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TEMPORAL VARIATION IN LARVAL ABUNDANCE OF THE MANGROVE OYSTER *Crassostrea* sp. IN AN ESTUARY OF SOUTHERN BRAZIL

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RESUMO: Uma alternativa para reduzir o grau de exploração de bancos naturais de ostras é a utilização de coletores artificiais de sementes. A eficácia dos referidos coletores de sementes, no entanto, depende do conhecimento dos ciclos e da abundância de larvas no plâncton. Este trabalho teve como objetivo estudar a presença de larvas do gênero *Crassostrea* na baía de Guaratuba, a qual está localizado na costa sul do Brasil, utilizando dois métodos complementares: 1) identificação visual seguido de contagem de larvas e 2) identificação das larvas no nível de espécie utilizando a biologia molecular. As medições de parâmetros abióticos relacionados com a qualidade da água (concentração e saturação de oxigênio dissolvido, pH, temperatura, salinidade e transparência) foram realizadas a cada duas semanas, em três pontos amostrais (Cabaraquara, Ilha da Sepultura e Parati) de janeiro de 2010 a abril de 2011 (n=32). Para a investigação larval, coletou-se plâncton em redes de 65 µm. Os parâmetros ambientais, particularmente o pH e a concentração de oxigênio dissolvido, influenciaram a presença de larvas de moluscos da baía de Guaratuba. As maiores concentrações de larvas no plâncton ocorreram entre setembro de 2010 e janeiro de 2011, e a variação na abundância larval foi observada principalmente durante as estações mais quentes do ano (primavera e verão).

Palavras-chave: reprodução; manguezais; moluscos bivalvos; variáveis abióticas, plâncton

ABSTRACT: An alternative for reducing the degree of exploitation of natural oyster banks is to use artificial seed collectors. The effectiveness of said seed collectors, however, depends on the knowledge of the cycles and abundance of larvae in the plankton. This work aimed to study the presence of larvae of the genus *Crassostrea* in the Guaratuba Bay, which is located on the southern coast of Brazil, using two complementary methods: 1) visual identification followed by larval counts and 2) identification of the larvae at the species level using molecular biology. Measurements of abiotic parameters related to water quality (concentration and saturation of dissolved oxygen, pH, temperature, salinity and transparency) were performed every two weeks at three sampling points (Cabaraquara, Ilha da Sepultura and Parati) from January 2010 to April 2011 (n=32), and plankton was collected in 65 µm nets for larval research. Environmental parameters, particularly pH and the concentration of dissolved oxygen, influenced the presence of mollusc larvae in the Guaratuba Bay. The highest concentrations of larvae in the plankton occurred between September 2010 and January 2011, and the variation in larval abundance was observed mainly during the warmer seasons of the year (spring and summer).

Key Words: reproduction; mangroves; bivalve mollusc; abiotic variables; plankton

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INTRODUCTION

Oysters of the genus *Crassostrea* (Sacco, 1897) are bivalve molluscs that belong to the family Ostreidae (Rios, 1994). These oysters inhabit shallow coastal waters and are found from the equatorial belt to moderately cold areas (Wakamatsu, 1973; Costa, 1985). The species of this genus are considered euryhaline (Paixão *et al.*, 2013; Pereira *et al.*, 2003), and they spawn intermittently throughout the year, are eurythermic, and are well adapted to estuarine environments (Galvão *et al.*, 2000; Christo, 2006). In Brazil, these oysters are popularly known as "mangrove oysters" (Amaral & Simone, 2014).

The reproductive cycle of *Crassostrea* is influenced by endogenous and exogenous factors and includes gametogenesis, larval development, attachment, and metamorphosis (Christo, 2006). Among the stages of larval development (trochophore, D larvae, veliger, and pediveliger), it is in the pediveliger stage that the larva undergoes metamorphosis, becoming benthic and receiving the denomination of "seed" (Grant *et al.*, 2013; Fabioux, 2004; FAO, 2004). When the oyster is in its sessile form, the substrate determines the growth pattern and external morphology of the oyster shell, which is a particularity that allows the animal to attach to different media (Absher, 1989). However, this morphological variation makes it difficult to confirm the identity of the species based on visual analysis (Ludwig, 2010).

