UNIVERSIDADE FEDERAL DO ESTADO DO PARÁ
INSTITUTO DE CIÊNCIAS BIOLÓGICAS
PROGRAMA DE PÓS-GRADUAÇÃO EM ECOLOGIA AQUÁTICA E PESCA

LEONARDO FERNANDES DA PAIXÃO

Estudo da Biologia reprodutiva de *Crassostrea gasar* (Adanson, 1757) no nordeste paraense.

BELÉM –PA
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Dissertação apresentada ao Programa de Ecologia Aquática e Pesca da Universidade Federal do Pará, como requisito parcial para a obtenção de grau de Mestre em ecologia Aquática e Pesca.

Orientador (a): Drª Rossineide Martins da Rocha – ICB/UFPA

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Dedico aos meus pais, que me incentivaram desde o início de minha vida, minha querida irmã Luciane Melo. A Michelle Failache pela compreensão e as professoras Rossineide e Auxiliadora pela orientação.

“Sem a religião, a ciência é capenga; sem a ciência, a religião é cega.”

Albert Einstein

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Sumário

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Introdução geral

O litoral brasileiro apresenta diferentes tipos de ecossistema, dentre eles os manguezais que são encontrados desde o Estado do Amapá até o de Santa Catarina (Souza-Filho, 2005). Este ecossistema possui espécies típicas e adaptadas ao substrato que sofre diariamente variações de maré o que acarreta oscilações dos fatores ambientais, como por exemplo a salinidade (Tomlinson, 1986); sendo um ambiente favorável para o desenvolvimento, crescimento e sobrevivência dos estágios iniciais do ciclo de vida de animais marinhos (Schuler *et al*. 2000). Peixes, moluscos (ostras e mexilhões) e crustáceos (caranguejos e camarões), são os componentes faunísticos dos manguezais que mais se destacam, pois são constituídos por expressiva biomassa, proporcionando excelentes fontes de proteína animal de alto valor nutricional (Kjerfve *et al.* 1997).

O cultivo de ostras constitui um importante recurso econômico por apresentar um excelente potencial de comercialização (Manzoni, 2001). É uma atividade que pode gerar recursos e oportunidades de negócios para empresas, comunidades e associações, criando empregos diretos e indiretos. Essa atividade apresenta as vantagens: do ponto de vista social promovendo a inclusão social, devido ao aumento da renda familiar, proporcionando igualdade dos gêneros e fortalecendo a dieta alimentar da família; do ponto de vista econômico, promovendo a abertura de novos mercados gerando empregos e rendas; e do ponto de vista ambiental, reduzindo a pressão extrativista sobre os estoques de populações naturais (Gomes *et al*. 2008).

O cultivo de ostras no Brasil é recente, iniciou nas décadas de 70 e 80, nos estados da Bahia, Rio de Janeiro, São Paulo e somente no início dos anos 90 se consolidou no Estado de Santa Catarina (Rupp *et al.* 2008). Dentre os dezessete Estados brasileiros localizados na região litorânea, apenas cinco constam como produtores de moluscos com cultivo em nível comercial. No Pará os experimentos pioneiros com cultivo de ostras com finalidades comerciais iniciaram nos anos de 2001 a 2003, na comunidade de Nova Olinda, município de Augusto Corrêa (Sampaio, 2007).

As populações de ostras estão adaptadas as condições específicas de seu habitat, pois cada estuário tem suas particulariedades, os fatores ambientais alteram o metabolismo do animal, influenciando no crescimento, desenvolvimento e reprodução das espécies (Wakamatsu 1973; Vilanova & Fonteles-Filho 1989).

As ostras de maior importância econômica pertencem ao gênero *Crassostrea*, o qual é dióico, não apresenta dimorfismo sexual, a sua reprodução é sexuada com fecundação externa. As gônadas têm um aspecto esbranquiçado e difuso e quando estão sexualmente maturas envolvem totalmente a glândula digestiva, sendo necessário o uso das técnicas histológicas para diferenciação entre machos e fêmeas (Christo & Absher, 2008).

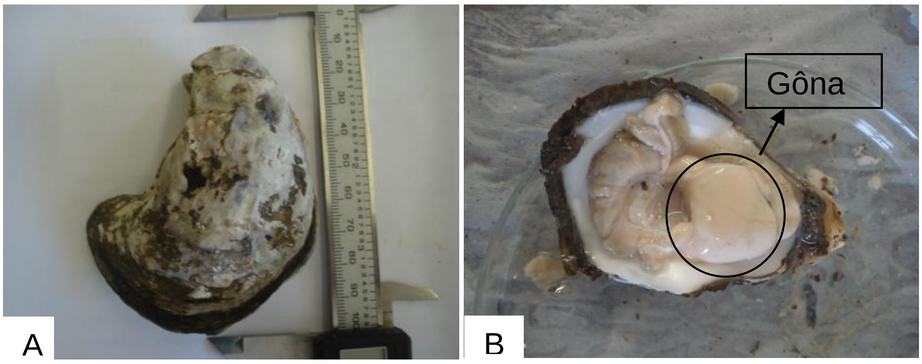

Figura 1 – Espécime de *Crassostrea gasar*, coletado no município de Augusto Corrêa - PA. A- valvas fechadas; B- Valvas abertas e região da gônada.

Na região amazônica dentre as espécies utilizadas no cultivo destaca-se *Crassotrea gasar* (Adanson, 1757) encontrada, principalmente, fixada as raízes aéreas do mangue vermelho (*Rhizophora mangle*) ou sobre zonas intertidais e costões rochosos (Nascimento, 1983). Podem ocorrer tanto na zona infralitoral, onde permanecem continuamente submersas, quanto na zona entre-marés, onde ficam alternadamente emersas pelas variações semi-diurnas da maré (Fernandes, 1979; Pereira *et al*. 1988; Rios, 1994).

