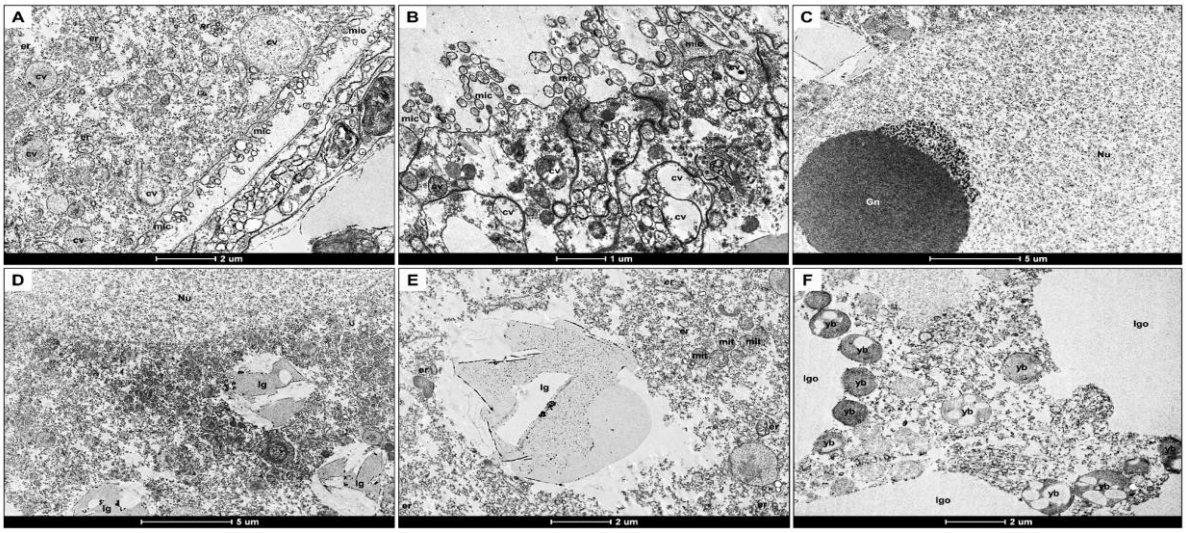

Fig. 5 Transmission photoelectron micrograph showing the internal components of *M. harttii* stage II oocytes. (A) General view: note the presence of microvilli (mic), cortical vesicles (cv), mitochondria (mit), and, endoplasmic reticulum (er). (B) Details of the evaginations of the more elongated microvilli (mic) with the cortical vesicles (cv) varying in shape and electron density. (C) Well-developed decondensed nucleus (Nu) with the presence of granular (gn) and fibrillar (fn) nucleoli near the periphery. (D) Maturing lipid granules (lg) distributed throughout the cytoplasm and close to the nucleus (Nu). (E) Details of the oval mitochondria (mit) next to the aggregating lipid granule (lg) and the presence of the endoplasmic reticulum (er) in the cytoplasm (lg). (F) Details of the yolk bodies (yb) with varying shapes and electron densities distributed around the lipid granules (lg).

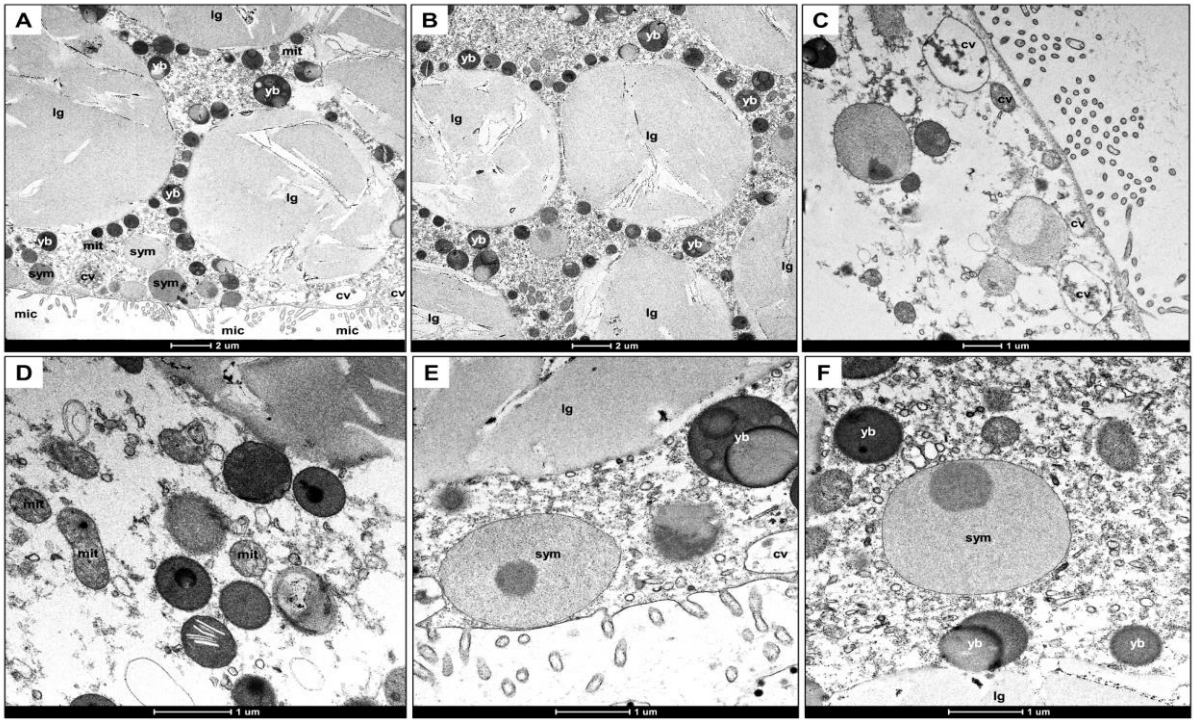

Fig. 6 Transmission photoelectron micrograph showing the internal components of the stage III or mature oocyte of *M. harttii*. (A) Internal components of the oocyte, including microvilli (mic), mitochondria (mit), cortical vesicles (cv), lipid granules (lg), yolk bodies (yb), and Symbiodinium-like cells (sym). (B) Spherical lipid granules (lg) surrounded by yolk bodies (yb) showing variations in size, shape, and electron density. (C) Cortical vesicle (cv) close to the plasma membrane. (D) Mitochondria (mit) are seen distributed throughout the cytoplasm of the oocyte. (E) Symbiodinium-like cells (sym) are located on the periphery of the oocyte. (F) Symbiodinium-like cells (sym) inside the cell, close to the lipid granules (lg) and yolk bodies (yb).

CAPÍTULO 3

ARTIGO 3 – "Ultrastructural evaluation of the oocytes and spermatozoa of the scleractinian coral *Mussismilia harttii*"

Publicado na revista: Tissue and Cell

Tissue and Cell 90 (2024) 102469

Contents lists available at ScienceDirect

Tissue and Cell

journal homepage: www.elsevier.com/locate/tice

Ultrastructural evaluation of the oocytes and spermatozoa of the scleractinian coral *Mussismilia harttii*

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ARTICLE INFO

Keywords:
Sexual reproduction
Gametes
Fertilization
Electron microscopy

ABSTRACT

Global coverage of living coral has declined by half since 1950s. Reef-building species have been severely impacted in this climate crisis scenario, compromising the future of coral reefs. Despite their importance, there is a lack of knowledge regarding the reproductive biology of scleractinian corals. In the present study, we evaluated through electron microscopy approaches, the gametes of the endemic Southwestern Atlantic coral *Mussismilia harttii*. We observed spherical oocytes with microvilli throughout the outer membrane. Fine granular material dispersed in cytoplasm, lipid granules, numerous yolk bodies, and mitochondria were identified in the oocytes. In addition, small Symbiodinium-like cells were observed, suggesting a vertical transmission from parental coral to oocytes. The spherical-head sperm presents a 9.3 ± 2.1 μm flagellum. The nucleus is located centrally in the head, and the centrioles are positioned between the nuclear base and the flagellar insertion, which is connected to the axoneme. This axoneme has a microtubular arrangement (9+2). Vesicles, underlining the inner plasma membrane, presented the same electron-dense pattern as the Golgi complex, and mitochondria positioned surrounding the axoneme. The vesicles present in the sperm may have a role as an acrosome since the oocytes do not develop any cell specialization for fertilization.

1. Introduction

Although the sexual reproduction of scleractinian corals has been studied for over 200 years, since Cavolini (1790, cited in De Lacaze-Duthiers, 1873), and substantial information has been made available through the studies of Fadlallah (1983), Harrison and Wallace (1990), Richmond and Hunter (1990), Richmond (1997), Harrison and Jamieson (1999), Kolinski and Cox (2003), Guest et al. (2005), Harrison and Booth (2007), and Baird and Guest (2009), there is still very limited information in the literature, considering that practically half of all coral species currently in existence have never been studied regarding their reproductive biology (Harrison, 2011).

Understanding the reproductive biology of coral species is essential to elucidate the processes underlying gametogenesis, which involves the generation of both male (spermatozoon) and female (oocyte) gametes. This process allows genetic recombination and the emergence of new genotypes, potentially increasing the resilience and survival of coral species (Harrison and Wallace, 1990; Harrison, 2011). The growing decline of reef-building species worldwide (Hughes et al., 2018, 2019) reinforces the need for more information on their reproductive biology and ecology. Ongoing research provides increasing evidence that the sexual reproductive processes of corals are highly sensitive to a wide range of natural and anthropogenic stressors, which cause sublethal stress, reduce fecundity, and hinder reproductive success (Loya and Rinkevich, 1980; Harrison and Wallace, 1990; Richmond, 1993; Richmond, 1997; Fabricius, 2005; Drury et al., 2019).

