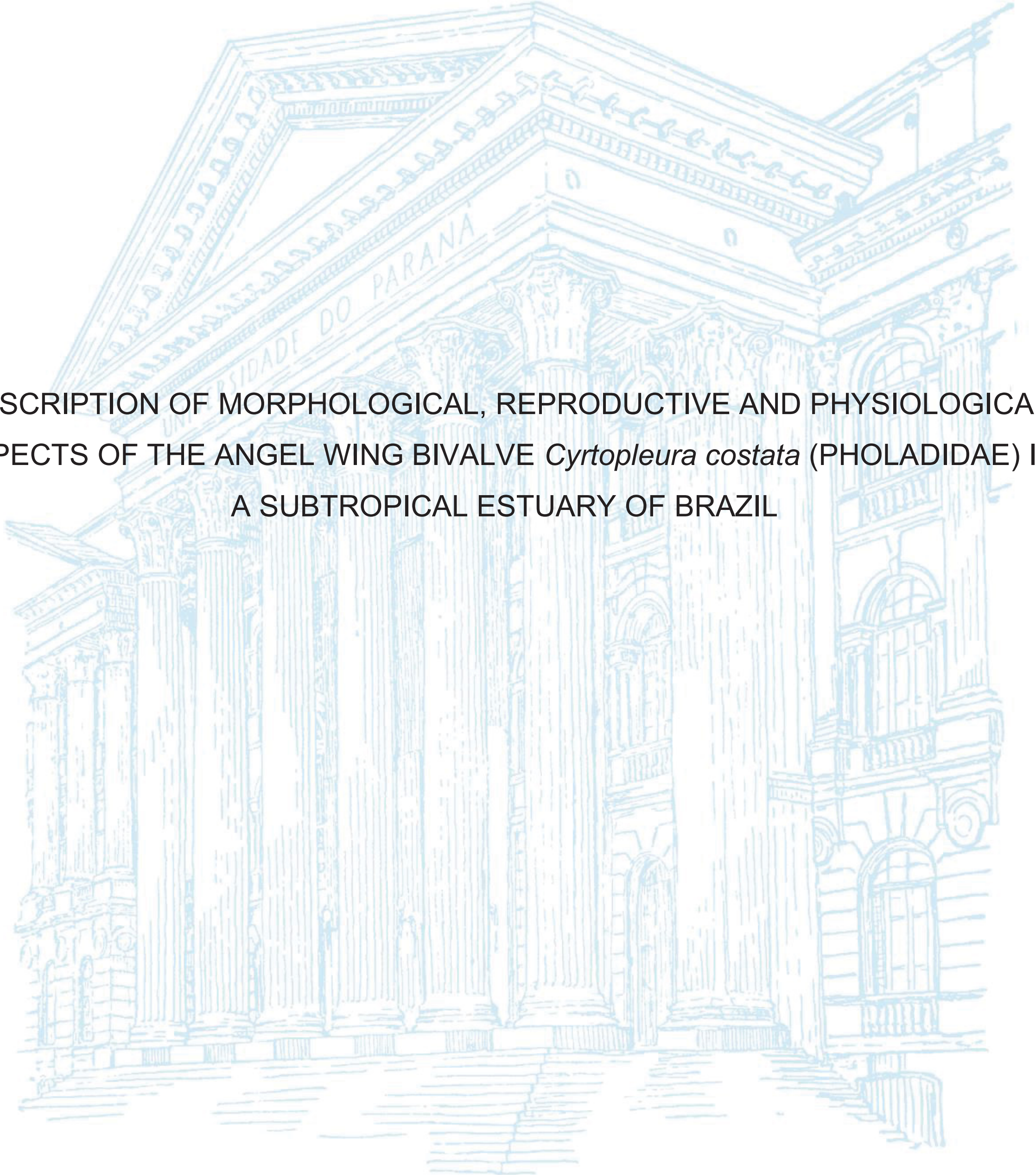
UNIVERSIDADE FEDERAL DO PARANÁ

NICOLE STAKOWIAN



DESCRIPTION OF MORPHOLOGICAL, REPRODUCTIVE AND PHYSIOLOGICAL ASPECTS OF THE ANGEL WING BIVALVE *Cyrtopleura costata* (PHOLADIDAE) IN A SUBTROPICAL ESTUARY OF BRAZIL

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

Orientadora: Profª Drª Carolina Arruda de Oliveira Freire
Co-orientador: Prof. Dr. Luiz Ricardo Lopes Simone

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TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em ZOOLOGIA da Universidade Federal do Paraná foram convocados para realizar a arguição da dissertação de Mestrado de **NICOLE STAKOWIAN** intitulada: **Description of morphological, reproductive and physiological aspects of the angel wing bivalve *Cyrtopleura costata* (Pholadidae) in a subtropical estuary of Brazil**, sob orientação da Profa. Dra. CAROLINA ARRUDA DE OLIVEIRA FREIRE, que após terem inquirido a aluna e realizada a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.

A outorga do título de mestre está sujeita à homologação pelo colegiado, ao atendimento de todas as indicações e correções solicitadas pela banca e ao pleno atendimento das demandas regimentais do Programa de Pós-Graduação.

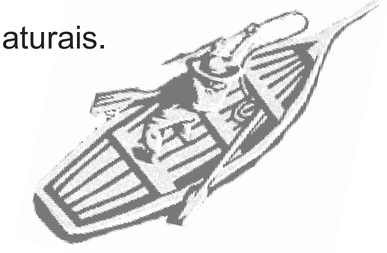
CURITIBA, 20 de Fevereiro de 2020.

CAROLINA ARRUDA DE OLIVEIRA FREIRE
Presidente da Banca Examinadora (UNIVERSIDADE FEDERAL DO PARANÁ)

YARA APARECIDA GARCIA TAVARES
Avaliador Externo (UNIVERSIDADE ESTADUAL DO PARANÁ)

MARTA MARQUES DE SOUZA
Avaliador Externo (UNIVERSIDADE FEDERAL DO RIO GRANDE)

Ao Romildo do Rosário, à Comunidade de São Miguel e a todos os pescadores e pescadoras de comunidades tradicionais que contribuem para o avanço da ciência e para a conservação dos recursos naturais.



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“*Tenho a impressão de ter sido uma criança brincando à beira-mar, divertindo-me em descobrir uma pedrinha mais lisa ou uma concha mais bonita que as outras [...]*”

Isaac Newton

RESUMO

Cyrtopleura costata, também conhecido como asa-de-anjo, ocorre em regiões estuarinas em tocas que podem alcançar até 40 cm de profundidade. A espécie vem sendo utilizada como recurso aquícola e isca de pesca por comunidades tradicionais brasileiras. Apesar disso, nenhum cultivo do asa-de-anjo ocorre no país e o seu uso é suprido por estoques naturais. Diante da importância ecológica e econômica potencial da espécie, o presente estudo buscou descrever a anatomia detalhada de *C. costata*, caracterizar o ciclo gametogênico e o período de maior investimento reprodutivo ao longo do ano e os processos fisiológicos envolvidos na sua osmorregulação. Nossos resultados apresentam as principais características morfológicas de *C. costata*, tais como formato das valvas e topologia dos órgãos internos. A população apresentou proporcionalidade entre os sexos, com uma tendência à prevalência de fêmeas nas classes de maiores tamanhos. A reprodução é contínua ao longo de todo o ano, embora entre o outono e o inverno tenha sido o período de maior investimento reprodutivo. A espécie demonstrou melhor desempenho entre as salinidades 25 – 40 ‰, sendo mais tolerante a estresse hiper- do que hipossalino em relação à água do mar normal. Em condições hiper-salinas, *C. costata* apresenta uma maior capacidade de regular a hidratação tecidual do que outros bivalves capazes de fechar completamente as valvas. Este estudo é inédito em sua abordagem e busca contribuir para o registro da biodiversidade e conhecimento da fisiologia de bivalves nativos da costa brasileira. Em suma, oferece dados sobre a anatomia, ecologia e fisiologia reprodutiva e osmorregulatória de um representante de Pholadidae, aspectos essenciais para avaliação de impactos de mudanças climáticas, ações antrópicas e potencial de exploração econômica sustentável do recurso.

Palavras-chave: Bivalvia. Ecofisiologia. Osmorregulação. Reprodução.

ABSTRACT

Cyrtopleura costata, also known as angel-wing, occurs in estuarine regions in burrows that can reach up to 40 cm in depth. The species has been used as an aquaculture resource and fishing bait by traditional Brazilian communities. Despite this, no cultivation takes place in the country and its use is supplied by natural stocks. Given the potential ecological and economic importance of the species, the present study sought to describe the detailed anatomy of *C. costata*, characterize the gametogenic cycle and the period of greatest reproductive investment throughout the year and the physiological processes involved in its osmoregulation. Our results present the main morphological characteristics of *C. costata*, such as the shape of the valves and the topology of the internal organs. The population showed proportionality between the sexes, with a tendency for the prevalence of females in the larger size classes. Reproduction is continuous throughout the year, although between the autumn and winter has been the period of greatest reproductive investment. The species demonstrated a better performance between salinities 25 - 40 ‰, being more tolerant to hyper than hyposaline-stress in relation to normal sea water. In hyper-saline conditions, *C. costata* has a greater capacity to regulate tissue hydration than other bivalves capable of completely closing the valves. This study is unprecedented in its approach and seeks to contribute to the registration of biodiversity and knowledge of the physiology of bivalves native to the Brazilian coast. In short, it offers data on the anatomy, ecology and reproductive and osmoregulatory physiology of Pholadidae species, essential aspects for assessing the impacts of climate change, human actions and the potential for sustainable economic exploitation of resource.

Keywords: Bivalvia. Ecophysiology. Osmoregulation. Reproduction.

RESUMO ESTENDIDO

Os moluscos são a segunda maior classe do filo Mollusca e constituem um grupo ideal para responder questões acerca de adaptações morfológicas, tolerâncias fisiológicas e estratégias reprodutivas, dada a ampla distribuição do grupo em diferentes ecossistemas (BIELER, 2006; BIELER; MIKKELSEN; GIRIBET, 2013; POURMOZAFFAR *et al.*, 2019). Os bivalves marinhos e estuarinos, em especial, são considerados fonte de subsistência para comunidades tradicionais de regiões costeiras de praticamente todo o mundo devido ao baixo custo de produção, alta rentabilidade e por serem fonte de proteínas e nutrientes. Isso faz com que este seja o principal grupo utilizado no cultivo de moluscos (BIN RAMLI; YUSOP, 2016; CAMILO *et al.*, 2018).

Bivalves da família Pholadidae apresentam um conjunto de características singulares que os permitem escavar diferentes tipos de substrato (lama, argila, madeira, rocha e calcáreo), tais como valvas acessórias, presença de apófise, redução ou perda do ligamento da charneira e músculo adutor anterior localizado externa e dorsalmente (ANSELL; NAIR, 1969; ITO, 2005; JEON *et al.*, 2012; MONARI, 2009). Foladídeos são consumidos pelas comunidades humanas desde os primeiros romanos e são comercializados em diversos lugares do mundo, tais como Hong Kong, Malásia, Tailândia e Filipinas (GUSTAFSON *et al.*, 1991; RONQUILLO; MCKINLEY, 2006; TURNER, 1954).

Entre as espécies de Pholadidae com interesse econômico está *Cyrtopleura costata*, também conhecido como “asa-de-anjo”, habitante infaunal de planícies lodo-arenosas do entre-marés (GUSTAFSON *et al.*, 1991; TURNER, 1954). A espécie ocorre desde o leste do Canadá até o sul do Brasil (CULLAIN *et al.*, 2018; RIOS, 2009). Suas tocas atingem cerca de 40 cm de profundidade, apresentam formato cônico com uma abertura estreita e uma câmara posterior que pode fornecer uma variedade de micro-hábitats e aumentar a biodiversidade local (PINN *et al.*, 2005; TURNER, 1954). *Cyrtopleura costata* já foi comercializado em Porto Rico e no Caribe, assim como em Cuba, onde já foi considerado alimento básico (GUSTAFSON *et al.*, 1991; TURNER, 1954). No Brasil, a espécie é consumida por comunidades tradicionais (TEIXEIRA; CAMPOS, 2019) e utilizada como iscas para a pesca de Miraguaia (*Pogonia cromis*, Sciaenidae) (RIBAS, 2014). Na costa brasileira nenhum cultivo dessa espécie foi registrado, sendo o consumo suprido pelos estoques naturais. Pesquisas em fisiologia de espécies nativas costeiras permite considerar a possibilidade de cultivá-las para gerar recursos e preservar os estoques naturais, e a biodiversidade.

Estudos de biologia básica, caracterizando os aspectos morfológicos, reprodutivos e fisiológicos permitem caracterizar os principais períodos do ciclo gametogênico, a estratégia reprodutiva da espécie e as condições favoráveis para o sucesso de suas populações naturais e de cultivos. Na ausência de dados que sustentem seu potencial para a aquicultura, torna-se impraticável a exploração do recurso. Diante disso, o objetivo do **Capítulo 1** foi descrever a anatomia detalhada (interna e externa) da espécie, o **Capítulo 2** buscou caracterizar o ciclo gametogênico de uma população natural e o **Capítulo 3** a tolerância e os processos fisiológicos envolvidos na osmorregulação do grupo, comparando com os mecanismos conhecidos para Bivalvia. Este estudo constitui o primeiro relato sobre a morfologia, a reprodução e a fisiologia osmorregulatória de *C. costata*.

Para isso, foram feitas coletas na Baía de Paranaguá (PR) (25º43’53,17”S / 48º44’63,05”W) entre Novembro de 2018 e Outubro de 2019. Os bivalves foram coletados sempre no período de maré baixa, observando o local de saída dos sifões ou pela presença de fezes em forma de moedas, característica da espécie. Dados de temperatura da água (termômetro portátil) e salinidade (refratômetro óptico) foram coletados *in situ*. Para os **Capítulos 1** e **2**, os bivalves foram relaxados em solução mentol por 30 minutos, fixados em formalina 10% e conservados em álcool 70%. Para o **Capítulo 3**, os animais foram envolvidos em espumas próprias para aquário (para evitar atrito e quebra das valvas) e transportados para o laboratório em galões contendo água do mar com aeração constante.

No **Capítulo 1**, os espécimes foram dissecados imersos em álcool e com o auxílio de um estereomicroscópio. Desenhos anatômicos foram produzidos com o uso de uma câmara clara. Secções transversais do palpo labial foram submetidas a análise histológica de rotina.

No **Capítulo 2**, após a fixação, todos os bivalves tiveram suas conchas mensuradas quanto ao comprimento, largura e altura, para relação do tamanho com a maturidade sexual. O peso úmido da carne foi quantificado para avaliação do Índice de Condição (peso úmido da carne / comprimento da concha x 100). Uma secção da massa visceral (corte transversal na base do processo pedioso) foi submetida a análise histológica de rotina. Análises biométricas foram realizadas para ambos os sexos. Para cada fêmea a área total de trinta ovócitos (apenas os com região nuclear evidente) aleatórios foi mensurada. Para machos, foram mensurados 10 folículos por indivíduo quanto sua área total, área ocupada por espermatozoides maduros e pela série radial de células iniciais da espermatogênese (SR).

Os experimentos do **Capítulo 3** foram divididos em duas partes:

In vivo: Os animais foram aclimatados por cerca de 5 dias a salinidade de 30‰, temperatura ambiente (20ºC ± 2), fotoperíodo natural e aeração constante. Os experimentos foram conduzidos com três indivíduos por tratamento, com um bivalve por recipiente com capacidade para 2L. Foi garantido que o animal permanecesse submerso durante todo o experimento e cada indivíduo foi testado apenas uma vez. Os bivalves foram submetidos a um aumento (35 - 50 ‰) ou diminuição (25 - 10 ‰) gradual (a cada 5 ‰) de salinidade a partir do controle (30 ‰). A cada uma hora toda a água do recipiente era trocada e amostras de água do tratamento, da água da cavidade do manto (expulsa pelo sifão) e da hemolinfa (punção pericárdica) eram coletadas para dosagem de osmolalidade e íons Na^+ e Cl^-. O experimento teve duração total de quatro horas e ao final (salinidades 10, 30 e 50 ‰) uma fração do músculo adutor anterior, do manto e do sifão de cada indivíduo foi coletada para avaliação do teor hídrico tecidual (TH).

In vitro: A aclimatação teve duração de cerca de dois dias sob as mesmas condições do experimento *in vivo*. Cada indivíduo foi crio-anestesiado por cerca de 20 minutos (envolto por gelo picado, sólido) e foram retiradas amostras triplicatas do músculo adutor anterior, do manto e do sifão. Imediatamente após a coleta, os tecidos foram acomodados em solução salina controle (30 ‰) por cerca de 15 minutos. Em seguida, cada tecido em um recipiente individual foi submetido à solução salina 10, 30 ou 50 ‰ por um tempo total de 120 minutos. Os tecidos foram pesados individualmente a cada 15 minutos em balança analítica (Bioprecisa® FA2104N, precisão 0,0001g) durante 1 hora e o peso final foi obtido no tempo 120'. Ao final do experimento, o mesmo procedimento para obtenção do teor hídrico tecidual (*in vivo*) foi aplicado.

No **Capítulo I** são apresentadas as principais características morfológicas de *C. costata*, tais como valvas ovais e alongadas com coloração bege, margem anterior apresentando o músculo adutor anterior unindo as valvas na região pré-umbonal. Duas valvas acessórias estão presentes: o mesoplax (calcificado) localizado na região umbonal, abaixo do adutor anterior e o protoplax (de aspecto córneo) cobrindo o músculo adutor anterior. A apófise é bem desenvolvida e oferece suporte aos palpos labiais e músculos do pé. Na ausência de um ligamento da charneira, o músculo adutor anterior (localizado dorsalmente) assume essa função. Além dos músculos adutores principais, há um músculo adutor auxiliar que se estende da base do sifão até a abertura pediosa. Os sifões são completamente unidos, sendo o inalante maior do que o exalante, com a margem bipregueada, tentáculos maiores dispostos internamente e os menores externamente. Apesar da ampla abertura pediosa, o pé é pequeno e sua inserção na massa visceral é superficial. O ventrículo é bem desenvolvido. O rim marrom/avermelhado se estende lateralmente na superfície dorsal. O saco do estilete é bem desenvolvido e destacado do estômago. O intestino apresenta inúmeras alças e curvas adentro da massa visceral. As fezes apresentam formato de moedas. As gônadas estão presentes por toda porção dorso-lateral da massa visceral. O gonóporo está localizado próximo ao ânus, na base da saída exalante.

De acordo com os resultados obtidos no **Capítulo II**, a população de *C. costata* estudada apresenta proporcionalidade entre os sexos, com uma tendência à prevalência de fêmeas nas maiores classes de tamanho. Isso pode ser positiva para a população, já que uma maior quantidade de ovócitos será produzida e poderá ser fertilizada. A espécie mostrou atividade reprodutiva ao longo de todo o ano, provavelmente devido à baixa amplitude de variação dos fatores abióticos em regiões subtropicais. Apesar disso, entre o outono e o inverno foi o período de maior atividade reprodutiva, em que ocorre o maior investimento na qualidade dos ovócitos, os maiores indivíduos são registrados e o índice de condição atinge seu máximo.

Quantos a seus aspectos fisiológicos, abordados no **Capítulo III**, a espécie demonstrou melhor desempenho entre as salinidades 25 – 40 ‰, sendo mais tolerante à estresse hiper- do que hipossalino. Para todos tecidos analisados (sifão, manto e músculo), a variação foi menor e alcançou alguma estabilidade apenas nos desafios hiperosmóticos. Em condições hipersalinas, *C. costata* apresenta uma maior capacidade de regular a hidratação tecidual do que outros bivalves capazes de fechar completamente as valvas. Essa característica provavelmente está relacionada com a história natural e evolutiva do grupo. Apesar disso, a tolerância a baixas salinidades pode ser favorecida pelo micro-habitat da toca, consequência do hábito escavador, e também pode estar relacionada à produção de urina, processo que merece investigação em Pholadidae.

Palavras-chave: Bivalvia. Ecofisiologia. Morfologia. Osmorregulação. Reprodução.

SUMÁRIO

1 **PROLOGUE**

1.1 The Class Bivalvia: economic interest and ecological importance

Mollusks constitute the second largest zoological group known to science, being represented by approximately 100,000 living species and 70,000 fossil species (COLLEY; SIMONE; SILVA, 2012). The group is one of the most evidently and closely related to human society, since prehistoric times. Their shells, now used as accessories and souvenirs, have been used as coins and symbols of power (COLLEY; SIMONE; SILVA, 2012; SIMONE, 2003). In addition, members of the group are frequently used for food, being considered a source of subsistence for traditional coastal communities, mainly because of their attractive taste and availability of protein and nutrients, as well as low production cost and high profitability (BIN RAMLI; YUSOP, 2016; CAMILO *et al.*, 2018).

Mollusks, in general, have intrinsic importance as protagonists in maintaining the ecosystems in which they are found (COLLEY; SIMONE; SILVA, 2012). Ecologically, bivalves are primary consumers and food sources for various species, natural filters – thus very relevant as bioindicators of water quality - and provide substrate and microhabitats for other organisms, increasing local biodiversity. On the other hand, perforating species can cause damage related to bioerosion, burrowing in coral and other bivalves (see Wong, 1982). Moreover, in several regions around the world, the superpopulation of some species (such as *Dreissena polymorpha*, *Corbicula* spp. and *Limnoperna fortunei*) render them as pests, causing ecological and economic damage (BOLTOVSKOY; CORREA, 2014; RICCIARDI; NEVES; RASMUSSEN, 1998).

