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Gonad morphology and reproductive cycle of the mangrove oyster *Crassostrea brasiliana* (Lamarck, 1819) in the baía de Guaratuba, Paraná, Brazil

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Abstract

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This study aimed to describe the gonadal histology and the reproductive cycle of *Crassostrea brasiliana* in the mangroves of Guaratuba Bay in southern Brazil. Adults were collected monthly from January 2010 to April 2011 from three sampling sites in intertidal oyster beds. The animals were evaluated using biometric and histological analyses of the gonads. The gonadal tissue samples were processed according to the standard histological procedures, and permanent slides were prepared using Harris' haematoxylin and eosin. The oysters were identified at the species level using a molecular protocol. Females (69%) predominated over males (26%), with 4% indeterminate and 1% hermaphroditic. Mature females were more prevalent in February, March and December 2010 and in March 2011. Mature males were more prevalent in February and April 2010 and in March 2011. The presence of hermaphroditic individuals was sporadic, and oysters in immature stages or sexual repose were observed in only a few collections between the months of May and October 2010. The reproduction of *C. brasiliana* in Guaratuba Bay occurs intermittently, but with greater intensity during the summer, with a larger number of females produced.

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Introduction

Among the oysters that occur in the Brazilian territory, two species stand out due to the economic interest related to their commercial exploitation: *Crassostrea brasiliana* (Lamarck, 1819) and *Crassostrea rhizophorae* (Guilding, 1828). Both species are commonly called 'mangrove oysters' ('ostras-do-mangue' in Portuguese) and are exploited for human consumption and to obtain aquaculture starter stock (young stages). These species are thus becoming important economic alternatives to traditional coastal populations (Pereira *et al.* 2001).

Until recently, *C. brasiliana* and *C. rhizophorae* were considered identical (Rios 1994); therefore, many reports of studies involving one species may have in fact involved specimens belonging to the other or both species.

Crassostrea brasiliana is a dioecious bivalve species that can change sex according to the environmental conditions, a trait known as alternating sex or sequential hermaphroditism (Galtsoff 1964; Wakamatsu 1973; Nascimento 1978; Solon 1984; Guo *et al.* 1998). According to Galtsoff (1964), the primary gonads are dioecious. However, simultaneously hermaphroditic individuals, that is, those with germ cells of male and female lineages in a single reproductive period, may be present in the population (Nascimento 1978). Because members of the genus *Crassostrea* do not show macroscopic sexual dimorphism, many authors use gonadal histology as a study tool (Solon 1984; Steele and Mulcahy 1999; Galvão *et al.*

2000; Lango-Reynoso *et al.* 2000; Fabioux 2004; Dridi *et al.* 2006; Ferreira *et al.* 2006; Normand *et al.* 2008; Lenz and Boehs 2011). However, there are a few studies about *C. brasiliana* reproduction that stand out Galvão *et al.* (2000), Christo (2006) and Christo and Absher (2006).

The present study aimed to analyse and describe the gonadal histology and reproductive cycle of *C. brasiliana* in the mangroves of Guaratuba Bay, an important centre for oyster fishery as well as aquaculture, in southern Brazil.

Materials and Methods

The studies were conducted in three sampling points, characterised as small natural beds (Ilha da Sepultura, Parati and Cabaraquara) in the Guaratuba Bay (25°52′S; 48°39′ W) (Fig. 1). From January 2010 to March 2011, oysters were collected monthly, with each sample consisting of approximately 10 adult specimens per sampling point, per month (n_{total} = 453). The collected individuals were transported alive under refrigeration, as recommended by *Codex Alimentarius* (1978), with a total journey time of no more than six hours, to the Laboratory of Histology and Microbiology of the Integrated Group on Aquaculture and Environmental Studies (GrupoIntegrado de Aquicultura e EstudosAmbientais – GIA) in Curitiba, Paraná, Brazil. In the laboratory, the oyster species were visually identified, as described by Castilho-Westphal (2012), after which their biometric measurements (height, width and length of the shell) were collected (Galtsoff 1964) (Fig. 2) and also collected tissue samples.

The adductor muscle fragments were preserved in a dimethyl sulphoxide (DMSO)–ethylenediaminetetraacetic acid (EDTA) buffer to identify the species via the polymerase chain reaction (PCR)-restriction fragment length polymorphisms (RFLP) protocol developed by Ludwig *et al.* (2011). Of the animals collected for gonadal histology, 58% were used to confirm their species identity. This subsample allowed the genetic analysis to be optimised by the assessment of a representative number of individuals.