The gamete morphology of 31 species of scleractinian corals have been described in the literature (Wallace, 1985; Steiner, 1991, 1993; Goffredo et al., 2000; Vargas-Ángel et al., 2006; Kawaroe and Soedharma, 2007; Padilla-Gamiño et al., 2011; Tsai et al., 2016; Lin et al., 2018), but only five of these studies presented ultrastructural details for the spermatozoa and oocytes (see Steiner, 1991; Steiner, 1993; Goffredo et al., 2000; Padilla-Gamiño et al., 2011; Tsai et al., 2016). This demonstrates a gap in basic knowledge about the biology of coral gametes

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https://doi.org/10.1016/j.tice.2024.102469
Received 18 December 2023; Received in revised form 7 July 2024; Accepted 8 July 2024
Available online 9 July 2024

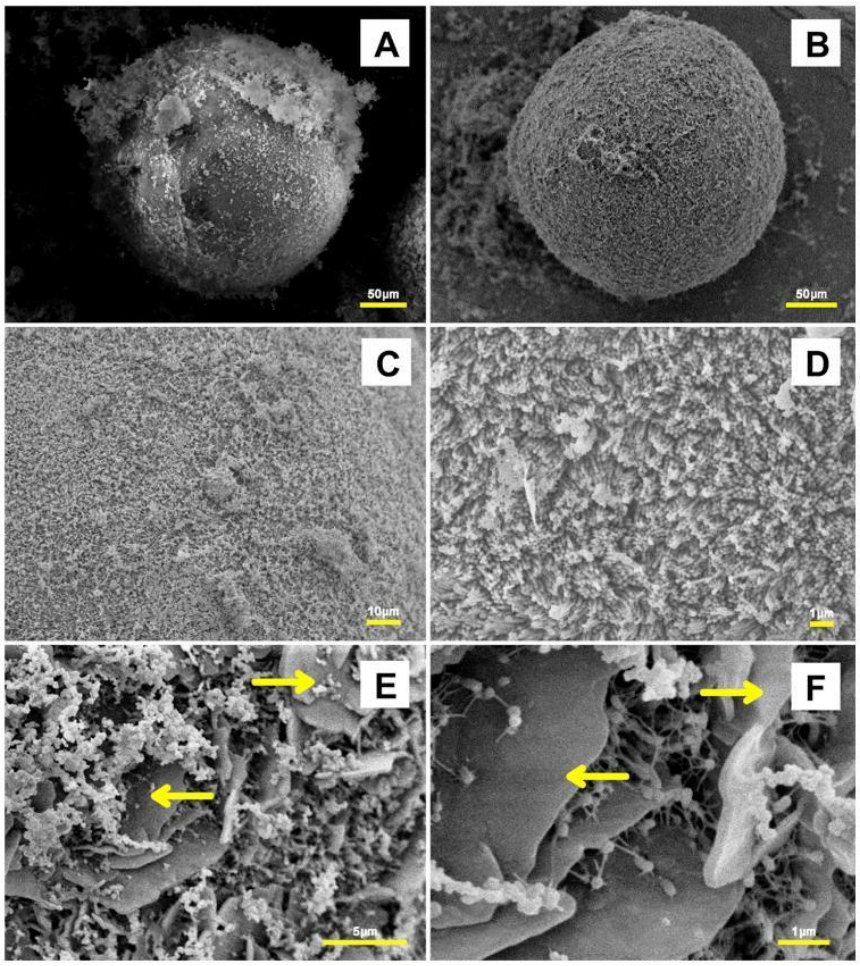

Fig. 1. Scanning electron microscopy of *M. harttii* oocytes **(a)** Spherical oocyte with a dense layer of mucus adhered to its surface. **(b)** Spherical oocyte with a thin layer of mucus on its surface. **(c)** Microvilli distributed throughout the oocyte and the presence of mucus adhered to them. **(d)** Detail of microvilli across the surface of the oocyte. **(e)** Presence of a scale-like structure on the surface of the oocyte (yellow arrows). **(f)** Detail of scale-like structures (yellow arrows).

through ultrastructural analyses.

The South Atlantic Ocean is inhabited by 16 species of shallow water scleractinian corals, five of which are endemic to Brazil (Castro and Zilberberg, 2016). The cauliflower coral (*Mussismilia harttii*) is among the main reef-building species in Brazil, though frequent and intense bleaching events triggered by increasing ocean temperatures over the past years (Castro and Pires, 1999; Leão et al., 2016; Teixeira et al., 2019; Pereira et al., 2022) and the occurrence of diseases (Francini-Filho et al., 2008) have placed the species under a status of endangered of extinction (LVFBAE., 2018). Knowledge about the sexual reproduction of scleractinian corals in the South Atlantic Ocean is limited to the pioneering studies carried out by Pires et al. (1999, 2011, 2016) and Neves and Pires (2002), who described the reproductive patterns, gametogenesis, and spawning of corals of the *Mussismilia* genus, using histological techniques and light microscopy.

Some techniques that are safe for coral conservation are related to knowledge on the biology of their gametes (Tsai et al., 2016). Understanding gametes at an ultrastructural level through electron microscopy enables a more detailed comprehension of their morphology, the organelles involved in their development, attraction, fertilization, and maintenance of their viability (Harrison, 2011; Tsai et al., 2016). In the present study, we developed a thorough investigation on the gametes of the coral *M. harttii* at an ultrastructural level, providing subsidies for a better understanding of the species' gametogenesis, formation of gamete bundles, and fertilization of scleractinian corals.

2. Material and methods

2.1. Collection of colonies and legal authorizations

Forty colonies of *Mussismilia harttii* were collected from the Municipal Marine Park of Recife de Fora (16°24'31″S; 038°58'39″W), Brazil, three weeks before the predicted spawning period for this species (Pires et al., 1999, 2016). The colonies were transferred to the Research Base of the Coral Vivo Project, identified, and placed in 1000-L tanks with periodical renewal of seawater. The research was approved by the Chico Mendes Institute for Biodiversity Conservation (ICMBio - SISBIO N° 63368–1) and by the Municipal Secretariat for the Environment of Porto Seguro (Authorization N° 01/2019).

2.2. Collection and separation of gametes

During the spawning nights (September/ 27–29/ 2019), the oocyte-sperm bundles were collected from the water surface in the tanks. Three

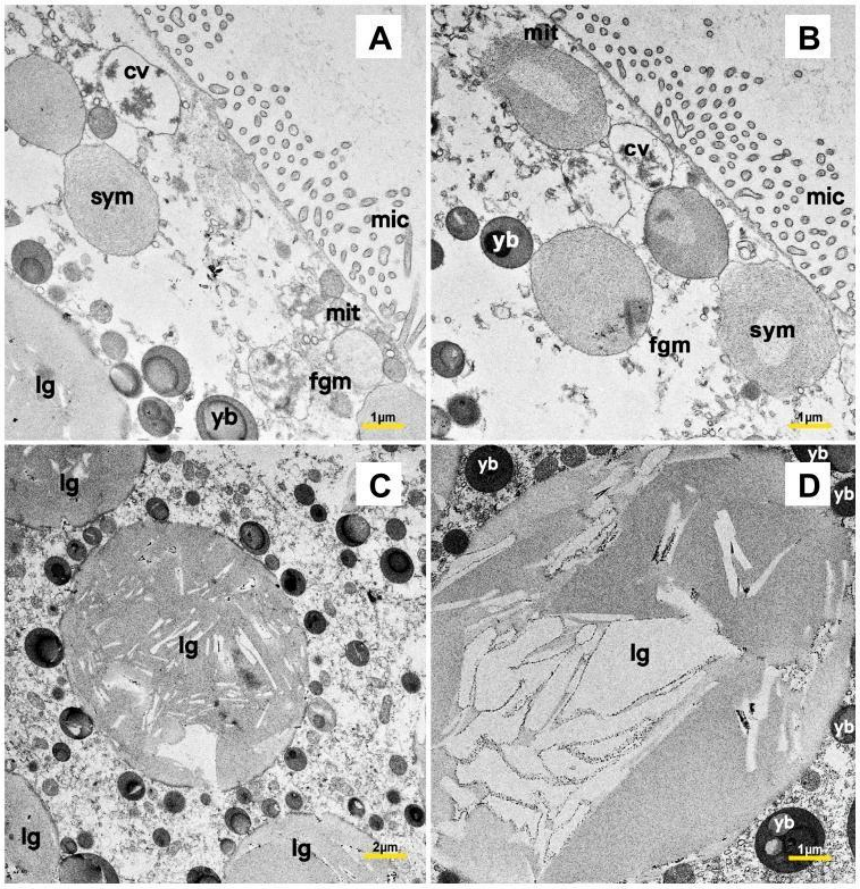

Fig. 2. Transmission electron microscopy showing the internal components of *M. harttii* oocytes. **(a)** Overview: note the presence of microvilli (mic), mitochondria (mit), cortical vesicles (cv), symbiodynium-like cells (sym), vitelline bodies (yb), lipid granules (lg), and fine granular material (fgm). **(b)** Microvilli (mic) surrounding the periphery of the oocyte in the form of dots due to the angle of the histological cut. **(c)** Spherical lipid granules (lg) with clear bands. **(d)** Detail of yolk bodies (yb) varying in size, shape and electron density.

bundles from different colonies (n = 6) were collected and immediately transferred to 10 mL tubes containing fixative solutions (n = 8) to investigate the morphology of the gametes when inside the bundles. Another 15 bundles were collected in 50 mL tubes (three bundles per tube) containing seawater, where they remained until complete dissociation, in order to later verify the morphology of oocytes and free-spawned sperm. The oocytes floated and occupied the surface of the tube, while the dense semen remained at the bottom. The oocytes were then isolated from the tube using a Pasteur pipette, washed in filtered seawater to remove any vestige of semen, and transferred to a fixative solution. Aliquots of 1 mL of semen were collected from the bottom of the tube and transferred directly into the fixative solution.

2.3. Scanning electron microscopy (SEM)

For the scanning electron microscopy, samples were fixed in 3 % glutaraldehyde and 0.1 M phosphate buffer, following the methodology described by Graham and Orenstein (2007). After fixing, the samples were washed in three baths (30 minutes each) with the same buffer solution to remove any excess fixative. Dehydration was then carried out in an incremental series of acetone (30–100 %) for 10 minutes. The material was desiccated using a Critical Point Dryer (Leica EM CPD030, Germany). After reaching the critical point, the pieces were placed on an aluminum stub, with the aid of a magnifier, to provide better visualization of the area of interest. For metallization, the material received gold and platinum conduction using a Super Cool Sputter Coater (Leica EM SCD050, Germany) and was taken for observation under a scanning electron microscope (Joel JSM 6060, EUA and Zeiss Evo 50, Germany).