Em qualquer organismo de interesse comercial o conhecimento da estrutura gonadal é fundamental permitem melhorar a qualidade produtiva desses animais. Em se tratando do gênero *Crassostrea* estudos sobre a estrutura gonadal estão limitados a algumas espécies como: *C. gigas* (Komaru *et al*. 1994; Dong *et al*. 2005; Franco *et al*. 2008; Yurchenko *et al*. 2009) e *C. rhizophorae* (Matos *et al*. 1999). Com relação a espécie *C. brasiliana* não há estudos sobre estrutura gonadal e dinâmica celular da linhagem germinativa. Afim de gerar informações a respeito da biologia reprodutiva do animal é necessário a definição dos estádios gonadais, do período reprodutivo e da relação entre a produção de gametas e os fatores ambientais para a espécie.

Nesse contexto, o presente trabalho teve como objetivo estudar a biologia reprodutiva e analisar a morfologia do gametogênese de *Crassostrea gasar* (Adanson, 1757) no estuário amazônico e influência dos fatores abióticos sobre a reprodução da espécie no município de Augusto Corrêa no Estado do Pará, onde foi implantado a atividade de cultivo em condições ainda inadequadas para o estabelecimento da produção em escala comercial.

OBJETIVO GERAL

- Estudar a biologia reprodutiva e analisar a morfologia da gametogênese de *Crassostrea gasar* (Adanson, 1757) no estuário amazônico.

OBJETIVOS ESPECÍFICOS

- Descrever os aspectos morfológicos e estruturais das gônadas de *Crassostrea gasar*.
- Caracterizar os estádios gonadais.
- Determinar a época da reprodução da espécie.
- Verificar a influência das variáveis físicas e químicas da água na reprodução da espécie.
- Caracterizar ultraestruturalmente a espermatogênese de *Crassostrea gasar*.
- Descrever a participação das proteínas do citoesqueleto na maturação gonadal de machos.

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CAPÍTULO I

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Effects of salinity and rainfall on the reproductive biology of the mangrove oyster (*Crassostrea gasar*): implications for farming practices.

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Resumo

O presente estudo analisou o ciclo reprodutivo e estabeleceu a relação dos fatores abióticos com os estádios de desenvolvimento gonadal de *Crassostrea gasar* criadas no estuário amazônico. Foram coletados mensalmente cerca de 20 ostras no período de agosto de 2009 a dezembro de 2010. Os meses de coleta foram agrupados em quatro períodos sazonais (seco, transicional seco/chuvoso, chuvoso e transicional chuvoso/seco). No local da coleta foram mensurados *in situ* salinidade, pH, temperatura e oxigênio dissolvido. A gônada foi dissecada e submetida ao procedimento histológico. Um total de 351 animais foram coletados, sendo 190 fêmeas, 161 machos e 2 hermafroditas. Histologicamente machos e fêmeas foram classificados em quatro estádios gonadais: I- imaturo, II - em maturação, III - maturo e IV - desovado (fêmeas) e espermiado (machos). Dentre os fatores abióticos analisados apenas a salinidade e a precipitação pluviométrica apresentaram diferenças estatisticamente significantes durante o estudo. Houve correlação entre esses dois fatores e a maturação gonadal, sugerindo que esses fatores estejam influenciando na reprodução, visto que foram encontrados predominância de indivíduos maturos (III) no período chuvoso e transicional chuvoso/seco (baixa salinidade e alta precipitação pluviométrica). Nos períodos seco e transicional seco/chuvoso (alta salinidade e baixa precipitação) foram encontrados indivíduos nos estágios imaturo (I), em maturação (II) e desovado/espermiado (IV). Por conseguinte, para o cultivo é indicado que a coleta de sementes seja feita nos períodos seco e transicional seco/chuvoso.

Palavras-chaves: reprodução, histologia, desenvolvimento gonadal, fatores abióticos e ostras

Abstract

The present study analyzed gonadal development in *Crassostrea gasar* from an Amazonian estuary and characterized the relationship between the reproductive cycle and the variation in abiotic factors. Samples of approximately 20 oysters were collected each month between August, 2009, and December, 2010. The study period was divided into four seasonal periods (dry, dry-rainy transition, rainy, and rainy-dry transition). The salinity, pH, temperature, and dissolved oxygen concentration of the water were measured *in situ* during the collection of specimens. The gonads were dissected and analyzed histologically. A total of 353 specimens were collected, of which 190 were female, 161 were male, and two were hermaphrodite. Histologically, the specimens

were classified in four gonadal stages: I – immature; II – maturing; III – mature, and IV – spawned (females) and spermiated (males). Of the abiotic factors analyzed, only salinity and rainfall varied significantly over the study period, with correlations being found between these factors and gonadal maturation, suggesting that they influence the reproductive cycle of the species. A predominance of mature (stage III) and maturing individuals were recorded during the rainy and rainy-dry periods (low salinity and high rainfall), whereas immature (I) and spawned/spermiated (IV) specimens were more common during the dry and dry-rainy periods (high salinity and low rainfall). Given this, the collection of seeds for farming would be recommended during the latter period, when spawned/spermiated oysters were more common.

Keywords: Histology, gonadal development, abiotic factors, oysters, Amazonian estuary

1. Introduction

Mangroves are among the most important ecosystems of tropical coastal zones in both biological and economic terms (Cintrón-Molero and Schaeffer-Novelli, 1999). They are considered to be essential nursery areas for many groups of animals, in particular fishes, crustaceans (crabs and shrimps) and mollusks (oysters and mussels). These animals are able to tolerate considerable variation in tidal conditions and salinity (Pereira et al., 2003).

