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Reproductive cycle of the mangrove oyster, *Crassostrea gasar* (Adanson, 1757), in tropical and temperate climates

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Abstract

The reproductive cycle of *Crassostrea gasar* oysters from a single origin (Federal University of Santa Catarina hatchery) was studied by culturing the species for 1 year in four distinct areas of Brazil; two of which were in tropical climates (02°44′S, 41°58′W; 02°47′S, 41°55′W) and two in temperate climates (26°28′S, 48°50′W; 27°35′S, 48°32′W). In each area, 20 individuals were collected each month for reproductive cycle analysis using standard histological techniques. Oysters cultivated in tropical climates demonstrated intermittent reproductive cycles, with individuals spawning throughout the year and rarely entering a resting stage. Oysters cultivated in temperate climates demonstrated spawning periods associated with increases in water temperatures, occurring in late spring and summer. Resting stages were clearly seen in samples from latitudes of 26 and 27 °S, with approximately 75% to 100% of the individuals in this stage between May and July.

KEYWORDS

Crassostrea gasar, mangrove oyster, reproductive investment, temperature

1 | INTRODUCTION

Brazilian oyster culture is concentrated in the state of Santa Catarina, which represents only 8% of the coastal line and yet accounts for 90% of total national production. This is due to an existing technological package for the Pacific oyster *Crassostrea gigas* culture, which was introduced in the state in 1987 (Melo et al., 2010). *Crassostrea gigas*, however, is restricted to cold seawater temperature areas (Poli, 2004). Thus, the expansion of oyster farming in Brazilian tropical waters is linked to the domestication and the development of technological basis for native species (Legat, et al., 2017).

The mangrove oyster, *Crassostrea gasar* (= *Crassostrea brasiliana*), inhabits most of the Brazilian coast, from the state of Pará to Santa Catarina (Melo et al., 2010), and is commonly found forming clusters in the mangrove ecosystem (Nascimento, 1991). Among indigenous oysters, *C. gasar* is the most cultivated species because it reaches greater size than *Crassostrea rhizophorae* (Christo & Absher, 2006) and presents similar growth to *C. gigas* in Europe and to *Crassostrea virginica* in the United States of America (Legat, et al., 2017).

As reproduction is one of the most important physiological processes in the life cycle of any bivalve (Enríquez-Díaz et al., 2009), the farming of oysters has led to many different studies being performed in various locations to better understand their annual reproductive cycles. A more thorough understanding of these reproductive cycles allows farmers to better determine the optimal moments for harvesting, with the aim of obtaining oysters with a higher meat weight before spawning and also seed collection.

Despite the environmental and economic importance of the Brazilian mangrove oyster, few studies have been conducted to evaluate its reproductive cycle. To the best of our knowledge, only two studies using standard histological techniques have previously been performed in field in Brazil's South Region (Castilho-Westphal

et al., 2015; Gomes et al., 2014), while only one study has been performed in the North Region (Paixão et al., 2013). Therefore, in the current study, we examined the gametogenesis cycle of *C. gasar* possessing the same age and origin but cultured in four distinctive marine and estuarine areas of different latitudes: two farming areas in northeast tropical climates and two in southern temperate climates.

2 | MATERIALS AND METHODS

2.1 | Obtaining seeds

Seeds were produced at the Laboratory of Marine Molluscs of the Federal University of Santa Catarina (LMM-UFSC) using the sixth generation obtained from a single population of *C. gasar* broodstock, which was free of notifiable parasites (Sühnel et al., 2016) and held in Praia do Sambaqui, Florianópolis (27°35′S, 48°32′W). Larvae culture and settlement were performed according to the methodology described by Silveira et al. (2011). The age of the animals used in this study refers to the number of days from fertilization to the sampling date and is described as 'days of age' (DA).

2.2 | Study sites and sampling

In June of 2012, oyster seeds with similar heights (5.85 ± 2.00 mm) and age (60 to 65 DA) were shipped in styrofoam boxes to our study sites: two areas in the State of Santa Catarina with temperate climates (São Francisco do Sul [SFS; 26°28′S, 48°50′W] and Sambaqui Beach [SB; 27°35′S, 48°32′W]) and two areas in Maranhão State with tropical climates (Morro do Meio [MM; 02°44′S, 41°58′W] and Torto [TT; 02°47′S, 41°55′W] (Figure 1). The grow-out systems were the same used by producers at each location. In Santa Catarina, we adopted long-line systems using lantern-nets located in the ocean near the coast in depths between 4 and 15 m. In Maranhão, we used off-bottom systems in estuarine riverbanks employing plastic mesh bags in depths between 3 and 4 m. We used a completely randomized design with four treatments (sites) and eight experimental units (EU), with 1,000 seeds per EU, totalling 8,000 seeds for each

