Factors associated with the prevalence of *Perkinsus marinus* in *Crassostrea virginica* from the southern Gulf of Mexico

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ABSTRACT: The protozoan *Perkinsus marinus* is considered the most important pathogen of the eastern oyster *Crassostrea virginica*, causing high mortality in natural and farmed oysters on the Atlantic coast of the US. In Mexico, no serious *P. marinus* epizootic has been reported. This study describes the current state of *P. marinus* prevalence in Terminos Lagoon (Mexico) associated with environmental factors including salinity, temperature, ammonium, nitrate, nitrite, silica, and phosphorus. In addition, the association of physiological (hemocyte density, protein concentration) and immunological (lysozyme, agglutination) parameters with the infection were studied. The prevalence was significantly different among seasons with mean values of 70, 23, and 7 % in the dry (February to May), rainy (June to September) and north-wind (October to January) seasons, respectively. Only light infection intensity (Mackin scale value <1) was observed. Prevalence of *P. marinus* was associated with seasonal salinity, phosphorus, and silica variations. Comparisons of oyster health demonstrates that the rainy and north-wind seasons are stressful periods. Redundancy analysis showed that only 34 % of the variation in seasonal *P. marinus* prevalence was explained by protein concentration (21 %), lysozyme (12 %), and agglutination (1 %). Overall, the data suggest that freshwater input associated with high nutrient concentrations during the rainy and north-wind seasons has a strong negative effect on *P. marinus* prevalence and also influences the oysters' physiology. It is probable that this seasonal stress was responsible for the absence of an epizootic event in Terminos Lagoon.

KEY WORDS: *Perkinsus marinus* · *Crassostrea virginica* · Environmental control · Salinity · Silica · Protein

INTRODUCTION

The eastern oyster *Crassostrea virginica* (Gmelin, 1791) is a major fishery species off the Mexican coast on the Gulf of Mexico. The exploitation of natural beds constitutes 90 % of national oyster production, mainly in the states of Veracruz and Tabasco (45.69 t yr^{-1}), followed by Tamaulipas and Campeche (2.4 t yr^{-1}; Amador del Angel et al. 2003).

Terminos Lagoon, the largest coastal lagoon in Mexico, supports commercial fisheries that are the main source of income to fishermen, as well as oil extraction on the adjacent continental shelf and agricultural activities in the watershed (Yáñez-Arancibia & Day 1988). In this region, the natural beds of *Crassostrea virginica* are situated on 260.4 ha and represent the oyster population of the extreme south of the Gulf of Mexico (Cabrera et al. 1999). Since 1969, oyster production has been declining (Amador del Angel et al. 2003) probably due to overfishing (MacKenzie & Wakida-Kusunoki 1997), pollution (Gold-Bouchot et al. 1995), and variations in seasonal salinity (Cabrera et al. 1999).

The protozoan *Perkinsus marinus* has been extensively studied in the US due to its association with

severe mortalities of commercial cultures of *Crassostrea virginica* (Burreson & Ragone Calvo 1996). In tropical areas, the behavior of *P. marinus* has not been widely documented. In Mexico, the first report of *P. marinus* was by Burreson et al. (1994) in Tabasco after a mortality event suspected to have been caused by pollutants from an oil refinery close to Carmen-Pajonal-Machona and Mecoacan Lagoons. These authors demonstrated the existence of *P. marinus* in the 3 lagoons with 90 to 100% prevalence at high salinity sites (30 psu) and 50% at low salinity sites (15 psu). Aguirre-Macedo et al. (2000) reported 50% prevalence in 8 coastal lagoons from Veracruz, Tabasco, and Campeche except in Terminos Lagoon, with 80% prevalence in the dry season. A common characteristic of *P. marinus* in these Mexican lagoons was the high to moderate prevalence of light infection intensity (Aguirre-Macedo et al. 2000) with the exception of one location in Mecoacan Lagoon where severe infection was registered (Burreson et al. 1994).

A key characteristic of the *Perkinsus marinus* epizootiology is the influence of environmental conditions on disease progression. Water temperature and salinity are the main environmental factors affecting the transmission and pathogenicity of the parasite in temperate climates (Ragone Calvo et al. 2000, Ragone Calvo & Burreson 2003, Audemard et al. 2006). It has been noted in Chesapeake Bay (USA) that high temperature and salinity are associated with parasite proliferation and disease outbreaks (Burreson & Ragone Calvo 1996). However, under cool water temperatures (10 to 15°C) and/or low salinity (less than 9 psu), the disease prevalence and the infection intensity remain low (Chu & La Peyre 1993). Environmental factors are also considered stressors because they influence the physiological condition of both the host and the parasite (Lenihan et al. 1999). The relative importance of these environmental conditions on *P. marinus* and its host's response is not well known in tropical areas of the Gulf of Mexico.

The main objective of the present study was to investigate the behavior of *Perkinsus marinus* on the tropical coast of the Gulf of Mexico with particular emphasis on (1) estimating the prevalence and intensity of infection of *P. marinus* in Terminos Lagoon during 3 seasons, (2) identifying the possible influence of the environmental factors on the prevalence of *P. marinus*, and (3) studying the oyster's physiological and immunological parameters associated with *P. marinus* infection in this tropical area.

