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Seasonal infection intensity cycle of the parasite *Perkinsus marinus* (and an absence of *Haplosporidium* spp.) in oysters from a South Carolina salt marsh*

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ABSTRACT: Investigation of the prevalence of the protozoan parasite *Perkinsus marinus* in populations of intertidal oysters (*Crassostrea virginica*) from a South Carolina salt marsh led to the finding of a distinct, 4 phase cycle of infection intensity. The cycle consisted of characteristic quiescent, pre-virulent, virulent and remission stages of infection. For the first time, *P. marinus* infection intensity in South Carolina oysters was found to have a statistically significant positive correlation with temperature and salinity, and a negative correlation with oyster condition index. Our data also indicated that *P. marinus* infection in adult oysters from South Carolina is not size dependent. Based on a limited, qualitative histopathological examination of oyster tissue samples, we found no evidence to indicate the presence of *Haplosporidium* spp. in the North Inlet salt marsh/estuary system. These results strongly suggest that highly localized mass mortalities of oysters in North Inlet have been due to virulent levels of *P. marinus* infections.

INTRODUCTION

Relatively high levels of very patchy, highly localized mortality of the American oyster *Crassostrea virginica* (Gmelin) were observed during early September 1987 in the North Inlet salt marsh/estuary, South Carolina, USA (qualitatively assessed by the authors to be ~80 % in some areas). Of all causes of oyster mortality (perhaps exclusive of harvesting), disease has been rated highest (Mackin 1959). We hypothesized that the oyster mortalities we observed were due to the apicomplexan protozoan *Perkinsus* (= *Dermocystidium*) *marinus* and possibly the haplosporidian protozoans *Haplosporidium* (= *Minchinia*) *nelsoni* and *H. costalis*. Descriptions and reviews of the taxonomy and pathology of these protozoans have been extensively presented elsewhere (Farley 1967, 1968, Perkins 1976, 1987, Levine 1978, Levine et al. 1980, Andrews 1982, Sparks 1985, Cheng 1988).

Mortalities due to disease are often cyclic with peak mortalities occurring during limited periods of the year. Variations in the timing and intensity of these cycles is likely heavily influenced by changes in environmental parameters of different geographic regions. Thus, the epizootiology for *Perkinsus marinus* in the Chesapeake Bay (Andrews 1965) may not be the same in a South Carolina salt marsh. The elucidation of site-specific disease cycles is essential in order to conduct and correctly interpret scientific research using oysters from a given region, as well as for making appropriate management decisions concerning possible prevention or termination of epizootic events (Van Banning 1988). Past field studies of disease prevalence in bivalves have generally been limited in their temporal sensitivity and/or duration (Ray 1963a, Andrews 1965, 1967, Goggin & Lester 1987, Craig et al. 1989). The potential for interaction between these protozoan diseases in bivalves has been rarely studied (Andrews 1965, 1967). There is a remarkable scarcity of reported data for these types of diseases in bivalves from South Carolina (Burrell et al. 1984) and a need for investiga-

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tions of the impact of *P. marinus* in South Carolina waters (Burrell et al. 1981).

Our original intent was to examine the seasonal prevalence and intensity cycles of both *Perkinsus marinus* and *Haplosporidium nelsoni* (and/or *costalis*) over a 2 yr period, at less than monthly intervals. The destruction of our laboratory and loss of stored histological oyster tissue slides and paraplast blocks by Hurricane Hugo in late September 1989 put an end to this study and prevented us from making a quantitative assessment of the prevalence and intensity of *Haplosporidium* spp. As a result, the purpose of this paper is to: (1) document the prevalence and 4 phase cycle of infection intensity for *P. marinus* in oysters from a relatively pristine South Carolina salt marsh; (2) report the limited qualitative results of histological examination for *Haplosporidium* spp. prevalence in these same oysters; and (3) discuss which protozoan is the likely cause of the observed oyster mortalities.

