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PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
PROGRAMA DE PÓS-GRADUAÇÃO EM RECURSOS PESQUEIROS E AQUICULTURA

FERTILIZAÇÃO ARTIFICIAL DE MOLUSCOS BIVALVES NATIVOS E APLICAÇÕES BIOTECNOLÓGICAS

Rodolf Gabriel Prazeres Silva Lopes

Tese apresentada ao Programa de Pós-Graduação em Recursos Pesqueiros e Aquicultura da Universidade Federal Rural de Pernambuco como exigência para obtenção do título de Doutor.

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Resumo

Bivalves nativos são identificados como espécies promissoras para a aquicultura tropical, no entanto, pouco se sabe sobre aspectos fisiológicos de reprodução em condições controladas. O estudo sobre o manejo da fertilização artificial em laboratório, a partir da compreensão dos eventos de indução a maturação gamética, taxa fertilização e do desenvolvimento embrionário, com destaque para os tempos de liberação corpuscular, podem fornecer importantes informações que auxiliem nos processos de larvicultura e de estruturação de protocolos de manipulação cromossômica. Neste estudo, foram conduzidos experimentos de fertilização artificial com as espécies *Anomalocardia flexuosa* e *Crassostrea rhizophorae.* Foram investigados os efeitos da salinidade em diferentes concentrações (15, 20, 25, 30, 35 e 40 gL^{-1}) na indução da Quebra do Vesícula Germinativa (GVBD) de ovócitos obtidos por stripped, na liberação de corpos polares (PB1 e PB2) e no desenvolvimento larval. Em ambos os experimentos, os resultados revelaram uma relação entre o aumento da salinidade e a porcentagem de maturação dos ovócitos. Para a espécie *A. flexuosa*, concentrações de salinidade entre 30 e 35 $g.L^{-1}$ proporcionaram melhores porcentagens de GVBD em até 120 minutos, e a incubação dos ovócitos na faixa de salinidade de 30-35 $g.L^{-1}$ por um intervalo de tempo de 80-120 minutos resultou em alta porcentagem de GVBD. Entretanto a salinidade de 40 $g.L^{-1}$ resultou no rompimento das estruturas celulares dos ovócitos, indicando o limite de tolerância hipersalina. Na análise pós-fertilização, a salinidade afetou a taxa de extrusão dos primeiros e segundos corpos polares (PB1 e PB2). A liberação de 50% dos PBs foi mais rápida na salinidade de 35 $g.L^{-1}$, apresentando maior potencial para um protocolo de indução a poliploidia. Para a espécie *C. rhizophorae*, a faixa mais eficaz na indução da GVBD ocorreu entre 30 a 40 $g.L^{-1}$ dentro do intervalo de tempo de hidratação de 70 a 120 minutos. A liberação de PB1 também foi mais rápida dentro deste intervalo salino, indicando a importância da sincronização da meiose para a reprodução bem-sucedida e oferecendo assim melhores recomendações para uso em metodologias de manipulação cromossômica. A viabilidade da indução química e da avaliação da triploidia na espécie *C. rhizophorae* foi testada a partir construção de um protocolo de calibração de leitura para citometria de fluxo. Foram testados dois métodos químicos de indução (Citocalasina B e 6-DMAP). Ambos os tratamentos apresentaram potencial de indução a triploidia, embora o 6-DMAP tenha sido mais eficiente. Esses são os primeiros resultados com manipulação cromossômica para espécie *Crassostrea rhizophorae*. A investigação sobre a dinâmica de preparação dos gametas e da fertilização artificial são fundamentais para a construção de uma estratégia de melhoramento genético para esta espécie.

Palavras-chave: Bivalve tropical, fertilização artificial, manipulação cromossômica.

Abstract

Native bivalves are identified as promising species for tropical aquaculture; however, little is known about the physiological aspects of reproduction under controlled conditions. Studying artificial fertilization management in a laboratory setting, starting from understanding gametic maturation induction events, fertilization rates, and embryonic development—particularly focusing on the times of corpuscular release—can provide crucial insights aiding in larviculture processes and the structuring of chromosomal manipulation protocols. In this study, experiments on artificial fertilization were conducted with the species *Anomalocardia flexuosa* and *Crassostrea rhizophorae*. The effects of salinity at different concentrations (15, 20, 25, 30, 35, and 40 $g.L^{-1}$) on the induction of Germinal Vesicle Breakdown (GVBD) in oocytes obtained through stripping, the release of polar bodies (PB1 and PB2), and larval development were investigated. In both experiments, the results revealed a relationship between increased salinity and the oocyte maturation percentage. For the *A. flexuosa* species, salinity concentrations between 30 and 35 $g.L^{-1}$ provided better GVBD percentages within 120 minutes, and incubating the oocytes in the salinity range of 30-35 $g.L^{-1}$ for a time interval of 80-120 minutes resulted in a high GVBD percentage. However, a salinity of 40 $g.L^{-1}$ resulted in the rupture of oocyte cellular structures, indicating the hypersaline tolerance limit. In the post-fertilization analysis, salinity affected the extrusion rate of the first and second polar bodies (PB1 and PB2). The release of 50% of PBs was faster at a salinity of 35 $g.L^{-1}$, showing greater potential for a polyploidy induction protocol. For the *C. rhizophorae* species, the most effective range in inducing GVBD occurred between 30 to 40 $g.L^{-1}$ within a hydration time interval of 70 to 120 minutes. The release of PB1 was also faster within this saline range, indicating the importance of meiosis synchronization for successful reproduction and providing better recommendations for use in chromosomal manipulation methodologies. The viability of chemical induction and triploidy assessment in the *C. rhizophorae* species was tested by constructing a flow cytometry reading calibration protocol. Two chemical induction methods were tested (Cytochalasin B and 6-DMAP). Both treatments showed potential for triploidy induction, although 6-DMAP was more efficient. These are the initial results involving chromosomal manipulation for the *Crassostrea rhizophorae* species. Investigating gamete preparation dynamics and artificial fertilization is crucial in constructing a genetic improvement strategy for this species.

Key words: Tropical bivalve, artificial fertilization, chromosomal manipulation.

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1. Introdução Geral

A produção mundial de moluscos, de acordo com o relatório da FAO (2020), está concentrada principalmente em espécies temperadas, com espécies tropicais representando apenas cerca de 1% da produção. Atualmente, a grande maioria dos moluscos bivalves consumidos são cultivados por países europeus, América do Norte, China e Chile (FAO 2022). E mesmo em países tropicais e subtropicais com diversidade de espécies de moluscos bivalves cultiváveis, ainda são as espécies de zona temperada que figuram no eixo principal do rancking de produção (Treviño et al. 2020; Garcia et al. 2022; Martínez-García et al. 2022).

Bivalves tropicais são considerados candidatos potenciais e recursos aquícolas subutilizados (MAZÓN-SUÁSTEGUI et al., 2017; Peter CHEN et al., 2013). O cultivo desses organismos tem um considerável valor econômico e ambiental, embora tenham recebido menos atenção e pesquisas de desenvolvimento quando comparado a outras espécies usadas na aquicultura (Willer and Aldridge 2020). A pouca produção de bivalves em regiões tropicais, aproximadamente 2 Mt de carne por ano (FAO 2022), relaciona-se aos níveis baixos de técnica de manejo específico, de financiamento e infraestrutura desse setor aquícola. Considerando as características inerentes às regiões litorâneas tropicais, estima-se que o uso para cultivo de 1% desse território poderia gerar uma produção de 120 Mt de carne de moluscos ao ano (Gentry et al. 2017).

Outro aspecto importante no contexto dos novos modelos de produção na aquicultura, está na valoração dos serviços ecossistêmicos prestados. A aquicultura de bivalves possui uma estimativa de retorno de US$ 30,5 bilhões por ano em serviços ecossistêmicos (van der Schatte Olivier et al. 2020), que associada ao potencial de desenvolvimento econômico sustentável, tornam a produção de bivalves uma alternativa promissora e viável para as zonas tropicais.

O crescimento da produção de bivalves tropicais perpassa pelo aprimoramento das técnicas de manejo a partir do desenvolvimento e implantação de tecnologias que possibilitem melhorias no desempenho zootécnico dos organismos cultivados. A produção de sementes representa o principal gargalo na expansão comercial do cultivo de moluscos (Wijsman et al. 2019), e por consequinte, pesquisas que explorem aspectos da reprodução, melhoramento e manejo, semelhantes aos realizados para espécies de zonas temperadas (Mao et al. 2019), são necessárias para produzir reprodutores com alto rendimento e

sementes de boa qualidade.

Pesquisas sobre a produção de sementes de moluscos bivalves tropicais em larviculturas são, portanto, um importante desafio, não só pela necessidade de garantir uma oferta de sementes confiável e contínua durante todo o ano (NOWLAND et al., 2021), mas também pela necessidade da diversificação das produções aquícolas a partir do desenvolvimento de tecnologias que proporcionem o aproveitamento de novas espécies potencialmente importantes para aquicultura (LAGREZE-SQUELLA et al., 2018).

No Brasil, a adequação de bivalves nativos com o objetivo de impulsionar aquicultura no país, ainda esbarra principalmente na falta de informações técnico-científicas. As espécies *Crassostrea rhizophorae* e *Crassostrea gasar* são apotandas como tendo grande potencial para ostreicultura, contudo, ainda há poucas informações especificas (SAMPAIO et al., 2019). Ao longo do litoral brasileiro vários esforços foram realizados para desenvolver o cultivo de ostras nativas em nível comercial, porém poucos obtiveram sucesso, principalmente pela dificuldade na obtenção de sementes, já que um percentual importante das ostras usadas para aquicultura são obtidas na natureza, usando coletores artificiais de sementes (NALESSO et al., 2008).

Outra espécie promissora para aquicultura é a *Anomalocarida flexuosa* (LAGREZE-SQUELLA et al., 2018), que apresenta um papel importante na alimentação e no incremento da renda de comunidades tradicionais (RIOS E.C., 2009), com sua cadeia produtiva limitada ao extrativismo. Esses organismos sofrem uma exploração intensa das comunidades tradicionais, sem que grandes alternativas venham sendo desenvolvidas para mitigar essa condição e possibilitar outros meios de produção (ARAÚJO, 2001). Sob essa perspectiva, metodologias de cultivo a partir do domínio dos eventos da alimentação, maturação, reprodução, larvicultura e assentamento em condições controladas tem sido desenvolvidas para essa espécie (LAGREZE-SQUELLA et al., 2018; LAVANDER et al., 2014; LIMA et al., 2018).

De acordo com Andrade (2016), o sucesso da cadeia produtiva na maricultura resulta da soma de esforços que tem como pilares de sustentação a pesquisa, a extensão e o desenvolvimento tecnólogico. É importante compreender que o incremento produtivo nas cadeias aquícolas relacionam-se diretamente à disseminação de bases técnologicas que viabilizem esse processo. O direcionamente de ferramentas biotecnológicas e avanços científicos com espécies nativas brasileiras são fundamentais para a superação dos desequilíbrios regionais e o uso eficiente de recursos disponíveis.

Sob essa perspectiva, a utilização de tecnologias de manipulação cromossômica foi um passo decisivo na consolidação da produção de bivalves no mundo (Piferrer et al. 2009). A poliploidia tem sido aplicada como uma das principais alternativas de qualificação do processo produtivo de moluscos (WADSWORTH et al., 2019) e de acordo com Rasmussen e Morrissey (2007), a poliploidia tem potencial para aprimorar o cultivo de moluscos por aumento da taxa de crescimento, em detrimento da capacidade de reprodução. Indivíduos poliploides podem ser definidos como sendo aqueles que possuem um ou mais conjuntos de cromossomos em relação ao que ocorre naturalmente na mesma espécie (PIFERRER et al., 2009). Dentre os de maior interesse para a aquicultura têm-se os organismos triploides, voltados para o cultivo e a comercialização e os organismos tetraploides usados como reprodutores para a obtenção de triploides.

Em moluscos, o uso de ferramentas de indução à poliploidia iniciou-se na década de 80 com a espécie de ostras *Crassostrea virginica* nos Estados Unidos (STANLEY et al., 1981). No decorrer dos anos, o cultivo de ostras triploides passou a ser uma alternativa de produção mundial (Wadsworth et al. 2019) e a poliploidia teve sua aplicação expandida a novas espécies de bivalves como *Crassostrea gigas* (Gagnaire et al. 2006; Normand et al. 2009; Melo et al. 2015), *Ostrea edulis* (Hawkins et al. 1994), *Saccostrea glomerata* (Hand et al. 2004), *Mulinia lateralis* (Yang and Guo 2006), *Mercenaria mercenaria* (El-Wazzan and Scarpa 2009; Yang and Guo 2018), *Mytilus edulis*, *Argopecten ventricosus* (Ruiz-Verdugo et al. 2000) e *Chlamys farreri* (Yang et al. 2000).

A poliploidia é ainda muito pouco explorada na malacocultura dos trópicos (Willer and Aldridge 2020). O nível de conhecimento biológico existente para a maioria das espécies de bivalves tropicais é insufuciente para apoiar a inserção de tecnicas que permitam uma experiência no desenvolvimento e teste de novos sistemas de produção (NOWLAND et al., 2020). O uso da manipulação cromossômica em moluscos bivalves pressupõe um domínio prévio dos mecanismos e estratégias de reprodução (Venier et al. 2019; Nowland et al. 2021). Todos esses processos estão intrinsicamente relacionados às características ambientais distintas e originais de cada espécie, que não se adequam a pacotes tecnológicos de espécies de zonas temperadas (Nowland et al. 2019).

Bivalves como a *Crassostrea gigas*, *Crassostrea Virgínica* ou *Mercenária mercenária* apresentam melhores condições de indução à reprodução logo após ao período de inverno (Gallager and Mann 1986; Fabioux et al. 2005). Zonas temperadas caracterizam-se por apresentar estações do ano bem definidas e as temperaturas mais baixas contribuem

para a desaceleração dos processos reprodutivos e para o acúmulo energético em bivalves. O aquecimento das águas, em consequências às mudanças sazonais são o importante sinalizador reprodutivo para essas espécies (Bayne 1976; Ruiz et al. 1992). Assim sendo, a indução à reprodução em larviculturas através de choques térmicos é uma das principais estratégias de obtenção de gametas na produção de sementes no mundo (Ludi 2011).

Zonas tropicais são conhecidas por exibirem altas temperaturas com variações mínimas ao longo do ano e as estações podem ser definidas em dois períodos, seco e chuvoso, onde ocorre importantes flutuações na salinidade (Paixão et al. 2013). Sob essas condições, espécies como *Crassostrea rhizophorae* e a *Anomalocardia flexuosa* apresentam liberação gamética parcial ao longo de todo o ano, havendo alguns momentos de picos reprodutivos que podem estar relacionados a outros fatores estimulantes que não só a temperatura (Barreira and Araújo 2005; Antonio et al. 2021). Pesquisas têm evidenciado a relevância da salinidade na modulação dos processos hormonais e do comportamento reprodutivo das ostras tropicais, constituindo, assim, um fator de considerável importância na condução e administração dessas espécies (Nowland et al. 2019; Nirchio and Vegas 2021). Aprofundar a compreensão da interação entre a salinidade e o ciclo reprodutivo de bivalves tropicais pode proporcionar uma base sólida para o desenvolvimento de estratégias mais eficazes de produção.

Os métodos de desova induzida em espécies tropicais de bivalves ainda são um desafio a ser dominado, particularmente em etapas de desova individualizada, de modo que a reprodução por stripping é o recurso mais prático na obtenção de gametas (Mouëza et al. 1999; Nowland et al. 2021). Entretanto, essa alternativa dá acesso a ovócitos imaturos, com a estrutura da vesícula germinal íntegra (Eudeline et al. 2000). A Quebra da Vesícula Germinal (QVG) é um indicador da maturação dos ovócitos em bivalves, e pode ser estimulada artificialmente por fatores ambientais como a salinidade, em um processo conhecido como “hidratação” (Allen and Bushek, 1992). A coordenação da meiose, promovida pela maturação induzida (QVG), tem o efeito de aumentar a taxa de fertilização e sincronizar a liberação dos corpúsculos polares (Qin et al. 2018). A saída do 1º e do 2º corpo polar (PB1 e PB2) são referências importantes no processo de manipulação cromossômica (Piferrer et al. 2009; Qin et al. 2018; Yang and Guo 2018), e, portanto, o controle dos mecanismos de influência na liberação constitui uma etapa fundamental para a construção de protocolos de poliploidia.

A investigação das melhores condições de fertilização artificial em laboratório em

espécies de bivalves nativos, a partir do domínio dos mecanismos de indução à maturação dos ovócitos, controle do tempo de liberação corpuscular, desenvolvimento embrionário e formação larval, podem representar um importante passo para a adequação do manejo de produção de semente e da inserção de biotecnologias de manipulação cromossômica como a produção de triploides.

2. Objetivos

2.1 Objetivo Geral

Determinar condições espécie-específica de fertilização artificial do marisco *Anomalocardia flexuosa* e da ostra *Crassostrea rhizophorea* como base para a construção de protocolos de manipulação cromossómica e a formação de ostras *Crassostrea rhizophorea* triploides por indução química.

2.2 Objetivos Específicos

- Avaliar o efeito de diferentes concentrações de salinidade no processo de desenvolvimento (pós-fertilização) embrionário inicial de *Anomalocardia flexuosa;*
- Avaliar o efeito de diferentes concentrações de salinidade no processo de desenvolvimento (pós-fertilização) embrionário inicial de *Crassostrea rhizophorae;*
- Avaliar a tecnologia de indução à triploidia em ostras *Crassostrea rhizophorae* por indução de choque químicos com citocalasina-B.

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CAPÍTULO 1

Effects of salinity on pre- and post-fertilization developmental events in the clam *Anomalocardia flexuosa* (Linnaeus, 1767)

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Effects of salinity on pre- and post-fertilization developmental events in the clam *Anomalocardia flexuosa* (Linnaeus, 1767)

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Abstract - The knowledge about the effect of salinity on the physiological mechanism of bivalve reproduction is fundamental to improve production strategies in hatcheries. The present work evaluated the influence of different salinity concentrations (15, 20, 25, 30, 35 and 40 $g.L^{-1}$) on pre- and post-fertilization development processes in the clam, *Anomalocardia flexuosa*, oocytes obtained by stripping. Salinity directly interfered with the germinal vesicle breakdown (GVBD) rate and in the cellular stability of unfertilized oocytes. Salinity concentrations between 30 and 35 $g.L^{-1}$ provided better percentages of stable GVBD within 120 min, and incubation of oocytes in the salinity range of 30-35 $g.L^{-1}$ for a time interval of 80-120 min provided > 80% GVBD. In the post-fertilization analysis, salinity affected the rate of the extrusion of the first and second polar bodies (PB1 and PB2). The release of 50% of the PBs was faster at a salinity of 35 $g.L^{-1}$, with an estimated time of 10 min for PB1 and 30 min for PB2. Thus, chromosome manipulation methodologies aiming triploids should be applied at 35 $g.L^{-1}$ salinity, with application of post-fertilization shock before 10 min for PB1 retention or before 30 min for PB2 retention.

Keywords: tropical bivalve reproduction, stripping, germinal vesicle breakdown, polar body.

1. Introduction

The production of bivalves in tropical regions has been consolidated as an important market for aquaculture (Nowland et al., 2020; Willer and Aldridge, 2020). However, tropical bivalve larviculture presents a series of obstacles that limits the continuous supply of seeds, essential to aquaculture and restocking (Nowland et al., 2021). One promising species for tropical aquaculture (Lagreze-Squella et al., 2018) is the clam Anomalocardia flexuosa, which is distributed from the Caribbean to Brazil (Abbott, 2011), and plays an important role in feeding and in the income of traditional communities (Rios, 2009). Hatchery production methodologies based on feeding, maturation, reproduction, larviculture and settlement events under controlled laboratory conditions have been developed for this species (Lavander et al., 2014; Lagreze-Squella et al., 2018; Lima et al., 2018).

The clam A. flexuosa, like most bivalve species, presents external fertilization. Stripping of the gonadal tissue gives access to undeveloped oocytes in maturation stages with intact germinal vesicle structure. This occurs because the oocytes of some species undergo a maturation process during the passage through the oviducts before their release (Colas and Dubé, 1998).

Germinal vesicle breakdown (GVBD) is a natural process occurring in the external environment when metaphase I oocytes are released (Guo et al., 1996). This process is considered as a sign of oocyte maturation. To continue the GVBD stage, the oocytes may go through a period of hydration in the external environment (Melo et al., 2015). The breakdown of the vesicle improves fertilization, decreases polyspermia, and increases efficiency in polyploidy induction processes (Qin et al., 2018), and has been exploited in the hatchery of bivalves (Dégremont et al., 2012).

The process of embryogenesis begins from the time the sperm enters the oocyte and is characterized by the release of two polar bodies, PB1 and PB2 (Colas and Dubé, 1998). Polyploidy can be achieved by the physical or chemical treatment of fertilized eggs to inhibit the exit of PB1 or PB2 (Piferrer et al., 2009).

Environmental factors such as salinity affect the incubation time and the extrusion of polar bodies (Lavander et al., 2014). The knowledge of the effect of environmental variables on oocyte GVBD and the timing of polar bodies release is critical to increase fertilization success in hatcheries, achieve greater control in subsequent embryonic developmental stages, and for adjusting protocols for the use and control of polyploidy (Qin et al., 2018).

This study evaluated the influence of salinity on the time of oocyte GVBD and on the extrusion of polar bodies in A. flexuosa.

2. Methods

Broodstock

A total of 150 adults were collected in estuarine zone of the state of Maranhão (2º30'03"S, 44º03'40"W) and stored at the State University of Maranhão for the experiments. Approval by the Ethics Council for the Use of Experimental Animals does not apply to the invertebrate group, in accordance with the Brazilian law 11.794/08 (Brasil, 2008).

Clams were cleaned with a sodium hypochlorite solution (2%) and then stored in a 100 L tank in water treated by cartridge filters (50, 25, and 5 μm), biological filter (containing different media and nitrifying bacteria), and UV. The organisms were maintained at a salinity of 30 g·L−1, temperature of 24 °C, and constant aeration for 4 h of depuration in a Recirculating Aquaculture System (RAS). After this period, the animals were left out of the water overnight at 24 °C to prevent the release of gametes into the water.

Gametes

For sex identification, each individual was carefully opened using a knife and the gonadal tissue was observed under an optical microscope. Gametes from 30 females were obtained by stripping using a surgical blade. Subsequently, they were mixed and filtered in a 100 μm mesh and rinsed in 25 μm. A dilution of the gametes was carried out to reach a final rate of 50 oocytes/mL in each experimental unit.