The tissues were fixed in Davidson's AFA (alcohol–formalin–acetic acid) solution (33% 95% alcohol, 22% formaldehyde, 11.5% acetic acid and 33.5% distilled water) for 48 h. After fixation, pieces of the gonad (1 cm^3) were removed and processed histologically by embedding them in histological paraffin and sectioning the blocks into 5-μm-thick sections. The permanent slides were stained with Harris' haematoxylin and eosin (HE) according to Behmer *et al.* (1976). Photomicrographs were produced using Leica QwinLite V 2.4 (Leica Microsystems Imaging Solution Ltd. Cambridge, UK 1993 – 2001) and ImageJ V 1.46a (Image Processing and Analysis in Java, 2011, Wayne Rasband, Public Domain, Bethesda, MD, USA) softwares.

The animals were classified histologically according to the type of germ cells present in their gonads into males, females, hermaphrodites (in which both oocytes and spermatozoa were found in the same individual as well as in the same acini) and

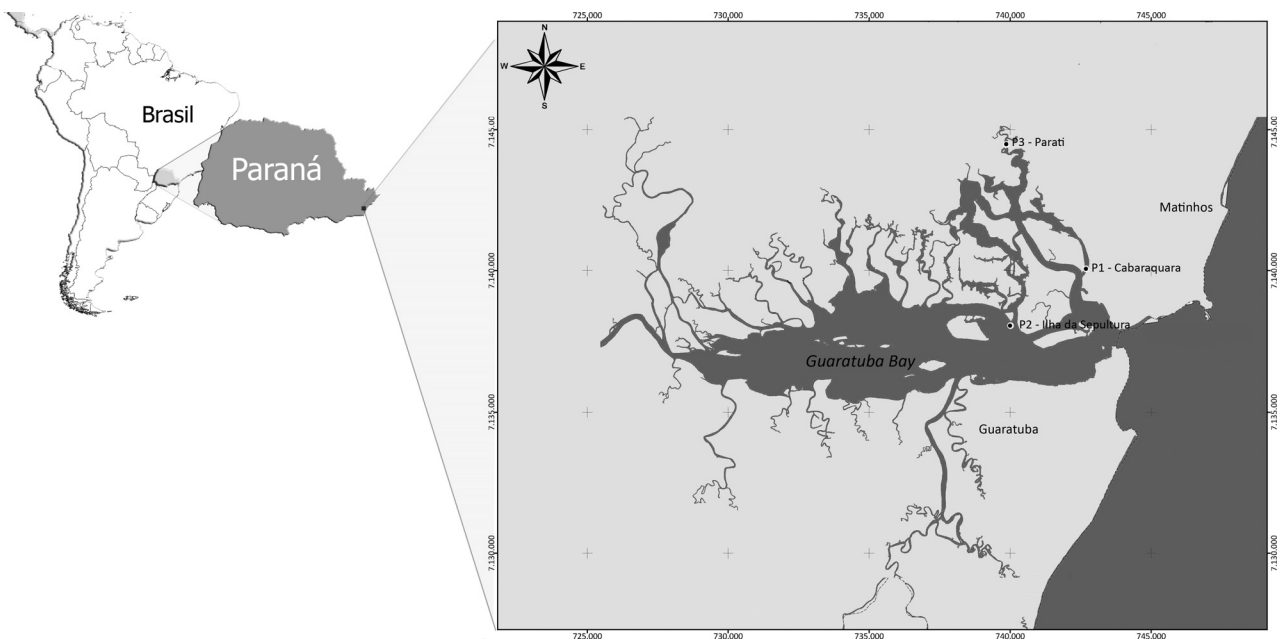

Figure 1—Sampling sites of oyster beds located in both intertidal and subtidal areas from the Guaratuba Bay, Paraná. The points on the map indicate the sampled oyster beds: Cabaraquara (25°49′59.8″ S, 048°34′41.6″ W), Ilha da Sepultura (25°51.154″ S, 048°36.481″ W) and Parati (25°47.866″S, 048°36.447″W).

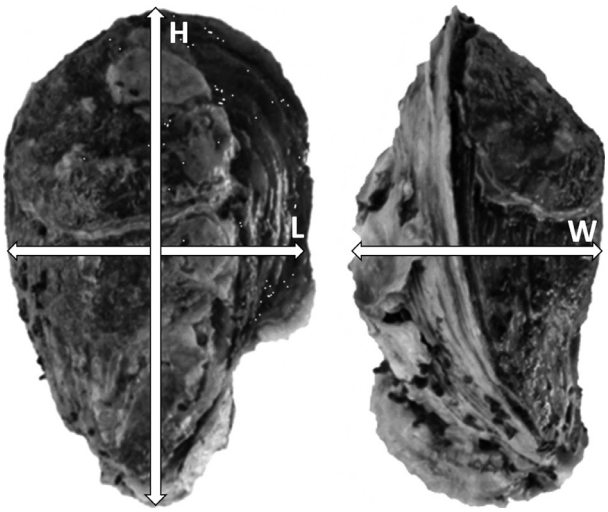

Figure 2—Schematic model for biometrics oyster proposed by Galtsoff (1964). H, height (centimetres), L, length (centimetres) and W, width (centimetres).

immature or in sexual repose [in which no germ cells were found in the gonads, making sex determination impossible, as per Lenz (2008)].