MATERIALS AND METHODS

Study area. Terminos Lagoon is located in Campeche, 18° 20′ to 19° 00′ N and 91° 10′ to 92° 00′ W (Fig. 1). It is approximately 70 km long and 30 km wide with a mean depth of 3.5 m and a surface area of 170 000 ha. The lagoon is connected to the sea by 2 inlets: Carmen Inlet on the western side (4 km long) and Puerto Real Inlet on the eastern side (3.3 km). The climate in this tropical region includes 3 distinct seasons: the north-wind season from October to January, the dry season from February to May, and the rainy season from June to September (Yáñez-Arancibia & Day 1988). The temperature shows low seasonal variations (27 to 33°C); however, the salinity varies between seasons due to river runoff. Three high-flow rivers (Candelaria, Chumpan, and Palizada) provide most of the freshwater input to the lagoon (378 m^3 s^{-1}) with weak circulation (Yáñez-Arancibia et al. 1983). Half of the water volume is renewed every 9 d, mostly as a result of tidal exchange. The tide is mainly diurnal, with a mean range of 0.3 m (David & Kjerfve 1998). Oysters are subtidal to about 1 m depth in the rainy season and 0.3 to 0.5 m in the dry season. The western side of the Lagoon is biologically more productive and accommodates most oyster beds (MacKenzie & Wakida-Kusunoki 1997, Bach et al. 2005). The spawning activity is not strictly associated with different seasons. There are 2 annual spawning periods: March to May (dry season) and September to November (rainy and north-wind seasons), both in the salinity range of 12 to 25 psu and 28 to 30°C (Rogers & García-Cubas 1981, Martínez et al. 1995).

Sample collection and processing. Oyster samples were hand-collected from 7 sites in the Terminos
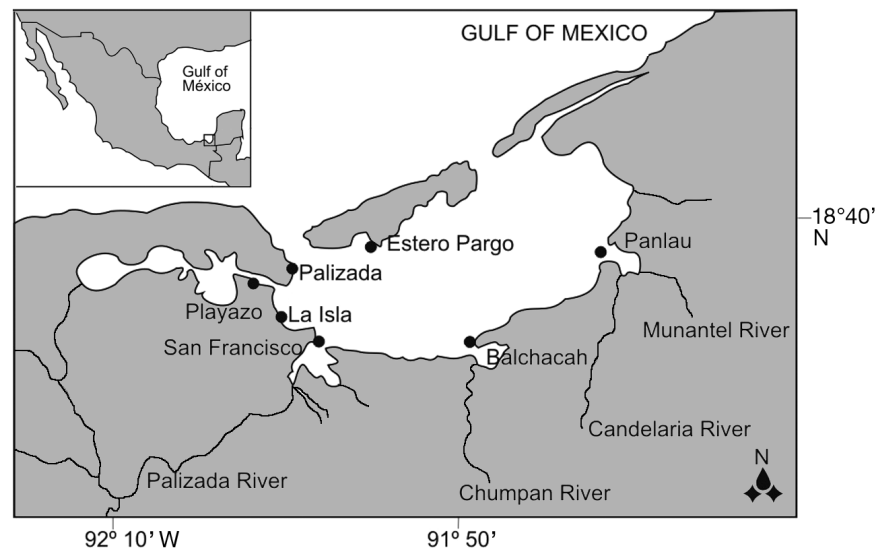

Fig. 1. Terminos Lagoon, Gulf of Mexico, showing the 7 sampling sites

Lagoon (Fig. 1) in the 'dry' (5 April 2005), 'rainy' (15 September 2005), and 'north-wind' (8 January 2006) seasons. These sampling months were selected because they reflect the environmental changes that occur at the end of each seasonal period. At each site, 45 oysters and 3 water samples were collected. In addition, temperature (°C) and salinity (psu) were taken *in situ* using a mercury thermometer and refractometer, respectively. Water samples were collected using Van Dorn bottles. The samples were stored at 10°C and transported to the Primary Production Laboratory of CINVESTAV. The oysters were placed into coolers containing cold ice packs and transported to the Aquatic Pathology Laboratory at CINVESTAV. In the laboratory, oysters were placed in tanks at similar salinity to their collection site.

Additional oyster samples (length = 6.35 ± 0.30 cm) were randomly taken from 25 points in a 5 m^2 area from each site until a tank (ca. 70 l) was full. Next, 100 oysters were classified as alive, empty shells (dead oysters with no soft tissue remaining, but with valves still attached at the hinge and little or no fouling), and gapers (dead oysters with valves attached containing soft tissue) to estimate *in situ* inter-seasonal mortality. Percentage mortality was calculated as [dead oysters (gapers + empty shells)/total oysters] × 100.

Oyster size and condition index (CI). Measurements of oysters were determined as height (tip of the bill to the hinge, along a line touching the ventral edge of the adductor muscle scar), length (measured on a line perpendicular to the height line through the anterior edge of the adductor muscle scar), and width (measured as the greatest distance between the 2 shells along a line perpendicular to both height and length; Galtsoff 1964). Following hemolymph withdrawal (see 'Hemolymph processing and hemocyte density'), oysters were shucked, and soft tissue was weighted to obtain the total wet weight. The CI was estimated using the formula proposed by Choi et al. (2002): CI = [wet tissue weight/(height × width × length)] × 100.

Prevalence and weighted prevalence of *Perkinsus marinus*. Infection intensity of *P. marinus* was assessed using the culture method of Ray (1966). Briefly, a small section of mantle, gill, and rectum (0.30 g) was removed from each oyster, placed in thioglycollate medium (FTM), and incubated at 24°C in the dark for 5 d. After staining with 0.5% Lugol's iodine, the tissue was examined under the light microscope at 400× magnification for the presence of *P. marinus* hypnospores. Infection intensity was ranked on a 0 to 5 point scale from uninfected to heavily infected for calculation of weighted prevalence (Mackin 1962).