The oysters of the genus *Crassostrea* are dioic, and present sexual reproduction with external fertilization. The gonads have a diffuse whitish aspect, but no sexual dimorphism, requiring histological analysis in order to differentiate males from females (Christo and Absher, 2008). In addition to the two native species of *Crassostrea* oyster

– *Crassostrea rhizophorae* (Guilding, 1828) and *Crassostrea gasar* (Adanson, 1757) – an exotic species, *Crassostrea gigas* (Thunberg, 1795), can be found on the Brazilian coast. The exotic *C. gigas* is farmed commercially in the south of the country (Varela et al., 2007). Molecular studies (partial sequencing of the COI gene) have revealed that the genus *Crassostrea* is present in the Amazon region, where it is represented by *Crassostrea gasar*, *Crassostrea rhizophorae*, and *Crassostrea* sp. (Melo et al., 2010). *C. gasar* is prevalent in the northeast of the Brazilian state of Pará, where it is now being farmed on a small scale.

As exogenous factors may affect the duration of gonadal stages, and thus the production of gametes, a good working knowledge of the relationship between environmental variables and gonadal development is an important prerequisite for the development of programs for the commercial exploitation of shellfish (Gosling, 2003). Given this, the present study analyzed the reproductive cycle of *C. gasar* to determine whether and to what extent variation in the physical and chemical of the water influence the reproduction of the species in an estuary in Pará. These results provide an important database for the development of effective farming and management techniques in the region.

2. Material and methods

2.1. Study area and specimen collection

Oysters, *C. gasar*, were farmed in rectangular oyster bags (1 m x 0.5 m) on the Emboraí River in the town of Nova Olinda (0°52’54” S, 46°26’54” W) located in the municipality of Augusto Corrêa in the Brazilian state of Pará (Fig. 2). An average of 20 oyster specimens were collected each month between September, 2009, and December,

2010. The study period was divided into four periods – the dry season (September-December, 2009, and August-December, 2010), dry-rainy transition period (January-March, 2010), rainy season (April-June, 2010), and the rainy-dry transition period (August, 2009, and July, 2010).

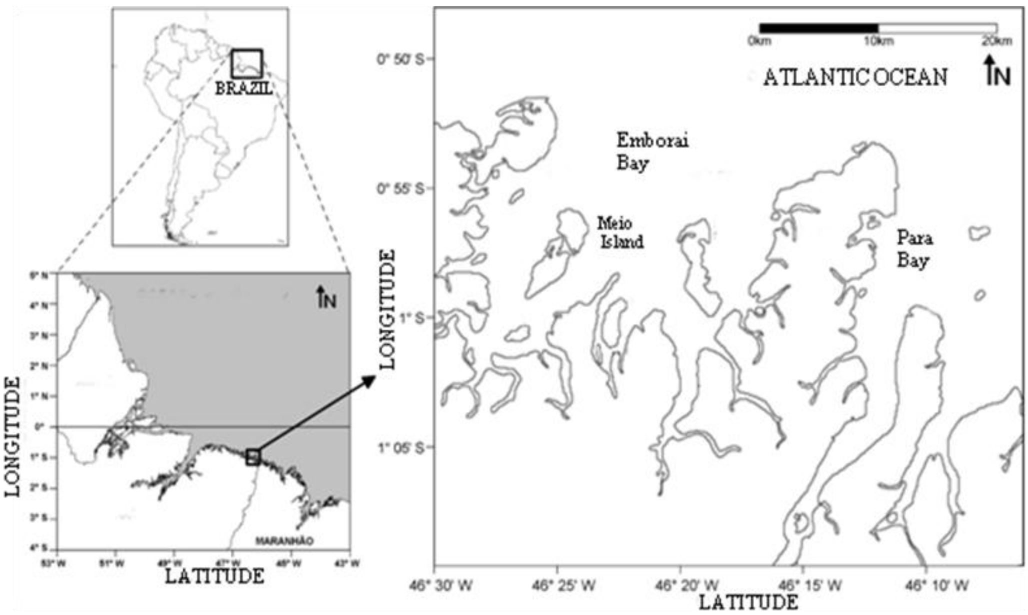

Fig. 2: The study area in Augusto Corrêa in the Brazilian state of Pará (modified from NOAA, National Oceanic Atmospheric Administration, 2010).

2.2. Environmental variables

During specimen collection, the salinity, pH, temperature (°C), and dissolved oxygen concentration (mg/L) of the water were measured *in situ* using a multi-parameter probe (Horiba U10). These measurements were taken during ebb spring tides. Rainfall data were obtained from the National Institute of Meteorology’s automatic meteorological station at Bragança (INMET, 2010).

2.3. Processing of the samples

Following collection, the oysters were packed in isothermal boxes for transportation to the laboratory, where they were opened for the removal of the gonads and processing for electronic and light microscopy.

2.4. Light microscopy

The gonad samples were fixed in Bouin's solution for 24 hours and then embedded in paraffin using standard histological techniques. Sections of 5 µm were obtained and stained with hematoxylin and eosin (HE) for analysis. Photomicrographs were taken with a Carl Zeiss optical microscope (Axiostar Plus 1169-151). Following this analysis, four gonadal stages were established, adapted from the classification of Rodríguez-Jaramillo et al. (2008).

2.5. Transmission electron microscopy (TEM)

Fragments of the gonads were fixed in Karnovsky's solution at pH 7.3 and 4°C for 24 hours and post-fixed in 1% osmium tetroxide. The samples were contrasted with uranyl acetate (1%), followed by dehydration in growing solutions of acetone and included in Epon. The ultra-thin cuts were contrasted with uranyl acetate and lead citrate and analyzed by transmission electron microscopy (LEO 906 E).