The methodology used to classify the gonadal maturation stages was based on the size of gonadal acini (follicle), the presence of interstitial tissue and the density and predominant stage of the germ cells. Therefore, the classification proposed herein used the methodology proposed for *C. rhizophorae* by Galvão *et al.* (2000) as a reference. Animals not belonging to the species *C. brasiliana* were excluded.

The data analysis was performed using Statistica 8.0 software (StatSoft, Inc., Tulsa, OK, USA; 1984–2007). The data normality was analysed using Kolmogorov–Smirnov–Lilliefors and Shapiro–Wilk tests. The variance homogeneity was evaluated using Cochran's C-test, Hartley test, Bartlett's test and Levene's test (ANOVA). The data did not fit these three tests simultaneously; therefore, they were subjected to nonparametric analysis using the multiple comparison test for independent variables using Kruskal–Wallis analysis of variance.

Results

Species identification

Oysters used in this research were identified initially by morphological characteristics and by the position they occupy in the water column, as described by Castilho-Westphal (2012). This preliminary identification was confirmed by sampling (n = 263) and further application of molecular protocols developed by Ludwig *et al.* (2011). Of these, 59% belonged to the *C. brasiliana* species, 38% belonged to *C. rhizophorae*, and 3% were classified as *Crassostrea* sp. (Table 1). The combined use of these methodologies was effective for identifying the oysters at the species level.

Gonadal histology

Among the specimens collected during the study period (n = 453), 108 were excluded from gonadal analyses because they were identified as belonging to *C. rhizophorae or Crassostrea* sp. (Table 2). The tissue analyses (n = 345) demonstrated a predominance of females (69%) over males (26%), indeterminates (4%) and hermaphrodites (1%) in a ratio of 2.65 F:1 M in the three sampling points (Fig. 3).

The number of females was significantly higher ($P < 0.05$) than that of the other individuals at all sampling sites. Thus, the biometric data exhibited little variation between the sites, with differences primarily observed among the females (Table 2). Of the collected animals, the minimum recorded height was 2.2 cm and the maximum was 12.8 cm, both corresponding to maturing males.

The animals that lacked germ cells undergoing gametogenesis or whose sex remained undifferentiated were classified as immature or indeterminate (S0). These individuals exhibited storage connective tissue (SCT) instead of gonadal tissue, which is characterised by poor staining with Harris' haematoxylin and eosin and by having an irregular globular shape (Fig. 4) with a mean connective tissue cell diameter of 7.4 ($\pm$1.9) μm (n = 77).

The male gonads were formed by microscopic acini, or sites at which spermatogenesis and the storage of spermatozoa occur. The diameter of the acini increased at the beginning of spermatogenesis and changed throughout the maturation stages. A range of acini were observed, from small acini interspersed with a large quantity of storage connective tissue to large and juxtaposed acini without interstitial tissue. The spermatogonia were found on the acinus walls and were differentiated into primary and secondary spermatocytes, spermatids

Table 1 Percentages and number of oysters (n = 263) identified as *Crassostrea brasiliana, Crassostrea rhizophorae* or *Crassostrea* sp. from the study area

	C. brasiliana	*C. rhizophorae*	*Crassostrea* sp.	Total analysed (specimens)
Cabaraquara	20% (18)[a]	80% (72)[b]	N	90
Parati	81% (79)[a]	13% (13)[b]	5% (5)[b]	97
Ilha da Sepultura	76% (58)[a]	21% (16)[b]	3% (2)[b]	76
Total (specimens)	59% (155)	38% (101)	3% (7)	263

N, not collected.
The different letters in the same column and the same row indicate significant differences ($P < 0.05$) according to Kruskal–Wallis test for relationships between species and study area, simultaneously.

Table 2 Medians, maxima and minima (in parentheses) for the biometric data measured in the animals subjected to histological analysis