Knowledge of larval cycles and abundance in plankton are essential for the implementation of measures that aim to reduce the exploitation of natural banks of oysters, which include the use of artificial seed collectors (substrates specially designed to allow the settlement and metamorphosis of the larvae, which will later be transferred to farming structures).

In a study conducted in the Guaratuba Bay, Paraná (PR), Christo (2006) found that oyster larvae of the genus *Crassostrea* were present in the plankton throughout the year and suggested that these animals exhibit a continuous reproduction pattern that is influenced by the ambient temperature.

However, a limitation of larval studies in the field is the difficulty of morphological differentiation of species, which often requires the use of electron microscopy techniques (Christo *et al.* 2010), often making its application unfeasible (Ludwig, 2010). For this reason, methods to molecularly detect mollusc larvae have been developed to overcome these methodological limitations and to provide greater specificity and sensitivity to the processing of samples (Claxton *et al.* 1997; Toro 1998; Pie *et al.* 2006; Boeger *et al.* 2007; Ludwig, 2010; Melo *et al.*, 2010; Okada *et al.*, 2013).

This work aimed to support to the use of artificial larvae collectors by studying the temporal variation in the abundance of oyster larvae of the genus *Crassostrea* in the Guaratuba Bay, which is located on the southern coast of Brazil, using two complementary analytical methods: visual identification followed by larval counts and larval identification at the species level using molecular biology.

METHODS

Study Area

The studies were conducted in the Guaratuba Bay (25°52'S 48°39'W; Paraná coast, southern coast of Brazil), which is part of the Atlantic Forest biome. The sampling points were defined based on their proximity to important natural oyster banks located in Ilha da Sepultura (25°51.154"S 048°36.481"W) and in the regions of

Parati (25°47.866"S 048°36.447"W) and Cabaraquara (25°49'59.8"S 048°34'41.6"W) (Figure. 1).

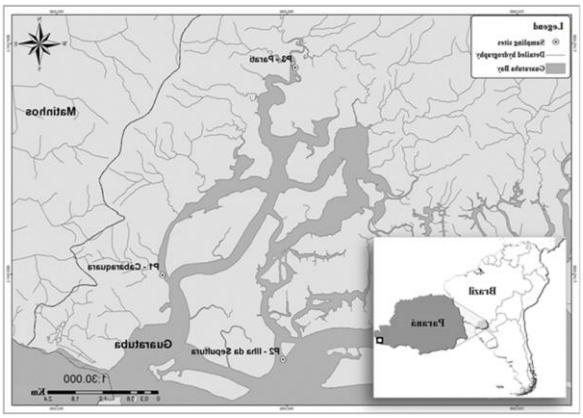

Figure. 1. Sampling sites in oyster beds located simultaneously in intertidal and subtidal habitats in the Guaratuba Bay, Paraná state. The points on the map indicate the banks: Cabaraquara, Ilha da Sepultura, and Parati.

Collection of oyster larvae in plankton

Plankton was collected every two weeks, from 19 January 2010 to 16 April 2011 (n=32) using a 0.5 HP motor pump (STIHL, P835 model, Brazil). Each sample was obtained by filtration of 1,000 L of water through a 65-µm mesh size plankton net. Three samples were collected at each sampling point: one for the quantification of mollusc larvae and two for the identification of molecular species of oyster larvae present in the plankton. The material retained in the net at each pumping was transferred into polyethylene flasks, fixed in 4% formalin solution buffered with monobasic and dibasic sodium phosphate, and stained with 1% Rose Bengal, to facilitate the quantification of the larvae, or fixed in 92° ethanol for the samples used for molecular larval identification.