Another sample of tissue (0.30 g) containing mantle, gill, and rectum was preserved in 95% ethanol and used to determine the prevalence by simple polymerase chain reaction (PCR) assay. Extraction of DNA was carried out using the phenol:chloroform method (Sambrook et al. 1989). The tissue samples were lysed in the presence of 10% sodium dodecyl sulfate (SDS) and 30% Proteinase K. The DNA was concentrated with 7.5 M ammonium acetate. PCR primers (5'-CACTTGTATTGTGA ACCC-3', 300F and 5'-TTGGTGACATCTCCAAATG AC-3', 300R) that amplify a 307 bp target region within the NTS domain between the 5S and small subunit rDNa (SSU rRNA) genes from *Perkinsus marinus* were used for the detection of the parasite (Marsh et al. 1995). All samples were subjected to identical reaction conditions in a Perkin Elmer 2400 thermal cycler. *Taq* DNA polymerase (1.5 U) was used in a 25 µl reaction with the manufacturer's reaction buffer. In addition, each assay contained 1.5 mM MgCl$_2$, 200 µM of each dNTP, 1 µM of each primer, and 1 µl (1 µg) of template DNA. The amplification profile described by Robledo et al. (1999) was used instead of the conditions described by Marsh et al. (1995), because the gradient of temperature significantly reduces the *P. marinus* amplification time. The temperature profile was 35 cycles at 91°C for 1 min, 58°C for 1 min (plus 1 s per cycle), and 72°C for 1 min (plus 2 s per cycle). The presence of the *P. marinus*-specific NTS target sequence was confirmed by sequencing the 307 bp PCR product, showing 99% similarity with the S78416 *P. marinus* sequence (Marsh et al. 1995). Since Aguirre-Macedo et al. (2000) had reported light infection in Terminos Lagoon and Robledo et al. (1998) described the sensibility difference in light infection between PCR and FTM, we analyzed the 945 samples by both techniques (PCR, FTM). The results obtained from PCR were used to estimate prevalence because they showed high sensibility (Table 1). The Ray/Mackin assay was used to estimate weighted prevalence because of its simplicity and accuracy at the population level (Bushek et al. 1994). Although FTM is a semi-quantitative method, it had been mainly used in fieldwork because of the large number of samples needed to estimate intensity and the use of the conventional Mackin's scale to compare results (Karolus et al. 2000, Carnegie & Burreson 2005).

The remaining tissues (digestive gland, mantle, intestine, labial palps) were also fixed in Davidson's fixative for histological examination.

Hemolymph processing and hemocyte density. The oysters were scrubbed clean of fouling organisms, and a grinder was used to notch the shells at the posterior edge adjacent to the adductor muscle. Hemolymph was withdrawn from the adductor muscle with a 3 ml syringe and a 22 gauge needle. To estimate the total hemocyte count, 10 µl of hemolymph were fixed in an equal volume of Baker's formol, containing 2% sodium chloride. Total cell counts were performed in duplicate using a Neubauer chamber.

Table 1. *Perkinsus marinus* infecting *Crassostrea virginica*. Prevalence and weighted prevalence of *P. marinus*, determined by thioglycollate medium (FTM), in oysters from Terminos Lagoon, Mexico

Sampling site	Location	Dry season (n = 45)			Rainy season (n = 45)			North-wind season (n = 45)		
		Percent infected		Weighted prevalence	Percent infected		Weighted prevalence	Percent infected		Weighted prevalence
		PCR	FTM		PCR	FTM		PCR	FTM	
Estero Pargo	18° 40.04′ N, 91° 44.32′ W	60	47	0.47	7	7	0.07	4	2	0.02
Palizada	18° 30.17′ N, 91° 51.35′ W	69	60	0.60	18	13	0.13	4	2	0.02
La Isla	18° 30.47′ N, 91° 47.60′ W	73	69	0.69	22	11	0.11	4	0	0.00
Balchacah	18° 25.55′ N, 91° 31.21′ W	96	89	0.89	24	18	0.18	0	0	0.00
San Francisco	18° 29.34′ N, 91° 46.27′ W	51	29	0.29	40	33	0.33	7	0	0.00
Playaso	18° 34.04′ N, 91° 53.15′ W	87	71	0.71	33	22	0.22	16	9	0.09
Panlau	18° 37.30′ N, 91° 17.22′ W	53	47	0.47	13	7	0.09	13	0	0.00

Protein concentration was determined by the method of Lowry (1951) with the sample diluted 10-fold in deionized water. Hemolymph (0.25 to 0.5 ml) from each individual oyster was placed in a plastic microfuge tube and centrifuged at 2900 × *g* for 5 min. The serum was held at 4°C for less than 1 wk before being tested for protein, lysozyme, and agglutinin content. Protein concentration of each sample was calculated from a standard curve generated from dilutions of bovine serum albumin (0 to 10 mg ml^{-1}).

Agglutination by hemolymph serum was tested using 0.9% NaCl-washed sheep erythrocytes (sheep red blood cells, SRBC). Hemolymph serum samples were diluted 1:1 in Alsever's solution. Serial 2-fold dilutions of 50 µl serum in 96-well microtiter plates were assayed with 50 µl of SRBC (7 × 10^6 cell ml^{-1}) suspension in 8.5 psu artificial seawater (Fisher & Di-Nuzzo 1991). Microtiter plates were recorded as the reciprocal of the highest dilution showing positive agglutination and expressed as log$_2$ values to reflect the 2-fold serial dilutions.