	F			M			ISEX			H		
Sampling sites	C	IS	P	C	IS	P	C	IS	P	C	IS	P
Number of animals	71[a]	75[a]	93[a]	17[b]	39[b]	35[b]	7[b]	3[b]	4[b]	0	0	3[b]
Height (cm)	9.0 (8.1–9.2)[abc]	6.9 (3.3–10.7)[bc]	6.0 (3.7–10.7)[abd]	6.1 (4.2–12.8)	6.5 (2.2–11.2)	5.6 (3.7–8.9)[ad]	7.5 (5.2–8.7)	6.5 (5.2–9.3)	6.3 (5.3–7.6)	–	–	4.5 (3.9–6.4)
Length (cm)	2.7 (2.1–3.1)[a]	5.1 (1.1–7.4)[b]	4.5 (1.3–7.2)	3.8 (1.7–6.0)	4.6 (1.3–7.4)	4.1 (1.5–8.3)	5.3 (4.1–9.4)	4.5 (4.1–6.4)	4.9 (3.5–5.7)	–	–	5.0 (3.2–5.1)
Width (cm)	5.9 (4.4–6.8)	2.9 (1.4–7.1)[b]	2.3 (1.1–8.7)[a]	2.2 (0.9–6.4)	2.5 (1.4–6.3)	1.8 (1.1–8.3)[a]	2.4 (2.1–5.5)	2.2 (1.9–3.1)	2.9 (1.9–3.9)	–	–	1.8 (1.4–2.0)

F, female; M, male; H, hermaphrodite; ISEX, indeterminate sex; C, Cabaraquara; IS, Ilha da Sepultura; P, Parati.
The different letters in the same row indicate significant differences ($P < 0.05$) according to Kruskal–Wallis test.

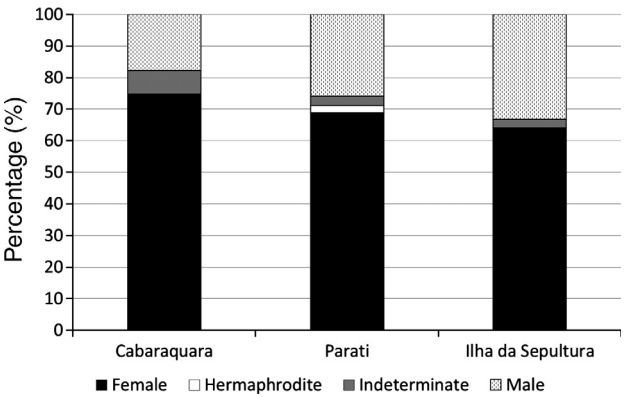

Figure 3—The percentage of individuals collected in each of the classes observed in the gonadal histology of the *Crassostrea brasiliana*.

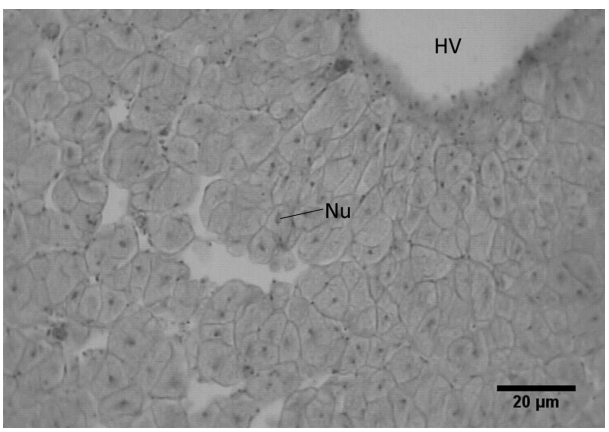

Figure 4—Photomicrograph of the storage connective tissue (SCT) from the *Crassostrea brasiliana* with the haemal vessel (HV) indicated in the top of the figure. The nucleus (Nu) of an SCT cell. HE staining.

Table 3 Developmental stages of the germ cells of male *Crassostrea brasiliana*

Cell	MD ± SD (µm)	*n*	Description
Spermatogonia	3.0 ± 0.7	107	Voluminous nucleus with chromatin dispersed in its interior
Spermatocytes	1.5 ± 0.3	144	Condensed nucleus in the primary stage and undergoing intense cell division in the secondary stage
Spermatids	1.0 ± 0.3	94	Resulting from meiotic division, with half the genetic material of the previous cells, which reduces the cell size and results in a more condensed nucleus
Spermatozoa	0.7 ± 0.2	130	Characterised by long eosin-stained flagella

MD, mean diameter; SD, standard deviation.

and spermatozoa as they approached the lumen of the acini (Table 3 and Fig. 5).

Based on the presence and quantity of different sperm cells, the maturation stages of the male *C. brasiliana* were classified as described in Table 4.

The tissue structure of the female gonads was similar to that found in the males with gonadal acini containing oogonia, immature oocytes in the maturation stage near the edge and mature oocytes in the follicle lumen (Fig. 6). The SCT was found surrounding the acini whose quantity was inversely proportional to the oocyte maturation stage (Table 5), that is, mature individuals had no or little interstitial tissue, whereas animals in the prematuration and mature stages displayed a large quantity of SCT (Table 6).

The statistical analysis of the gonadal development exhibited no significant difference between the collection sites. Thus, the data from the three sites were pooled.