GVBD at different salinities

The effect of salinity on GVBD was evaluated at salinities of 15, 20, 25, 30, 35, and 40 g·L−1, with five replicates per treatment. The marine water used in the experiment was collected at sea (original concentration of 42 g.L−1), filtered and sterilized in a UV filter in the laboratory. The dilution for the different saline concentrations was carried out by adding autoclaved freshwater.

The experimental units were thermostat regulated at 26 °C, which was considered as

the ideal temperature for meiosis of the species (Lavander et al., 2017). Diluted oocytes were distributed in 100 mL beakers, from which 1 mL aliquot was removed with the aid of a pipette for observation of the progression of GVBD (Figure 1). The germinal vesicle breakdown process was observed over a period of 120 min, from the observation of 1ml samples (approximately 50 oocytes) taken every 10 minutes for counting and recording.

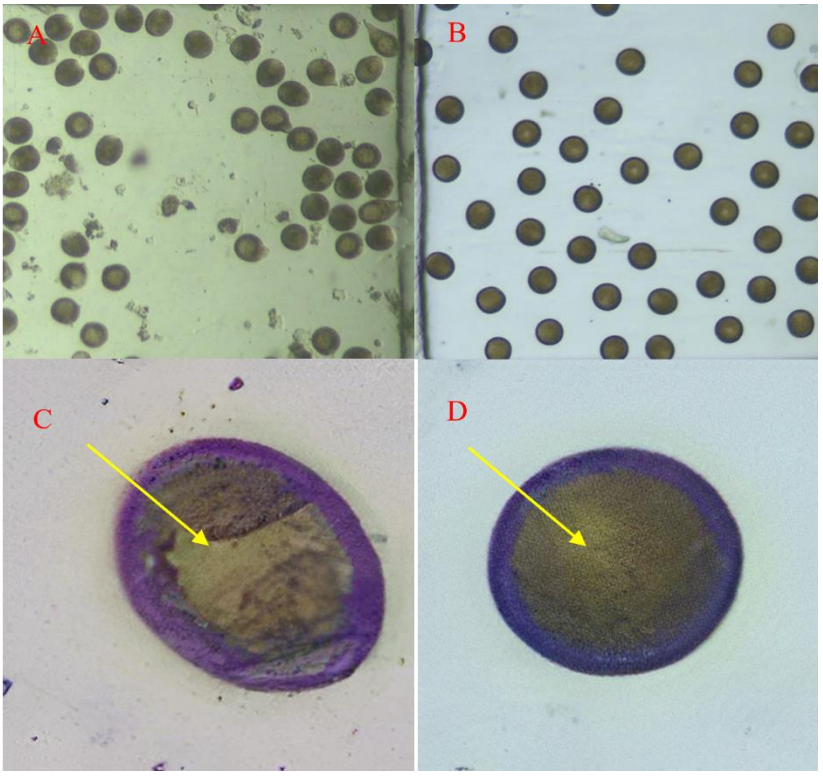

Figure 1: Images registered in the program Mosaic V.2.2.1 with a camera attached to the microscope showing the appearance of Anomalocardia flexuosa oocytes before and after germinal vesicle breakdown (GVBD); A: Image of a set of oocytes without uniformity soon after undergoing the stripping process observed under 4X lens; B: Set of oocytes after hydration showing the GVBD under 4X lens; C: Image of a magnified oocyte before hydration with an arrow in yellow highlighting the structure of the intact germinal vesicle observed under 10X lens; D: Image of an oocyte magnified after hydration and with a yellow arrow indicating the uniformity of the nucleus as a function of the GVBD observed at 10X lens.

Extrusion of PB1 and PB2 in different salinities

The spermatozoa from 30 males were obtained by stripping, mixed, filtered using 80 µm mesh and quantified by optical microscopy. After 2 h of oocyte hydration for GVBD in different salinities (15, 20, 25, 30, 35, and 40 g·L−1) and at a constant temperature of 26 °C, the spermatozoa were added to each experimental unit at a ratio of 7 spermatozoa: 1 oocyte,

according to the previously described methodology (Melo et al., 2015). The first and second polar bodies (PB1 and PB2) extrusion rates were monitored at 5 min intervals over a period of 60 min using 1 ml samples (approximately 50 oocytes).

The average time to obtain the 50% of PB1 and PB2 extrusions for each treatment were determined as previously recommended (Melo et al., 2015; Lavander et al., 2017).

Statistical analyses

The averages of the five replicates of each salinity treatment were used to estimate the GVBD and the extrusion of PB1 and PB2 at the different time interval. The trends of the percentages of GVBD and PB released with time at each salinity concentration were estimated by fitting the best likelihood model with a generalized additive model (GAM) and considering a binomial distribution. A two-way analysis of variance (ANOVA) was used to measure treatment effects, and averages were compared using Tukey's post-hoc test at a $p \leq 0.05$ significance level.

Statistical analyses were performed in R studio version 4.1.0, and the package "MGCV" (Wood, 2011) was used to run the models. The steps for model selection were performed as previously described (Zuur, 2012; Zuur et al., 2009).

3. Results

Effect of salinity on GVBD

None of the salinity treatments resulted in 100% GVBD, however, high values were found at 30 and 35 g·L−1 salt concentrations (93.26 ± 1.04% and 93.76 ± 4.98%, respectively) at 120 min. Also, at 40 g·L−1 salt concentration 93.99% ± 7 were detected at 50 min, followed by a decrease in the breakdown in subsequent intervals. Salinity treatments at 15, 20 and 25 g·L−1 presented inferior values <85% after 120 min (Table 1 and Figure 2).

Table 1: Germinal vesicle breakdown of Anomalocardia flexuosa oocytes under salinity

influence.

Time	Percentages of GVBD in the interval from 0 to 120 minutes					
	15 g·L^{-1}	**20 g·L^{-1}**	**25 g·L^{-1}**	**30 g·L^{-1}**	**35 g·L^{-1}**	**40 g·L^{-1}**
T10 (min)	3.24±1.09 ^{b}	17.7±1.9 ^{ab}	21.84±4.72 ^{a}	24.69±10.29 ^{a}	17.07±6.33 ^{ab}	27.22±12.06 ^{a}
T20 (min)	14.95±2 ^{c}	25.33±1.32 ^{bc}	27.43±7.25 ^{bc}	37.16±6.5 ^{ab}	31.58±14.65 ^{b}	47.63±15.25 ^{a}
T30 (min)	22.51±1.5 ^{d}	30.36±1.51 ^{cd}	34.39±9.73 ^{bcd}	48.97±10.94 ^{b}	42.72±17.34 ^{bc}	73.75±11.55 ^{a}
T40 (min)	32.02±4.42 ^{c}	35.37±3.97 ^{c}	46.8±8.39 ^{bc}	51.48±9.89 ^{b}	52.52±22.4 ^{b}	86.29±12.87 ^{a}
T50 (min)	42.62±4.44 ^{c}	39.37±1.98 ^{c}	53.4±8.6 ^{bc}	60.71±8.76 ^{b}	64.72±15.24 ^{b}	93.99±7 ^{a}
T60 (min)	47.39±4.97 ^{cd}	42.96±2.56 ^{d}	60.17±7.84 ^{bc}	69.54±7.86 ^{b}	70.79±16.85 ^{b}	91.88±5.82 ^{a}
T70 (min)	52.12±2.55 ^{cd}	44.96±3.41 ^{d}	64.31±3.95 ^{bc}	74.38±9.14 ^{ab}	80.11±15 ^{a}	80.08±14.35 ^{a}
T80 (min)	54.5±3.96 ^{b}	48.6±3.79 ^{b}	70.52±2.65 ^{a}	82.74±11.44 ^{a}	82.66±12.78 ^{a}	70.57±10.95 ^{a}
T90 (min)	58.24±1.12 ^{bc}	55.81±2.13 ^{c}	72.22±4.5 ^{ab}	87.24±8.89 ^{a}	86.96±10.74 ^{a}	61.41±6.01 ^{bc}
T100(min)	61.6±2.27 ^{bc}	61.89±3.19 ^{bc}	75.4±4.84 ^{ab}	88.39±6.97 ^{a}	90.2±8.27 ^{a}	55.39±5.79 ^{c}
T110(min)	68.84±3.58 ^{c}	67.91±3.51 ^{c}	78.48±6.72 ^{bc}	91.15±3.23 ^{ab}	93.76±4.98 ^{a}	51.23±7.05 ^{d}
T120 (min)	73.75±1.86 ^{b}	75.12±2.74 ^{b}	82.4±7.06 ^{ab}	93.26±1.04 ^{a}	93.76±4.98 ^{a}	44.8±6.13 ^{c}

The mean number of oocytes analyzed per time interval was 50. Different superscript letters in each indicator indicate statistical differences ($p < 0.05$).

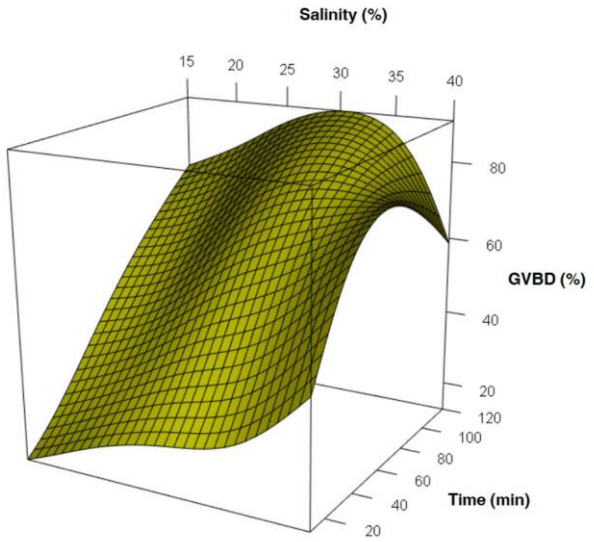

Figure 2: Mean of the percentages of germinal vesicle breakdown of oocytes obtained by stripping from Anomalocardia flexuosa.

Most of the treatments showed an increase in the percentage of GVBD as salinity raised, but in the 40 g·L−1 salinity treatment, the oocytes started to break up after 60 min to generate a significant loss until 120 min.

Effect of salinity on the extrusion of PB1 and PB2

Fertilization was conducted right after 120 min to guarantee maximum level of GVBD considering the previous section results. The extrusion of 50% PB in 20, 25, 30, and 40 g.L^{-1} treatment groups occurred nearly within the first 15 min. For the 15 g.L^{-1}, the average time was 25 min, while for 35 g.L^{-1} treatment group it happened in the first 10 min (Table 2 and Figure 3).

Table 2: Percentages of polar body 1 (PB1) extrusion of Anomalocardia flexuosa at each 5 min interval.

Time	**Time to reach 50% of PB1 release**					
	15 g·L^{-1}	**20 g·L^{-1}**	**25 g·L^{-1}**	**30 g·L^{-1}**	**35 g·L^{-1}**	**40 g·L^{-1}**
T5 (min)	16.46±4.39 ^{bc}	15.03±1.63 ^{bc}	12.92±4.5 ^{c}	14.17±4.1 ^{c}	29.81±8.89 ^{a}	23.68±0.17 ^{ab}
T10 (min)	28.06±3.18 ^{c}	29.24±2.23 ^{c}	39.44±1.13 ^{b}	34.75±4.83 ^{bc}	52.22±5.05 ^{a}	40.67±7.73 ^{b}
T15 (min)	37.68±5.13 ^{c}	50.77±1.97 ^{b}	46.39±2.29 ^{bc}	48.89±3.06 ^{b}	74.57±4.61 ^{a}	52.28±8.22 ^{b}
T20 (min)	43.69±3.01 ^{d}	66.83±6.16 ^{b}	53.87±3.26 ^{c}	58.45±4.89 ^{bc}	78.2±3.96 ^{a}	63.13±4.56 ^{b}
T25 (min)	51.73±0.9 ^{e}	78.58±1.99 ^{ab}	60.83±1.37 ^{de}	63.4±3.2 ^{cd}	87.12±5.6 ^{a}	71.78±6.56 ^{bc}
T30 (min)	57.09±0.97 ^{e}	87.53±2.92 ^{ab}	71.19±5.12 ^{d}	75.18±4.73 ^{cd}	92.12±1.69 ^{a}	82.49±5.27 ^{bc}
T35 (min)	61.17±0.94 ^{d}	92.63±1.98 a	78.17±3.82 ^{c}	81.81±2.54 ^{bc}	96.3±3.38 ^{a}	90.07±2.87 ^{ab}
T40 (min)	70.84±3.62 ^{d}	95.58±1.67 ^{ab}	83.66±3.83 ^{c}	87.38±1.33 ^{bc}	100±0 a	96.44±0.83 ^{ab}
T45 (min)	72.32±3.95 ^{b}	98.57±1.25 ^{a}	91.12±1.3 ^{a}	93.69±1.9 ^{a}	100±0 ^{a}	100±0 ^{a}
T50 (min)	82.79±9.5 ^{b}	98.57±1.25 ^{a}	95.18±2.1 ^{a}	97.73±1.87 ^{a}	100±0 ^{a}	100±0 ^{a}
T55 (min)	86.71±8.28 ^{b}	100±0 ^{a}	96.5±3.04 ^{a}	98.86±1.97 ^{a}	100±0 ^{a}	100±0 ^{a}
T60 (min)	86.93±9.98 ^{b}	100±0 ^{a}	98.17±3.18 ^{a}	98.86±1.97 ^{a}	100±0 ^{a}	100±0 ^{a}

The mean number of oocytes analyzed per time interval was 50. Different superscript letters indicate

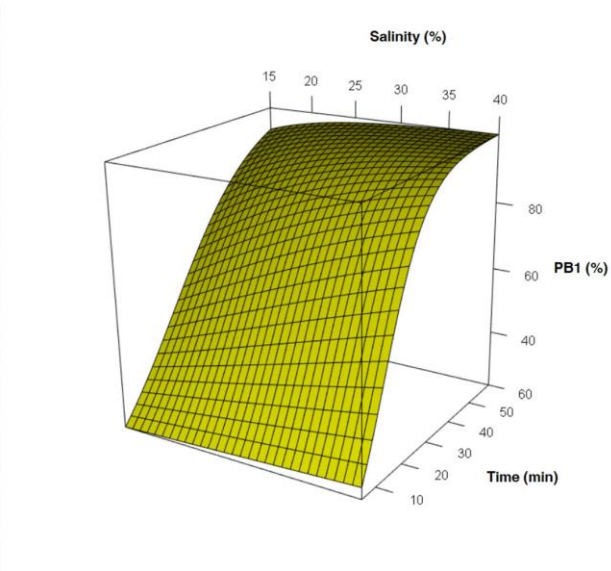

Figura 3: Mean of the percentages of polar body 1 release from fertilized eggs of Anomalocardia flexuosa.

For the extrusion of PB2, in treatments at 20, 25, and 30 g.L^{-1}, nearly 50% was achieved in the first 35 min, while that rate was accomplished in the first 30 min for 35 and 40 g.L^{-1} s (Figure 4 and Table 3). Yet for 15 g.L^{-1}, extrusion was accomplished later, in 45 min.

Table 3: Percentages of polar body 2 (PB2) extrusion of Anomalocardia flexuosa at each 5 min interval.

Time	**Time to reach 50% of PB2 release**					
	15 g·L^{-1}	**20 g·L^{-1}**	**25 g·L^{-1}**	**30 g·L^{-1}**	**35 g·L^{-1}**	**40 g·L^{-1}**
T5 (min)	1.15±1.99	4.32±3.15	4.59±2.57	0±0	12.14±2.94	5.52±0.68
T10 (min)	5.08±4.45	6.71±4.91	13.1±6.19	5.23±1.99	17.88±3.99	15.29±3.1
T15 (min)	10.15±8.91 ^{b}	15.46±5.71 ^{ab}	21.26±6.59 ^{ab}	9.78±2.39 ^{b}	28.49±5.85 ^{a}	20.04±6.7 ^{ab}
T20 (min)	12.72±4.59 ^{c}	24±3.96 ^{abc}	30.58±6.93 ^{ab}	20.61±7.78 ^{bc}	35.27±6.81 ^{a}	25.33±4.39 ^{abc}
T25 (min)	16.64±8.67 ^{c}	30.13±1.25 ^{abc}	36.22±5.26 ^{ab}	27.87±7.64 ^{bc}	43.94±12.61 ^{a}	34.5±8.47 ^{ab}
T30 (min)	21.51±5.32 ^{c}	39.28±0.65 ^{ab}	41.32±0.96 ^{ab}	35.16±4.23 ^{bc}	50.48±11.94 ^{a}	45.39±4.43 ^{ab}
T35 (min)	25.43±9.17 ^{c}	46.48±6.37 ^{ab}	44.91±2.25 ^{b}	47.26±7.31 ^{ab}	60.38±6.48 ^{a}	58.7±0.99 ^{ab}
T40 (min)	33.08±9.37 ^{c}	56.59±5.76 ^{ab}	47.44±1.29 ^{b}	55.58±6.27 ^{ab}	68.72±10.66 ^{a}	67.31±2.07 ^{a}
T45 (min)	45.58±6.23 ^{d}	62.85±6.71 ^{bc}	56.66±3.92 ^{cd}	60.17±6.42 ^{c}	78.7±9.45 ^{a}	75.07±3.92 ^{ab}
T50 (min)	63.21±5.54 ^{b}	70.62±6.75 ^{b}	65.97±4.32 ^{b}	70.32±8.47 ^{b}	87.18±5.23 ^{a}	86.57±7.52 ^{a}
T55 (min)	71.33±8.08 ^{b}	76.09±6.97 ^{b}	73.98±7.75 ^{b}	73.77±6.93 ^{b}	95.11±2.43 ^{a}	91.16±7.92 ^{a}
T60 (min)	73.63±4.15 ^{b}	86.05±4.41 ^{ab}	81.96±4.38 ^{b}	78.82±5.2 ^{b}	100±0 ^{a}	96.66±3.06 ^{a}

The mean number of oocytes analyzed per time interval was 50. Different superscript letters indicate statistical differences ($p < 0.05$).

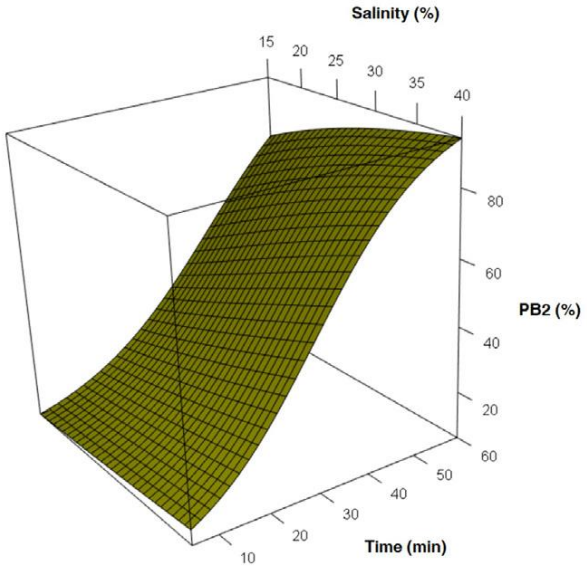

Figure 4: Mean percentage of polar body 2 release from fertilized eggs of Anomalocardia flexuosa.

4. Discussion

Effect of salinity on GVBD

Sodium-potassium antagonism is largely responsible for the oocyte germinal vesicle maintenance (Allen, 1953). The presence of Ca^{2+} ions, on the other hand, is essential for triggering vesicle degradation (Colas; Dubé, 1998). Thus, the salt concentration during hydration of oocytes obtained by stripping directly influences the GVBD (Qin et al., 2018). According to Allen and Bushek, (1992), the "immersion time" in seawater is important for synchronization of oocytes that are in prophase I. Thus, GVBD is a signal that meiosis is preparing to advance to the metaphase I stage until fertilization (Eudeline; Allen; Guo, 2000).

The present study suggests the existence of an association between increasing salinity concentrations and GVBD rates. The obtained results suggested that there are important aspects of biochemical and biophysical characteristics of A. flexuosa oocytes, such as plasma membrane permeability and osmotic tolerance that interfere on GVBD rates. In

hypertonic medium, the cells first react by shrinking owing to the exit of water from the cellular structure. This phenomenon is followed by cell swelling following the entry of water to maintain the osmotic balance (Salinas-Flores; Adams; Lim, 2008).

A. flexuosa is characterized as a species tolerant to variation in high salinities (Lagreze-squella et al., 2018) and, therefore, can adapt its reproduction to the environmental conditions. Based on our results, however, the oocyte stability seems to be associated with a tolerance limit for high saline concentrations. The osmotic tolerance limit may be exceeded if cells undergo excessive shrinkage or swelling that may lead to irreversible injury (Salinas-Flores; Adams; Lim, 2008). Although the salinity of 40 $g.L^{-1}$ had a higher catalytic ability for GVBD in our study, the effort to maintain the osmotic balance seemed to be critical for oocytes, leading to the rupture of the cell structure after 60 min.

Different studies have already pointed out that there are factors that can disturb embryogenesis and larval development such as gonadal maturation, oocyte maturation, and polyspermia in bivalves (Lavander et al., 2011; Qin et al., 2018). The larviculture of A. flexuosa is characterized as a stage strongly affected by factors such as temperature and salinity, water quality, feeding, and management (Oliveira et al., 2016). In this sense, identifying the best conditions for fertilization can be a tool to help seed production in hatchery. For A. flexuosa, 30 and 35 $g.L^{-1}$ salinity in the time between 80 and 120 min were the optimum range of hydration with higher percentages of GVBD.

Effect of salinity on the 50% release of PB1 and PB2

The use of polyploidy has become popular in bivalve aquacultur (Ma; Wang; Yu, 2019) e, either due to gonadal sterility (Piferrer et al., 2009; Dheilly et al., 2014; Zhang et al., 2017), to the increase in the cell volume and lack of compensation of cell number (Guo; Allen, 1994), or even to the increased heterozygosity that promote larger and faster growth in bivalves (Guo et al., 2009; Yang; Guo, 2018).

There are different alternatives to obtain triploid bivalve individuals. One of them refers to the possibility of interrupting the meiotic process by inhibiting the extrusion of PB1 or PB2 (Piferrer et al., 2009). For this purpose, the determination of the moment of the exit of 50% of PB1 or PB2 after fertilization is important as a reference for the application of inducers (Guo; Debrosse; Allen, 1996; Lavander et al., 2017; Melo et al., 2015). As in GVBD, salinity can also impact post-fertilization processes such as the timing of release or

inhibition of polar bodies in bivalve mollusks (Ma; Wang; Yu, 2019).