2.4. Transmission electron microscopy (TEM)

For the analyses using transmission electron microscopy, the samples were fixed in a solution containing 2,5 % glutaraldehyde, 2 % paraformaldehyde, and 0.1 M phosphate buffer (Karnovsky, 1965). The pre-fixed material was washed three times (30 min each) in 0.2 M phosphate buffer to remove any excess fixative solution. Post-fixation was carried out in 2 % OsO_4 in 0.2 M phosphate buffer (45 min) followed by three baths in the same buffer (15 minutes each). Dehydration was then carried out in an incremental series of acetone (30–100 %) for 10 min and pre-infiltration was carried out in baths, mixing the dehydrant with resin (Epoxy Embedding kit) at gradual and increasing proportions of resin, with a minimum duration of 2 hours in each bath. Infiltration was carried out in a resin bath (100 %) over the course of 24 h and inclusion was carried out in silicon molds with pure resin in a hot air oven (60 °C) for 72 h. Ultrafine sections (80 nm) were deposited in grids, contrasted with an aqueous solution of 2 % uranyl acetate and lead citrate, analyzed, and photographed using transmission electron

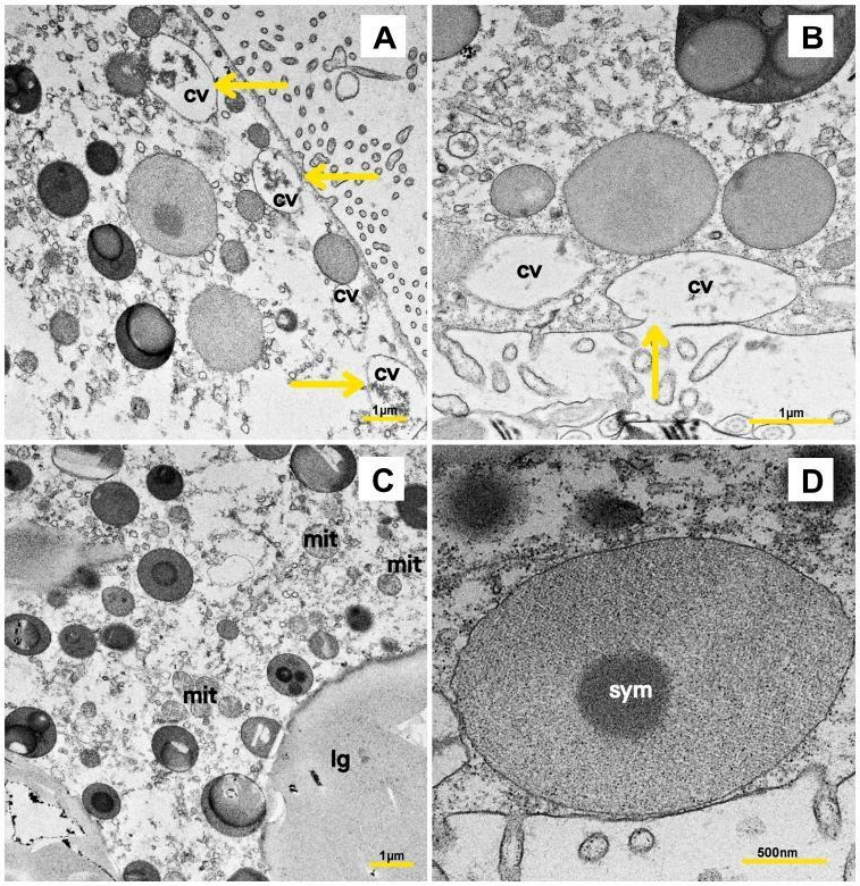

Fig. 3. Transmission electron microscopy detailing some internal components of *M. harttii* oocytes. **(a)** Homogeneous ovoid cortical vesicles (cv) with homogeneous filamentous content close to the oocyte plasma membrane (yellow arrows). **(b)** Cortical vesicle (cv) fused to the membrane and possibly releasing extracellular material (yellow arrow). **(c)** Presence of mitochondria (mit) distributed throughout the oocyte cytoplasm. **(d)** Symbiodinium-like cell (sym) near the periphery of the oocyte.

microscopy (FEI COMPANY, Tecnai G^2 20 S-TWIN, USA).

2.5. Image analyses

The images obtained through SEM and TEM were digitized and converted using Adobe Photoshop CS3 software (A. S. I, 345 Park Avenue, San Jose, California, USA), where they were also adjusted regarding focus, contrast, brightness, and gray scale. Qualitative and morphometric studies were performed on these oocytes (14 cells) and spermatozoa (44 cells) images, collecting information on the various components of the gametes. Morphometric analyses were performed using the ImageJ software (Microsoft Java 1.1.4.), with quantitative data presented as mean ± standard deviation. The following equation was used to determine the oocyte diameter (D):

$$D = \frac{larger\ diameter + smaller\ diameter}{2}$$

3. Results

3.1. Oocyte ultrastructure

The SEM analyses showed that the oocytes have a spherical shape, measuring 275.3 ± 0.02 µm in diameter (Fig. 1A-B) and are covered by a layer of mucus. Beneath the layer of mucus, microvilli are present along the oocytes' membrane (Fig. 1C-D). Among the typical microvilli, a scale-like membrane specialization was also observed (Fig. 1E-F).

In the oocytes evaluated by TEM, the presence of microvilli was observed in the external region. Inside, we observed cortical vesicles, lipid granules, yolk bodies of different sizes, shapes, and electron densities. In addition, fine granular material was found dispersed in the cytoplasm. Furthermore, we found mitochondria and Symbiodinium-like cells (Fig. 2A). Microvilli were observed in the external region of the oocyte plasma membrane. Due to the spherical shape of the oocytes and the angle from which the section was obtained, the microvilli, which are often shown as elongated finger-shaped projections, were seen as a series of dots surrounding the periphery of the oocyte (Fig. 2B). Inside the oocytes, the lipid granules were spherical and contained clear bands (12.1 ± 1.34 µm in diameter) (Fig. 2C) and numerous vitelline bodies around them (1.23 ± 1.21 µm in diameter) (Fig. 2C). The cortical vesicles (1.73 ± 1.25 µm in diameter) generally had an ovoid shape, homogeneous filamentous content and were located close (Fig. 3A) or fused to the plasma membrane (Fig. 3B). Mitochondria (0.43 ± 2.02 µm in diameter), were observed throughout the oocyte cytoplasm (Fig. 3C). Furthermore, small cells, described as Symbiodinium type (see Leite et al., 2017), were identified underlining the inner region of the plasma membrane (Fig. 3D).

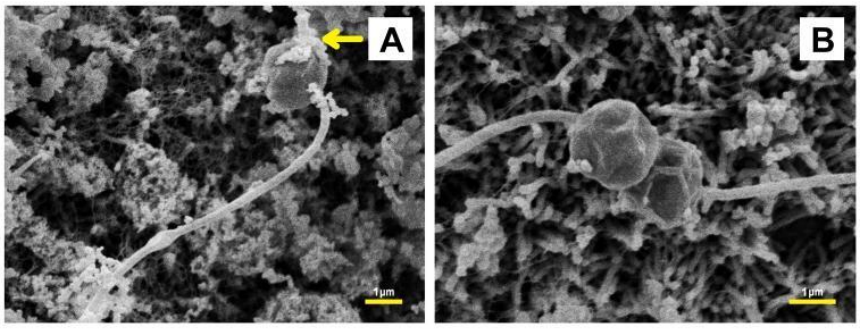

Fig. 4. Scanning electron microscopy of *M. harttii* sperm **(a)** Sperm with extended flagellum and mucus adhered to the head (yellow arrow). **(b)** Detail of the sperm head.

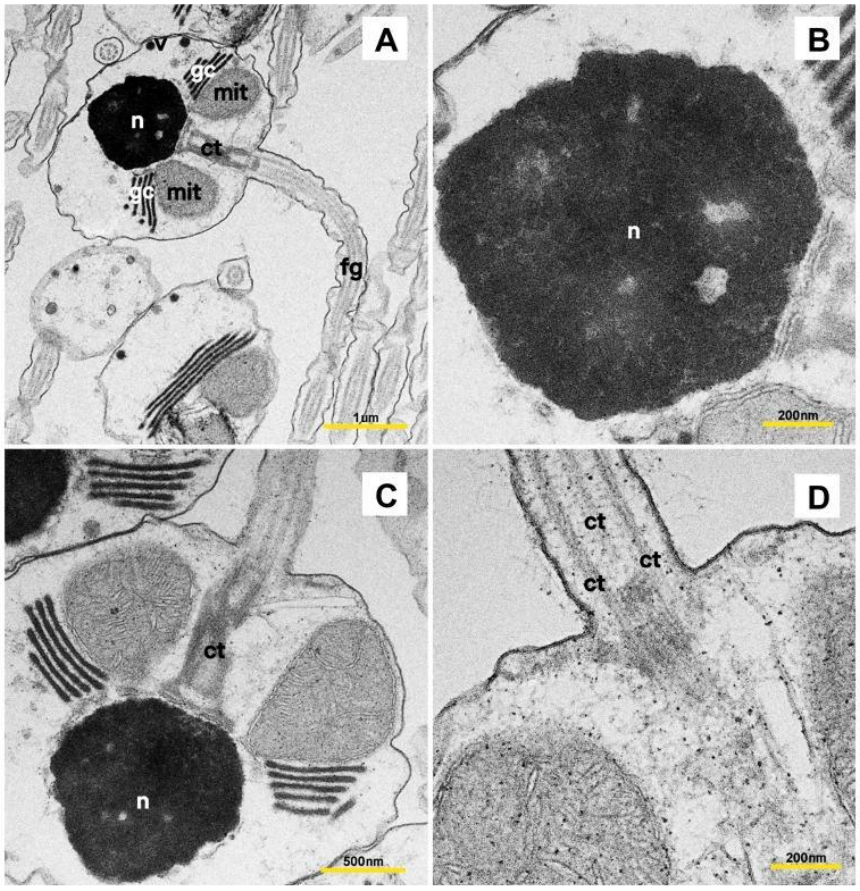

Fig. 5. Transmission electron microscopy of *M. harttii* spermatozoa. **(a)** Overview: Note the nucleus (n), the centriolar complex (ct), electrodense vesicles (v), Golgi complexes (gc), mitochondria (mit), and flagellum (fg). **(b)** Detail of the drop-shaped nucleus (n) with heterogeneous electron-dense genetic material. **(c)** The proximal and distal centriole (ct) positioned between the nuclear base and the flagellar pole of the cytoplasm. **(d)** Microtubules (ct) with central doublets and peripheral microtubule doublets.