Lysozyme was quantified by measuring the ability of oyster serum to degrade a suspension of the bacterium *Micrococcus lysodeikticus* (Sigma). The bacteria were re-suspended in potassium phosphate buffer (0.1 M, 0.09% NaCl), and the optical density was adjusted to 0.7 absorbance units at 560 nm with a spectrophotometer. Serum (25 µl well^{-1}) and 175 µl of *M. lysodeikticus* suspension were placed in a 96-well plate. The decrease in turbidity was traced for 4 min at 560 nm. The rates of degradation were compared to a curve of standard solutions prepared from hen egg-white lysozyme (0 to 30 µg ml^{-1}; Sigma). Lysozyme activity was expressed in µg ml^{-1} of hen egg-white lysozyme equivalent and adjusted to protein concentration.

Water quality. For analysis of dissolved inorganic nutrients, the water samples were filtered through a 0.45 µm membrane filter. Ammonium (NH$_4^+$) was determined by the phenol hypochlorite method (Solorzano & Sharp 1980). Soluble reactive silica (SRSi) was determined by the molybdenum blue method, with ascorbic acid as a reductant. Nitrogen in the form of nitrite (NO$_2^-$) was determined by sulfanilamide in an acid solution method, and nitrate (NO$_3^-$) was measured as NO$_2^-$ after its reduction in a Cd-Cu column. Soluble reactive phosphorus (SRP) was determined using the molybdenum blue method (Strickland & Parsons 1972).

Statistical methods. Two-way analysis of variance (ANOVA) was conducted to relate the response variable (prevalence) to the main effects of the season and sampling sites and to test for possible interactions. Bonferroni/Dunn post hoc tests were used to differentiate between overall season and site means. Physiological data were processed using a nonparametric Kruskal-Wallis test. The significance between PCR and FTM methods was assessed by a chi-squared test. Multivariate statistical methods were used to determine the variability of environmental and physiological parameters. Canonical variant analysis (CVA) was used to assess whether the environmental parameters were sufficiently different to discriminate the 3 seasons (Ter Braak & Smilauer 2002). The same analysis was used to classify the oysters from the different sites into groups based on their physiological characteristics. The forward selection procedure was performed to select the predictors included in the CVA plot. The individual predictors were accepted with a type I error probability estimated less than α/Nc (Bonferroni correction), where Nc is the number of independent variables used in the analysis (Legendre & Legendre 1998). Redundancy analysis (RDA; Leps & Smilauer 2003) was used to determine the amount of variability in the *Perkinsus marinus* infection data explained by the physiological condition of the oysters. A Monte Carlo test with 5000 permutations was used to select the variables to include in the RDA. The condition index was used as a co-variable to control oyster size over the physiological variables. To describe the dependence of *P. marinus* prevalence (response variable) on the environmental parameters, we used a

generalized linear model (GLM) that made less specific assumptions about the variance compared to the general linear regression method (McCullagh & Nelder 1989). Analyses were performed using CANOCO 4.5 software, and plots were made with the Canodraw 4.0 graphics software (Ter Braak & Smilauer 2002).

RESULTS

Prevalence and weighted prevalence

Prevalence (percent infected) determined by PCR and intensity (weighted prevalence) determined by FTM is shown in Table 1. There were differences in the number of oysters infected in the 3 seasons (2-way ANOVA; $F_{(6,2)} = 109$, $p < 0.0001$) with 70, 23, and 7% prevalence in the dry, rainy, and north-wind seasons respectively. No significant differences were registered in the interaction of site and season ($F_{(6,2)} = 1.87$, $p = 0.066$) indicating that independent of the site, prevalence increased in the dry season, declined in the rainy season, and became lower in the north-wind season. Spherical black cells from 10 to 200 µm diameter typical *of Perkinsus marinus* were observed as a result of the FTM assay. PCR and FTM differed significantly by 104 samples ($\chi^2_{(0.95, 1)} = 49.9$, $R^2 = 0.86$) in prevalence

estimation. Only light infection intensity was registered (Mackin scale value <1) with no significant difference (ANOVA; $F_{(6,2)} = 1.88$, $p = 0.100$) among sites (Table 1). No severe histological changes were found in the oysters with the exception of *Nematopsis* spp. in 8% of samples.

Environmental data and mortality

The seasonal variation of environmental parameters and oyster mortality are shown in Tables 2 & 3, respectively. The environmental conditions were sig-

Table 3. *Crassostrea virginica.* Percentage mortality in Terminos Lagoon, Mexico

Site	Dry (n = 100)	Rainy (n = 100)	North-wind (n = 100)
		Season	
Estero Pargo	0	5	9
Palizada	13	40	30
La Isla	21	43	45
Balchacah	12	61	52
San Francisco	22	30	36
Playaso	10	29	39
Panlau	18	40	48

Table 2. Environmental parameters (mean ± SD) measured at 7 sites in Terminos Lagoon, Mexico. SRP: soluble reactive phosphorus; SRSi: soluble reactive silica

