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Reproductive investment of *Perna perna* (Mytilida: Mytilidae) in subtropical regions: combining several methods

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Abstract. In general, tropical/subtropical populations of marine invertebrates reproduce continuously. Nevertheless, nuances in reproductive investment can be detected when reproductive effort is observed through quantitative analysis applied to the traditional histological methods. The aim of this study was to detect such nuances for the *Perna perna* species through qualitative and quantitative analysis. Mussels (n=116; 80 ± 20.7mm) were collected on the coast of Paraná between September/2013 and July/2014. A fraction of gonadal tissue was processed by histological techniques and the reproductive cycle could be described through a maturity scale and biometric analysis. The frequency of gametogenic phases was established and the sex ratio tested by X^2 (α=0.05). The spermatozoids occupation in the follicles were evaluated by ANOVA. The population showed sex ratio of 1:1. Individuals (7.8%) were castrated by *Bucephalus* sp. mainly during the spring/2013. Oocyte diameter ranged from 12.8 to 55.5µm. The lesser occupation of the follicle by spermatozoids was in fall/2014. Although *P. perna* exhibits gonadal activity throughout all periods of the year, our results, for the first time, showed that there are two greater spawning events during the year: one in early spring and one in mid-summer.

Key words: bivalve; reproduction; biometry; ecology; gametogenesis; clam; invertebrate.

Resumo: **Investimento reprodutivo de Perna perna (Mytilida: Mytilidae) em regiões subtropicais: combinando vários métodos.** Em geral as populações de invertebrados marinhos tropicais/subtropicais, se reproduzem continuamente. No entanto, nuances no investimento reprodutivo podem ser detectadas através de análises quantitativas aplicadas aos métodos histológicos tradicionais. O objetivo deste estudo foi detectar tais nuances para a espécie *Perna perna* através de análises qualitativa e quantitativa. Mexilhões (n=116; 80 ± 20.7mm) foram coletados na costa do Paraná, entre setembro/2013 e julho/2014. Uma fração do tecido gonadal foi processada por técnicas histológicas e o ciclo reprodutivo pôde ser descrito através de uma escala de maturidade e análise biométrica. A frequência das fases gametogênicas foi estabelecida e a razão sexual testada por X^2 (α=0.05). A ocupação do folículo por espermatozoides foi avaliada por ANOVA. A população apresentou razão sexual de 1:1. Indivíduos (7,8%) foram castrados por *Bucephalus* sp. principalmente durante a primavera/2013. O diâmetro do ovócito variou de 12,8 a 55,5µm. A menor ocupação do folículo por espermatozoides foi no outono/2014. Embora *P. perna* exiba atividade gonadal em todos os

períodos do ano, nossos resultados, pela primeira vez, mostraram que há dois maiores eventos de desova durante o ano: um no início da primavera e outro no meio do verão.

Palavras-chave: bivalve, reprodução, biometria, ecologia, gametogênese.

Introduction

Knowledge about species biology reproductive is fundamental to establish farming management strategies, indicate the best times for collection and production of juveniles and for conserving and restoring natural beds. Mussels have an outstanding functional role in most marine intertidal and shallow shelf environments providing a wide variety of ecosystem services (Carranza *et al.* 2009). In South America, overexploitation occurs either for subsistence or for artisanal or industrial fisheries (Nishida *et al*. 2006, Narvarte *et al.* 2007). Among the wide diversity of shellfish species harvested, the extraction of several mussel beds may have harmful consequences for the rest of the community, because they provide habitat and recruitment sites for many other species (Fernandez *et al.* 2000).

The extraction of living resources used as source of livelihood has been a common practice in the Brazilian coast communities, in particular for mussels of genus *Mytilus, Perna* and *Mytella*, and this is mainly due to the high protein value of its flesh (Furlan *et al.* 2011, Camilo *et al.* 2018). *P. perna* is an important species farmed and artisanal hand-gathering in southern Brazil. However, such practices are not any more sustainable activities. Along this time, the extractivism activity presented predatory pressure on natural banks of these species, contributing to stock reduction is increasing and this pressure adds to the chronic impact of historical exploitation of these shellfish (Lage & Jablonki 2008, Carranza *et al.* 2009, Souza *et al.* 2019). For these species, it is essential to know the reproductive patterns and strategies of the populations throughout their distribution.

Perna perna (Linnaeus, 1758) is regarded as the largest mussel of Brazil, potentially reaching 182 mm of length (Resgalla 2008). In South America, it can be found from Venezuela to Uruguay (Gardner *et al.* 2016) and its introduction is still a matter of discussion (see Pierri *et al.* 2016, Oliveira *et al.* 2017, Silva *et al.* 2018). The studies in Brazil about reproductive cycle and development of *P. perna* are focused in the states of São Paulo and Santa Catarina (23.3 – 29.5º S), located in south and southeast region of Brazil (Henriques *et al.* 2001, Fagundes *et al.* 2004, Abessa *et al.* 2005, Bainy *et al.* 2006, Galvão *et al.* 2006, Marenzi & Branco 2006).

For marine invertebrates that exhibit wide geographic distributions, the optimal reproductive time varies according to the latitude and local conditions (Giese 1959, Corte 2015). It is well-reported that populations of temperate regions display synchronous reproduction with an annual cycle in response to markedly contrasting seasonal environment conditions. Alternatively, populations of tropical/subtropical regions in general reproduce continuously, since they get “triggers” all year long, with individuals producing gametes and spawning along the whole year (see Freites *et al.* 2010) such as *P. perna* in the Brazilian coast (Marenzi & Branco 2006). Nevertheless, nuances in reproductive investment can be detected through quantitative analysis applied to the traditional histological methods (Sarkis *et al.* 2006, Peixoto *et al.* 2018).

Reproductive investment refers to the amount of energy expended for the production of gametes and their energy reserves and is usually measured through “maternal investment” (Honkoop *et al.* 1999, Moran & McAlister 2009, Brunner 2013), a topic widely discussed for Echinodermata (*e.g.* Lessios 1990, Ross *et al*. 2013, Moran *et al*. 2013). These studies demonstrate that larval performance is closely related to the energy investment of females, where large eggs generate larvae with greater chances of survival (Honkoop *et al.* 1999, Marshal & Keough 2008, Moran & McAlister 2009, Marshal *et al.* 2010, Powell *et al.* 2011, Brunner 2013). The oocyte size is a proxy for assessing maternal investment in marine invertebrates (Bolognini *et al.* 2017). This is an easier parameter to be taken than fertility, fertilization and recruitment, for example (Moran 2004, Marshal & Keough 2008).

The subjectivity in determining the gametogenic stages of bivalves is a constant, making it difficult to standardize the characterization of the reproductive cycle within and between species. In addition to allowing an evaluation of the population's reproductive investment, quantitative analysis provides more robust results and a stages classification scale standardizes the assessment of the species' reproductive activity, which can be applied to different populations. Although we are experiencing an increase in the number of studies

that consider quantitative analysis in the evaluation of the bivalve reproductive cycle and investment (*e.g.* Phillips 2007, Moran *et al.* 2013, Corte 2015, Joaquim *et al.* 2016), such approach has not yet been applied to *P. perna*, an important fishing resource throughout its distribution.