3.2. Spermatozoa ultrastructure

The mean total length of *M. harttii* spermatozoon is 12.24 ± 2.51 µm, with a spherical head (2.02 ± 0.22 µm) and a long flagellum (9.3 ± 2.1 µm), which represents 90.82 % of its total size (Fig. 4A-B).

The nucleus in the head of the spermatozoon is featured by a drop-like shape and a heterogeneous electron-dense genetic material (Fig. 5A-B). The proximal centriole is positioned between the nuclear base, while the distal centriole connects to the flagellar pole of the cytoplasm (Fig. 5C-D) and is connected to the axoneme. A single axoneme, whose microtubule arrangement is nine peripheral doublets and one central doublet (9+2), was involved by the invagination of the plasma membrane, giving rise to the flagellar membrane (Fig. 6A-B). Underlining the inner plasmatic membrane, electron-dense vesicles

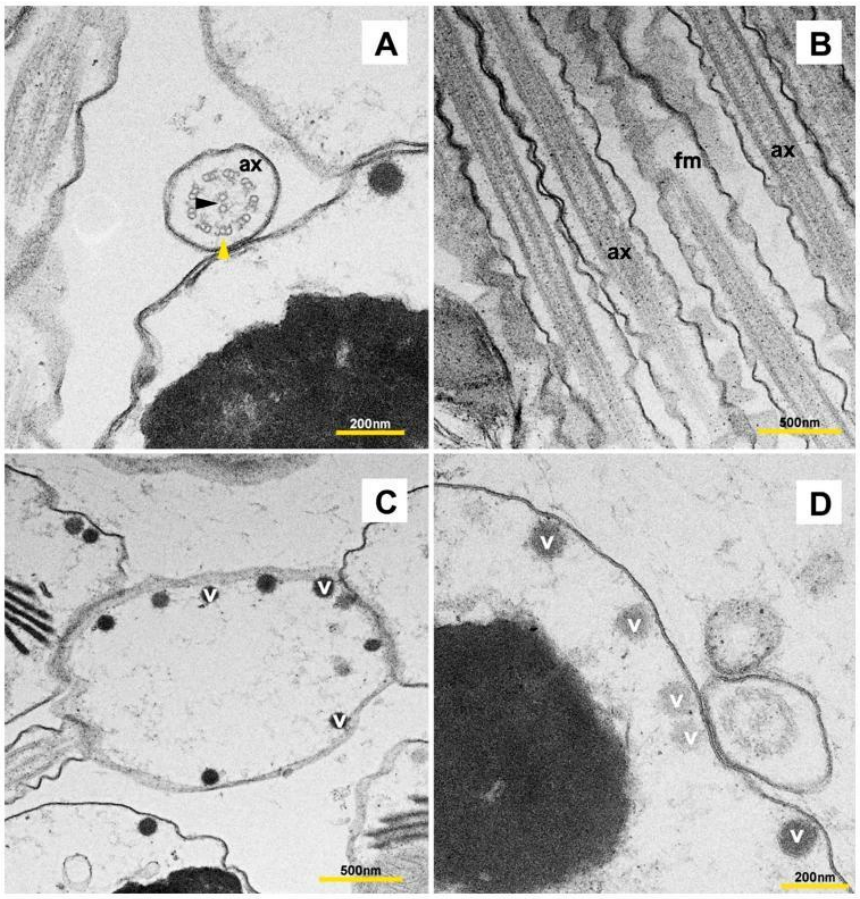

Fig. 6. Transmission electron microscopy of *M. harttii* spermatozoa showing **(a)** the microtubules, with the arrangement (9+2) with the nine peripheral doublets (yellow arrow) and central doublets (black arrow) of the axonemes (ax) **(b)** Detail of the longitudinal section of the flagellum showing the axoneme (ax) surrounded by the invagination of the plasma membrane (fm). **(c)** Vesicles (v) underlined in the inner plasma membrane of the sperm. **(d)** Details of the vesicles (v) delimited by lamellae similar to those found in association with Golgi complexes (gc).

were observed (Fig. 6C-D). These vesicles, delimited by lamellae, were very close to those found in association with Golgi complexes (Figs. 5A, 6C-D). In this regard, Golgi complex is distributed across the equatorial axis of the cell, which depicts a perpendicular angle with the flagellum (Fig. 5A-C; 7A-B). Mitochondria were observed occupying the quadrants delimited between the flagellum insertion and the Golgi complex (Fig. 7C-D), resembling a collar around the axoneme. Some sperm had condensed and other decondensed chromatin (Fig. 8). Based on the two-dimensional ultrastructural analysis, a schematic representation of *M. harttii* spermatozoon was proposed (Fig. 9).

4. Discussion

4.1. Oocyte ultrastructure

The present study brings new information about the morphology of gametes from the Brazilian coral *Mussismilia harttii*. We carried out a detailed ultrastructural morphological description of its gametes, including their organelles and their associated functions. The spherical shape of the oocyte, which was observed through SEM images, is expected for those that are dissociated from the gamete bundles and means they were already exposed to the seawater milieu, acquiring suitable conditions for fertilization (Valente et al., 2023). The microvilli of the *M. harttii* oocytes are similar to those found for oocytes in another six species of stony corals (Tsai et al., 2016) and in *Junceella juncea* (Tsai et al., 2014) and *Heteroxenia fuscescens* (Benayahu et al.,1989) soft corals species. These microvilli presented a slender and spaced structure and were distributed over the entire surface of the oocytes. The presence of these microvilli corroborates the findings of Padilla-Gamiño et al. (2011) for mature oocytes of the coral *Montipora capitata*.

In addition to indicating oocyte maturity, we believe that the microvilli observed could have a protective and nutritional role in the oocyte of *Mussismilia harttii*, as already pointed out by Tsai et al. (2014, 2016). Ultrastructural observations in oocytes of some aquatic invertebrates, such as the mollusks *Lymnaea stagnalis* (Eckelbarger and Davis, 1996) and *Haliotis varia* (Najmudeen, 2008), show that the microvilli function as a protective barrier, avoiding damages caused by the friction between female gametes during oogenesis and by physical shocks when released into the external environment. The molecular and histological studies conducted by Tan et al. (2020) indicate that microvilli may play a role in the capture of nutrients by the oocytes during the vitellogenesis of the coral *Acropora tenuis*. Moreover, other studies have also shown that microvilli may be associated with the fertilization process (Matsunaga et al., 2002). The morphological and ultrastructural observations regarding the oocytes of the sea urchin *Arbacia punctulata* demonstrate that microvilli assist in the formation of the fertilization cone, a protoplasmic extension that forms at the point of contact between the spermatozoon and oocyte to prevent polyspermy

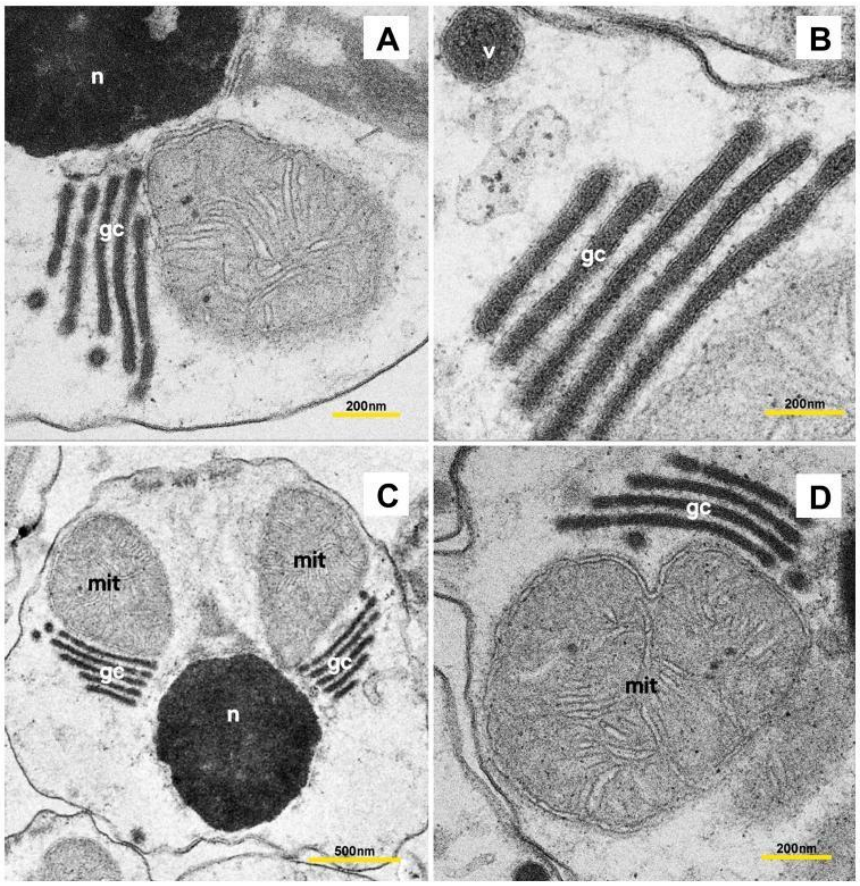

Fig. 7. Transmission electron microscopy of *M. harttii* spermatozoa. (a) Golgi complex (gc) distributed along the equatorial axis of the cell, forming a perpendicular angle to the flagellum. (b) Details of the Golgi complex (gc), showing lamellae similar to those found in association with vesicles (v). (c) Mitochondria (mit) occupying the quadrant delimited between the intersection of the flagellum and the Golgi apparatus (gc). (d) Detail of a mitochondrion (mit), showing cristae and double membrane.