In the present study, we were unable to detect an explicit association between increasing salinity and the speed of events, as observed in GVBD evaluation. However, there was an optimal range for the extrusion of both polar bodies that occurred from 20 to 35 g.L−1 salinity at an interval of 10 to 20 min for PB1 and 30 to 40 min for PB2. The time to initiation of post-fertilization treatment and its duration are the main aspects influencing the success rate of polyploidy and embryo survival (Allen and Bushek, 1992; Eudeline et al., 2000).

Salinities of 15 $g.L^{-1}$ may hold back the induction to triploidy because the longer the process time for the release of PB1 or PB2 during chromosome manipulation, the higher are the chances of anomalies occurrence (Qin et al., 2018). Although 40 $g.L^{-1}$ concentration showed high percentages of extrusion of PB1 and PB2, the anticipated cell lysis process before fertilization made this treatment unfeasible. A previous report (Guimaraes et al., 2008) showed that salinity above 40 $g.L^{-1}$ can be lethal in the early life of estuarine bivalves.

Another study with A. flexuosa Lavander et al., (2017) also observed that the highest salinity tested (35 $g.L^{-1}$) resulted in better extrusion rates of PB1 and PB2 as compared to 15 and 25 $g.L^{-1}$, corroborating the hypothesis that salinity interferes with the exit time of polar bodies. On the other hand, under the same conditions of temperature and salinity (26□C and 35 $g.L^{-1}$), these authors found higher extrusion rates, such as of 70% for PB1 until the first 10 min and 62.67% for PB2 after 16 min of fertilization. The discrepancy in the results may be attributed to the methodological differences of observation, prediction analysis, and mainly the methodology of obtaining gametes. Using stripping instead of natural spawning to obtain gametes configures a more controlled strategy for the various treatments applied. On the other hand, oocytes obtained by stripping will be in different stages of maturation (Downing and Allen, 1987), contrasting to those obtained by natural or induced release (Lavander et al., 2017).

Regardless of the method applied for spawning, either natural or stripping, the induction of triploidy is most suitable at 35 $g.L^{-1}$ salinity, with the possibility of carrying post-fertilization shocks before 10 min for PB1 retention or before 30 min for PB2 retention. According to a previous study (Yang and guo, 2018), the formation of triploid clams by retention of both PB1 and PB2 is feasible.

Unlike bivalves from temperate climate where temperature largely influences spawning (Camacho et al., 2011; O'connor et al., 2008), those from tropical regions are more

influenced by salinity in the reproductive cycle (Nowland et al., 2021) because temperature does not change much throughout the year (Paixão et al., 2013). A previous study Lavander et al., (2011) identified that A. flexuosa species presented the fullest gonads during the dry period with salinity > 35 $g.L^{-1}$ and released gametes during the rainy period with salinities between 25 and 35 $g.L^{-1}$. Thus, the species may present a synchronous behavior with the rainy period.

The information that spawning occurs more frequently in monsoon and at intermediate salinities (Lavander et al., 2011) along with the results of the present work that there is an optimal range for GVBD and post-fertilization processes corroborate the idea that there is a reproductive strategy of this animal that is intrinsically related to salinity.

It is also necessary to consider the influence of genetic factors of the population under analysis. Different geographic populations of the same species accumulate alleles that reflect environmental adaptability (Kim et al., 2014; Ren et al., 2016). Organisms with external fertilization can develop their gametic plasticity to adapt to environmental conditions (Lettieri et al., 2019). However, the results presented herein provide an important information for the control of the reproductive process and on chromosome manipulation of the species to achieve triploidy.

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CAPÍTULO 2

Effects of salinity on pre- and post-fertilization developmental events in the oyster *Crassostrea rhizophorae* (GUILDING, 1828)

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Effects of salinity on pre- and post-fertilization developmental events in the mangrove oyster *Crassostrea rhizophorae* (GUILDING, 1828)

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Abstract - The mangrove oyster *Crassostrea rhizophorae* is identified as a potentially valuable species for tropical aquaculture, however, information on the physiological mechanisms of reproduction under laboratory conditions for this species is limited. This study investigated the effects of salinity at different concentrations (15, 20, 25, 30, 35, and 40 g/L) on the induction of germinal vesicle breakdown (GVBD) of oocytes obtained through stripping, the release of polar bodies (PB1 and PB2), and the larval development of the mangrove oyster. The results revealed a relationship between salinity and the percentage of GVBD, with the most effective range being 30 to 40 g/L within the hydration time frame between 70 to 120 minutes. The release of 50% of PB1 was detected within this salinity range, while for the release of 50% of PB2, the saline treatments of 35 and 40 g/L showed the best results. Overall, the salinity range of 30-40 g/L is suggested as the most suitable of

polyploidy induction methodologies through the retention of PB1 or PB2. Regarding larval hatching, while salinities between 25 and 40 g/L presented similar percentages, at 15 g/L no hatching was observed. This study demonstrated that salinity is a key factor in early pre- and post-fertilization stages for the successful reproduction of mangrove oyster in hatcheries and that the percentages of oocyte maturation and artificial fertilization can be optimized by adjusting salinity.

Keywords: tropical bivalve, maturation induction, artificial fertilization, polar body.

1. Introduction

Marine aquaculture has experienced significant growth in recent years, driven by increasing demand for food, especially in tropical and subtropical countries (Nowland et al. 2020; Willer and Aldridge 2020). In Brazil, although there is a diversity of native bivalve species with aquaculture potential, there is limited technical-scientific information available to develop management strategies and implement biotechnologies to support the production of these organisms (Sampaio et al. 2019). The mangrove oyster, *Crassostrea rhizophorae*, is identified as a promising species for aquaculture; however, it has been practiced with limited technological sophistication (Nalesso et al. 2008). The mangrove oyster exhibits external fertilization (Antonio et al. 2021). When released into the water, the oocytes are in a state of incomplete meiosis (Colas and Dubé 1998). Upon fertilization, they progress through a series of embryonic stages that include the formation and expulsion of the 1st and 2nd polar bodies, the onset of cell division, the formation of the D larva, and subsequently reaching the fixation or seed stage (Longwell and Stiles 1968; Legat et al. 2021). The production of native oysters often begins with seed collection from the wild, which is later transferred to cultivation areas, where they are monitored and maintained until reaching commercial size (Pereira et al. 2003). The production of high-performance seeds, such as polyploid seeds, requires the control of reproductive events including oocyte maturation (Colas and Dubé 1998), the optimal time for gamete collection (Antonio et al. 2021), artificial fertilization (Awaji et al. 2022), and chromosomal manipulation (Qin et al. 2018). Polyploidy induction is a technique that increases the number of chromosomes in an organism. In bivalve aquaculture, this can result in individuals with advantageous traits such as larger size, disease resistance, and tolerance to environmental variations (Wadsworth et al. 2019). Applications in polyploidy require the determination of optimal conditions in

hatchery production. Induced spawning methods in the mangrove oyster are still challenging, making “stripping” the most practical method for obtaining gametes. However, this alternative provides access to immature oocytes with intact germinal vesicle structure (Eudeline et al. 2000). Germinal vesicle breakdown (GVBD) is an indicator of oocyte maturation in bivalves and can be artificially stimulated by environmental factors such as salinity through a process known as "hydration" (Allen and Bushek 1992).

Tropical regions are known to exhibit minimal temperature variations throughout the year but experience significant fluctuations in salinity between dry and rainy seasons (Paixão et al. 2013). These unique environmental conditions can significantly influence the reproductive cycle of tropical oysters (Nowland et al. 2021), and they may experience maturation and gamete release changes, affecting their reproduction. Studies have shown that salinity plays a crucial role in hormonal regulation and reproductive behavior of tropical oysters, making it an essential factor to consider when breeding these species (Nowland et al. 2019; Nirchio and Vegas 2021). A better understanding of the relationship between salinity and the reproductive cycle of tropical oysters can contribute to more effective conservation and management strategies for these organisms in tropical environments. Oyster oocytes can be fertilized even if they have not completed GVBD. However, when completed, the synchronization of meiosis and the release of polar bodies are greatly improved (Qin et al. 2018). The release of the 1st and 2nd polar bodies (PB1 and PB2) is crucial in chromosomal manipulation, as triploidy induction can be promoted by inhibiting either PB1 or PB2 (Piferrer et al. 2009; Qin et al. 2018; Yang and Guo 2018).

Therefore, the goal of this study was to determine the optimal salinity condition and hydration time for inducing GVBD and the release of polar bodies for captive breeding for future triploidy induction in the mangrove oyster.

2. Materials and methods

2.1.Preparation of oysters

The oysters used in this study were collected from the Paciência River estuary, Maranhão island, northeast coast of Brazil (Figure 1). The breeders were transferred to the Marine Organisms Physieocology, Reproduction and Culture Laboratory of the State University of Maranhão. These specimens underwent disinfection with a 2% sodium hypochlorite solution and were then stored in a 100 L tank with filtered treated water (50, 25, and 5 μm cartridge filters), biological filter, and ultraviolet light. They were maintained at a salinity of 35 g/L, temperature of 24°C, and constant aeration for 4 hours for acclimatation. After this period, the animals were removed from water and kept at 24°C overnight to prevent gamete release. Approval by the Ethics Council for the Use of Experimental Animals does not apply to the invertebrate group, in accordance with the Brazilian law 11.794/08 (BRASIL 2008).

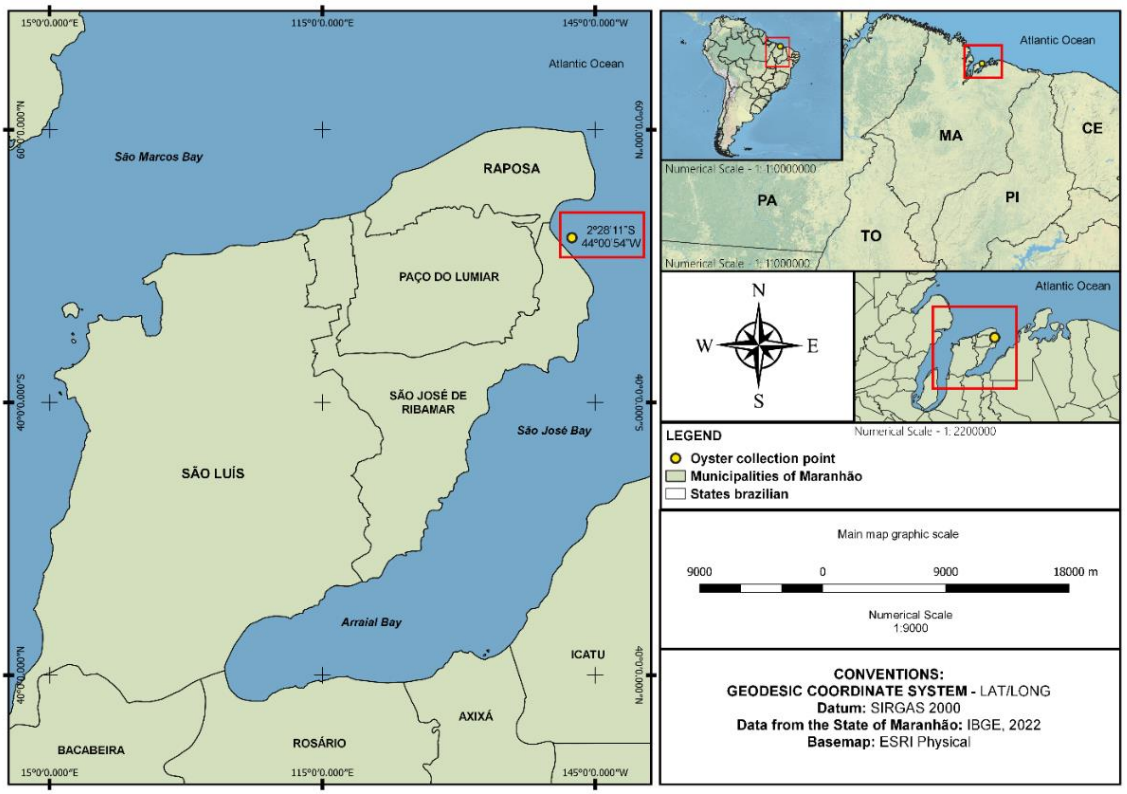

Figure 1: Location of the recruitment point for oysters, Crassostrea rhizophorae, in the estuary of the river Paciência, state of Maranhão, Brazil.

2.2.Genetic Identification

To genetically identify the collected species, muscle tissue samples were taken from the specimens used for molecular analysis. DNA extraction was carried out using the saline protocol by [22], and genetic identification was obtained using the species-specific multiplex PCR technique described by [23]. All specimens were identified as Crassostrea rhizophorae (Figure 2).

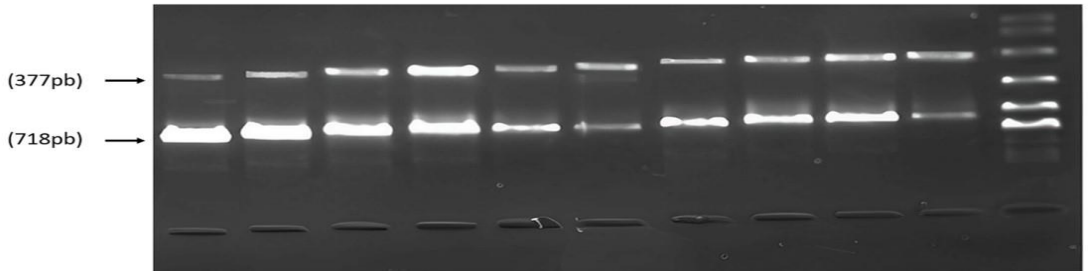

Figure 2: Species-specific Multiplex PCR with amplifications of two bands corresponding to the Crassostrea rhizophorae species: 377 bp from the COI region and 718 bp from the ITS 1 region.

2.3.Gamete Collection

Gametes were obtained through stripping using a slide and subjected to observation under an optical microscope for sexual identification following the methodology of [11]. Gametes from six females were used, approximately 1,750,000 oocytes. The oocytes were filtered through 100 µm mesh sieves to separate debris and rinsed through a 25 µm mesh screens..

2.4. Experimental Design

Experimental Design Germinal Vesicle Breakdown at different salinities

The effect of salinity on germinal vesicle breakdown was evaluated at salinity treatments of 15, 20, 25, 30, 35, and 40 g L^{-1}, with three simultaneous replicates for each treatment. The experimental units were maintained at 28°C, corresponding to the collection environment's natural average temperature (Antonio et al. 2021). For each treatment evaluation, the total volume was distributed into 2L beakers, from which 1 mL aliquots were taken to observe the progression of GVBD. The percentage of GVBD was calculated as follows:

$$\% \text{ GVBD} = G \times 100 / A$$

A: total number of observed oocytes; G: number of oocytes that underwent GVBD.

The observation of the germinal vesicle breakdown process took place over a two-hour period, with counting and recording intervals for vesicle breaks every 10 minutes. To establish a recommendation for oocyte maturation induction, the best conditions of time x salinity in hydration were defined, where GVBD percentages were equal to or greater than 80% (Li et al. 2021; Lopes et al. 2023).

Release of PB1 and PB2 in different salinities and embryonic development

A total of four males were identified, and their semen were collected through stripping and mixed, filtered through a 100µm mesh, and observed under an optical microscope for quantification. After a two-hour hydration period for GVBD at different salinities (15, 20, 25, 30, 35, and 40 g/L) and constant temperature of 28°C, spermatozoa were added to each

experimental unit at a ratio of 7 spermatozoa: 1 oocyte, following the methodology of (Melo et al. 2015). The release rates of the 1st and 2nd polar bodies (PB1 and PB2) were monitored over a 60-minute period, with observation intervals every 5 minutes. The average time in each treatment to achieve 50% release of PB1 and 50% of PB2 was determined (Melo et al. 2015; Barreto-Hernández et al. 2018; Yang and Guo 2018).

To assess the larval formation at each treatment, gametes were transferred to 15L compartments, and embryonic development was monitored for the following 4 hours after the release of the PB2, with confirmation of formed D larvae after 12 hours of fertilization (Figure 3). The fertilization rate and larval formation were defined as follows:

% fertilization = F × 100 / A; and % hatchability = L * 100 / F

F: number of eggs that exhibited polar body release; A: total number of observed oocytes; L: number of formed D larvae.

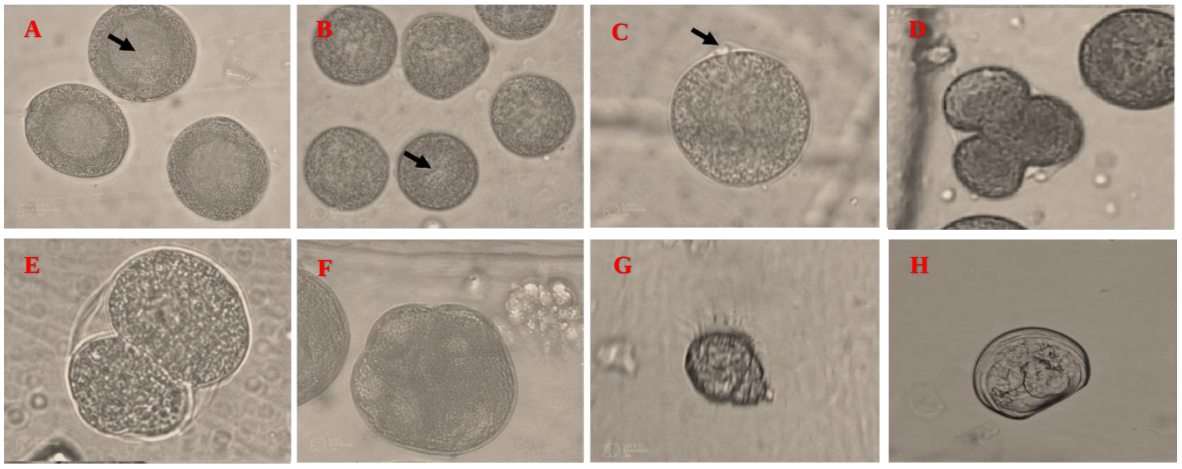

Figure 3: Images of pre- and post-fertilization in Crassostrea rhizophorae recorded A: Germinal Vesicle Identification; B: Highlight of GVBD; C: Release of PB1; D: First cleavage; E: 2 cells; F: 4 cells; G: trochophore larva; H: D-larva.

2.5.Statistical analysis

The data consisted of three repetitions per treatment. The average of the replicates of each treatment were used to estimate the release time of PB1 and PB2 at different proportions. Trends in the percentages of GVBD and released PB over time in each salinity

condition were estimated by fitting the best-likelihood model with a Generalized Additive Model (GAM) on the data, considering a binomial distribution. A two-way analysis of variance (ANOVA) was used to measure the effects of salinity and time, and means were compared using the Tukey post hoc test at a significance level of $p \leq 0.05$.

The statistical relationship between the percentage of unfertilized oocytes, fertilized oocytes, and larval formation was evaluated. Normality and homogeneity of data were checked using the Shapiro-Wilk and Levene tests, respectively. A one-way analysis of variance (ANOVA) and the Tukey test were performed. All analyses were conducted in RStudio version 4.1.0, and the "MGCV" package (Wood 2011) was used to execute the models. The model selection steps were performed as outlined in (ZUUR 2012) and (Zuur et al. 2009).

3. .Results

3.1 Effect of Salinity on Germinal Vesicle Breakdown

The salinity of 40 g/L exhibited the best GVBD performance across all time intervals, followed by the salinity of 35 and 30 g/L. On the other hand, salinities of 25 g.L -1, 20 g/L and 15 g/L had gradually lower performances in terms of maturation, suggesting an increasing effect relationship between salinity and GVBD (Table 1).

Table 1: Germinal vesicle breakdown of Crassostrea rhizophorae oocytes under salinity influence.

Time (min)	GVBD percentages in the range of 10 to 120 minutes					
	15 g/L	**20 g/L**	**25 g/L**	**30 g/L**	**35 g/L**	**40 g/L**
10	0±0 ^{c}	0.6±1.03 ^{bc}	0±0 ^{c}	1.75±3.04 ^{bc}	3.85±0.15 ^{ab}	7.11±1.1 ^{a}
20	0.6±1.03 ^{d}	2.81±1.11 ^{d}	8.1±0.85 ^{c}	7.67±0.57 ^{c}	14.63±1.42 ^{b}	25.2±0.73 ^{a}
30	4.45±0.79 ^{d}	6.69±2.02 ^{d}	12±1 ^{c}	15.35±1.13 ^{bc}	16.73±1.32 ^{bc}	38.4±1.39 ^{a}
40	6.38±0.66 ^{c}	9.98±0.74 ^{c}	19.57±1.91 ^{b}	19.6±3.42 ^{b}	18.17±0.3 ^{b}	52.08±0.34 ^{a}
50	10.24±0.41 ^{e}	14.89±0.55 ^{d}	27.92±0.97 ^{c}	31.78±1.68 ^{b}	26.28±0.55 ^{c}	65.41±2.25 ^{a}
60	14.1±0.17 ^{e}	19.83±1.26 ^{d}	31.2±3.77 ^{c}	36.36±3.2 ^{b}	38.45±0.45 ^{b}	73.07±1.42 ^{a}

70	16.02±0.04 [f]	29.17±1.04 [e]	35.72±0.99 [d]	40.87±2.8 [c]	49.31±2.2 [b]	82.42±3.88 [a]
80	21.14±1.03 [e]	34.2±0.85 [d]	44.79±1.18 [c]	58.55±0.95 [b]	59.6±1.74 [b]	91.77±4.25 [a]
90	25±1 [e]	40.73±1.1 [d]	56.9±0.85 [c]	72.06±1.91 [b]	71.1±2.6 [b]	95.88±2.39 [a]
100	30.12±0.21 [e]	49.17±0.76 [d]	67.54±0.4 [c]	78.32±0.55 [b]	78.8±2.38 [b]	97.64±0.81 [a]
110	35.9±0.17 [e]	55.2±2.46 [d]	74.31±1.13 [c]	83.03±2.68 [b]	85.89±1.21 [b]	98.79±1.07 [a]
120	44.21±0.37 [f]	61.03±1.7 [e]	78.17±0.76 [d]	87±2 [c]	93.58±1.12 [b]	100±0 [a]

A sample of 50 oocytes was used to calculate the percentages. Different superscript letters indicate statistical differences ($p<0.05$) at each time interval.

The obtained data allowed delimiting a salinity and time interval where the GVBD percentages were above 80%. The range between 30 and 40 g/L salinities in the period between 70 min and 120 min provided better oocyte maturation results (Figure 4).