The objective of this study was to compare the utility of the various methods for characterization of the gonadal development and of the investment reproductive of a population subtropical of *P. perna*. For that, we use the physiological index Condition Index, the macroscopic aspect of the flash and cytometric scale to categorize gametogenic events. We characterize the reproductive cycle, propose a cytometric scale to both sexes and compare it with the macroscopic aspect of the flash, evaluating especially the maternal investment (oocyte size) and compare some reproductive aspects with other Brazilian populations. Our results pointed out the fundamental importance of the combined use of several methods and contributed not only to establish the reproductive pattern of *P. perna*, but to demonstrate nuances of the reproductive activity. Although it is well established that the species has a continuous gametogenic cycle, we expected to observe variations in maternal investment in response to the subtropical climate experienced by the assessed population.

Material and methods

Around 30 mussels were monthly hand-collected between September/2013 and July/2014 in a nautical establishment located in the euhaline sector of the Paranaguá Bay, Pontal do Paraná city, southern Brazil (25º31'S, 48º32'W). The specimens were placed in containers with seawater, they had their shells scraped to remove epibionts/fouling organisms and were fixed in formalin at 10%. Environmental data were measured punctually *in situ*: water temperature (portable thermometer), salinity (refractometer) and water transparency (Secchi disk). To better represent the monthly variation of abiotic factors, additional air temperature data collected daily near (~10 km) the study region (Ilha do Mel) were obtained from Instituto Nacional de Meteorologia (INMET, inmet.gov.br).

In the laboratory, all specimens were weighted and had the shell length (SL) (anteroposterior axis, see Bailey 2009) measured with a digital caliper (0.02 mm accuracy). Fifteen adult individuals/month (SL > 70 mm) were treated for the assessment of reproductive cycle and the other (not sexed) were used for temporal assessment of the Condition Index (CI), according Kagley *et al*. (2003), by the formula: CI (%) = FWW/SL x 100, where FWW is flesh wet weight (g) and SL is shell length (mm), demonstrated by Galvao *et al*. (2015) as the one with the best resolution to assess the contribution of flesh in *P. perna*.

Organisms were eviscerated and photographed for assessment of macroscopic aspects of the mantle. Fractions of gonadal tissue were submitted to a histological routine, including dehydration, diaphanization and inclusion in paraffin (Borzone *et al.* 2001, Borzone *et al*. 2003, Meyer *et al*. 2018). Digital images of the histological sections were taken and analyzed monthly in order to undertake the sex diagnosis and the assessment of the degree of gonadal development of the individuals.

For the assessment of the oocytes' diameter, around 30 cells/individual were measured (average value of the distances between the axles larger and smaller of the cell surface). In males, 30 follicular sections were deemed, and it was estimated the occupation percentage by spermatic series and by spermatozoids (adapted from Borzone *et al.* 2001). In both was employed the ImageJ software (Schneider *et al.* 2012). Histograms of the frequencies of the average diameter of oocytes were built using the method of Sturges' rule (Sturges 1926). The Bhattacharya's (1967) method was utilized to separate oocyte cohorts (adapted from Castillo-Durán *et al*. 2013). The description of the stages of gonadal development here identified was adapted from Borzone *et al*. (2001) and Borzone *et al*. (2003).

The significance of the monthly variances of the of the CI, mean oocyte diameter and occupation percentage by spermatic series/spermatozoids were tested using Analysis of Variance (ANOVA) one way on ranks, and significant differences were detected by Tuckey or Dunn's test ($p<0.05$). The sex ratio was analyzed by X^2 test. To detect the relationship between the abiotic variables and the mean, standard deviation, kurtosis and skewness of the percentage of occupation by spermatozoids and by radial spermatic series, the diameter of the oocyte and the Condition Index were used pairwise Pearson's correlations; only correlations with $r > 0.7$ were considered.

Results

During December/2013, the salinity was abnormally low (20 ‰) when compared with other

Table I. Monthly values of air (data obtained from Instituto Nacional de Meteorologia) and seawater temperature, salinity and transparency of the seawater in the collection site obtained in the marked months between the years of 2013 and 2014.

Months	Air temperature (ºC)	Seawater temperature (ºC)	Salinity (‰)	Transparency (cm)
Sep/2013	17	18	30	100
Oct/2013	20	20	30	75
Nov/2013	22	*	30	75
Dec/2013	24	22	20	80
Mar/2014	24	25	28	40
May/2014	20	23	31	19
Jun/2014	19	21	32	28
Jul/2014	17	18	30	150

* Data not available.

months, mainly in a euhaline region of the estuary, ranging from to 28 a 32 ‰ (Table I). Transparency was the most variable parameter, ranging from 19.5 to 150 cm. The air and seawater temperature varied in similar ways, between 17º C in September/2013 and 25º C in March/14.

The biological material used for analysis of the reproductive cycle was composed of 106 individuals of *P. perna*, with average length of 80 mm (standard deviation = ±20.7 mm), being 49 males (42.2%) and 48 females (50%), assuming the sex ratio of 1:1 (X^2= 0,34; GL= 3; p = 0.4394). In the other hand, sexing was not possible for nine (n=9) castrated individuals (7.8%) (Fig. 1) due the presence of intense parasitic infestation by the trematode *Bucephalus* spp., being readily identified macroscopically by the presence of orange filaments on the mantle and evidenced by the microscopic analysis (Fig. 2).

The diameter of the oocytes ranges from 13.6 µm (October/2013) to 53.4 µm (March/2014). In the spring months of 2013, the oocyte population was composed of cells with sizes between 21.7 and 31.6 µm (62.2%), with 23.6% of the cells > 31.7 µm. Values between 21.7 and 31.6 µm were prevailing in summer/2014 (50.6%), with 47% of the cells > 31.7 µm; for the autumn/2014, most of the cells were between 21.7 and 31.6 µm (62.6%), with 35% of the cells > 31.7 µm, and in winter/2014 46.6% of the cells were between 21.7 and 31.6 µm, with 48.1% of the oocytes > 31.7 µm (Fig. 3). December/2013 and June/2014 were the months with highest mean of the

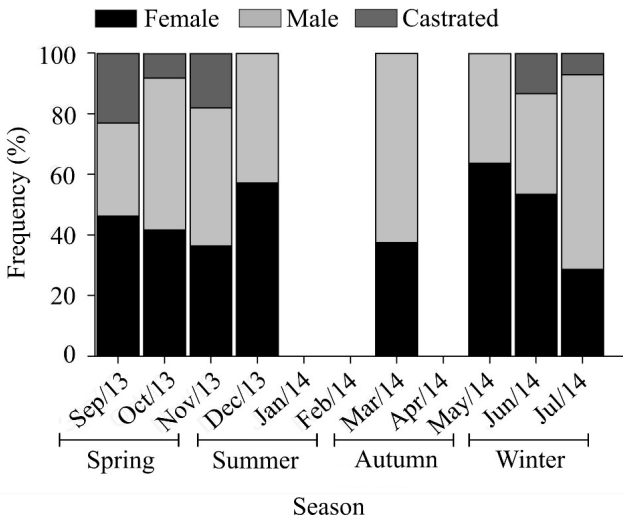

Figure 1. Frequency of males, females and indeterminate individuals of *Perna perna* collected on the coast of Paraná, southern Brazil.

oocyte diameter (p < 0.05), followed by March/2014. All other months were grouped with a mean oocyte diameter < 30 µm (Fig. 4).