2.6. Statistical analysis

The physical and chemical variables were analyzed using ANOVA and the Kruskal-Wallis (K-W) nonparametric analysis of variance, depending on the normality of the data. A univariate analysis was then employed to test the possible relationship between environmental factors and the stages of gonadal development in male and female *C. gasar*. The SIMPER analysis was used to verify the contribution of each gonadal stage within each seasonal period. The Chi-square test with Yates' correction (Snedecor & Cochran, 1980) was used to determine the significance ($p < 0.05$) of deviations in the sex ratio. The analyses were run in the Statistica V 6.0 and Primer 6.0 programs.

3. Results

Monthly rainfall ranged from 0 to 360 mm, with significantly higher values being recorded in the rainy season (K-W, $p < 0.05$). Salinity varied from 19 to 39 and was significantly higher during the dry season (ANOVA, $p < 0.05$). Temperature, pH, and dissolved oxygen concentrations all fluctuated slightly during the course of the study period, but did not vary significantly between seasons. Water temperature ranged between 28.4 and 30.1°C, pH from 7.3 to 8.35, and dissolved oxygen concentrations from 3.62 to 5.95 mg/L (Fig. 3).

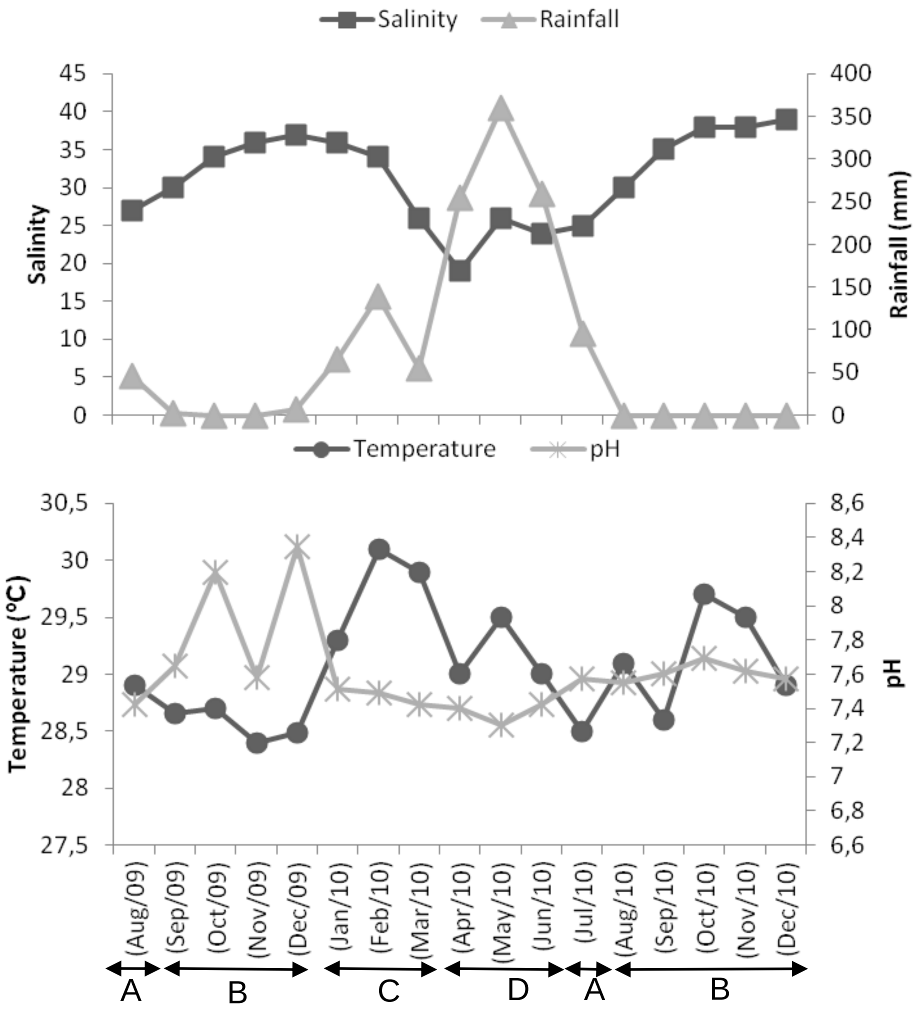

Fig. 3: Seasonal variation in (a) salinity and rainfall and (b) temperature (^{o}C) and pH in Augusto Corrêa PA during the study period. A – rainy-dry transition period; B – dry season; C - dry-rainy transition period and D - rainy season.

A total of 353 individuals were analyzed. No sexual dimorphism was observed macroscopically. Histological examination of the specimens identified 190 (55%) females, 161 (45%) males, and two (0.5%) hermaphrodites. While the sex ratio favored the females – 1.1:1 – the difference was not significant (Chi-square: $p > 0.05$).

The males and females were classified in four gonadal stages. Females: 1) Immature: follicles containing pre-vitellogenic oocytes (I) characterized by scarce cytoplasm and well-defined nucleus; some oocytes in vitellogenesis, identified as basophilic cells with prominent cytoplasm, and evident nucleoli and nuclei (Fig. 4A); 2) Maturation stage: increase in follicle diameter and three cell types: pre-vitellogenic oocytes (I), predominance of oocytes in vitellogenesis (II), and some vitellogenic oocytes, characterized by very evident acidophile cytoplasm and nucleus (III) (Fig. 4C); 3) Mature stage: ovarian follicles filled by vitellogenic or mature oocytes (IV). At this stage, oocytes in vitellogenesis were also found (II) (Fig. 4E); and 4) Spawned stage: follicles with highly visible lumen containing pre-vitellogenic oocytes (I) and oocytes in vitellogenesis (II) (Fig. 4G).