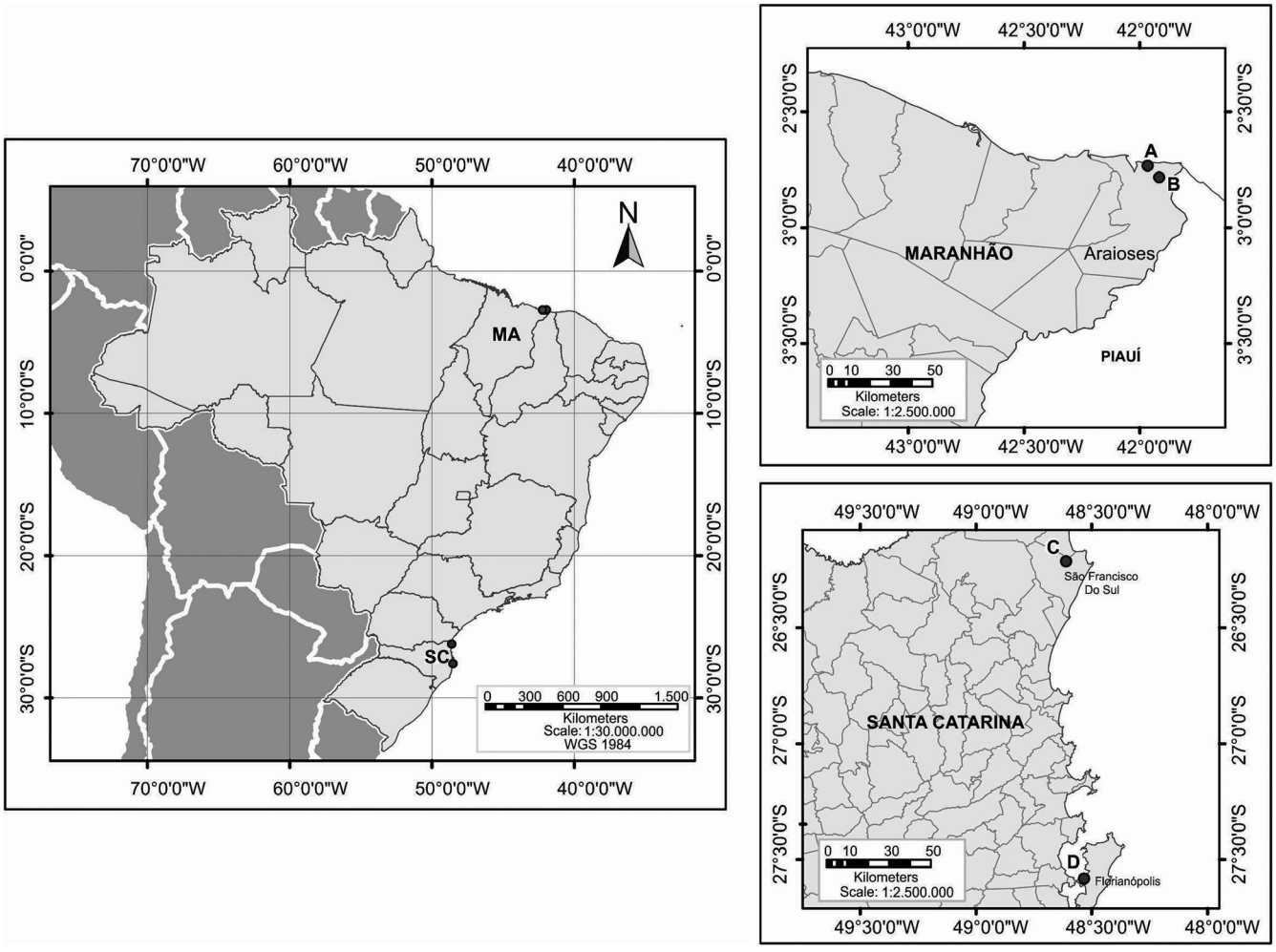

FIGURE 1 Location of the mangrove oyster, *Crassostrea gasar*, culture fields in the Brazillian states of Maranhão (a,b) and Santa Catarina (c,d). A = MM (Morro do Meio; 02°44′S, 41°58′W); B = TT (Torto; 02°47′S, 41°55′W); C = SFS (São Francisco do Sul; 26°28′S, 48°50′W) and D = SB (Sambaqui; 27°35′S, 48°32′W)

sites. The seed stocking density was standardized by 100% occupancy of each EU during all experimental period. Handling consisted of fouling removal and stocking density control.

Sampling was performed monthly from July of 2012 until July of 2013. Each sample from the individual sites consisted of 20 oysters and was collected by staff from either the Brazilian Agricultural Research Corporation (Embrapa) in Maranhão sites or the LMM-UFSC in Santa Catarina sites. A total of 260 oysters from each site were collected and sent to the laboratory for measuring height (according to Galtsoff, 1964), whole weight, dissection and histological analyses. The temperatures (at a depth of 20 cm) and salinities of each site were recorded at the moment of the sampling using thermometer and manual refractometer.

2.3 | Histological and statistical analysis

Sections of oyster gonads were fixed in Davidson's solution and stored in 70% ethanol. The sections were then routinely processed for histology using the method described by Sühnel et al. (2014). Briefly, samples were bathed six times in ethanol (ranging from 70% to 100%) and once in xylol, and then, the tissues were embedded in paraffin. The blocks containing the tissue samples were sliced into segments 5 μm thick, mounted on slides and then stained with HHE. The slides were then examined under a light microscope to determine the sex and gametogenic stage of the sample. The gametogenic stage was determined using a reproductive scale adapted from Sühnel et al. (2010) and Ramos et al. (2014), which consisted of five different stages that were defined according to their histological characteristics: 1) immature, 2) gametogenesis, 3) pre-spawning {subdivided in initial (A) and advanced (B)}, 4) spawning {subdivided in initial (A) and advanced (B)} and 5) resting/spent (Table 1).