| Site	Temperature (°C)	Salinity (psu)	NO_3^- (mM)	NO_2^- (mM)	NH_4^+ (mM)	SRP (mM)	SRSi (mM)
Dry season							
Estero Pargo	32 ± 0.0	45 ± 0.0	1.14 ± 0.08	0.18 ± 0.16	10.0 ± 1.41	0.12 ± 0.09	50.09 ± 0.72
Palizada	30 ± 0.0	25 ± 0.5	2.07 ± 0.48	0.30 ± 0.16	14.19 ± 1.08	0.36 ± 0.14	70.86 ± 0.45
La Isla	28 ± 0.5	28 ± 0.0	6.14 ± 0.22	0.07 ± 0.10	13.92 ± 0.27	0.06 ± 0.04	68.47 ± 0.16
Balchacah	28 ± 0.0	30 ± 0.6	1.48 ± 0.18	0.3 ± 0.03	42.30 ± 6.29	0.26 ± 0.26	85.31 ± 0.60
San Francisco	28 ± 0.0	1 ± 0.6	3.32 ± 1.30	0.16 ± 0.15	20.85 ± 2.49	0.41 ± 0.11	112.04 ± 3.17
Playaso	28 ± 0.0	34 ± 0.8	3.57 ± 2.38	0.71 ± 1.22	10.53 ± 1.08	0.05 ± 0.01	53.96 ± 0.45
Panlau	28 ± 0.5	30 ± 0.0	1.95 ± 0.58	0.01 ± 0.00	9.82 ± 0.67	0.06 ± 0.05	101.20 ± 13.61
Rainy season							
Estero Pargo	32 ± 0.0	23 ± 2.3	2.60 ± 0.40	1.13 ± 0.04	20.33 ± 11.42	1.47 ± 0.40	107.37 ± 2.10
Palizada	30 ± 0.0	5 ± 0.0	2.69 ± 0.67	1.14 ± 0.08	63.35 ± 59.31	1.48 ± 0.03	223.07 ± 1.45
La Isla	30 ± 0.0	3 ± 2.9	2.46 ± 0.35	1.14 ± 0.15	64.82 ± 72.23	1.52 ± 0.08	152.80 ± 15.65
Balchacah	28 ± 0.6	2 ± 2.5	7.14 ± 0.27	1.42 ± 0.05	75.87 ± 50.39	1.90 ± 0.03	140.60 ± 1.55
San Francisco	28 ± 0.0	1 ± 1.5	4.21 ± 0.03	1.29 ± 0.01	28.50 ± 19.06	1.46 ± 0.05	157.33 ± 13.99
Playaso	31 ± 0.0	12 ± 1.7	1.94 ± 0.06	1.01 ± 0.00	22.76 ± 9.44	1.41 ± 0.05	187.23 ± 4.58
Panlau	28 ± 0.0	7 ± 5.8	2.92 ± 0.32	1.21 ± 0.09	18.34 ± 13.42	1.84 ± 0.08	208.13 ± 4.23
North-wind season							
Estero Pargo	26 ± 0.0	26 ± 0.0	2.25 ± 0.67	0.14 ± 0.04	9.76 ± 4.24	0.21 ± 0.16	74.63 ± 5.23
Palizada	27 ± 0.0	7 ± 1.2	2.00 ± 0.16	0.05 ± 0.04	0.25 ± 0.13	0.05 ± 0.02	127.07 ± 5.51
La Isla	26 ± 0.0	3 ± 0.0	5.60 ± 0.69	0.14 ± 0.02	0.39 ± 0.25	0.44 ± 0.13	112.27 ± 4.98
Balchacah	24 ± 0.0	15 ± 0.0	2.22 ± 0.66	0.26 ± 0.09	2.61 ± 1.23	0.10 ± 0.07	125.45 ± 1.73
San Francisco	23 ± 0.5	3 ± 1.0	6.82 ± 0.25	0.29 ± 0.09	0.10 ± 0.00	0.97 ± 0.65	139.43 ± 5.66
Playaso	26 ± 0.0	10 ± 1.3	1.57 ± 0.29	0.22 ± 0.15	3.77 ± 3.18	0.31 ± 0.18	113.07 ± 9.90
Panlau	25 ± 0.0	5 ± 0.0	2.79 ± 0.42	0.31 ± 0.09	1.73 ± 0.63	0.13 ± 0.12	145.87 ± 0.29
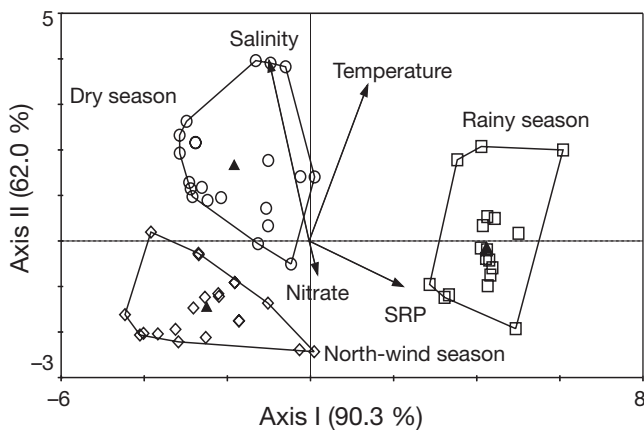
Fig. 2. Canonical variant analysis (CVA) based on 7 environmental parameters. Canonical scores of the 4 significant environmental variables (arrows) plotted on the first 2 discriminating axes (Monte Carlo test with 5000 permutations, p = 0.001). Eigenvalues shown in parentheses. The diagram was adjusted by differentiating line style for sample group envelopes. SRP: soluble reactive phosphorus; ▲: centroids of each data group; □: rainy season samples; ◇: north-wind season samples; ○: dry season samples

nificantly different between seasons (Fig. 2). SRP, temperature, salinity, and nitrate showed the highest seasonal variation (84, 52, 11, and 6%, respectively), contributing to the CVA variance. Temperature variation was clearly related to different climatic periods. In the dry season, when the prevalence was high, the temperature reached 29 ± 2°C (mean ± SD). The highest temperature (30 ± 2°C) was recorded in the rainy season and the lowest (25 ± 1°C) in the north-wind season. SRP exhibited the highest concentration (1.58 ± 0.20 µM) in the dry season and the lowest in rainy season (0.19 ± 0.15 µM) contrary to SRSi, which had the highest concentration in the rainy season (168 ± 40 µM) and the lowest in the dry season (77.4 ± 23 µM). Salinity exhibited marked seasonal variations, ranging from 8 ± 8 psu in the rainy season to

29 ± 10 psu in the dry season. The geographic position of the oyster bed determined the salinity difference among the sampling sites and also the high standard deviation (Fig. 1, Table 2). Results of the GLM showed that annual *Perkinsus marinus* prevalence was positively associated with salinity ($F_{(1,61)}$ = 31.79, p < 0.00001, R^2 = 0.40), and negatively associated with SRSi ($F_{(1,61)}$ = 23.47, p = 0.00002, R^2 = 0.30), and SRP ($F_{(1,61)}$ = 6.17, p = 0.01, R^2 = 0.20; Fig. 3). Temperature, ammonium, nitrite, and nitrate concentration were not significant predictors.