(Tilney and Jaffe, 1980). When evaluating the microvilli structure in oocytes of the sea star *Patiria pectinifera* at different maturity stages, Santella et al. (2020) observed that when the oocytes reach an optimal period for fertilization, they became shorter and a process of structural maturation began, promoting the migration and accumulation of cortical granules towards the cortex of the oocyte. In fact, our results show the presence of symbiotic cells close to the plasma membrane. These findings make us believe that microvilli could be related to nutrient intake. Therefore, it is clear that greater attention should be given to this subject and that more detailed and specific studies are needed to assist in better comprehending the role of microvilli in coral oocytes.

In some regions, it was possible to observe among the microvilli a structure that was similar to scales. Based on observations regarding the surface of *M. harttii* oocytes through SEM, we believe that this scale-like structure could in fact be remnants of the hexagonal structures where the spermatozoa are stored before the bundle is dissociated (Valente et al., 2023), which must remain adhered to the oocyte with mucus.

TEM images demonstrated that most of the volume of *M. harttii* oocytes consists of vitelline materials, such as vitelline bodies (spherical, electron-dense and granulated), lipid granules (spherical, larger and with light bands) and vesicles. Vitelline bodies and lipid granules are associated with the energy reserve of oocytes (Tsai et al., 2016). Furthermore, the presence of a large number of these structures in the cytoplasm of oocytes suggests that these organelles may be associated with the buoyancy of gametes, after dissociation of the bundles. In addition to these vitelline materials, numerous vesicles were also found dispersed in the cytoplasm. These vesicles may serve to transport nutrients from the endoplasmic reticulum to vitelline materials (Tsai et al., 2016).

Observations regarding the lipid content within the oocytes of different coral species reveal that lipidic granules are also used as an energy source for tissue growth, through the reabsorption of immature oocytes by the end of oogenesis (Davies, 1991). The decrease of lipid content during oocytes development of *Junceella juncea* and *Junceella fragilis* (Lin et al., 2012) supports the importance of lipid substrate as an energy source during oogenesis in coral species. In addition, lipidic granules are used as metabolic energy (Imbs et al., 2006) and supply energy for larval development after fertilization (Edmunds and Davies, 1986).

Studies involving marine invertebrates, such as the sea anemone *Bunodosoma cavernata* (Dewel and Clark, 1974) and the sea urchin *Arbacia lixula* (Monroy, 1953), showed cortical vesicles fused to the plasma membrane and releasing cortical granules to the extracellular region of the oocyte, after contact with seawater. No ultrastructural study has assessed the possible cortical reactions in coral oocytes after contact with water and the subsequent fertilization yet. In this regard, the electron density observed for the granules present inside the cortical

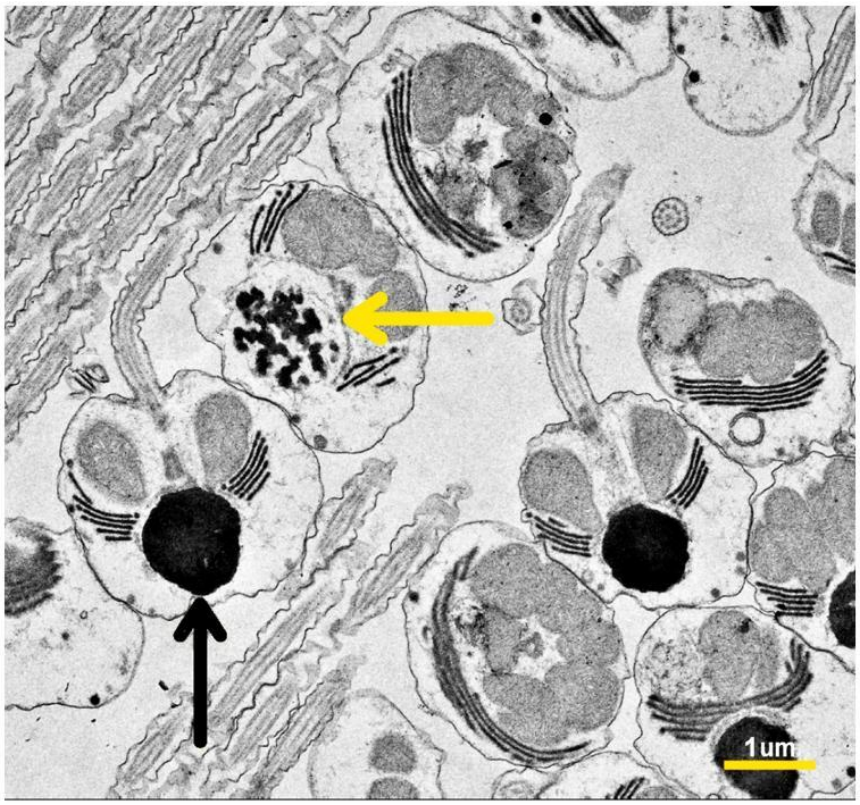

Fig. 8. Transmission electron microscopy of *M. harttii* spermatozoa. Sperm with decondensed chromatin (yellow arrow) next to sperm with condensed chromatin (black arrow).

vesicles and in the mucous layer (extracellular) of *M. harttii* bundles suggests that these vesicles may also assist in the formation of the oocyte-sperm bundles. The study by Padilla-Gamiño et al. (2011) also showed that the electron density of granules present within the oocytes of the scleractinian coral *Montipora capitata* was similar to the mucus that forms the oocyte-sperm bundles of this species.

The mitochondria were widely distributed throughout the cytoplasm of the *M. harttii* oocytes, given their main role of supplying the necessary energy for oocyte development (Tsai et al., 2016). The distribution of mitochondria across the whole oocyte seems to be a common characteristic among many marine invertebrates, such as the ascidia *Ciona intestinalis* (Tosti et al., 2003), the mollusk *Bolinus brandaris* (Pérez et al., 2004), and the anemone *Actinio fragacea* (Larkman, 1984).

Scleractinian corals have an endosymbiotic relationship with dinoflagellates of the genus *Symbiodinium* (Muscatine, 1990). The acquisition of *Symbiodinium* by the gastrodermal cells of the coral usually occurs through either vertical or horizontal transmission (Lin et al., 2018). The offspring of certain coral genera, such as *Montipora*, *Porites*, and *Pocillopora* (Padilla-Gamiño et al., 2012; Sharp et al., 2012; Ceh et al., 2012), inherit the *Symbiodinium* from the parental lineage (vertical transmission), though this phenomenon is more common among brooding corals. Species that release gamete bundles usually require that their gametes or larvae obtain the *Symbiodinium* from the water column (horizontal transmission). Inheriting *Symbiodinium* through oocytes could influence settlement behavior and fertilization success (Boulotte et al., 2016).

Although the occurrence of Symbiodinium in the oocyte cytoplasm is still a finding little reported in the literature for broadcast spawning corals, Leite et al. (2017) when evaluating the ultrastructure of the gamete bundle of *Mussismilia hispida* found small cells called *Symbiodinium-like* in the peripheral region of the oocytes. These findings corroborate our observations by TEM, which revealed the presence of *Symbiodinium-like* cells in *M. harttii* oocytes. Thus, based on findings of Leite and colleagues, we believe that the transmission of these cells in *M. harttii* occurs vertically, from parental colonies to oocytes. However, further studies are needed to better understand the mechanisms associated with symbiotic cell transmission in this species.

4.2. Spermatozoa ultrastructure

Perhaps one of the reasons for the scarce knowledge on the morphology of mature spermatozoa in broadcast spawners is because of their rapid dilution and dispersion in the ocean. Herein we present new information about the morphology and ultrastructure of male gametes.

The head of *M. harttii* spermatozoon is devoid of an acrosome vesicle, however, some electron-dense vesicles were found distributed in the peripheral region. At the antiflagellar cell pole, electron-dense vesicles present close to the inner membrane of the male gamete may have a role similar to that of the acrosome. We believe that these electron-dense vesicles may be involved in the fusion of male and female gametes. This hypothesis is corroborated by the morphology of the oocytes, since we did not observe the presence of a germinative pore (micropyle) in the female gamete of *M. harttii*. When evaluating the ultrastructure of the spermatozoa of four species of scleractinian corals in the Caribbean, Steiner (1991, 1993) observed that the species *Siderastrea siderea* presented electron-dense membranous vesicles (called proacrosomal vesicles) near the plasma membrane. Electron-dense vesicles were also found in the gonochoric black corals *Cirrhipathes* sp. (Gaino and Scoccia,

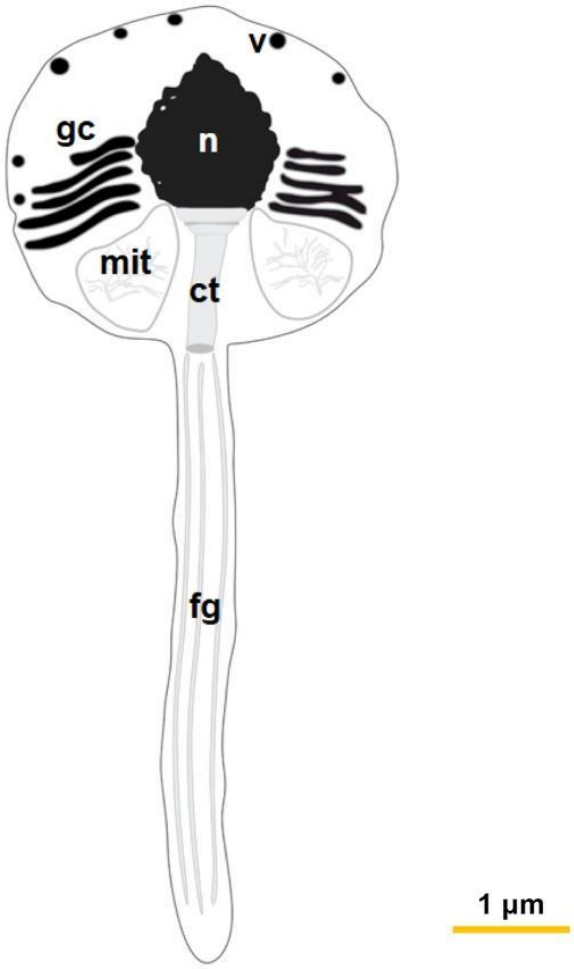

Fig. 9. Schematic representation of *M. harttii* spermatozoa based on two-dimensional ultrastructural analysis. Proacrosomal vesicles (v), nucleus (n), Golgi complexes (gc), mitochondria (mit), centriole (ct) and flagellum (fg).