Males: 1) Immature stage: testicular parenchyma characterized by spermatic tubules irregular in form and size. Spermatogonia adjacent to the tubule wall (Fig. 4B); 2) Maturation stage: increase in the diameter of the tubule, which contains spermatogonia, spermatocites, spermatids, and spermatozoids (Fig. 4D); 3) Mature stage: spermatic tubules wider in diameter with a predominance of spermatozoids, but some spermatogonia still anchored to the tubule wall (Fig. 4F); and 4) Spermiated stage: characterized by a reduction of the tubule diameter and the presence of few spermatozoids in the lumen (Fig. 4H).

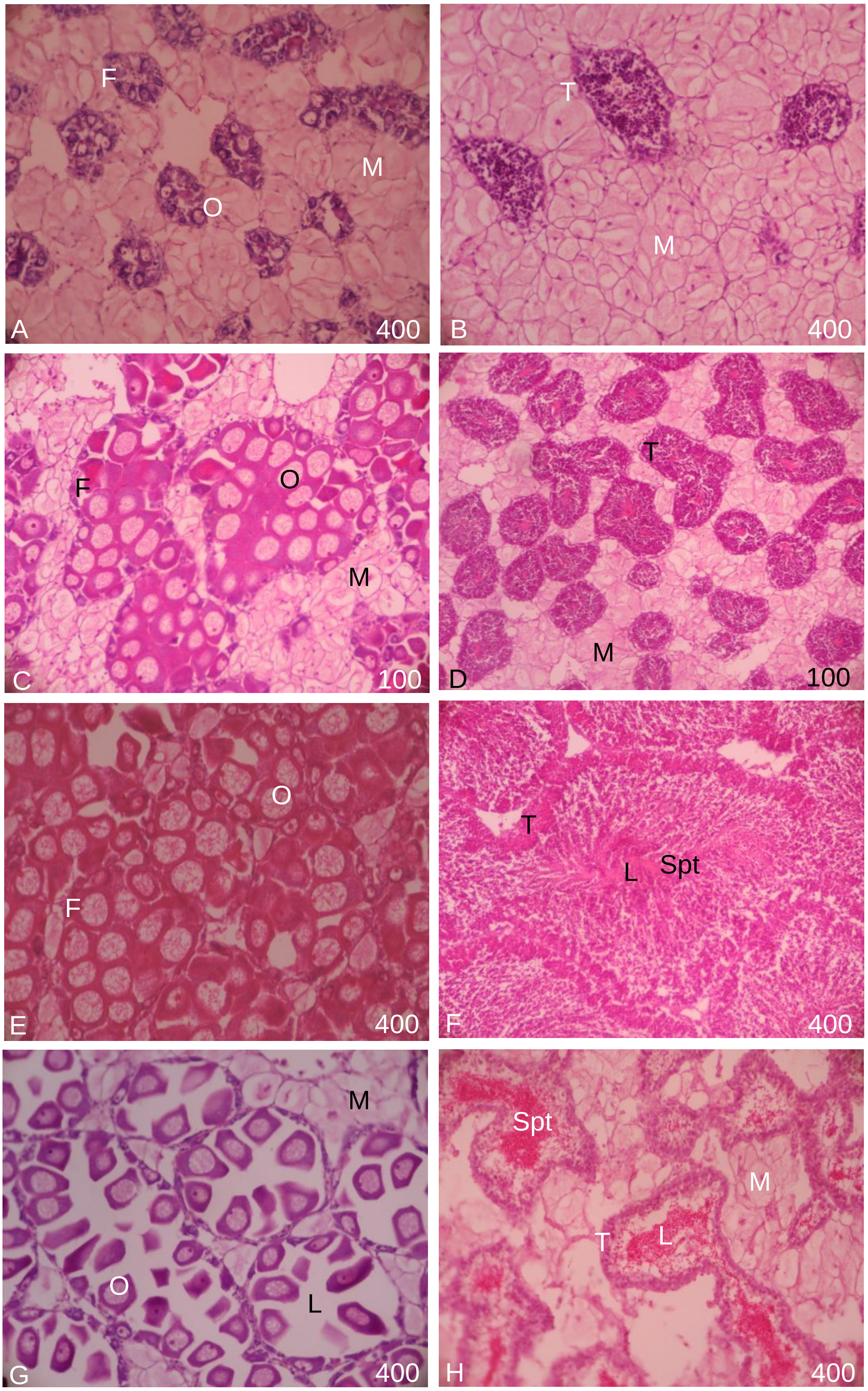

Fig. 4: Gonadal stages of female and male *C. gasar*: A – Immature female; B- Imature male; C – Maturing female; D – Maturing male; E – Mature female; F – Mature male; G – Spawned female; H – Spawned male. M – mantle; F – Follicles; T – spermatic tubules; L – lumen; O – oocytes; Spt – sperm.

Two different hermaphrodite forms were observed: 1) spermatic tubules with free mature oocytes in the lumen and spermatogonia fixed to the wall of the tubule; 2) ovarian follicles with spermatozoa in the lumen and oocytes in vitellogenesis fixed to the wall of the follicle. However, only two hermaphrodites were observed (0.5%), and their presence in the sample did not affect the sex ratio or classification of the gonadal stages (Fig. 5).