1.2 Reproductive cycle

The reproductive system of marine bivalves consists of paired gonoducts, larger genital channels and numerous smaller channels that end in a chain of follicles. It is common for the gonads to reach the maximum pre-spawning volume and occupy much of the visceral mass. In many species the gonads are diffuse and may penetrate other organs, especially the mantle. In general, bivalves do not have copulatory organs or accessory sexual glands (SASTRY, 1979).

Sex determination in bivalves can be defined by the environment, resulting in distinct sexual proportions and incidence of hermaphroditism, although most species are dioecious with an approximate 1:1 sex ratio (FREITES *et al.*, 2010; MORTON, 1991; RONQUILLO; MCKINLEY, 2006). Sexual dimorphism is rare, except when the sex can be distinguished by differences in shell size. In general, sex analysis occurs through histological examination of the gonads or observations during spawning (SASTRY, 1979).

The reproductive cycle of bivalves, like that of other marine invertebrates, is cyclic and can be annual, semi-annual or continuous and can be divided into three main phases: gonadal development, spawning and oocyte development and growth (CORTE, 2015; LUZ; BOEHS, 2011). These phases, working in coordination with seasonal environmental changes, produce the reproductive pattern of a species. The synchronization of the reproductive phases with the most favorable environmental conditions for juvenile development and growth is obviously significant for the reproductive success of the group (FREITES *et al.*, 2010; MORTON, 1991; SASTRY, 1979).

Different criteria for categorizing reproductive stages in bivalves are used, based on histological sections of the gonads. The main gametogenic stages are categorized as: proliferation (or growing), maturation, spawning and resting (BORZONE *et al.*, 2001; CORTE, 2015; MORSAN; KROECK, 2005). These stages can be synchronized across all individuals in a population, or individuals are in the same period at different stages of the cycle (SASTRY, 1979). The main factors that influence bivalve reproduction are water temperature and salinity (BOEHS; ABSHER; CRUZ-KALED, 2008; CAMILO *et al.*, 2018; CEUTA; BOEHS, 2012; GUSTAFSON *et al.*, 1991), in particular for inhabitants of the intertidal region, subject to large fluctuations of these factors. Abiotic stress (changes in temperature, salinity and exposure to contaminants) does not alter the stages of the reproductive cycle, but may alter their duration, the number and quality of the produced gametes and the sex ratio (CEUTA; BOEHS; SANTOS, 2010; COE, 1943; HAMDANI; SOLTANI-MAZOUNI, 2011).

Studies on the reproductive cycle of marine bivalves off the Brazilian coast are available mainly for *Crassostrea* spp. (CHRISTO; ABSHER, 2006; LENZ; BOEHS, 2011), *Anomalocardia flexuosa* (LUZ; BOEHS, 2011), *Perna perna* (ABESSA *et al.*, 2005; FAGUNDES *et al.*, 2004), *Donax trunculus* (HAFSAOUI *et al.*, 2016), *Mytella* spp. (CAMILO *et al.*, 2018; CHRISTO; FERREIRA-JR; ABSHER, 2016), and *Tagelus plebeius* (CEUTA; BOEHS, 2012; CEUTA; BOEHS; SANTOS, 2010).

1.3 Physiology of estuarine bivalves

Many species of mollusks have radiated along the coast, where food supply is abundant and varied. Currently, most of the group's representatives inhabit the marine and estuarine environment (COLLEY; SIMONE; SILVA, 2012). Estuarine animals undergo daily changes in temperature, salinity, pH and, for intertidal organisms, periods of exposure to air (BREAUX *et al.*, 2019). All of these factors control species distribution and abundance (LAVAUD *et al.*, 2017). The main factors affecting bivalve growth and reproductive success are temperature, salinity and availability of organic matter (SANTANA; ROCHA-BARREIRA, 2018; TURNER, 1954; WONG, 1982).

For mollusks in general, behavioral changes can provide primary protection from environmental fluctuations, preventing the use of metabolic resources to maintain homeostasis. Bivalves can isolate their soft tissues with complete valve closure, through anaerobic mechanisms, decreased ciliary beating and controlling the opening of inhalant and exhalant siphons (DEATON, 2009; POURMOZAFFAR *et al.*, 2019; VEIGA *et al.*, 2015). When behavioral responses are not sufficient, osmoregulatory mechanisms are triggered, essentially through regulation of tissue hydration and variably effective mechanisms of control of ionic concentrations (DEATON, 2009; NARVÁEZ *et al.*, 2008; PIERCE, 1982; POURMOZAFFAR *et al.*, 2019).

Marine invertebrates are able to regulate cell volume according to the ionic concentration of the environment (i.e., water salinity), where cells increase or decrease in size by controlling the pool of organic and inorganic (ions) osmolytes exchanged between the intra- and extracellular compartments (DEATON, 2009; PIERCE, 1971, 1982). The ability of species to regulate volume is directly related to their degree of euryhalinity tolerance limits, especially for osmoconformers such as mollusks in general (DEATON, 2009; FREIRE *et al.*, 2008; PIERCE, 1971).

1.4 Pholadidae family

Bivalves of the Pholadidae family display a set of features that allow them to excavate different substrates (clay, rock, wood, limestone or mud). These are: an anterior reflection of valves and the positioning of the anterior adductor muscle externally, an apophysis offering support to the foot muscles and visceral mass and the reduction or absence of a ligament (PURCHON, 1955; TURNER, 1954). Its burrows are conical in shape, with the diameter of the opening possibly related to the age of the bivalve; the posterior chamber (where the bivalve is located) provides a microhabitat, increasing the complexity of the local ecosystem (PINN *et al.*, 2005; TURNER, 1954). Moreover, their burrows never intercommunicate; when in large agglomerations, their burrows may take on more complex shapes to ensure their isolation from each other (PINN *et al.*, 2005; WONG, 1982). Despite being an important group from the ecological (trophic chain, microhabitat, bioerosion) and economic (gastronomic utilization, source of income) aspects, we know little about its basic biology/physiology and ecology (MONARI, 2009; PINN *et al.*, 2005).

Pholadidae molluscs are commonly used as baits for fish and culinary dishes and are marketed in various parts of the world such as Hong Kong, Malaysia, Thailand and the Philippines, and are consumed still today in parts of Europe and Asia (GUSTAFSON *et al.*, 1991; PINN *et al.*, 2005; RONQUILLO; MCKINLEY, 2006; TURNER, 1954). Studies on foladid species are available mainly for *Pholas dactylus* (ARIAS; RICHTER, 2012; PINN *et al.*, 2005; TRIGO; DIESTE; ROLÁN, 2010), *P. orientalis* (BIN RAMLI; YUSOP, 2016), and *Barnea candida* (ALEXANDROV, 2017; TRIGO; DIESTE; ROLÁN, 2010).

Considering the economic importance of pholadids, their considerable biomass, and their role in the ecosystems in which they are found and the little knowledge we have about these species, including groups to be described and identified, the preservation of these populations should be a priority. *Pholas dactylus*, in Europe, has become rare due to habitat destruction and pollution, overfishing for human consumption and use as fish bait. The species is currently included in several biodiversity protection programs (see Arias & Richter, 2012). For *P. orientalis*, the danger of extinction for some natural populations is predictable, as in the case of the Philippines, due to overexploitation and unregulated extraction activities (BIN RAMLI; YUSOP, 2016).

1.5 The angel swing clam *Cyrtopleura costata*

In the Americas, among the pholadids of economic interest we find *C. costata*, a burrower bivalve in its early ontogenetic stages, also known as "angel wing clam", due to the appearance of its valves. The species occurs from eastern Canada down to the Brazilian coast (CULLAIN *et al.*, 2018; RIOS, 2009; VELÁSQUEZ, 2017), and is found in intertidal regions that have sandy-mud sediment.

Juveniles of *C. costata* less than 15 mm long bury in the sediment and remain there, growing inside the burrow. They are unable to bury as adults, only moving on a vertical axis and maintaining the structure of the burrow (GUSTAFSON *et al.*, 1991; TURNER, 1954). Their burrows are about 40 cm deep, conical in shape; shell parts are attached to the wall and a posterior chamber hosts the bivalve (PINN *et al.*, 2005; ROTHSCHILD, 2004). The fused inhalant and exhalant siphons project about 1 cm above the surface and circulate water and suspended organic matter to the gills. Due to their size and fragile shells, the valves cannot close completely, as in other bivalves, thus the animal remains vulnerable especially to fluctuations of in salinity, but also in temperature (TURNER, 1954).

Studies about *C. costata* are scarce, but among them we find Turner (1954), who makes some notes about the habit, consumption, morphology, physiology and reproduction of the species; Castagna & Chanley, (1973) evaluated the behavior of *C. costata* to changes in salinity; Gustafson et al. (1991) assessed the combined effects of temperature and salinity on larval and juvenile development; Albuquerque (2010), in his thesis, explored cultivation techniques of the species and Nur Syazwani et al. (2015, 2017, 2019) and Marwan & Indarti (2016) have been using *C. costata* shells as catalysts for biodiesel production.

In Puerto Rico and the Caribbean, *C. costata* has already been marketed, as well as in Cuba, where it was once considered a basic food (see Gustafson et al., 1991). In addition, high juvenile growth rates were reported in Florida (EUA) by Gustafson et al. (1991) and in Santa Catarina (Brazil) by Albuquerque (2010). Given this, *C. costata* appears to be a good candidate for aquaculture. In the Brazilian coast, *C. costata* is used as food and bait for fishing by traditional communities (RIBAS, 2014; TEIXEIRA; CAMPOS, 2019), being the consumption supplied by natural stocks. In some coastal communities of Paraná and Santa Catarina, according to fishermen reports, the species is no longer found.

Given the ecological and economic importance of the species and the vulnerability of its populations, there is a need to prioritize basic biology studies about the morphological description, gametogenic cycle and physiological processes that allow the success of natural populations. In order to offer subsidies for sustainable use of the resource, the first chapter of this paper describes for the first time the external and internal morphology of *C. costata*, the second chapter characterizes the gametogenic cycle of the species and the reproductive investment throughout the year in a population of the coast of Paraná, and the third chapter addresses the physiological responses of this bivalve under saline challenges, aiming at understanding how a burrowing bivalve that is unable to isolate itself from the environment through the complete closure of the valves accomplishes to dwell under the challenging variable salinity regimes of the intertidal flat.

2 CHAPTER 1

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Morphological description of *Cyrtopleura costata* (Bivalvia: Pholadidae) from southern Brazil

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ABSTRACT

The aim of the study is to describe in detail, for the first time, the internal and external anatomy of *Cyrtopleura costata*, which displays oval and elongated valves with beige periostracum, the anterior adductor muscle unites the valves in the pre-umbonal region, with abduction capacity in its dorsal half, sparing the ligament. Two accessory valves are identified: the mesoplax (calcified) located in the umbonal region; and the protoplax (corneus) above the anterior adductor muscle. Internallu there is a pair of well-developed apophysis that supports the labial palps and the pedal muscles, and support part of the gills. The posterior half of mantle ventral edge is fused and richly muscular, working as auxiliary adductor muscle. The siphons are completely united with each other, the incurrent being larger than the excurrent. The foot is small (about 1/8 the size of the animal). The kidneys extend laterally on the dorsal surface, solid, presenting a brown/reddish color. The style sac is well developed and entirely detached from the adjacent intestine. The intestine has numerous loops and curves within the visceral mass. The fecal pellets are coin-shaped.

INTRODUCTION

The bivalves are the second greatest class of the phylum Mollusca in diversity and, accordingly, of high economic and ecological importance. They constitute an ideal group to answer biological questions around morphological adaptations that allow them to dig, to drill, to swim and to attach on several substrates (Bieler, 2006; Bieler, Mikkelsen & Giribet, 2013).

Although the available knowledge about bivalves is relatively extensive and dates back to several centuries, there are still many gaps to be filled. Moreover, many species are still to be described and identified, as well as the known ones must be better defined (Bieler, Mikkelsen & Giribet, 2013). The magnitude of the group hampers large-scale research and, for morphological researches, it is usual that these are conducted based on a single organ or structure, or even generalizations are made from other groups and specimens. In addition, scant attention is given for the anatomy of the soft parts, focusing only on description of the shell, although internal organs display abundant characters of taxonomic importance. Studies describing soft anatomy are still scattered for Pholadidae (e.g. Purchon (1955)).

Pholadidae family is able of burrow into different substrates such as clay, limestone, rocks or mud. The group has a set of features that reflects the punch/burrowing habit, such as accessory valves, apophyses, the presence of thorns adorning the surface of the valves, reduction or loss of the hinge ligament and anterior adductor muscle entered dorsally, which allows the valves to move on a dorsoventral axis. This arrangement is exclusive of this family (Ansell & Nair, 1969; Ito, 2005; Monari, 2009; Jeon *et al.*, 2012).

Pholadids are an important source of protein and several species are eaten nowadays by coastal communities in Brazil (Ribas, 2014), Europe (Arias & Richter, 2012) and Philippines (bin Ramli & Yusop, 2016). Among them, *Cyrtopleura costata* is a burrowing organism in its early ontogenetic stages, inhabitant of muddy infauna of intertidal regions from the east coast of Canada to southern Brazil (Rios, 2009; Velásquez, 2017; Cullain *et al.*, 2018). Its galleries have a narrow opening and an internal chamber that contributes to increased complexity of the ecosystem, acting as ecosystem engineers (Pinn *et al.*, 2005; Haider, Sokolov & Sokolova, 2018). Despite being one of the most conspicuous species of Pholadidae, with easily recognizable large individuals (Turner, 1954), little is known about its anatomy. Dall (1889) produced a brief note on the its basic anatomy, while Kellogg (1915) approached the ciliary mechanism of the species, and Turner (1954) presented a general overview of external anatomic features.

Descriptive morphology can provide new characters for phylogenetic studies, contributing towards the description of new species and for the survey of local biodiversity, besides serving as the basis for further researches. In view of that, this study aimed at describing the morphology of the shell and soft parts of specimens of a population of *C. costata* located in southern Brazil, Paranaguá Estuarine Complex, Paraná.

MATERIAL AND METHODS

The specimens were collected in Paranaguá Bay (State of Paraná, Southern Brazil) (25º43'53,17"S / 48º44'63,05"W), were then shortly after relaxed in menthol for 30 minutes, and fixed in formalin 10% for at least 24 hours, with posterior conservation in ethanol 70%. Dissection was performed using standard techniques (Simone et al., 2015), with animals immersed in alcohol, with the aid of a stereomicroscope. Internal structures were observed by removing the integument from the visceral mass and scraping the gonadal tissue. Transverse sections of the labial palp were submitted to histological procedures (adapted from Borzone, Vargas, Pezzuto, & Tavares, 2001). The drawings were done with the aid of a camera lucida. Additional conchological material ("shells") was examined when soft tissues were not available.

The collected and dissected bivalves (treated as *specimens* in material examined) were deposited at the Museu de Zoologia da Universidade de São Paulo (MZSP 143461). Shells (without the soft parts) from other places, deposited in the same collection, were also examined (treated as *shells*).

SYSTEMATIC DESCRIPTION

Cyrtopleura costata **Linnaeus, 1758**

(Figs 1-2)

Pholas costatus Linnaeus, 1758: 669; Lamarck, 1818: 445; 1835: 45; Tryon, 1862: 201; Schepman, 1887: 156, 164.

Capulus shreevei Conrad, 1869: 105, pl. 13, fig. 3.

Scobina costata: Bayle, 1880: 242.

Pholas (*Barnea*) *costatus:* Grant & Gale, 1931: 431.

Cyrtopleura (*Scobinopholas*) *costata:* Turner, 1954: 35, pls. 17-18; Altena, 1968: 156, 176; 1971: 78, pl. 6, figs. 10-11; Pacaud, 1998: 64-65, figs. 21-22.

Cyrtopleura costata: Perry & Schwengel, 1956: 94, pl. 19, fig. 124; Moore, 1961: 49; Turgeon, 1968: 71, fig. 43; Hoagland, 1983: 44; Spencer & Campbell, 1987: 54, pl. 9, figs. 19-20.

Material examined: EUA. Florida, Pierce, Bathtub Beach, MZSP 46850, 1 shell. BRAZIL. Bahia, Ilhéus, Olivença, MZSP 101530, 1 shell; Rio de Janeiro, Ibicuí, Almirante Aratanha, MZSP 144243, 1 shell; São Paulo, Iguape, MZSP 20106, 1 shell; Paraná, Baía de Paranaguá, Comunidade de São Miguel, MZSP 143461, 8 specimens.

Shell (Fig. 1A-F): Opaque beige to white, thin, fragile, with vertical adornments extending from umbo region to ventral edge; more protruded in anterior and posterior regions (Figs 1A, B, E). Shape oval and elongated rounded at both ends (Figs 1A, C). Posterior region narrowing gradually, extending beyond base of siphonal process. Anterior edge of valves reflected, anterior to umbo; insertion of anterior adductor muscle upwards, external to valves (Figs. 1D: am. Sockets are formed below and posteriorly to reflection, where mantle flaps (Fig. 2B) are inserted. Periostracum brown, translucent, well-developed near edges. Pallial line apparent, pallial sinus wide and shallow (Fig. 1C). Apophysis robust, long (~half valve width), spoon-shaped. Scar of posterior adductor muscle is smaller than the anterior, close to dorsal edge. Two accessory plates present: protoplax chitinous and large, anterior to umbo (Fig. 1A: pt), covering anterior adductor muscle; mesoplax calcareous, located posterior to umbo, below anterior adductor (Fig. 1F: mt). Ligament absent.

Main muscle system (Figs. 2A, D, E): Proportions and location of adductor muscles as described above. Paired anterior pedal retractor muscles originates into outer edge of apophysis. Fusion of ventro-posterior edge of pallial edge thick muscular, mostly with transverse muscles, possibly working as auxiliary adductor muscle. Siphonal retractor muscle arranged in bundles and well-demarcated on mantle surface; origin like fan in siphonal base (Fig. 2D: sm).

Foot: relatively small (~1/8 of animals' size) (Fig. 1E: ft) origin in visceral mass slightly "superficial" .

Mantle: Edges mostly fused, through inner fold (Fig. 2C). Siphons completely merged with each other; base surrounded by, but not connected to, periostracum layer. Incurrent siphon ~double than excurrent (Fig. 1H), with folded edge on itself (Fig. 1I: it), baring larger tentacles more internally arranged, with smaller situated more externally (Fig. 1J: ot); internal longitudinal folds present. Excurrent siphon smooth, with tiny tentacles on its margin. Both siphons possessing brownish-red pigment. Siphon reaching about 3 times or longer than valves' length. Pedal gap slightly smaller than half of ventral mantle edge (Fig. 1D).

Pallial cavity (Fig. 2D): Labial palps very thick occupying ~1/3 of pallial area (Fig. 1G; 2D: op, ip) located inside the umbonal cavity. Hemipalps triangular and asymmetrical, being outer slightly smaller than inner one, both with posterior distal tip. Inner demibranch passing between hemipalps, larger than outer demibranch, and with ciliary connection with visceral mass. Outer demibranch smaller, showing no contact with labial palps, displaying tissue connection with visceral mass (Fig. 2G). Both dorso-ventrally short, ~half of pallial cavity in length, gradually becoming wider towards posterior region.

Visceral mass (Fig. 2F): Stomach located ventrally at mid region. Intestinal looping throughout visceral mass. Gonad fulfilling entire remaining visceral space.

Circulatory and excretory systems (Fig. 2E): Pericardium compressed dorsally; just anterior to posterior adductor muscle. Auricles with rose tone, walls very thin. Ventricle large, rectum passing through center of it (Fig. 2E: rt). Kidney underdeveloped, spongy, solid; extending laterally on dorsal surface, color reddish-brown. Nephropore located near base of excurrent siphon.

Digestive system (Fig. 2F): Labial palp described above. Mouth close to anteroventral surface of anterior adductor muscle. Esophagus narrow, attached to adductor at intersection of labial palps, running horizontally along dorsal visceral mass, inserting in anterodorsal side of stomach. Digestive gland lying on each side. Style sac well-developed, apart from the stomach; extending until anterior end of visceral mass; almost as long as visceral sac (Fig. 2F: ss). Intestinal origin large, located in anteroventral region. Intestine relatively narrow, with numerous loops as in Fig 2F, becoming gradually thinner towards posterior. Fecal pellets stacked up, coin-shaped along posterior portion of intestine.

Reproductive system: Gonad described above. Genital duct not detected. Genital pore located close excurrent siphon, anterior to anus, under and slightly posterior to nephropore.

Nervous system: Cerebropleural and pedal ganglia remained undetected. Visceral ganglia located on anteroventral side of posterior adductor muscle. Cerebrovisceral connectors crossing visceral mass.