Identification of larvae

Plankton samples intended for larval identification were sent to the Laboratory of Molecular Ecology and Evolutionary Parasitology (LEMPE) of the Federal University of Paraná (UFPR). In the laboratory, the samples were filtered with the aid of a manual vacuum pump, thus concentrating the organisms on Millipore® filter paper for subsequent molecular larval identification, as developed and described by Ludwig *et al.* (2011). This method was chosen because it allows the detection of the presence of larva(e) of the genus *Crassostrea* in a sample, although it has the limitation of being unable to quantify the larvae.

Quantification of larvae

The bivalve mollusc larvae were counted using the methodology proposed by Boltovskoy (1999). The analysis was conducted using a Leica DMLS optical microscope and a Sedgewick-Rafter counting chamber. The number of larvae present in each sample was initially quantified by screening 25% of the sample. Based on the results, two different procedures were adopted: 1) in situations where less than 30 larvae were counted in 25% of the sample, the analysis continued until a minimum of 30 larvae were observed or until 100% the sample was analysed; 2) in situations where counts were equal to or greater than 30 larvae, the analysis was ended after 25% of the sample was analysed and was thus completed during the screening step.

Abiotic variables

The following abiotic variables were quantified biweekly at the moment the water was pumped for plankton collection at each of the sampling points: dissolved oxygen concentration (in mg/L and as a percentage of saturation) and temperature (both using an oximeter - YSI 550A, USA), pH (pH metre - pHtek, Brazil), salinity (refractometer - Instrutemp ITREF10, Brazil), and transparency (Secchi disk). Both the water analysis and the sample

collections were performed in the morning, during the flood tide, and at a depth of approximately 1.0m.

Statistical analysis

Statistical analyses were performed using the Statistica Software 8.0 (Statsoft®, 1984-2007). Data on biotic and abiotic variables were grouped, and the normality of the distribution was tested using the Kolmogorov-Smirnov, Lilliefors, and Shapiro-Wilk tests. Once the non-parametric distribution of the data ($p<0.05$) was confirmed, a comparison analysis between multiple independent variables was performed using the Kruskal-Wallis analysis of variance. The proximity of the sampling points based on the quantified abiotic data was assessed by grouping the data using multivariate cluster analysis and the simple Euclidean distance metric. The same method was used to establish the relationship between sampling points according to the number of larvae observed. The correlation between the amount of bivalve mollusc larvae in the plankton and the abiotic variables was also evaluated by principal component analysis (PCA).

RESULTS

Abiotic variables

During the 16-month sampling period, abiotic parameters were measured 32 times in each of the three monitored sample areas. There was no difference in temperature and pH among the sampling points. The Parati site had lower dissolved oxygen concentrations and lower salinity compared with the remaining points. The Cabaraquara site showed higher salinity compared with the remaining sites and a lower transparency than at Ilha da Sepultura (**Erro! Fonte de referência não encontrada.**1 and Figure. 2).

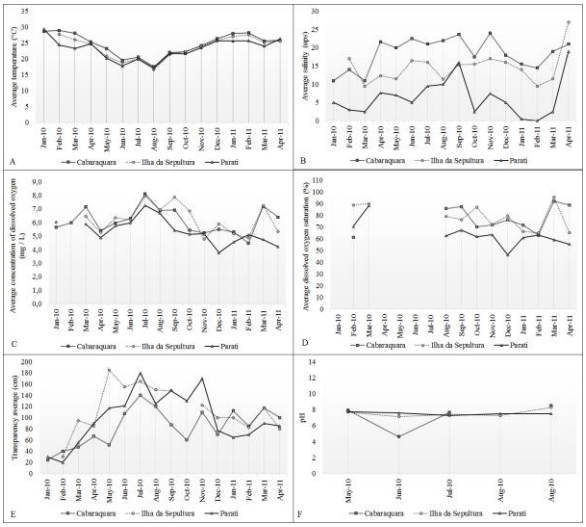

Figure. 2. Abiotic data collected at three sampling points in Guaratuba Bay, Paraná state. A) Monthly average temperature (°C). B) Monthly average salinity (psu). C) Monthly average concentration of dissolved oxygen (mg/L). D) Monthly average dissolved oxygen saturation (%). E) Monthly average transparency (cm). F) pH values.