MATERIALS AND METHODS

Beginning in March 1988, 12 adult oysters (*Crassostrea virginica*) were collected from the lower intertidal zone at each of 2 collection sites in the salt marsh of North Inlet estuary, South Carolina, USA. North Inlet is a pristine system that has been a National Science Foundation long-term ecological research site for the past 10 yr. Collections were made every 2 to 3 wk. After each oyster was scrubbed clean of all epibionts, individual shell heights and live whole animal volume were determined. Oysters were then opened, the soft body tissue removed from the shell and a transverse cross section of tissue (~ 4 mm thick with dorsal edge of cut beginning at the junction of the palps and gill) taken for histopathological examination for *Haplosporidium* spp. Wet weight of the tissue cross section was determined prior to placing the tissue in a formalin acetic acid (FAA) fixative (917 ml 50 % ethyl alcohol + 23 ml glacial acetic acid + 60 ml 40 % formalin). The tissue was then processed for histological examination according to methods outlined by Howard & Smith (1983). Tissues were taken for histological examination at approximately 8 wk intervals, while examination of tissues for *Perkinsus marinus* occurred every 2 to 3 wk. The labial palps and terminal portion of the rectum were removed for *P. marinus* infection intensity determinations (see below for technique). The remaining soft body tissue was dried (80 °C, 48 h) and weighed, while the cleaned, empty shell volume was determined. The wet weight of the labial palps was determined prior to being placed into the culture medium. Dry weights of palps and tissue taken for histopathology were determined via the regression equation:

$$\log_{10} \text{dry wt} = 0.427 + (0.571)(\log_{10} \text{wet wt}) \quad (1)$$
$$p < 0.0001,\ r = 0.922,\ R^2 = 0.851$$

and added to the soft body tissue dry weight for an accurate measure of total soft body tissue dry weight. The amount of dry weight lost by removal of the terminal portion of the rectum (~ 3 to 4 mm in length) was not significant. In May 1989, a Kendall rank correlation analysis demonstrated a significant ($p < 0.001$) correlation between *P. marinus* infection intensities found in an individual oyster's palps and rectum tissues. In addition, a Mann-Whitney U analysis revealed a significantly higher ($p < 0.001$) mean ranking of rectum infection intensities over palps intensities. Thus, beginning in June 1989, palps tissues were no longer excised for *P. marinus* infection intensity determination so that the number of oysters collected from each site and examinations for rectal infection intensities could be doubled ($n = 25$ for each site). The study was terminated at the end of September 1989 by Hurricane Hugo.

Initially, condition index (CI) was determined volumetrically via the equation:

$$CI = \frac{\text{dry soft body tissue (g)} \times 1000}{\text{internal shell cavity vol. (ml)}} \quad (2)$$

However, a concurrent study (Crosby & Gale in press) demonstrated that a gravimetric methodology was superior to volumetric methods in reducing errors, increasing levels of sensitivity and reducing time of measurements. Gravimetric and volumetric methods were also found to be highly correlated and not significantly different. Beginning in June 1989, therefore, we elected to begin using gravimetric methods to determine CI via the equation recommended by Crosby and Gale (in press), i.e.

$$CI = \frac{\text{dry soft body tissue (g)} \times 1000}{\text{internal shell cavity capacity (g)}} \quad (3)$$

The culturing technique and examination for *Perkinsus marinus* consisted of incubation of the sample tissue in fluid thioglycolate media as described by Ray (1963b). Infection intensity level for each oyster was assigned on a scale from 0 to 6 as described by Quick & Mackin (1971), with 0 being an absence of hypnospores and 6 indicating all fields almost black with stained hypnospores. Monthly mean infection intensity includes all oysters sampled in a given month. Monthly mean water temperatures and salinities were derived from daily sampling at a site in North Inlet, midway between each of the oyster sampling sites. All statistical analyses were performed with a Macintosh SE computer using the StatView 512+ statistical package (Gagnon & Feldman 1986).