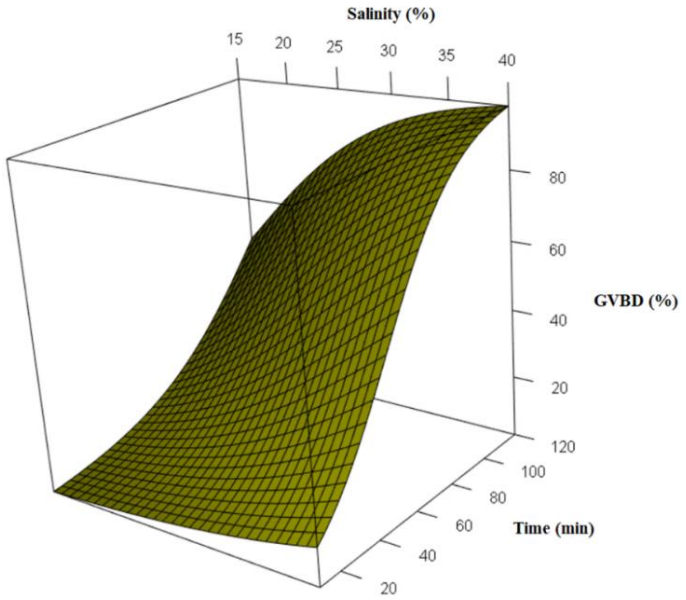

Figure 4: Mean percentage of GVBD of oocytes obtained by stripping Crassostrea rhizophorae.

3.2 Effect of salinity on the release of 50% of PB1 and PB2 and larval formation

Except for the 15 g/L treatment, all other salinities showed a potential release of 50% of PB1 in the period of 60 min, following GVBD. The release time of the first polar body at

35 and 40 g/L salinity was significantly lower than for other salinities, and the reference value of 50% was reached after 35 min (Table 2 and Figure 5).

Table 2: Percentages of polar body 1 (PB1) extrusion of Crassostrea rhizophorae at each 5 min interval

Time (min)	Time to reach 50% PB1 release					
	15 g/L	**20 g/L**	**25 g/L**	**30 g/L**	**35 g/L**	**40 g/L**
5	3.86±3.71	4±3.46	3.31±1.13	3.43±3.45	3.97±3.49	8.16±0.27
10	5.77±3.76 ^{b}	8.67±3.06 ^{ab}	6.63±1.19 ^{ab}	8.37±1.81 ^{ab}	9.21±1.06 ^{ab}	13.65±2.86 ^{a}
15	10.87±3.24	14±4	13.91±2.01	14.46±2.26	14.46±2.15	17.55±1.38
20	13.35±1.09 ^{b}	18±4 ^{b}	17.88±0.2 ^{b}	16.82±1.24 ^{b}	26.31±2.07 ^{a}	27.6±1.45 ^{a}
25	14.64±1.88 ^{d}	25.33±4.16 ^{bc}	21.84±1.77 ^{cd}	18.53±1.62 ^{cd}	31.59±2.12 ^{ab}	33.2±2.07 ^{a}
30	18.38±0.46 ^{c}	30.67±5.03 ^{b}	28.48±1.34 ^{b}	32.16±6.51 ^{b}	42.13±1.81 ^{a}	43.36±1.18 ^{a}
35	21.57±1.28 ^{e}	36.67±7.02 ^{cd}	34.44±3.15 ^{d}	43.07±1.54 ^{bc}	51.33±2.31 ^{a}	50.28±0.49 ^{ab}
40	24.75±3.79 ^{d}	40±2 ^{c}	40.41±5.1 ^{c}	50.26±3.85 ^{b}	54.62±3.07 ^{ab}	59.11±1.02 ^{a}
45	26.11±6.71 ^{d}	41.33±1.15 ^{c}	47.69±6.02 ^{c}	58.07±1.21 ^{b}	60.49±3.95 ^{ab}	65.93±3.41 ^{a}
50	31.76±5.1 ^{d}	44.67±1.15 ^{c}	51.67±3.78 ^{c}	63.96±2.06 ^{b}	68.36±4.09 ^{ab}	74.66±3.04 ^{a}
55	36.29±5.12 ^{e}	47.33±2.31 ^{d}	56.95±3 ^{c}	68.82±0.76 ^{b}	72.97±4.55 ^{b}	82.25±2.38 ^{a}
60	40.54±1.46 ^{e}	50±2 ^{d}	58.94±3 ^{c}	72.89±3.16 ^{b}	75.62±4.88 ^{b}	89.94±0.1 ^{a}

A sample of 50 oocytes was used to calculate the percentages. Different superscript letters in each indicator indicate statistical differences ($p<0.05$) in each time interval.

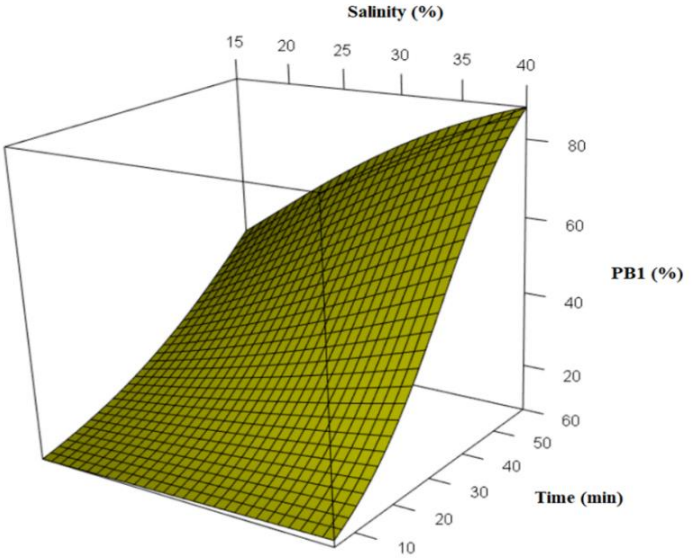

Figure 5: Mean percentages of PB1 release from fertilized Crassostrea rhizophorae eggs.

The trend of faster polar body release at higher salinities was also observed in the release of PB2. The treatments at 35 and 40 g/L reached 50% release at 50 min after PB1 release, while the others seemed to reach this mark well beyond 60 min (Table 3 and Figure 6).

Table 3: Percentages of polar body 2 (PB2) extrusion of Crassostrea rhizophorae at each 5 min interval

Time (min)	**Time to reach 50% PB2 release**					
	15 g/L	**20 g/L**	**25 g/L**	**30 g/L**	**35 g/L**	**40 g/L**
5	0±0	0±0	0±0	0±0	0±0	0±0
10	0.69±1.2	0.67±1.15	0±0	0±0	0±0	2.56±1.25
15	0.69±1.2	2.67±1.15	0.65±1.13	1.15±1.99	2±3.46	5.59±3.17
20	1.91±0.15 ^{b}	6.67±1.15 ^{b}	3.29±2.24 ^{b}	4.08±1.8 ^{ab}	7.33±2.31 ^{ab}	10.16±7.15 ^{a}
25	2.6±1.36 ^{d}	8.67±1.15 ^{abc}	5.96±0.07 ^{cd}	7.12±1.25 ^{bcd}	12±0 ^{ab}	13.95±7.23 ^{a}
30	3.2±1.2 ^{c}	11.33±1.15 ^{b}	8.61±3.07 ^{bc}	10.62±4.02 ^{b}	14±2 ^{b}	20.68±1.15 ^{a}
35	3.79±1.79 ^{d}	16.67±3.06 ^{bc}	11.91±3.39 ^{c}	18.34±3.77 ^{b}	18.67±1.15 ^{b}	27.04±3 ^{a}
40	5.68±3.57 ^{e}	20±4 ^{c}	13.9±1.85 ^{d}	23.09±4.42 ^{bc}	27.33±3.06 ^{ab}	32.63±2.28 ^{a}
45	7.58±3.56 ^{e}	22.67±2.31 ^{d}	19.87±0.23 ^{d}	28.61±2.34 ^{c}	38.67±2.31 ^{b}	44.59±2.24 ^{a}
50	8.8±3.49 ^{d}	28±2 ^{bc}	25.18±1.43 ^{c}	33.46±1.3 ^{b}	50±2 ^{a}	54.75±1.09 ^{a}
55	12.63±1.59 ^{e}	32±3.46 ^{d}	29.79±1.69 ^{d}	40.68±1.48 ^{c}	60±2 ^{b}	67.7±0.36 ^{a}

60	14.54±1.56 [e]	33.33±1.15 [d]	33.78±2.04 [d]	45.43±1.81 [c]	65.67±0.58 [b]	80.45±2.15 [a]

A sample of 50 oocytes was used to calculate the percentages. Different superscript letters in each indicator indicate statistical differences ($p<0.05$) in each time interval.

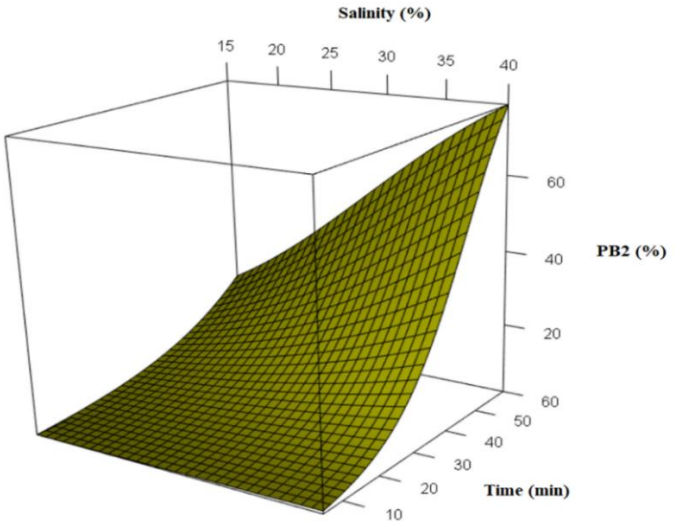

Figure 6: Average percentages of PB2 release from fertilized Crassostrea rhizophorae eggs.

Fertilization conducted at salinities ranging from 20 to 40 g/L resulted in the formation of normal D larvae. The ratio between fertilized oocytes and larval hatch indicated a similar yield within the range above 25 g/L, although the number of fertilized oocytes was considerably higher in the 40 g/L treatment. No larval formation was observed in the 15 g/L treatment (Figure 7).

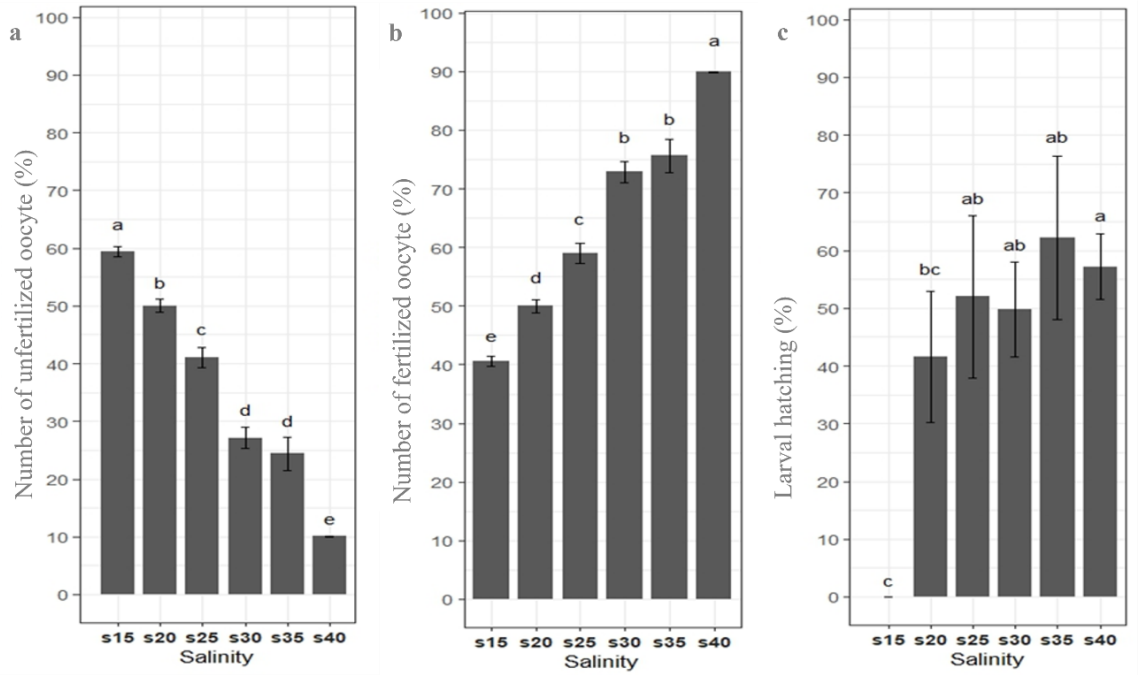

Figure 7: Results of the Crassostrea rhizophorae fertilization experiment at different salinities. % of unfertilized oocytes (a) (mean ± SD, n = 3 repetitions), % of fertilized oocytes (b) (mean ± SD, n = 3 repetitions), and % of formed larvae (mean ± SD, n = 3 repetitions) (c) of each saline treatment. Different subscript letters indicate a significant difference (p <0.05) between salinities.

4. Discussion

4.1 Effect of Salinity on Germinal Vesicle Breakdown

Euryhaline bivalve organisms are characterized as osmoconformers capable of regulating their extracellular and intracellular hemolymph fluids with changes in the environment, such as the natural salinity fluctuations in estuarine environments (Sokolov and Sokolova 2019). This ability has been made possible by developing sophisticated adaptive mechanisms to cope with salinity fluctuations (Pourmozaffar et al. 2020). However, metabolic changes in the osmotic equilibrium process can interfere with physiological aspects (Pourmozaffar et al. 2020; Zhou et al. 2022).

This study demonstrated a relationship between increased salinity concentrations and GVBD rates. Oocyte hydration was most efficient within the salinity range of 30 to 40 g/L,

within a time frame of 70 to 120 minutes. In a specific study on the correlation between salinity and GVBD, (Li et al. 2021) demonstrated higher GVBD percentages at salinities between 24 - 32 g/L and significantly lower ratios at salinities ≤ 20 g/L for the species *C. gigas* and *C. ariakensis*. However, (Qin et al. 2018) showed that GVBD induction for *C. hongkongensis* was more efficient at low salinities (15 g/L). This variation in salinity effects reinforces the importance of determining how the gradual increase would impact GVBD rates under laboratory conditions to ensure higher percentages.

Although studies on the artificial fertilization of tropical oyster species in the laboratory have incorporated the oocyte hydration step into their methodologies (Dos Santos and Nascimento 1985; Legat et al. 2017), these protocols do not rely on species-specific information. Tropical regions are characterized by minimal temperature variation throughout the year but high salinity variations (Paixão et al. 2013). Oysters from the Paciência River estuary region, where the specimens for this study were collected, are exposed to high salinities, with annual variations ranging from 31 g/L (rainy season) to 42 g/L (dry season), and constant temperatures, with no significant difference throughout the seasons, at 28-29 °C (Funo et al. 2019; Antonio et al. 2021). The higher GVBD percentages obtained in this experiment suggest that the best conditions for oocyte maturation were those with salinities close to the local of origin. (Nirchio and Vegas 2021) have already pointed out that environmental salinity has a selective and significant effect on reproductive adaptations in the mangrove oyster. Therefore, hatchery production for this species should consider the salinity of origin of the broodstock used for reproduction.

4.2 Effect of salinity on the release of 50% of PB1 and PB2 and larval formation

High or low salinity levels can cause osmotic stress and interfere with the normal functioning of cells and tissues in the mangrove oyster (Nirchio and Vegas 2021). In

particular, changes in salinity can affect the synchronization of meiotic division, including the formation and release of polar bodies in oysters and other bivalve mollusks (Qin et al. 2018; Li et al. 2021). In our results, the release of 50% of PB1 in the mangrove oyster eggs was faster within the salinity range of 30 to 40 g/L, where the highest percentage of GVBD also occurred, highlighting the importance of meiotic synchronization in stripped oocytes for post-fertilization processes.

Control over the polar body release is crucial in chromosomal manipulation methodologies. Identifying the conditions, in terms of salinity factor, under which 50% of PB1 is released after fertilization in a faster and more uniform manner is crucial to ensure a higher percentage of triploids (Guo et al. 1996; Melo et al. 2015; Qin et al. 2018). In these processes, the longer the time for PB1 release, the higher the chances of anomalies to occur (Qin et al. 2018). Although the treatments at 20 and 25 g/L allowed for the release of 50% of PB1, the time was significantly longer than that of the other treatments, occurring between 50 and 60 minutes after fertilization. The release of the second polar body can provide information about the duration of immersion time in chemical or physical induction treatments (Yang and Guo 2018). We observed that only salinities of 35 and 40 g/L released 50% of PB2 during the observed period. Based on these results, it is recommended that for the mangrove oyster hydration must be conducted within the interval of 70 to 100 minutes in the salinity range of 35-40 g/L and that triploidy induction shocks should be conducted between 15 to 25 minutes to retain 50% to 80% of PB2) in this salinity range.

Salinity is a determining factor throughout the embryonic and larval development stages (Pourmozaffar et al. 2020). We found that the higher the salinity, the higher the number of fertilized oocytes, however, for larval hatching, similar percentages were detected for either moderate or high salinities (25-40 g/L). This suggests that higher salinities are crucial for the early pre- and post-fertilization stages, but it is not at the later stage of larval

hatching. Several studies under laboratory conditions of the mangrove oyster provide different optimal salt concentrations, which can be 20 to 25 g/L (Rampersad and Ammons 1992), 28 to 30 g/L (Rodríguez, J. ; Frías 1992), 25 to 30 g/L (Miranda and Guzenski 1999), or 25 to 37 g/L (Dos Santos and Nascimento 1985). These differences suggest that there may be other related factors, such as temperature, pH, broodstock origin, or even correct species identification, given that until recently, the taxonomy of the *C. rhizophorae* and *C. gasar* species was problematic (Ferreira et al. 2023).

According to (Romo et al. 2010), salinity and seawater temperature are the main factors controlling the life cycle, distribution, and physiology of aquatic organisms. However, studies have reached different conclusions regarding the salinity-temperature relationship for the larval development of bivalves. Some authors consider that both factors need to be combined (Doroudi et al. 1999; de Albuquerque et al. 2012; Nowland et al. 2019), others highlight the greater importance of temperature (His et al. 1989; Joyce et al. 2013; Santerre et al. 2013), and others emphasize salinity (Taylor et al. 2004; Xu et al. 2011; Huo et al. 2014; Lopes et al. 2023). In this research, we evaluated the effect of salinity on fertilization and larval development of the mangrove oyster under laboratory conditions in a tropical area. Furthermore, the effect of sperm motility variations under different environmental conditions should be considered. Vigor and duration of motility are regulated by the concentration of ions such as K+, Ca2+, Na+, and pH in the water (Alavi et al. 2014), which, combined with oocyte maturation conditions, can interfere with the fertilization process and the timing of polar body release.

5. Conclusions

In conclusion, based on both current and previous results for the mangrove oyster, salinity is a key factor for successful hatchery reproduction, and the percentages of oocyte

maturation and artificial fertilization can be optimized by adjusting salinity. However, further research focusing on laboratory production, should evaluate other aspects in artificial fertilization of native oysters, such as the effect of water pH, ion concentration, the combination of salinity-temperature effects, sperm motility, and assessment of genetic and enzymatic markers, particularly in the mangrove oyster.

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CAPÍTULO 3

Efeito da Citocalasina B (CB) e do 6-Dimetilaminapurina (6-DMAP) na indução à triploidia e sobrevivência de larvas de Crassostrea rhizophorae (GUILING, 1828)

Comunicação curta.

Efeito da Citocalasina B (CB) e do 6-Dimetilaminapurina (6-DMAP) na indução à triploidia e sobrevivência de larvas de *Crassostrea rhizophorae* (GUILING, 1828)

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Resumo - A *Crassostrea rhizophorae* é uma ostra tropical com importante potencial para aquicultura. Com o objetivo de obter larvas triploides da espécie, foi avaliado o efeito dos agentes químicos indutores Citocalasina B (1mg.L^{-1}) e 6-DMAP (450 µmols.L^{-1}). Para a realização deste experimento; foram utilizados reprodutores da ostra coletadas no litoral do Maranhão. Para obtenção de gametas foi utilizada a técnica de stripping (raspagem do tecido gonadal), seguida de indução à maturação dos ovócitos em água do mar. A fertilização artificial e as respectivas 1ª e 2ª meioses foram monitoradas para aplicação de dois indutores químicos aos 35 minutos pós-fertilização, com duração de 25 minutos de imersão em cada tratamento. A confirmação da triploidia foi realizada em análise de citometria de fluxo 48 horas após a indução, constatando-se que tanto a CB como o 6-DMAP produziram larvas triploides, com percentual médio de 28,33% e 75,23%, respectivamente. Essa pesquisa traz os primeiros dados de viabilidade tecnológica da manipulação cromossómica na espécie nativa *Crassostrea rhizophorae*.

Palavras-Chaves: Bivalve tropical, manipulação cromossômica, indução química.

1. Introdução

A triploidia é uma condição caracterizada pela presença de três conjuntos completos de cromossomos em células somáticas e tem sido amplamente explorada na aquicultura de moluscos, para se obter um melhor crescimento (Stanley et al. 1981; Piferrer et al. 2009; Peachey and Allen 2016, Wadsworth et al. 2019). Algumas teorias são propostas para explicar o crescimento aprimorado nos moluscos triploides (Li et al. 2022). A primeira hipótese sugere que a esterilidade dos triploides redireciona a energia antes destinada à reprodução para favorecer o crescimento (Guo et al. 1996). A segunda teoria aponta para um aumento na heterozigosidade dos triploides, especialmente naqueles gerados retendo-se a 1ª meiose, comparados aos produzidos retendo-se a 2ª meiose (Stanley et al. 1981a; Hawkins et al. 1994; Li et al. 2022). Por fim, a terceira hipótese indica um potencial aumento no tamanho das células dos triploides, devido ao acréscimo de 50% no conteúdo de DNA dessas células (Rasmussen and Morrissey 2007).

Embora o cultivo de ostras poliploides esteja amplamente disseminado em muitas regiões do mundo como nos EUA, Europa e Asia (Willer and Aldridge 2020), espécies tropicais de bivalves ainda carecem de biotecnologias direcionadas às suas particularidades que permitam o desenvolvimento de protocolos eficientes. A ostra *Crassostrea rhizophorae* é uma espécie identificada como promissora para aquicultura (Paixão et al. 2013), entretanto a sua taxa de crescimento tende a ser mais lenta quando comparada a outras espécies de ostras nativas, resultando em um tempo de cultivo mais longo para alcançar o tamanho comercial (Pereira, 2003). Nesse contexto, a triploidia é uma abordagem biotecnológica promissora para superar as limitações de crescimento.