Physiological parameters

CI and hemocyte concentration (THC) showed a seasonal pattern for all sampling sites (Table 4). The highest CI was observed in the dry season (median = 5.10) and decreased in the rainy season (2.80; p < 0.0001). The THC showed the same pattern, with a median of 3.0×10^6 and 1.0×10^6 cells ml^{-1} in the dry and rainy seasons, respectively. Protein and lysozyme concentrations showed a strong seasonal pattern at all sites (Table 4), with the highest concentrations in the dry season (medians = 5.39 and 13.72 mg ml^{-1}, respectively) and the lowest in rainy season (protein = 0.66 mg ml^{-1}, lysozyme = 4.98 mg ml^{-1}). Agglutination had only a significant seasonal variation in the San Francisco and Panlau sites (Table 4). From RDA, only 34.0% of the seasonal *Perkinsus marinus* infection intensity can be explained by the canonical axis. The variables that explained the most consecutive variance (conditional effects) were protein concentration (21% of total inertia), lysozyme (12%), and agglutination (1%). The hemocyte density was not a significant predictor. CVA using 4 physiological variables (protein, lysozyme, THC, agglutination) showed that oysters from different sampling sites aggregated into a single population independent of their geographic origin (Fig. 4).
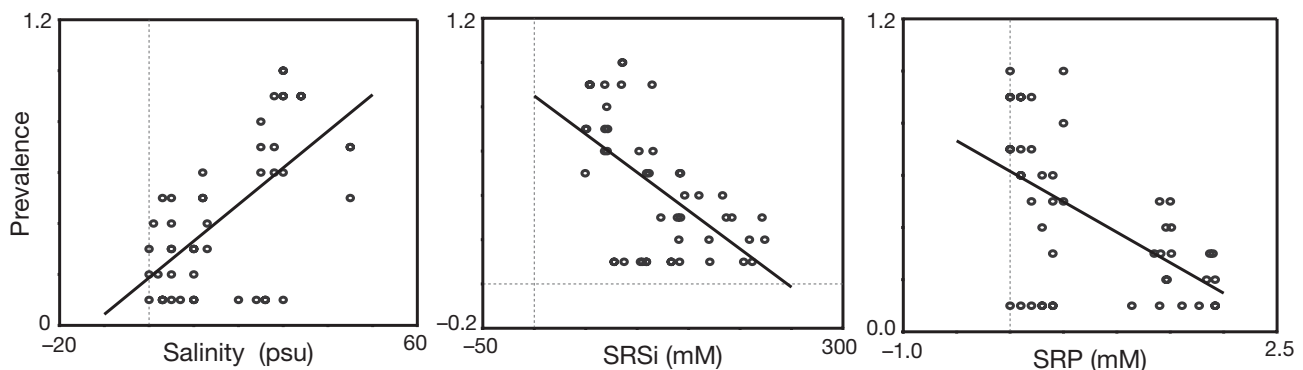

Fig. 3. *Perkinsus marinus.* Generalized linear models (GLM) of prevalence, and environmental variables. SRSi: soluble reactive silica; SRP: soluble reactive phosphorus

Table 4. *Crassostrea virginica.* Seasonal effects on physiological and immuno-logical parameters of oysters collected in Terminos Lagoon, Mexico. Different letters indicate significant differences between groups of each parameter. Statistical analysis was performed using a Kruskal-Wallis test. THC: hemocyte concentration