Table 1. Mean (and SE) monthly *Perkinsus marinus* infection intensity in rectal tissue of *Crassostrea virginica*, total number of oysters sampled (N), condition index (CI), dry soft tissue weight (in g), water temperature (T) and salinity (S)

Month	*P. marinus*	N	CI	Tissue	T (°C)	S (‰)
Jan	2.62 (0.19)	24	90.97 (5.01)	1.32 (0.09)	11.1	34.2
Feb	2.04 (0.13)	48	75.89 (4.25)	1.11 (0.06)	11.5	33.9
Mar	2.00 (0.15)	48	76.22 (4.41)	1.05 (0.04)	13.5	29.9
Apr	1.53 (0.12)	72	93.09 (6.07)	1.16 (0.05)	17.9	29.3
May	2.50 (0.20)	48	81.51 (5.34)	1.08 (0.06)	22.1	30.2
Jun	3.28 (0.10)	148	81.80 (2.68)	1.00 (0.03)	26.9	32.6
Jul	3.29 (0.12)	96	74.12 (2.80)	1.02 (0.04)	28.2	33.0
Aug	3.81 (0.10)	148	65.57 (1.70)	0.95 (0.03)	28.2	33.1
Sep	3.74 (0.12)	98	61.45 (1.98)	1.00 (0.03)	26.1	31.6
Oct	3.78 (0.31)	23	69.45 (6.44)	1.03 (0.07)	19.2	34.6
Nov	2.88 (0.30)	24	95.04 (9.47)	1.00 (0.06)	15.9	34.2
Dec	2.67 (0.30)	24	80.70 (7.84)	0.89 (0.08)	9.8	34.9

Table 2. Mean (and SE) monthly *Perkinsus marinus* infection intensity in rectal tissue of *Crassostrea virginica*, number of oysters (N), range of observed intensity levels, and prevelence of infection in oyster. Months with infection intensity levels found to be not significantly different via ANOVA with Fisher's PLSD comparisons are given the same letter under the heading PLSD. Level of significance set at $p \leq 0.05$

Month	*P. marinus*	N	Range	Prevelence (%)	PLSD
Jan	2.62 (0.19)	24	1 to 4	100.00	A, B
Feb	2.04 (0.13)	48	0 to 5	95.83	B, C
Mar	2.00 (0.15)	48	0 to 5	93.75	C
Apr	1.53 (0.12)	72	0 to 5	87.50	
May	2.50 (0.20)	48	0 to 5	97.02	A, B
Jun	3.28 (0.10)	148	1 to 6	100.00	D, E
Jul	3.29 (0.12)	96	1 to 6	100.00	D, E
Aug	3.81 (0.10)	148	1 to 6	100.00	F
Sep	3.74 (0.12)	98	1 to 6	100.00	F
Oct	3.78 (0.31)	23	1 to 5	100.00	D, F
Nov	2.88 (0.30)	24	1 to 5	100.00	A, E
Dec	2.67 (0.30)	24	1 to 5	100.00	A

Table 3. Kendall rank correlation coefficient matrix between *Perkinsus marinus* infection intensity in rectal tissue of *Crassostrea virginica*, condition index (CI), height (HT), dry tissue weight (Tissue), water temperature (T °C), and salinity (S, ‰). Abbreviations are: (t) tau corrected for ties; (p) level of significance; (n) no. cases; (ns) not significant at $p > 0.05$

	S	T	Tissue	HT	CI
P. marinus					
t	0.094	0.283	−0.098	ns	−0.167
p	<0.001	<0.001	<0.001		<0.001
n	801	801	753		716
CI					
t	ns	ns	0.277	−0.128	
p			<0.001	<0.001	
n			761	739	
HT					
t	ns	−0.099	0.236		
p		<0.001	<0.001		
n		824	776		
Tissue					
t	−0.112	−0.063			
p	<0.001	<0.01			
n	777	777			
T					
t	0.060				
p	<0.02				
n	825				

