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Environmental significance of freshets in reducing *Perkinsus marinus* infection in eastern oysters *Crassostrea virginica*: potential management applications

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ABSTRACT: The effects of extreme freshwater events on *Perkinsus marinus–Crassostrea virginica* interactions remain unexplored. The effects of freshwater events on *P. marinus* infection in *C. virginica* and oyster survival were therefore examined in controlled laboratory experiments and a field study. For the laboratory experiments, oysters were collected in spring, summer and winter from an area in Louisiana where *P. marinus* is endemic. Oysters were placed in 2 recirculating water systems at a salinity and temperature similar to their collection site. They were subjected to 2 salinity treatments (freshet and control). Freshet events were simulated by reducing the water to salinities of 0 to 1 ppt over a 48 h period, and maintained for a 21 d period. Control oysters were maintained at the initial salinity. Thirty oysters were sampled prior to the freshet event, and 30 oysters per treatment group (freshet, control) were sampled on Days 7, 14 and 21 after initiation of the freshet event. Oyster mortality, *P. marinus* infection intensities, oyster condition index and oyster plasma osmolality were measured in weekly samples. All 3 simulated freshet events (i.e. spring, summer, winter) resulted in a significant reduction in *P. marinus* infection intensity, but failed to eliminate infection. The failure of the oyster plasma to reach very low osmolality (< 50 mOsm kg^{-1}) provides a likely explanation for the lack of complete *P. marinus* elimination. The field study involved sampling oysters monthly in the Caloosahatchee estuary, Florida, from September 2000 to February 2002, and determining *P. marinus* weighted prevalence and condition index of wild oysters, and growth and survival of caged juvenile oysters. The data strongly support the contention that the numerous freshwater releases to the Caloosahatchee River kept *P. marinus* infection intensities in oysters at low levels, resulting in an overall low weighted prevalence, low oyster mortality and good growth. Data from our field study appear to support the hypothesis that repetitive and well-timed freshet events can prevent infection of oysters with *P. marinus* or at least maintain *P. marinus* infections at non-lethal intensities (e.g. $<10^6$ parasites g^{-1} wet tissue) in oyster populations. The use of an adaptive management approach involving control of freshwater inflows could be invaluable to the oyster industry in areas close to freshwater diversion projects.

KEY WORDS: Dermo · *Perkinsus marinus* · *Crassostrea virginica* · Freshet · Infection intensity · Mortality · Osmolality · Condition index

INTRODUCTION

Modern ecological synthesis recognizes that variability is an inherent part of natural systems: natural and human induced change and disturbance are considered the norm rather than the exception (Odum 1969, Pickett et al. 1992). Nonetheless, in attempts to establish causal relationships among various environmental factors, many natural-system studies assume that systems are in a state of equilibrium that categorically ex-

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cludes short-term and catastrophic events. While environmental conditions have long been held as critical controls on host-parasite interactions through their impacts on the physiological condition, reproduction and survival of both hosts and parasites, little attention has been paid to the effects of short-term events on host-parasite interactions. Understanding the role these short-term events may play in influencing host-parasite interactions is critical to the design, application and evaluation of disease management strategies for enhanced production of wild commercially important species, such as the eastern oyster *Crassostrea virginica*.

The oyster parasite *Perkinsus marinus* is considered to be a major cause of mortality in Gulf Coast (Craig et al. 1989, Soniat 1996) and East Coast subtidal *Crassostrea virginica* populations (Burreson & Ragone Calvo 1996, Ford 1996). In the Chesapeake Bay region, *P. marinus* has decimated oyster populations (Burreson & Ragone Calvo 1996). In the Gulf of Mexico, the market-size component of oyster populations suffers an estimated 50% yearly mortality from *P. marinus* (Mackin 1962, Hofstetter 1977, Powell et al. 1996). Temperature and salinity are generally held to be the dominant environmental factors controlling both the survival and growth of oysters and *P. marinus* independently, and it is likely that they influence the host-parasite interaction (Soniat 1985, Soniat & Gauthier 1989, Chu & La Peyre 1993, Chu et al. 1993, Ragone & Burreson 1993, Burreson & Ragone Calvo 1996).