In males, the percentage occupation by spermatozoids was higher in September/2013 and July/2014 (> 90 %, p < 0.05) and lower in March and June/2014 (< 80 %, p < 0.05) (Fig. 5). The occupation by the spermatic radial series has always been antagonistic to the occupation by spermatozoids.

Three reproductive stages were identified for females and two for males, these are described in Table II. The qualitative analysis considered the

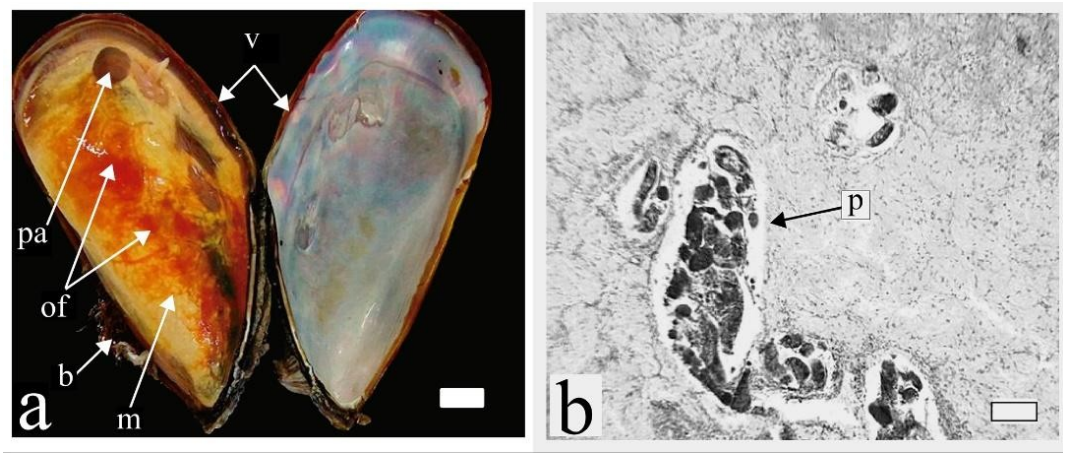

Figure 2. Macroscopic (a) and microscopic (b) aspects of the mantle of a parasitized specimen. b: byssus. m: mantle. pa: posterior adductor muscle. of: orange filaments (indicating parasitic infestation). p: parasitic forms. (Scale bars: a = 10 mm; b = 10 µm).

aspect of the gonadal tissue, cell types, shape of the follicles and occupation by the connective tissue. Quantitative analysis allowed to characterize different stages of gametogenic development for males and females. For females, based on the Bhattacharya's method, the oocyte population was divided into 3 main cohorts, used to define the stages using a cytometric scale: (1) <21.6 µm, (2) 21.7 – 31.6 µm and (3) > 31.7 µm. When > 10 % of oocytes were < 21.6 µm, it was categorized as *Partial spawning and proliferation* (since the species presents mature gametes throughout the year, when an expressive amount of proliferative cells was observed, this was considered); when > 60 % of the cells had diameters between 21.7 and 31.6 µm, it was categorized as *Partial spawning and intermediate maturation* and when > 40% of the cells had a diameter > 31.7 µm as *Partial spawning and maximum maturation* (see representation of the stages in Fig. 6). For males, when > 10 % of the follicle was occupied by the radial spermatic series and < 80 % was occupied by mature sperm, the *Emission with proliferation* stage was considered, when the radial series occupied < 10 % and the spermatozoids mass occupied > 80 %, the *Maturation* was assumed.

The highest frequency of females in the stage of *Partial spawning and proliferation* was observed in September/2013 (50%), October/2013 (60%), November/2013 (50%) and July/2014 (75%). In December/2013, March/2014 and June/2014 the stage of *Partial spawning and maximum maturation* was more evident (75, 50 and 87.5%, respectively). The stage of *Partial spawning and intermediate maturation* occurs over the period studied with the other two stages, mainly in September/2013, November/2013, March/2014 and May/2014 (Fig. 9a). In the males, the stage of *Maturation* was observed in all the months analyzed, always with a prevalence greater than 60%. The stage of *Emission with proliferation* was recorded in December/2013 (16.7%), March/2014 (40%) and June/2014 (40%) (Fig. 9b).

The input of the visceral mass reflected by CI ranged from 11.15% in October/2013 to 22.82% in December of same year. Two well-defined groups can be identified, one of spring and summer, with higher values of flesh contribution (> 25%, $p < 0.05$), and another of autumn and winter, with the lowest values (< 25%, $p < 0.05$) (Fig. 10).

The Pearson's correlation evidenced that the oocytes had positive asymmetric distribution in warmer months ($r = 0.78$, $p < 0.05$), that the highest percentage of occupation by spermatozoids occurs in periods with greater transparency and lower seawater temperature ($r = 0.72$ and -0.81, respectively; $p < 0.05$) and did not indicate any relationship between the CI and the reproductive cycle of *P. perna* (tables with all the results of the correlations in the supplementary material).

Discussion

Our results, for the first time, integrated different tools (macroscopic scale, qualitative histological analysis, cytometric scale and physiological index (Condition Index) to evaluate the reproductive activity and investment of *P. perna*. Although it is known that the species reproduces all year round, there are peaks of intensity in reproductive investment (detected here through maternal investment), these being observed, fundamentally, through cytometric scales.