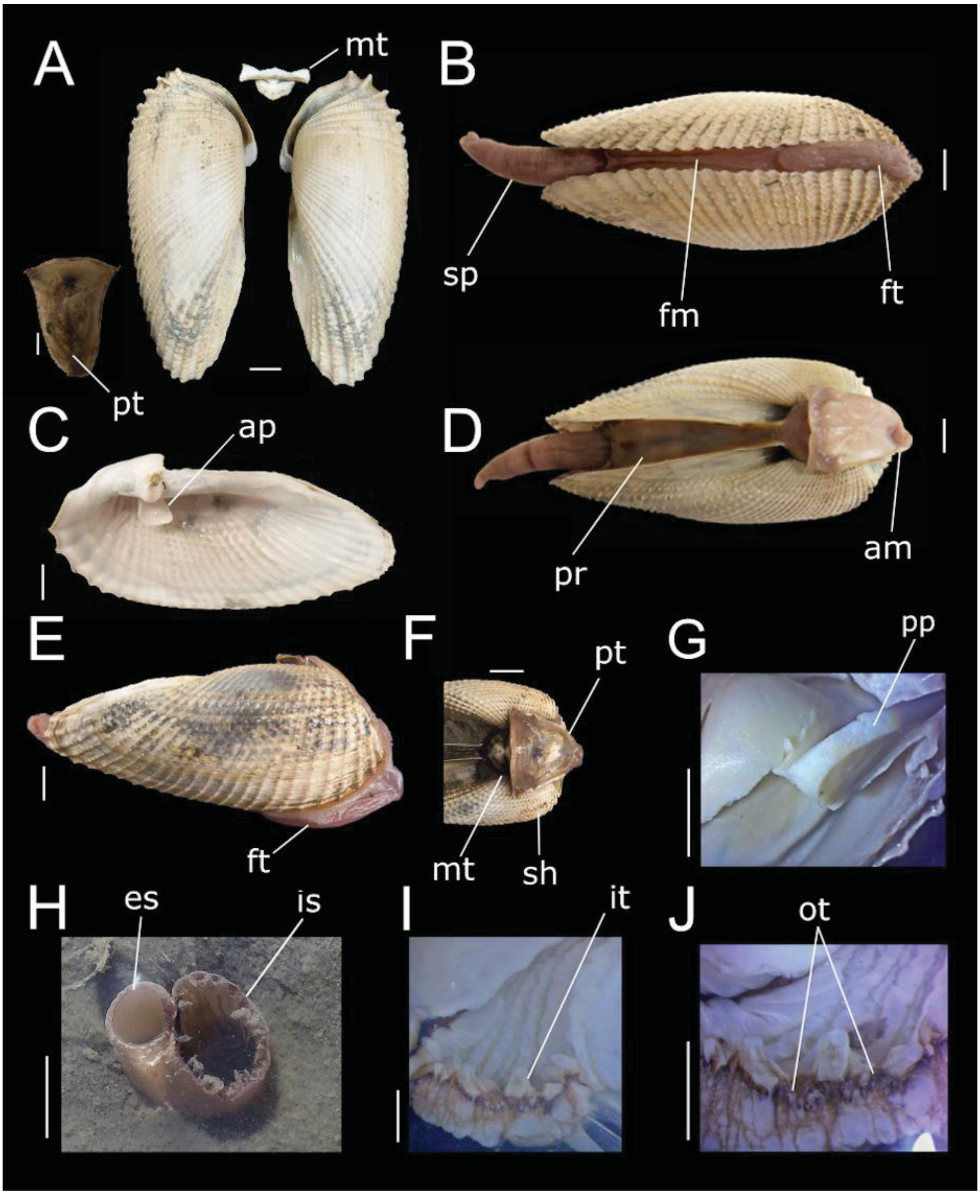

Figure 1. Shell and anatomic aspects of *Cyrtopleura costata* (MZSP 143461): **A**, valves and accessory plates, external view; **B**, ventral whole view; **C**, left valve, internal view; **D**, dorsal

whole view; **E**, left whole view; **F**, detail of the location of accessory plates, anterior region, dorsal view; **G**, left outer hemipalp cross section; **H**, siphon *in situ* opening out of gallery; **I**, incurrent siphon tip, opened longitudinally; **J**, incurrent siphon margin detail. Abbreviations: **am**, anterior adductor muscle; **ap**, apophyses; **es**, excurrent siphon; **fm**, fusion between left and right mantle lobes; **ft**, foot; **is**, incurrent siphon; **it**, inner tentacle; **mt**, metaplax; **ot**, outer tentacle; **pp**, labial palp; **pr**, pericardial region; **pt**, protoplax; **sh**, shell; **sp**, siphon. Scales bar: A-H = 1 cm; I-J = 1 mm.

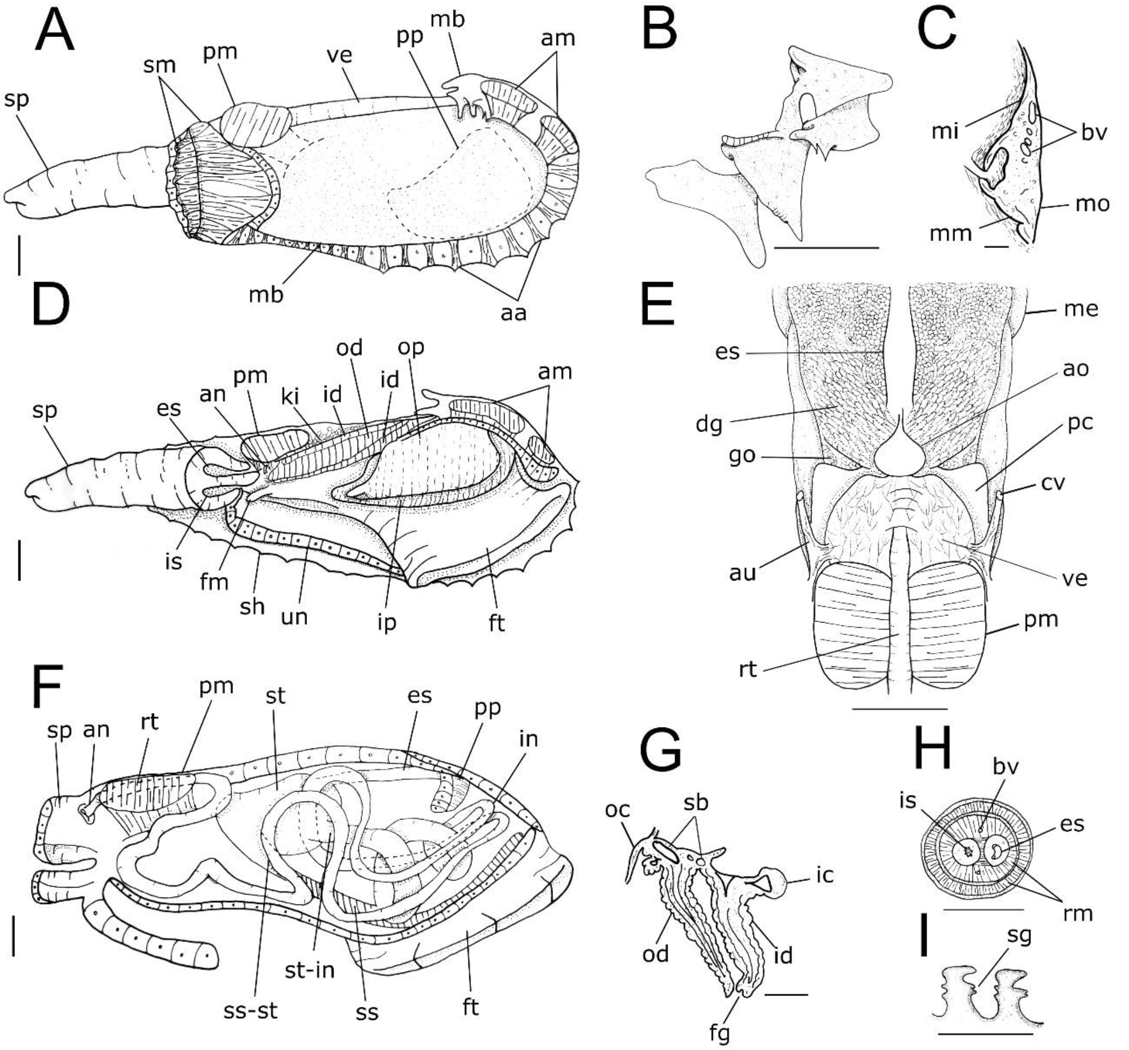

Figure 2. *Cyrtopleura costata* general anatomy: **A**, whole right view shell removed, ; **B**, flaps of mantle in region just posterior to umbo, right view; **C**, transverse section of right mantle edge; **D**, pallial cavity whole right view, fusion of both mantle lobes sectioned longitudinally, right mantle lobe removed; **E**, pericardial region, dorsal view; **F**; digestive system *in situ*, right view, topologies of some adjacent structures also shown; **G**, gill, cross section at mid-region; **H**, siphons, transverse section in their middle level; **I**, labial palp profile in cross section. Abbreviations: **aa**, auxiliary adductor muscle; **am**, anterior adductor muscle; **an**, anus; **ao**, aorta; **au**, auricle; **bv**, “blood” vessel; **cv**, ctenidial (efferent) vessel; **dg**, digestive gland; **es**, esophagus; **es**, excurrent siphon; **fg**, food grooves; **fm**, flap of visceral mass; **ft**, foot; **go**, gonad; **ic**, inner ciliary connection of gill; **id**, inner demibranch; **id**, insertion of outer demibranch in

mantle; **in**, intestine; **ip**, inner hemipalp; **is**, incurrent siphon; **ki**, kidney; **mb**, mantle border; **me**, mantle; **mi**, mantle border inner fold; **mm**, mantle border middle fold; **mo**, mantle border outer fold; **oc**, outer tissue connection of gill; **od**, outer demibranch; **op**, outer hemipalp; **pc**, pericardium; **pm**, posterior adductor muscle; **pp**, labial palp; **rm**, radial siphonal muscle; **rt**, rectum; **sb**, suprabranchial chamber; **sg**, selection groove; **sh**, shell; **sm**, siphonal retractor muscle; **sp**, siphon; **ss**, style sac; **ss-st**, conjoined style sac-stomach; **st**, stomach; **st-in**, conjoined style stomach-intestine; **un**, fusion between left and right mantle lobes; **ve**, ventricle. Scales bar: A, B, D-F = 1 cm; C, G, H = 2 mm; I = 200 µm.

Distribution: From eastern Canada, USA, México, Cuba, and South America from Venezuela to southern Brazil (Rios, 2009; Velásquez, 2017; Cullain *et al.*, 2018).

DISCUSSION

Cyrtopleura costata has typical radially crimped shaped valves of the Pholadidae, which may vary due to age, population size and sort of substrate (Turner, 1954; Purchon, 1955; Wong, 1982). Like other representatives of the family, *C. costata* has sculpture on the surface of the valves that are employed in abrasion and may indicate a preference for a particular type of substrate, as well as it reflects phylogenetic relationships (Ito, 2005). Only one individual had pink coloration in the umbonal region and on the anterior adductor muscle; this seems to be more common in certain populations, such as those from the west coast of Florida (Turner, 1954). About the two accessory valves, the mesoplax is chitinous, which may be due to the low abrasion suffered by the valves within of the burrowed sediment (Morton, 1973; Turner & Santhakumaran, 1989; Fiori, Simonetti & dos Santos, 2012; Voight & Segonzac, 2012).

Burrowing pholadid molluscs are known to have an apophysis that protrudes from the umbonal region toward the visceral mass. For *Barnea parva*, *B. candida*, *Pholas dactylus*, *Pholadidae loscombiana*, *Zirfaea crispata* and *Z. subconstricta*, the apophysis was considered a support for the insertion of the pedal muscles (Purchon, 1955; Ito, 2005). Despite *C. costata* is not burrowing in in adult phase, it has a well-developed apophysis that supports the labial palps, the pedal muscles, the gills, and, provably, the visceral mass.

A ligament was not observed in *C. costata*, as seems to be common for Pholadidae, as in, e.g., *B. candida*, *P. dactylus* and *P. loscombiana* (Purchon, 1955). It is suggested that the degree of reduction or loss of the ligament is related to the rate of boring activity – highly burrowing pholadids do not have ligament

– as such reduction allows valves to move along a vertical axis (Purchon, 1955). This way, the articulation of the valves is related to the anterior adductor muscle, positioned dorsal and externally, which takes the position of the ligament (Simone, 2019; Turner & Santhakumaran, 1989).

The diductor function in the group occurred through the dorsal displacement and expansion of the anterior adductor, which boosted the burrowing habit, since it allows the adductors to operate oppositional to each other, which is a feature typical of pholadids (Wong, 1982; Savazzi, 1987; Ito, 2005). The protection given by the walls of the burrows allows the muscle to be exposed, as observed in *C. costata*, where the anterior adductor muscle extends to the outer surface of the umbonal region, being protected only by the protoplax and the thin pallial layer that builds it. Although it is known that there is a dependence of the substrate to support the shell (Rothschild, 2004), *C. costata* has a strong and well-developed muscular system that moves its valves.

For Pholadidae, two stages in the life cycle are considered: the first is recognized as “active burrowing” and the second as “non-burrowing”, thus having a stunted foot. Most pholadid species, if removed from the substrate, are unable to produce a new burrow (Ansell & Nair, 1969; Ito, 2005). This information is supported by the features of the adult *C. costata*, which has a shortened foot despite a wide pedal aperture, suggesting the conclusion of the active burrowing stage. After going through the active burrowing stages, adult individuals of *Penitella penita* and *Aspidopholas obtecta* have the foot stunted and the visceral mass expanded, allowing more space for the development of the gonads (Evans, 1970; Wong, 1982). After the burrowing phase is over, *C. costata* does not build a callus (calcium accumulation in the anterior region between the valves), feature presented by other pholalids at the end of the burrowing phase (Turner, 1954).

The siphons are completely merged in *C. costata*, as well as in *Barnea parva*, *B. candida*, *Pholas dactylus*, *Pholalidea loscombiana* and *Zirphea crispata*. The periostracal layer is not attached to the siphon surface, which allows it to be trapped into the pallial sinus. Like in other pholadids, even when totally retracted, the siphon cannot be entirely trapped into the infrabranchial chamber. This feature is considered indicator of a burrowing habit into hard substrates (such as in *Z. crispata*), where the siphons suffer low or no abrasion (Purchon, 1955). Despite presenting large siphons, which cannot be entirely trapped, *C. costata* is inhabitant of soft substrates. Moreover, although it presents substrate preference similar to that of *B. candida*, *C. costata* displays a substantially more sturdy siphon (Purchon, 1955), which is probably related to the great depth of the burrow of the species (Turner, 1954), which can reach almost 1 meter.

An extension at the posterior end of the visceral mass (Fig. 2, fm) probably is what Turner (1954) calls the "collecting membrane", also present in *Barnea* (Purchon, 1955). This collects the excess sediment and deposits near the siphon to be expelled, which allows animals to feed all times, even in muddy waters (Turner, 1954).

The well-developed ventricle in *C. costata*, already recorded for other pholadids (Purchon, 1955), may be associated to a long siphonal process, which requests hydraulic pressure to permit its extension until the surface. The amount of intestinal loops may suggest access to food of relatively poor nutritional value.

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3 CHAPTER 2

Manuscript formatting: Marine Biology

Reproductive biology of *Cyrtopleura costata* (Pholadidae) in southern Brazil

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ABSTRACT

Cyrtopleura costata, a burrowing bivalve also known as "angel's wing", is consumed by traditional Brazilian communities and used as fish bait. However, no efforts for its culture have been recorded, and its consumption is supplied by natural stocks. This study aimed to describe, for de first time, the gametogenic cycle of a natural population of *C. costata* in Paranaguá Estuarine Complex (PR) and to contribute to the knowledge of the diversity in reproductive patterns of bivalves. Monthly collections were performed between November/2018 and October/2019. All specimens had their shells measured for analysis of the relationship between size with gonadal maturity; wet meat weight was quantified for Condition Index evaluation, and a section of visceral mass was submitted to histological analysis. Biometric parameters of gonadal tissue were determined for both sexes. A total of 129 individuals have been sampled ($\bar{x}$=85.82, SD=13.27). Sex ratio was of 1:1, although the largest individuals were all females. Six gametogenic stages were identified in the gonads of *C. costata* adults: *Proliferation*, *Early ripe*, *Late ripe*, *Spawning with proliferation*, *Spawning with resting*, and *Resting*. Mature animals have been observed in practically all year round. Between late summer and mid-autumn, both sexes exhibit a synchronized spawning peak. For females, an intense spawning is repeated during the winter which releases the largest oocytes produced in the year, followed by a period of reproductive rest. The mean oocyte diameter ranged from 3.63 (June/19) to 39.29µm (July/19). As menores temperaturas estiveram relacionadas ao período de maior investimento reprodutivo da população. The reproductive pattern of *C.*

costata from Paranaguá Bay agreed with that observed for bivalves in tropical and subtropical regions, although Pholadidae exhibits a wide range of reproductive patterns and strategies that do not appear to be related to latitude. The continuous reproduction of this species allows its exploitation throughout the year, except in the winter months.

INTRODUCTION

Studies on the biology and, specifically, reproductive strategies are an important starting point for decision-making in monitoring, management and conservation, since they make it possible to predict recruitment and spawning times besides provide insights into the structure and dynamics of the populations and their relationships with the environment (Oyarzún et al. 2016; Purroy et al. 2019).

Marine bivalves are considered a source of subsistence for traditional coastal communities because of their low production costs, high profitability and protein and nutrient supply. This makes this the main group used in shellfish farming (bin Ramli and Yusop 2016; Camilo et al. 2018).

Pholadidae family are infaunal burrowing bivalves of several type of substrates, from soft mud to rocks and limestone (Turner 1954). In addition to their ecological importance as bioengineers and responsible for maintaining the ecosystems where they live (Haider et al. 2018), pholadids are widely eaten by human communities (Turner 1954; Gustafson et al. 1991; Ronquillo and McKinley 2006).

Among the species of economic interest is the socalled “angel wing clam” *Cyrtopleura costata*, inhabitant of the infauna of intertidal sandy-mud regions (Turner 1954; Gustafson et al. 1991) from eastern Canada to southern Brazil (Rios 2009; Cullain et al. 2018). Its burrows reach about 40 cm deep and, as well other bivalves, can provide microhabitats for other marine invertebrates, as crab symbionts (Ferreira-Jr et al. 2015; Acarlı et al. 2019), increasing the local biodiversity (Pinn et al. 2005).

Cyrtopleura costata has already been commercialized in several regions throughout its distribution (see Gustafson et al., 1991 and Turner, 1954) and your valves have been used as catalyzer in biodiesel production due to the availability of Calcium oxide (CaO) (Nur Syazwani et al. 2015, 2017, 2019; Marwan and Indarti 2016). In Brazil, the species is consumed by traditional communities (Ribas 2014; Teixeira and Campos 2019) and no culture has ever been recorded; all consumption is supplied by natural stocks.

Life cycle studies, with emphasis on reproductive investment, allow to characterize the main periods of the gametogenic cycle and the reproductive strategy of the species, useful information in order to conserve natural populations and subsidize their sustainable economic exploitation. Despite its ecological and economic importance, due to his cryptic (Fiori, Simonetti, & dos Santos, 2012) habit little is known about the ecology and biology of this species (Turner 1954; Gustafson et al. 1991). Investigating the reproductive patterns of poorly studied species allows us to fill in gaps on the broad spectrum of reproductive strategies within Bivalvia (Collin 2013).

This study aimed to describe the gametogenic cycle and the Condition Index of a natural population of *C. costata* in Paranaguá Estuarine Complex (PR) and constitutes the first report about the reproductive cycle of this group. It does also contribute to the knowledge of the diversity in reproductive patterns of bivalves in general, and pholadids in particular. Is expected that the reproductive pattern observed to be similar to that of other infaunal bivalves of the intertidal environments of tropical and subtropical regions, which are exposed to the same exogenous factors.

MATERIAL AND METHODS

The collections were held monthly between November/2018 and October/2019 in the euhaline sector of the Paranaguá Estuarine Complex (25º43'53,17"S / 48º44'6305"W), which is located close to a series of legally protected areas where extractive fishing is a vital activity for the survival of local riverside communities (FUNDEPAG 2015).

Based on previous studies on bivalve reproductive cycles (Pinn et al. 2005; Ceuta et al. 2010; Freites et al. 2010; Christo et al. 2016; Hafsaoui et al. 2016; Camilo et al. 2018), the capture was limited to 10 individuals / month, performed manually and randomly, through active search observing the opening of the siphons on the soft bottom.

Relevant environmental data for the reproductive cycle of marine bivalves such as water temperature (portable thermometer) and salinity (optical refractometer) (Gustafson et al. 1991; Ceuta and Boehs 2012; Camilo et al. 2018) were collected *in situ*. In addition, bimonthly seawater temperature and salinity data collected at Paranaguá Bay were provided by the “TCP Expansion Works Complementation Project” (Acquaplan 2019).

After capture, the animals were anesthetized (30 minuts in menthol and seawater solution) and subsequently fixed in 10% formalin and preserved in 70% alcohol. In the laboratory, all specimens were weighed (accuracy 0,0001g) and their shells were measured (digital caliper) for length, height and width (Fig. 1) (Bailey 2009) for evaluation of sexual dimorphism and to analyze the relationship between size and gonadal maturity. The flesh wet weight was measured for Condition Index (CI) evaluation, according to Kagley et al. (2003), by the formula:

$$CI\ (\%)\ = \frac{FWW}{SL} \times 100$$

where FWW is flesh wet weigh (g) and SL is shell length (mm).