RESULTS

Differences between sites in *Perkinsus marinus* infection intensities were examined for each month by analyses of variance (ANOVA). Results indicated no significant difference between sites for monthly intensity levels. Data from the 2 sites were therefore, pooled for further analyses. Monthly means for infection intensity, CI, dry soft body tissue weight, water temperature and salinity are given in Table 1. An ANOVA with Fisher's protected least squares difference (PLSD) comparison was conducted in order to determine any significant differences between monthly mean infection intensities. Results of this analysis, along with monthly mean and range of observed infection intensity levels are presented in Table 2. Total *P. marinus* prevalence for all oysters examined was 98.1 %. Only 4 mo exhibited prevalence levels less than 100 %: 95.8 % in February, 93.8 % in March, 87.5 % in April and 97.9 % in May.

A results matrix of the Kendall rank correlation analyses between infection intensity, CI, shell height, dry soft body tissue weight, water temperature and salinity are presented in Table 3. Salinity and temperature were positively correlated with infection intensity. Dry soft-body tissue weight and CI were negatively correlated with infection intensity. No significant correlation

was found between shell height and infection intensity. Regression analyses indicated that only 2% of dry soft body tissue and 5% of CI variability was explained by infection intensity. Infection intensity combined with shell height explained 13.9% of dry soft body tissue and 8.8% of CI variability. Shell heights ranged from 6.7 to 16.5 cm, with a mean of 10.1 cm (SE = 1.0 cm). However, 63% of examined oysters had shell heights between 8.7 and 11.6 cm, and 88% were between 7.7 and 12.6 cm. Temperature was found to explain 16.7%, while salinity explained only 3.6% of the infection intensity variability.

Random histological samples of oysters collected in June 1988 were examined microscopically prior to the loss of these samples in Hurricane Hugo. No histological evidence for the presence of *Haplosporidium* spp. was seen in the approximately 25 samples surveyed.

DISCUSSION

Examination of the *Perkinsus marinus* infection intensity data presented in Table 1 and 2 has led to our conclusion that the epizootiology of *P. marinus* in South Carolina oysters exists in a 4 phase cycle which we present in Fig. 1. Phase 1 of this cycle may be viewed as a period of quiescence. The quiescent phase of infection intensity (February, March, April) is characterized by the lowest levels of both mean intensity and prevalence, as well as each month in the phase having individual oysters exhibiting no infection at all. Phase 2 is the pre-virulent period (May, June, July) and is marked by a dramatic and rapid increase in monthly mean intensity levels along with a transition from a maximum observed intensity level of 5 to the maximum, 6. Phase 3 is the virulent stage of *P. marinus*

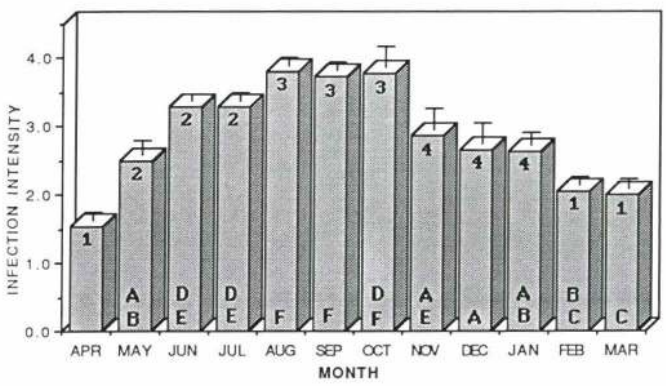

Fig. 1. Mean monthly (+ SE) *Perkinsus marinus* infection intensity in oysters from North Inlet, South Carolina. Months having the same letter were not found to be significantly different via ANOVA with Fisher's PLSD comparisons. Numbers within each monthly column indicate the infection cycle phase, where: (1) quiescent; (2) pre-virulent; (3) virulent; and (4) remission. (See text for further details)

infection in *Crassostrea virginica*. The virulent phase (August, September, October) is characterized by the peak of infection intensity and no occurrence of oysters free of infection in each of the inclusive months. The fourth phase of the cycle (November, December, January) is a remission stage when monthly mean infection intensity drops dramatically and all oysters examined had intensity levels of less than 6.