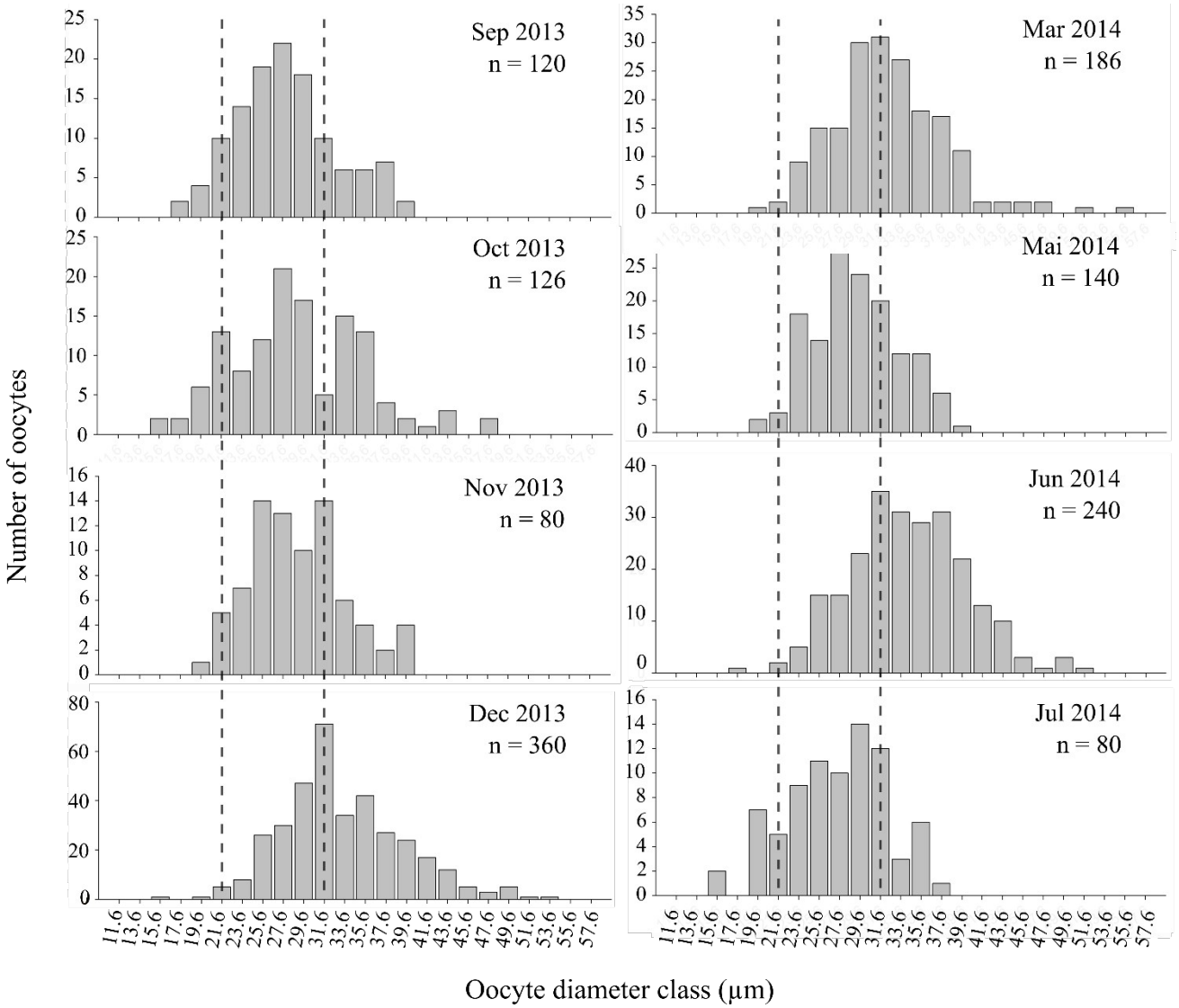

Figure 3. Monthly distribution of the frequency of the average diameter of oocytes of *Perna perna* on the coast of Paraná. The dotted lines represent the cohorts based on Bhattacharya's method.

Reproductive cycle in the Paraná coast: As already noted, (see Corte 2015), populations from nearby regions may present varied reproductive strategies, which can be considered a "regional adaptation". The study of the reproductive cycle in response to different environmental conditions experienced by the species allows establishing the group's reproductive pattern and the wide variety of reproductive strategies that can be adopted by the bivalves.

The population of *P. perna* from the coast of Paraná had proportionality between sexes and no event of hermaphroditism was registered as seen at the other populations on the southeastern and southern coast of Brazil (Magalhães 1998, Souza *et al.* 2019). The same occurs in other species of the genus *Perna* (*e.g.* Lee 1988, Alfaro *et al.* 2001). In some populations of the Brazilian coast there may also be a small prevalence of males within a few months throughout the year (*e.g.* Galvão *et al.* 2006), except Angra dos Reis (RJ), where it was recorded a prevalence of females at certain times (Silvestri *et al.* 2018). However, in this last, the solely macroscopic analysis of the individuals may have led to misinterpretation of the sex of some individuals.

The trematode *Bucephalus sp.* is the main organism responsible for parasitosis known as "orange disease", being the only with potential interference in reproductive activity of the mussels (Calvo-Ugarteburu & McQuaid 1998, Hausen 2006). In several specimens were detected cell structures identified as development stages of parasites, which filled the mantle area corresponding to gonadal

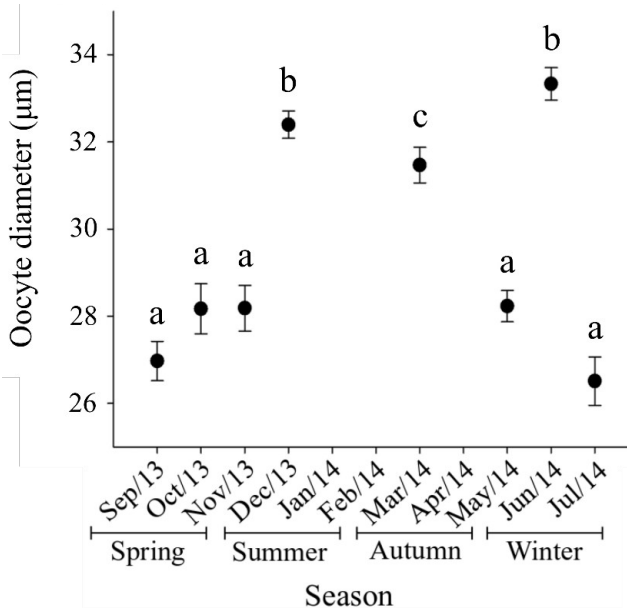

Figure 4. Mean and standard error of oocyte diameter (µm) of *Perna perna* on the coast of Paraná, southern Brazil. Letters denote homogenous groups by post hoc comparison of means (α = 0.05).

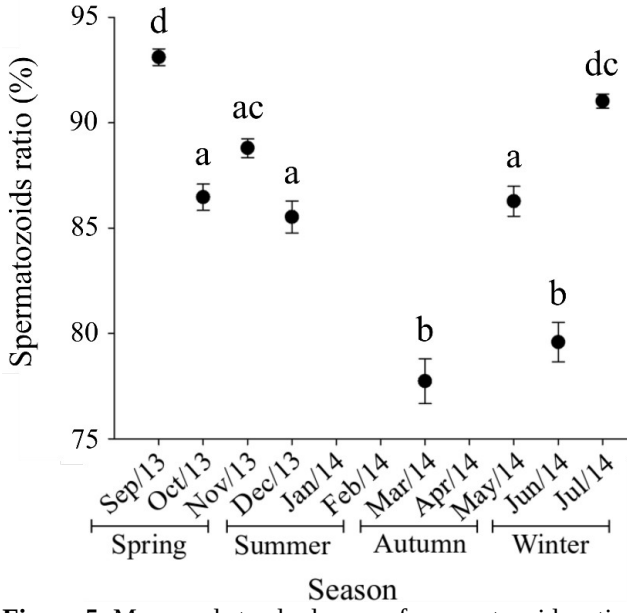

Figure 5. Mean and standard error of spermatozoids ratio (%) of *Perna perna* on the coast of Paraná, southern Brazil. Letters denote homogenous groups by post hoc comparison of means (α = 0.05).

tissue. Individuals in different levels of infestation were observed and, in some cases, the disease has become so intense to the extent of leading to the castration and reproductive downtime of the mussel. The largest number of castrated individuals occurs during periods when the Partial spawning and proliferation stage prevails. Probably at that moment the energy used for proliferation is used by the parasite (Magalhães 1998, Galvão *et al.* 2006).