The immature oysters or those in sexual repose were observed primarily between the months of May and October 2010. The highest peak (July 2010) coincided with the period following spawning, which was observed in 62% of the females sampled and in 80% of the males sampled in June 2010.

The temporal distribution of the relative frequencies (%) of the female *C. brasiliana* (Fig. 7A,B) demonstrated the presence of individuals in the prematuration stage, primarily from May to September 2010, with two periods of maturation, the first between the January and May 2010 and the second between August and December 2010.

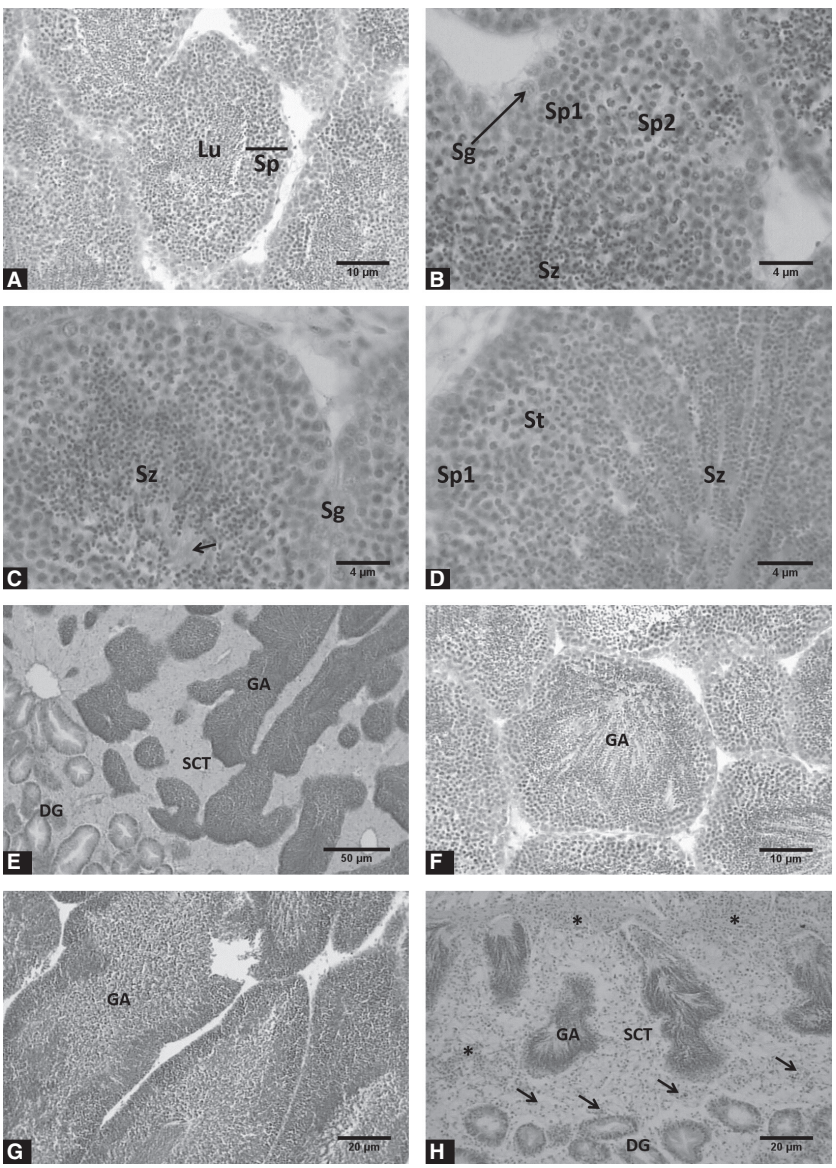

Figure 5—Photomicrographs of the male gonad of a *Crassostrea brasiliana*. The stages of spermatogenesis in a gonadal acinus: — **A.** Lumen (Lu) containing spermatozoa surrounded by spermatogenic cells (Sp); — **B.** primary spermatocytes (Sp1) and secondary spermatocytes (Sp2), spermatogonia (Sg) and spermatozoa (Sz); — **C.** spermatogonia (Sg) and spermatozoa (Sz) with eosin-stained flagella (arrow); — **D.** primary spermatocyte (Sp1), eosin-stained spermatids (St) and spermatozoa (Sz). gonadal maturation stages: — **E.** prematuration with small gonadal acini (GA), interspersed with storage connective tissue (SCT), near the digestive gland (DG); — **F.** gonad in prematuration with germ cells at different stages of spermatogenesis in the gonadal acinus (GA); — **G.** mature gonad with GA occupying a large area of the gonad, an absence of interstitial tissue and a lumen filled with spermatozoa; — **H.** a small GA undergoing cell regression with a large quantity of SCT. The gonad next to the DG. Presence of brown cells (arrows) and a large quantity of haemocytes (*). HE staining.