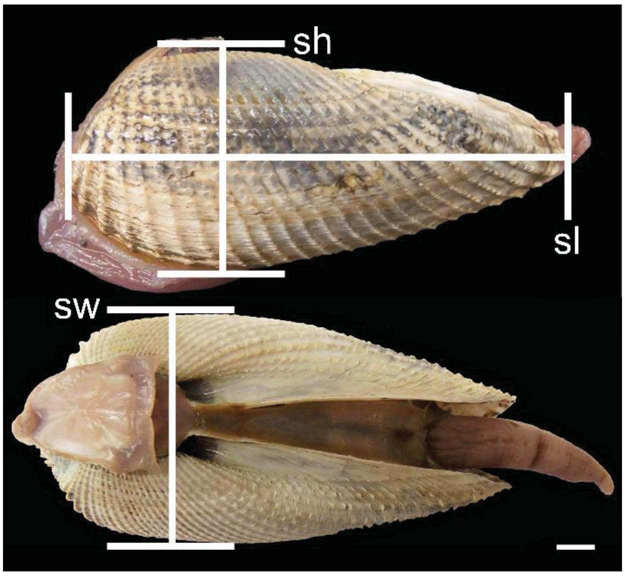

Figure 1. Shell measuring axes of *Cyrtopleura costata*. **sh**: shell height (axis between the umbo and the opposite end; **sl**: shell length (anteroposterior axis); **sw**: shell width (axis of greatest distance between the outer surface of each valve. Scale bar = 1 cm.

A longitudinal section of the visceral mass was submitted to routine histological analysis, as adapted from Borzone et al. (2001). Histological images were captured for sex diagnosis (adapted from Borzone et al. 2001; Corte 2015 and Jeon et al. 2012) and biometric analysis of gametogenesis using ImageJ software (Schneider et al. 2012).

Biometric analysis (adapted from Lazzara et al. 2012) was performed for both sexes. For each female, a total area of thirty random oocytes (only those with a visible germinal vesicle) was measured. For each males, 10 follicles were measured in terms of their total area, area occupied by mature spermatozoids and by spermatical radial series. All area measurements were converted to diameter (d) ($d = 2.\sqrt{\text{total area}/\pi}$) to enable comparison with other studies.

Sexual dimorphism in shell biometric data and Condition Index (CI) were tested using Student's t-tests. The sex ratio was tested by χ^2. Shell and oocyte size classes were defined from the Sturges' rule (Sturges 1926). Bhattacharya' method (Bhattacharya 1967) was employed to decompose the oocyte diameter frequency distribution. The relationship between CI and shell length was tested using linear regression. A multivariate procedure (Principal Component Analysis: PCA) evaluated the relationship of the gametogenic cycle with environmental factors (seawater temperature and salinity). All analyzes were performed using RStudio and SIGMAPLOT 11.0 software and a significance level of 95 % was assumed.

RESULTS

In Paranaguá Bay's euhaline sector, area of occurrence of *C. costata, water* temperature ranged from 20.5 (April/19) to 29.9 °C (January/19) and salinity from 20 (February/19) to 29‰ (December/18) (Fig. 2).

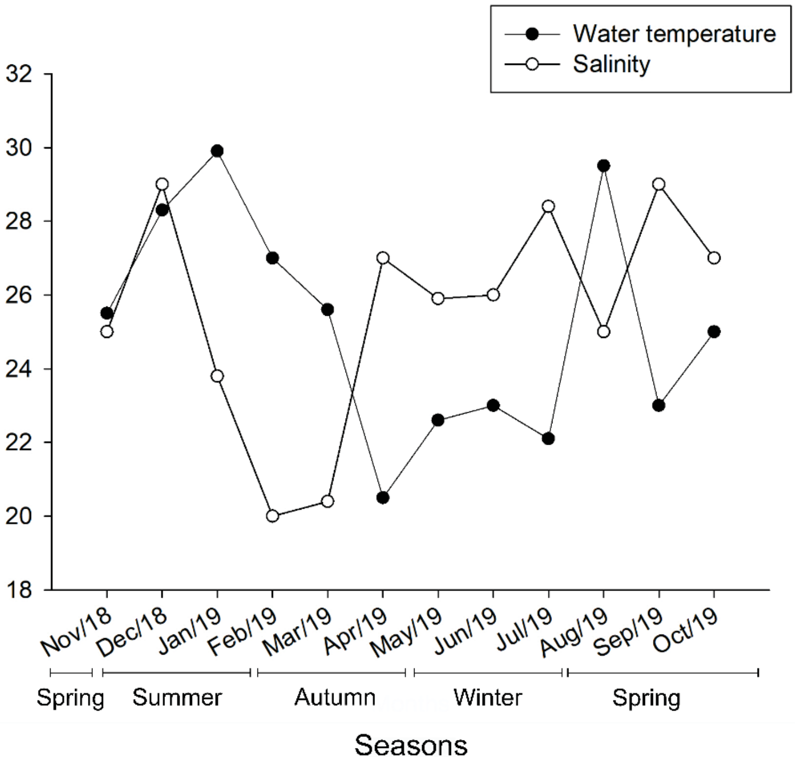

Figure 2. Monthly variation of temperature and salinity of water in Paranaguá Bay between November/2018 and October/2019.

A total of 129 bivalves have been analyzed between November/18 and October/19. Shell length ranged from 39.8 (May/19) to 112.8 mm (April/19) ($\bar{x}$ = 85.8, SD = 13.2). April, June and September/19 months were different (p <0.001) from the other, with the largest bivalves reported (Fig. 3). The Sturges' method separated the population into 12 size classes (every 4.5 mm), from which 8 classes (68.5–72.9 mm), comprising 88.06% of the individuals, were considered for subsequent analyzes.

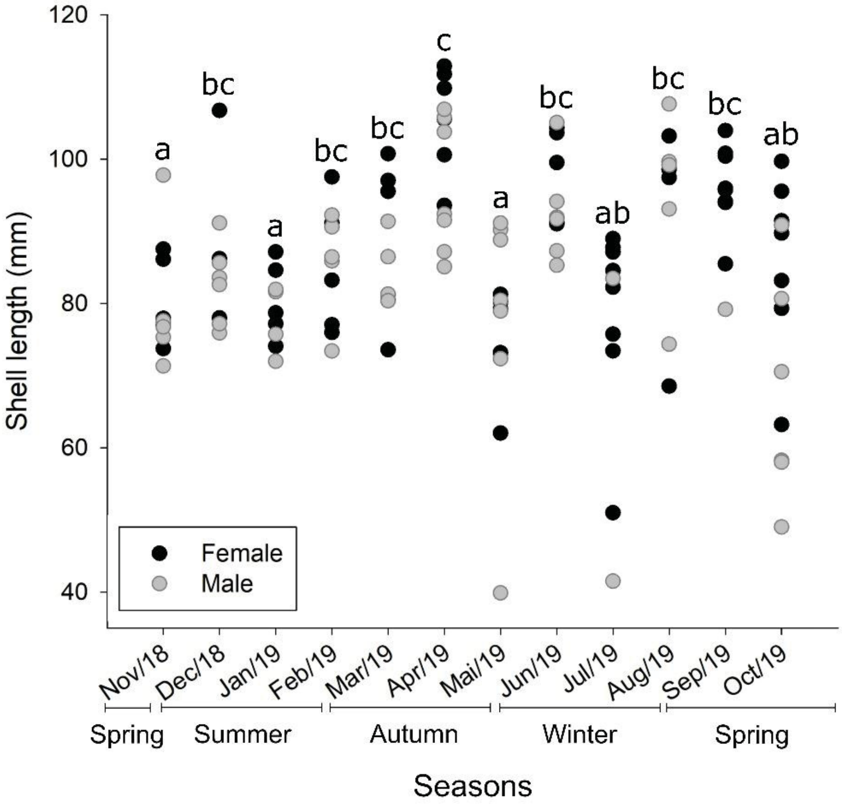

Figure 3. Shell length (N = 10 – 13/month) variation of *Cyrtopleura costata* in Paranaguá Bay throughout the sampling period.

There was no macroscopic sexual dimorphism for any biometric parameters (length, width and height) (p> 0.05). When analyzed monthly, the sex ratio was 1:1 in all months analyzed (p <0.05), except in September/19 where a predominance of females (1:9) was observed (Fig. 4A). When analyzed by size class, the sex ratio was 1:1 for the first seven classes (68.5 – 95.5 mm) (p <0.05). In the last two classes (100.0 - 108.9 mm) there was a predominance of females (1:8 and 1:4, respectively) (Fig. 4B).

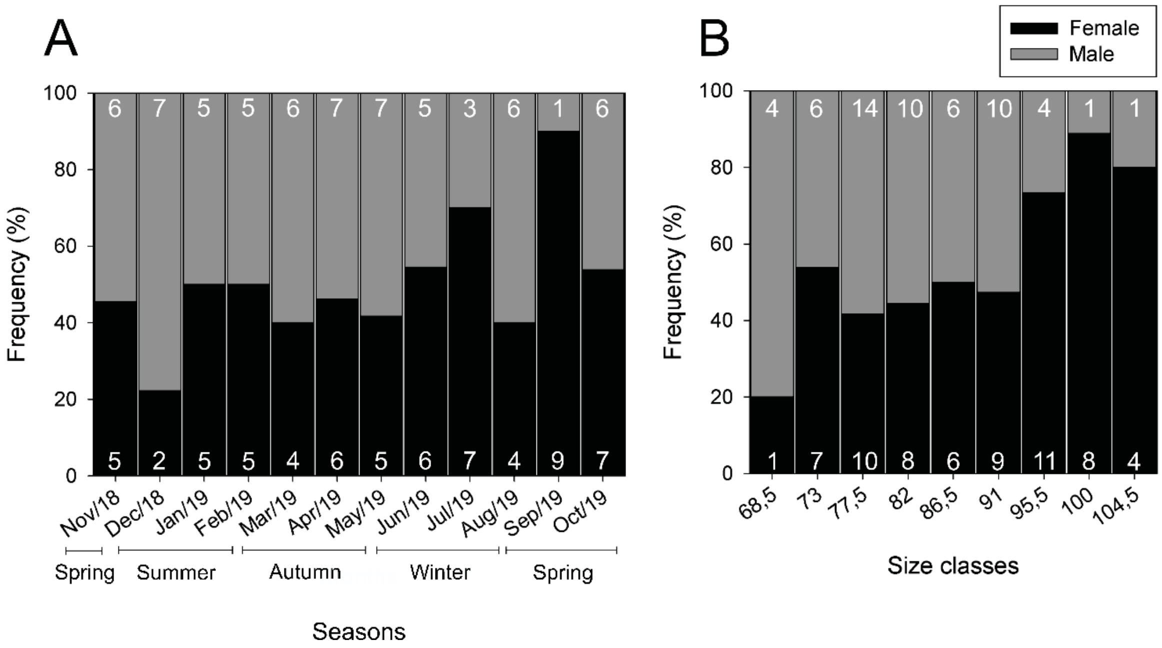

Figure 4. Sex ratio monthly (a) and by size class (b) of *Cyrtopleura costata* in Paranaguá Bay between November/18 and October/19. White numbers represent the N of each sex in each month.

Six different gametogenic stages were identified for females: *Proliferation*, *Early ripe*, *Late ripe*, *Spawning with proliferation*, *Spawning with resting* and *Resting* (Tab. 3 and Fig. 5), only the first four being observed for males (Tab. 3 and Fig. 6). When more than one stage was present in the same individual, the most prevalent (visually) was considered.

Table 3. Description of the gametogenic stages of *Cyrtopleura costata* males and females throughout of a year in the euhaline sector of Paranaguá Bay, southern Brazil.

Female (Fig. 5)	
Proliferation	Many oogonia fill follicle walls, visible lumen, large amount of connective tissue.
Early ripe	Distended follicles, decreasing the amount of connective tissue. Small oocytes, close to or attached to the follicle wall. Some mature cells can already be identified in the lumen.
Late ripe	Ripening-like appearance except for the follicle lumen that becomes full of mature oocytes of circular shape and uniform size.
Spawning with proliferation	Follicles continue to occupy large portions of the gonadal tissue. Mature oocytes are still observed in the lumen. Follicle wall is thick and with large amount of germ cells.
Spawning with resting	Follicular contraction is evident, mature cells can still be identified in the lumen. Follicle wall becomes thinner due to absence of germ cells. Connective tissue is expanded.

Resting	Small or absent follicles, absence of germ cells. Residual and resorbing oocytes may be observed. Increased amount of connective tissue.
Male (Fig. 6)	
Proliferation	Small and deformed follicles, with no apparent cellular organization, intense proliferation of spermatogonia near the follicle wall, which may occupy the follicular lumen. Large amounts of connective tissue.
Early ripe	Follicles distend, decreasing the amount of connective tissue. Radial series becomes thick and some spermatozoa can be seen in the center of the follicular lumen.
Late ripe	Follicles occupy all the gonadal tissue, essentially there is no connective tissue. Reduced or absent radial series, the mass of mature spermatozoa fills the entire lumen and may take the form of swirls.
Spawning with proliferation	Follicles are contracted and tissue disorganization can be identified, empty spaces can be observed in the lumen, but residual spermatozoa are present and, according to follicle size, in large quantities. Radial series becomes thick again and increases the amount of connective tissue.

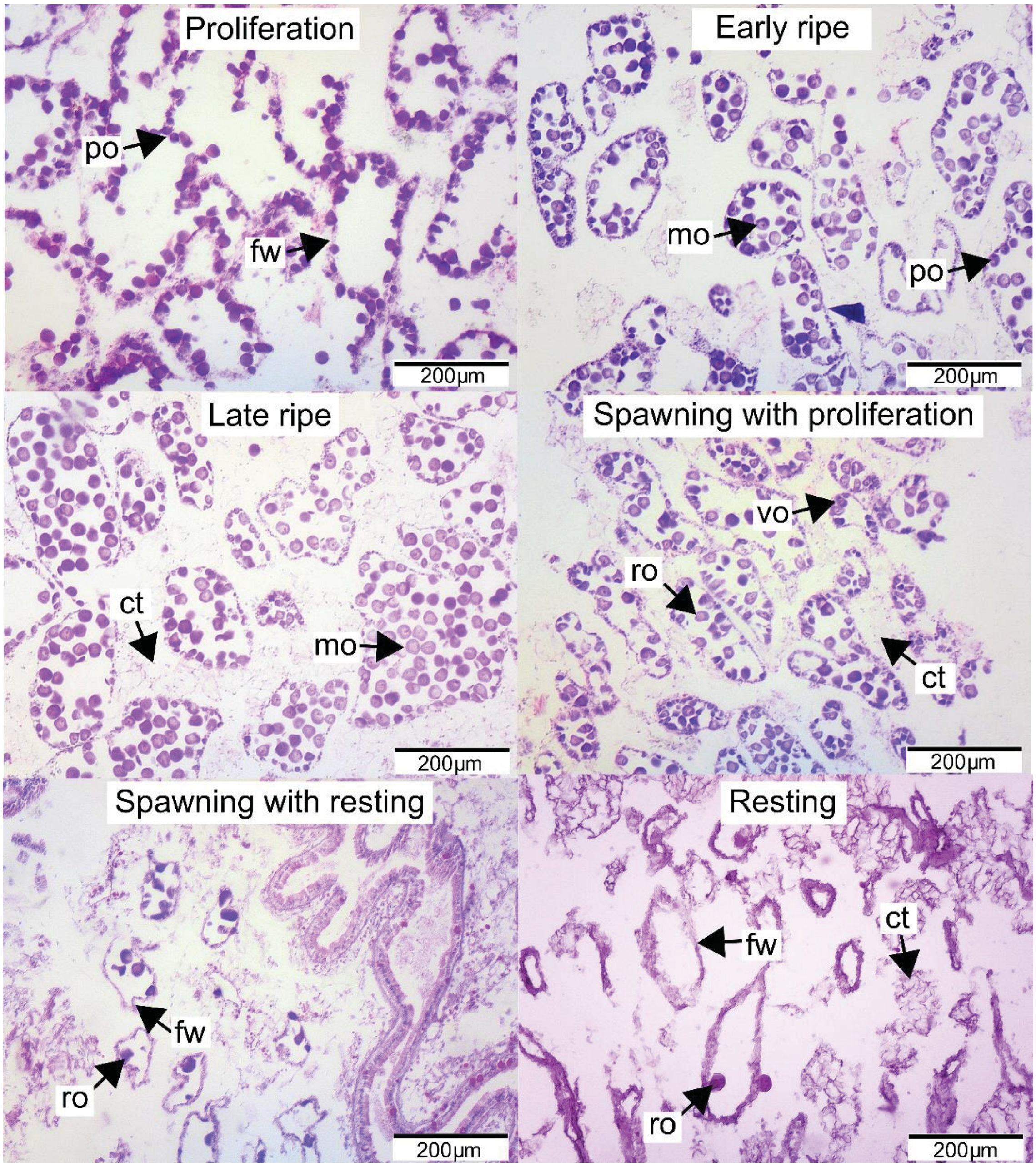

Figure 5. Microscopic aspects of the gametogenic stages of female *Cyrtopleura costata*. Abbreviations: **ct**: connective tissue; **fw**: follicle wall; **mo**: mature oocyte; **po**: previtellogenic oocyte; **ro**: residual oocyte; **vo**: vitellogenic oocyte.

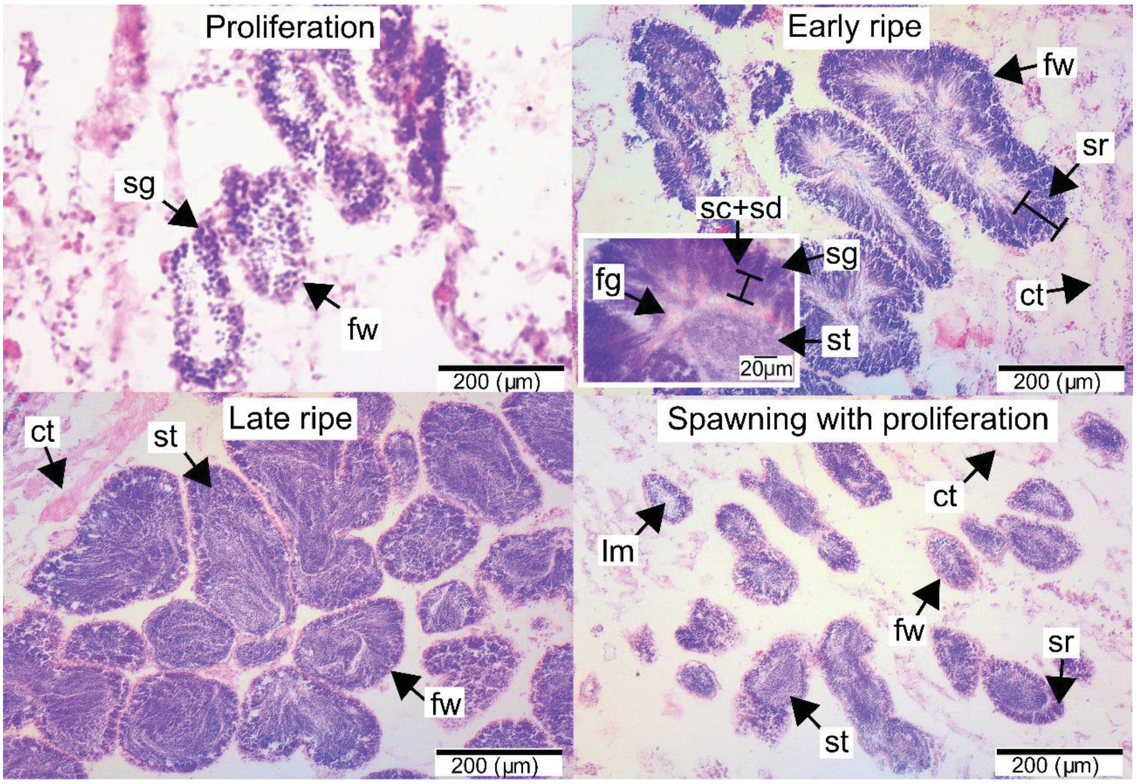

Figure 6. Microscopic aspects of spermatogenic stages of male *Cyrtopleura costata*. Abbreviations: **ct**: connective tissue; **fg**: flagella; **fw**: follicle wall; **lm**: lumen; **sc**: spermatocytes; **sd**: spermatids; **sg**: spermatogonia; **sr**: spermatic radial series; **st**: spermatozoids.

The reproductive cycle of *C. costata* was characterized at least two to three simultaneous stages per month. For females, investment in *Proliferation* occurred in late summer (February/19 with 20.0% of them at this stage), early winter (June/19, 16.7%) and spring (September/19, 10.0%). *Ripe* females (*early* or *late*) were observed throughout practically the whole year, mainly in early summer (December/18 and January/19, with 100.0% and 80.0%, respectively), late autumn (May/19 with 80.0%) and early spring (50.0 and 100.0% in September and October/19). With the exception of February/19 and during winter (June, July and August), in which the *Spawning with proliferation* (80.0% in February/19; 50.0% and 62.5% in June and July/19, respectively) and *Resting* stages (33.3%, 25.0 % and 66.7% in June, July and August/19) were predominant (Fig. 7).