Lipids represent an important energy reserve of edible oysters and mussels species because of their high caloric contents, they are mainly used in chronic stress conditions and can be used during gametogenic processes (Radic *et al.* 2014). Changes in fatty acid composition are closely related to available food and correspond with good nutritional conditions. According to Ruano *et al.* (2012), changes in lipid contents was inexorably associated with loss with the nutritional quality and may be used to evaluate on their Condition Index (CI). The occurrence of the infection for this population was 7.8%, similar to what is found in other studies of the Brazilian coast (*e.g.* Galvão *et al.* 2006), suggesting that there was low inhibition of gametogenesis, apparently not affecting the reproductive potential of the population. Although it is well known that in species of commercial interest diseases can lead to large financial losses (Markowitz *et al.* 2016, Castinel *et al.* 2019).

Along the southeast and south Brazilian coast, from Rio Janeiro (22ºS) to Santa Catarina (27ºS), the reproductive activities of *P. perna* is uninterrupted all year around with a longer period of spawning occurring usually in spring, summer and autumn. Although reproduction of the species be described as continuous, with constant presence of mature and spent individuals, it is common to describe one mainly period of gamete release. Authors have reported that the timing of proliferation (gametogenesis or recovery events), maturation, spawning are different between populations. However, these periods are alternated at least six months, indicating that there are preferred seasons devoted to maximization and recuperation of the individual's reproductive efforts (Table III).

It is important to note that the inference of the period of greatest reproductive activity for the Brazilian coast so far has not taken into account the size of the oocyte. Since the species reproduces throughout the year in subtropical regions, it is natural for spawning events to be recorded in all seasons, but reproductive intensity and investment can change from one event to another.

In this study, the period of greatest participation of proliferative cells in females (Partial spawning with proliferation) was in mid-spring and winter. Mature oocytes of intermediate size (Partial spawning with intermediate maturation) occurred mainly in spring and autumn, while the largest oocytes (Partial spawning with

Table II. Description of the gametogenic stages of *Perna perna* males and females collected in the euhaline sector of the Paranaguá Bay, southern Brazil.

	Female (Fig. 7)	
	Macroscopic features	*Microscopic events*
Partial spawning and proliferation	Mantle becomes less orange and turgid, acquiring a yellowish tone according to the degree of emptying (Fig. 7a).	There is a greater occupation by connective tissue, the follicles are separated from each other. Growing oocytes attached to the follicular wall and residual oocytes are present (Fig. 7b). > 10 % of the cells are < 21.6 µm.
Partial spawning and intermediate maturation	Thick, reddish-orange mantle (Fig. 7c).	The follicles are distended, little connective tissue can be observed. The follicular lumen is not completely filled, it is possible to identify mature cells with a circular shape and apparent vitellogenic vesicle (Fig. 7d). > 60 % of oocytes are between 21.7 and 31.6 µm.
Partial spawning and maximum maturation	Macroscopic appearance is no different from intermediate maturation.	Little or no connective tissue can be seen. The follicles were abundant and distended, with some overlap between them. Mature oocytes fill the entire follicle and are polyhedral in shape (Fig. 7e). > 40 % of oocytes are > 31.7 µm.
	Male (Fig. 8)	
	Macroscopic features	*Microscopic events*
Maturation with proliferation	Thick and creamy-white mantle (Fig. 8a).	Little or no interfollicular space, it is possible to observe homogeneity in the gonadal tissue. Follicles completely filled with mature spermatozoids mass that occupy > 80 % of the follicle, a poorly developed radial series and occupying < 10 % of the intra-follicular space (Fig. 8b).
Emission with proliferation	Slender and, according to the degree of emptiness, fully transparent mantle (Fig. 8c).	Spaced and contracted follicles with empty spaces in the center, well developed and apparent radial spermatic series arranged toward the follicular lumen (Fig. 8d). At this stage, the radial series occupies > 10 % of the follicle, while < 80 % is occupied by mature spermatozoids.

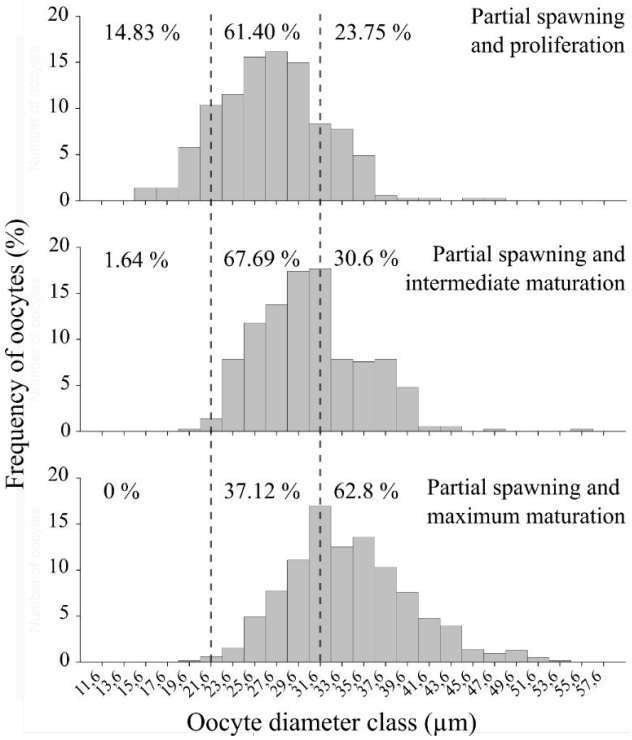

Figure 6. Frequency of oocyte diameter in each gametogenic stage of *Perna perna* in Paranaguá Bay, southern Brazil.

maximum maturation) were frequent in summer and winter, which may represent better quality larvae/seeds (better survival and performance). For the males was observed, in early autumn and mid-winter the highest percentage of occupation of the follicle by the spermatic radial series (Emission with proliferation), which may be an indicative of a time of year when some recovery of the gonadal tissue occurs. The higher investment in spermatozoids output (Maturation) happened in the spring. This pattern demonstrates the continuous reproductive activity of the population, as well established for the species, although with variations in the intensity and quality of the gametes, this can be analysed only through quantitative and integrated approaches, as discussed below.