Table 4 Stages of gonadal maturation in male *Crassostrea brasiliana*

Gonadal maturation stage	Description
Prematuration (S1)	When large numbers of spermatogonia and gonadal acini were present, the size was reduced. Storage connective tissue interspersed between acini
In maturation (S2)	Large gonadal acini containing spermatids and spermatozoa in the lumen and spermatogonia and spermatocytes at its margins
Mature (S3)	The gonadal acini reached the maximum size with a layer of spermatocytes in the edge of the acini and the lumen densely filled with spermatozoa. The interstitial tissue was scarce
Empty or spent (S4)	The gonadal acini were in regression with the presence of cells undergoing atresia. Brown cells and a migration of the haemocytes to the tissue reserve were observed

Mature animals were primarily found from February to April 2010 and from December 2010 to March 2011. Spent or spawning females were more frequent from April to July 2010 and from December 2010 to February 2011 (Fig. 7A,B).

Table 5 Stages of oocyte development observed in female *Crassostrea brasiliana*

Cell	MD ± SD (μm)	*n*	Description
Oogonia	2.9 ± 0.9	53	Cytoplasm reduced to a size close to that of the nucleus, basophil cell
Immature oocyte	3.0 ± 0.6	111	Basophil, usually with a peripheral polyhedral-shaped nucleolus
Maturing oocytes	5.3 ± 0.9	84	Lightly eosinophilic, polyhedral-shaped voluminous nucleus, usually with a peripheral nucleolus
Mature oocyte	9.46 ± 1.8	111	Eosinophilic, voluminous nucleus, located near the follicle lumen

MD, mean diameter; SD, standard deviation.

Males in the prematuration stage were observed between May and September 2010. The S2 stage was observed during the warmer months of the year (January to February 2010 and September 2010 to February 2011); the same was true for the S3 stage (primarily in April 2010 and March 2011).

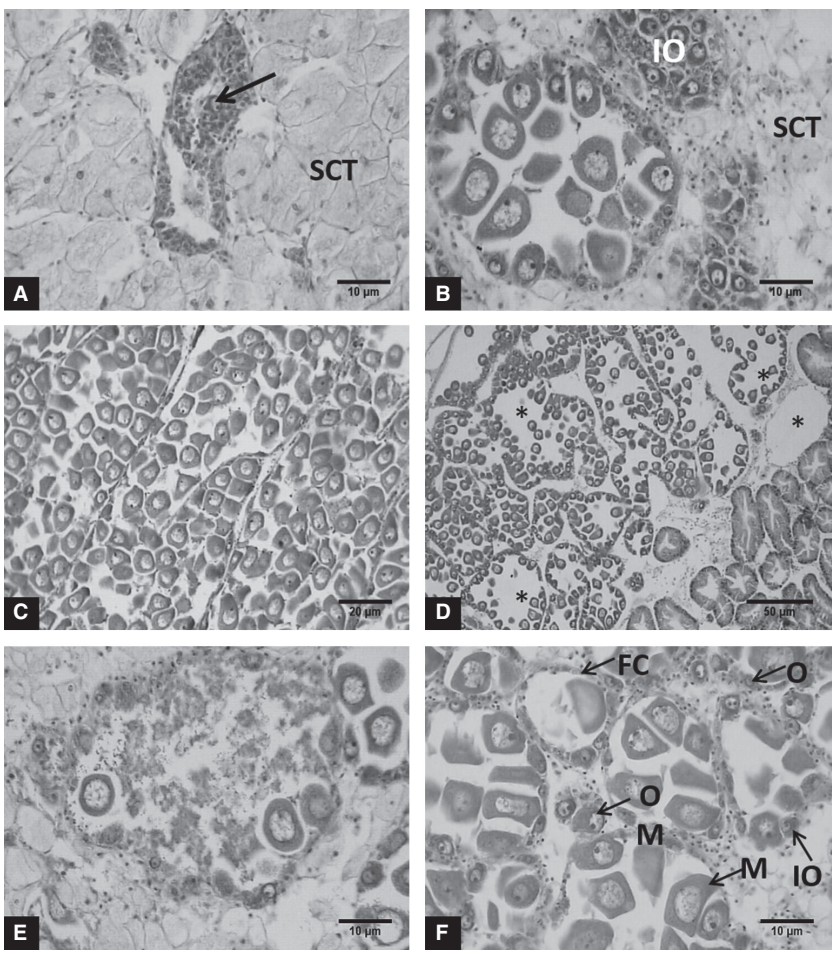

Figure 6—Photomicrographs of the female gonad of *Crassostrea brasiliana* at different stages of development. HE staining. — **A**. Oyster in prematuration stage with only one immature oocyte in the follicle lumen surrounded by storage connective tissue (SCT). — **B.** Prematuration stage with many immature oocytes (IO) and SCT. Mature oocytes can already be observed at the follicle lumen. — **C.** Mature stage characterised by follicles filled with mature oocytes and an absence of interstitial tissue. — **D.** Oyster in the process of spawning with multiple voids (*), due to the absence of oocytes in the lumen of the follicles. The same image shows tubules of the digestive gland (DG). — **E.** The follicle during the reabsorption process, visible in the spent or spawning stage. — **F.** Ovarian follicles surrounded by follicular cells (FC) and containing germ cells at different stages of development (O, oogonium; IO, immature oocyte; OM, oocyte in maturation; and M, mature oocyte).