For males, investment in *Proliferation* was punctually recorded in late autumn (14.3% in May/19). *Ripe* males (*early* or *late*) were observed throughout the year, mainly in late spring (November) and early summer (December) of 2018 and during spring of 2019 (September and October), with 100.0% of individuals at this stage. *Spawning with proliferation* occurred between January and July/19, being more expressive from the middle of summer (60.0 and 80.0% in January and February/19, respectively) and in the beginning of autumn (83.3% in March/19) (Fig. 7).

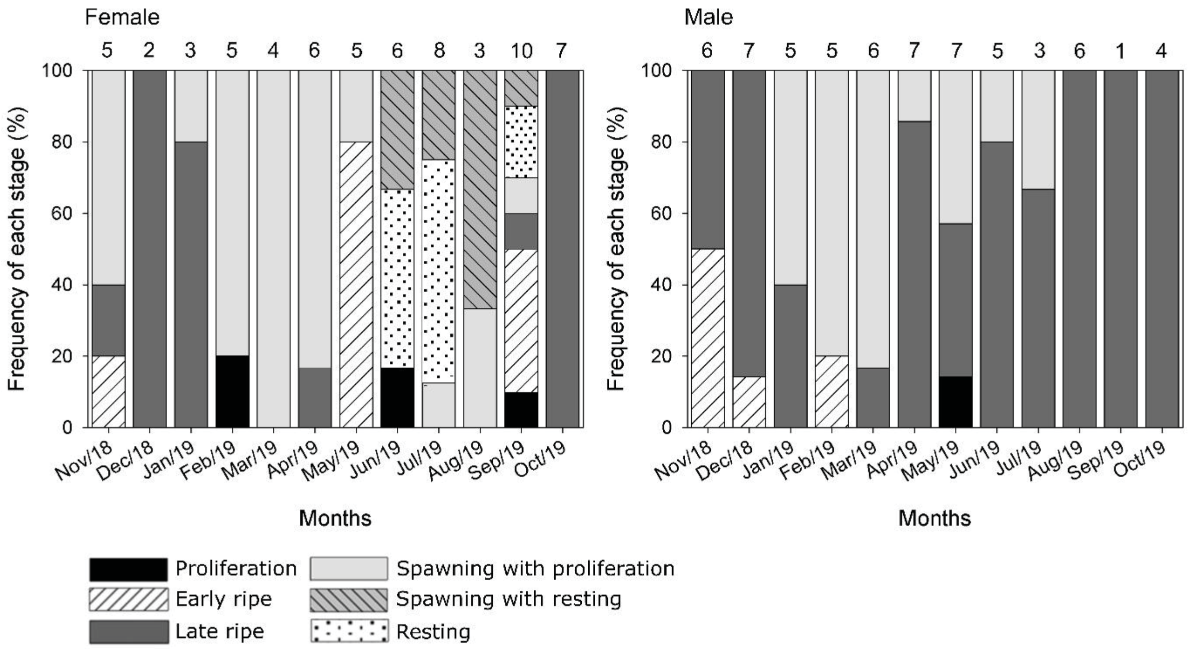

Figure 7. Gametogenic stages of female (right) and male (left) of *Cyrtopleura costata* throughout the sampling period. Numbers above the columns indicate the sample number of each month.

A total of 1,950 oocytes with a diameter ranging from 3.6 µm (June/19) and 39.2µm (July/19) ($\bar{x} = 23.3$, SD = 6.1) were measured. The largest cells were registered in June and July/19, with 28.3 and 36.1% of oocytes > 30µm, respectively. The smallest were observed in June and August/19, with 23.1 and 22.5% of cells < 13.5µm, respectively (Fig. 7). Based on the Bhattacharya's method of frequency distribution decomposition, the oocyte population was divided into 4 groups and stages were defined based on oocyte diameter. Oocytes <13.5µm were considered as in *Proliferation* stage, 13.6 – 22.4µm as *Early ripe*, 22.5 – 30.0µm *Late ripe* and >30µm *Spawning*. It was possible to observe a modal shift in the frequency distribution histograms of the oocyte diameter (Fig. 8) that partially reflects the stages described in the qualitative analysis (Fig. 7). Two points must be taken into account when comparing the distribution of stages between quantitative and qualitative analyzes: (1) not all oocyte diameters were sampled in a few months (as in November/18, February, March, April and May/19) and (2) in the qualitative analysis, when more than one stage was present, it was considered the most prevalent, not reflecting the variety of oocyte diameters (as in February, March, April, July, August and October/19).

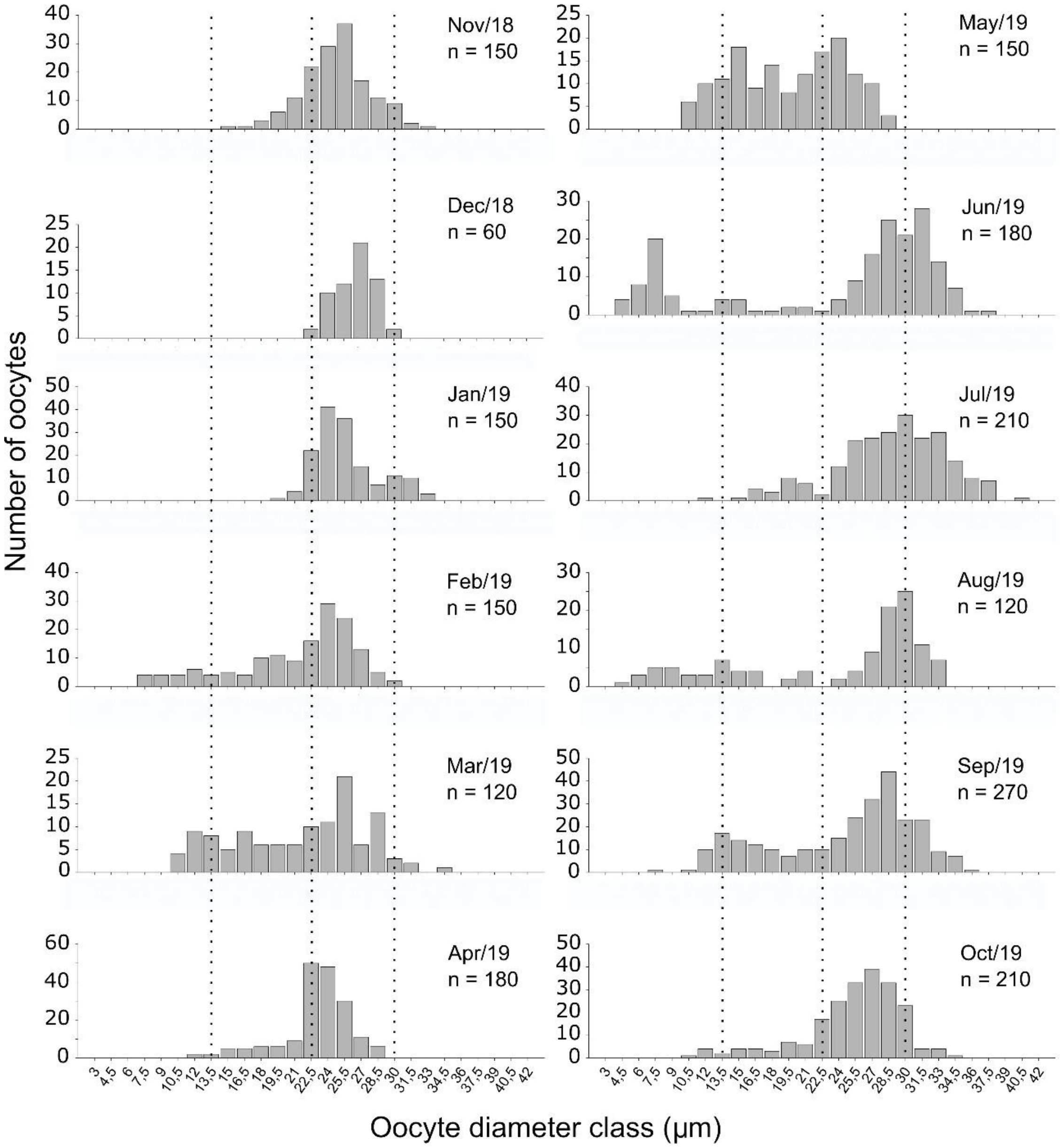

Figure 8. Monthly relative frequency of oocyte diameter of *Cyrtopleura costata* at Paranaguá Bay. The dotted lines represent the cohorts determined by the Bhattacharya's method.

For males, 604 follicles were measured. The percentage of follicle occupation by radial series was reverse to the percentage of occupation by spermatozoids, except in the months of September and October/19, which had no difference between SR and spermatozoa occupation ($p > 0.05$). The months with the lowest amount of spermatozoa were June and July/19, while the highest spermatozoa count was recorded in February and March/19 (Fig. 9).

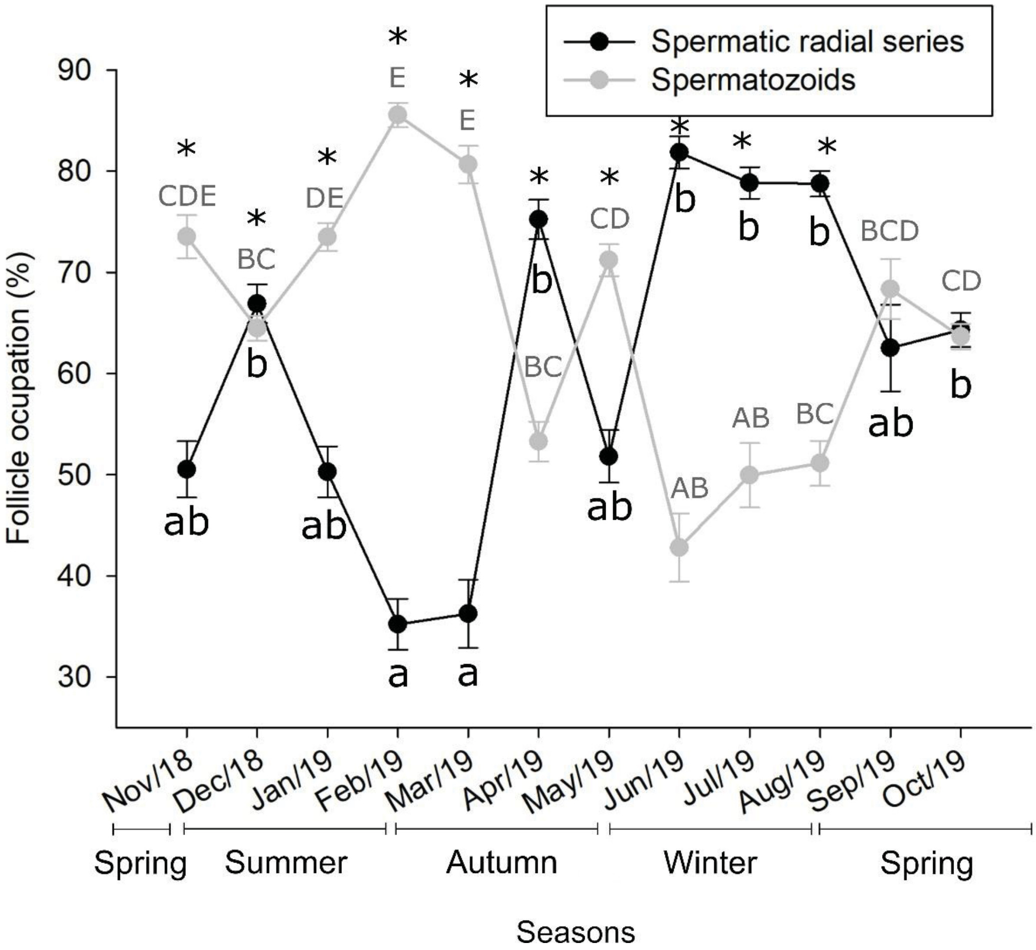

Figure 9. Monthly variation of the follicle occupation by spermatic radial series and by mature spermatozoids in *Cyrtopleura costata* throughout the sampling period. Data presented are mean ± standard error. Letters indicate statistical differences in occupation by radial series (black) and spermatozoids (gray) between months, asterisks indicate difference between occupancy percentage by radial series and spermatozoids.

The Condition Index of *C. costata* ranged from 6.8% (October/19) to 46.8% (September/19) ($\bar{x} = 29.3$, SD = 8.4). There was no significant difference between females and males ($p > 0.05$). The highest index values were recorded in June, August and September/19, which were different from the all others months ($p < 0.001$) (Fig. 10). Linear Regression' analysis showed that Condition Index varies with bivalve size ($p < 0.001$, F = 156.1, Rsqr = 0.5) as expected, but for animals larger than 80 mm the dispersion tends to increase (Fig. 11).

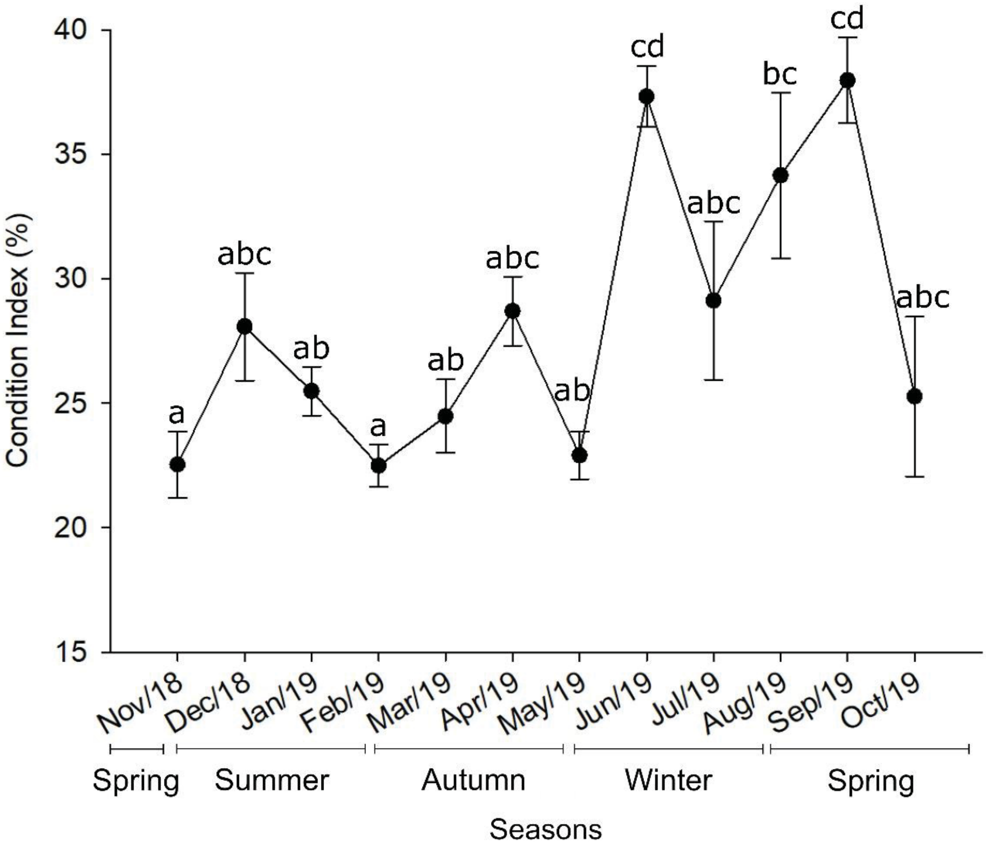

Figure 10. Monthly means of the Condition Index of *Cyrtopleura costata* at Paranaguá Bay. Data presented are mean ± standard error.

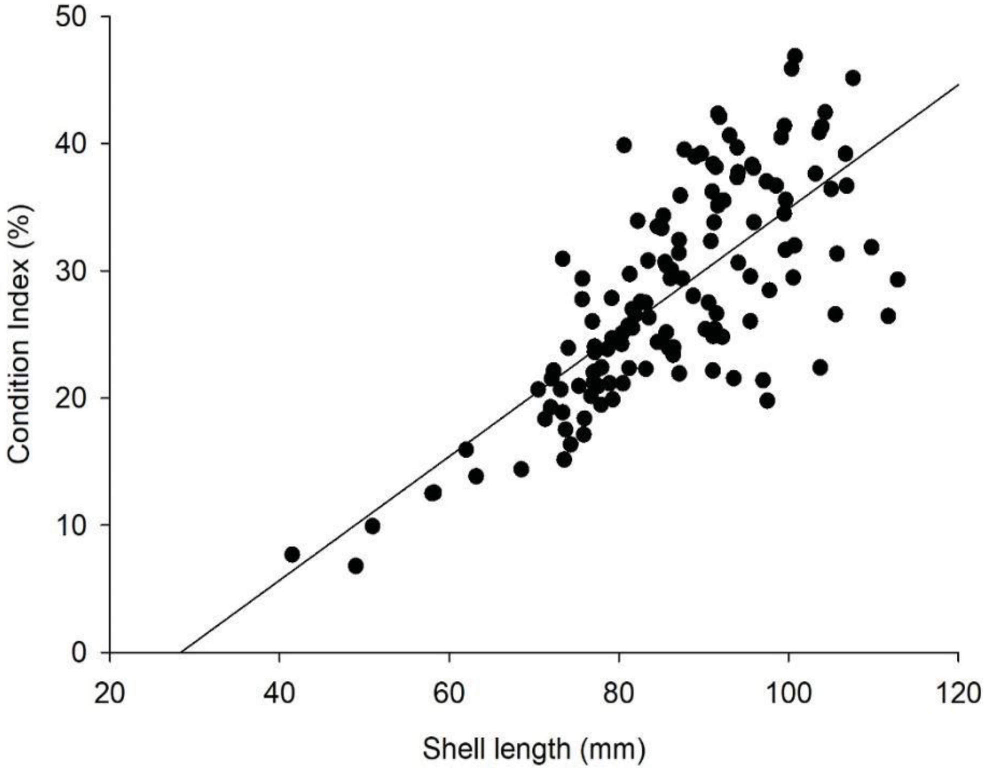

Figure 11. Condition Index and shell length relation in *Cyrtopleura costata* throughout the sampling period.

The PCA in the first dimension (38.5% of explicability) indicated a relationship between the increase in Condition Index (score 0.6) and the spawning period of the females (0.5). In the second dimension (22.4%), the decrease in temperature (-0.5) was related to the increase in the amount of mature sperm (0.7) and also to the spawning of females (-0.4).

DISCUSSION

The reproductive pattern of *C. costata* from Paranaguá Bay was in agreement with that observed for bivalves from tropical and subtropical regions inhabiting the intertidal environment. The population varied between 39.8 to 112.8 mm, the sexes were proportional, except in the classes of larger sizes, which presented a prevalence of females. Investments in proliferation were observed in February, June and September/19 for females and in May/19 for males. Mature animals were observed in practically all the year, except for a reproductive rest of the females during the winter (June, July and August/19), observed in the qualitative analysis. Between late summer and mid-autumn (from February to April/19), both sexes exhibit a synchronized spawning peak. For females, specifically, an intense spawning is repeated during winter (from June to August/19) which, although to a lesser extent, releases the largest oocytes produced in the year.

Abiotic factors

For marine invertebrates that reproduce sexually, exogenous factors regulate gametogenesis and ensure synchronicity between the sexes (Subramoniam 2018). Bivalves, in general, exhibit a high phenotypic plasticity in response to environmental conditions (see Collin 2013). The main factors that control marine bivalve gametogenesis are temperature and salinity (Corte 2015). Variations of these factors act exhibit a series of reproductive patterns that may vary within a single species according to the environment (Sastry 1979; Morton 1991; Enríquez-Díaz et al. 2009). Despite this, little is known about the extent of variation of these factors reflected in the gametogenic cycle, since most reproductive cycle works are carried out with point collections in the field. Laboratory experiments should be conducted to investigate which changes serve as triggers for species and, even, how climate changes affect their reproductive patterns. Without this information, the prediction about the success of populations is uncertain.

In the subtropical region studied (25° S), the climate is characterized by cold polar masses in winter (June – August) and stationary warm fronts during summer (December – February). A rainy season occurs in late spring and can stay during the summer. The dry season occurs from late autumn to late winter (Lana et al. 2001). In the present study, the highest values of water temperature and salinity were recorded in summer and spring. The lowest salinity values occurred in late summer and early autumn. Between mid-autumn and late winter, the lowest temperatures were observed, along with the dry season. In addition to the data obtained during sampling of the animals, additional data were added, from sampling events carried out in neighboring areas (Acquaplan 2019), for a more accurate representation of the annual variation of temperature and salinity of the estuary.

Temperature is one of the main factors affecting the growth of pholadids (Wong 1982; Gustafson et al. 1991). For *C. costata* of Florida it influenced the survival and growth of larvae, which were impaired when temperature was too high (~30 ºC), along with high salinity values (> 30‰, Gustafson et al. 1991). In addition to being related to the transfer of reserves between tissues of the females (Luz and Boehs 2011), temperature fluctuations not only interfere in plankton composition and food availability, but also forsters gamete maturation (Jeon et al. 2012; Morán et al. 2018).

Shell length and populational dynamics insights

Although this species lacks data for comparison, *C. costata* from the Northern Hemisphere (Florida, USA) seems to reach larger sizes, reaching 183.0 mm (see Turner 1954). This difference does not seem to be related to latitudinal gradient, since the coast of Florida and Paraná are at 25º (to the north and south, respectively).

