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GAMETOGENIC CYCLE OF Lytechinus variegatus (LAMARCK, 1816) (ECHINOIDEA: TOXOPNEUSTIDAE) IN NORTHEASTERN BRAZIL

Ciclo gametogênico de *Lytechinus variegatus* (Lamarck, 1816) (Echinoidea: Toxopneustidae) no Nordeste do Brasil

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ABSTRACT

Some reproductive aspects of *Lytechinus variegatus* were described including sex ratio, gonad index (GI) and histological examination of the gonads. Ninety individuals (63.44 mm ± 6.17) were sampled from March 2017 to January 2018 randomly collected by hand at Enseada da Ribeira (municipality of Salvador, northeastern Brazil: 12º54'488''S, 38º29'872''W). The sex ratio did not differ from 1:1 and the GI showed no significant differences (p>0.05) for both sexes. Males and females continuously release its gametes, presenting a greater reproductive effort during the dry and warmer periods of the year. A wide range of food supply for the individuals guarantees the maintenance of the long reproductive period. Immediate metabolic expenditure may explain the lack of variation in GI, as gonads are not storage organs in this species. *L. variegatus* populations present variability in strategies for using and allocating energy resources in response to environmental interactions *in situ*, regardless of latitude. Along the Brazilian coast, an inverse relationship is observed between oocyte size and the effort devoted to reproduction in different populations of this species. Quantitative oogenesis evaluations over time are assertive analytical techniques for assessing the reproductive cycle of echinoids

Keywords: *Lytechinus variegatus*, sea urchin, histology, reproductive cycle, gonadal index, cytometric analysis.

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RESUMO

Alguns aspectos reprodutivos de Lytechinus variegatus *foram descritos, incluindo proporção sexual, índice gonadal (IG) e o exame histológico das gônadas. Foram amostrados 90 indivíduos (63,44 mm ± 6,17) entre março de 2017 e janeiro de 2018, coletados manualmente de forma aleatória na Enseada da Ribeira (município de Salvador, Nordeste do Brasil: 12º54'488''S, 38º29'872''W). A proporção sexual não diferiu de 1:1 e o IG não apresentou diferenças significativas (p>0,05) para ambos os sexos. Machos e fêmeas liberam seus gametas continuamente, apresentando maior esforço reprodutivo nos períodos secos e mais quentes do ano. Uma ampla oferta de alimentos aos indivíduos garante a manutenção do longo período reprodutivo. O gasto metabólico imediato pode explicar a falta de variação no IG, uma vez que as gônadas não são órgãos de armazenamento nesta espécie. Populações de* L. variegatus *apresentam variabilidade no uso de recursos energéticos e estratégias de alocação em resposta a interações ambientais in situ, independentemente da latitude. Ao longo da costa brasileira observa-se uma relação inversa entre o tamanho dos ovócitos e o esforço dedicado à reprodução em diferentes populações desta espécie. Avaliações quantitativas da oogênese ao longo do tempo são técnicas analíticas assertivas para avaliar o ciclo reprodutivo dos equinóides.*

Palavras-chave*: Lytechinus variegatus, ouriço de mar, histologia, ciclo reproductivo, índice gonadal, análise citométrica.*

INTRODUCTION

Echinoid echinoderms are essential components of coastal-marine biodiversity. In Brazil, the Northeastern coast has the largest representation of this group, particularly in shallow water environments (Gondim *et al.*, 2018). Many species, such as *Lytechinus variegatus* (Lamarck, 1816), have a wide latitudinal distribution (Ventura *et al.*, 2003). Their populations are recorded from North Carolina (USA) to Rio Grande do Sul (Brazil). They inhabit areas of unconsolidated substrate, colonized by macroalgae and marine phanerogams, generally up to 20 m deep (Lopes and Cerqueira, 2007; Tavares, Borzone, 2015; Carvalho-Souza *et al.*, 2018).

Studies on bioecological aspects, such as the reproductive cycle of marine invertebrates, are among the main recommendations of Brazilian government agencies for endangered species. An example is *L. variegatus*, which has been categorized in the last decade as "vulnerable". Researchers gathered at the XXVII Brazilian Zoology Congress in 2008 reported a reduction in this echinoid population, mainly on the northeastern coast (Carvalho-Souza *et al.,* 2018; Tavares, 2020).

Echinoids reproductive cycles are typically annual or semi-annual. In addition, their gametogenesis can be influenced by environmental, endogenous, or biological factors (Lawrence 2007; Tavares, Borzone, 2006; Tavares, Borzone, 2015). Although, in Brazil, there are no published records of the commercial exploitation of this species, its use in ecotoxicological assays is already known (Leite *et al.,* 2012; Warner *et al.,* 2021).

Sporadic consumption practices were observed at Enseada da Ribeira (municipality of Salvador, Bahia, Northeastern Brazil) by researchers W. R. P. Cerqueira and Y. A. G. Tavares. On low tide days, the site is used as a collection area by residents looking for various benthic animals used for subsistence (Lopes, Cerqueira, 2007). Thus, recognizing reproductive strategies is essential in determining populational attributes for the sake of exploitation and management practices and a deeper understanding of the life histories of

marine biota in the South Atlantic.

Hence, the main objective of this study was to characterize the gametogenic events in the reproductive cycle of *L. variegatus* in a stretch of the Northeastern Brazilian coast. The study is grounded on cytometric analysis and describes the gonadal development stages for gametogenic events in males and females. Furthermore, the proportionality between the sexes and the participation of the gonads in the total weight of the animals were evaluated.