Early studies concluded that oysters can exist and grow vigorously in salinities slightly lower than the minimum tolerated by *Perkinsus marinus*, although it was concluded at the time that the differences were so minimal that for practical purposes they did not exist (Mackin 1956). Despite this contention, along both the East and Gulf Coasts, locations characterized by the regular occurrence of freshet events, are noteworthy for their lack of *P. marinus*-infected oysters (Brooks et al. 1988, Chu & Greene 1989, Soniat & Gauthier 1989). The term freshet is used broadly to denote a rapid and short-term freshwater event in normally saline waters. Our overall goal is to identify the significance of freshet events in reducing *P. marinus* infection in their host, the eastern oyster.

There is a paucity of manipulative field and laboratory studies examining effects of short-term acute events such as freshets on oysters, *Perkinsus marinus* and oyster–*P. marinus* interactions. For example, only recently have we acquired *in vitro* evidence to suggest that acute freshwater events may be important in oyster–*P. marinus* interactions. The most relevant recent laboratory study tested the effects of acute exposure (24 h) of *in vitro* cultured *P. marinus* parasites to low salinities and found that mortality was greater than 99% (Burreson et al. 1994). Field surveys and studies that have discussed short-term events concluded that variation in oyster physiology (Fisher et al. 1996), oyster production (Livingston et al. 2000) and disease incidence (Soniat 1985) were related to storm events and ephemeral impacts of human activities.This laboratory and field evidence combined suggests that the use of environmental averages (e.g. monthly averages) to fully understand and predict oyster and *P. marinus* survival, and interactions in a natural environment may be limited: *P. marinus*–*Crassostrea virginica* dynamics may be controlled more by extreme events in the environment (i.e. freshets) than yearly means (i.e. salinity means).

Extreme variations in salinity frequently occur in coastal areas due to both natural events (e.g. seasonal rains, heavy rainstorms and El Niño Southern Oscillation events) and human actions (e.g. land development, water management) (e.g. Livingston et al. 2000). In particular, in southwest Florida, extreme salinity variations are common due to management practices designed to accommodate watershed land uses and development (Volety et al. 2001a,b). Typically, these water management practices include a cessation of weir openings during dry months to conserve water for human consumption (e.g. agricultural, residential), and frequent weir openings during the rainy season to prevent upriver flooding. This pattern of weir openings results in a general freshening of estuaries in the summer (rainy months) as large freshwater flows are allowed into the estuary, contrasted with higher salinities in the winter (dry season), when weirs are not opened and very little fresh water enters the estuary.

The Caloosahatchee River in south Florida, USA, is dominated by a water management approach involving the extensive use of weirs. The Caloosahatchee River basin is located in southwest Florida and drains 3700 km^2. The entire watershed is dominated by agricultural and rangeland land uses in the upper reaches of the basin and urban developments in the lower reaches of the basin. Water releases into the Caloosahatchee Estuary from Lake Okeechobee are controlled by the opening and closing of weirs and follow the typical seasonal pattern described above. This water management practice results in extreme variations of salinity in the estuary ranging from 0 to 37 ppt (depending on time of year and location), providing an ideal setting to investigate effects of water management and freshet events on oyster survival and *Perkinsus marinus* infection.

The main objective of this study was to investigate the significance and potential role of freshet events in reducing *Perkinsus marinus* infection in their host, the eastern oyster. This study focused on subtidal oysters as these are the dominant oysters along the Gulf Coast. This study reports the results of (1) controlled laboratory experiments in which we determined the effects of

simulated freshet events occurring in the spring, summer and winter on oyster *P. marinus* infection intensity, condition index, mortality and plasma osmolality, and (2) a field study quantifying the related effects of season and freshet events on oyster *P. marinus* weighted prevalence, condition index, mortality and growth.