Eight size groups were selected for the population studied, which not appear to be an increase in the average shell length throughout the year, except during late summer (January – February) and early autumn (March) which seems to culminate in the largest recorded individuals in the mid-autumn (April). Winter (June – August) and spring (September – November) months smallest bivalves are recorded (here treated as "young", although they have already started reproductive activity), suggesting a population renovation (Allen 1969; Wong 1982). The small number of young individuals throughout the year suggests that newly established bivalves only balance the mortality (older animals) and this pattern was also observed for *Z. crispata* (Allen 1969).

The smallest individual sampled (39.86 mm) already had developed gonads, despite occupying the smallest space in the visceral mass. The difficulty of finding pholadid recruits was pointed by Allen (1969), and new reports on this subject have not been recorded. Our hypothesis is that the opening of the burrows may be too small and imperceptible during gatherings, mainly due to the movement of the substrate.

During the summer (December – February), the population of *C. costata* studied maintained a mean size of ~80.0 – 90.0 mm, with no young (<70.0 mm) and rare individuals with shells close to 100.0 mm. High temperatures seem to interfere not only with larval development (as noted by Gustafson et al. (1991)), but also with the growth of adult specimens.

Interestingly, the largest sizes of the population of *Zirphaea crispata* (a pholadid of temperate regions) of Northumberland (≅ 55º N), were also observed in the autumn, in addition to this population also being divided into 8 cohorts of size (Allen 1969). A similar result was observed for *Aspidopholas obtecta* of Deep Bay (Hong Kong, ≅ 22º N) (Wong 1982). Adult pholadids, in general, seem to prefer low temperatures. Despite inhabiting tropical/subtropical regions (Turner 1954), their performance in growth and reproduction seems to be reduced in the hot months along the distribution of populations.

A similar response has already been recorded for other infaunal species inhabiting of the intertidal regions (e.g. populations of *Cerastoderma edule* and *Tapes decussatus* of Spain, ≅ 43º N), which high temperatures can induce high metabolic rates, leading to stagnation or decreased somatic growth (Navarro and Iglesias 1995; Zhang et al. 2020).

For larval stages, the condition may be different, since Gustafson (1991), when carrying out experiments in the laboratory with larvae of *C. costata* from Florida (25º N), observed higher growth rates at 30ºC. This can be an adaptive characteristic that favors the dispersion of the larvae (Hadfield and Strathmann 1996).

Sex ratio

Sexual dimorphism is rare among bivalves (Sastry 1979), and accordingly, no differences between females and males was observed for *C. costata*. There were no differences between biometric parameters (length, width and height), nor in the coloration of the gonads in different stages. However, some authors observed macroscopic differenciation of ripe gonads for Pholadidae, as already noted for other bivalves (e.g. Veneridae). When mature, the female gonad of *B. candida* is white in color, while the male is grayish (Duval 1963). For *P. orientalis*, the female gonad becomes reddish and the male whitish (Ng et al. 2009).

Although gonad color is commonly used for the sexual determination of marine bivalves, this is not a safe method for all stages of the reproductive cycle. During the period of sexual maturation, female gonads may become reddish due to lipid inclusions in oocytes of mussels (Lunetta 1969). In other gametogenic stages, this feature may not be observed. In addition, the soft tissue coloration may be altered according to the individual's health (Grimm et al. 2016; Carella et al. 2019).

Our results demonstrated that *C. costa* is a dioecious species, an ancestral characteristic of Bivalvia (Collin 2013), with no record of hermaphrodite individuals. The sex ratio was 1:1 in essentially the whole sampling period, as common for marine and estuarine bivalves (Coe 1943; Morton 1991), although the largest individuals of *C. costata* were all females. The month of September/19 was the only in which the sex ratio was not proportional for males and females (1:9), which coincides with one of the months with the largest recorded bivalves. This variations in sex ratio may suggest early male-linked mortality (Coe 1943; Morton 1991) or sexual reversal, as observed in *Crassostrea gigas* (Park et al. 2012).

Sequential hermaphroditism is common for Bivalvia and generally occurs in the most derived clades (Collin 2013), this agree with the great diversification of Imparidentia, the superorder Pholadidae is inserted in (Bieler et al. 2014). Other pholadids also have a biased sex ratio between size classes. *Z. crispata* and *B. davidi* exhibit a prevalence of males with the increase in shell length (Allen 1969; Jeon et al. 2012). Futhermore, for a natural population of *Pholas orientalis* of Philippines (10º N), individuals were simultaneous functional hermaphrodites, and self-fertilization seemed to be the rule (Ronquillo and McKinley 2006).

Size differences between males and females and biased sex ratio are strong indicators of sexual change in invertebrates in general, especially when sexual dimorphism and sex-related mortality are ruled out (Coe 1943; Jeon et al. 2012; Collin 2013). For the studied population, no stage of gonadal transition from male to female was registered, although a high sample N is necessary for this (Collin 2013). Protandrous mollusks usually exhibit a series of characteristics such as: external fertilization (evidenced mainly by Gastropoda), sedentary lifestyle, gregarious habit and the ability to reach large sizes (see Collin (2013)). All these characteristics are present in *C. costata*, and protandry should be better investigated for this species.

Qualitative analysis and reproductive cycle

Cyrtopleura costata ends its burrowing phase when it reaches approximately 15.0 mm in length (Gustafson et al. 1991), after which the foot becomes small and insertion into the visceral mass is superficial, as observed in this study. The same occurs in the pholadids *Penitella penita* and *A. obtecta*, whose foot is stunted and the visceral mass is expanded with fully developed gonads (Evans 1970; Wong 1982). This indicates increased reproductive activity/investment when the burrowing phase is over. By limiting energy expenditure with burrow activity, all available energy is directed to gamete formation (Evans 1970).

The fertilization of *C. costata* is external and no adults were observed retain embryos, as already observed for other pholadids (see Turner (1954)). The gonad is not a discrete organ, but rather, it extends from the anterior extremity to the base of the foot, interspersing between the loops of the digestive system. Despite occupying the entire visceral mass, the follicles are separated from each other and, for females, the follicles were never completely filled with oocytes, even when mature.

This may indicate a low fertility (Morsan and Kroeck 2005). The same happens with the pholadid of intertidal regions *Z. crispata* (Allen 1969) and with the venerid *Amiantis purpurata*, inhabitant of tidal flats (Morsan and Kroeck 2005). This is probably due to the high variation in biological processes (such as high competitiveness, food availability and oxygen) of these ecosystems. In more stable regions, where less energy is expended to maintain other physiological functions, fertility tends to be higher (Corte 2015). Thus, low fecundity of bivalves of intertidal flats appears to be common (Schaffer 1974; Morsan and Kroeck 2005; Corte 2015).

Reproductive patterns are not phylogenetically related and had many independent origins (Subramoniam 2018). Coastal populations, especially in the intertidal environment, tend to have a certain periodicity of reproductive events in response to the environment, unlike deep-sea populations in which the clues as to temperature, salinity and increased availability of nutrients are not always noticeable (Scheltema and Williams 2009). Here, only bivalves from coastal environments will be considered.

Bivalvia exhibits a wide range of reproductive strategies (see Collin (2013)). The bivalve of hard substrate *Modiolus barbatus* (Mytilidae) from Croatia (≅ 42º N), for example, spawns during summer and early autumn (Mladineo et al. 2007). The infaunal *Mactra stultorum* (Mactridae) (Tunisia, ≅ 35º N) presents a spawning peak at the end of the summer (Chetoui et al. 2019), as well as *Scrobicularia plana* (Semelidae) from Bourgneuf (46º N) (Mouneyrac et al. 2008). Beukema et al. (2001), after analyzing the recruitment period of the infaunal bivalves *Cerastoderma edule*, *Mya arenaria*, *Mytilus edulis* and *Macoma balthica* for 3 decades in Balgzand (≅ 52º N), observed that the longest recruitment period of the juveniles occurred after the coldest periods, suggesting a strategy that avoids predators and competition, which is lower in this period. Cabiles and Soliman (2019), investigating the determinant environmental factors in the reproduction of bivalves Pectinidae found that the majority of species (from both tropical and temperate climates) spawned in the warmer months, which would probably be related to the high photosynthetic activity and availability of food in that period.

For females of *C. costata* of Paranaguá Bay (25° S) the proliferation occurred in late summer (February), early winter (June) and spring (September). Mature gametes were observed practically every month, suggesting small continuous spawning. During winter, the largest gametes produced throughout the year were released and residual oocytes and follicles were absorbed, leading to a slight period of resting phase, probably a moment of recovery after several spawning events and, especially, after this last most important. This phase was directly influenced by the low temperatures in that period (PCA analysis). For the males, the proliferation was only recorded in late autumn. Probably spermatogenesis occurs more quickly, as observed for other bivalves (e.g. *Tagelus plebeius*; see Hamdani and Soltani-Mazouni (2011); Ceuta and Boehs (2012); Camilo et al. (2018) and Antonio and Camacho (2019)), and the proliferation has not been sampled with the constancy in which it occurs. Like females, mature individuals have been observed throughout the year. The resting period was not observed, and the reproductive activity was continuous. The largest reproductive investment of this population occurred between autumn and winter (March - August), which both sexes exhibit synchronized spawning. According to our initial hypothesis, the reproductive pattern of *C. costata* is similar to that of bivalves in tropical and subtropical regions, where reproduction is continuous with periods of greater reproductive investment (Corte 2015; Christo et al. 2016; Camilo et al. 2018).

When we compare our results with the patterns observed in Pholadidae, we realize how varied the group's reproductive strategies can be. For tropical/subtropical regions, *C. costata* from Florida (25° N) kept in the laboratory, spawning peaks were in autumn and spring (Gustafson et al. 1991). *A. obtecta* from Hong Kong (≅ 22° N) spawns in the summer (Wong 1982), as well as *B. davidi* from South Korea (35° N) (Jeon et al. 2012). For temperate regions, *B. similis* from New Zealand (≅ 40° S) spawning during winter and spring (Booth 1983). *Z. crispata* from Malpeque Bay (≅ 46° N), the largest amount of veligers in plankton (after the incubation period, indicating spawning) was during the summer (Sullivan 1948) and, for another population from Northumberland (≅ 55° N), spawning peak occur during the autumn (Allen 1969).

This studys, although old (these patterns may have changed), reflect a wide range of reproductive patterns among pholadids that do not appear to be being driven only by latitude. The influence of this factor on the reproductive patterns of bivalves has long been a matter of debate (see Freites et al. (2010) and Corte (2015)). Other factors directly influence the maturation and release of gametes in addition to temperature, such as food availability, species sensitivity/tolerance and the ability to assimilate available nutrients (Navarro and Iglesias 1995; Cabiles and Soliman 2019). The evolutionary history of the group and endogenous factors are determinant in the reproduction not only of bivalves, but of marine invertebrates in general (Navarro and Iglesias 1995; Subramoniam 2018), this allows a wide intra and interspecific diversity of reproductive patterns. Most likely, all of these factors act together offering signals for the moment of maturation and release of gametes, as observed by Corte (2015), making each population respond according to the particularities of the environment.

Quantitative analysis

Quantitative analyzes allow to broaden the ecological approach of the study and increase the reliability of the results, as these will not be based solely on qualitative analyzes, which may incur error (Corte 2015). For the determination of the reproductive stage of marine invertebrates, the biometric categorization of stages should be a standard to be followed.

For males, the occupation by radial series has always been reverse to occupation by spermatozoa, since the maturation of these cells occupies the entire intrafollicular space (Sastry 1979). Exceptions were recorded in September and October/19, where radial series and spermatozoids occupied similar portions of the follicle. This condition may indicate a time for spermatide maturation (Souza et al. 2019) when, although the radial series is still thick, a reasonable amount of mature sperm can already be observed. The greater amount of mature sperm coincided with the decrease in temperature (PCA analysis), probably due to the high reproductive activity of the population in this period.

Oocyte size is a good indicator of the gamete quality and, consequently of reproductive investment (Morsan and Kroeck 2005; Corte 2015), since a gamete with a higher nutrient reserve increases the likelihood of larval success (George 1990; Massapina et al. 1999). According to biometric analysis, throughout the spring (September – November), oocytes tend to remain between 21 and 30µm, so that in summer (December – February) there will be a reduction in reproductive investment, probably due to high temperatures, as discussed earlier. Even late summer (February) females do not invest in large oocytes or young cells. From there, histograms show a bimodal tendency, indicating continuous spawning with proliferation (Borzone et al. 2001; Corte 2015). In winter (June – August) and early spring (September) the highest amounts of cells <13 and >30µm were observed, suggesting that this period was the one with the highest gametogenic activity.

For females, the description of the gametogenic cycle was more concise using as parameters the appearance of the gonadal tissue, the occupation by connective tissue and the thickness of the follicular wall, in addition to biometric analyzes. To the quantitative method reflect all stages, we suggest that all oogenesis cells of a given amount of follicles be considered. In the present study, random oocytes from female gonadal tissue were measured, which may have caused biased measurements that not adequately representing the oocyte size frequencies of all stages observed.

When compared to other bivalves, *C. costata* exhibits a much smaller average oocyte diameter (23.3µm). *Megallana gigas* (Ostreidae; Spain, ≅ 42º N), for example, has an average oocyte diameter of 46.5µm (Antonio and Camacho 2019) and *Modiolus barbatus* (Mytilidae; Croatia, ≅ 42º N) 48.3µm (Mladineo et al. 2007). *Anomalocardia flexuosa* (Veneridae; Araçá Bay, ≅ 23º S), an infaunal bivalve much smaller in length than *C. costata*, has an average oocyte diameter of 48.9µm (Corte 2015). For the infaunal *Amiantis purpurata* (Veneridae; Patagonia, 40º S), of a substrate and habit similar to that of *C. costata*, the maximum oocyte size was 33.1µm (Morsan and Kroeck 2005). One explanation for the small oocytes of *C. costata* may be the low fertility (Morsan and Kroeck 2005; Corte 2015), but also the high availability of food in sheltered environments (such as estuaries) that makes oocytes not need ample reserves (Mladineo et al. 2007), and may be smaller in size. Studies that focus on population dynamics of this species can help to clarify which explanation is the most suitable.

Condition Index

The condition index (CI) indicates the period of higher yield of the soft part of the bivalves (Chávez-Villalba et al. 2019). The formula used in this study was selected as it provides the best resolution for bivalve mussels (Galvao et al. 2015). In addition, it allows histological analysis to be performed with the same tissue, since the dry weight of the flesh is not required. The CI showed no differences between females and males, as it happened in the pholadid *B. davidi* (Jeon et al. 2012).

The months with the highest CI were during winter (June – August) and early spring (September), coinciding with the spawning peak and resting of the females (corroborated by the PCA analysis). Our results suggest that the condition CI is not completely associated with the reproductive cycle of *C. costata*, and may represent nutrient storage processes (Bi et al. 2016; Wang et al. 2017), mainly during a recovery period . Studies considering seasonal fluctuations in carbohydrate and lipid concentrations in reserve tissues (muscle, digestive gland and gonads) can confirm this information (Hamdani and Soltani-Mazouni 2011; Nie et al. 2016; Wang et al. 2017).

When compared to the CI of other bivalves, *C. costata* presents very high values (up to 46%). The highest index of the infaunal bivalve *Mactra stultorum* (Mactridae), for example, was 14.4% (Chetoui et al. 2019), of *Chionista fluctifraga* (Veneridae) 24.2% (Chávez-Villalba et al. 2019), *Chione pubera* (Veneridae) 22.0% (Borzone et al. 2001) and, for the mitilids *Mytela charruana* and *M. guyanensis*, 17.6 and 13.2%, respectively (Christo et al. 2016). This is probably due to the thin, fragile and light weight shell of this species. In addition to the soft parts growing beyond the length of the shell, making a reasonable portion of the total weight, in fact, consist of flesh. This pattern can probably also be expected for other pholadids.

Conclusions

The population varied between 39.8 to 112.8 mm, the sexes were proportional, except in the classes of larger sizes, which presented a prevalence of females. The smallest individual sampled (39.86 mm) already had developed gonads. The sex ratio was 1: 1 in essentially the whole sampling period, although the largest individuals were all females. Protandry should be further investigated in this species.

Integrated analysis considering gonadal tissue appearance, connective tissue occupancy, follicle wall thickness, cell types, CI and quantitative methods ensured an accurate description of the reproductive pattern of the population. Mature animals have been observed in practically all year round. Between late summer and mid-autumn (February – April), both sexes exhibit a synchronized spawning peak. For females, specifically, an intense spawning is repeated during winter (June – August) which, although to a lesser extent, releases the largest oocytes produced in the year, followed by a period of reproductive rest. The lowest temperatures recorded are related to the period of the largest reproductive investment of the population (spawning of the largest oocytes and the highest amount of sperm).

Some characteristics of *C. costata* deserve to be mentioned: (1) the size of the oocyte much smaller than that of other bivalves; the question to be answered is whether this is due to the low fertility of the population or the high availability of food in the estuary that makes the oocytes not need ample reserves, and (2) flesh yield (CI) higher than other bivalves; probably due to the characteristics of the shell and the growth of soft parts to beyond the length of the valves, a pattern that can be expected for other pholadids. In addition, the highest rates observed seem to be related not to the reproductive cycle, but to the storage of nutrients.

The reproductive pattern of C. costata from Paranaguá Bay was in agreement with that observed for bivalves in tropical and subtropical regions inhabiting the tides. Despite this, Pholadidae exhibits a wide range of reproductive patterns and strategies that do not appear to be related to latitude. The evolutionary history of the group and endogenous factors of the populations must be considered. The continuous reproduction of the species allows its exploitation throughout the year, except in the autumn and winter months, where the establishment of younger individuals occur, that in future will contribute to the reproductive input of the population and, mainly, due to the spawning peak in this period. In addition, crops should be encouraged for the conservation of natural populations, for this we suggest that adults be kept at a temperature between ≅ 20 - 25°C for better performance and reproduction of the species in artificial farming.

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4 CHAPTER 3

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Osmoregulatory physiology of the angel wing bivalve *Cyrtopleura costata* (Pholadidae), an intertidal burrowing mollusk

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ABSTRACT

Cyrtopleura costata is a native bivalve of the Brazilian coast, found in tidal flats, from eastern Canada down to Southern Brazil. It inhabits burrows about 40 cm deep and, unlike other bivalves, cannot isolate its soft tissues with complete valve closure, thus being more susceptible to the challenges of tidal fluctuations. The literature brings no data on its osmoregulatory behavior. The hypothesis tested here was that it has a good capacity of regulation of tissue hydration, higher than that found in bivalves which are capable of completely closing their valves during low tides. The physiological responses of *C. costata* to osmotic challenges was here evaluated through an *in vivo* experiment in which osmolality, [Na^+], and [Cl^-]of its mantle cavity fluid and hemolymph have been assayed, upon exposure of the animal to salinities between 10 - 50‰. In addition, the ability of isolated slices of 3 different tissues (siphon, mantle, and muscle) to regulate their hydration under osmotic challenges has been evaluated through an *in vitro* experiment, using salines corresponding to the salinities of 10, 30, and 50‰. *C. costata* showed better performance between 25-40‰, salinities within the normal range found in their habitat (~20-30‰), being more tolerant to hyper- than to hyposaline stress. In spite of this, the bivalve continues to circulate water to the mantle cavity even at salinities < 20‰. *C. costata* indeed has shown a greater capacity to regulate tissue hydration than bivalves capable of completely isolating themselves from the medium, under hyper-saline situations. Though not especially able to regulate tissue hydration under low salinity stress, the bivalve tolerates seawater dilution using the microhabitat of the burrow and some capacity to remain hyperosmotic to the environment. We demonstrate that the angel wing clam responded to the osmotic challenges as a typical euryhaline osmoconformer and suggest that the process of urine production in osmotic maintenance be further investigated in Pholadidae.

Keywords: Ecophysiology, Bivalvia, Osmoregulation, Tissue hydration

INTRODUTION

Estuarine environments provide high productivity and nutrient availability to the organisms that can cope with these highly variable systems. Estuarine and intertidal organisms are routinely exposed to wide variations in salinity, temperature, pH and amount of dissolved oxygen (Willmer, Stone & Johnston, 2009; Breaux *et al.*, 2019; Kennish, 2019). These environmental factors pose selective pressure and control the distribution and success of species in these ecosystems (Lavaud *et al.*, 2017). Bivalves, as well as other marine and estuarine invertebrates, depend on environmental conditions such as the ideal amount of organic matter, temperature and salinity to supply their metabolic demands and succeed in growth and reproduction (Santana & Rocha-Barreira, 2018).

Given the cyclical fluctuation in all these abiotic parameters, at the rhythm of the tides, avoidance behavior is quite often observed. Avoiding the change provides a primary response to abiotic challenges, saving crucial metabolic resources to maintain internal homeostasis. Bivalves can isolate their soft tissues with shell closure, altering the opening diameter of the siphons, decreasing the gill ciliary beat and adopting anaerobic mechanisms (Deaton, 2009; Roden, 2018; Woodin *et al.*, 2020). Behavioral escape responses are expected to be time-limited, to various degrees, depending on the animal and habitat characteristics. However, after some time, which can be from minutes to hours, other compensatory physiological mechanisms are normally triggered (Verdelhos, Marques & Anastácio, 2015; Lavaud *et al.*, 2017; Rola, Souza & Sandrini, 2017; Pourmozaffar *et al.*, 2019).