For statistical analyses, we did not consider initial and advanced subdivisions of pre-spawning and spawning. Thus, the sexual stages of samples (260 oysters from each site) were quantified using a numeral scale (1 = immature; 2 = gametogenesis, 2 = pre-spawning, 4 = spawning e 5 = resting) and compared between South and Northeast regions, sites and months using the chi-square test and a SAS® program.

2.4 | Ethical approval

According to Brazilian law, authorization for the use of invertebrates, including oysters, is not required in the conduct of scientific experiments.

3 | RESULTS

3.1 | Seawater parameters

Seawater temperatures taken in the Northeast Region were similar for both culture areas (MM and TT) and remained constant throughout the year, ranging from 27 to 30°C in TT and from 26 to 29°C in MM (Figure 2a). The salinity recordings at the sites ranged from 5 to 32 mg/L in TT and from 25 to 37 mg/L in MM (Figure 2b).

Similarities were also observed in seawater temperatures taken from the two sites in the South Region; however, extreme values varied up to 12°C depending on the season. Seawater temperatures at the SFS site ranged from 17 to 28°C, while at the SB site they ranged from 15 to 27°C (Figure 2a). The lowest temperatures were observed between June and August 2012. Seawater temperatures increased during the months of September and October, remaining relatively constant until March, at which point temperatures began to decline until sampling ended in July of 2013. Salinity recordings taken from the SB site remained relatively constant, ranging from 33 to 36 mg/L, while those from the SFS site varied from 25 to 33 mg/L (Figure 2b); however, these measurements were between 30 and 33 mg/L for most of the study period.

3.2 | Sex ratio, height and weight

We observed balanced sex proportions during the first maturation of *C. gasar*. A total of 1,040 oysters were analysed across all our experimental sites, of which 40% were female, 39% were male, 20% were sexually undifferentiated, and 1% were simultaneous hermaphrodites. The sex ratios (number of females: number of males) and numbers of hermaphrodites and specimens of undetermined sex from each culture area are presented in Table 2.

Throughout our experimental period, the heights of sampled individuals ranged from 6 to 98 mm, while whole weights ranged from 0.06 to 106.15 g. Maximum, minimum and average values for whole weight and height from each sample area are presented in Table 3.

3.3 | Annual reproductive cycle

All the stages described in Table 1 were observed in the four locations. The characteristics of each stage are presented in Figures 3 and 4, except the immature stage, which did not present any differences between males and females.

A statistically significant difference ($\chi^2_{df=4;\alpha=0.05;} = 4.49$; $p < .0001$) was observed between the reproductive cycle stages in the South (temperate climate) and Northeast Regions (tropical climate) (Table 4).

In the temperate waters of the South Region, oysters started gametogenesis in the late winter and early spring (August and September), which was 60 to 90 days after the cultures began and when the oysters were 120 to 150 DA (Figure 5). The smallest individuals to be observed in the gametogenesis stage from the SB and SFS sites measured 21 and 33 mm respectively. The smallest individuals in pre-spawning stage measured 27 mm shell height in SB and 31 mm in SFS.

The pre-spawning stage took place from October onwards (180 DA). At the SB site, spawning began in November (210 DA), reached

TABLE 1 Histological descriptions of the gonad stages in the reproductive cycle of the mangrove oyster, *Crassostrea gasar*, adapted from Sühnel et al. (2010) and Ramos et al. (2014)

Stage	Female	Male
Immature	Connective tissue evident; absence or rare presence of forming follicles; total absence of gametes, making a distinction between the sexes impossible.	
Gametogenesis	Few observed oocytes, consisting of various sizes and a heterogeneous aspect; presence of inter-follicle tissue; non-juxtaposed follicle walls; thicker follicle walls (presence of internal oogonia); empty inter-follicle and intra-follicle spaces.	High presence of germ cells near the inner walls of the follicles; non-juxtaposed follicle walls; thicker follicle walls (presence of internal spermatogonia); empty inter-follicle and intra-follicle spaces.
Initial pre-spawning	Little evidence of connective tissue; many follicles; follicles full of gametes; elongated oocytes; clear but recognizable follicle walls; empty tube.	Scarce presence of connective tissue; follicles with juxtaposed walls and a high concentration of sperm with lumen-oriented flagella.
Advanced pre-spawning	More oocytes per follicle than the previous phase; extremely long and elongated oocytes; very juxtaposed and not clearly visible follicle walls; many follicles; empty tube; a slight stimulus or cut releases the gametes; no empty inter-follicle or intra-follicle spaces.	More follicles and higher sperm concentration than the previous phase; very juxtaposed and not clearly visible follicle walls; many follicles; empty tube; a slight stimulus or cut releases the gametes; no empty inter-follicle or intra-follicle spaces.
Initial spawning	Initial phase of oocyte elimination; visible and not completely juxtaposed follicle walls with an irregular form; presence of oocytes in the genital tubes; intra-follicle and inter-follicle empty spaces.	Initial phase of sperm elimination; visible and not completely juxtaposed follicle walls with a flaccid aspect; presence of sperm in the genital tubes; intra-follicle and inter-follicle empty spaces.
Advanced spawning	Empty follicles present, with irregularly formed follicles due to recent spawning; completely or partially empty follicles; few remaining gametes.	
Resting/spent	Abundant inter-follicle connective tissue; scarce follicles with small diameter; either rare occurrence of residual gametes allowing distinction between the sexes, or absence of follicles and gametes making distinction between the sexes impossible.	