MATERIALS AND METHODS

Laboratory experiments. ***Oysters and site:*** Infected subtidal eastern oysters *Crassostrea virginica*, 6 to 10 cm in length, were obtained from the Louisiana Sea Grant oyster hatchery in Grand Isle, Louisiana. The Grand Isle area is endemic for *Perkinsus marinus*, ensuring that oysters collected from this area will have been exposed naturally to *P. marinus* during their grow-out phase. All oysters in this region are subtidal. At the time of collection, water temperature and salinity were recorded. Oysters were transported to Louisiana State University (LSU), Baton Rouge, in April, July and November 2001 and evenly distributed between 2 recirculating water systems.

Experimental design and exposure system: The experiment was conducted as a controlled laboratory experiment with 3 seasons (spring, summer, winter) and 2 salinity treatments (control and freshet). Each season 280 oysters were randomly placed, in groups of 13 to 14 oysters, into a total of 21 separate containers. These 21 containers were divided between the 2 recirculating systems holding 1000 l artificial seawater (Hawaiian Marine Imports), resulting in 10 and 11 containers per system. Initial salinity and temperature conditions in each system were established that were similar to conditions at Grand Isle at the time of oyster collection (April: 18°C, 23 ppt; July: 28°C, 20 ppt; November, 16°C, 26 ppt). Water in each system was filtered through 10 and 1 µm filters to eliminate cross-contamination of parasites between containers. Water in each system was recirculated at least 4 times h^{-1}, except during feeding, and was constantly aerated. The oysters were fed daily with the marine algae *Isochrysis galbani* (Reed Mariculture) for about 4 h, during which time water bypassed each container. After 1 wk of acclimation, 30 oysters from 3 containers (2 containers from the system with 11 containers, and 1 container from the system with 10 containers) were sampled to determine initial (Day 0) *Perkinsus marinus* infection intensities, oyster condition index and plasma osmolality. At Day 0, one system, designated the treatment system, was subjected to a simulated freshet event: salinity was reduced to 0–1 ppt over a period of 48 h. Water used for the reduction of salinity was held in a separate tank and filtered through a carbon filter to dechlorinate the water. Throughout the course of the experiment, the other system was maintained at the initial salinity and temperature as a control. Oyster mortality and water quality (pH, NO_2, NH_3) were measured daily in both control and treatment systems in order to ensure that water quality parameters were similar among systems, and not a cause of mortality in oysters. *P. marinus* infection intensities, oyster condition index and plasma osmolality were determined in 30 treatment and 30 control oysters collected from 3 containers in each system on Days 7, 14 and 21. When the number of live treatment or control oysters in the 3 randomly chosen containers was less than 30, all oysters remaining in the selected containers were sampled.

Perkinsus marinus infection intensity: The number of parasites per gram of oyster tissue was determined using the whole-oyster procedure as described by Fisher & Oliver (1996) and modified by Coates et al. (1999). The whole-oyster procedure, although labor intensive, provides an accurate measure of infection intensity in individual oysters (Bushek et al. 1994). All chemicals were from Sigma Chemical unless otherwise indicated. Briefly, each oyster was weighed and homogenized in alternate fluid thioglycollate medium (ARFTM) supplemented with 16 g marine salts (Hawaiian Marine Imports) and 5% of a commercial lipid concentrate 100× at a ratio of 1 g oyster tissue per 50 ml of ARFTM. After 1 wk of incubation in ARFTM, samples were centrifuged at 1500 × *g* for 10 min and the supernatant discarded. The resulting pellets were incubated in 2 N NaOH at 60°C to digest oyster tissues, leaving the parasites intact. The samples were rinsed with 0.1 M phosphate buffer saline containing 0.5 mg ml^{-1} of bovine serum albumin to prevent parasite clumping. Samples were then serially diluted in 96-well plates and parasites stained with Lugol's solution. The number of parasites was counted from wells containing 100 to 400 parasites (i.e. hypnospores) with an inverted microscope at a magnification of 200×. Infection intensity of individual oysters is reported as number of parasites per gram of oyster tissue.