Salinity variation is one of the major challenges in estuaries and intertidal habitats. Marine molluscs are typically found to be osmoconformers, with subtle and mild capacities for vectorial salt transport across their epithelia (Pierce, 1982; Leader, Hall & Bedford, 1986; Deaton *et al.*, 1989; McFarland, Donaghy & Volety, 2013; Rola, Souza & Sandrini, 2017). That means that they are essentially, when open to water circulation, fairly permeable, allowing osmotic and ionic fluxes between external water and their mantle cavity water and hemolymph. Those that inhabit tidal habitats are by necessity euryhaline, thus relying on good capacity of their cells to perform volume regulation and thus tolerate wide salinity variation along the tidal cycles (Pierce, 1982; Ruiz & Souza, 2008; Rola, Souza & Sandrini, 2017). Cell volume regulation is also called "intracellular isosmotic regulation" (IIR), when changes in the intracellular pool of organic molecules (free amino acids - FAA's) and/or inorganic ions (Na^+, K^+, or Cl^-), exchanged with the extracellular compartment, results in either influx (Regulatory Volume Increase, RVI), or efflux (Regulatory Volume Decrease, RVD) (Pierce, 1982; Deaton, 2009; Pourmozaffar *et al.*, 2019). Cells exposed to a hyperosmotic medium tend to lose water, and volume regulation may occur through absorption of osmolytes and water (RVI), partially or totally returning to their initial volume. Otherwise, when exposed to hypoosmotic shocks, cells tend to gain water and may exhibit RVD through the release of osmolytes and then water into the extracellular fluid (Freire *et al.*, 2008; Deaton, 2009; Pourmozaffar *et al.*, 2019).

Physiological mechanisms to deal with salinity changes demand metabolic energy. Thus, less stressful conditions mean that more energy can be directed to growth and reproduction, as it is required, for instance, in aquaculture (Pourmozaffar *et al.*, 2019). In addition, research on such a yet unexplored resource also contributes to the ever more urgent investigation on the effects of climate change in coastal tidal habitats (Helmuth *et al.*, 2002; Rivera-Ingraham & Lignot, 2017; Ducrotoy *et al.*, 2019). It is essential to know how these species, each with its specific ecological role within these crucial ecosystems, will possibly deal with this scenario, their tolerance limits and physiological mechanisms used to deal with wide and rapid environmental variation, and even extreme weather events (Paalvast & van der Velde, 2011).

Bivalves of the family Pholadidae are highly adapted for the burrowing habit, are strictly marine or estuarine, with few exceptions (Turner, 1954). Unlike other bivalves (e.g. Mytilidae), pholadids are unable to isolate their soft tissues with complete valve closure, making them susceptible to environmental oscillations. Although widely distributed and economically relevant, both in gastronomic use and in damage caused by wood- and rock-boring (Wong, 1982; bin Ramli & Yusop, 2016; Alexandrov, 2017), very little is known about their physiology. Important notes made by Turner (1954) allow us to understand some physiological aspects of pholadids, such as the ability of many species to store calcium, the exclusive occurrence in marine or estuarine environments, with some species being tolerant to dilutions of seawater down to 50% for short periods of time.

The pholadid *Cyrtopleura costata* is native of the Brazilian coast, but with a very wide latitudinal distribution, from eastern Canada to southern Brazil (Rios, 2009; Velásquez, 2017; Cullain *et al.*, 2018). The species is found in tidal flats that have sandy-mud sediment, in burrows that can reach up to 40 cm deep (Gustafson *et al.*, 1991; Rothschild, 2004). When exposed, the inhaling and exhaling siphons protrude about 1 cm above the surface and circulate water and suspended organic matter into the gills.

Despite being a very relevant group in ecological (trophic chain and provision of micro-habitats, ecosystem services) and economic (fishing resource by traditional communities) terms, we know little about its ecophysiology. Available knowledge about its physiology is the research reported by Castagna

& Chanley (1973) who evaluated the behavior of *C. costata* to changes in salinity, noting that when transferred directly from 20 ‰, filtration was reduced to 12.5 ‰ and completely stopped from 7.5‰, with 5‰ the minimum survival salinity of the species. In addition, Gustafson et al. (1991) assessed the combined effects of temperature and salinity on the growth of larvae of *C. costata*, reporting that the best larval shell growth was at 30°C and 20‰ in the first eight days and at 30°C and 25‰ in the next 16 days of the period larval.

It is clear that additional studies on the physiology of osmoregulation of *C. costata* will contribute to knowledge on this ecologically relevant group of bivalves (Yennawar *et al.*, 1999; bin Ramli & Yusop, 2016). Our study goes beyond simple detection of tolerance to salinity variation in this species. Our hypothesis is that this bivalve, which cannot fully isolate itself from the water, has a good capacity to regulate tissue hydration, better than that commonly reported for common bivalves capable of completely isolating themselves from the environment by closing the valves.

MATERIAL AND METHODS

Study area and animals collection

Collections were performed manually by active search in Paranaguá Estuarine Complex, State of Paraná in Southern Brazil (25º43'53,17 ”S / 48º44'6305” W). They occur in sandy/mud tidal flats and were obtained during low tide. Their burrows are recognized through of the opening of siphons or the presence of coin-shaped feces, which is a characteristic of the species (personal observation). The angel wing's shells are thin and delicate and can break during transportation, then all individuals (total N ≅ 56, mean length = 81.1mm (SD = 9.1), mean weight = 48.7g (SD = 17.3), the sex was not identified) were carefully removed from their burrow and wrapped in aquarium-specific styrofoams (to avoid friction and valve breakage) (Albuquerque, 2010). Animals were then transported in gallons containing seawater from the collection site (salinity between 20 - 29‰), under constant aeration, at a maximal density of 15 animals/730g per 10 liters of seawater. Practically all animals transported in this manner survived the displacement period (~ 2 h) and subsequent procedures.

Physiological experiments

The bivalves were divided into two experimental procedures: *in vivo*, using the whole animal, and *in vitro*, using isolated tissue slices:

In vivo Experiments. The animals were acclimated for ~5 days at 30‰, room temperature (20 ºC ± 2), natural photoperiod and constant aeration. Each replica (4 in total) was conducted with 3 individuals per condition (hypoosmotic, control and hyperosmotic), with each bivalve tested individually per 2 L container. The animal remained submerged throughout the duration of the experiment, and each individual was tested only once. Every 1 hour, all the water in the container was replaced, following a protocol of 5‰-steps of either salinity decrease (hyposmotic challenge: 30, 25, 20, 15, 10‰) or salinity increase (hyperosmotic challenge: 30, 35, 40, 45, 50‰). Control animals (isosmotic conditions) were subject to the same protocol of water replacement for a total of 4 hours, but salinity remained as 30‰. Samples of the treatment water, mantle cavity water (expelled by the siphon) and hemolymph (pericardial puncture) were collected for osmolality, Na^+ e Cl^- assays, every hour, when the treatment water was exchanged.

Osmolality was measured (Wescor® 5520 VAPRO vapor pressure osmometer, USA) in undiluted samples. Sodium concentration was measured using a flame photometer (Digimed® - DM 61, Brazil) in samples appropriately diluted with deionized water. Labtest® (Brazil) commercial kits were used for the assay of chloride levels; absorbances were read on a spectrophotometer (Amersham Pharmacia Biotech® - Ultrospec 2100 PRO, Sweden). Samples were also appropriately diluted in deionized water, so that absorbances would remain within linearity limits of the colorimetric assay.

At the end of the experiments (after 4 hours), when bivalves had remained for 1 hour in the final salinity of 10, 30, or 50‰, the animals were photographed and a fraction of their anterior adductor muscle, mantle, and siphon of each individual (Fig. 1) was collected. These tissues were dehydrated at 60 °C for 48 hours, weighed, and the formula for the evaluation of tissue water content (TWC) was applied:

$$TH\ (\%) = \frac{WW - DW}{WW} \times 100$$

where WW is wet weight and DW is dry weight, both in grams (g).

In vitro Experiments. Bivalves were acclimated in the laboratory for ~2 days, under the same conditions described for the *in vivo* experiment. The experimental procedure was adapted from Freire et al. (2008) and Veiga et al. (2015). Each individual was cryo-anesthetized for about 20 minutes buried in crushed ice, without contact with melted water. Then, 3 tissue samples (~ 75 mg) were taken from each of the following tissues: the anterior adductor muscle, mantle, and siphon (Fig. 1). Immediately after collection, the tissue slices were immersed in control saline (30‰) for ~15 min. Each replica (4 in total) was performed on a multiwell plates with capacity for 5 mL and each of the 3 slices of the same tissue was immersed separately in a well containing one of the following salines, corresponding to seawater of salinities: 10 (hyposmotic), 30 (isosmotic), or 50‰ (hyperosmotic), for a total time of 120 min. Table 1 provides the composition of the 3 salines. Each tissue was then weighed every 15 minutes using an analytical balance (Bioprecisa® FA2104N, accuracy 0.0001g), for 1 hour. Final weight was obtained at time 120 min, and the slice had then its tissue water content determined, as described above for the *in vivo* experiment.

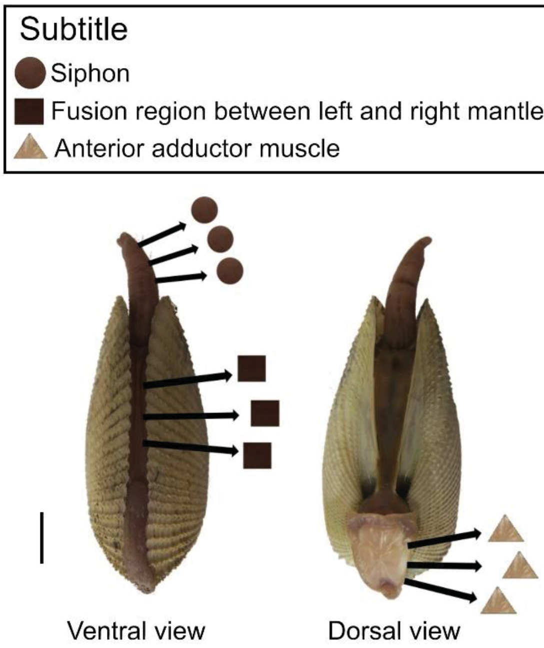

Figure 1. Tissues sampled (in triplicates) for assessment of water content in the *in vivo* experiment, and for regulation of tissue hydration in the *in vitro* experiment. Scale bar = 1 cm.

Tabela 1. Composition of control (isosmotic) and experimental (hypo- and hyperosmotic) salines of the *in vitro* experiment.

Salines	**NaCl**	**$MgCl_2$**	**KCl**	**$CaCl_2$**	**Measured osmolality (mOsm/kg.H2O)**
Isosmotic (30‰)	402,86	46,28	8,57	8,57	928
Hyposmotic (10‰)	134,33	15,33	3	3	231
Hyperosmotic (50‰)	671,66	76,66	15	15	1398

Concentration of all salts in mM. Components of constant concentration in all salines: glucose (5 mM), $NaHCO_3$ (2 mM), HEPES acid (5 mM) and glycine (5 mM); pH always set at 7.75.

Statistical analyses

For the *in vivo* experiment, the effect of the experimental salinities (25-10‰, hyposmotic challenge, and 35-50‰, hyperosmotic challenge) was tested, for each kind of fluid (water, mantle cavity water, and hemolymph), by one-way repeated measures ANOVA, followed by Holm-Sidak *post -hoc* test. Samples of fluid from experimental and control salinities were compared using Mann-Whitney rank sum test. Fluid samples from a same salinity/treatment were also compared using one-way ANOVA (Kruskal-Wallis for non-parametric data). Differences between the ionic concentration (Na^+ and Cl^-) of the mantle cavity water and the hemolymph were revealed using T-test, differences between salinities (10, 30 and 50‰) were revealed using Kruskal-Wallis one-way, followed by Dunn's test. Confidence interval analyzes were applied to assess the osmoregulatory response based on standard ionic values of seawater (obtained in Prosser, 1943). For the *in vitro* experiment, differences in tissue hydration/weight, considering the 3 osmotic conditions and the 5 exposure times were tested by two-way repeated measures ANOVA followed by Holm-Sidak *post-hoc* test. All tests had the limit of significance at 0.05 and were performed using the software RStudio and SigmaPlot 11.0 .

The comparison of *C. costata* performance in regulating tissue hydration, with that of other species from other studies that used the same methodology was performed using the index (Tissue Hydration Regulation Index, THRI) calculated as indicated below:

$$THRI = \frac{vv\ (\%)}{OC\ (\%)} \times 100$$

where vv is volume/weight variation, and OC the osmotic challenge offered. The lower the THRI of a species/tissue, the greater its ability to regulate its hydration when osmotically challenged.

RESULTS

In vivo – Osmolality, sodium and chloride concentrations

The three fluids did not differ from each other between 25 and 40 ‰. Under hypo- (≤ 20 ‰) and hyper-saline (≥ 45 ‰) conditions, hemolymph was more concentrated or more diluted than treatment, respectively. The mantle cavity did not differ from treatment in any of the experimental conditions and was not significantly different from hemolymph only in 10 ‰ salinity (Fig. 2).

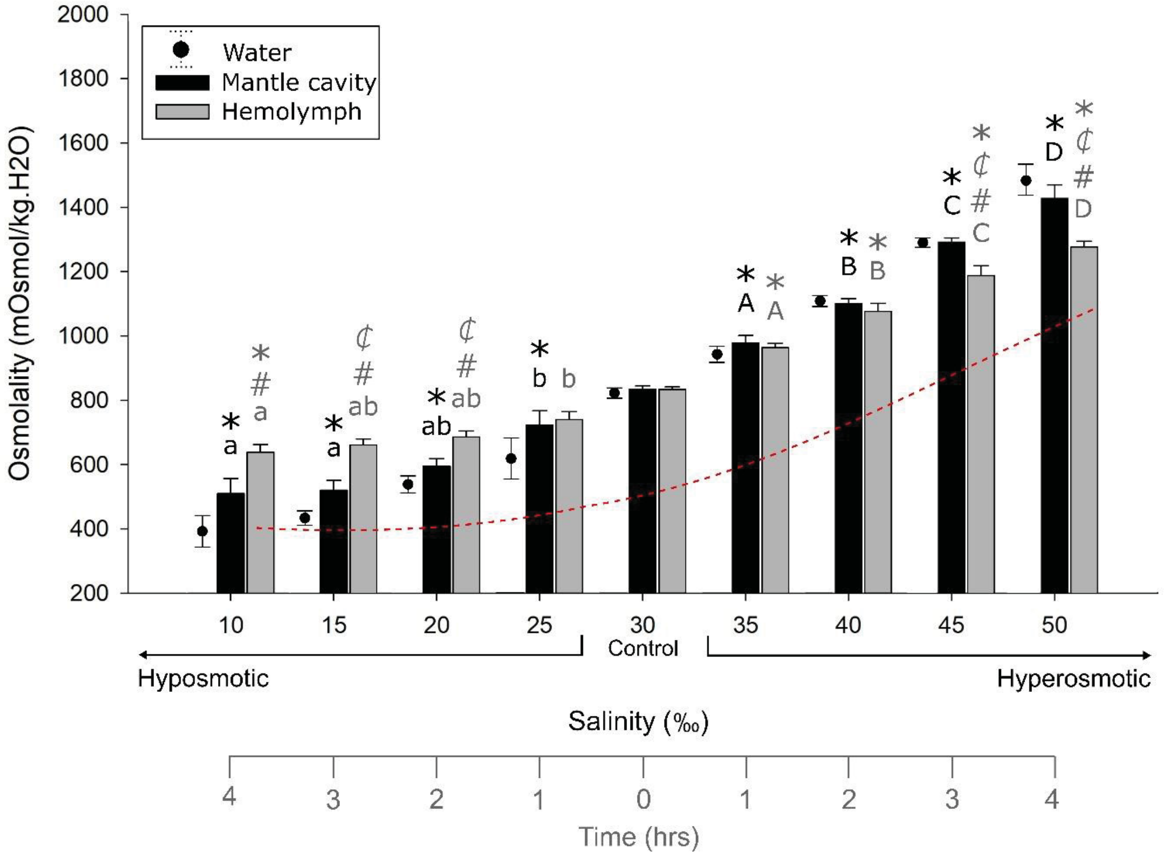

Figure 2. Aquarium water, mantle cavity water and hemolymph osmolality of *Cyrtopleura costata* at experimental (25-10‰, 35-50‰) and control (30‰) salinities (N = 11-13 for each condition). Data presented are mean ± standard error. Red dotted line demonstrates the tendency to increase hemolymph osmolality according to salinity. Different letters indicate statistical difference for a same fluid among salinities. Hashtag (#) indicate difference of the water in the same salinity. Capital letter C with stroke (₵) indicate difference between the osmolality of the hemolymph and mantle cavity water in the same salinity. Asterisk (*) indicate difference of the control.

The [Na^+] were lower than control (30 ‰) at both experimental salinities (10 and 50 ‰). Differences in the mantle cavity water concentration and in the hemolymph were observed at 30 and 50 ‰. According to the confidence interval analyzes, *C. costata* hyperregulates Na^+ in the hyposaline (10 ‰) and control (30 ‰) conditions, and hypo-regulates in the hyper saline condition (50 ‰) (Fig. 3).

The [Cl^-] in the mantle cavity water was different from the control (30 ‰) in both experimental salinities (10 e 50 ‰). The hemolymph was different from the control only in the hyper-saline condition (50 ‰). Differences between the mantle cavity water and hemolymph were observed only at 10 ‰. Based on confidence interval analyzes, *C. costata* hypo-regulates under all conditions analyzed (experimental and control) (Fig. 3).

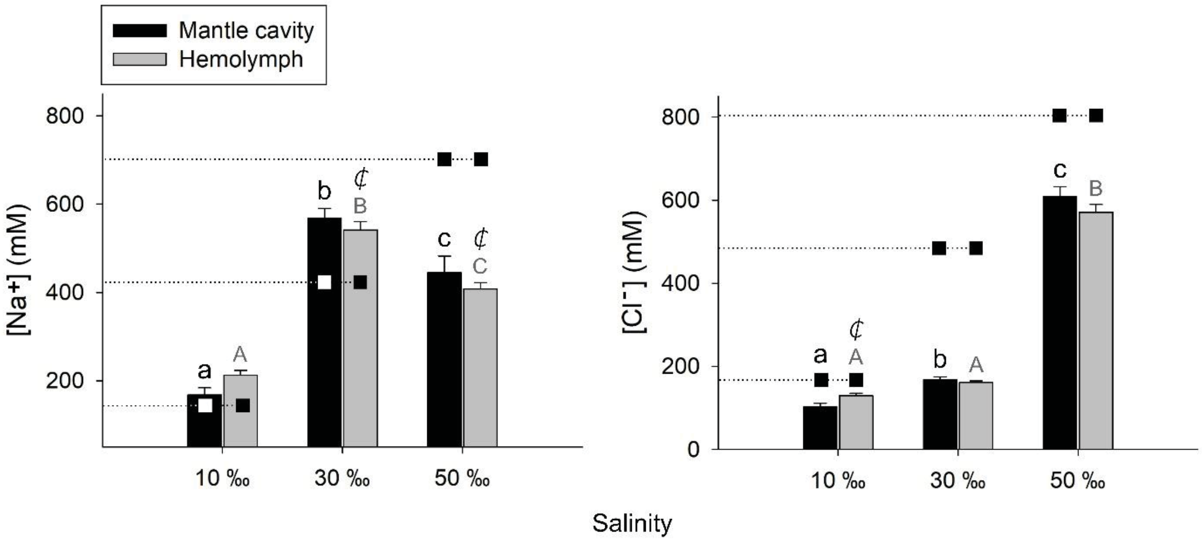

Figure 3. Concentration of Na$^+$ (left) and Cl$^-$ (right) ions of the mantle cavity water and hemolymph of *Cyrtopleura costata* in experimental (10 and 50 ‰, N = 12 for each condition) and control (30 ‰, N = 11) salinities. Data presented are mean ± standard error. Dotted lines indicate predicted seawater levels of the respective ions, sodium and chloride, based on standard ionic values of seawater (Prosser, 1973) and squares indicate osmotic regulation according to the confidence interval analyzes. ₵ indicate statistical difference between the ionic concentration of the hemolymph and mantle cavity water in the same salinity. Letters indicate statistical difference in the ionic concentration of each fluid between salinities.

In vitro – Tissue hydration regulation

The three isolated tissues analyzed responded differently to hypo- and hyper-osmotic shocks (Fig. 4). All 3 tissues showed very stable controls in saline 30. Stability even after 2 hours (120 min) indicate viability of the handled tissues along the experiment. The siphon and the mantle slices gained water along the whole time course of the experiment, when exposed to saline 10, and lost water when exposed to the hyperosmotic saline (50). Further water was gained in 10 and lost in 50, after 120 min. The muscle slices, however, swelled and gained water in saline 10, but did not lose water in saline 50, even after 120 min. Siphon gained water more rapidly that either mantle or muscle, and to a higher degree of swelling. Muscle slices gained less water and were stable after 30 min of exposure to saline 10. As all 3 graphs are shown with a same scale of weight change, a gradient of capacity for weight/hydration maintenance is clear: Muscle > mantle > siphon.