2008) and *Rhipidipathes reticulata* (Gaino and Scoccia, 2010). Positioned on the flagellar pole of the spermatozoon head, centriole and axoneme were connected and stablished the classical microtubule arrangement (9 + 2), which constitutes the flagellar axis. The presence of electron-dense vesicles and axonemes in such arrangement in the flagella of the spermatozoa were already reported as typical characteristics of primitive spermatozoa, which carry out external fertilization (Reunov, 2005).

The qualitative evaluation of electron density of the very well-developed Golgi complex and the sprouting vesicles that migrates toward the anti-flagellar pole of the sperm head, indicates the Golgi potential origin of the proacrosomal vesicles in *M. harttii*. The presence of mitochondria surrounding the spermatozoon flagellum is a well-known characteristic, preserved among the species of vertebrates and invertebrates already investigated (Padilla-Gamiño et al., 2011). Mitochondria are associated with the supply of energy for flagella movement of the spermatozoon during spawning (Cummins, 2009). Regardless of the middle piece formation, mitochondria in *M. harttii* were strategically positioned close to the flagellum implantation site, assuming a ring-like disposition. The large mitochondria, as observed in the present study, seem to be a mature spermatozoa characteristic, as already reported for the gonochoric coral *Cirrhipathes* sp. In this species, numerous small mitochondria were observed in the immature spermatozoa, and during the spermatogenesis process, these organelles fuse to each other, originating a larger mitochondrion (Gaino and Scoccia, 2008). Only a few spermatozoa of *M. harttii* showed the small mitochondria type, which is in accordance with the mature status of the sampled cells evaluated in the present study. Thus, we suggest that as the spermatogenesis advances, the small mitochondria present int the immature spermatozoon tend to fuse together forming the large mitochondrion of the mature gamete. According to Steiner (1991), the partial or total fusion of mitochondria in scleractinian corals occurs to increase the inner and decrease the outer membranes. This strategy is used to decrease barriers, promoting a more efficient movement in the inner substrate of the mitochondrial matrix (Baccetti and Afzelius, 1976). The presence of free axonemes, small mitochondria, and euchromatic nucleus were stated as morphological markers of spermatozoa immaturity in black corals *Cirrhipathes* sp. and *Cupressopathes pumila* (Gaino and Scoccia, 2008, 2009). Similarly, in our study, the spermatic cells exhibiting nuclear dispersed chromatin may be an indicative that some spermatozoa during the spawning of *M. harttii* were still immature.

In summary, the present study is the first report on the ultrastructure of the spermatozoa and oocytes of a scleractinian coral that is endemic to the South Atlantic Ocean. Analysis through SEM and TEM are essential to understand in detail the biology and physiology of coral gametes. The findings described herein can support future, and much needed, studies that seek to better comprehend the gametogenesis, formation of gamete bundles, and the fertilization of scleractinian corals. Moreover, reproductive biotechnologies, such as gamete cryopreservation and *in vitro* fertilization can also make good use of the information reported.

CRediT authorship contribution statement

Jener Alexandre Sampaio Zuanon: Writing – review & editing, Writing – original draft, Validation, Supervision, Conceptualization. **Cláudia Kelly Fernandes da Cruz:** Writing – review & editing, Writing – original draft, Formal analysis. **Wanderson Valente:** Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Methodology, Formal analysis, Data curation, Conceptualization. **Gleide Fernandes de Avelar:** Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Formal analysis, Conceptualization. **Leandro Godoy:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of Competing Interest

The authors declare no competing interests.

Data availability

Data will be made available on request.

Acknowledgments

The Coral Vivo Project and its sponsors, Petrobras, through the Petrobras Socioenvironmental Program, and Arraial d'Ajuda Eco Parque, are acknowledged for financing the field work and allowing the use of the research station installations. WV received a research scholarship from the Brazilian Biodiversity Fund (FUNBIO) and from the Humanize Institute (N° 121/2019). We thank the financial support from the Boticário Group Foundation for Nature Protection (1138–2018/2). LG is a research grantee of CNPq-Brazil (308784/2022–7).

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DISCUSSÃO GERAL

O conhecimento a respeito da biologia reprodutiva em corais escleractíneos é crucial para sua sobrevivência e resiliência, diante das constantes mudanças climáticas (Ayalon et al., 2019). Os recifes de coral suportam alta biodiversidade de espécies e atuam em diversos serviços ecossistêmicos, incluindo proteção costeira, pesca e turismo (Wild et al., 2011; Anthony et al., 2014). Um entendimento detalhado da biologia reprodutiva dos corais escleractíneos auxiliará no desenvolvimento de estratégias de conservação eficazes, aumentando a resiliência e assegurando a sobrevivência dos recifes de coral.

No capítulo 1, exploramos a gametogênese em profundidade utilizando técnicas histomorfométricas, imunofluorescência e imuno-histoquímica. Essas análises permitiram entender a organização e o arranjo das gônadas, além de corroborar sobre a sua estratégia reprodutiva. Também investigamos os processos de maturação dos gametas e os fatores ambientais que influenciam a gametogênese.

De forma geral, observamos que as gônadas masculinas na *Mussismilia harttii* estão localizadas próximas à região oral, enquanto as gônadas femininas se encontram na região aboral do pólipo. A *M. harttii* é uma espécie hermafrodita, com três estágios de maturação dos gametas e desova sincrônica. Estudos mostram que, o sucesso reprodutivo em corais escleractíneos é significativamente influenciado por sua organização gonadal, hermafroditismo e fatores ambientais (Kerr et al., 2010). A organização das gônadas dentro dos pólipos facilita a formação dos pacotes de gametas e a liberação dos espermatozoides e oócitos durante a desova (Baird et al., 2009). O hermafroditismo aumenta o sucesso reprodutivo a partir da variabilidade genética pelo processo de fecundação cruzada (Rapuano et al., 2017). Além disso, devido às pressões ambientais, as mudanças na temperatura da água podem afetar a viabilidade dos gametas durante as desovas (Harrison, 2010).

A gametogênese nos corais escleractíneos é complexa e envolve uma série de estágios de desenvolvimento que são regulados sincronicamente por mecanismos biológicos internos e sinais ambientais externos (Tarrant et al., 2004; Maboloc et al., 2015; Shikina et al., 2020). A sincronização durante a gametogênese aumenta a probabilidade de uma fertilização bem-sucedida, pois garante que os espermatozoides e os oócitos estejam presentes no mesmo local de desova (Craggs et al., 2017; Fang et al., 2023). A liberação dos gametas geralmente ocorre em eventos curtos, durante alguns dias no ano e normalmente é alinhado com as fases da lua (O'Neil et al., 2021; Sakai, 2024).

No capítulo 2, conduzimos uma análise detalhada da ultraestrutura da gametogênese na *M. hartti* utilizando microscopia eletrônica de varredura (MEV) e de transmissão (MET). As investigações ultraestruturais possibilitaram compreender como a morfologia celular e as organelas atuam na maturação e no desenvolvimento dos gametas.

De acordo com as análises de MET da espermatogênese, observamos a presença de núcleos, axonemas, mitocôndrias, flagelos, complexos de Golgi e vesículas elétron-densas semelhantes ao acrossoma nas células germinativas masculinas. Os núcleos em invertebrados marinhos são responsáveis pelos processos transcricionais e passam por mudanças morfológicas significativas como a condensação da cromatina, que é importante para a integridade dos espermatozoides (Sperry, 2012). Além disso, a interação entre o núcleo e outras organelas, como o complexo de Golgi e as mitocôndrias, são necessárias para o transporte e a montagem de proteínas necessárias na funcionalidade do gameta masculino (Liu et al., 2021). O axonema é empregado na motilidade flagelar, crucial para o movimento dos espermatozoides (Qü et al., 2020). As vesículas apresentam funções semelhantes ao acrossoma, estão envolvidas no armazenamento de enzimas responsáveis na reação de fusão dos gametas (Kaneda, 2023).

No contexto da espermatogênese, Shikina et al., (2017), destacam que os flagelos no coral *Euphyllia ancora*, por meio da MET, ocorrem em diferentes estágios, incluindo nas espermatogônias e espermatócitos. Além disso, Shikina et al., (2020), demonstram a partir de análises ultraestruturais dos tecidos gastrodérmicos de *Euphyllia ancora*, a ocorrência de apoptose em células somáticas, importantes no suporte nutricional aos gametas em desenvolvimento.

Já as análises de MET durante a oogênese, observamos a presença de microvilosidades, vesículas corticais, grânulos lipídicos, corpos de vitelo, mitocôndrias e células semelhantes a *Symbiodinium* nas células germinativas femininas. As microvilosidades aumentam a área de superfície do oócito, facilitando a absorção de nutrientes e a interação entre as células circundantes (Sathananthan et al., 2006). Vesículas corticais, estão localizadas logo abaixo da membrana do oócito e são essenciais para prevenir a polispermia (Kanagaraj et al., 2014). Grânulos lipídicos e corpos de vitelo são vitais para o fornecimento de energia e nutrientes ao gameta em maturação e ao embrião em desenvolvimento. A microscopia eletrônica revelou que essas estruturas são frequentemente associadas ao retículo endoplasmático e ao complexo de Golgi, destacando seu papel no armazenamento e transporte de vesículas (Grier, 2012; Tsai et al., 2014). As mitocôndrias são cruciais para a produção de energia e regulação metabólica dentro dos oócitos (Grier, 2012; Manandhar et al., 2005). Finalmente, a presença de células semelhantes aos *Symbiodinium*, indicam uma interação entre esses organismos fotossintetizantes e os oócitos sobre os aspectos do fornecimento de energia e nutrição (Tsai et al., 2014).

Nesse contexto, estudos sobre a oogênese em corais escleractíneos demonstram que a microscopia eletrônica tem sido amplamente utilizada para analisar os estágios iniciais e finais do desenvolvimento dos oócitos no coral *Junceella juncea*. Essa abordagem permitiu a identificação de componentes celulares essenciais, como grânulos citoplasmáticos e o

complexo de Golgi, que desempenham papeis fundamentais na maturação dos oócitos e no armazenamento de nutrientes (Tsai et al., 2014).