In the studied population, high temperatures (December/2013 and March/2014) were related to the occurrence of the large oocytes and to the increase in occupation by the radial spermatic series, indicating a greater investment in large oocytes in females and the proliferative phase in males. In addition to temperature, the only abiotic parameter that was associated with the gametogenic cycle was the seawater transparency, often used as a proxy to

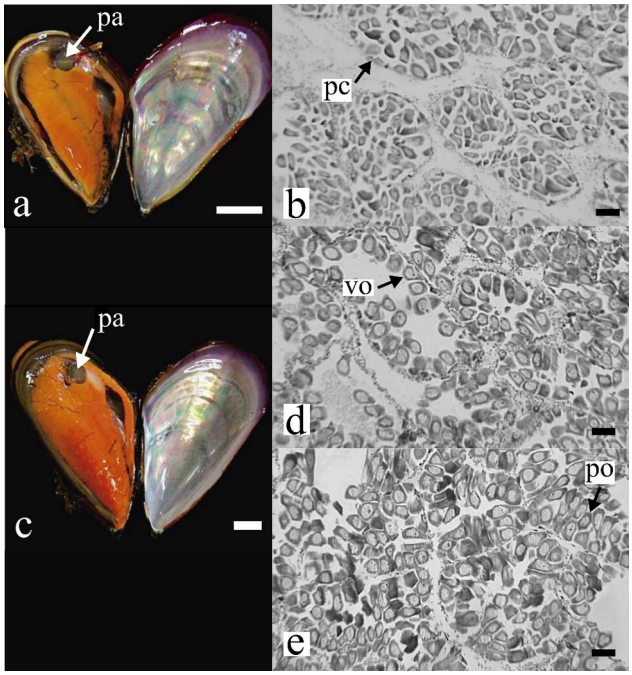

Figure 7. Macro and microscopic aspects of the gonads of females of *Perna perna* in the stages of Partial spawning and proliferation (a, b), Partial spawning and intermediate maturation (c, d) and Partial spawning and maximum maturation (e). See Table II for descriptions. pa: posterior adductor muscle. pc: primary cells. po: polyhedral oocytes. vo: vitellogenic oocyte. (Scale bars: a, c = 10 mm; b, d, e = 10 µm).

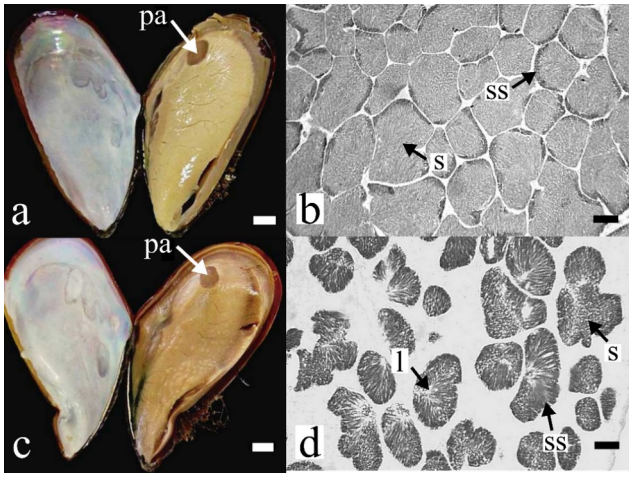

Figure 8. Macro and microscopic aspects of the gonads of males of *Perna perna* in the stages of Maturation with proliferation (a, b) and Emission with proliferation (c, d). See Table II for descriptions. pa: posterior adductor muscle. l: lumen. s: spermatozoids. ss: spermatic radial series. (Scale bars: a, c = 10 mm; b, d = 10 µm).

measure the amount of organic matter (nutrients) available. Periods of greater seawater transparency (September/2013 and July/2014) were observed to the higher occupation by spermatozoids.

The mussels reply metabolically to environmental changes like other marine invertebrates (Ross *et al.* 2013, Moran *et al.* 2013). The adjustment of reproductive events is associated with the internal control of the individuals (endogenous factors) and with environmental factors (exogenous factors), being the temperature and salinity of the seawater, and the food availability (phytoplankton development) the main regulators factors (Asaduzzaman *et al.* 2019). These factors act in an isolated way or in association such as triggers, which synchronize or direct the breeding season (Narváez *et al.* 2008, Hafsaoui *et al.* 2016). With this in mind, the reproductive biology of these species must be well known and monitored in view of the rapid climatic changes that the planet has been experiencing, changing the physical-chemical composition of the oceans and, consequently, the reproductive pattern of the species (Oyarzún *et al.* 2018, Monaco & McQuaid 2019, IPCC 2019).

Cytometric scale and gametogenic stages: Qualitative analysis is widely used to describe the reproductive cycle of populations of *P. perna*. Some studies consider only the macroscopic aspect and Condition Index, which can classify adults as females or males depending on the color of the mantle, and as in the stages of spawning or maturation depending on the weight and turgidity of the soft parts (Hausen 2006, Marenzi & Branco 2006, Silvestri *et al.* 2018). Others perform histological analysis of the gonads as a basic condition for understanding the sexual cycle. For this gametogenesis events are classified into stages according to the subjective (qualitative) analysis of the histologic slides, taking into account the aspect of the tissue, cellular components and development of the interfollicular connective tissue (Magalhães 1998, Galvão *et al.* 2006). Most of Brazilian edible mussels, oysters and clams studies consider qualitative evaluations of gametogenesis using the criteria of Lunetta (1969) for reproductive development. Up to 3 consecutive developmental stages were identified: I (Immature), II (Differentiation) and III (Sexual maturity). In the last adults individuals are still categorized into three substages: IIIA (Maturation), IIIB (Partial or total Elimination) IIIC (Recovering) and IIID (End of gametogenesis or exhaustion) (see Lunetta 1969 for a complete description of each stage of gonadal development). Here we renamed IIIC stage as Proliferative.

In addition to the low resolution of the cycle when only qualitative methods are used, it is not

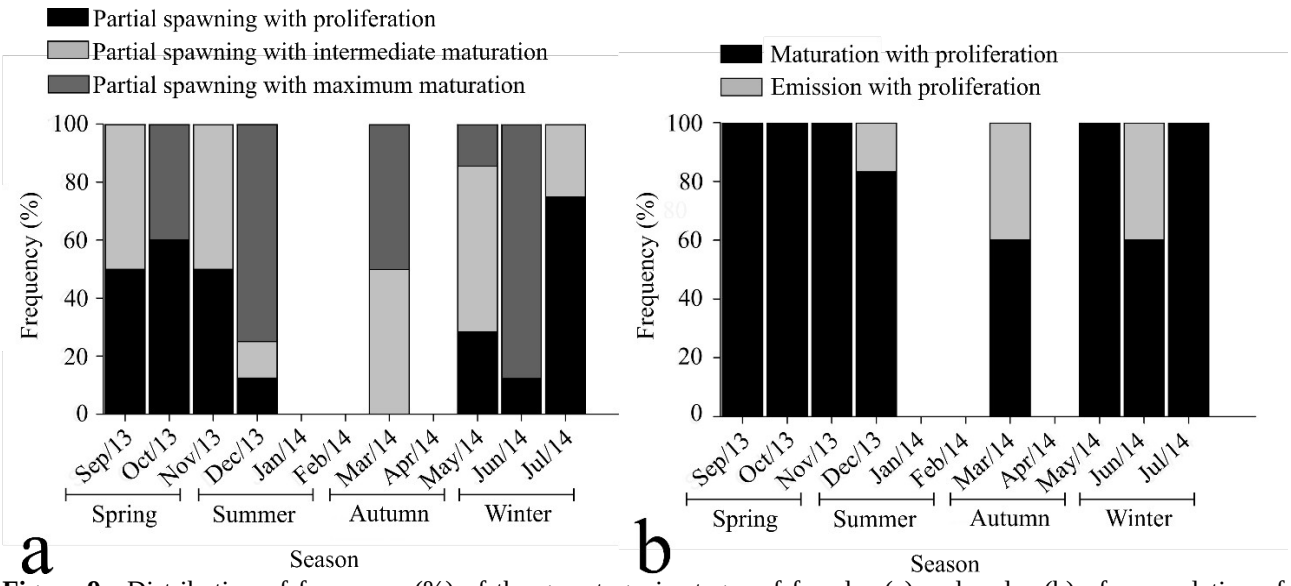

Figure 9. Distribution of frequency (%) of the gametogenic stages of females (a) and males (b) of a population of *Perna perna* of the coast of Paraná, southern Brazil.