Table 6 Stages of gonadal maturation in female *Crassostrea brasiliana*

Maturation stage	Description
Prematuration (S1)	Large SCT quantities, small rounded follicles. Large quantities of oogonia and oocytes in previtellogenesis and some oocytes in vitellogenesis
In maturation (S2)	Reduced quantity of interstitial tissue due to follicle growth and the presence of mature cells in the lumen
Mature (S3)	The follicles were at the peak of their development and displayed many mature cells. In this stage, as in all others, the oogonia were present, although in lower numbers
Empty or spent (S4)	The follicles were smaller and emptier; some atretic oocytes were observed

The S4 animals were prevalent between March and August 2010 (Fig. 7C,D).

The presence of hermaphroditic individuals in the sampled population was sporadic and was observed only in the months of September and December 2010. During both events, a predominance of spent individuals was observed, with 33% of the individuals collected in December 2010 undergoing the maturation process.

Discussion

The observation that the smallest individual captured (2.2 cm) was already reproductively active is consistent with the literature on *Crassostrea* oysters. Several studies have indicated that individuals larger than 2.0 cm are able to reproduce (Nascimento 1978; Santos 1978; Galvão *et al.* 2000). In fact, Galvão *et al.* (2000) found reproductively active individuals smaller than 2.0 cm.

The sex ratio among the groups, which exhibited a predominance of females over the other classes, was also observed in other species of the genus *Crassostrea* by other researchers including Lenz (2008), in *C. rhizophorae* from Camamu (Bahia, BA) by Lenz and Boehs (2011), in *C. rhizophorae* from Guaratuba Bay by Christo (2006), in *Crassostrea gigas* from France by Lango-Reynoso *et al.* (1999) and in *C. gigas* from Korea by Lee *et al.* (2010).

Guo *et al.* (1998) used dioecious and hermaphroditic *C. gigas* individuals to determine the genetic control of the coexistence of protandric sex change and observed different ratios of females. The ratios, according to these authors, were related to the age of the animal: a significant proportion of the oysters matured first as males and changed to females in later years. They also concluded that the primary sex was