a peak in December, and then continued through February to reach a new peak in March. At the SFS site, spawning was observed from December until May, with a prolonged peak occurring between January and February. After the spawning stage started, some oysters demonstrated characteristics of the pre-spawning stage, while others demonstrated those of the spawning in advanced stage. In the advanced spawning stage, follicles with releasable gametes can be observed, but there was also a high number of collapsed follicles with a significant amount of connective tissue, indicating that these animals will enter the resting stage.

From the beginning of autumn (March in SFS and April in SB) until early winter (June in both locations), the percentage of oysters in the spawning stage gradually decreased while the percentage of animals in the resting stage increased, at which point all sampled oysters were in the resting stage. In the winter months (June and July), animals in the resting stage represented between 75% and 100% of the oysters sampled. Both resting and gametogenesis stages were observed in samples collected during the month of July.

In the tropical waters of the Northeast Region, oysters began maturing in July, which was 30 days after the culture began (90 DA) (Figure 5). The smallest individuals observed in the gametogenesis stage from the TT and MM sites measured 11 mm and 14 mm respectively. The smallest individuals in pre-spawning stage measured 20 mm shell height in MM and 22 mm in TT.

The number of pre-spawning animals gradually increased in samples from August and September (120 to 150 DA), while the first oysters in the spawning stage were observed in samples from October (180 DA). In the months that followed, most specimens were found to be in the spawning stage. At the MM site, peaks in the number of spawning stage oysters were observed in December, February, June and July, while at the TT site these peaks were in November, December and between May and July. After spawning began in the Northeast Region, the animals presented part of the empty follicles and gametes in the genital channels and presented follicles in stages of gametogenesis and pre-spawning. Thus, as mature gametes were being released, individuals continued producing new gametes with no evidence of gender alternation in oysters undergoing continuous gametogenesis. In contrast to samples from the South Region, very few oysters cultured in the Northeast Region were found to be in the resting stage, with only 3.0% of the samples from the TT site and 1.9% from the MM site observed to be at this stage.

Synchronized development through the reproductive cycle was observed on males and females in the four culture areas, as the same stages occurred in both sexes at similar times. We did not observe any evidence of sex reversal after the spawning periods in this study. Oysters classified as hermaphrodites were found to produce both male and female gametes simultaneously during their first sexual maturation (Figure 6). Hermaphrodite samples from sites in both regions were observed in stages of gametogenesis, pre-spawning and spawning, although these animals were not included in our reproductive cycle analyses.

4 | DISCUSSION

Mackie (1984) previously described temperature-latitude effects on bivalves that result in different breeding strategies along latitudinal