A eficiência da indução à triploidia varia com diferentes espécies e técnicas, e a indução química é identificada como uma das principais ferramentas de indução (Gerard et al. 1994; Melo et al. 2015; Peachey and Allen 2016; Barreto-Hernández et al. 2018). O presente trabalho propõe explorar o efeito da Citocalasina B (CB) e do 6-Dimetilamino-purina (6-DMAP) na obtenção de larvas triploides de *C. rhizophorae.*

2. Material e métodos

2.1. Preparação das ostras e indução à maturação dos ovócitos

Para a realização destes experimentos foram utilizados exemplares de ostra *C. rhizophorae* coletadas no estuário do rio Paciência, na Ilha do Maranhão, Brasil. Os reprodutores foram mantidos no Laboratório de Fisioecologia, Reprodução e Cultivo de Organismos Marinhos da Universidade Estadual do Maranhão em um tanque de cultivo de 100L, com temperatura de 24ºC, salinidade de 35 $g.L^{-1}$ e aeração constante.

A identificação sexual e a obtenção dos gametas se deram pelo mecanismo de *stripping,* com visualização em microscópio ótico. Foram utilizados gametas de 4 fêmeas e 2 machos, em cada repetição. Os ovócitos foram filtrados em peneiras de malhas de 100 µm para a separação de detritos e pedaços do tecido gonadal, filtrados em telas de 25 µm, quantificados, diluídos a uma densidade de 50 ovócitos/ml em um bequer com capacidade para 2L, em salinidade de 35 $g.L^{-1}$, seguido de um período de 100 minutos para a hidratação e monitoramento da quebra da vesícula em microscópio. Em seguida, foram adicionados aos ovócitos espermatozoides diluídos em 100 ml de água do mar filtrada.. A mistura de gametas foi separada em três béqueres de 500 ml, correspondente a uma unidade experimental de cada tratamento e do controle. Triplicatas de amostras com 1ml (50 ovócitos/ml) foram observados em microscópio ótico para o acompanhamento da fertilização, sinalizada pela liberação da 1º meiose (saída do primeiro corpo polar). A taxa de fertilização foi definida do seguinte modo:

$$\textbf{\% de fertilização} = F \times 100 / A$$

F: número de ovos que apresentaram liberação do corpúsculo polar; A: número total de ovócitos observados;

2.2 Indução à triploidia

Os ovos foram colocados em tubos cilíndricos de PVC com uma tela transversal com malha de 25 µm conectada para permitir a retenção dos ovos nas etapas de imersão (Figura 1). Aos 35 min, aproximadamente, após a fertilização e com uma liberação de 50% do primeiro corpo polar (CP1) (Lopes et al., 2024), os ovos foram submetidos ao tratamentos químicos de indução à triploidia com Citocalasina B (CB) a 1 $mg.L^{-1}$ e com 6-dimetilaminopurina (6-DMAP) a 450 $\mu mols.L^{-1}$ (Normand et al. 2009; Melo et al. 2015;

Lavander et al., 2018). Ovos não tratados com indutores químicos foram usados como controle.

O tempo de imersão nos dois tratamentos foi de 25 minutos, que corresponde ao tempo de 60 minutos (35 + 25 min) indicado por Lopes et al., (2024) como sendo correspondente ao tempo em que 50% do segundo corpo polar foi expelido. Após o tratamento com CB, os ovos retidos na tela foram lavados e ressuspendido em solução de 0,05% de DMSO em água do mar, por 15 min, como na figura 1, conforme Melo et al., (2015).

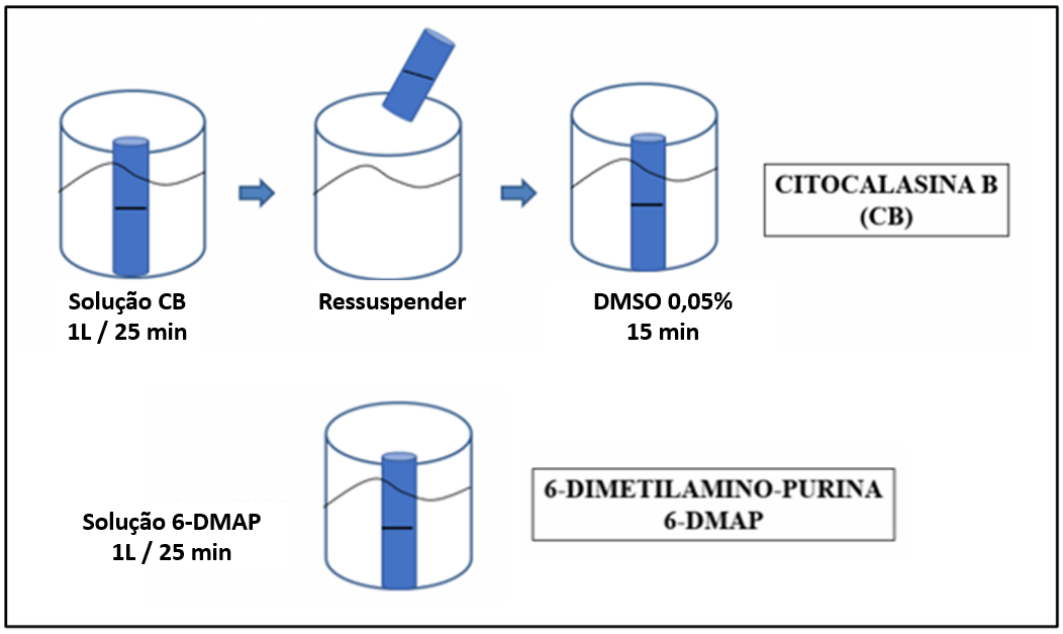

Figura 1: Etapas de tratamento químico de indução à triploidia com Citocalasina B e 6DMAP.Tubo cilíndrico de PVC destacado em azul.

Os embriões tratados e não tratados foram transferidos para tanques de 20L para o acompanhamento do desenvolvimento e formação larval. Após 24h da fertilização seguida da indução química, a porcentagem de larvas "D" normais foi calculada a partir de três amostras de 1 mL de cada unidade experimental com o uso de microscópio óptico e câmara de Sedgwick-Rafter (Legat et al. 2017). A taxa de formação larval foi definida do seguinte modo:

$$\% \text{ de eclosão} = L * 100 / F$$

F: número de ovos que apresentaram liberação do corpúsculo polar; L: número de larvas D formadas.

A análise de cada tratamento quanto ao processo de indução à triploidia foi realizada

48 h após a etapa da fertilização. Amostras contendo um "pool" de 2.000 larvas de cada repetição do tratamento foram separadas, por centrifugação e depois maceradas e fixadas em placas de petri contendo um 1 mL do tampão de isolamento WPB, de acordo com a metodologia adaptada de Loureiro et. al (2007).

As amostras foram filtradas através de um filtro descartável de malha de 22 μm e, ao final, o conteúdo de DNA foi corado com uma solução de iodeto de propídeo (1mg/mL). Para o processo de calibração do equipamento de citometria pelo grau de fluorescência das amostras, foram utilizados os espermatozoides, como controles haploides, em um teste de "*threshold*" onde foram avaliadas diferentes concentrações de iodeto de propídeo, de 1 μg a 1000 μg. As amostras foram analisadas no citômetro de fluxo Guava® easyCyte e os dados gerados foram processados no software FlowJo 7.6.

2.3 Análise estatística

Foi avaliada a relação estatística do percentual de ovócitos não fertilizados, ovócitos fertilizados e formação larval. Avaliou-se também o desempenho dos dois tratamentos de indução à triploidia. A normalidade e homogeneidade dos dados foi verificada pelos testes de Shapiro-Wilk e Bartlett's, respectivamente e foi realizado uma análise de variância (ANOVA) e o teste de Tukey. Todas as análises foram conduzidas no R studio versão 4.1.0.

3 Resultados e Discussão

3.2 Preparação das ostras e indução à maturação dos ovócitos

A porcentagem de larvas D formadas após 24 horas da fertilização variou de 35% a 66% entre os dois tratamentos (Tabela 1) e houve um efeito significativo dos tratamentos sobre a porcentagem de formação larval (Figura 2). O tratamento com 6-DMAP proporcionou uma maior formação larval, com uma média de 49% de eclosão.

Tabela 1: Resultados do experimento de fertilização artificial e indução à triploidia em Crassostrea rhizophorae utilizando Citocalasina B(CB) e 6-Dimetilaminapurina (6-DMAP)

Número de reprodutores	Média do Nº de ovócitos	Média da Taxa de fertilização (%)	Tratamentos	Taxa de eclosão larval após 24hrs (%)	Média do Nº de Larvas Formadas após 24hrs	Percentual de Triploidia após 48hrs (%)
12 ♀: 6 ♂	1.062.333	78	CB	35±2,2	88.191	28±2,8
			6-DMAP	49±4,4	127.985	75±6,1
			Controle	66±2,8	161.540	1±1,01

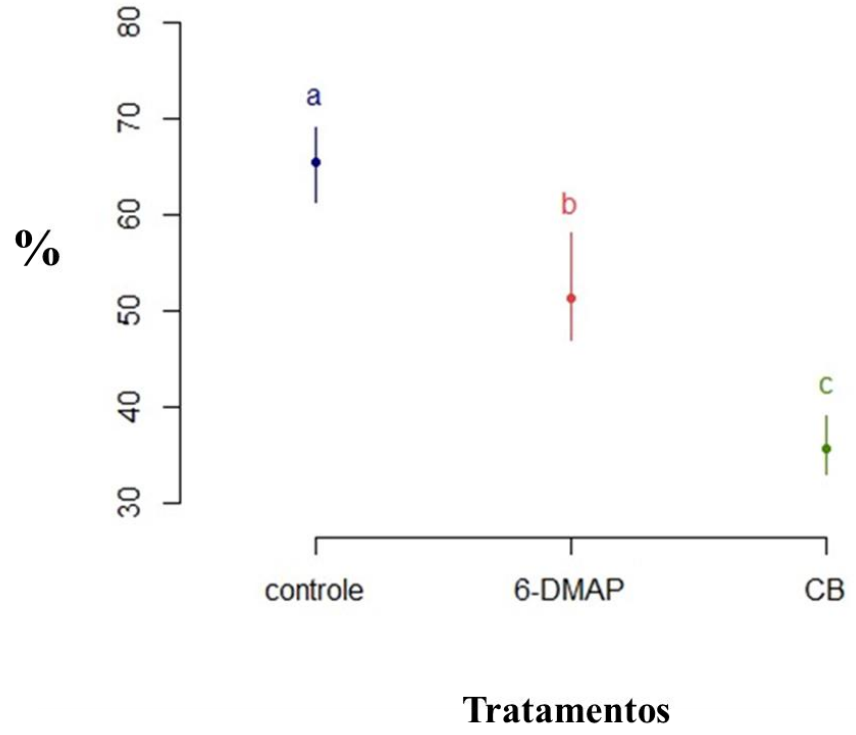

Figura 2: Porcentagem de eclosão larval obtidas nos diferentes tratamentos e no controle. Letras diferentes indicam diferenças significativas (P > 0,05).

Eventuais anomalias no processo de indução química à triploidia em bivalves podem repercutir na formação larval já nas primeiras 24 horas após a fertilização (Lavander et al.,

2018). O modo de atuação de cada reagente pode ser determinante no rendimento final da taxa de formação larval (Melo et al. 2015; Peachey and Allen 2016; Barreto-Hernández et al. 2018).

A Citocalasina B é uma micotoxina produzida por fungos que tem a capacidade de encurtar os filamentos de actina, essenciais para a divisão citoplasmática, impedindo que as células se dividam (Stanley et al. 1981b; Downing and Allen 1987). Além disso, a Citocalasina B pode causar extrusão nuclear, ou deformação do núcleo da célula, pelo mesmo mecanismo (Guo et al. 1992), podendo afetar o rendimento de larvas viáveis. Por outro lado, o 6-DMAP é um inibidor da maturação dos ovócitos, impedindo a conclusão da 2ª meiose, podendo comprometer os microtúbulos durante a divisão celular, resultando em um ovo com um conjunto extra de cromossomos (Piferrer et al. 2009; Melo et al. 2015). O modo de atuação tende a interferir menos diretamente na divisão celular, o que pode resultar em menos impacto imediato na sobrevivência das larvas.

A concentração e o tempo de exposição ao agente também desempenham um papel crucial no rendimento das larvas poliploides (Liang et al. 2023), de modo que concentrações inadequadas ou tempos prolongados de exposição podem ser prejudiciais ao desenvolvimento, independentemente do composto usado. No presente estudo, avaliou-se uma única concentração de cada reagente com base em protocolos de outras espécies de bivalves (McCombie et al. 2005; Melo et al. 2015; Peachey and Allen 2016; Lavander 2018). O tempo de exposição aos reagentes teve por base o estudo prévio de maturação e fertilização artificial para espécie *C. rhizophorae* (Lopes et al., 2024), adequando o início da indução ao tempo de liberação de 50% do primeiro corpúsculo polar e finalizando os tratamentos no tempo correspondente a retenção de mais de 60% do segundo corpo polar.

3.2 Indução e análise à triploidia

O teste de concentrações do marcador (iodeto de propídeo) apontou que a melhor concentração em células da ostra *Crassostrea rhizophorae* foi de 300µg/ml, gerando picos mais limpos. As análises de citometria de fluxo permitiram a determinação dos diferentes níveis de poliploidia. Os grupos controle, com espermatozoides e larvas diplóides, e os grupos tratados, com larvas diploides e triplóides foram detalhadas na figura 3.

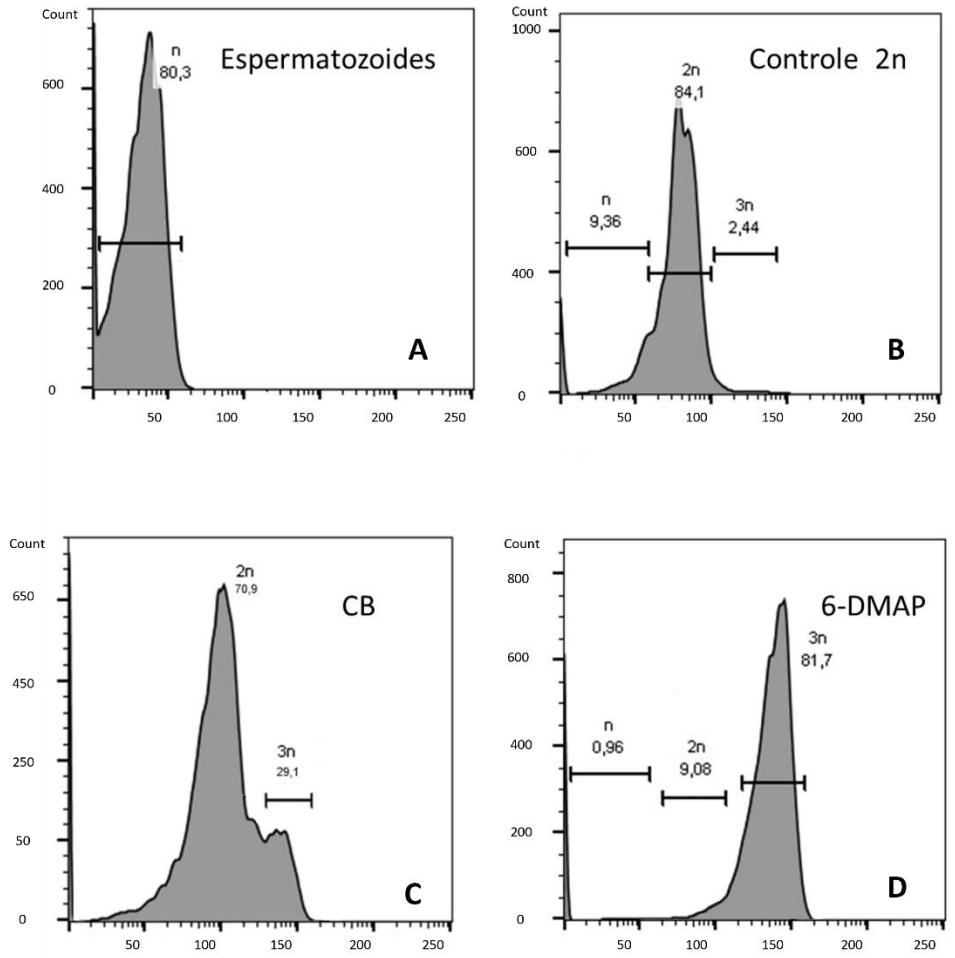

Figura 3: Histograma de frequência com conteúdo relativo de DNA para nível de ploidia em células de Crassostrea rhizophorae. A: Padrão haploide (Espermatozoide); B: padrão diploide (larvas controle);C: Tratamento com Citocalasina B (CB); D; Tratamento com 6-Dimetilaminapurina (6-DMAP).

Ambos os tratamentos (CB e 6-DMAP) produziram larvas triploides, mas a porcentagem foi significativamente maior no tratamento com o 6-DMAP (Tabela 1 e Figura 4). O percentual de triploidia identificado nas amostras controles, embora muito baixas ou nulas, podem estar relacionadas a uma sobreposição de células no momento da leitura. Em citometria de fluxo, a sobreposição de eventos ocorre quando duas ou mais células passam pelo feixe de laser simultaneamente, o que pode levar à interpretação incorreta dos resultados (Hoshino et al., 2019).

A grande diferença de resultados entre os tratamentos testados pode estar relacionada ao fato de existir uma maior dificuldade das células eliminarem a CB, dando continuidade à sua ação bloqueadora. A CB tem um peso molecular maior que o 6-DMAP, e, portanto, há

a necessidade do uso de um detergente, como o DMSO, que ajude na permeabilidade da membrana, auxiliando a saída da CB (Trendowski et al. 2015; Barreto-Hernández et al. 2018). Uma melhor adequação do tempo de imersão em cada etapa de indução pode resultar em melhores percentuais de triploidia por CB (Gérard et al. 1999).

O 6-DMAP demonstrou ser um indutor significativamente mais eficiente na produção de larvas triploides nas condições testadas. O melhor potencial de indução conferido pelo 6-DMAP quando comparado ao CB, no processo de triploidia, também foi observado em outras espécies como *Crassostrea gigas, Crassostrea virginica* e *Argopecten nucleus* (Peachey and Allen 2016; Barreto-Hernández et al. 2018; Melo et al. 2022). De modo geral, o 6-DMAP é considerado menos tóxico do que a CB (Peachey and Allen 2016), e, portanto, mais seguro para o operador.

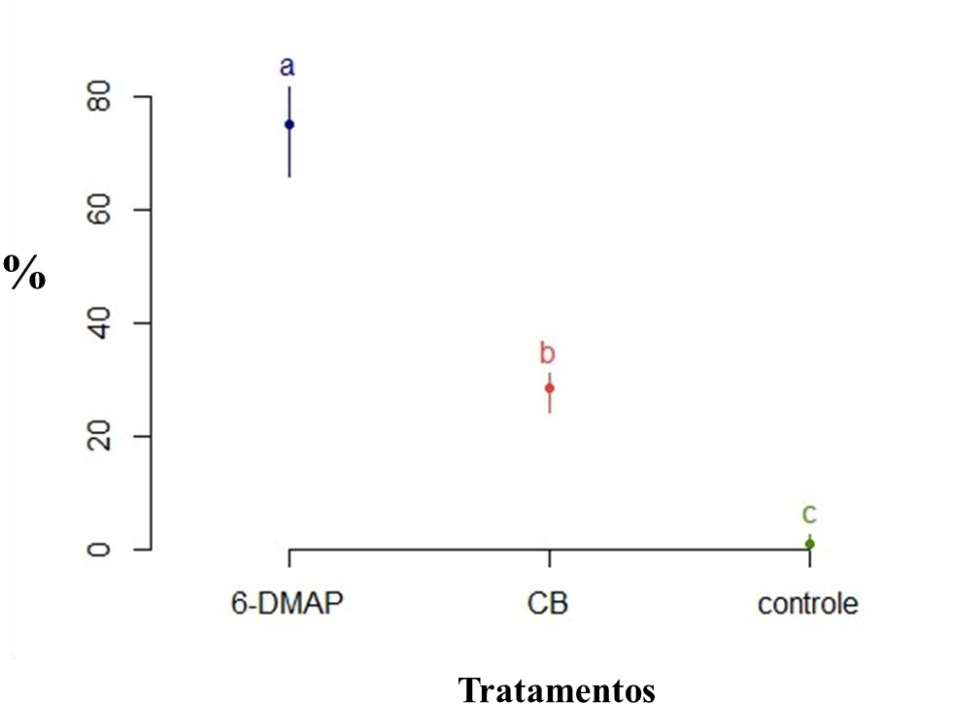

Figura 4:Porcentagem de larvas triploides de Crassostrea rhizophorae obtidas por indução química por 6-Dimetilaminapurina (6-DMAP)e Citocalasina B (CB). Letras diferentes indicam diferenças significativas (P > 0,05).

A determinação de ploidia em ostras por citometria de fluxo permite a análise do conteúdo de DNA de muitas células em um curto espaço de tempo, possibilitando distinguir e classificar diferentes níveis de ploidia (Allen 1983). O iodeto de propídeo é utilizado como marcador para quantificar o conteúdo de DNA nas células e a intensidade de sua fluorescência permite distinguir os diferentes níveis de ploidia, células diploides, triploides e outros (Barreto-Hernández et al. 2018). Concentrações elevadas podem gerar um sinal de fluorescência muito intenso, saturando os detectores do citômetro de fluxo, enquanto as muito baixas; comprometem a detecção da fluorescência (Allen 1983).

A padronização prévia de indução à maturação dos ovócitos e da sincronização da etapa de liberação corpuscular demonstraram ser essenciais na condução dos testes de indução à triploidia. Os momentos pós-fertilização indicados por Lopes et al., 2024 de 35 minutos para a saída de 50% do primeiro corpo polar e de 60 minutos para a saída de 50% do segundo corpo polar foram determinantes para aplicar o indutor químico aos 35 minutos e mantê-lo por 25 minutos para a obtenção de larvas triploides da ostra *Crassostrea rhizophorae* com o uso de 6-DMAP com percentuais >70%. Este é, portanto, o primeiro protocolo para a obtenção de triploides da ostra tropical *C. rhizophorae* . No entanto, considerando que o fundamento principal das metodologias de triploidia em bivalves está relacionado ao desempenho zootécnico final no cultivo, se faz necessário que novos estudos avaliem o efeito da triploidia sobre a sobrevivência e crescimento nas etapas subsequentes do desenvolvimento, queserão cruciais para a elaboração de um protocolo comercial de produção de triploides de *C. Rhizophorae*.