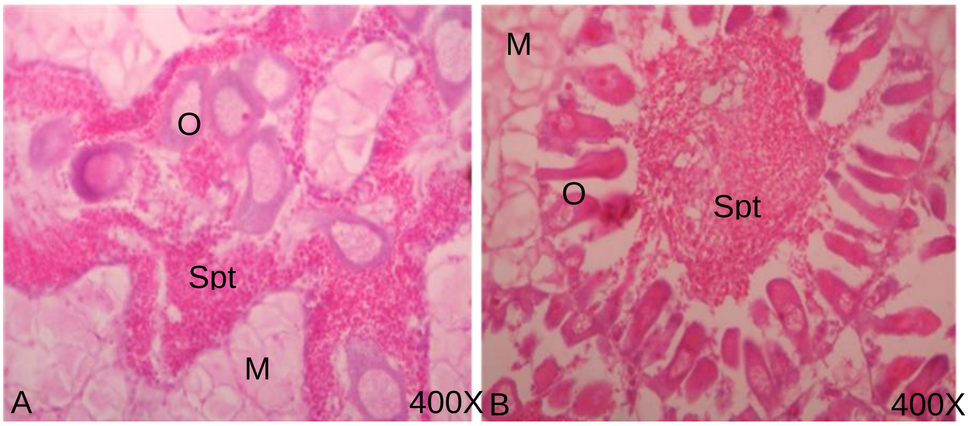

Fig. 5: Hermaphroditic gonad of *C. gasar*. A – Spermatic tubules containing mature oocytes (O) and B – ovarian follicles with spermatozoids in the lumen and oocytes in vitellogenesis.

Immature females were present only in the rainy-dry transition, dry season and dry-rainy transition (January-March, 2010), whereas all other phases were observed throughout the study period. Maturing females were present throughout the study period, but were also more frequent during the dry season. Mature females were more common during the rainy season and the rainy-dry transition (August, 2009, and July, 2010), while the highest frequency of the spawning phase was recorded in the dry season (Fig. 6 and 7). The frequency of immature males was similar to that of immature females, although they were relatively more abundant during the dry season. Maturing males were observed throughout the study period except for the rainy-dry transition. The highest frequencies of mature males were recorded in the rainy season and rainy-dry transition. By contrast, spermiated males were relatively more common in the dry season and dry-rainy transition of 2009 (Fig. 8 and 9).

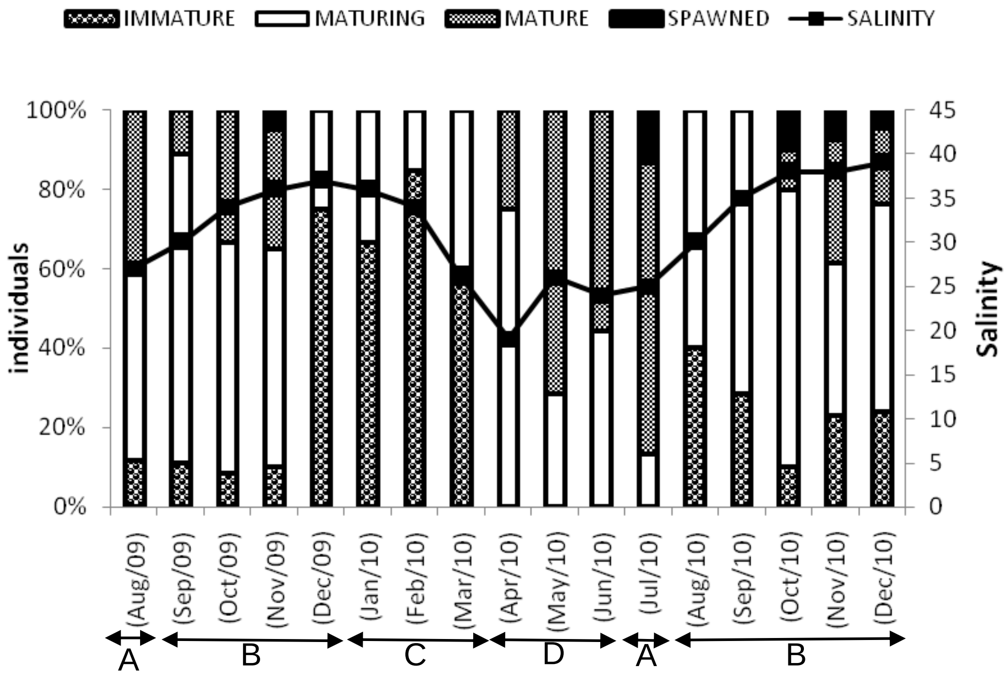

Fig. 6: Seasonal variation in salinity during the 2009 and 2010 reproductive cycles of *C. gasar* femalesA – dry-rainy transition period; B – dry season; C - rainy-dry transition period and D - rainy season.