The 2 physical factors that were examined in this study, temperature and salinity, were very highly correlated ($p < 0.001$) with *Perkinsus marinus* infection intensity in *Crassostrea virginica*. It was earlier determined (see Results) that changes in salinity could only account for 3.6% of the variability in infection intensity, while temperature accounted for much more (16.7%) of infection intensity changes. When monthly mean temperature and infection intensity are plotted together (Fig. 2) it becomes apparent that increases and decreases in water temperature 'lead' similar changes in infection intensity. Although past reports of general, non-statistical (Mackin 1962, Andrews 1965, 1967) and more specific, yet still non-statistical (Chu & Greene 1989) studies have indicated a positive correlation between water temperature and *P. marinus* infection intensity levels in *C. virginica*, the limited number of field studies using statistical analyses of the data have found no statistically significant relationship between water temperature and infection intensity (Burrell et al. 1984, Craig et al. 1989). The contrasting finding in our study of a statistically significant ($p < 0.001$) positive relationship between water temperature and infection intensity supports the inferred correlations of the above studies. The difference of our results from those of Burrell et al. (1984) and Craig et al. (1989) are likely due to the manner in which water temperature data was collected in the 3 studies. Both Burrell et al. and Craig et al. measured temperature on the day of oyster collection, which yields only an instantaneous point of measurement in time. Our water temperature data were an integration of daily measurements over month-long periods of time, and as such are much more representative of the history of environmental exposure which both the protozoan and oyster have been exposed to prior to sampling. The importance of having a knowledge of recent environmental exposure history is graphically evident in Fig. 2.

Our study is the first to document a statistically significant relationship between *Crassostrea virginica* CI and *Perkinsus marinus* infection intensity levels in South Carolina. A negative correlation between spawning and CI in South Carolina oysters has been previously inferred (McNulty 1953). However, oysters in North Inlet have a peak spawning event in April/May (Kenny et al. unpubl. data) during the quiescent and early pre-virulent phases of *P. marinus* infection.

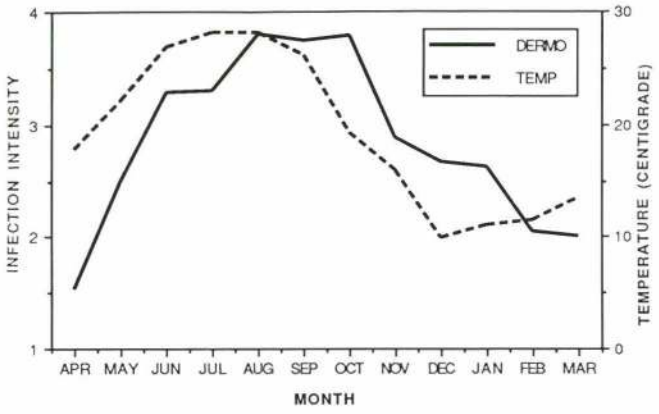

Fig. 2. Water temperature fluctuations associated with observed *Perkinsus marinus* (DERMO) infection intensity cycle in oysters from North Inlet, South Carolina

Both oyster dry soft body tissue weight and CI were significantly ($p < 0.001$) negatively correlated with *P. marinus* infection intensity level. These relationships agree with the results of previous published reports on *P. marinus* parasitism in *C. virginica* outside of South Carolina (Menzel & Hopkins 1955, Quick & Mackin 1971, Craig et al. 1989). The exact mechanism(s) by which *P. marinus* causes decreases in *C. virginica* soft-body tissue and CI have not yet been clearly elucidated. There is evidence that *P. marinus* infection alters fat metabolism (Stein & Mackin 1955) and free amino acid composition (Soniat & Koenig 1982) in *C. virginica*. It is possible that our findings are a result of systemic invasion by the protozoan with damage occurring to the oyster's blood sinuses and connective tissue (Mackin 1951, Sparks 1985). The oyster must then reallocate energy from growth and normal maintenance metabolism to resisting the parasitism, i.e. increase in phagocytotic activity and repairing the tissue damage, while concurrently having a compromised system with which to carry out defensive and repair activities.