Variables analysed	Cabaraquara	Ilha da Sepultura	Parati
Dissolved oxygen (mg/L)	6.2 (4.1-8.2)^{b}	6.2 (3.6-9.5)^{b}	5.3 (3.3-8.2)^{a}
Dissolved oxygen (%)	76.2 (58.2-98.9)^{b}	75.2 (36.7-123.8)^{b}	62.6 (38.5-88.3)^{a}
pH	7.8 (4.6-8.5)	7.4 (7.1-8.9)	7.5 (7.3-7.8)
Salinity	20.0 (7.0-28.0)^{a}	13.5 (0.8-27.0)^{b}	5.0 (0.0-20.0)^{c}
Temperature (°C)	25.0 (16.1-31.4)	24.0 (15.1-29.0)	23 (15.0-29.5)
Transparency (cm)	85.0 (24.5-160.0)^{a}	100.0 (30.0-240)^{b}	95.0 (20.0-230.0)^{ab}

Table 1. Median, maximum, and minimum values of the environmental variables measured at the sampling points. The letters indicate significant differences by the Kruskal-Wallis test ($p<0.05$).

In the multivariate analysis, the sampling sites were grouped according to the quantified abiotic variables; it was found that the monitored sites showed small Euclidean distances from one another, with an even smaller distance between the Parati and Cabaraquara sites.

Presence of oyster larvae in plankton

Throughout the study period, 92 samples were collected for the

quantification of larvae. In these samples, 9,336 bivalve mollusc larvae were recorded, 71% of which were from the Cabaraquara site, 25% were from Ilha da Sepultura, and 4% were from Parati. When the sampling sites were grouped by multivariate analysis according to the amount of larvae present in plankton, a greater proximity was observed between Sepultura Island and Parati.

A comparison analysis of multiple independent variables using the Kruskal-Wallis analysis of variance found differences in the distribution of data obtained between the Parati and Cabaraquara sites, with the latter site exhibiting greater variation in the amount of larvae observed. In some cases, over 1,600 larvae per sample were recorded (Fig. 3). The Ilha da Sepultura site, in contrast, did not differ significantly from the other sites ($p>0.05$).

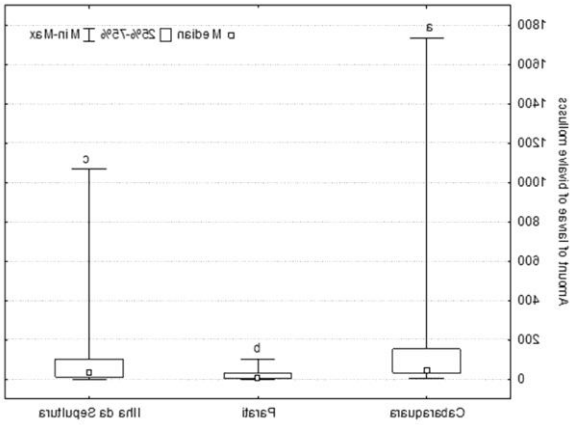

Figure. 3. Distribution of the number of larvae observed during the study period at each sampling site. The letters indicate the significant differences found using the Kruskal-Wallis test ($p<0.05$).

The prevalence of mollusc larvae at the Cabaraquara site was higher in the months of February and April 2010 and September 2010 to February 2011, with the highest peaks observed in the latter range. To identify the larvae quantified under a light microscope, genetic analyses were performed with samples collected in March and October 2010 and in October 2011. On these occasions, oyster larvae were detected in most samples (Table 2).