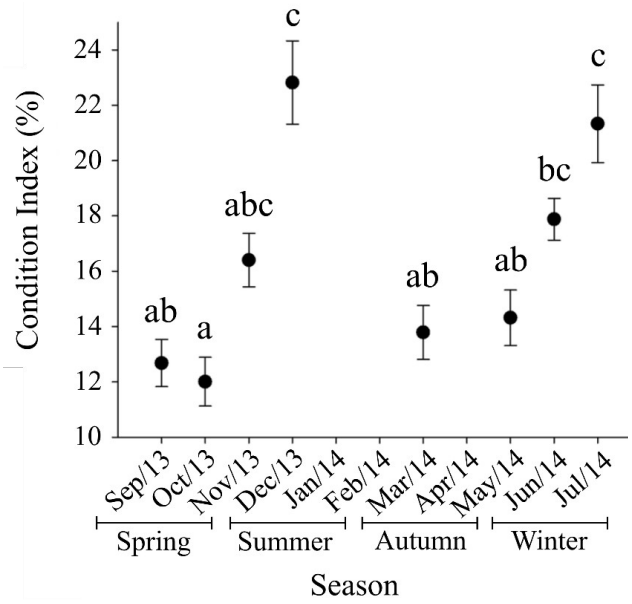

Figure 10. Mean and standard error of Condition Index of males and females of *Perna perna* of the coast of Paraná, southern Brazil. Letters denote homogenous groups by post hoc comparison of means (α = 0.05).

possible to standardize the classifications of stages for different populations. Thus, each author classifies the stages subjectively, impairing an adequate comparison between populations over the wide distribution of the species. Despite of the contributions of this studies to the understanding of the reproductive pattern of the species, such analyses are insufficient considering that *P. perna* reproduces continuously in tropical and subtropical regions (see Araújo 2001 for a broad discussion on this subject). The nuances of reproductive investment and periods of availability of the best gametes and seeds cannot be detected without the use of a complementary quantitative method. Taking this into account, the adoption of a cytometric scale to assess gametogenic development allows not only to identify the nuances of the population's reproductive investment, but also to standardize the method of classifying stages.

In the present study, through qualitative analysis of histological slides and cytometry, it was possible to establish a quantitative scale for the classification of *P. perna* gametogenic stages (Table IV).

As the gametogenic cycle of the species is continuous, it is natural that all stages have spawning/emission characteristics (partial spawning) and proliferative cells (proliferation) present. In this case, must take into account the other constituents of the tissue, their occupancy rate and cell size.

The Partial spawning and proliferation stage had already been identified by Borzone et al. (2001) and Borzone et al. (2003) for other bivalves. The two other stages of gonadal development (Partial spawning and intermediate maturation and Partial spawning and maximum maturation) propose a division of female maturation into two types, based on the size of the oocyte (cytometry). Such separation, confirmed by the analysis of variance (see Fig. 4), confirms the difference in the reproductive investment of a species of continuous reproduction, being the largest maternal investment in December/2013, March and June/2014. Such results have never been demonstrated before for *P. perna*.

Table III. Brazilian literature review of some biological and reproductive features of *P. perna*. The squares marked in gray indicate the period in which the author considered the period of greatest reproductive activity of the assessed population. The symbol ">" represents a higher frequency of the stage; The symbol "=" represents the same frequency of the stages; SL: shell lenght (mm); ML: first maturation lenght (mm); OD (µm): mature oocyte mean diameter; SR: sex ratio (female:male); PI: median physiological index; P%: parasite frequency; U%: undeterminated frequency; P (proliferation); M (maturation); MProl (male maturation with proliferation); S (spawning); SProl (female parcial spawning with proliferation); SInt (femmale parcial spawning with intermediate maturation); SMax (female parcial spawning with maximum maturation) and EProl (male emission with proliferation).

LATITUD/ REFERENCE		REPRODUCTIVE FEATURES						SEASONS			
		SL	OD	SR	PI%	P%	U%	SUMMER	AUTUMN	WINTER	SPRING
22ºS	1 N	69	-	1.2:1	-	-	-	M	S>M>P	S>M>P	M>S>P
23ºS	2 N	-	67.5*	-	-	-	-	M>S>P	M>S= P	M>S= P	M>S> P
	3 N	37	-	-	-	-	-	S	-	S	S
	4 C	70	-	0.6:1	12-19**	-	-	P>M>S	P>S>M	P>S=M	P>S>M
24ºS	5 N	49	28.3*	1.2:1 1.6:1	-	4-7.7	-	S>M	M=S>P	S>M	S>M
25ºS	6*** A	80	33.2	1:1	11-23	-	7.8	$S^{Max} > S^{Prol} = S^{Int}$	$S^{Int} > S^{Max} > S^{Prol}$	$S^{Int} > S^{Max} > S^{Prol}$	$S^{Prol} > S^{Max} > S^{Int}$
								$M^{Prol} > E^{Prol}$	$M^{Prol} > E^{Prol}$	$M^{Prol} > E^{Prol}$	M^{Prol}
27ºS	7 N/C	<80	40.2*	1:1	11 N	1.8-5.9 / 2.0	-	S>M>P	M>S=P	M>P>S	M=S>P
	8 C	>40	30.7	-	12	-	-	$P > M^{Prol} > S$	$M^{Prol} > S > P$	$M^{Prol} > P$	$P > M^{Prol}$

1: Mesquita et al. (2001); 2: Lunetta (1969); 3: Marques et al. (1991); 4: Silvestri et al. (2018); 5: Galvão et al. (2006); 6: Present Study; 7: Magalhães (1998); 8: Hausen (2006)
(A) artificial substrate; (N) natural substrate; (C) cultive; (-) no data; (*) approximate value; (**) cooked flesh index; (***) the columns divided into two parts represent the females in the upper row and the males in the lower row.

Table IV. Cytometric scale proposed of the gametogenic stages in a Brazilian subtropical population of *P. perna*. OD: mean oocyte diameter.