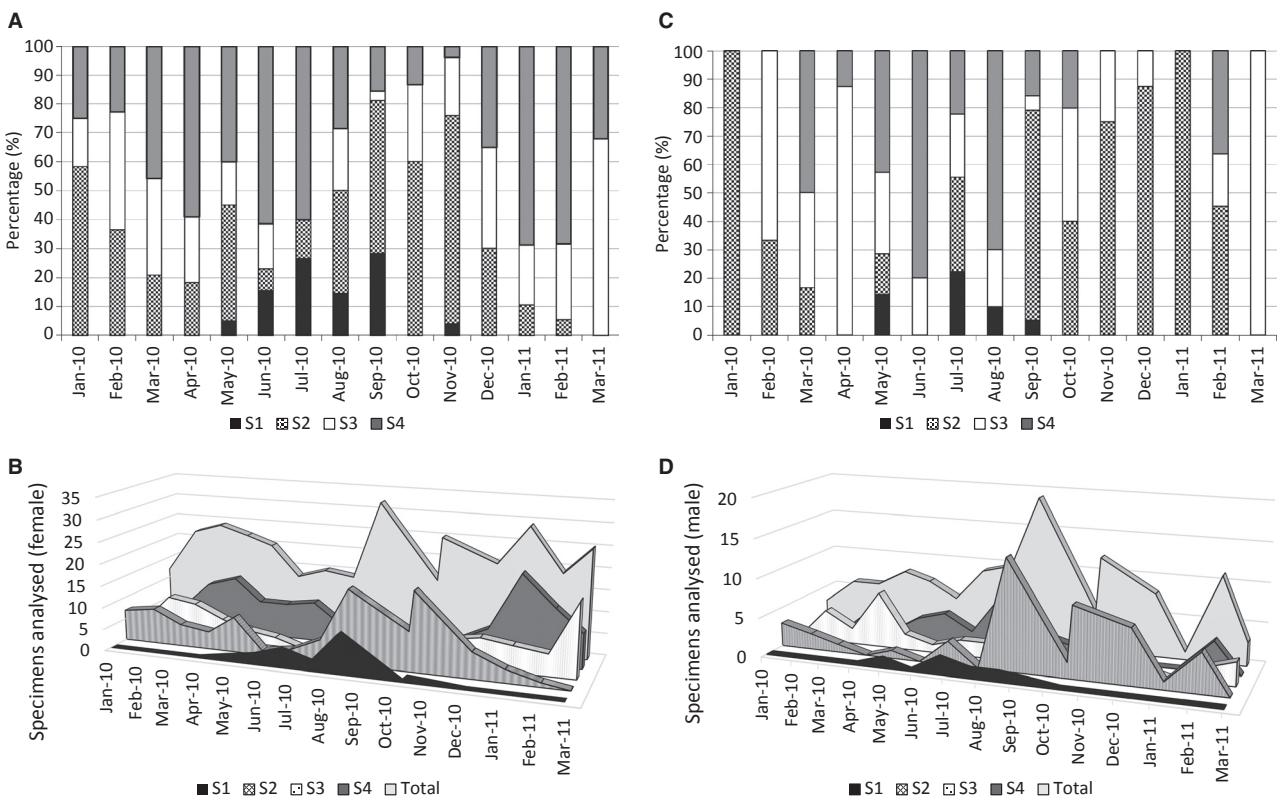

Figure 7—— **A.** and **B.** Temporal distribution of the gonadal maturation stages in female *Crassostrea brasiliana* collected between January 2010 and March 2011 from the Guaratuba Bay, Paraná, Brazil. S1 = premature, S2 = maturing, S3 = mature, S4 = spawning. — **C.** and **D.** Temporal distribution of the gonadal maturation stages of male *Crassostrea brasiliana* collected between January 2010 and March 2011 from the Guaratuba Bay, Paraná, Brazil. S1 = premature, S2 = maturing, S3 = mature, S4 = spawning or spent.

determined by a dominant male allele (M) and a protandric female allele (F); thus, MF individuals were true males, and FF were protandric females capable of changing sex. The rate of sex change in FF individuals was influenced by their secondary genes and/or environmental factors (Guo *et al.* 1998; Lango-Reynoso *et al.* 1999). The effects of maternal and paternal variables on the sexual maturation and spawning period were also suggested by Guo *et al.* (1998), given that the females required greater food availability and higher temperatures (Lango-Reynoso *et al.* 1999). Therefore, the presence of large amount of female oysters in Guaratuba Bay suggests a favourable ambient condition in this site.

Given the sexual traits of the *Crassostrea* oysters, Lango-Reynoso *et al.* (1999) stated that hermaphroditism could be considered a transitional stage between the different sexes, with a strong tendency towards becoming female. Furthermore, the frequency of hermaphroditism varied with the age of the animals and the environment in which they were found (Galtsoff 1964; Galvão *et al.* 2000).

Because they exist in an ephemeral stage, hermaphrodites are generally found in smaller proportions than the other classes, as observed in the present study and in studies by Menzel (1951), Dinamani (1974), Nascimento (1978), Sá (1980), Paniagua-Chávez *et al.* (1995), Lango-Reynoso *et al.* (1999), Galvão *et al.* (2000), Christo (2006) and Lenz (2008).

However, the presence of gonads with both male and female gametes may indicate a pathological finding. In this case, the animals were termed 'intersex' by Lee *et al.* (2010), a phenomenon that, according to the authors, induced by aquatic pollutants and chemical endocrine disruptors. These authors also suggested that the increase in intersex individuals within a population could serve as a bioindicator of environmental quality. There are few studies on environmental quality in the study area, and although oxygen depletion may occur (Mizerkowski *et al.* 2012), levels of pollutants such as organochlorines are low (Combi *et al.* 2013).

The lower proportion of males compared with females poses no risk to population maintenance within the environment because, according to Lenz (2008), males may release gametes more frequently than females due to faster gonadal recovery. The predominance of animals of indeterminate sex in a population varied throughout the study period, as shown by other authors such as Dinamani (1974) in New Zealand from March to September and Lenz (2008) in north-east of Brazil, mainly from August to September.

The collection of sexually mature animals, producing and releasing gametes during nearly every month of the year, regardless of the class to which they were assigned, can also be considered normal behaviour for the species because intermittent spawning has been described for *C. brasiliana* in south-east Brazil by Galvão *et al.* (2000) as a characteristic of the species. Several authors have described intermittent spawning in other *Crassostrea* spp. (Vélez 1977; Nascimento 1978; Zamora *et al.* 2003; Cardenas *et al.* 2007; Lenz 2008; Lenz and Boehs 2011).

Therefore, one can conclude that the reproduction of *C. brasiliana* in Guaratuba Bay occurs intermittently, but with greater intensity during the warmer months of the year, producing a predominantly female population.

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