Sampling sites	Cabaraquara			Ilha da Sepultura			Parati		
Species	B	R	S	B	R	S	B	R	S
April 2010	2	1	2	4	5	0	2	0	0
May 2010	2	2	0	0	0	0	3	1	0
June 2010	0	1	0	0	0	1	1	0	0
Total number of samples	**4**	**4**	**2**	**4**	**6**	**1**	**6**	**1**	**0**

Table 2. Number of plankton samples positive for the presence of oyster larvae of the genus *Crassostrea*. B = *Crassostrea brasiliana*, R = *C. rhizophorae* (Guilding, 1928), S = *Crassostrea* sp..

Among the three sampling sites studied, the Parati site exhibited the smallest amount of larvae, with discrete peaks in April and July 2010, from September 2010 to December 2010, and from February to April 2011 (Figure. 4.).

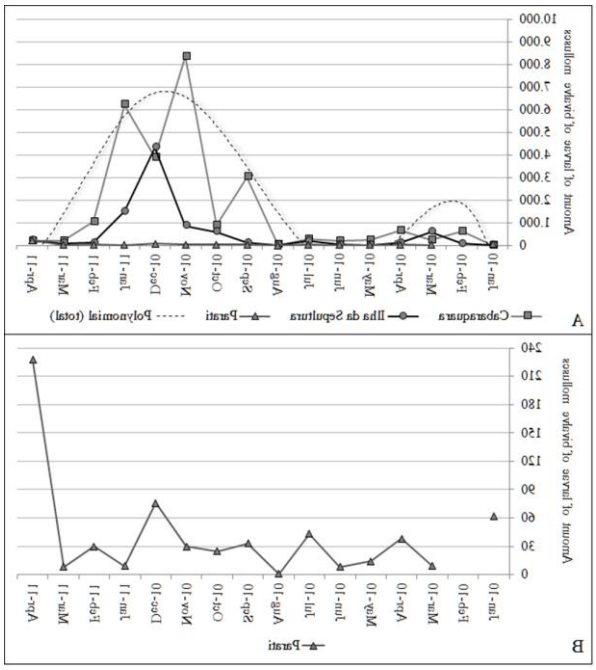

Figure. 4. Temporal distribution of bivalve mollusc larvae. The values shown correspond to the total volume pumped monthly (2,000 L). A) Plankton samples collected at the three sampling points in Guaratuba Bay, Paraná state. B) Plankton samples collected at the Parati site.

The PCA showed that the presence of plankton larvae (BML) was mostly correlated with the dissolved oxygen concentrations (mg/L), pH, and dissolved oxygen saturation (%), in that order, and to a lesser extent with salinity, temperature (°C), and water transparency (cm) (Figure. 5.).

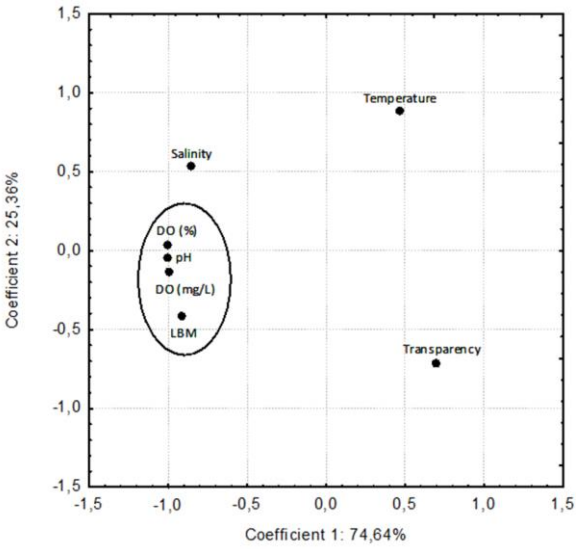

Figure. 5. Graphical representation of the principal component analysis of the number of bivalve mollusc larvae in plankton and the abiotic variables measured at the sampling sites during the study period. The circled group corresponds to the more highly correlated variables. DO = dissolved oxygen, BML = bivalve mollusc larvae.