Sex	Cytometric scale	Stage definition
Female	above 10 % of the cells with OD < 21.6 µm	*Partial spawning and proliferation*
	above 60 % of cells with 21.7<OD< 31.6 µm	*Partial spawning and intermediate maturation*
	above 40 % of cells with OD> 31.7 µm.	*Partial spawning and maximum maturation*
Male	Spermatozoids mass occupy > 80 % of the follicle and radial spermatic series < 10%	*Maturation with proliferation*
	Spermatozoids mass occupy < 80 % of the follicle and radial spermatic series > 10%	*Emission with proliferation*

Reproductive investment: Perna perna has a quick larval life and recruitment occurs in about 5 weeks after fertilization, with 20 mm size shell the individual could be attached to the substrate (Fernandes 1985). For planktotrophic larvae (such as those of *P. perna*), the maternal investment, reflected in the size of the oocyte/egg and its lipid reserves are also essential for survival and larval performance (Honkoop *et al*. 1999, Moran & McAlister 2009, Marshal *et al.* 2010, Brunner 2013). Previous work has shown that the largest oocytes have a greater amount of nutritional reserves in bivalves (Morsan

& Kroeck 2005, Liu *et al*. 2013, Corte 2015, Balić *et al.* 2020). Thus, the size of the oocyte can be used as a proxy to measure maternal investment.

Genetic variations determine the limits of oocyte size, and also allow cells of intermediate size to ensure the balance between quality (size) and the number of eggs released into the environment (fertility) (Lango-Reynoso *et al.* 2000, Moran 2004, Moran & McAlister 2009, Powell *et al*. 2011). This condition is quite clear in the population assessed here. As the species has continuous reproduction throughout the year, an intermediate size of the oocyte is always observed (considered here between 21.7 and 31.6 µm and called "intermediate maturation"), and then environmental changes can offer selective pressures for the production of larger oocytes than those currently produced (Moran & McAlister 2009, Powell *et al*. 2011). The largest percentage of larger oocytes (considered here > 31.7 µm and called "maximum maturation") observed in summer (December/2013) and winter (June/2014) represent the moment of greatest maternal investment and are related with the higher temperatures, which seems to work as a trigger for females. For males, fewer stages were identified, which is common, since male gametogenic activity is much faster than female and not all stages are always recorded. Paternal investment is little explored among marine invertebrates, but there is evidence that the quantity and availability of spermatozoids also directly affects maternal investment (Marshal & Keough 2008).

On mussels farms, changes in the condition of brood stock, presence of larvae, and post-larval settlement are used to determine the reproductive cycle of the species (Ferreira *et al.* 2006, Hausen 2006, Silvestri *et al.* 2018). In Brazilian coast, the state of Santa Catarina, for example, is the largest producer of the cultivated *P. perna* and the production is closely related to obtaining seeds (juveniles) (Marenzi & Branco 2005, Hausen 2006, Silva 2007, Aquini *et al.* 2013). This knowledge that here we provide about periods of higher reproductive efficiency combined with quality of oocytes can enable greater productivity for aquaculture sectors (Bordon *et al.* 2014, Silvestri *et al.* 2018).

It is worth mentioning that this investment can vary within the same population, from individual to individual and even within the same individual (Marshal & Keough 2008, Moran & McAlister 2009). Oocytes from the same female may have varying amounts of reserves (see Lango-Reynoso *et al.* 2000 and Moran & McAlister 2009) and, in addition, selective environmental pressures change from year to year. Finally, the size of the oocyte will be a consequence of the synergistic relationship between the availability of spermatozoids and nutrients, larval survival rate and abiotic factors (Moran 2004, Marshal & Keough 2008, Powell *et al*. 2011). Such considerations demonstrate that marine invertebrate mothers have more control over the offspring's survival and dispersion potential than we thought (Marshal & Keough 2008, Moran & McAlister 2009).

Condition Index as a probable response of allocation for reproduction: For commercially exploited bivalves, the CI provides the higher value of the flesh weight regarding gross weight, thereby indicating the best time for harvest and a better use of the flesh (see Galvao *et al.* 2015). The weight of the mussels can be related with the stage of sexual maturity; during maturation, the mussel is commonly known as "fat mussel" or "full mussel", due to the large amount of reserve substances - mainly glycogen - that are stored along with the gametes. This is the point at which the animal exhibits their maximum value of CI (Magalhães 1998, Hausen 2006, Galvão *et al.* 2006, Galvao *et al.* 2015).

In this study, the CI was not related to the population's reproductive events. Other physiological functions are also reflected in these values, such as the storage of nutrients, which can be used to support continuous reproductive activity (Borzone *et al*. 2001). In addition, investment in gametes and storage can occur concurrently, requiring a biochemical assessment to clarify the seasonal variation in CI values. For the population of Paraná, the input of visceral mass is greater in the beginning of summer and mid-winter and is in line with what was observed in other studies in the southern region of Brazil, summarized in table 5.3 of Resgalla (2008). Greater consistency in the sampling effort may clarify how these values relate to the gametogenic cycle.

Indications for tools to describe the reproductive cycle of bivalves: In order to simplify the identification of the reproductive characteristics of culture bivalves, other studies have already suggested macroscopic scales to identify when spawning occurs in *Perna* (see McDonald *et al*. 2018). The macroscopic aspect used here was sufficient to distinguish sexually mature from post-spawned individuals, as well as parasitized bivalves. The cytometric scale allows to standardize the

description of the reproductive cycle of the species and, mainly, to elucidate the period of greater maternal investment and, consequently, of better seed quality (Lango-Reynoso *et al.* 2000, Moran & McAlister 2009, Brunner 2013). The CI is a well-established tool to observe the variation in the reproductive cycle of bivalves (Hausen 2006, Galvão *et al.* 2006, Galvao *et al.* 2015), but in this study we did not identify a good relation to reproductive activity, requiring complementary biochemical analyzes. In view of the methods used here and the results obtained for a population with a subtropical climate, we strongly encourage the adoption of multiple methods for the evaluation of the reproductive activity of bivalves in tropical and subtropical regions. In addition to improving our understanding of the reproductive strategies adopted by the populations, it allows the standardization of the classification of gametogenic stages of the group.

Conclusion

For bivalves, as well as for other marine invertebrates, in addition to latitude, local factors are fundamental in determining the reproductive strategy presented by the population (Corte 2015, Rola *et al.* 2017). Thus, the evaluation of the reproductive behavior of the species in different regions allows to establish the reproductive pattern of the group and to understand the range of variation (plasticity) of strategies that can be adopted.

In the Paraná coast, the number of females and males was proportional, the frequency of individuals castrated by parasitosis does not seem to affect the population's reproductive potential. The largest oocytes, representing a better quality of the gamete and, consequently, greater larval survival and performance, were observed in December/2013, March and June/2014. Males presented spermatozoids throughout the year. Proliferative stages were observed in the spring and winter for females and in autumn and winter for males.

The macroscopic aspect used here was sufficient to distinguish sexually mature from post-spawned individuals, as well as parasitized bivalves. The cytometric scale that we propose allows not only to identify the nuances of the population's reproductive investment, but also to standardize the gametogenic stages classification method. The CI was not related to the reproductive events of the population, requiring a biochemical evaluation to clarify the seasonal variation shown.

In view of the methods here discussed and of the results obtained, we strongly encourage the adoption of multiple methods for the evaluation of the reproductive activity of bivalves in tropical and subtropical regions that tend to present continuous gametogenic activity. The recognition of nuances in reproductive investment can assist in the management of farms and indicate the best period for capturing seeds and must be taken into consideration when considering the conservation of natural stocks.

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