Site Parameter	Dry season (n = 315) Median	Rainy season (n = 315) Median	North-wind season (n = 315) Median	p
Balchacah				
Condition index	5.50 a	2.40 b	5.30 a	<0.0001
Agglutination titer	5.30 a	4.90 a	5.60 a	0.2156
Protein (mg l^{-1})	6.11 a	0.54 b	0.65 b	<0.0001
Lysozyme (mg l^{-1})	17.42 a	3.54 b	4.88 c	<0.0001
THC (cell ml^{-1})	3×10^6 a	7.1×10^5 b	2.8×10^6 a	<0.0001
Estero Pargo				
Condition index	5.80 a	2.00 b	3.80 c	<0.0001
Agglutination titer	4.90 a	4.90 a	5.30 a	0.1462
Protein (mg l^{-1})	6.78 a	6.87 a	5.78 b	0.0200
Lysozyme (mg l^{-1})	5.61 a	4.43 b	4.06 b	<0.0001
THC (cell ml^{-1})	2.6×10^6 a	8.9×10^5 b	3.2×10^6 a	<0.0001
La Isla				
Condition index	3.60 a	4.30 b	4.30 b	0.0147
Agglutination titer	5.30 a	4.90 a	5.30 a	0.1734
Protein (mg l^{-1})	2.60 a	0.53 b	0.86 c	<0.0001
Lysozyme (mg l^{-1})	14.41 a	8.62 b	9.04 b	<0.0001
THC (cell ml^{-1})	1.8×10^6 a	1.7×10^6 b	3.2×10^6 c	<0.0001
Palizada				
Condition index	5.50 a	5.60 a	5.20 a	0.1498
Agglutination titer	4.90 a	4.90 a	4.30 a	0.5166
Protein (mg l^{-1})	7.55 a	3.18 b	0.66 c	<0.0001
Lysozyme (mg l^{-1})	14.52 a	3.97 b	3.89 b	<0.0001
THC (cell ml^{-1})	3.8×10^6 a	7.9×10^5 b	3.2×10^6 c	<0.0001
Panlau				
Condition index	6.00 a	1.80 b	2.40 c	<0.0001
Agglutination titer	5.30 a	4.90 b	3.30b	<0.0001
Protein (mg l^{-1})	6.11 a	0.53 b	0.49 b	<0.0001
Lysozyme (mg l^{-1})	13.73 a	3.75 b	4.62 b	<0.0001
THC (cell ml^{-1})	3.0×10^6 a	7.5×10^5 b	3.6×10^6 a	<0.0001
Playaso				
Condition index	4.70 a	2.70 b	4.10c	<0.0001
Agglutination titer	5.30 a	4.30 a	3.30 a	0.2985
Protein (mg l^{-1})	2.60 a	0.67 b	4.44a	<0.0001
Lysozyme (mg l^{-1})	15.09 a	7.88 b	8.35 b	<0.0001
THC (cell ml^{-1})	3.6×10^6 a	1.6×10^6 b	3.0×10^6 c	<0.0001
San Francisco				
Condition index	4.30 a	3.70 b	4.70 a	0.0329
Agglutination titer	5.30 a	4.30 b	4.30 b	<0.0001
Protein (mg l^{-1})	2.83 a	0.59 b	1.00 c	<0.0001
Lysozyme (mg l^{-1})	14.57 a	7.94 b	9.63 b	<0.0001
THC (cell ml^{-1})	3.2×10^6 a	1.6×10^6 b	3.0×10^6 a	<0.0001

DISCUSSION

The prevalence of *Perkinsus marinus* in Terminos Lagoon exhibited a strong seasonal pattern reflecting the influence of environmental conditions on the infection. Although temperatures and salinities were high enough to support a seasonal outbreak, *P. marinus* was not associated with disease in Terminos Lagoon during the study period. The difference in seasonal oyster mortality was attributed to the direct effect of environmental conditions on oyster physiology. Even though previous studies reported *P. marinus* from the Mexican coast, little research has been done over a long-term period in this tropical area. When the parasite was discovered by Burreson et al. (1994) in Tabasco, few samples were assayed (mainly gapers), and despite the high prevalence (60 to 100%), the infection intensity was light to moderate except in one location in Machona Lagoon. Because water samples and/ or hemolymph were not collected, it is difficult to draw a definite conclusion about different environmental or physiological conditions with respect to Terminos Lagoon. To date, no serious *P. marinus* epizootic has been reported in Mexican lagoons; however, the pattern of high prevalence and light infection intensity found in the present study is consistent with previous findings in the field by Aguirre-Macedo et al. (2000) in 8 Mexican coastal lagoons.

We observed the absence of a direct relationship between weighted prevalence and temperature, in contrast with the US Gulf of Mexico, where multi-year climate cycles have a significant influence on *Perkinsus marinus* prevalence and infection intensity (Powell et al. 1996). On the southern Mexican coast, the seasonality related to temperature is not evident (annual mean, 28 ± 3°C). However, since previous studies from temperate climates showed that *P. marinus* sporulation in seawater occurs from 2 to 4 d at 28°C (Chu & Green 1989) and even very light infections can increase to lethal levels in a 30 d period at 25°C (Fisher et al. 1992, Ragone Calvo & Burreson 1994, Brousseau & Baglivo 2000), we had expected to find high weighted prevalence in Terminos Lagoon, at least in high salinity sites. In the dry season, with the onset of warmer temperature (29 ± 2°C) and higher salinity conditions (29 ± 10 psu) during 4 consecutive months, the low *P. marinus* intensity (Mackin scale <1) in the oyster population is surprising. Similar results
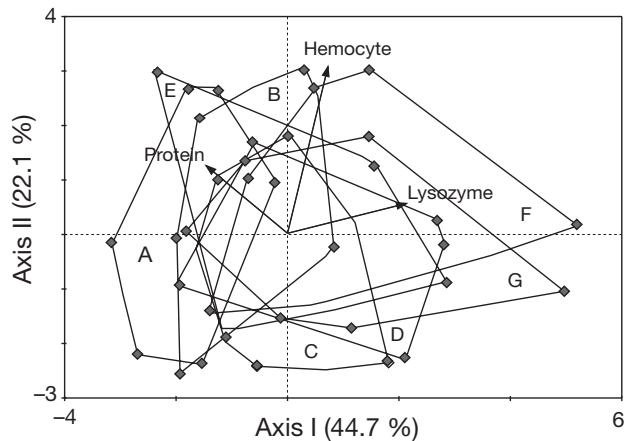
Fig. 4. *Crassostrea virginica*. Canonical variant analysis (CVA) based on 4 physiological variables (lysozyme and protein concentration, agglutination, hemocyte density) of dry season oysters (n = 315). Arrows: significant physiological variables (Monte Carlo test with 5000 permutations, p = 0.001). Diagram was adjusted by differentiating polygons representing different oyster beds. Letter inside each polygon indicates the oyster site name. A: Estero Pargo; B: Palizada; C: La Isla; D: Balchacah; E: San Francisco; F: Playaso; G: Panlau. Eigenvalues for Axes I and II shown in parentheses

were found by Aguirre-Macedo et al. (2007), who observed low *P. marinus* intensity (Mackin scale <1) in the dry and rainy seasons in Terminos Lagoon, with 80 and 50% prevalence, respectively. On the other hand, the correlation reported by Burreson & Ragone Calvo (1996) between water temperature and *P. marinus* intensity approximately 2 to 3 mo after the annual temperature maximum was not evident in this tropical climate. Our results suggest that other environmental factors are controlling *P. marinus* intensity in tropical climates, without eliminating the possibility of race variation of either host or parasite due to different environmental adaptations, as has been suggested by other authors (Bushek & Allen 1996, Gaffney & Bushek 1996).