MATERIAL AND METHODS

Study area

Enseada da Ribeira (12º54'488''S, 38º29'872''W) is located in Todos-os-Santos Bay (municipality of Salvador, Bahia, Brazil). Due to the geography of the bay, the hydrodynamic in the area is weak (Alves, Cerqueira, 2000) and the substrate can be essentially sandy or mixed (sandy, muddy, and gravel) with various shell fragments and no reef outcrops (Ventura, Cruz, 2016).

The regional climate is tropical with an average annual temperature of 25.3ºC, rainfall of 2.086 mm, and an average salinity of 37 with a small vertical variation (Cirano, Lessa, 2007). The area presents moderate environmental degradation caused by urban waste and chemical contaminants from petrochemical plants in adjacent municipalities (detailed descriptions of the study area see Cirano, Lessa, 2007).

Field and laboratory procedures

The echinoids were collected bimonthly from March 2017 to January 2018 by active search at mean low water spring levels, with a minimum mature size of test diameter up to 40 mm (Lopes, Cerqueira, 2007). As far as possible, a minimum number of 15 specimens per month were randomly sampled. Sea urchins were placed in recipient holding seawater, transported to the laboratory where whole individuals were preserved in 10% phosphate-buffered formalin for at least one week and then preserved in 70% alcohol solution.

All animals were measured for the largest test diameter (0.02mm) and the greatest distance from the elliptical axis (Lewis, Storey, 1984; Hammer *et al.,* 2004) as a primary parameter of the organisms' shape. Then, they were weighed (total wet weight: 0.01g) and eviscerated to remove the gonads.

A fraction of gonad tissue ($3cm^3$) was removed and processed with sequential submersions in graded ethanol for dehydration followed by xylene for clarification and impregnation with paraffin wax at 60ºC. After that tissue samples were embedded in 100% (v/v) paraffin and they were cut with a thickness of 7 µm using a manual rotative microtome and stained with Harris' Haematoxylin and alcoholic Eosin Y (yellowish) solutions (Behmer *et al.,* 1976).

Microscopic observations performed using an Olympus® CX43 light optical microscope equipped with an Olympus® Microscope Camera and the combined EP50 v. 2019 software for monitor display. Digital images of the histological sections (n=5 per gonad) were captured for sex diagnosis and gonadal development stages diagnosis (Tavares, Borzone, 2015).

Data analysis

The contribution of the gonads to the total weight of the animal was determined by the gonad index (GI) formula: (wet weight of the gonads/total wet body weight) x 100. The sex ratio (number of females/males) was statistically analyzed utilizing the Chi-square test (χ^2).

Citometrics data as average oocyte diameter (OD) was measured by establishing two transverse sections through the center of the gamete. Only the oocytes with a visible germinal vesicle were considered. From these data, intervals of the OD were determined using Sturges' Rule (Sturges, 1926). The representative modes of OD were determined through the modal progression analysis of the frequency distributions employing the Bhattacharya method (Bhattacharya, 1967). In males, all intact follicles per field were also measured to estimate the relative amount of mass sperm (SO%) to the total cross-sectional area (Tavares, Borzone, 2015). All measurements were made using ImageJ software (Schneider *et al.* 2012).

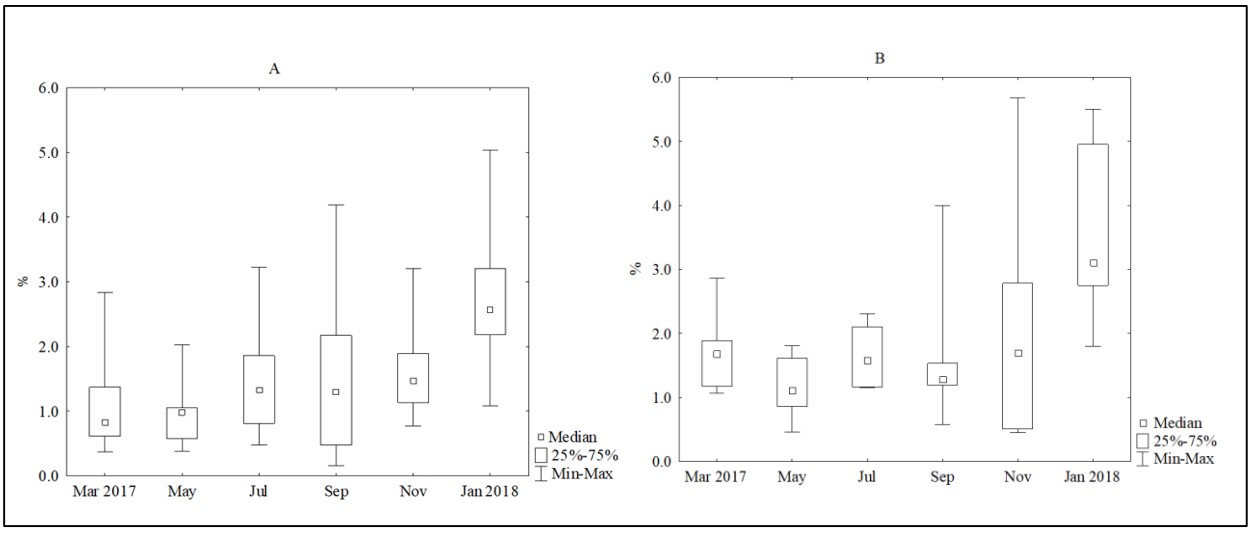
Statistical comparisons of GI and SO% values were tested for normality (Kolmogorov–Smirnov test) and homogeneity of variance prior to deciding upon the appropriate statistical test, using a confidence of 95%. If data violated the test assumptions of normal distributions and homoscedasticity, a non-parametric test was performed.