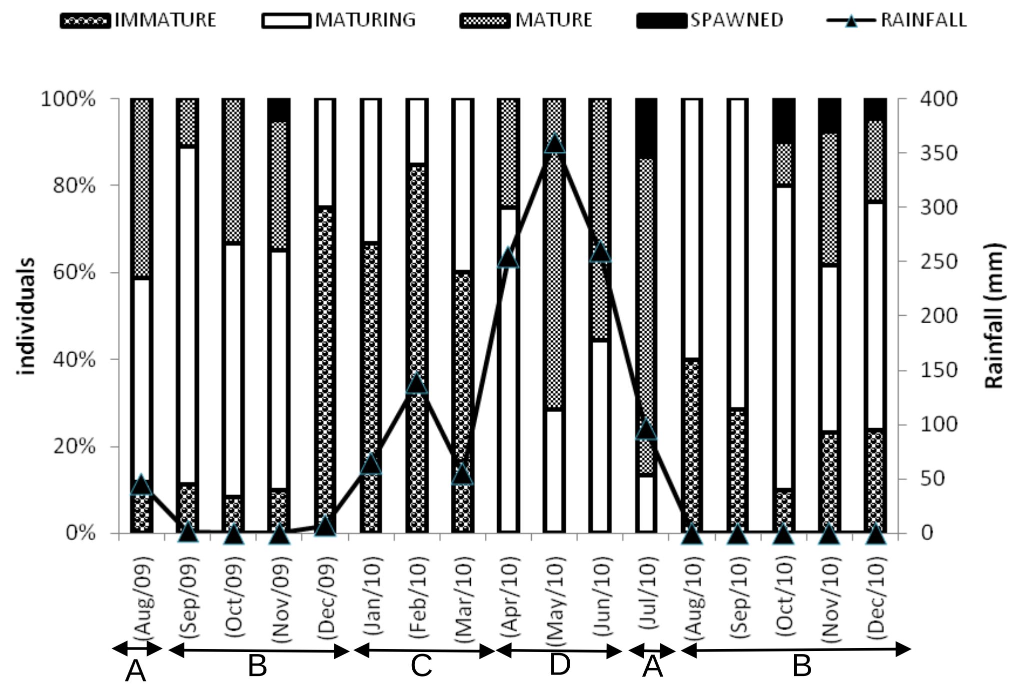

Fig. 7: Seasonal variation in rainfall during the 2009 and 2010 reproductive cycles of *C. gasar* females. A – dry-rainy transition period; B – dry season; C – rainy-dry transition period and D - rainy season.

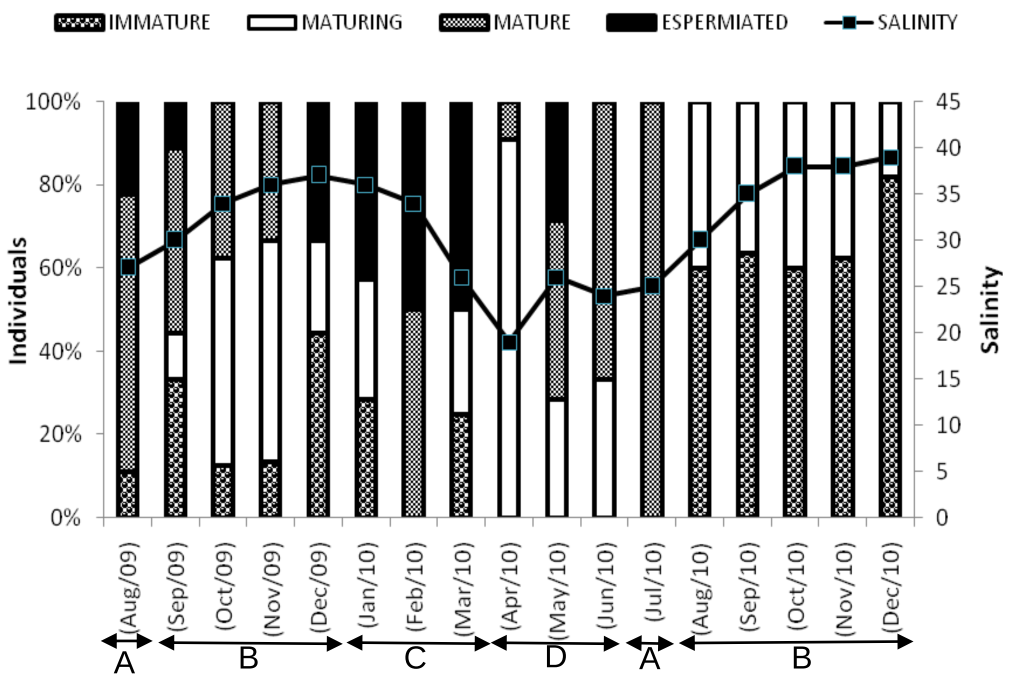

Fig. 8: Seasonal variation in salinity during the 2009 and 2010 reproductive cycles of *C. gasar* males. A – dry-rainy transition period; B – dry season; C – rainy-dry transition period and D - rainy season.

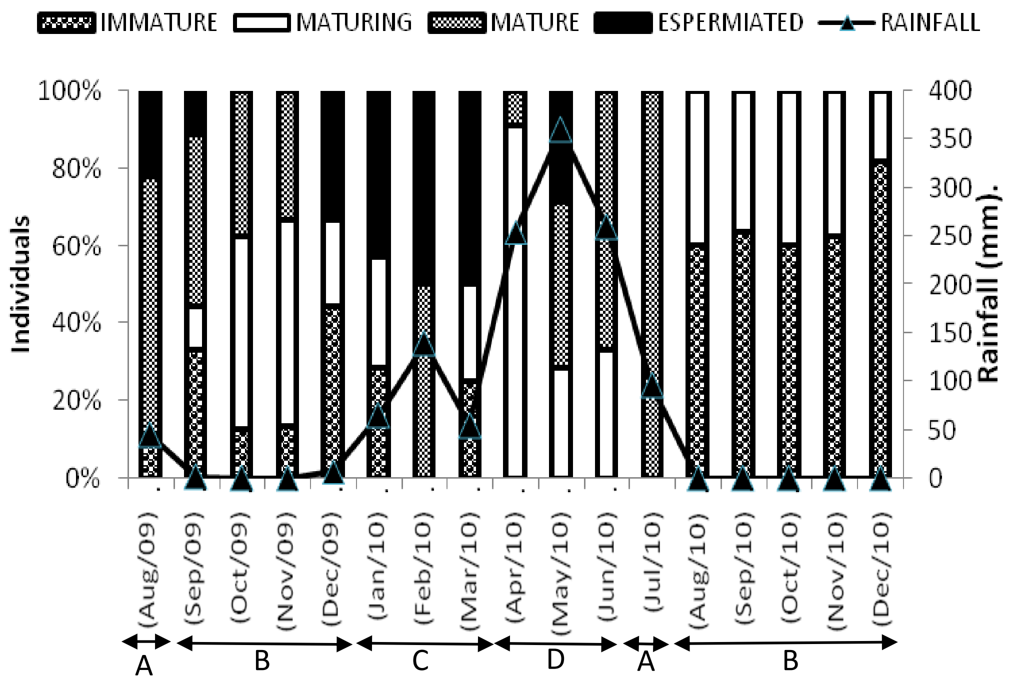

Fig. 9. Seasonal variation in rainfall during the 2009 and 2010 reproductive cycles of *C. gasar* males. A – dry-rainy transition period; B – dry season; C – rainy-dry transition period and D - rainy season.