Our results show that inter-seasonal variation in salinity, although important, is not enough to explain the decreased prevalence in the rainy and north-wind seasons, and suggest that inter-seasonal nutrient levels play an important role in *Perkinsus marinus*-weighted prevalence. The GLM (Fig. 3) shows that together with the positive correlation with salinity, *P. marinus* prevalence has an important negative correlation with SRSi and SRP. On the Atlantic coast of the US, *P. marinus* appears tolerant to salinities as low as 6 psu, and >12 psu is usually described as the salinity required for lethal infections (Ford & Tripp 1996). In tropical climates, the interaction between *P. marinus* prevalence and salinity seems to be differ-

ent. In the dry season, high prevalence in sampling sites was expected considering the high salinity (29 ± 10 psu) and temperature (29 ± 2°C) conditions. However, in the rainy season when the prevalence decreased, the salinity change did not suffice to explain the decrease at Estero Pargo (marine site) and at Playaso (Table 1), where the salinity and temperature conditions remained high enough for *P. marinus* replication (Table 2). Some authors have emphasized that, although important, temperature and salinity do not explain most of infection levels observed in the field, suggesting water quality as a possible influence of *P. marinus* prevalence (Soniat 1985, Craig et al. 1989). According to La Peyre et al. (2003), the repetitive and well-timed freshet can maintain *P. marinus* infections at non-lethal intensities. Moreover, in Terminos Lagoon, the freshwater inputs carry high nutrient concentrations (SRP, SRSi) from the lowlands of Tabasco and the highlands of Chiapas (Yáñez-Arancibia et al. 1983, Ramos-Miranda et al. 2006) that could be controlling *P. marinus* replication. In this context, it is very difficult to define with precision the importance of each nutrient separately when the variables are observed in nature; nevertheless, our data suggest that in Terminos Lagoon, *P. marinus* replication is controlled at least by the combination of the water parameters measured in this study, if not by an even more complex set of parameters.

Our data support that inter-seasonal salinity variation and inter-seasonal nutrient levels have important effects on the oyster's physiological condition. Oyster populations at all sites showed a seasonal pattern in CI and protein concentration with the highest values in the dry season and the lowest in the rainy season (Table 4), suggesting that dry season conditions favor oyster growth. Mortality was higher in rainy and north-wind seasons than in the dry season (Table 3). Since *Perkinsus marinus* weighted prevalence was <1.0 during the study year, it is unlikely that oyster mortality in the rainy season is associated with the parasite (Andrews 1988). Because temperature in tropical areas is not a stress factor for *Crassostrea virginica* survival (Helm et al. 2004), the inter-seasonal salinity variation, especially from the dry (29 ± 10 psu) to the rainy season (8 ± 8 psu) seems to be the main factor associated with mortality. Oyster mortality from excessive freshwater runoff has been reported by several authors (Caffey & Schexnayder 2002, Volety et al. 2003, Tolley & Volety 2005), and this situation is exacerbated when periods of runoff coincide with periods of high temperature because of metabolic energy demands (Shumway 1996). Therefore, one could conclude that oysters that die during this rainy period are the most infected; however, the low *P. marinus* intensity before the freshwater input, added to the reduc-

tion in the oyster CI in the rainy season, greatly limit the applicability of this hypothesis. In addition, mortality from the rainy season and the fact that re-establishment of parasite populations lag behind the re-establishment of host populations (Ray 1987) would be the cause of the low prevalence in the subsequent north-wind season (Table 1).

Establishing a correlation between physiological parameters and disease status was difficult in this fieldwork because of the light infection intensity registered. CVA confirmed that the physiological condition of the oyster population was the same independent of the bed location (Fig. 4). By observing the trend of the humoral oyster parameters, the ordination diagram showed a weak association between *Perkinsus marinus* intensity and physiological parameters (Axis I explained only 34% of the association). The conditional effects show protein and lysozyme as main parameters associated with the dry season, where the salinity condition was significantly high. It is probable that environmental variables have a more important influence over the physiological parameters than *P. marinus* infection. In the future, it will be invaluable to study these physiological and immunological parameters in oyster populations with moderate to heavy *P. marinus* infections to clarify doubts regarding their contribution to defense against the parasite in tropical climates. Nevertheless, the information collected in this study includes a useful first description of *Crassostrea virginica* health in the tropical areas of the Gulf of Mexico.

Based on our discrete seasonal sampling, the results show epizootiological differences between *Perkinsus marinus* behavior in tropical and temperate climates. Our data support the fact that changes in salinity exert an important effect on both the parasite and the oysters' physiological condition, and suggests that freshwater input, which results in high SRP and SRSi concentrations, are controlling the *P. marinus* weighted prevalence in the Terminos Lagoon. The data reported here further contribute to the characterization of perkinsosis in tropical climates but do not refute the likelihood that other factors associated with the host, parasite, or environment can influence the epizootiology of *P. marinus* on the Mexican coast.

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