DISCUSSION

The Guaratuba Bay is part of the Environmental Protection Area (Área de Proteção Ambiental - APA) of Guaratuba, a protected area with sustainable use of natural resources of nearly 200,000 hectares, which includes part of the municipal districts of Guaratuba, São José dos Pinhais, Tijucas do Sul, Morretes, Paranaguá, and Matinhos, in Paraná state, Brazil (SEMA *et al.*, 2003; TNC *et al.*, 2008). Despite the existence of laws and decrees aimed at protecting these areas, the number of well-preserved habitats has decreased significantly in this coastal-estuarine region (TNC *et al.*, 2008).

The environmental variables monitored here exhibited oscillations (mainly at sites 1 and 2 (Cabaraquara and Ilha da Sepultura)) within the standard values expected for the region, thus suggesting a relative stability of the system in regards to the variation limits of the variables analysed. The average annual temperature of the water in Guaratuba Bay, which ranged between 23 and 25°C during this study, remained within the limits quantified by others (Chaves and Bouchereau, 1999; Christo, 2008). The water transparency values were similar to those obtained by Christo (2008), whereas the salinity remained within the limits described for the study area based on the data presented by Chaves and Bouchereau (1999), Christo (2008) and Santos *et al.* (2008). This stability, however, did not apply to the Parati site, which lies in an innermost area of Guaratuba Bay and receives a higher contribution of inland waters compared with the other sites. Moreover, salinity was naturally lower at the Parati site than in other sampling sites, reaching 0.0 psu on some occasions.

Thus, the analysis of these data and the fact that a large amount of larvae were quantified in plankton during the collections confirms the great environmental plasticity of these native bivalve molluscs. The molluscs were collected both in relatively low dissolved oxygen concentrations (below 5.0 mg/L) and in situations of varying pH range (4.6 to 8.5).

Although the methodology proposed by Ludwig (2010) enabled the differentiation of larvae of the genus *Crassostrea* in water samples, this method does not permit the quantification of larvae of each species present in the samples. Thus, molecular

analyses were performed to confirm the presence of each species of the *Crassostrea* genus identified in Guaratuba Bay in the collected samples. Therefore, only samples with high purity were considered, and those that had large amounts of nucleic acids from other organisms were discarded. This procedure greatly increased the reliability of the results obtained regarding the specific identity of the collected larvae.

It is known that the dispersal of oyster larvae estuarine regions occurs with the ecological purpose of selecting new habitats, thus allowing for genetic variability and a safe place for the larvae that is rich in food and free of predators (Levin, 2006). However, there is no consensus on the physical and biological mechanisms that determine the distribution patterns of oyster larvae in these locations (Boehs, 1996). It is believed that larval dispersal and retention result from the interaction between passive transport, which is caused by the action of currents and tides, and the active transport of larvae by swimming (Silva and Boehs, 2007). It is also known that temperature may influence the number of larvae of this genus that are recruited (Philippart, C. J. M. *et al.*, 2012; Dutertre *et al.*, 2010). In this study, the PCA showed that there was an effect of abiotic variables, mainly dissolved oxygen and pH, on the number of bivalve mollusc larvae present in the plankton of Guaratuba Bay, Paraná state.

Among the samples analysed, a higher prevalence of oyster larvae of the genus *Crassostrea* was observed between the months of September and March, suggesting that there is a marked seasonal effect on determining the reproductive period, which is more intense during the warmer seasons (spring and summer). These findings are consistent with the descriptions by Christo (2008), Castilho-Westphal (2012), and Castilho-Westphal *et al.* (2013). The latter two authors found a higher prevalence of *Crassostrea brasiliana* (Lamarck, 1819) in the final stage of gonadal maturation and in the spawning stage during that same period. Thus, it is suggested that artificial oyster seed collectors be installed in regions close to natural banks between the months of December and January.

CONCLUSÃO

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