Multivariate analysis specifically an ordination technique, the Principal Component Analysis or PCA was used to search for relationships month average data corresponding to the study period for abiotic (air temperature and precipitation data, available at http://www.inmet.gov.br/portal) and biological parameters: gonad index, gametogenic stages frequency (male and female), OD intervals, relative amount of mass sperm, number of oocytes per month, oocyte mature diameter. The results were graphically represented in a hyperspace established by perpendicular axes, whose relative contributions (eigenvalues of the data matrix) represented most of the data variation (Karamizadeh *et al.,* 2013).

RESULTS

Ninety individuals of *L. variegatus* with a test diameter of 37.3 to 76.6 mm (63.44 ± 6.17) were processed. Among them, 46 females and 44 males presented a sex ratio of 1:1.1 (χ^2=0.76, Df=1, p<0.05). The gonad index (GI) did not indicate statistically significant differences over time (p>0.05) in both sexes, with average values ranging from 0.2 to 5.7% (Figure 1A, B).

Figure 1 – Gonad index (%) in the *Lytechinus variegatus* population at Enseada da Ribeira (Bahia, Brazil). A: Female. B: Male

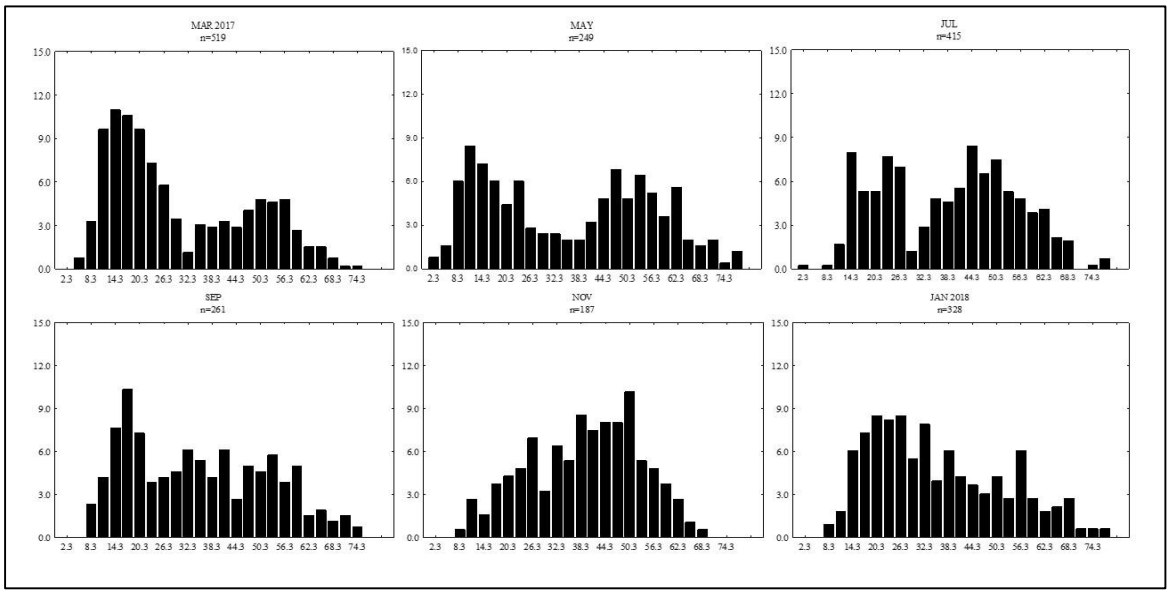


A total of 1,955 oocytes and 620 male follicles were counted. The Bhattacharya method (Bhattacharya, 1967) established two distinct and representative cohorts for both OD (16 and 50 µm). Three cytometric intervals were established for oocyte diameter (OD): (1) growing: OD<16 µm, (2) premature: 16<OD<50 µm and (3) when were observed vitellogenic oocytes (or completely mature): OD>50 µm. Along the year the optimum maturity size is represented by

oocytes with OD of up to 60 µm.

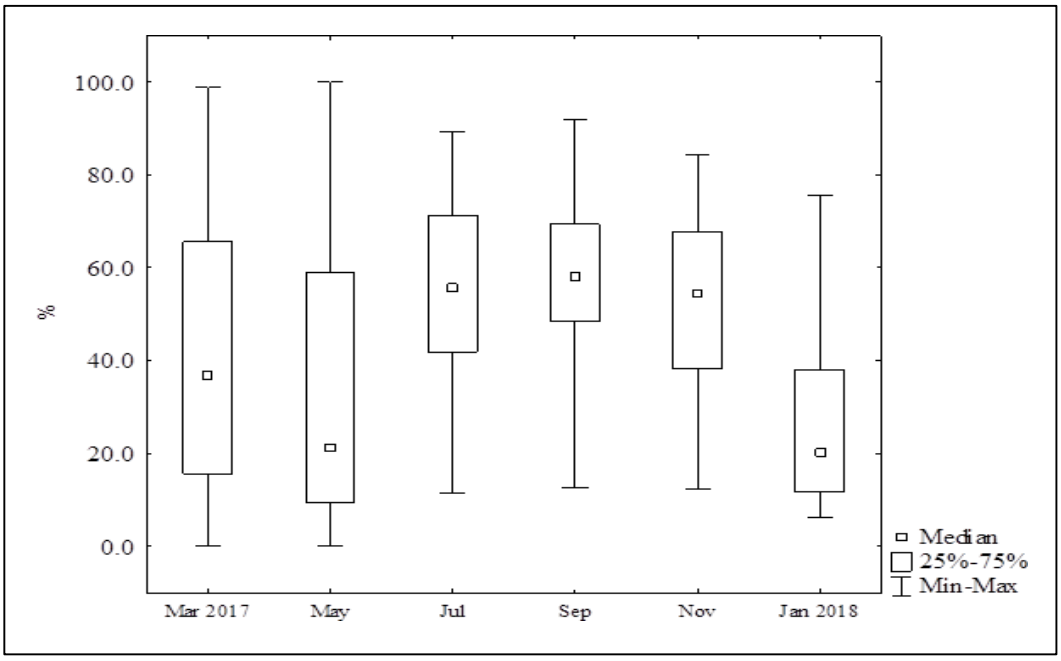
Grow phenomena (interval 1) were frequent in March (24.7%), May (16.8%) and September (14.2%) (Figure 2). The vitellogenesis process (interval 3) is continuous and is most expressive from July 2018 to January 2018 (>60%). The largest oocyte size classes (interval 3) occurred in May (38.5%) and July (30.6%). Oocyte diameter up to 70 µm were not expressive along the year (< 2,5%).