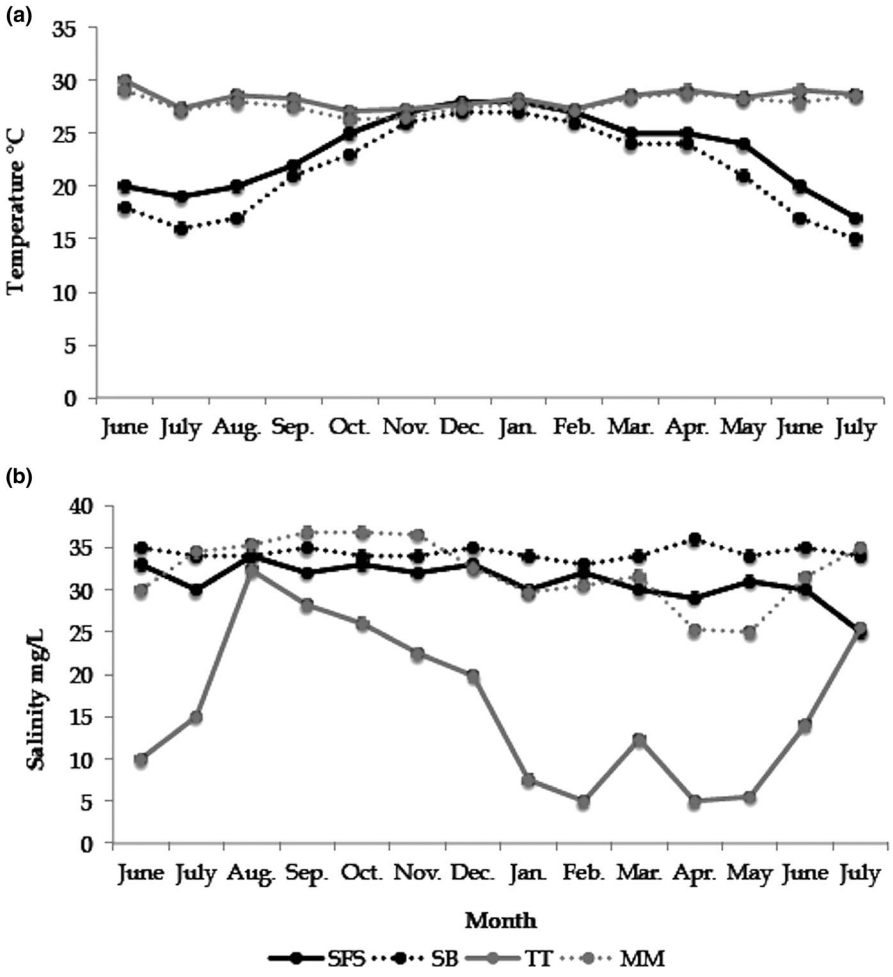

FIGURE 2 Temperature (a) and salinity (b) in culture areas between June 2012 and July 2013. Where SFS, São Francisco do Sul; SB, Sambaqui; TT, Torto; and MM, Morro do Meio

gradients, characterized by a single synchronous spawning per year in cold waters, two synchronous spawning events per year in temperate climates and year-round spawning in tropical climates. In this work, we observed similar patterns in *C. gasar* cultivated in the temperate and tropical waters of the Brazilian coast.

TABLE 2 Sex ratios, hermaphrodites and specimens of undetermined sex for each culture area

	Sexing (%)			
	Culture area			
Sex	SFS	SB	TT	MM
Female	25.4	35.8	55.4	43.5
Male	30.4	30.8	41.5	53.5
Simultaneous hermaphrodite	1.2	0.8	1.2	1.1
Sexually undifferentiated	43.0	32.6	1.9	1.9
Sex ratio (F:M)	1:1.2	1.2:1	1.3:1	1:1.2

Abbreviations: MM, Morro do Meio; SB, Sambaqui; SFS, São Francisco do Sul; TT, Torto.

The most noticeable differences in the reproductive cycle of *C. gasar* between the South and Northeast Regions were the time elapsing from the beginning of the first maturation and the gamete production sequence, both of which can be attributed to variations in water temperature in the study areas. In the Northeast Region, seawater temperatures remained above 26°C throughout the year, and 80% of the oysters sampled from these areas were in the gametogenesis stage 30 days after the experiment began. In the South Region, gametogenesis occurred later, taking 120 days after the experiment began at the SB site, and 150 days at the SFS site, coinciding with seawater temperatures reaching values above 20°C. Thus, the temperature can also explain difference in size of individuals in gametogenesis and pre-spawning stages between Regions. These results corroborate previous findings that gametogenesis is positively correlated with temperature, as noted during field experiments on *C. gasar* by Castilho-Westphal et al. (2015) and Gomes et al. (2014), as well as in laboratory experiments conducted by Ramos et al. (2014).

Regarding reproductive strategy, in the Northeast Region we observed an intermittent reproductive cycle with spawning stages occurring throughout the year, while in the South Region a well-defined resting stage was observed. Nascimento (1991) described a similar pattern for *C. rhizophorae*, in which gametogenesis occurs

TABLE 3 Shell height and whole weight of oysters sampled for histological analysis

	Culture area			
	SFS	SB	TT	MM
Shell height (mm)				
Minimum	8.0	6.0	10.0	10.0
Maximum	98.0	70.0	69.0	68.0
Mean ± *SD*	53.8 ± 22.0	43.37 ± 15.43	42.25 ± 13.25	32.90 ± 10.5.1
Whole weight (g)				
Minimum	0.08	0.06	0.13	0.10
Maximum	106.15	55.01	48.40	77.0
Mean ± *SD*	34.46 ± 28.02	16.91 ± 12.39	13.51 ± 9.63	8.68 ± 9.48

Abbreviations: MM, Morro do Meio; SB, Sambaqui; SFS, São Francisco do Sul; TT, Torto.