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Considerações finais:

- Os resultados de indução à Quebra da Vesícula Germinal evidenciaram o seu potencial de sincronização e aceleração do ritmo na liberação dos corpos polares em ambas as espécies em certos intervalos de salinidade.
- Houve uma clara indicação de uma relação entre o aumento das concentrações de salinidade e as taxas de QVG tanto para *A. flexuosa* como para *C. rhizophorae*..
- A fertilização artificial na espécie *A. flexuosa* pode ser conduzida, com melhores percentuais de QVG, nas salinidades entre 25 a 35 $g.L^{-1}$. Entretanto, considerando o tempo da liberação do 1º e 2º Corpúsculo polar, as salinidades de 30 e 35 $g.L^{-1}$ proporcionaram melhores resultados para metodologias voltadas para a manipulação cromossômica.
- A fertilização artificial da espécie *C. rhizophorae* indicou que as melhores condições de QVG ocorreram na faixa de 30 a 40 $g.L^{-1}$, em intervalo de tempo 80 a 120 minutos de hidratação. Entretanto a formação larval é viável em salinidades de 20 a 40 $g.L^{-1}$. A sincronização da meiose e a liberação dos corpos polares foram mais eficientes nas salinidades de 30 e 35 $g.L^{-1}$, sendo as mais indicadas a serem adotadas em metodologias de manipulação cromossômica.
- A produção de larvas triploides de *C. rhizophorae* por meio de indução química é viável. Esse é o primeiro registro de indução a triploidia para esta espécie.

ANEXOS

Animal Reproduction

ORIGINAL ARTICLE

Effects of salinity on pre- and post-fertilization developmental events in the clam *Anomalocardia flexuosa* (Linnaeus, 1767)

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Abstract

The knowledge about the effect of salinity on the physiological mechanism of bivalve reproduction is fundamental to improve production strategies in hatcheries. The present work evaluated the influence of different salinity concentrations (15, 20, 25, 30, 35 and 40 g·L^{-1}) on pre- and post-fertilization development processes in the clam, *Anomalocardia flexuosa*, oocytes obtained by stripping. Salinity directly interfered with the germinal vesicle breakdown (GVBD) rate and in the cellular stability of unfertilized oocytes. Salinity concentrations between 30 and 35 g·L^{-1} provided better percentages of stable GVBD within 120 min, and incubation of oocytes in the salinity range of 30-35 g·L^{-1} for a time interval of 80-120 min provided > 80% GVBD. In the post-fertilization analysis, salinity affected the rate of the extrusion of the first and second polar bodies (PB1 and PB2). The release of 50% of the PBs was faster at a salinity of 35 g·L^{-1}, with an estimated time of 10 min for PB1 and 30 min for PB2. Thus, chromosome manipulation methodologies aiming triploids should be applied at 35 g·L^{-1} salinity, with application of post-fertilization shock before 10 min for PB1 retention or before 30 min for PB2 retention.

Keywords: tropical bivalve reproduction, stripping, germinal vesicle breakdown, polar body.

Introduction

The production of bivalves in tropical regions has been consolidated as an important market for aquaculture (Nowland et al., 2020; Willer and Aldridge, 2020). However, tropical bivalve larviculture presents a series of obstacles that limits the continuous supply of seeds, essential to aquaculture and restocking (Nowland et al., 2021). One promising species for tropical aquaculture (Lagreze-Squella et al., 2018) is the clam *Anomalocardia flexuosa*, which is distributed from the Caribbean to Brazil (Abbott, 2011), and plays an important role in feeding and in the income of traditional communities (Rios, 2009). Hatchery production methodologies based on feeding, maturation, reproduction, larviculture and settlement events under controlled laboratory conditions have been developed for this species (Lavander et al., 2014; Lagreze-Squella et al., 2018; Lima et al., 2018).

The clam *A. flexuosa,* like most bivalve species, presents external fertilization. Stripping of the gonadal tissue gives access to undeveloped oocytes in maturation stages with intact germinal vesicle structure. This occurs because the oocytes of some species undergo a maturation process during the passage through the oviducts before their release (Colas and Dubé, 1998).

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Germinal vesicle breakdown (GVBD) is a natural process occurring in the external environment when metaphase I oocytes are released (Guo et al., 1996). This process is considered as a sign of oocyte maturation. To continue the GVBD stage, the oocytes may go through a period of hydration in the external environment (Melo et al., 2015). The breakdown of the vesicle improves fertilization, decreases polyspermia, and increases efficiency in polyploidy induction processes (Qin et al., 2018), and has been exploited in the hatchery of bivalves (Dégremont et al., 2012).

The process of embryogenesis begins from the time the sperm enters the oocyte and is characterized by the release of two polar bodies, PB1 and PB2 (Colas and Dubé, 1998). Polyploidy can be achieved by the physical or chemical treatment of fertilized eggs to inhibit the exit of PB1 or PB2 (Piferrer et al., 2009).

Environmental factors such as salinity affect the incubation time and the extrusion of polar bodies (Lavander et al., 2014). The knowledge of the effect of environmental variables on oocyte GVBD and the timing of polar bodies release is critical to increase fertilization success in hatcheries, achieve greater control in subsequent embryonic developmental stages, and for adjusting protocols for the use and control of polyploidy (Qin et al., 2018). This study evaluated the influence of salinity on the time of oocyte GVBD and on the extrusion of polar bodies in *A. flexuosa.*

Methods

Broodstock

A total of 150 adults were collected in estuarine zone of the state of Maranhão (2º30'03"S, 44º03'40"W) and stored at the State University of Maranhão for the experiments. Approval by the Ethics Council for the Use of Experimental Animals does not apply to the invertebrate group, in accordance with the Brazilian law 11.794/08 (Brasil, 2008).

Clams were cleaned with a sodium hypochlorite solution (2%) and then stored in a 100 L tank in water treated by cartridge filters (50, 25, and 5 µm), biological filter (containing different media and nitrifying bacteria), and UV. The organisms were maintained at a salinity of 30 $g \cdot L^{-1}$, temperature of 24 °C, and constant aeration for 4 h of depuration in a Recirculating Aquaculture System (RAS). After this period, the animals were left out of the water overnight at 24 °C to prevent the release of gametes into the water.

Gametes

For sex identification, each individual was carefully opened using a knife and the gonadal tissue was observed under an optical microscope. Gametes from 30 females were obtained by stripping using a surgical blade. Subsequently, they were mixed and filtered in a 100 µm mesh and rinsed in 25 µm. A dilution of the gametes was carried out to reach a final rate of 50 oocytes/mL in each experimental unit.

GVBD at different salinities

The effect of salinity on GVBD was evaluated at salinities of 15, 20, 25, 30, 35, and 40 $g \cdot L^{-1}$, with five replicates per treatment. The marine water used in the experiment was collected at sea (original concentration of 42 $g.L^{-1}$), filtered and sterilized in a UV filter in the laboratory. The dilution for the different saline concentrations was carried out by adding autoclaved freshwater.

The experimental units were thermostat regulated at 26 °C, which was considered as the ideal temperature for meiosis of the species (Lavander et al., 2017). Diluted oocytes were distributed in 100 mL beakers, from which 1 mL aliquot was removed with the aid of a pipette for observation of the progression of GVBD (Figure 1). The germinal vesicle breakdown process was observed over a period of 120 min, from the observation of 1ml samples (approximately 50 oocytes) taken every 10 minutes for counting and recording.

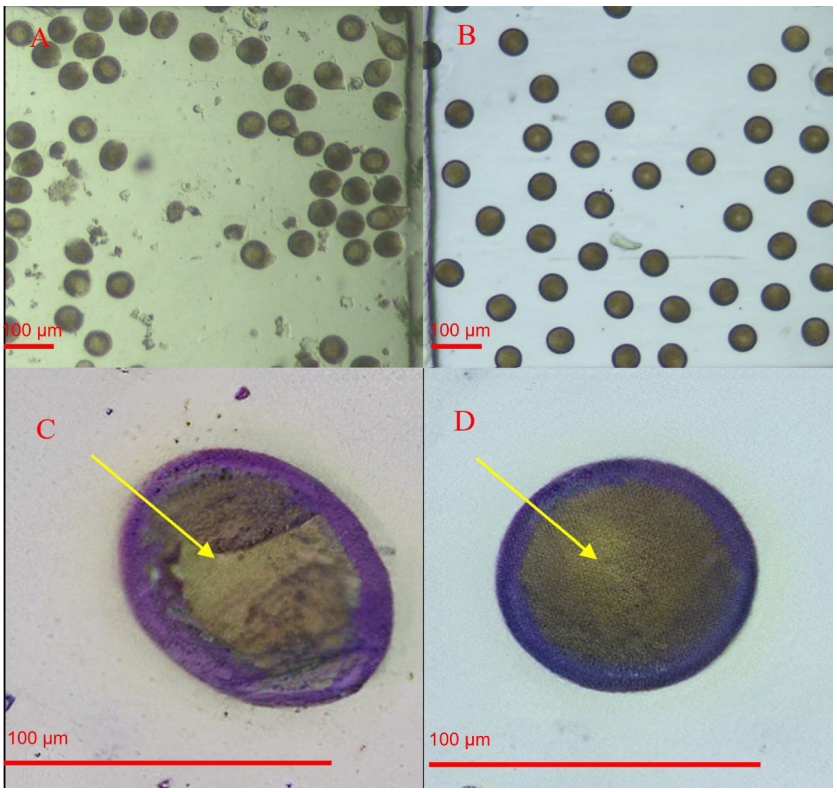

Figure 1. Images registered in the program Mosaic V.2.2.1 with a camera attached to the microscope showing the appearance of *Anomalocardia flexuosa* oocytes before and after germinal vesicle breakdown (GVBD); **A**: Image of a set of oocytes without uniformity soon after undergoing the stripping process observed under 4X lens; **B**: Set of oocytes after hydration showing the GVBD under 4X lens; **C**: Image of a magnified oocyte before hydration with an arrow in yellow highlighting the structure of the intact germinal vesicle observed under 10X lens; **D**: Image of an oocyte magnified after hydration and with a yellow arrow indicating the uniformity of the nucleus as a function of the GVBD observed at 10X lens.

Extrusion of PB1 and PB2 in different salinities

The spermatozoa from 30 males were obtained by stripping, mixed, filtered using 80 µm mesh and quantified by optical microscopy. After 2 h of oocyte hydration for GVBD in different salinities (15, 20, 25, 30, 35, and 40 $g \cdot L^{-1}$) and at a constant temperature of 26 °C, the spermatozoa were added to each experimental unit at a ratio of 7 spermatozoa: 1 oocyte, according to the previously described methodology (Melo et al., 2015). The first and second polar bodies (PB1 and PB2) extrusion rates were monitored at 5 min intervals over a period of 60 min using 1 ml samples (approximately 50 oocytes).

The average time to obtain the 50% of PB1 and PB2 extrusions for each treatment were determined as previously recommended (Melo et al., 2015; Lavander et al., 2017).

Statistical analyses

The averages of the five replicates of each salinity treatment were used to estimate the GVBD and the extrusion of PB1 and PB2 at the different time interval. The trends of the percentages of GVBD and PB released with time at each salinity concentration were estimated by fitting the best likelihood model with a generalized additive model (GAM) and considering a binomial distribution. A two-way analysis of variance (ANOVA) was used to measure treatment effects, and averages were compared using Tukey's post-hoc test at a $p \leq 0.05$ significance level.

Statistical analyses were performed in R studio version 4.1.0, and the package “MGCV” (Wood, 2011) was used to run the models. The steps for model selection were performed as previously described (Zuur, 2012; Zuur et al., 2009).

Results

Effect of salinity on GVBD

None of the salinity treatments resulted in 100% GVBD, however, high values were found at 30 and 35 g·L^{-1} salt concentrations (93.26 ± 1.04% and 93.76 ± 4.98%, respectively) at 120 min. Also, at 40 g·L^{-1} salt concentration 93.99% ± 7 were detected at 50 min, followed by a decrease in the breakdown in subsequent intervals. Salinity treatments at 15, 20 and 25 g·L^{-1} presented inferior values <85% after 120 min (Table 1 and Figure 2).

Table 1. Germinal vesicle breakdown of *Anomalocardia flexuosa* oocytes under salinity influence.

Time	Percentages of GVBD in the interval from 0 to 120 minutes					
	15 g·L^{-1}	20 g·L^{-1}	25 g·L^{-1}	30 g·L^{-1}	35 g·L^{-1}	40 g·L^{-1}
T10 (min)	3.24±1.09 ^{b}	17.7±1.9 ^{ab}	21.84±4.72 ^{a}	24.69±10.29 ^{a}	17.07±6.33 ^{ab}	27.22±12.06 ^{a}
T20 (min)	14.95±2 ^{c}	25.33±1.32 ^{bc}	27.43±7.25 ^{bc}	37.16±6.5 ^{ab}	31.58±14.65 ^{b}	47.63±15.25 ^{a}
T30 (min)	22.51±1.5 ^{d}	30.36±1.51 ^{cd}	34.39±9.73 ^{bcd}	48.97±10.94 ^{b}	42.72±17.34 ^{bc}	73.75±11.55 ^{a}
T40 (min)	32.02±4.42 ^{c}	35.37±3.97 ^{c}	46.8±8.39 ^{bc}	51.48±9.89 ^{b}	52.52±22.4 ^{b}	86.29±12.87 ^{a}
T50 (min)	42.62±4.44 ^{c}	39.37±1.98 ^{c}	53.4±8.6 ^{bc}	60.71±8.76 ^{b}	64.72±15.24 ^{b}	93.99±7 ^{a}
T60 (min)	47.39±4.97 ^{cd}	42.96±2.56 ^{d}	60.17±7.84 ^{bc}	69.54±7.86 ^{b}	70.79±16.85 ^{b}	91.88±5.82 ^{a}
T70 (min)	52.12±2.55 ^{cd}	44.96±3.41 ^{d}	64.31±3.95 ^{bc}	74.38±9.14 ^{ab}	80.11±15 ^{a}	80.08±14.35 ^{a}
T80 (min)	54.5±3.96 ^{b}	48.6±3.79 ^{b}	70.52±2.65 ^{a}	82.74±11.44 ^{a}	82.66±12.78 ^{a}	70.57±10.95 ^{a}
T90 (min)	58.24±1.12 ^{bc}	55.81±2.13 ^{c}	72.22±4.5 ^{ab}	87.24±8.89 ^{a}	86.96±10.74 ^{a}	61.41±6.01 ^{bc}
T100(min)	61.6±2.27 ^{bc}	61.89±3.19 ^{bc}	75.4±4.84 ^{ab}	88.39±6.97 ^{a}	90.2±8.27 ^{a}	55.39±5.79 ^{c}
T110(min)	68.84±3.58 ^{c}	67.91±3.51 ^{c}	78.48±6.72 ^{bc}	91.15±3.23 ^{ab}	93.76±4.98 ^{a}	51.23±7.05 ^{d}
T120 (min)	73.75±1.86 ^{b}	75.12±2.74 ^{b}	82.4±7.06 ^{ab}	93.26±1.04 ^{a}	93.76±4.98 ^{a}	44.8±6.13 ^{c}

The mean number of oocytes analyzed per time interval was 50. Different superscript letters in each indicator indicate statistical differences ($p < 0.05$).

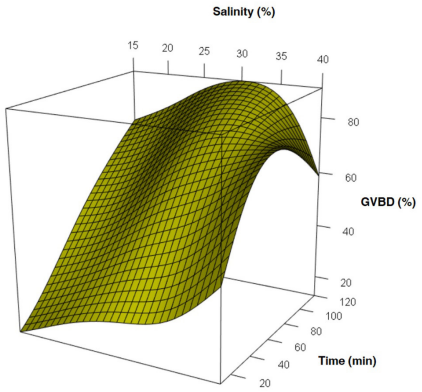

Figure 2. Mean of the percentages of germinal vesicle breakdown of oocytes obtained by stripping from *Anomalocardia flexuosa*.

Most of the treatments showed an increase in the percentage of GVBD as salinity raised, but in the 40 g·L^{-1} salinity treatment, the oocytes started to break up after 60 min to generate a significant loss until 120 min.

Effect of salinity on the extrusion of PB1 and PB2

Fertilization was conducted right after 120 min to guarantee maximum level of GVBD considering the previous section results. The extrusion of 50% PB in 20, 25, 30, and 40 g·L^{-1} treatment groups occurred nearly within the first 15 min. For the 15 g·L^{-1}, the average time was 25 min, while for 35 g·L treatment group it happened in the first 10 min (Table 2 and Figure 3).

Table 2. Percentages of polar body 1 (PB1) extrusion of *Anomalocardia flexuosa* at each 5 min interval.

Time	Time to reach 50% of PB1 release					
	15 g·L^{-1}	20 g·L^{-1}	25 g·L^{-1}	30 g·L^{-1}	35 g·L^{-1}	40 g·L^{-1}
T10 (min)	28.06±3.18 ^{c}	29.24±2.23 ^{c}	39.44±1.13 ^{b}	34.75±4.83 ^{bc}	52.22±5.05 ^{a}	40.67±7.73 ^{b}
T15 (min)	37.68±5.13 ^{c}	50.77±1.97 ^{b}	46.39±2.29 ^{bc}	48.89±3.06 ^{b}	74.57±4.61 ^{a}	52.28±8.22 ^{b}
T20 (min)	43.69±3.01 ^{d}	66.83±6.16 ^{b}	53.87±3.26 ^{c}	58.45±4.89 ^{bc}	78.2±3.96 ^{a}	63.13±4.56 ^{b}
T25 (min)	51.73±0.9 ^{e}	78.58±1.99 ^{ab}	60.83±1.37 ^{de}	63.4±3.2 ^{cd}	87.12±5.6 ^{a}	71.78±6.56 ^{bc}

The mean number of oocytes analyzed per time interval was 50. Different superscript letters indicate statistical differences ($p < 0.05$).

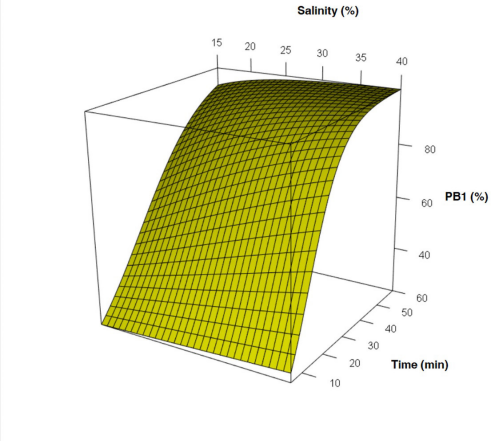

Figure 3. Mean of the percentages of polar body 1 release from fertilized eggs of *Anomalocardia flexuosa*.

For the extrusion of PB2, in treatments at 20, 25, and 30 g·L^{-1}, nearly 50% was achieved in the first 35 min, while that rate was accomplished in the first 30 min for 35 and 40 g·L^{-1} s (Table 3 and Figure 4). Yet for 15 g·L^{-1}, extrusion was accomplished later, in 45 min.

Table 3. Percentages of polar body 2 (PB2) extrusion of *Anomalocardia flexuosa* at each 5 min interval.

Time	Time to reach 50% of PB2 release					
	15 g·L^{-1}	20 g·L^{-1}	25 g·L^{-1}	30 g·L^{-1}	35 g·L^{-1}	40 g·L^{-1}
T30 (min)	21.51±5.32 ^{c}	39.28±0.65 ^{ab}	41.32±0.96 ^{ab}	35.16±4.23 ^{bc}	50.48±11.94 ^{a}	45.39±4.43 ^{ab}
T35 (min)	25.43±9.17 ^{c}	46.48±6.37 ^{ab}	44.91±2.25 ^{b}	47.26±7.31 ^{ab}	60.38±6.48 ^{a}	58.7±0.99 ^{ab}
T40 (min)	33.08±9.37 ^{c}	56.59±5.76 ^{ab}	47.44±1.29 ^{b}	55.58±6.27 ^{ab}	68.72±10.66 ^{a}	67.31±2.07 ^{a}
T45 (min)	45.58±6.23 ^{d}	62.85±6.71 ^{ab}	56.66±3.92 ^{cd}	60.17±6.42 ^{c}	78.7±9.45 ^{a}	75.07±3.92 ^{ab}

The mean number of oocytes analyzed per time interval was 50. Different superscript letters indicate statistical differences ($p < 0.05$).

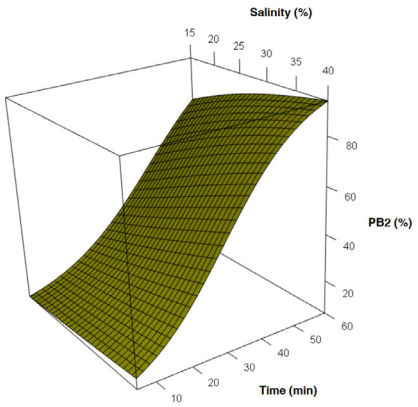

Figure 4. Mean percentage of polar body 2 release from fertilized eggs of *Anomalocardia flexuosa*.

Discussion

Effect of salinity on GVBD

Sodium-potassium antagonism is largely responsible for the oocyte germinal vesicle maintenance (Allen, 1953). The presence of Ca^{2+} ions, on the other hand, is essential for triggering vesicle degradation (Colas and Dubé, 1998). Thus, the salt concentration during hydration of oocytes obtained by stripping directly influences the GVBD (Qin et al., 2018). According to Allen and Bushek (1992), the "immersion time" in seawater is important for synchronization of oocytes that are in prophase I. Thus, GVBD is a signal that meiosis is preparing to advance to the metaphase I stage until fertilization (Eudeline et al., 2000).

The present study suggests the existence of an association between increasing salinity concentrations and GVBD rates. The obtained results suggested that there are important aspects of biochemical and biophysical characteristics of *A. flexuosa* oocytes, such as plasma membrane permeability and osmotic tolerance that interfere on GVBD rates. In hypertonic medium, the cells first react by shrinking owing to the exit of water from the cellular structure. This phenomenon is followed by cell swelling following the entry of water to maintain the osmotic balance (Salinas-Flores et al., 2008).

A. flexuosa is characterized as a species tolerant to variation in high salinities (Lagreze-Squella et al., 2018) and, therefore, can adapt its reproduction to the environmental conditions. Based on our results, however, the oocyte stability seems to be associated with a tolerance limit for high saline concentrations. The osmotic tolerance limit may be exceeded if cells undergo excessive shrinkage or swelling that may lead to irreversible injury (Salinas-Flores et al., 2008). Although the salinity of 40 $g \cdot L^{-1}$ had a higher catalytic ability for GVBD in our study, the effort to maintain the osmotic balance seemed to be critical for oocytes, leading to the rupture of the cell structure after 60 min.