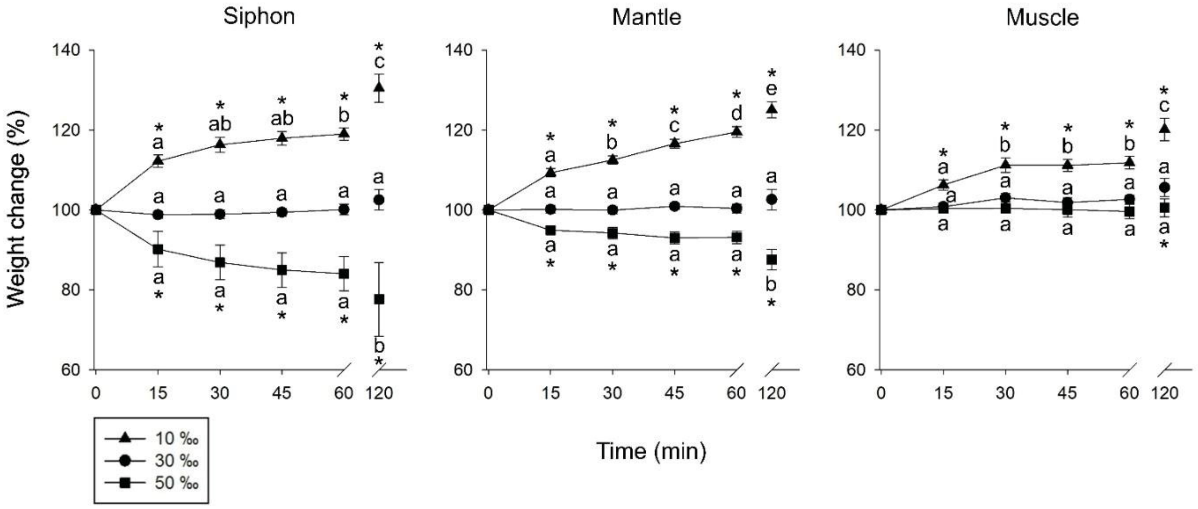

Figure 4. Time course of tissue hydration of siphon, mantle and anterior adductor muscle of *Cyrtopleura costata* under both hyposmotic (10‰) and hyperosmotic (50‰) shocks (15–60 min, N = 20; 120 min, N = 9). Data presented are mean ± standard error. Lower case letters indicate statistical differences over time in each condition. The asterisks indicate statistical difference when compared to respective control, at the same time of exposure (30‰).

Tissue water content – In vivo and in vitro

Photographs of *C. costata* showing the exposed siphon show, visually demonstrate the swelling at the end of the *in vivo* experiment in salinity 10, and the shrinking at the end of the *in vivo* experiment in salinity 50 (Fig. 5). Final values of tissue water content also revealed that the muscle was the tissue, among the 3 tested, more resilient to water fluxes, tighter in the maintenance of percent hydration (Fig. 6). In the end of the *in vivo* experiment, after 4 hours, the siphon water content differed from the control (30‰) only in the hypersaline condition (50‰), just like the mantle. Differences in water content between *in vivo* and *in vitro* experiments were in general observed (Fig. 6).

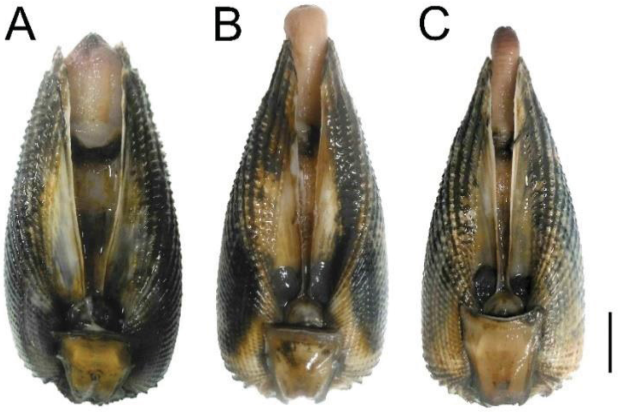

Figure 5. *Cyrtopleura costata* at the end of the *in vivo* experiments (4 hours total) with gradual dilution or concentration of salinity. Different degrees of valve opening and siphon turbidity can be observed. **A**, 10‰; **B**, 30‰ (control condition); **C**, 50‰. Scale bar = 1 cm.

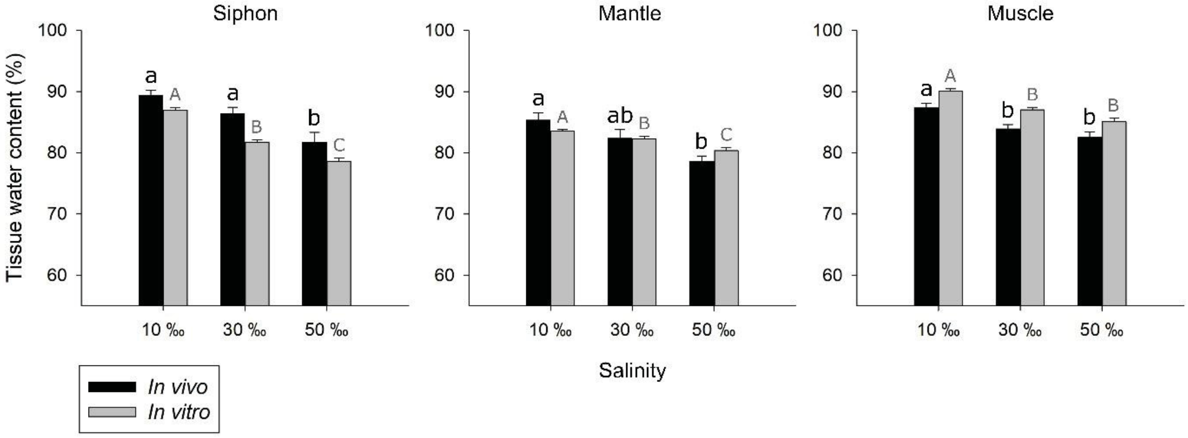

Figure 6. Water content (%) of the siphon, mantle and anterior adductor muscle of *Cyrtopleura costata* at the end of the *in vivo* (N = 6-7, for each salinity) and *in vitro* (N = 20, for each salinity) experiments. Data presented are mean ± standard error. There was no difference between *in vivo* and *in vitro* experiments for any of the conditions (10, 30 and 50‰). Different letters indicate statistical difference in the *in vivo* or *in vitro* experiment between salinities.

DISCUSSION

Salinity is the main factor controlling the abundance and distribution of marine invertebrates (Breaux *et al.*, 2019; Pourmozaffar *et al.*, 2019). Over millions of years, bivalves are able to tolerate a wide range of variations in abiotic factors, exhibiting great physiological plasticity (Yuan *et al.*, 2010; Carvalho, Romano & Poersch, 2015; Rola, Souza & Sandrini, 2017). Several physiological and behavioral mechanisms may be involved in maintaining homeostasis in the face of salinity fluctuations in estuarine environments: isolation within the shell, changes in free amino acid and inorganic ion concentration, altered metabolic rate, endocrine responses and expression of special genes (see Deaton (2009) and Pourmozaffar *et al*. (2019)).

Within the Heterodonta subclass (to which the Pholadidae family belongs) some representatives are extremely tolerant to diluted water (e.g. *Polymesoda caroliniana, P. maritima* and *Rangia cuneate*, see Deaton, 1981). Virtually all representatives of Pholadidae are strictly marine or brackish inhabitants (Turner, 1954; Deaton, 1981; Wong, 1982). *C. costata* live in estuarine environments protected with muddy substrate, is unable to excavate after 15 mm in length and its only movement is to move up and down along a well-established burrow (Turner, 1954). Their populations occur in salinities between 16 and 40‰; there are no records of the species in salinities < 10‰ (Castagna & Chanley, 1973), which is compatible with the results of the *in vivo* experiment performed here.

In vivo – Osmolality of hemolymph

At salinities between 25-40‰, the osmolality of the mantle cavity and hemolymph remain isosmotic to the environment and the animals remained active. Salinities between 20-30‰ are usual conditions of the polyhaline sector where the species is found. In Santa Catarina, southern Brazil, the highest larval survival rates of these bivalves (>70%) occur under salinities between 15-35‰ (Albuquerque, 2010), corroborating the range well tolerated by adults.

On the other hand, faced with hypoosmotic challenges (≤20‰), the bivalves retracted and kept the siphons tightly closed (see Fig. 5). This behavior is reflected in the osmolality of hemolymph in relation to the environment at salinities ≤20‰, remain hyperosmotic. Despite this, *C. costata* does not appear to disrupt the circulation of water in the mantle cavity, as it has been isoionic with respect to the medium in all tested salinities. This suggests that, despite the unpleasant condition, these bivalves may continue to feed and perform gas exchange, unlike bivalves that completely isolate themselves between the valves (Deaton, 2009; Rola, Souza & Sandrini, 2017; Pourmozaffar *et al.*, 2019).

Probably other mechanisms are used to preserve hemolymph osmolality. In fact, evidences suggest that mollusks are not always completely conformers; they may display evidences of salt absorption in dilute medium, and effective hyper-regulation, with maintenance of significant gradients. *Perna perna*, for example, is able to maintain hyperosmotic hemolymph relative to the environment with open valves (Rola, Souza & Sandrini, 2017), as well the gastropod *Stramonita brasiliensis* can maintain hyperosmotic hemolymph without closing the operculum (Veiga *et al.*, 2015). The abalone *Haliotis discus hannai* changes hemolymph concentration even without an operculum or any other calcified structure to isolate its soft parts (Gao *et al.*, 2017).

Interestingly, the anatomy of *C. costata* has a large pericardial region, an elongated kidney, and gills that extend from the anterior region to the posterior extremity. The kidney is known to be involved in osmotic balance and the gills may act as an extrarenal uptake site of ions, in addition, urine formation through hemolymph filtration may occur in the pericardial cavity (see review Deaton (2009) and Pourmozaffar *et al.* (2019)). The recorded urine output for marine osmoconformers may be of the same order of magnitude as freshwater animals (Deaton, 2009), suggesting that the balance of water and ions in marine species may also rely on this mechanism (Pourmozaffar *et al.*, 2019). The role of urine in ability of *C. costata* to maintain the hyperosmotic hemolymph in relation to the environment under hyposaline conditions should be investigated.

Sodium and chloride concentrations

Inorganic ions (as Na^+ and Cl^-) are responsible for providing protection against rapid changes in media salinity. Generally, shellfish keep Na^+ and Cl^- concentrations lower than the environment, although they vary in parallel with it (Gao *et al.*, 2017). This was not entirely true for *C. costata* since the $[Na^+]$ was lower than the control under both experimental conditions and the $[Cl^-]$ at 10‰ was close to the control value.

Decreased external osmolality may be reflected in the low $[Cl^-]$ in *C. costata* hemolymph at 10‰. Under hyposmotic conditions (<20‰), this bivalve hyper-regulates Na^+, but not Cl^-. Low $[Cl^-]$ appears to be common for mollusks in general (Pierce, 1971; Veiga *et al.*, 2015). On the other hand, an increase in salinity produces a higher concentration of NaCl (see review Pourmozaffar *et al.* (2019)), what is in according with the results presented here.

The increase of Na^+ and Cl^- in hypersaline conditions may be related to a volume regulation response (Ruiz & Souza, 2008; Torre *et al.*, 2013). Although Na^+ and Cl^- concentrations increase at 50‰ exposure, *C. costata* hyporegulates both ions in this condition, with Na^+ concentration at 50 ‰ lower than in the control. Similar results were recorded for the bivalves *Mya arenaria*, *C. largillieri*, *Erodona mactroides* and *Amarilladesma mactroides*, which hyporegulate the hemolymph chloride concentration during hyperosmotic exposure (Deaton, 2009; Medeiros, Faria & Souza, 2020).

The soft parts of *C. costata* are always exposed and only the mantle fills the space between the valves. Given the flexibility of the body wall, osmotic shocks can cause muscle contraction (as observed in the *in vivo* experiment), which can reduce water and ion permeability and may establish an ionic gradient (Castellano, Santos & Freire, 2016). The ability to control the gradient of inorganic ions is species-specific and defined according to the occupied niche (Medeiros, Faria & Souza, 2020).

In vitro - Tissue hydration regulation

Maintaining cell volume is essential for a variety of functions, such as metabolic regulation, cell pH maintenance, and transmembrane transport (Pierce, 1982; Deaton, 2009). Although the cell volume regulation methodology adopted here did not use isolated cells, differences in weight throughout the experiment reflect the movement of water and osmolytes to / from tissues (see discussion in Freire et al., 2008, 2013). Changes in tissue weight will be considered herein as regulation of cell volume.

For the tissues of *C. costata*, both under hypoosmotic and hyperosmotic challenge, the muscle was exhibit greatest capacity to regulate volume, followed by the mantle and by siphon. Interestingly, the echinoderm *Holothuria grisea*, submitted to the same methodology for volume regulation evaluation, presented results very similar. Authors explain that muscle was the most robust among the tissues analyzed, which may have conferred less variability in hydration (Castellano, Santos & Freire, 2016). Although the *C. costata* siphon is quite robust, perhaps as much as the muscle, we should consider that it is also formed by the mantle, which presented considerable volume variability. Thus, differences may be ascribed to intrinsic variability in transporters expressed in the cell membranes of these distinct tissues (Ruiz & Souza, 2008; Deaton, 2009; Torre *et al.*, 2013; Castellano, Santos & Freire, 2016).

Different tissues do respond differently to saline challenges, some may recover their original volume, others partially recover and others not it (Ruiz & Souza, 2008). The muscle tissue of *C. fluminea* (freshwater), for example, although increasing or decreasing in volume, reaches stability after 90 minutes of exposure in both hypo- and hyperosmotic conditions (Ruiz & Souza, 2008). *S. brasiliensis* shows no return to pre-stress saline weight, presenting "controlled swelling" compatible with partial RVD (Veiga *et al.*, 2015), in accordance with our results for *C. costata*. Although it gains or loses volume, a partial regulation of tissue hydration is sufficient to avoid the deleterious effects of a large increase or decrease in hydration (Torre *et al.*, 2013). Few cells recover the original volume, and partial volume recovery is the rule (Pierce, 1982).

For all tissues of *C. costata* analyzed (siphon, mantle and muscle), the variation was smaller and reached some stability only in hyperosmotic challenges. This may explain the avoidance behavior (contraction) of *C. costata* at low salinity (*in vivo* experiment), which probably is associated with poor capacity to regulate volume under hyposmotic conditions (Castellano, Santos & Freire, 2016).

The asymmetry between hypo- and hyperosmotic stress responses seems to be universal (Deaton, 2009). Bivalves appear to be more tolerant to increase than decrease in salinity. Chronically faced with hyposaline stress, the pholadid *Aspidopholas obtecta* and the mussel *M. edulis* show reduced growth (Wong, 1982; Maar *et al.*, 2015), *M. edulis* also shows lower efficacy of the immune system (Bussell *et al.*, 2008), *C. virginica*, *Phacoides pectinatus* and *A. flexuosa* have impaired reproductive cycle (Lima *et al.*, 2009; Lavaud *et al.*, 2017; Santana & Rocha-barreira, 2018), *M. arenaria* has the muscle functional capacity affected, reducing the burrowing capacity (Haider, Sokolov & Sokolova, 2018; Haider *et al.*, 2019), the fertilization and performance of larvae of the giant clam *Tridacna gigas* becomes compromised (Sayco *et al.*, 2019) and mortality of *Mesodesma mactroides*, *C. edule* and *S. plana* is recorded at <15% (Carvalho, Romano & Poersch, 2015; Verdelhos, Marques & Anastácio, 2015). Rare are the species of sea / estuarine bivalves that are more tolerant to hypo- that hypersaline challenges, such as *C. gasar*, which has high tolerance to low salinities, with representatives surviving up to 6 months at 5 ‰ salinity (Horodesky *et al.*, 2019).

Tissue water content – In vivo and in vitro

Regulate tissue hydration is critical for osmoconformers to inhabit places such as estuaries and intertidal zones (Pierce, 1971; Freire *et al.*, 2008; Pourmozaffar *et al.*, 2019). The results of tissue hydration were in agreement with the results obtained in the in vitro experiment, with the muscle showing less variation, followed by the mantle and the siphon. In addition, it should be noted that in the *in vivo* experiment was used sea water (diluted or concentrated), unlike the *in vitro* experiment. Despite this, the results obtained were very similar, indicating effectiveness of the method using saline solutions.

Low water permeability are adaptive characteristics of bivalves of the high intertidal habitat (Deaton, 1992), which is not the case with *C. costata*. Nevertheless, even highly permeable body-walled molluscs may not be in full osmotic balance with the environment (Leader, Hall & Bedford, 1986). *M. arenaria*, for example, is a highly permeable bivalve (Deaton, 1992) and protects itself from dissection and variations in the water column by burying itself in the substrate (Haider, Sokolov & Sokolova, 2018), which may serve as a buffering agent for infaunal bivalves (Carvalho, Romano & Poersch, 2015). The same mechanism can be used by *C. costata*, burrowing about 40 cm in the mud.

Tissue Hydration Regulation Index

In hyposmotic challenges, *C. gigas* and *P. perna* perform better in regulating cell volume, with a THRI of 16.4 and 17.4, respectively. Under these conditions, *C. costata* achieves a THRI of nearly double for the mantle (41.1) and muscle (32.3), reaching 49.2 for the siphon. On the other hand, in hyperosmotic challenges, *C. costata* has the lowest THRI values, followed by *C. fluminea*, *C. gigas* and *P. perna* (Table 2).

Table 2. Tissue Hydration Regulatory Index (THRI) of *Crassostrea gigas*, *Perna perna,* and *Cyrtopleura costata*.

Reference	**Species**	**Habitat**	**Tissue**	**Hyposmotic challenge (%)**	**THRI (%) (Hypo-)**	**Hyperosmotic challenge (%)**	**THRI (%) (Hyper-)**
Veiga, 2013	*Perna perna*	Marine	Mantle	50	16.4	50.0	53.6
	Crassostrea gigas	Marine/ Estuarine	Mantle	50	17.4	50.0	51.2
Ruiz e Souza, 2008	*Corbicula fluminea*	Freshwater	Foot muscle	20	78.0	30	41.0
This study	*Cyrtopleura costata*	Estuarine	Mantle	66	41.1	66	23.2
			Siphon	66	49.2	66	34.4
			Muscle	66	32.3	66	20.1

The performance of *C. costata* in the face of hypoosmotic challenges was lower than that of *P. perna* and *C. gigas*, but when exposed to hypersaline conditions, it has greater capacity to regulate cell volume. Thus, our initial hypothesis that this species might be more effective in regulating tissue hydration than commonly reported for bivalves that can isolate their soft tissues from the environment has been proven for hyper-saline conditions.

Conclusions

The best performance of the *C. costata* seems to be between 25 and 40‰, where the bivalve conforms and is able to maintain homeostasis at no additional energy cost. At salinities < 20‰ the bivalve continues to circulate water to the mantle, but apparently to a lesser extent. This tolerance to low salinities can be achieved due to burrow microhabitat (in the natural environment) as a consequence of the burrowing habit and with the addition of physiological mechanisms, such as change in body wall permeability through muscle contraction and urine production, processes that deserve investigation in Pholadidae.

Our results agree with the pattern observed in estuarine bivalves and with the results obtained in larval development studies of the species (Gustafson et al., 1991; Albuquerque, 2010). The angel wing clam responded to the osmotic challenges as a typical euryhaline osmoconformer. The hypothesis that C. costata has a greater ability to regulate tissue hydration than bivalves capable of completely closing valves has been confirmed for hyper-saline situations. This feature is probably related to the natural and evolutionary history of Pholadidae.

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5 EPILOGUE

The method used for angel wing clam collection and transport proved to be efficient for all necessary procedures, comparing with the difficulties with valve rupture and mortality already registered. The main anatomical characteristics of *C. costata* reflect the excavating habit, although the activity is ended in adult period. In addition, morphological adaptations may be linked to physiological functions and act on osmoregulation, such as kidney topology and pericardial region.

The reproductive pattern of *C. costata* from Paranaguá Bay was in agreement with that observed for bivalves in tropical and subtropical regions inhabiting the tides. The reproductive activity occurs throughout most of the year, with greater investment in autumn and winter. The size of the oocyte of this species is much smaller than that of other bivalves, this what can be due to the low fertility of the population or the high availability of food in the estuary that makes the oocytes not need ample reserves. The Condition Index is higher than other bivalves, probably due to the characteristics of the shell and the growth of soft parts to beyond the length of the valves, a pattern that can be expected for other pholadids. In addition, the highest rates of flesh yield observed seem to be related not to the reproductive cycle, but to the storage of nutrients.

The angel wing clam responded to the osmotic challenges as a typical euryhaline osmoconformer. Our initial hypothesis that *C. costata* has a greater ability to regulate tissue hydration than bivalves capable of completely closing valves has been confirmed for hyper-saline situations. The best performance of the species was between 25 and 40‰, where the bivalve conforms and is able to maintain homeostasis at no additional energy cost. At salinities < 20‰ the bivalve continues to circulate water to the mantle, but apparently to a lesser extent. This tolerance to low salinities can be achieved due to burrow microhabitat and with the addition of physiological mechanisms. The urine production is a processes that deserve investigation in Pholadidae.

According to the results obtained here and data available in the literature, for optimal development and growth of *C. costata* larvae the ideal conditions are salinity between 20 and 25 ‰ at 30 °C and, for best performance, adults should be kept in a range between 25 and 40 ‰. The continuous reproduction of the species allows its exploitation throughout the year, except in the autumn and winter months, in which the largest reproductive investment of the population occurs.

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