Figure 2 – Oocyte diameter distribution (%) of *Lytechinus variegatus* in Enseada da Ribeira (Bahia, Brazil). The number of oocytes measured is indicated for each month analyzed



In males, the relative amount of mass sperm (SO%) ranging from 25,3% (January 2018) to 57.3% (September 2017) (Fig. 3). SO% values above 50% predominated from July to November (p<0.05).

Figure 3 – Relative amount of mass sperm (%) of *Lytechinus variegatus* in Enseada da Ribeira (Bahia, Brazil)



Five gametogenic stages were described for *L. variegatus* population: growing (observed only in males), premature, mature, spawning and resting (Table 1, Figures 4 and 5). Spawning events occurred in every month of the year for both sexes. Female premature stage was only observed in January, and mature stage occurred in a few individuals in July. Follicles with reserve accumulation phenomena (resting stage) were more observed in November. Male growing stage was observed in March and January and premature stage from September to January. Mature individuals occurred in May, July and September and resting stage only in May.

Table 1 - Gametogenic events for males and females of *Lytechinus variegatus* at Enseada da Ribeira (Bahia, Brazil)

Stage	**Diagnosis**
Growing	Expanded follicles, mostly filled with nutritive phagocytes. Presence of primary cells in the process of cellular differentiation (spermatogonia, spermatocytes, and spermatozoa) on the follicle periphery. Masses of sperm can be found, but they are still sparse and surrounded by reserve tissue (Figure 4B).
Premature	Reduction in the number of nutritive phagocytes in the follicles, confined to a still evident layer on the follicle's periphery. Progressive increase in oocytes' size and sperm mass in the lumen. Elements of the germline still adhered to the follicle wall, and the spermatogenic series are radially arranged in stacked columns (Figures 4A and 4D).
Mature	Maximum follicular expansion. Nutritive phagocytes are virtually absent or forming a thin peripheral layer next to primary sex cells in the acinus wall. In females, there are oocytes of varying sizes (different stages of the vitellogenic process). Great number of mature cells in the lumen exhibit polygonal shape with a cortical granules at their surface (Figure 4C). In the males, a dense mass of sperm is evident. Growing processes are commonly observed in the germinative line (Figure 4F).
Spawning	Follicular contraction with the elimination of mature oocytes and sperm and an apparent empty lumen. Germinative line of the follicle wall thickening with or without associated primary cells (Figures 4E and 4H). Remaining vitellogenic oocytes may show processes of cell lysis. Primary cells can be observed. In males, the sperm mass showed apparent spatial disorganization.
Resting	Increased presence of reserve tissue (nutritive phagocytes) in the lumen. Relict ova (whole or not) and mass sperm can be observed. No sign of reproductive activity (Figures 4G, 4I and 4J).

Figure 4 – Gametogenic stages of *Lytechinus variegatus* at Enseada da Ribeira (Bahia, Brazil). A: Female Premature, B: Male Growing, C: Female Mature, D: Male Premature, E: Female Spawning, F: Male Mature, G: Female Resting* (with total oocyte atresia), H: Male Spawning; I: Female Resting** (residual oocytes still intact), J: Male Resting). Abbreviatures: GL: germinative line; L: lumen: NP: nutritive phagocyte OV: vitelogenic oocyte; PC: primary cells; PVO: previtellogenic oocyte; RO: relitic oocyte; SPTZ: sperm mass. (100X; HE)