Different studies have already pointed out that there are factors that can disturb embryogenesis and larval development such as gonadal maturation, oocyte maturation, and polyspermia in bivalves (Lavander et al., 2011; Qin et al., 2018). The larviculture of *A. flexuosa* is characterized as a stage strongly affected by factors such as temperature and salinity, water quality, feeding, and management (Oliveira et al., 2016). In this sense, identifying the best conditions for fertilization can be a tool to help seed production in hatchery. For *A. flexuosa*, 30 and 35 $g \cdot L^{-1}$ salinity in the time between 80 and 120 min were the optimum range of hydration with higher percentages of GVBD.

Effect of salinity on the 50% release of PB1 and PB2

The use of polyploidy has become popular in bivalve aquaculture (Ma et al., 2019), either due to gonadal sterility (Piferrer et al., 2009; Dheilly et al., 2014; Zhang et al., 2017), to the increase in the cell volume and lack of compensation of cell number (Guo and Allen, 1994), or even to the increased heterozygosity that promote larger and faster growth in bivalves (Guo et al., 2009; Yang and Guo, 2018).

There are different alternatives to obtain triploid bivalve individuals. One of them refers to the possibility of interrupting the meiotic process by inhibiting the extrusion of PB1 or PB2 (Piferrer et al., 2009). For this purpose, the determination of the moment of the exit of 50% of PB1 or PB2 after fertilization is important as a reference for the application of inducers (Guo et al., 1996; Lavander et al., 2017; Melo et al., 2015). As in GVBD, salinity can also impact post-fertilization processes such as the timing of release or inhibition of polar bodies in bivalve mollusks (Ma et al., 2019).

In the present study, we were unable to detect an explicit association between increasing salinity and the speed of events, as observed in GVBD evaluation. However, there was an optimal range for the extrusion of both polar bodies that occurred from 20 to 35 $g \cdot L^{-1}$ salinity at an interval of 10 to 20 min for PB1 and 30 to 40 min for PB2. The time to initiation of post-fertilization treatment and its duration are the main aspects influencing the success rate of polyploidy and embryo survival (Allen and Bushek, 1992; Eudeline et al., 2000).

Salinities of 15 g·L^{-1} may hold back the induction to triploidy because the longer the process time for the release of PB1 or PB2 during chromosome manipulation, the higher are the chances of anomalies occurrence (Qin et al., 2018). Although 40 g·L^{-1} concentration showed high percentages of extrusion of PB1 and PB2, the anticipated cell lysis process before fertilization made this treatment unfeasible. A previous report (Guimarães et al., 2008) showed that salinity above 40 g·L^{-1} can be lethal in the early life of estuarine bivalves.

Another study with *A. flexuosa* (Lavander et al., 2017) also observed that the highest salinity tested (35 g·L^{-1}) resulted in better extrusion rates of PB1 and PB2 as compared to 15 and 25 g·L^{-1}, corroborating the hypothesis that salinity interferes with the exit time of polar bodies. On the other hand, under the same conditions of temperature and salinity (26°C and 35 g·L^{-1}), these authors found higher extrusion rates, such as of 70% for PB1 until the first 10 min and 62.67% for PB2 after 16 min of fertilization. The discrepancy in the results may be attributed to the methodological differences of observation, prediction analysis, and mainly the methodology of obtaining gametes. Using stripping instead of natural spawning to obtain gametes configures a more controlled strategy for the various treatments applied. However, oocytes obtained by stripping will be in different stages of maturation (Downing and Allen, 1987), contrasting to those obtained by natural or induced release (Lavander et al., 2017).

Regardless of the method applied for spawning, either natural or stripping, the induction of triploidy is most suitable at 35 g·L^{-1} salinity, with the possibility of carrying post-fertilization shocks before 10 min for PB1 retention or before 30 min for PB2 retention. According to a previous study (Yang and Guo, 2018), the formation of triploid clams by retention of both PB1 and PB2 is feasible.

Unlike bivalves from temperate climate where temperature largely influences spawning (Camacho et al., 2011; O'Connor et al., 2008), those from tropical regions are more influenced by salinity in the reproductive cycle (Nowland et al., 2021) because temperature does not change much throughout the year (Paixão et al., 2013). A previous study (Lavander et al., 2011) identified that *A. flexuosa* species presented the fullest gonads during the dry period with salinity > 35 g·L^{-1} and released gametes during the rainy period with salinities between 25 and 35 g·L^{-1}. Thus, the species may present a synchronous behavior with the rainy period.

The information that spawning occurs more frequently in monsoon and at intermediate salinities (Lavander et al., 2011) along with the results of the present work that there is an optimal range for GVBD and post-fertilization processes corroborate the idea that there is a reproductive strategy of this animal that is intrinsically related to salinity.

It is also necessary to consider the influence of genetic factors of the population under analysis. Different geographic populations of the same species accumulate alleles that reflect environmental adaptability (Kim et al., 2014; Ren et al., 2016). Organisms with external fertilization can develop their gametic plasticity to adapt to environmental conditions (Lettieri et al., 2019). However, the results presented herein provide important information for the control of the reproductive process and on chromosome manipulation, suggesting that for achieving triploidy in animals maintained at 35 g·L^{-1} salinity, the application of post-fertilization shocks should be carried out before 10 min for PB1 or before 30 min for PB2 retentions, respectively.

Conclusion

We concluded that in the tropical clam (*Anomalocardia flexuosa*) salinity plays a key role on the pre- and post-fertilization stages with best ranges between 30 and 35 g·L^{-1} for GVBD and between 20 to 35 g·L^{-1} for PB1 and PB2 extrusions. These findings have implications not only for chromosome manipulation approaches aiming triploidy as well as for the reproductive management in hatcheries.

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Author contributions

RGPSL: Conceptualization, Methodology, Formal analysis, Writing – original draft; APR: Investigation, Methodology; SMJG: Investigation, Methodology; TR: Investigation, Methodology; ÍGA: Conceptualization, Methodology, Resources, Funding acquisition, Writing – review & editing; MRMC: Conceptualization, Methodology, Supervision, Writing – original draft, Writing – review & editing.

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Original Research Article

Effects of salinity on pre- and post-fertilization developmental events in the mangrove oyster *Crassostrea rhizophorae* (GUILDING, 1828)

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ABSTRACT

The mangrove oyster *Crassostrea rhizophorae* is identified as a potentially valuable species for tropical aquaculture, however, information on the physiological mechanisms of reproduction under laboratory conditions for this species is limited. This study investigated the effects of salinity at different concentrations (15, 20, 25, 30, 35, and 40 g/L) on the induction of germinal vesicle breakdown (GVBD) of oocytes obtained through stripping, the release of polar bodies (PB1 and PB2), and the larval development of the mangrove oyster. The results revealed a relationship between salinity and the percentage of GVBD, with the most effective range being 30–40 g/L within the hydration time frame between 70 and 120 min. The release of 50 % of PB1 was detected within this salinity range, while for the release of 50 % of PB2, the saline treatments of 35 and 40 g/L showed the best results. Overall, the salinity range of 30–40 g/L is suggested as the most suitable of polyploidy induction methodologies through the retention of PB1 or PB2. Regarding larval hatching, while salinities between 25 and 40 g/L presented similar percentages, at 15 g/L no hatching was observed. This study demonstrated that salinity is a key factor in early pre- and post-fertilization stages for the successful reproduction of mangrove oyster in hatcheries and that the percentages of oocyte maturation and artificial fertilization can be optimized by adjusting salinity.

1. Introduction

Marine aquaculture has experienced significant growth in recent years, driven by increasing demand for food, especially in tropical and subtropical countries [1,2]. In Brazil, although there is a diversity of native bivalve species with aquaculture potential, there is limited technical-scientific information available to develop management strategies and implement biotechnologies to support the production of these organisms [3]. The mangrove oyster, *Crassostrea rhizophorae*, is identified as a promising species for aquaculture; however, it has been practiced with limited technological sophistication [4]. The mangrove oyster exhibits external fertilization [5]. When released into the water, the oocytes are in a state of incomplete meiosis [6]. Upon fertilization, they progress through a series of embryonic stages that include the formation and expulsion of the 1st and 2nd polar bodies, the onset of cell division, the formation of the D larva, and subsequently reaching the fixation or seed stage [7,8]. The production of native oysters often begins with seed collection from the wild, which is later transferred to cultivation areas, where they are monitored and maintained until reaching commercial size [9]. The production of high-performance seeds, such as polyploid seeds, requires the control of reproductive events including oocyte maturation [6], the optimal time for gamete collection [5], artificial fertilization [10], and chromosomal manipulation [11]. Polyploidy induction is a technique that increases the number of chromosomes in an organism. In bivalve aquaculture, this can result in individuals with advantageous traits such as larger size, disease resistance, and tolerance to environmental variations [12]. Applications in polyploidy require the determination of optimal conditions in hatchery production. Induced spawning methods in the mangrove oyster are still challenging, making "stripping" the most practical method for obtaining gametes. However, this alternative provides access to immature oocytes with intact germinal vesicle structure [13]. Germinal vesicle breakdown (GVBD) is an indicator of oocyte maturation in bivalves and can be artificially stimulated by environmental factors such as salinity through a process

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known as “hydration” [14].

Tropical regions are known to exhibit minimal temperature variations throughout the year but experience significant fluctuations in salinity between dry and rainy seasons [15]. These unique environmental conditions can significantly influence the reproductive cycle of tropical oysters [16], and they may experience maturation and gamete release changes, affecting their reproduction. Studies have shown that salinity plays a crucial role in hormonal regulation and reproductive behavior of tropical oysters, making it an essential factor to consider when breeding these species [17,18]. A better understanding of the relationship between salinity and the reproductive cycle of tropical oysters can contribute to more effective conservation and management strategies for these organisms in tropical environments. Oyster oocytes can be fertilized even if they have not completed GVBD. However, when completed, the synchronization of meiosis and the release of polar bodies are greatly improved [11]. The release of the 1st and 2nd polar bodies (PB1 and PB2) is crucial in chromosomal manipulation, as triploidy induction can be promoted by inhibiting either PB1 or PB2 [11,19,20].

Therefore, the goal of this study was to determine the optimal salinity condition and hydration time for inducing GVBD and the release of polar bodies for captive breeding for future triploidy induction in the mangrove oyster.

2. Materials and methods

2.1. Preparation of oysters

The oysters used in this study were collected from the Paciência River estuary, Maranhão island, northeast coast of Brazil (Fig. 1). The breeders were transferred to the Marine Organisms Physieocology, Reproduction and Culture Laboratory of the State University of Maranhão. These specimens underwent disinfection with a 2 % sodium hypochlorite solution and were then stored in a 100 L tank with filtered treated water (50, 25, and 5 μm cartridge filters), biological filter, and ultraviolet light. They were maintained at a salinity of 35 g/L, temperature of 24 °C, and constant aeration for 4 h for acclimatation. After this period, the animals were removed from water and kept at 24 °C overnight to prevent gamete release. Approval by the Ethics Council for the Use of Experimental Animals does not apply to the invertebrate group, in accordance with the Brazilian law 11.794/08 [21].

2.2. Genetic identification

To genetically identify the collected species, muscle tissue samples were taken from the specimens used for molecular analysis. DNA extraction was carried out using the saline protocol by Ref. [22], and genetic identification was obtained using the species-specific multiplex PCR technique described by Ref. [23]. All specimens were identified as *Crassostrea rhizophorae* (Fig. 2).

2.3. Gamete collection

Gametes were obtained through stripping using a slide and subjected to observation under an optical microscope for sexual identification following the methodology of [11]. Gametes from six females were used, approximately 1,750,000 oocytes. The oocytes were filtered through 100 μm mesh sieves to separate debris and rinsed through a 25 μm mesh screens.

2.3.1. Experimental design germinal vesicle breakdown at different salinities

The effect of salinity on GVBD was evaluated at salinity treatments of 15, 20, 25, 30, 35, and 40 g/L, with three replicates, resulting in 18

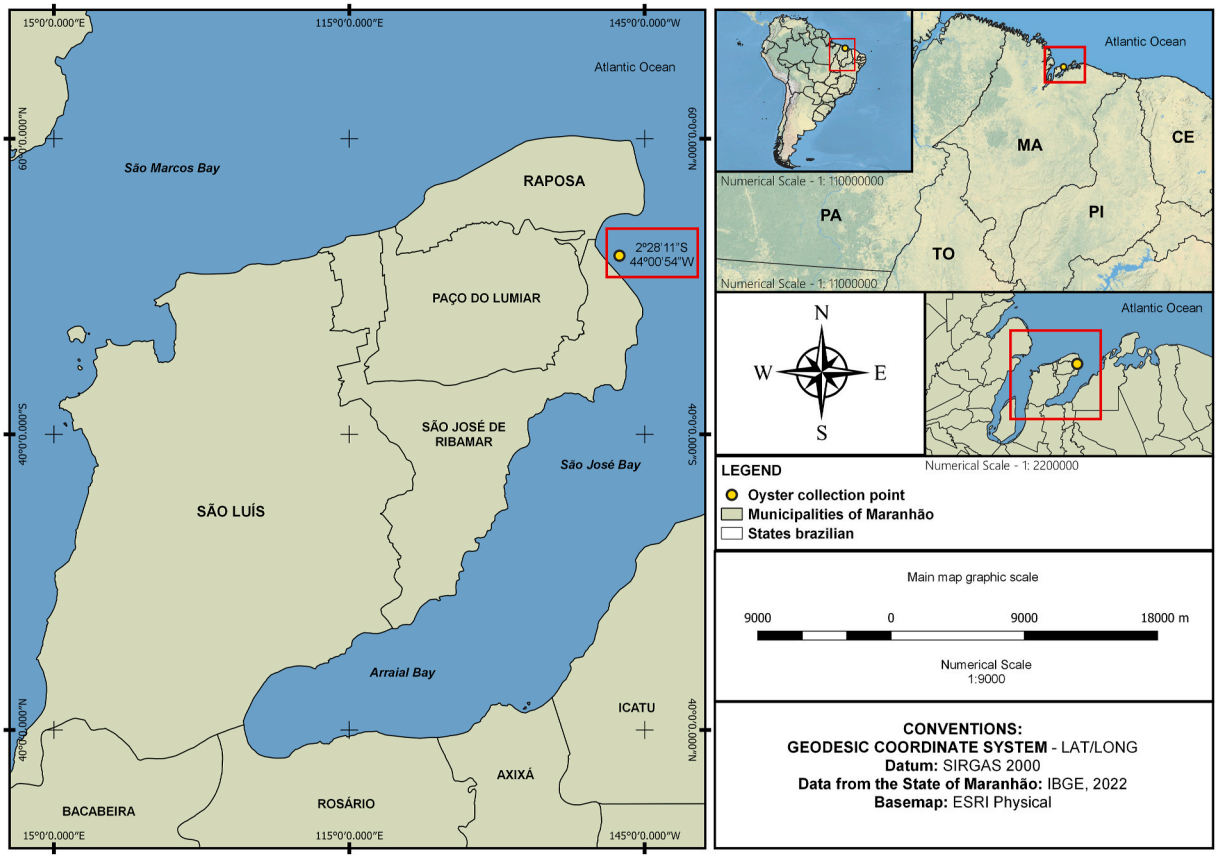

Fig. 1. Location of the recruitment point for oysters, *Crassostrea rhizophorae*, in the estuary of the river Paciência, state of Maranhão, Brazil.

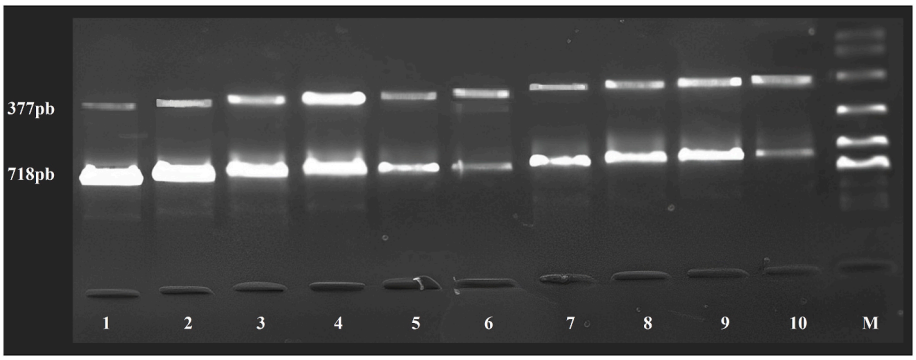

Fig. 2. *Species-specific Multiplex PCR with amplifications of two bands corresponding to the Crassostrea rhizophorae species: 377 bp from the COI region and 718 bp from the ITS 1 region* (1-10). M = DNA Marker – 1000 pb.

experimental units, maintained at 28 °C, which corresponds to the temperature of the oyster's original sampling location [5]. For each experimental unit, the total oocyte volume was distributed into 2 L beakers with an approximate final concentration of 50 oocytes/mL, from which 1 mL aliquots were taken to observe the progression of GVBD. The percentage of GVBD was calculated as follows:

$$\% \text{ GVBD} = G \times 100 / A$$

A: total number of observed oocytes; G: number of oocytes that underwent GVBD.

The observation of the germinal vesicle breakdown process took place over a 120 min period, with count records at every 10 min.

2.3.2. Release of PB1 and PB2 in different salinities and embryonic development

A total of four males were identified, and their semen were collected through stripping and mixed, filtered through a 100 μm mesh, and observed under an optical microscope for quantification. After a 2-h hydration period for GVBD at different salinities (15, 20, 25, 30, 35, and 40 g/L) and constant temperature of 28 °C, spermatozoa were added to each experimental unit at a ratio of 7 spermatozoa: 1 oocyte, following the methodology of [24]. The release rates of the 1st and 2nd polar bodies (PB1 and PB2) were monitored over a 60-min period, with observation intervals every 5 min. The average time in each treatment to achieve 50 % release of PB1 and 50 % of PB2 was determined [20,24,25].

To assess the larval formation at each treatment, gametes were transferred to 15L compartments, and embryonic development was monitored for the following 4 h after the release of the PB2, with confirmation of formed D larvae after 12 h of fertilization (Fig. 3). The fertilization rate and larval formation were defined as follows:

$$\% \text{ fertilization} = F \times 100 / A; \text{ and } \% \text{ hatchability} = L * 100 / F$$

F: number of eggs that exhibited polar body release; A: total number of observed oocytes; L: number of formed D larvae.

2.4. Statistical analysis

The data consisted of three repetitions per treatment. The average of the replicates of each treatment were used to estimate the release time of PB1 and PB2 at different proportions. Trends in the percentages of GVBD and released PB over time in each salinity condition were estimated by fitting the best-likelihood model with a Generalized Additive Model (GAM) on the data, considering a binomial distribution. A two-way analysis of variance (ANOVA) was used to measure the effects of salinity and time, and means were compared using the Tukey post hoc test at a significance level of $p \leq 0.05$.

The statistical relationship between the percentage of unfertilized oocytes, fertilized oocytes, and larval formation was evaluated.

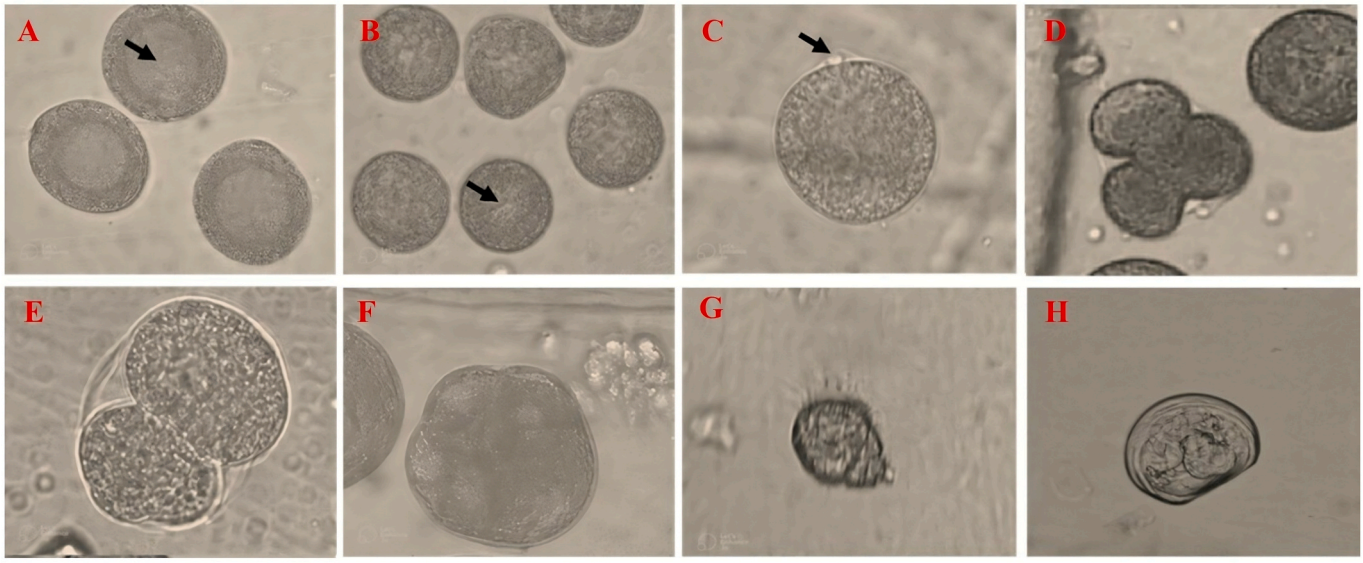

Fig. 3. Images of pre- and post-fertilization in *Crassostrea rhizophorae* A: Germinal Vesicle Identification; B: Highlight of GVBD; C: Release of PB1; D: First cleavage; E: 2 cells; F: 4 cells; G: trochophore larva; H: D-larva.

Normality and homogeneity of data were checked using the Shapiro-Wilk and Levene tests, respectively. A one-way analysis of variance (ANOVA) and the Tukey test were performed. All analyses were conducted in RStudio version 4.1.0, and the "MGCV" package [26] was used to execute the models. The model selection steps were performed as outlined in Refs. [27,28].

3. Results

3.1. Effect of salinity on germinal vesicle breakdown

The salinity of 40 g/L exhibited the best GVBD performance across all time intervals, followed by the salinity of 35 and 30 g/L. On the other hand, salinities of 25 g/L, 20 g/L and 15 g/L had gradually lower performances in terms of maturation, suggesting an increasing effect relationship between salinity and GVBD (Table 1).

The obtained data allowed delimiting a salinity and time interval where the GVBD percentages were above 80 %. The range between 30 and 40 g/L salinities in the period between 70 min and 120 min provided better oocyte maturation results (Fig. 4).