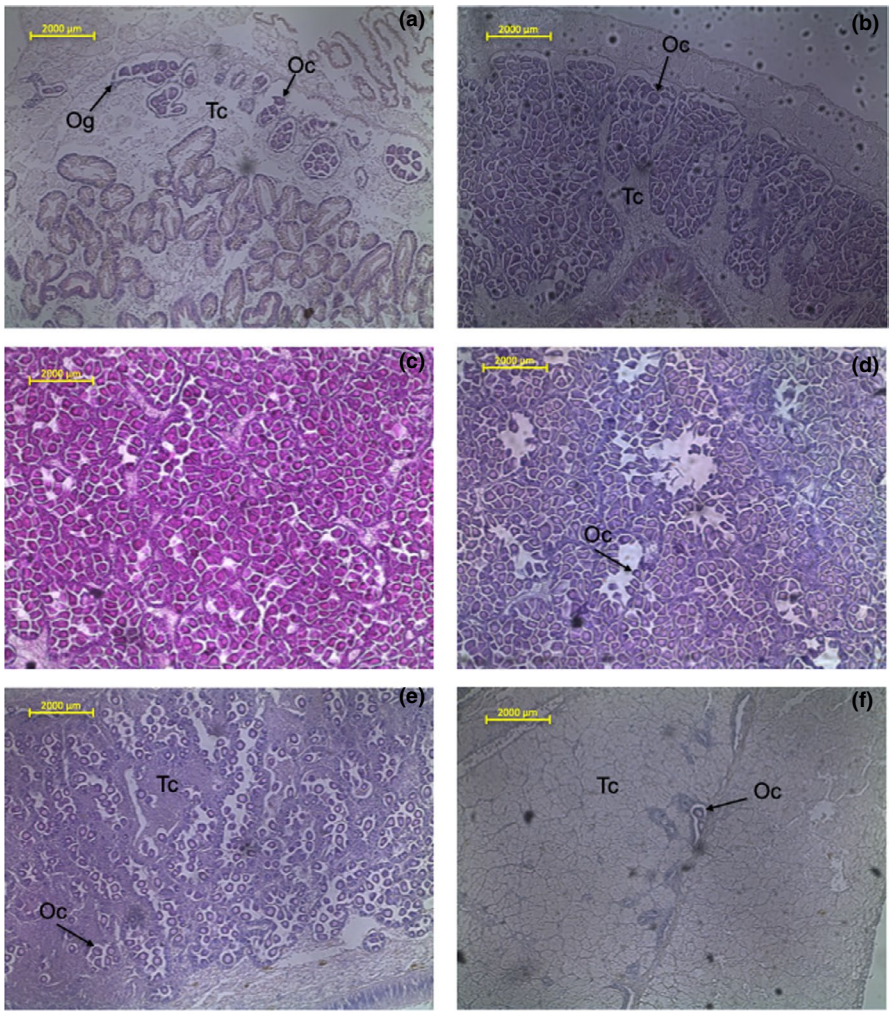

FIGURE 3 Histological sections of *Crassostrea gasar* female gonads demonstrating the different stages of the reproductive cycle: (a) gametogenesis; (b) initial pre-spawning; (c) advanced pre-spawning; (d) initial spawning; (e) advanced spawning; (f) resting. ct, connective tissue; og, oogonia; oc, oocytes; bar represents 2000 microns

year-round without a resting stage in tropical waters of the North and Northeast Regions of Brazil.

The reproductive cycle we observed in SB site are similar to those described by Gomes et al. (2014), who conducted experiments in the same region and noted two spawning peaks and a well-established resting period. In contrast, our experiments at the SFS site revealed a single, prolonged spawning peak followed by a resting stage. In experiments conducted in Paraná State (South Region, 25°52′S), Castilho-Westphal et al. (2015) describe an intermittent *C. gasar* reproductive cycle, with greater intensity during the summer months and resting stages between May and October.

Similar to our results, studies by Paixão et al. (2013) and Gomes et al. (2014) have also observed synchronism between male and female reproductive cycles. Our reporting of samples from the Northeast Region as small as 11 mm found to be in the gametogenesis stage correspond to the smallest size