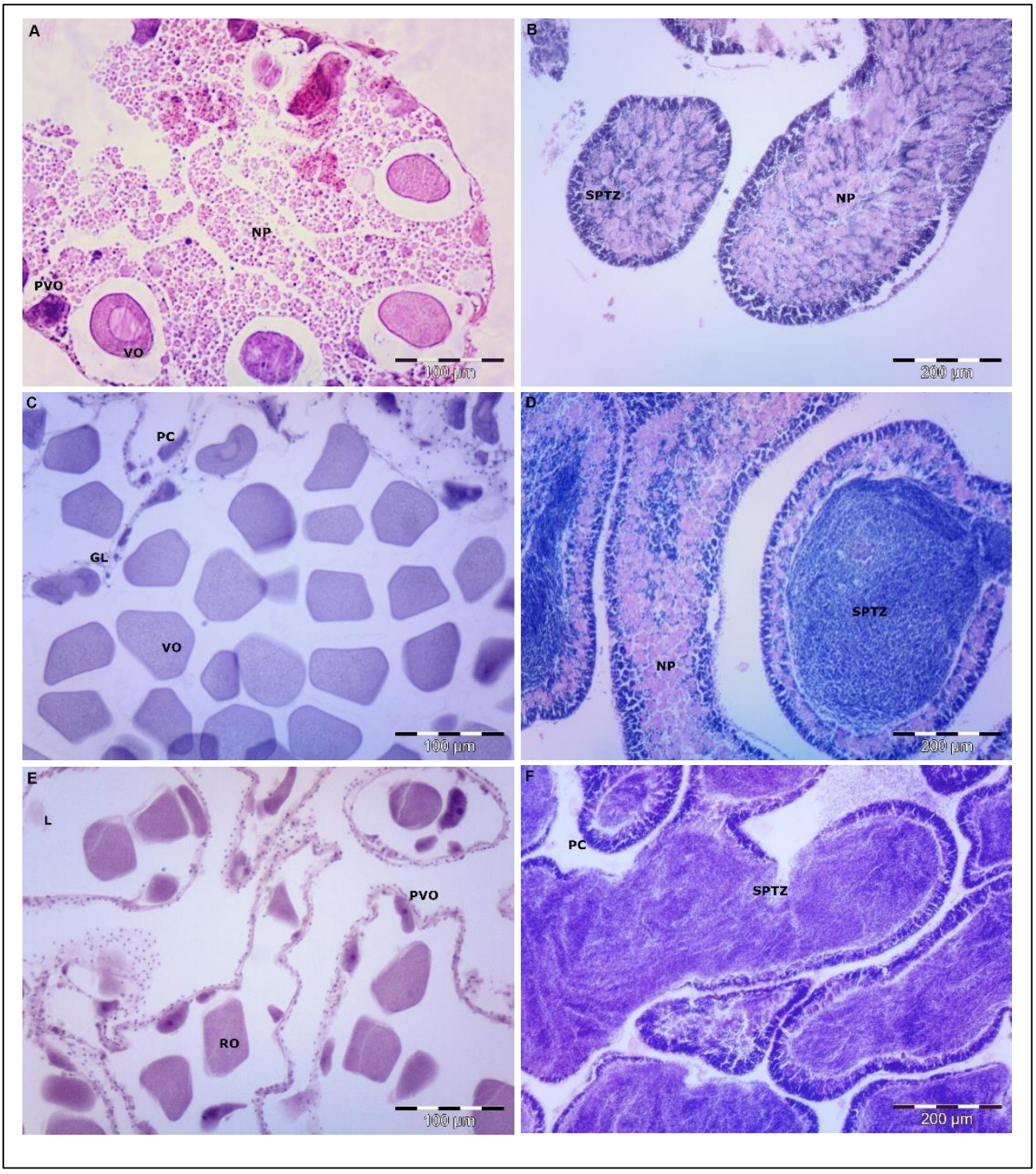


Figure 4 (cont.) – Gametogenic stages of *Lytechinus variegatus* at Enseada da Ribeira (Bahia, Brazil). A: Female Premature, B: Male Growing, C: Female Mature, D: Male Premature, E: Female Spawning, F: Male Mature, G: Female Resting* (with total oocyte atresia), H: Male Spawning; I: Female Resting** (residual oocytes still intact), J: Male Resting). Abbreviatures: GL: germinative line; L: lumen: NP: nutritive phagocyte OV: vitelogenic oocyte; PC: primary cells; PVO: previtellogenic oocyte; RO: relitic oocyte; SPTZ: sperm mass. (100X; HE)