No capítulo 3, trazemos informações inéditas sobre a morfologia dos gametas maduros do coral *Mussismilia harttii*. Nós realizamos uma descrição morfológica ultraestrutural detalhada, avaliando as organelas presentes no interior dos gametas bem como suas funções associadas. O formato arredondado dos oócitos e suas microvilosidades alongadas indicam maturidade e podem ter funções protetoras e nutricionais, como observado em outros invertebrados aquáticos (Padilla-Gamiño et al., 2011). Os corpos de vitelo e os grânulos de lipídeos são fontes importantes de energia para desenvolvimento e flutuabilidade do gameta (Tsai et al., 2016). Vesículas corticais, localizadas próximas à membrana plasmática, liberam grânulos em resposta ao contato com a água, sugerindo participação em processos como a formação de pacotes de gametas (Padilla-Gamiño et al., 2011). Mitocôndrias distribuídas pelo citoplasma fornecem energia essencial ao desenvolvimento (Pérez et al., 2004). Foi observada uma relação simbiótica com dinoflagelados do gênero *Symbiodinium*, fornecendo energia e influenciando no desenvolvimento larval (Lin et al., 2018).

Talvez uma das razões para o pouco conhecimento morfológico acerca dos espermatozoides maduros em corais escleractíneos liberadores de gametas seja devido a sua rápida diluição e dispersão no oceano. Assim, as informações inéditas sobre a morfologia e ultraestrutura dos gametas masculinos maduros foram registradas. Os espermatozoides apresentam cabeça ovoide com núcleo central e vesículas elétron-densas, com possível função acrossômica, formadas pelo complexo de Golgi (Gaino & Scoccia, 2010). Os axonemas apresentam arranjo típico (9+2), característico de espermatozoides primitivos de fecundação externa (Gaino et al., 2008). Uma única mitocôndria circundante ao axonema fornece energia ao flagelo (Cummins, 2009). A microscopia revelou detalhes importantes sobre a formação e

função das estruturas dos espermatozoides, contribuindo para o entendimento da fecundação em corais escleractíneos.

Estudar a morfologia e a ultraestrutura dos espermatozoides e oócitos maduros é essencial para uma compreensão abrangente de sua biologia reprodutiva e suas adaptações evolutivas. Esse conhecimento não apenas aprimora nossa compreensão da biologia reprodutiva dos corais, mas também informa os esforços da conservação que visam preservar esse ecossistema marinho.

A sincronização na liberação de gametas durante os eventos de desova é uma estratégia reprodutiva essencial que aumenta as taxas de fertilização e a sobrevivência larval (Twan et al., 2005). Entender esses processos por meio de estudos ultraestruturais podem informar estratégias de conservação, especialmente à luz das ameaças representadas pelas mudanças climáticas (Kirk et al., 2013). Características morfológicas dos gametas podem fornecer informações sobre as adaptações evolutivas. A presença de estruturas especializadas, como os flagelos indicam caminhos evolutivos e estratégias reprodutivas que se desenvolveram em resposta a pressões ambientais (Shikina et al., 2020).

Portanto, a integração da microscopia avançada e técnicas histológicas é essencial para entender a biologia reprodutiva na *Mussismilia hartti* e contribuir para sua conservação. A microscopia eletrônica permite visualizar detalhes ultraestruturais, para compreender a formação dos gametas, processos reprodutivos, produção dos pacotes de gametas e fertilização na *M. hartti*. Métodos por marcação de imuno-histoquímica e imunofluorescência auxiliam no conhecimento sobre os aspectos bioquímicos dos gametas, identificando proteínas e marcadores envolvidos na maturação das células germinativas primordiais. Esses avanços permitem prever resultados promissores sobre a biologia reprodutiva na *M. hartti* e conservação dos recifes de coral ao redor do mundo.

CONCLUSÕES

ARTIGO 1 – Morphofunctional evaluation of gametogenesis in the endemic South Atlantic reef-builder Mussismilia harttii"

- Os mesentérios foram observados na região oral ao aboral, contendo gônadas masculinas e femininas, entre as camadas celulares do endoderma;
- As gônadas masculinas estavam predominantemente próximas à região oral (superior), enquanto as femininas localizavam-se na região aboral (inferior) do pólipo;
- A espécie é hermafrodita, apresentando gônadas femininas e masculinas no mesmo indivíduo;
- A taxa de ocupação dos gametas durante o período de maturação celular foi de aproximadamente 90% dos mesentérios, e a distribuição entre as gônadas masculinas e femininas foi bastante semelhante;
- A gametogênese apresenta três estágios de maturação dos gametas;
- Os gametas femininos e masculinos atingiram a maturidade sincronicamente;
- A distribuição das áreas gametogênicas femininas e masculinas nos mesentérios e a flutuação da temperatura da água do mar foram significativamente correlacionados.

ARTIGO 2 – Evaluating the maturation of male and female gametes in the South Atlantic endemic coral Mussismilia harttii using ultrastructural analysis

- A presença dos cistos espermáticos fornece um ambiente propício para o desenvolvimento dos gametas masculinos;
- A espermiogênese ocorre no interior dos cistos espermáticos;
- A espermatogênese apresenta três estágios de maturação;
- As células germinativas masculinas apresentam características morfológicas e organelas

distintas no decorrer da espermatogênese;

- A oogênese consiste em três estágios de desenvolvimento;
- As células germinativas femininas sofrem mudanças morfológicas e estruturais no decorrer de toda a oogênese.

ARTIGO 3 – Ultrastructural evaluation of the oocytes and spermatozoa of the scleractinian coral Mussismilia harttii

- Os oócitos maduros apresentam microvilosidades na região externa e estas estão associadas a nutrição e fecundação;
- Os grânulos lipídicos e os corpos vitelinos são abundantes e estão envolvidos na flutuabilidade e no fornecimento de energia ao embrião após a fecundação;
- As vesículas corticais localizadas próximas ou fundidas à membrana plasmática estão relacionadas na polispermia e formação dos pacotes de gametas;
- As mitocôndrias distribuídas por todo o citoplasma produzem energia aos oócitos;
- As células semelhantes aos *Symbiodinium*, fornecem suporte energético para o oócito através da fotossíntese;
- O espermatozoide apresenta uma cabeça esférica e um flagelo longo, que representa 90,82% do seu tamanho total;
- O núcleo, localizado centralmente na cabeça do espermatozoide é responsável pelos processos transcricionais;
- O axonema, cujo arranjo de microtúbulos é de (9+2), foi envolvido pela membrana plasmática, dando origem ao flagelo, responsável pelo batimento durante a fecundação;
- O complexo de Golgi é responsável pela formação das vesículas pró-acrossomais;
- A mitocôndria está envolvida no fornecimento de energia durante o batimento flagelar;
- As vesículas pró-acrossomais auxiliam no processo de fusão dos gametas.

Portanto, as análises por meio das técnicas histológicas, imuno marcação e microscopia eletrônica são essenciais para compreendermos em detalhes a biologia reprodutiva dos corais. Os achados aqui detalhados darão suporte para futuros e necessários estudos que buscam compreender melhor a reprodução sexual e a fecundação em corais escleractíneos em todo o mundo.

Esta tese lança luz sobre uma área pouco explorada que pode auxiliar em um melhor entendimento sobre a fertilização de corais hermafroditas, bem como fornecer suporte para estudos futuros sobre biotecnologias reprodutivas para a conservação de corais.

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ANEXOS

Ministério do Meio Ambiente - MMA
Instituto Chico Mendes de Conservação da Biodiversidade - ICMBio
Sistema de Autorização e Informação em Biodiversidade - SISBIO

Autorização para atividades com finalidade científica

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Dados do titular

Nome: LEANDRO CESAR DE GODOY	CPF: 049.140.259-73
Título do Projeto: Estudo morfofuncional da gametogênese do coral Mussismilia harttii (Verrill, 1868)	
Nome da Instituição: UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL	CNPJ: 92.969.856/0001-98

Cronograma de atividades

#	Descrição da atividade	Início (mês/ano)	Fim (mês/ano)
1	Reunião mensal da equipe	07/2021	12/2024
2	Aquisição de reagentes e materiais	07/2021	12/2023
3	Coleta das amostras na APA Costa dos Corais	08/2021	08/2023
4	Microscopia óptica e imunohistoquímica	01/2022	11/2023
5	Relatório final	11/2024	12/2024
6	Redação e submissão de artigos científicos	06/2022	11/2024
7	Processamento e análise dos dados	02/2022	08/2024
8	Microscopia eletrônica de varredura e transmissão	10/2021	07/2023

Equipe

#	Nome	Função	CPF	Nacionalidade
1	GLEIDE FERNANDES DE AVELAR	Pesquisadora	034.574.126-93	Brasileira
2	Pedro Henrique Cipresso Pereira	Pesquisador	014.998.396-42	Brasileira
3	Gislaine Vanessa de Lima	Pesquisadora	088.878.044-35	Brasileira
4	Wanderson Valente dos Santos	Pesquisador	112.261.696-19	Brasileira
5	ANTONIO VITOR DE FARIAS PONTES	Pesquisador	105.004.734-63	Brasileira
6	Amanda Pereira de Amaral	Pesquisadora	020.254.792-20	Brasileira

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Nome da Instituição: UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL	CNPJ: 92.969.856/0001-98