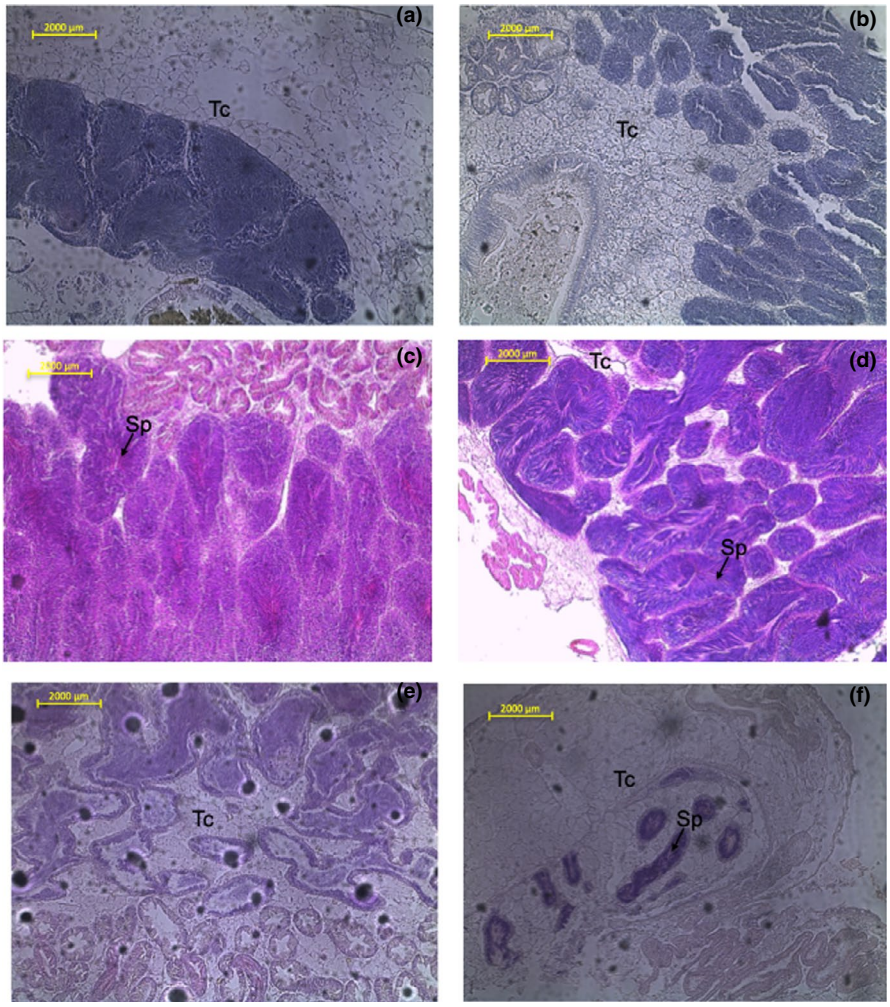

FIGURE 4 Histological sections of *Crassostrea gasar* male gonads demonstrating the different stages of the reproductive cycle: (a) gametogenesis; (b) initial pre-spawning; (c) advanced pre-spawning; (d) initial spawning; (e) advanced spawning; (f) resting. ct, connective tissue; sp, spermatozoa; bar represents 2000 microns

TABLE 4 Difference observed between the reproductive cycle stages in the South (temperate climate) and Northeast Regions (tropical climate)

	Stages of the reproductive cycle					
Region	**Immature**	**Gametogenesis**	**Pre-spawning**	**Spawning**	**Resting**	**Line total**
Northeast						
N	7	56	154	284	13	514
%	6.09	56.57	73.33	66.2	7.39	
South						
N	108	43	56	145	163	515
%	93.91	43.43	26.67	33.8	92.61	
Column total	115	99	210	429	176	1,029

Statistic	**Value**	**DF**	***p*-value**
Chi-square	309	4	<.0001

recorded in the literature for *C. gasar*, while the smallest size we recorded in the South Region (21 mm) corroborates previous studies of *Crassostrea* sp. (Galvão et al., 2000; Nascimento & Lunetta, 1978).

Although we observed no evidence of salinity influencing the reproductive cycle of *C. gasar* in the present study, field and laboratories experiments conducted by others have shown how this parameter may affect reproduction of the species. Paixão et al. (2013) conducted studies in a tropical monsoon climate region, with high temperatures and well demarcated periods of high and low rainfall, in Pará State (North Region, 0°52′S). They observed an increase in the number of mature individuals (pre-spawning stage) during periods of high rainfall and low salinity, while in months of low rainfall, when salinity increased, they found oysters