It is generally assumed (Ray 1963a, Burrell et al. 1984) that *Perkinsus marinus* infection is positively correlated with oyster age (= height). White et al. (1989) reported that large oysters (> 5 cm) were infected 3 to 4 times as frequently by *P. marinus* as smaller oysters, while Andrews (1967) presented evidence that disease-free oysters require 1 to 3 yr to acquire the disease. Our study found no significant relationship between *Crassostrea virginica* age and infection intensity. The minimum oyster shell height in our study was 6.7 cm (see Results), so that the minimum age oyster was 2-yr-old (Crosby unpubl. data). Although all oysters examined in our study were greater than 2 yr of age, it is nevertheless interesting that over a size range of 10 cm no significant relationship was found between age and infection intensity. Our data then, indicate that *P. marinus* infection in adult oysters from South Carolina is not size dependent. In order for a definitive Conclusion regrading the lack of correlation between oyster age and infection, however, a single time point study across a sufficiently large number of oysters of different ages may be required.

On the basis of our very limited, qualitative histopathological examination of oyster tissue samples, we have no evidence to indicate the presence of *Haplosporidium* spp. in the North Inlet salt marsh/estuary system. This parasite has been found to the north (Andrews 1982) and, to a limited degree, to the south (Kern pers. comm.) of South Carolina. To date, only $\ll 1$ % of a sample of *Crassostrea virginica* from the region of the South Carolina-Georgia border have shown any *H. nelsoni* in South Carolina (Burrell pers. comm.). If *Haplosporidium* spp. were infecting oysters in North Inlet one would expect much less patchy, non-localized high levels of mortality to have occurred. This is due to the *Haplosporidium* spp. parasite not being host-density dependent (Perkins 1987), as well as it killing it's host rapidly and dispersing quickly within a system (Andrews 1982). The negative histopathological examination for *Haplosporidium* spp. and the lack of characteristic epizootiology of *Haplosporidium* spp. being noted in the oyster mortality patterns we observed in North Inlet are strong evidence that the significant contributing factor for the patchy, localized mortalities was virulent levels of *Perkinsus marinus* infection rather than *Haplosporidium* spp. However, the histopathological samples we examined were only from the month of June, which is not a period that *Haplosporidium* spp. would be expected to be most prevalent, and a 'border-line' area of *Haplosporidium* spp. infestation may exhibit patchy mortalities (Kern pers. comm.). We therefore appreciate the need for, and recommend that histopathological surveys for *Haplosporidium* spp. be re-instated in North Inlet, in particular, and be expanded throughout the South Carolina region.

Although water temperature and salinity account for approximately 20 % of the variability in *Perkinsus marinus* infection intensity levels in *Crassostrea virginica*, much of the remaining intensity variability is likely to due to the physiological state, i.e. scope for growth, of both oyster and protozoan and the interaction between the 2 with their environment. To more clearly understand the mechanisms of marine bivalve diseases unique to South Carolina, studies dealing with these interactions, such as Andrews (1967), Ford (1985), Newell (1985), Fisher (1987, 1988), Barber et al. (1988), and Chu & Greene (1989), need to be conducted in this geographic region. One of us (M. P. C.) has just completed a nearly 3 yr investigation of the in situ ecophysiology of oyster populations from the North Inlet collection sites examined in this study, the results

of which are being prepared for publication. It is hoped that the combined efforts of our 2 studies will serve as a basis for a much needed future expansion of studies dealing with the ecological effects of diseases in bivalves in the South Carolina region.

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