3.2. Effect of salinity on the release of 50 % of PB1 and PB2 and larval formation

Except for the 15 g/L treatment, all other salinities showed a potential release of 50 % of PB1 in the period of 60 min, following GVBD. The release time of the first polar body at 35 and 40 g/L salinity was significantly lower than for other salinities, and the reference value of 50 % was reached after 35 min (Table 2 and Fig. 5).

The trend of faster polar body release at higher salinities was also observed in the release of PB2. The treatments at 35 and 40 g/L reached 50 % release at 50 min after PB1 release, while the others seemed to reach this mark well beyond 60 min (Table 3 and Fig. 6).

Fertilization conducted at salinities ranging from 20 to 40 g/L resulted in the formation of normal D larvae. The ratio between fertilized oocytes and larval hatch indicated a similar yield within the range above 25 g/L, although the number of fertilized oocytes was considerably higher in the 40 g/L treatment. No larval formation was observed in the 15 g/L treatment (Fig. 7).

Table 1
Germinal vesicle breakdown of the mangrove oyster *Crassostrea rhizophorae* oocytes under salinity influence.

Time (min)	GVBD percentages in the range of 10–120 min					
	15 g/L	20 g/L	25 g/L	30 g/L	35 g/L	40 g/L
10	0 ± 0^{c}	0.6 ± 1.03 ^{bc}	0 ± 0^{c}	1.75 ± 3.04 ^{bc}	3.85 ± 0.15 ^{ab}	7.11 ± 1.1^{a}
20	0.6 ± 1.03^{d}	2.81 ± 1.11^{d}	8.1 ± 0.85^{c}	7.67 ± 0.57^{c}	14.63 ± 1.42^{b}	25.2 ± 0.73^{a}
30	4.45 ± 0.79^{d}	6.69 ± 2.02^{d}	12 ± 1^{c}	15.35 ± 1.13 ^{bc}	16.73 ± 1.32 ^{bc}	38.4 ± 1.39^{a}
40	6.38 ± 0.66^{c}	9.98 ± 0.74^{c}	19.57 ± 1.91^{b}	19.6 ± 3.42^{b}	18.17 ± 0.3^{b}	52.08 ± 0.34^{a}
50	10.24 ± 0.41^{e}	14.89 ± 0.55^{d}	27.92 ± 0.97^{c}	31.78 ± 1.68^{b}	26.28 ± 0.55^{c}	65.41 ± 2.25^{a}
60	14.1 ± 0.17^{e}	19.83 ± 1.26^{d}	31.2 ± 3.77^{c}	36.36 ± 3.2^{b}	38.45 ± 0.45^{b}	73.07 ± 1.42^{a}
70	16.02 ± 0.04^{f}	29.17 ± 1.04^{e}	35.72 ± 0.99^{d}	40.87 ± 2.8^{c}	49.31 ± 2.2^{b}	82.42 ± 3.88^{a}
80	21.14 ± 1.03^{e}	34.2 ± 0.85^{d}	44.79 ± 1.18^{c}	58.55 ± 0.95^{b}	59.6 ± 1.74^{b}	91.77 ± 4.25^{a}
90	25 ± 1^{e}	40.73 ± 1.1^{d}	56.9 ± 0.85^{c}	72.06 ± 1.91^{b}	71.1 ± 2.6^{b}	95.88 ± 2.39^{a}
100	30.12 ± 0.21^{e}	49.17 ± 0.76^{d}	67.54 ± 0.4^{c}	78.32 ± 0.55^{b}	78.8 ± 2.38^{b}	97.64 ± 0.81^{a}
110	35.9 ± 0.17^{e}	55.2 ± 2.46^{d}	74.31 ± 1.13^{c}	83.03 ± 2.68^{b}	85.89 ± 1.21^{b}	98.79 ± 1.07^{a}
120	44.21 ± 0.37^{f}	61.03 ± 1.7^{e}	78.17 ± 0.76^{d}	87 ± 2^{c}	93.58 ± 1.12^{b}	100 ± 0^{a}

A sample of 50 oocytes was used to calculate the percentages. Different superscript letters indicate statistical differences ($p < 0.05$) at each time interval.

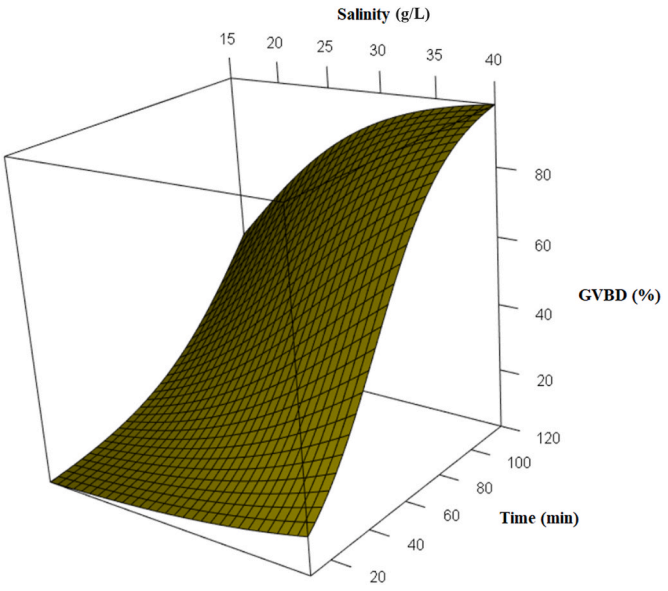

Fig. 4. Mean percentage of GVBD of oocytes obtained by stripping *Crassostrea rhizophorae*.

Table 2
Percentages of polar body 1 (PB1) release of the mangrove oyster *Crassostrea rhizophorae* at each 5 min interval after GVBD.

Time (min)	Time to reach 50 % PB1 release					
	15 g/L	20 g/L	25 g/L	30 g/L	35 g/L	40 g/L
5	3.86 ± 3.71	4 ± 3.46	3.31 ± 1.13	3.43 ± 3.45	3.97 ± 3.49	8.16 ± 0.27
10	5.77 ± 3.76^{b}	8.67 ± 3.06 ^{ab}	6.63 ± 1.19 ^{ab}	8.37 ± 1.81 ^{ab}	9.21 ± 1.06 ^{ab}	13.65 ± 2.86^{a}
15	10.87 ± 3.24	14 ± 4	13.91 ± 2.01	14.46 ± 2.26	14.46 ± 2.15	17.55 ± 1.38
20	13.35 ± 1.09^{b}	18 ± 4^{b}	17.88 ± 0.2^{b}	16.82 ± 1.24^{b}	26.31 ± 2.07^{a}	27.6 ± 1.45^{a}
25	14.64 ± 1.88^{d}	25.33 ± 4.16 ^{bc}	21.84 ± 1.77 ^{cd}	18.53 ± 1.62 ^{cd}	31.59 ± 2.12 ^{ab}	33.2 ± 2.07^{a}
30	18.38 ± 0.46^{c}	30.67 ± 5.03^{b}	28.48 ± 1.34^{b}	32.16 ± 6.51^{b}	42.13 ± 1.81^{a}	43.36 ± 1.18^{a}
35	21.57 ± 1.28^{e}	36.67 ± 7.02 ^{cd}	34.44 ± 3.15^{d}	43.07 ± 1.54 ^{bc}	51.33 ± 2.31^{a}	50.28 ± 0.49 ^{ab}
40	24.75 ± 3.79^{d}	40 ± 2^{c}	40.41 ± 5.1^{c}	50.26 ± 3.85^{b}	54.62 ± 3.07 ^{ab}	59.11 ± 1.02^{a}
45	26.11 ± 6.71^{d}	41.33 ± 1.15^{c}	47.69 ± 6.02^{c}	58.07 ± 1.21^{b}	60.49 ± 3.95 ^{ab}	65.93 ± 3.41^{a}
50	31.76 ± 5.1^{d}	44.67 ± 1.15^{c}	51.67 ± 3.78^{c}	63.96 ± 2.06^{b}	68.36 ± 4.09 ^{ab}	74.66 ± 3.04^{a}
55	36.29 ± 5.12^{e}	47.33 ± 2.31^{d}	56.95 ± 3^{c}	68.82 ± 0.76^{b}	72.97 ± 4.55^{b}	82.25 ± 2.38^{a}
60	40.54 ± 1.46^{e}	50 ± 2^{d}	58.94 ± 3^{c}	72.89 ± 3.16^{b}	75.62 ± 4.88^{b}	89.94 ± 0.1^{a}

A sample of 50 oocytes was used to calculate the percentages. Different superscript letters indicate statistical differences ($p < 0.05$) at each time interval.

4. Discussion

4.1. Effect of salinity on germinal vesicle breakdown

Euryhaline bivalve organisms are characterized as osmoconformers capable of regulating their extracellular and intracellular hemolymph fluids with changes in the environment, such as the natural salinity fluctuations in estuarine environments [29]. This ability has been made

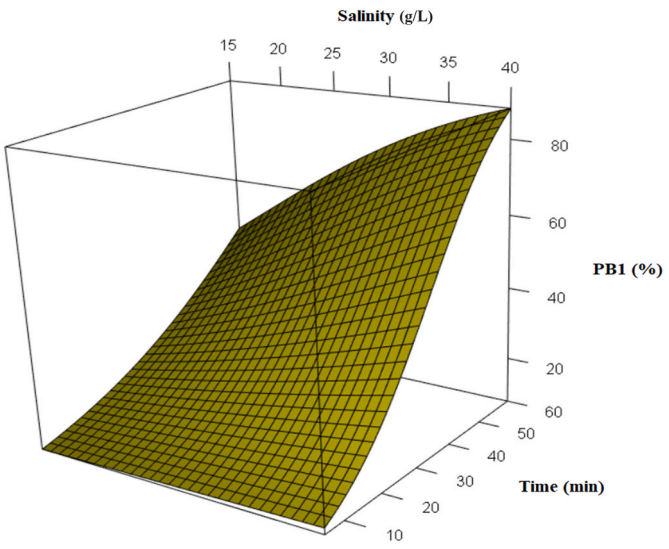

Fig. 5. Mean percentages of PB1 release from fertilized *Crassostrea rhizophorae* eggs.

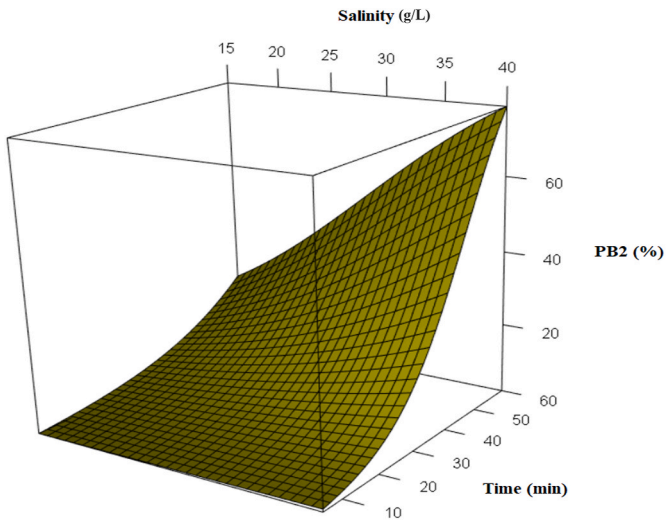

Fig. 6. Average percentages of PB2 release from fertilized *Crassostrea rhizophorae* eggs.

Table 3
Percentages of polar body 2 (PB2) release of the mangrove oyster *Crassostrea rhizophorae* at each 5 min interval after PB1 release.

Time (min)	Time to reach 50 % PB2 release					
	15 g/L	20 g/L	25 g/L	30 g/L	35 g/L	40 g/L
5	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
10	0.69 ± 1.2	0.67 ± 1.15	0 ± 0	0 ± 0	0 ± 0	2.56 ± 1.25
15	0.69 ± 1.2	2.67 ± 1.15	0.65 ± 1.13	1.15 ± 1.99	2 ± 3.46	5.59 ± 3.17
20	1.91 ± 0.15^{b}	6.67 ± 1.15^{b}	3.29 ± 2.24^{b}	4.08 ± 1.8 ^{ab}	7.33 ± 2.31 ^{ab}	10.16 ± 7.15^{a}
25	2.6 ± 1.36^{d}	8.67 ± 1.15 ^{abc}	5.96 ± 0.07 ^{cd}	7.12 ± 1.25 ^{bcd}	12 ± 0 ^{ab}	13.95 ± 7.23^{a}
30	3.2 ± 1.2^{c}	11.33 ± 1.15^{b}	8.61 ± 3.07 ^{bc}	10.62 ± 4.02^{b}	14 ± 2^{b}	20.68 ± 1.15^{a}
35	3.79 ± 1.79^{d}	16.67 ± 3.06 ^{bc}	11.91 ± 3.39^{c}	18.34 ± 3.77^{b}	18.67 ± 1.15^{b}	27.04 ± 3^{a}
40	5.68 ± 3.57^{e}	20 ± 4^{c}	13.9 ± 1.85^{d}	23.09 ± 4.42 ^{bc}	27.33 ± 3.06 ^{ab}	32.63 ± 2.28^{a}
45	7.58 ± 3.56^{e}	22.67 ± 2.31^{d}	19.87 ± 0.23^{d}	28.61 ± 2.34^{c}	38.67 ± 2.31^{b}	44.59 ± 2.24^{a}
50	8.8 ± 3.49^{d}	28 ± 2 ^{bc}	25.18 ± 1.43^{c}	33.46 ± 1.3^{b}	50 ± 2^{a}	54.75 ± 1.09^{a}
55	12.63 ± 1.59^{e}	32 ± 3.46^{d}	29.79 ± 1.69^{d}	40.68 ± 1.48^{c}	60 ± 2^{b}	67.7 ± 0.36^{a}
60	14.54 ± 1.56^{e}	33.33 ± 1.15^{d}	33.78 ± 2.04^{d}	45.43 ± 1.81^{c}	65.67 ± 0.58^{b}	80.45 ± 2.15^{a}

A sample of 50 oocytes was used to calculate the percentages. Different superscript letters indicate statistical differences ($p < 0.05$) at each time interval.

possible by developing sophisticated adaptive mechanisms to cope with salinity fluctuations [30]. However, metabolic changes in the osmotic equilibrium process can interfere with physiological aspects [30,31].

This study demonstrated a relationship between increased salinity concentrations and GVBD rates. Oocyte hydration was most efficient within the salinity range of 30–40 g/L, within a time frame of 70–120 min. In a specific study on the correlation between salinity and GVBD, [32] demonstrated higher GVBD percentages at salinities between 24 and 32 g/L and significantly lower ratios at salinities ≤20 g/L for the species *C. gigas* and *C. ariakensis*. However, [11] showed that GVBD induction for *C. hongkongensis* was more efficient at low salinities (15 g/L). This variation in salinity effects reinforces the importance of determining how the gradual increase would impact GVBD rates under laboratory conditions to ensure higher percentages.

Although studies on the artificial fertilization of tropical oyster species in the laboratory have incorporated the oocyte hydration step into their methodologies [33,34], these protocols do not rely on species-specific information. Tropical regions are characterized by minimal temperature variation throughout the year but high salinity variations [15]. Oysters from the Paciência River estuary region, where the specimens for this study were collected, are exposed to high salinities, with annual variations ranging from 31 g/L (rainy season) to 42 g/L (dry season), and constant temperatures, with no significant difference throughout the seasons, at 28–29 °C [5,35]. The higher GVBD percentages obtained in this experiment suggest that the best conditions for oocyte maturation were those with salinities close to the local of origin. [18] have already pointed out that environmental salinity has a selective and significant effect on reproductive adaptations in the mangrove oyster. Therefore, hatchery production for this species should consider the salinity of origin of the broodstock used for reproduction.

4.2. *Effect of salinity on the release of 50 % of PB1 and PB2 and larval formation*

High or low salinity levels can cause osmotic stress and interfere with the normal functioning of cells and tissues in the mangrove oyster [18]. In particular, changes in salinity can affect the synchronization of meiotic division, including the formation and release of polar bodies in oysters and other bivalve mollusks [11,32]. In our results, the release of 50 % of PB1 in the mangrove oyster eggs was faster within the salinity range of 30–40 g/L, where the highest percentage of GVBD also occurred, highlighting the importance of meiotic synchronization in stripped oocytes for post-fertilization processes.

Control over the polar body release is crucial in chromosomal manipulation methodologies. Identifying the conditions, in terms of salinity factor, under which 50 % of PB1 is released after fertilization in a faster and more uniform manner is crucial to ensure a higher percentage of triploids [11,24,36]. In these processes, the longer the time for PB1 release, the higher the chances of anomalies to occur [11]. Although the treatments at 20 and 25 g/L allowed for the release of 50 % of PB1, the time was significantly longer than that of the other treatments, occurring between 50 and 60 min after fertilization. The release of the second polar body can provide information about the duration of immersion time in chemical or physical induction treatments [20]. We observed that only salinities of 35 and 40 g/L released 50 % of PB2 during the observed period. Based on these results, it is recommended that for the mangrove oyster hydration must be conducted within the

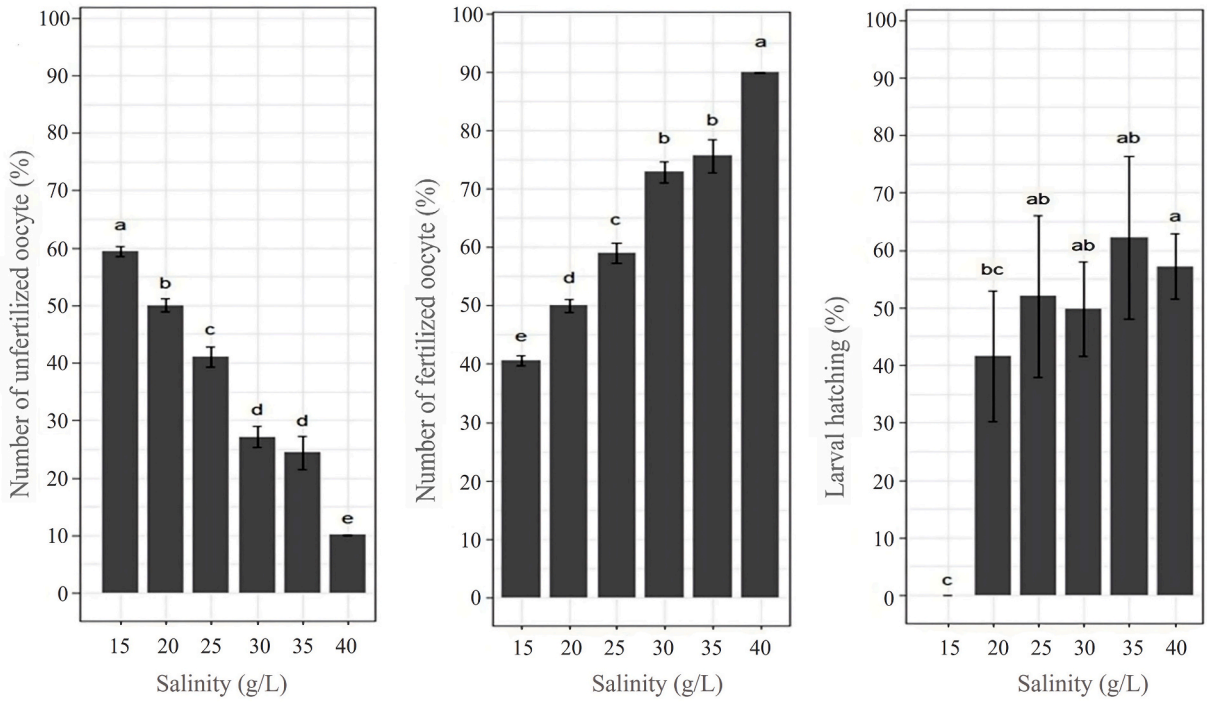

Fig. 7. Results of the *Crassostrea rhizophorae* fertilization experiment at different salinities. % of unfertilized oocytes (a) (mean ± SD, n = 3 replicates), % of fertilized oocytes (b) (mean ± SD, n = 3 replicates), and % of formed larvae (mean ± SD, n = 3 replicates) (c) of each saline treatment. Different subscript letters indicate a significant difference ($p < 0.05$) between salinities.

interval of 70–100 min in the salinity range of 35–40 g/L and that triploidy induction shocks should be conducted between 15 and 25 min to retain 50 %–80 % of PB2) in this salinity range.

Salinity is a determining factor throughout the embryonic and larval development stages [30]. We found that the higher the salinity, the higher the number of fertilized oocytes, however, for larval hatching, similar percentages were detected for either moderate or high salinities (25–40 g/L). This suggests that higher salinities are crucial for the early pre- and post-fertilization stages, but it is not at the later stage of larval hatching. Several studies under laboratory conditions of the mangrove oyster provide different optimal salt concentrations, which can be 20–25 g/L [37], 28–30 g/L [38], 25–30 g/L [39], or 25–37 g/L [33]. These differences suggest that there may be other related factors, such as temperature, pH, broodstock origin, or even correct species identification, given that until recently, the taxonomy of the *C. rhizophorae* and *C. gasar* species was problematic [40].

According to Ref. [41], salinity and seawater temperature are the main factors controlling the life cycle, distribution, and physiology of aquatic organisms. However, studies have reached different conclusions regarding the salinity-temperature relationship for the larval development of bivalves. Some authors consider that both factors need to be combined [17,42,43], others highlight the greater importance of temperature [44–46], and others emphasize salinity [47,48–50]. In this research, we evaluated the effect of salinity on fertilization and larval development of the mangrove oyster under laboratory conditions in a tropical area. Furthermore, the effect of sperm motility variations under different environmental conditions should be considered. Vigor and duration of motility are regulated by the concentration of ions such as K+, Ca2+, Na+, and pH in the water [51], which, combined with oocyte maturation conditions, can interfere with the fertilization process and the timing of polar body release.

5. Conclusions

In conclusion, based on both current and previous results for the mangrove oyster, salinity is a key factor for successful hatchery reproduction, and the percentages of oocyte maturation and artificial fertilization can be optimized by adjusting salinity. However, further research focusing on laboratory production, should evaluate other aspects in artificial fertilization of native oysters, such as the effect of water pH, ion concentration, the combination of salinity-temperature effects, sperm motility, and assessment of genetic and enzymatic markers, particularly in the mangrove oyster.

CRediT authorship contribution statement

Rodolf Gabriel Prazeres Silva Lopes: Writing – review & editing, Writing – original draft, Methodology, Data curation, Conceptualization. **Ana Paula Rego:** Methodology. **Sabrina Melo de Jesus Gomes:** Methodology. **Ícaro G. Antonio:** Writing – original draft, Methodology, Conceptualization. **Thaís Brito Freire:** Methodology, Formal analysis, Data curation. **Maria Raquel Moura Coimbra:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare no conflict of interest.

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