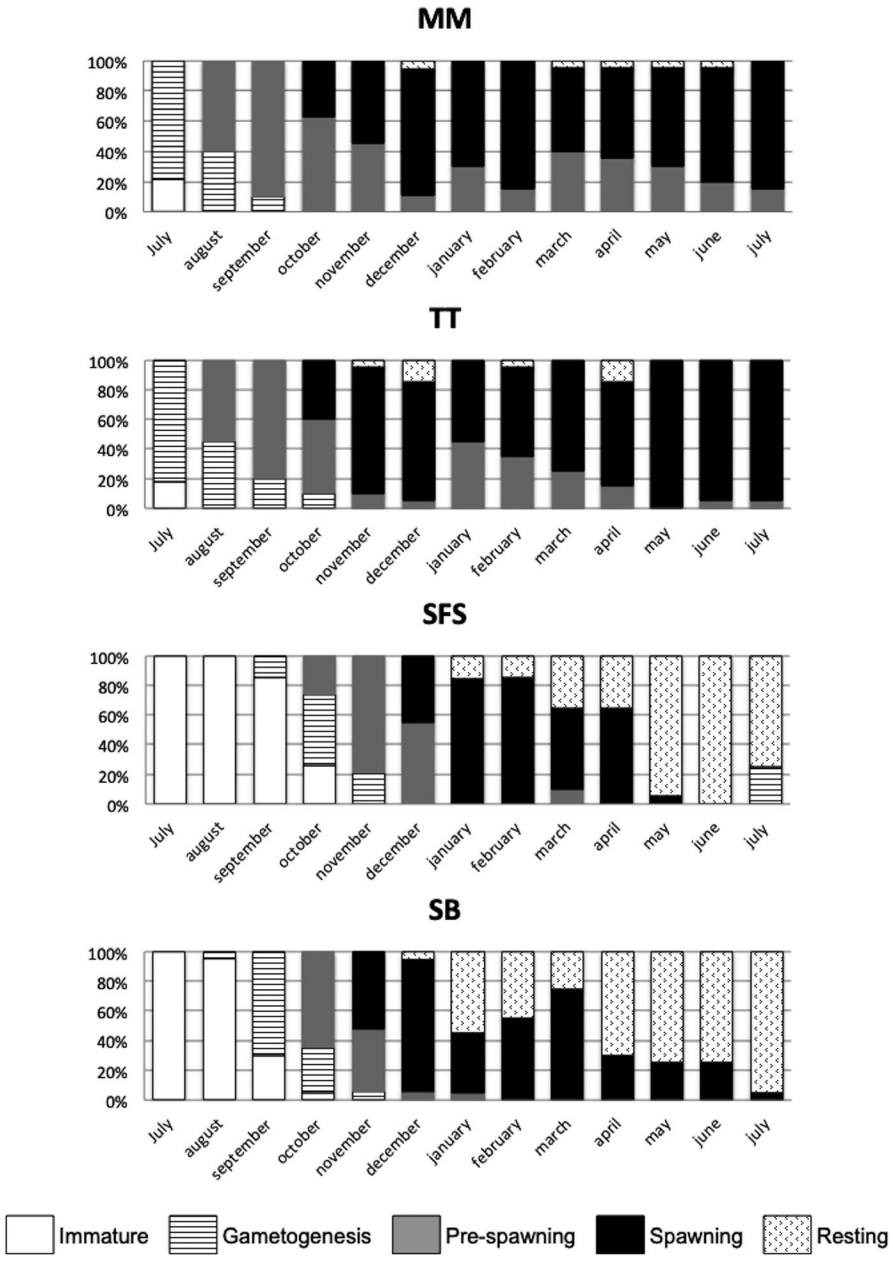

FIGURE 5 Stages of the reproductive cycle of the mangrove oyster, *Crassostrea gasar*, observed in our culture fields. MM = Morro do Meio (02°44′S, 41°58′W); TT = Torto (02°47′S, 41°55′W); SFS = São Francisco do Sul (26°28′S, 48°50′W) and SB = Sambaqui (27°35′S, 48°32′W). For a better understanding and visualization in these graphs, pre-spawning includes both the initial and advanced pre-spawning stages, while spawning includes the initial and advanced spawning stages

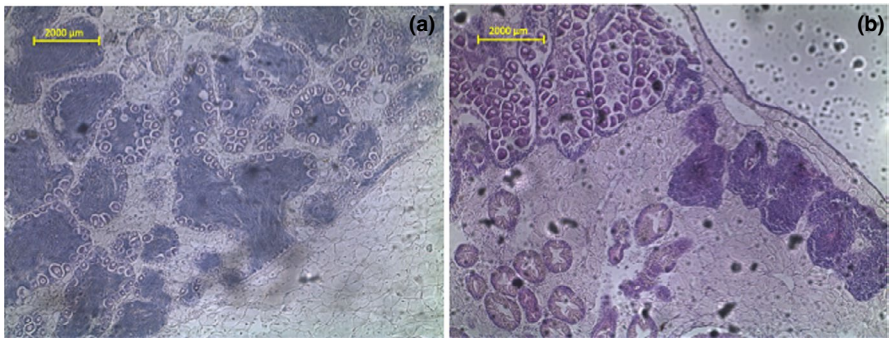

FIGURE 6 Histological sections from *Crassostrea gasar* hermaphrodites. (a) follicles with spermatozoa rounded by oocytes; (b) follicles containing spermatozoa or oocytes. oc, oocytes; sp, spermatozoa; bar represents 2000 microns

in the spawning stage. Additionally, Gomes et al. (2014), conducting maturation experiments in a laboratory setting, demonstrated pronounced gonadal tissue development in *C. gasar* oysters conditioned in salinity 24 when compared to oysters conditioned in salinity 34. Legat, et al. (2017) evaluated the effects of salinity on fertilization and the larval development of *C. gasar*, reporting that fertilization and larva-D development occurred between salinities 21 and 35, yet only larvae cultivated in salinities 28 and

35 reached the settlement stage. Additionally, in the laboratory setting, both fertilization and development of the embryos and larvae were enhanced in salinity 28. These findings indicate that, even in environments with constant temperature, periods of low salinity may interfere with the spawning stages of *C. gasar*, acting in a similar way to low temperatures.

Despite the differences in temperature and salinity between the culture areas, we observed similar sex ratios at the four locations (MM, TT, SFS and SB). Thus, these parameters do not appear to influence the sexual differentiation into male or female variants of *C. gasar*. The sex ratios observed in this study were similar to those found by Paixão et al. (2013), who observed a 1.1F:1M in Pará State. Christo and Absher (2006) observed a similar sex ratio (1.4F:1M) in Paraná State, while Castilho-Westphal et al. (2015) observed a higher proportion of females compared to males (2.6F:1M) in Paraná State.

The lack of evidence supporting sex alternation in oysters with a continuous gametogenesis cycle suggests that *C. gasar* is a dioecious species. The possibility of asynchronous hermaphroditism in oysters participating in a resting stage should be assessed by sexing and monitoring the reproductive cycle in marked specimens.

The low percentage of simultaneous hermaphrodites that we observed is similar to results from previous studies that have reported values of 0.5% (Paixão et al., 2013), 1.0% (Castilho-Westphal et al., 2015) and 1.1% (Ramos et al., 2014). Although hermaphrodites have been identified in distinct stages of the reproductive cycle, including spawning, the possibility of these animals being reproducibly viable should be investigated on specific fertilization and larviculture studies.

According to ours findings we suggest the use of artificial collectors to obtain *C. gasar* seeds from the environment between November and March in Brazilian South Region and along the year at the Northeast Region. However, local experiments and monitoring must be conducted due to the occurrence of fouling and overlapping settlement periods with other oyster species.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

AUTHOR CONTRIBUTIONS

We declare that all listed authors contributed to obtaining and processing data and preparing the article.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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