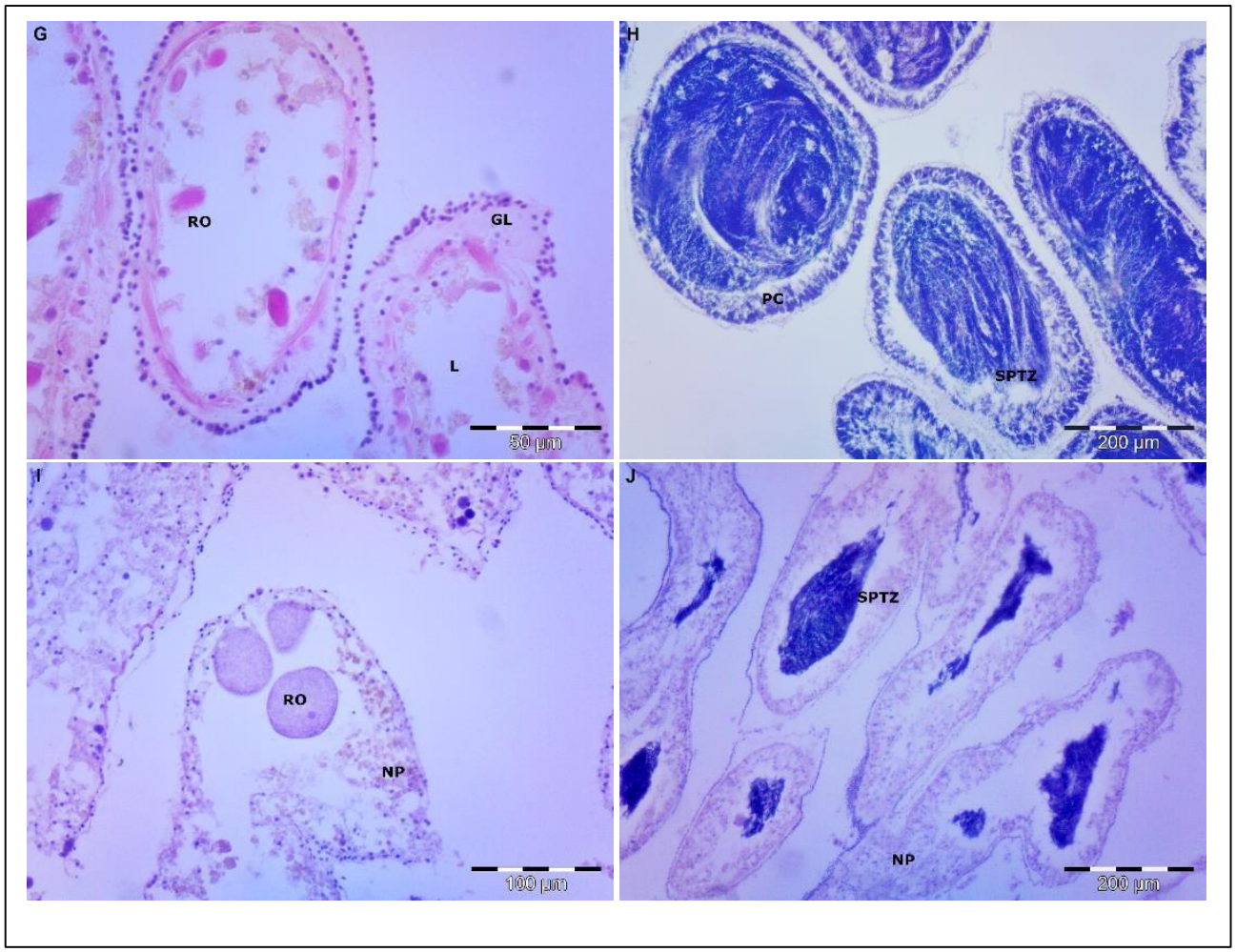
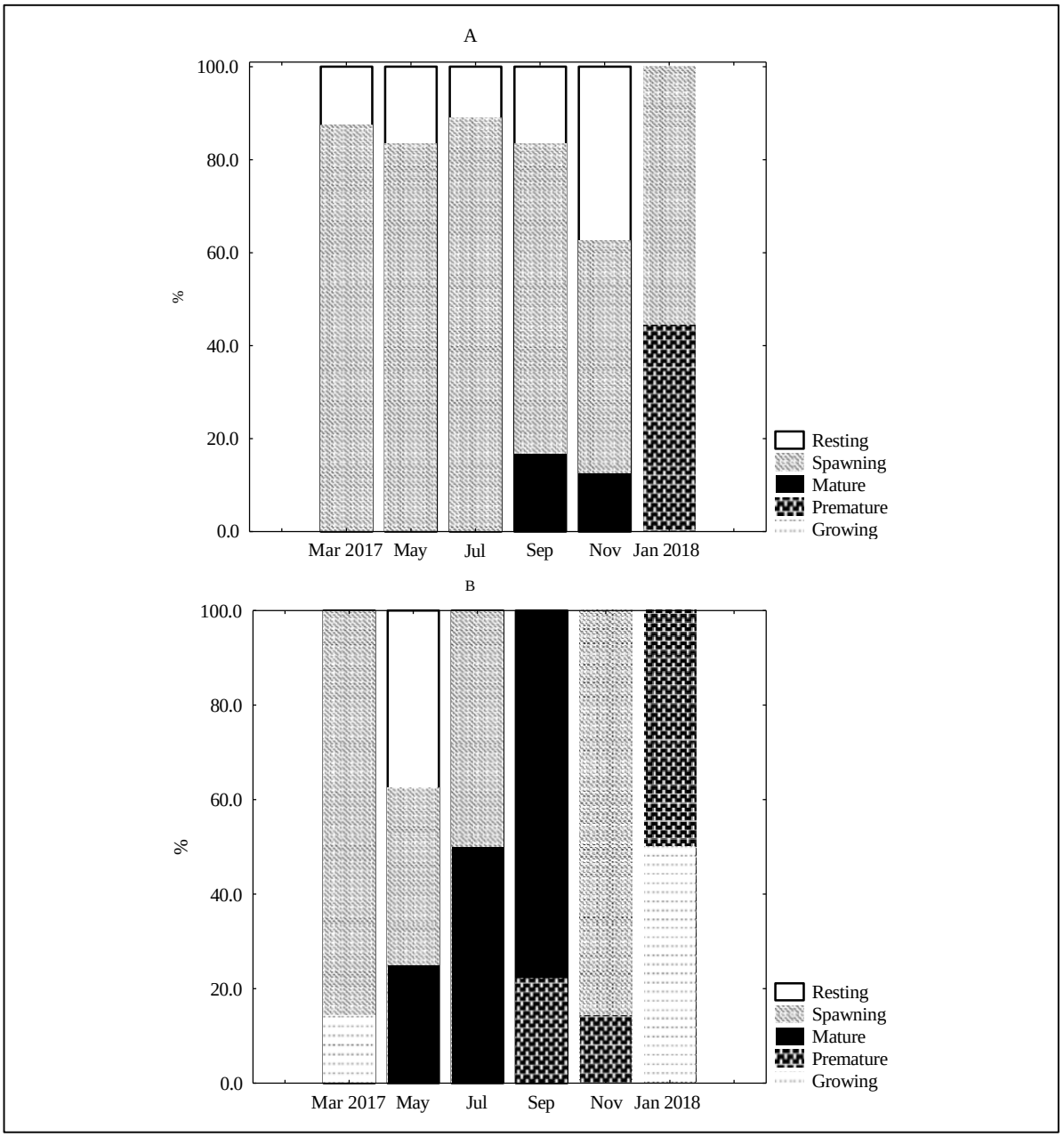