As noted above, only salinity varied significantly during the study period, presenting an inverse relationship with rainfall. During periods of low rainfall and high salinity, all stages of the gametogenic cycle were observed in both male and female specimens, except mature stage. During periods of reduced salinity (high rainfall), however, the proportion of mature specimens of both sexes increased. While that of immature and spermiated/spawned individuals decreased.

The proportion of mature specimens peaked in the rainy-dry transition, with males reaching 100% of the sample and females, 73.37% (Table 1). In this case, the difference between males and females was significant.

Table 1: Relative frequency of gametogenic stages in male and female *C. gasar* during the different seasonal periods.

Sex	Stage	Contribution (%) during the season:			
		Rainy-Dry	Dry	Dry-Rainy	Rainy
Female	Immature		22.68	71.76	-
	Maturing	26.63	65.47	28.24	49.53
	Mature	73.37	10.39	-	50.47
	Spawned	-	-	-	-
Male	Immature	-	52.86	14.07	-
	Maturing	-	47.14	14.07	60.35
	Mature	100.00	-	-	39.65
	Spermiated	-	-	71.85	-

4. Discussion

The histological analysis of the *C. gasar* specimens collected in the present study revealed a predominance of females in the population, although there was no significant deviation in the sex ratio. A similar situation was recorded by Lenz and Boehs (2011) in a Brazilian population of *C. rhizophorea*. Le Dantec (1968) has suggested that the proportion of females tends to increase in areas that suffer oceanic influences, which results in increased salinity. A similar scenario was observed in the present study of *C. gasar*, even though the study area was an estuary, in which salinity levels fluctuate considerably through evaporation, precipitation, and the inflow of fluvial discharge.

Hermaphroditism – regarded as a transitional stage between the male and female condition (Baghurst & Mitchell, 2002; Lango-Reynoso et al., 2006) – was observed only rarely in the present study, and was characterized by the presence of spermatozoa in the female gonads, and oocytes in the male gonads, confirming the transition from male to female or female to male, respectively.

In general, gametogenesis in *C. gasar* follows the typical pattern of bivalve mollusks, with the exception of the undifferentiated stage reported in *C. corteziensis* (Rodrigues-Jaramillo et al., 2008), *C. gigas* (Enríque-Díaz et al., 2009), and *Ostrea edullis* (Cano et al., 1997). In *C. gasar*, it was necessary to employ TEM in order to characterize the gametic cells, and thus permit the identification of ultra-structural differences between males and females.

The gonadal development of *C. gasar* was classified in four stages (immature, maturing, mature, and spawned/spent). There is some divergence in the number of gonadal stages identified by other authors – in *C. gigas*, for example, and Lango-Reynoso et al. (2000) and Chávez-Villalba et al. (2007) describe four stages in females (initial gametogenesis, gonad growth, mature, and degeneration), but only three in the males (initial gametogenesis, gonad growth and mature), although these authors described the same histological characteristics of gonadal development as those observed in the present study.

The increase in the frequency of mature oysters during the rainy and rainy-dry transition (low salinity) may be related to the higher concentrations of organic matter found in the water during this period (Dittmar and Lara, 2001; Dittmar et al., 2001). While this parameter was not quantified in the present study, a number of studies of the Amazon coast have shown phytoplankton biomass increases during the rainy season. The data suggest that rainfall influences the composition, density, biomass, and diversity of phytoplankton (Costa et al., 2011). Given the observed pattern, we suggest that organic matter in suspension is the primary source of food for *C. gasar* and that these nutrients are necessary for gonadal maturation, given that the production of gametes requires a considerable input of energy (Galtsoff, 1964; Andrews, 1979; Lunetta and Grotta, 1982).

The present study found no evidence of fluctuations in water temperature that may have affected reproductive patterns in *C. gasar*. This contrasts with findings from temperate regions, where well-defined seasonal variation in temperature appears to affect reproductive patterns in *C. gigas* (Massapina et al., 1999; Fabioux et al., 2005; Chávez-Villalba et al., 2007; Enríque-Díaz et al., 2009), *C. virginica* (Lango-Reynoso et al., 2000), *C. corteziensis* (Mazón-Suástegui et al., 2002; Rodríguez-Jaramillo et al., 2008), and *P. carnea* (Narváez et al., 2000). By contrast, the results of the present study indicated that increased salinity during the dry season was a factor in the release of *C. gasar* gametes. This conclusion is reinforced by the relative frequency of immature and spawned/spermiated specimens in the dry season and dry-rainy transition period. However, Lenz and Boehs (2011) observed spawned and spermiated individuals throughout the year in a Brazilian population of *Crassostrea rhizophorae*.

The results of the present study also indicate a degree of synchronization between males and females in all stages of gonadal maturation. Massapina et al. (1999)

recorded a similar pattern in *C. gigas*, which appears to favor the probability of the reproduction process in these oysters.

The reproductive cycle of *C. gasar* is regulated by the typical tropical seasonality of the Amazon region, in which the marked increase in precipitation during the rainy season provokes a decrease in salinity levels in estuarine regions, such as that of the study area. The results of the present study indicate that the dry season or dry-rainy transition months is the optimum period for the collection of embryos or larvae for cultivation, given the higher frequency of spawned/spermiated individuals found during these months.

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