Observações e ressalvas

1	A autorização não eximirá o pesquisador da necessidade de obter outras anuências, como: I) do proprietário, arrendatário, posseiro ou morador quando as atividades forem realizadas em área de domínio privado ou dentro dos limites de unidade de conservação federal cujo processo de regularização fundiária encontra-se em curso; II) da comunidade indígena envolvida, ouvido o órgão indigenista oficial, quando as atividades de pesquisa forem executadas em terra indígena; III) do Conselho de Defesa Nacional, quando as atividades de pesquisa forem executadas em área indispensável à segurança nacional; IV) da autoridade marítima, quando as atividades de pesquisa forem executadas em águas jurisdicionais brasileiras; V) do Departamento Nacional da Produção Mineral, quando a pesquisa visar a exploração de depósitos fossilíferos ou a extração de espécimes fósseis; VI) do órgão gestor da unidade de conservação estadual, distrital ou municipal, dentre outras.
2	O pesquisador somente poderá realizar atividade de campo após o término do estado de emergência devido à COVID-19, assim declarado por ato da autoridade competente.
3	Esta autorização NÃO libera o uso da substância com potencial agrotóxico e/ou inseticida e NÃO exime o pesquisador titular e os membros de sua equipe da necessidade de atender às exigências e obter as autorizações previstas em outros instrumentos legais relativos ao registro de agrotóxicos (Lei nº 7.802, de 11 de julho de 1989, Decreto nº 4.074, de 4 de janeiro de 2002, entre outros).
4	Esta autorização NÃO libera o uso da substância com potencial agrotóxico e/ou inseticida e NÃO exime o pesquisador titular e os membros de sua equipe da necessidade de atender às exigências e obter as autorizações previstas em outros instrumentos legais relativos ao registro de agrotóxicos (Lei nº 7.802, de 11 de julho de 1989, Decreto nº 4.074, de 4 de janeiro de 2002, entre outros)
5	Este documento somente poderá ser utilizado para os fins previstos na Instrução Normativa ICMBio nº 03/2014 ou na Instrução Normativa ICMBio nº 10/2010, no que especifica esta Autorização, não podendo ser utilizado para fins comerciais, industriais ou esportivos. O material biológico coletado deverá ser utilizado para atividades científicas ou didáticas no âmbito do ensino superior.
6	As atividades de campo exercidas por pessoa natural ou jurídica estrangeira, em todo o território nacional, que impliquem o deslocamento de recursos humanos e materiais, tendo por objeto coletar dados, materiais, espécimes biológicos e minerais, peças integrantes da cultura nativa e cultura popular, presente e passada, obtidos por meio de recursos e técnicas que se destinem ao estudo, à difusão ou à pesquisa, estão sujeitas a autorização do Ministério de Ciência e Tecnologia.
7	O titular de licença ou autorização e os membros da sua equipe deverão optar por métodos de coleta e instrumentos de captura direcionados, sempre que possível, ao grupo taxonômico de interesse, evitando a morte ou dano significativo a outros grupos; e empregar esforço de coleta ou captura que não comprometa a viabilidade de populações do grupo taxonômico de interesse em condição in situ.
8	Este documento não dispensa o cumprimento da legislação que dispõe sobre acesso a componente do patrimônio genético existente no território nacional, na plataforma continental e na zona econômica exclusiva, ou ao conhecimento tradicional associado ao patrimônio genético, para fins de pesquisa científica, bioprospecção e desenvolvimento tecnológico. Veja maiores informações em www.mma.gov.br/cgen.
9	Esta autorização NÃO exime o pesquisador titular e os membros de sua equipe da necessidade de obter as anuências previstas em outros instrumentos legais, bem como do consentimento do responsável pela área, pública ou privada, onde será realizada a atividade, inclusive do órgão gestor de terra indígena (FUNAI), da unidade de conservação estadual, distrital ou municipal, ou do proprietário, arrendatário, posseiro ou morador de área dentro dos limites de unidade de conservação federal cujo processo de regularização fundiária encontra-se em curso.
10	Em caso de pesquisa em UNIDADE DE CONSERVAÇÃO, o pesquisador titular desta autorização deverá contactar a administração da unidade a fim de CONFIRMAR AS DATAS das expedições, as condições para realização das coletas e de uso da infraestrutura da unidade.
11	O titular de autorização ou de licença permanente, assim como os membros de sua equipe, quando da violação da legislação vigente, ou quando da inadequação, omissão ou falsa descrição de informações relevantes que subsidiaram a expedição do ato, poderá, mediante decisão motivada, ter a autorização ou licença suspensa ou revogada pelo ICMBio, nos termos da legislação brasileira em vigor.

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Nome da Instituição: UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL	CNPJ: 92.969.856/0001-98

Outras ressalvas

1	A restrição de acesso ao interior das Unidades de Conservação (UC?s) Federais determinada pela Portaria Nº 227, de 22 de março de 2020 se aplica às atividades com finalidades científicas e didáticas. Portanto, ficam vedados os acessos ao interior das UC?s para fins de expedições de campo e demais atividade dos projetos de pesquisa. Assim sendo, o pesquisador somente poderá realizar atividades de campo após o término do estado de emergência devido à COVID-19, assim declarada por ato da autoridade competente.	CEPENE Tamandaré/PE
2	O pesquisador deverá anexar no relatório apresentado ao SISBIO toda a publicação científica resultante de suas atividades na APA Costa dos Corais. Não serão permitidas atividades dentro das Zonas de Preservação da Vida Marinha (ZPVM) e Zonas de Visitação (ZV). Se houver previsão de coleta nessas zonas o pesquisador precisará de autorização específica, formulário disponível em: http://www.icmbio.gov.br/apacostadoscorais/o-que-fazemos/pesquisa-cientifica.html	APA Costa dos Corais

Locais onde as atividades de campo serão executadas

#	Descrição do local	Município-UF	Bioma	Caverna?	Tipo
1	Área de Proteção Ambiental da Costa dos Corais	PE	Marinho	Não	Dentro de UC Federal

Atividades

#	Atividade	Grupo de Atividade
1	Coleta/transporte de amostras biológicas in situ	Dentro de UC Federal
2	Coleta/transporte de amostras biológicas ex situ	Atividades ex-situ (fora da natureza)

Atividades X Táxons

#	Atividade	Táxon	Qtde.
1	Coleta/transporte de amostras biológicas ex situ	Mussismilia harttii	-
2	Coleta/transporte de amostras biológicas in situ	Mussismilia harttii	-

A quantidade prevista só é obrigatória para atividades do tipo "Coleta/transporte de espécimes da fauna silvestre in situ". Essa quantidade abrange uma porção territorial mínima, que pode ser uma Unidade de Conservação Federal ou um Município.

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Materiais e Métodos

#	Tipo de Método (Grupo taxonômico)	Materiais
1	Amostras biológicas (Invertebrados Aquáticos)	Outras amostras biológicas(Pólipos)

Destino do material biológico coletado

#	Nome local destino	Tipo destino
1	Universidade Federal de Minas Gerais	Laboratório

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Registro de coleta imprevista de material biológico

De acordo com a Instrução Normativa nº03/2014, a coleta imprevista de material biológico ou de substrato não contemplado na autorização ou na licença permanente deverá ser anotada na mesma, em campo específico, por ocasião da coleta, devendo esta coleta imprevista ser comunicada por meio do relatório de atividades. O transporte do material biológico ou do substrato deverá ser acompanhado da autorização ou da licença permanente com a devida anotação. O material biológico coletado de forma imprevista, deverá ser destinado à instituição científica e, depositado, preferencialmente, em coleção biológica científica registrada no Cadastro Nacional de Coleções Biológicas (CCBIO).

Táxon*	Qtde.	Tipo de Amostra	Qtde.	Data

* Identificar o espécime do nível taxonômico possível.

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19-Mar-2025

Dear Dr. Avelar:

We are pleased to inform you that your paper REP-25-0032 entitled "Morphofunctional evaluation of gametogenesis in the endemic South Atlantic reef-builder Mussismilia harttii" has received generally favourable comments, and we would be delighted to receive a revised manuscript, accompanied by a detailed point by point response explaining the changes made in response to the comments of the Associate Editor and Expert Reviewers.

Although acceptance is not guaranteed at this stage, we expect that your paper would be acceptable after appropriate changes.

Please find below the comments of the AE and Reviewers. These comments are followed by important information regarding the process of resubmission, which we encourage you to read before beginning your revisions.

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Associate Editor's Comments to Author:

Associate Editor
Comments to Authors:
This manuscript from Valente et al nicely characterizes gametogenesis in the reef-builder coral Mussismilia harttii, an endangered species found in the South Atlantic Ocean. I agree with both reviewers that this study was thoroughly

performed and will be quite useful for conservation efforts. However, there are issues that the reviewers indicate that need to be addressed. As pointed out by reviewer 2, the discussion is too long, and repeats what is indicated in the results section, so please remove all the repetition.

Reviewers' Comments to Author:

Reviewer: 1

Comments to the Author
Valente et al. present a manuscript nicely describing a histological analysis of gametogenesis in a reef-building coral. I see no major issues with the manuscript and congratulate the authors on their thorough work, particularly given the language barrier.

A few minor points:
-the details for the primary antibody negative controls should be included in the main methods
-the main methods section should indicate the existence of the supplementary methods

Reviewer: 2

Comments to the Author
The manuscript by Valente and collaborators explores the basic gametogenic patterns of the threatened coral Mussimilia harttti. The manuscript presents relevant data, carefully imaged and properly analysed mostly, but it needs some shortening, especially the discussion, and some proper justification in some analysis. I believe it can be published with minor revisions, and I provide some recommendations below.

There is an extensive use of the word germinative, which has connotations applicable to plants, and I suggest the authors change it to germinal.

Methods:
The authors say that they have conducted morphometric analyses, but these involve much more precise measurements (landmarks, etc), and I believe they mean morphological analyses.

There is an entire section about statistical analyses that is not presented in detail in the results. Actually, I don't really see the point of statistical analyses in such a basic description of the gametogenic cycle of the coral. My recommendation is to remove it.

Results:
In the text it Is said that the early spermatogenic cysts were first detected in June, but Figure 3 shows a continuous detection of male regions or mesenteries, but it is not clear what is in the mesenteries to be identified as male if spermatogenic cysts are only seen from June to August. Also, Figure 3B is not very clear, and I suggest the authors plot the number or diameter of reproductive elements per month instead of this diagram that is better suited for a presentation than a paper (just moving the graphs from the other figures here would be enough).

Page 12. Line 248. The authors say that the mesoglea outline the cysts, but do they mean collagen? What type of structure is enveloping the spermatogonial cells?

Line 259, What are the needed tools for fertilisation the authors refer to here?

Discussion
The discussion section is too long and the sections regarding spermatogenesis and oogenesis can be combined into a single one.
Also, the section about General aspects of gametogenesis lack a bit of detail. The VASA antibody is an interesting tool that also serves the purpose of identifying the cells that give rise to gametes. Are these Interstitial cells? What is the

insight obtained from applying immunohistochemistry here?

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