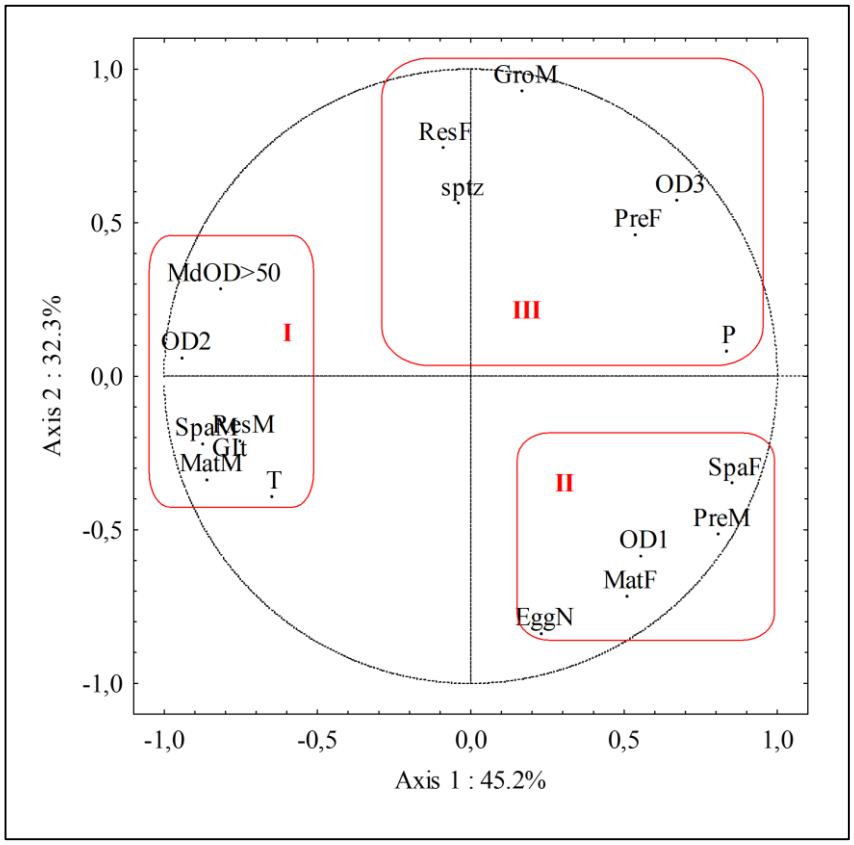
Figure 5 – Gametogenic stages frequency of *Lytechinus variegatus* in Enseada da Ribeira (Bahia, Brazil). A: Female. B: Male.



The PCA first and second axis from the 2-D plot accounted for 42.2 and 32.3% of the total variance respectively (Fig.6). The first axis was strongly correlated with precipitation (P), gonad index (both sexes) (GIt), intervals 2 and 3 of oocyte diameter (OD_2, OD_3), month average oocyte mature diameter (Md_{OD}>50), premature, mature, spawning and resting stages for males (PreM, MatM, SpaM, ResM) and spawning stage for females (SpaF). The second axis also was strongly correlated only with water temperature (T), number of oocytes per month (EggN), interval 1 of oocyte diameter (OD_1), growing stage for males (GroM) and mature and resting stages for females (MatF, ResF).

Data analysis revealed that three groups were negatively related with each other: group I (T, GIt, MatM, SpaM, ResM and OD_2), group II (EggN, MatF, OD_1, PreM, SpaF) and group III (P, sptz, ResF, GroM, PreF and OD_3).

Figure 6 – Plot of PCA for abiotic and biological month values of *Lytechinus variegatus* in Enseada da Ribeira (Bahia, Brazil). Abbreviatures: air temperature (T), precipitation (P), gonad index average (both sexes) (GIt), OD<17.3 interval (OD1), 17.3<OD<50.3 interval (OD2), OD>50.3 interval (OD3), mature oocyte value up to 50 (MdEgg>50), Relative amount of mass sperm (sptz) growing stage frequency for males (GroM), premature stage frequency for males (PreM), mature stage frequency for males (MatM), spawning stage frequency for males (SpaM), resting stage frequency for males (ResM), premature stage frequency for females (PreF), mature stage frequency for females (MatF), spawning stage frequency for females (SpaF), resting stage frequency for females (ResF)



DISCUSSION

Lytechinus variegatus individuals in Bahia coast release their gametes almost continuously. In the Enseada da Ribeira locality, the gametogenic cycle is characterized by a greater reproductive effort from the second half of the year (July, September and November), coinciding with the reduction in rainfall and warmer periods. The simultaneous presence of developing sex cells and these stages point to a rapid renewal of the gametic population in the studied population, a pattern also observed in the Brazilian Southeast (Junqueira, 1998).

In Bahia, the first months of the year (January and March) indicate a brief recovery period of the reproductive activity with the exclusive stages of growing (only in males) and premature. Females storing nutritional reserves (resting stage) in the gonads along the year with a marked period in November. For the other hand, males the resting stage was observed only in May showing the sexes not presenting exhausted gonads simultaneously towards the year. This pattern differs somewhat as seen by Tavares and Borzone (2015) in the southern Brazilian population (September and October).

Some temporal differences in gametogenic events between the sexes of this echinoid can

occur even in continuous cycles (Junqueira, 1998). Asynchronous periods in gametic production are described as related to female's high energy requirements due to the particularities of oocytes such as their size and capacity to store biomolecules during vitellogenesis (Bishop, Watts, 1994).

In Enseada da Ribeira's population, gonochoric individuals were always recorded in the same sex ratio, confirming observations along the Brazilian coast (Junqueira, 1998; Lage *et al.,* 2011, Tavares, Borzone, 2015) and other tropical and subtropical regions (Moore *et al.,* 1963; Quijano, Gómez, 2005). At the maturity stage, it is usual to notice some differentiation between the sexes by visualizing their gonad's color (orange for females and white for males) (Junqueira, 1998; Lage *et al.,* 2011), which was confirmed during the dissection of the individuals in the present study.

L. variegatus individuals are recognized for presenting rapid growth (larval settlement in 5 weeks), early maturity, reaching 40 mm test diameter in 1 year and short longevity (Quijano, Gómez, 2005). Life histories such as this species tend to develop strategies that maximize gonadal production under ideal conditions, as pointed out by Hammer *et al.* (2004). In their experiments, it was possible to observe the emergence of multiple cohorts of gametes (accelerated gamete growth) in response to a rich protein diet.

Hence, it is possible to hypothesize that the rapid phenomena of maturation and longer period of release of gametes observed in Enseada da Ribeira would be correlated to an ample (and continuous) food supply for the individuals. In particular for males short periods of nutrient accumulation would be sufficient to maintain a long period of gamete elimination.

Immediate metabolic expenditure may also explain the lack of significance of GI oscillations in this population. Thus, the gonads of *L. variegatus* do not represent a great storage structure as observed in another equinoids as *Tripneustes ventricosus* (Machado, 2007) or *Echinometra lucunter* (Tavares, 2004; Mariante *et al.,* 2009).

Even though the use of GI as an indirect measure of echinoid maturity is a common practice in echinoid studies (Ernest, Blake, 1981; Bishop, Watts, 1994; Ventura *et al.,* 2003), its application to the different *Lytechinus variegatus* populations (Junqueira, 1998; Hammer *et al.,* 2004; Tavares, Borzone, 2015) should be employed with caution, mainly when the arguments are not supported by microscopic observations of cellular events (Lopes, Cerqueira, 2007; Lage *et al.,* 2011), which makes any comparison unfeasible.

The concept of phenotypic plasticity in the face of the contrasting environmental conditions to which *L. variegatus* populations are subjected is well exemplified when analyzing the reproductive patterns of the species along the latitudinal gradient on the Brazilian coast. According to Junqueira (1998), the species has a genetic potential with sufficient flexibility for adaptive strategies in different environments (Table 2).

Intraspecific reproductive patterns of echinoids with extensive geographical distribution, such as *L. variegatus*, usually lead to inferences about the environmental seasonality of factors such as temperature, salinity, photoperiod, and the lunar cycle, among others, as modulators of reproductive intensity and periodicity (Tavares, Borzone, 2006; Langue *et al.,* 2011; Brogger *et al.,* 2010).

Differences in the gonadal development in each population may also indicate the strategies' variability for using and allocating energy resources in response to environmental interactions *in situ* (feed supply, physical factors, hydrodynamic stress, predation, anthropogenic pressure, among others), regardless of latitude. Extremely low percentages (less than 3% GI expression) were recorded in the northeastern and southern Brazilian populations. In southeastern populations, rates up to four times higher were recorded.

Changes in salinity seem to affect the gonadal development by delaying the gamete maturation process, as observed for *Paracentrotus lividus* by Santos *et al.* (2022). In Bahia dry and warmer periods seem to represent more favorable conditions for optimal reproductive effort. Data obtained by Lopes and Cerqueira (2007) in the same location indicated reductions in the GI in months of higher rainfall (June, July, and August). However, *L. variegatus* may occupy estuarine environments (Junqueira *et al.,* 1997; Sánchez-Jérez *et al.,* 2001; Tavares, Borzone, 2015) and show hyper-conforming behavior to maintain osmolality (Vidolin *et al.,*

2007).

For marine invertebrates, the qualitative and quantitative characteristics of the female gamete's energy content are descriptors of investment degree in the vitellogenesis process. They are considered an essential component of interspecific and intraspecific life history, such as the successful production of offspring (larvae and juveniles) (Marshall, Keough, 2007; Moran, McAlister, 2009; Moran *et al.,* 2013). When observing the size of the female gamete in different echinoids populations along the Brazilian coast (Table 2) an inverse relationship is observed between the oocyte's size and the effort devoted to reproduction.

Table 2 - Brazilian literature review of some biological and reproductive features of *Lytechynus variegatus*. Abbreviatures: TD: test diameter, GI: gonadal index, MOd: mature oocyte diameter.

Latitud/Reference		Habitat	TD (mm)	Sex ratio	GI (%)	MOd (µm)	Gametogenic Events			
							Growing	Premature/ Mature	Spawning	Resting
12ºS	Enseada da Ribeira (Salvador, Bahia) **Present study**	intertidal sandy	37-76	1.1:1	0.2-5.7	>50	Jan ♂	all year		Nov ♀
	Enseada da Ribeira (Salvador, Bahia) Lopes and Cerqueira (2006)	mud/ gravel bottom	40-42	-	0.7-4,0	-	-	Apr-Jun	Jul-Sep	-
20ºS	Ilha do Boi (Vitória, Espírito Santo) Lage *et al.* (2011)	sublittoral rocky beach	55-87	1.2:1	7.0-13.0	-	-	-	May-Oct*	-
22ºS	Lagoa de Araruama (Araruama, Rio de Janeiro) Junqueira (1998)	intertidal sandy bottom	60-70	1:1	4.0-10.0	57**	Mar, Jun	Apr, May, Jul	all year	Feb, Apr
23ºS	Baia Ilha Grande (Angra dos Reis, Rio de Janeiro) Junqueira (1998)	sublittoral rocky shore	60-70	1.1:1	3.0-9.0	56**	Apr, Aug	Jul, Aug, Oct, Dec	Mar, Apr, Oct, Dec	Apr, Jul
25ºS	Baia de Paranaguá (Paranaguá, Paraná) Tavares and Borzone (2015)	intertidal sandy/ mud bottom	51-77	1:1	0,2-2,8	>60	Apr, Sep, Dec ♂	Apr, Feb ♀ Feb, Sep ♂	Feb, Jun ♀ May, Jun ♂	Sep, Oct ♀ May, Jun ♂

*Assessments based on the lunar cycle, **average value

So that future comparisons can be made, and as a proposal for standardization in other echinoderm species on the Brazilian coast, quantitative evaluations are also suggested. In particular, the measurement of the female gamete size with modal progression analysis over the time as assertive analytical techniques for evaluating the oogenesis process. Furthermore, integrated tools (physiological indices, quali and quantitative microscopic analysis) can be useful to better understand echinoids life history strategies in South Atlantic Ocean.

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