Condition index: A 10 ml aliquot of oyster tissue homogenate in ARFTM was dried at 65°C for 48 h and the dry weight determined by subtracting the weight of ARFTM only. The dry weight for the whole oyster was calculated based on the total volume of homogenized tissue in ARFTM. The ratio of the dry weight of tissue to the dry weight of the shell was calculated and multiplied by 100 to determine oyster condition index (CI). This index has been recommended for measuring the condition of adult oysters and other bivalves (Mann 1978, Lucas & Beninger 1985).

Oyster plasma osmolality: Oyster plasma osmolality was measured because plasma thoroughly bathes oyster tissues where *Perkinsus marinus* proliferate extracellularly. Oyster hemolymph (0.2 ml) was withdrawn

from the pericardial cavity using a 27 gauge needle, following careful removal of the shell of all oysters sampled in July and December. The sampled hemolymph was immediately transferred into vials on ice and the cell-free hemolymph or plasma was obtained by centrifugation of hemolymph at 600 × *g* for 15 min at 4°C. The osmolality of the plasma was measured using a vapor pressure osmometer (Wescor). During the July 2001 experiment, we measured osmolality on Days 2, 4 and 6 using 10 extra oysters in a separate container each time in order to examine the immediate change in plasma osmolality as a response to the simulated freshet event.

Statistical analyses: Results of *Perkinsus marinus* infection intensity, condition index and osmolality for each season were analyzed using a 2-factor analysis of variance (i.e. treatment and sampling time) followed by Student-Newman-Keul's test (SNK) when significant differences were found ($p < 0.05$) (Zar 1984). To test for seasonal differences in initial *P. marinus* infection levels (i.e. pre-freshet event), we ran a 1-factor ANOVA followed by SNK when significant differences were found ($p < 0.05$). All data were checked for assumptions of ANOVA and transformed as needed. Data for *P. marinus* body burden were log transformed to achieve normality and homogeneity of variance. Mortality data were analyzed as percent mortality using chi-square tests.

Field study. ***Oysters and site:*** Ten eastern oysters (6 to 12 cm) were collected monthly from September 2000 through February 2002 from 2 sampling stations near the Caloosahatchee River, Florida. Piney Point sampling station is located approximately 2 km upstream from the river mouth while the more estuarine influenced station, Bird Island, is located approximately 6 km downstream from the river mouth (Fig. 1). The total length of the river, from the upstream weir to the mouth of the river before opening into San Carlos Bay, and ultimately into the Gulf of Mexico, is 42 km. Discrete measures of temperature and salinity data at both sampling sites were obtained on each day of sampling. Salinity data were also obtained throughout the study from continuous water quality monitoring stations maintained by the South Florida Water Management District (SFWMD) at Shell point, located at the river mouth, also at Fort Myers, 20 km upstream of the river mouth, and in Sanibel, 10 km southwest of the river in San Carlos Bay. Daily freshwater discharge data via the weir from Lake Okeechobee into the Caloosahatchee River were also obtained from the SFWMD (Fig. 1).

Perkinsus marinus weighted prevalence: Oysters collected monthly were assayed for the presence of *P. marinus* using Ray's fluid thioglycollate medium (FTM) technique (Ray 1954). Samples of gill and digestive diverticulum were incubated in FTM for 5 d, smeared on a slide and the enlarged parasites stained with Lugol's solution. The intensity of infection was rated according to the categories of Ray (1954) by estimating the percentage of tissue occupied by the parasite, with 0 = no infection, 1 = light infection, 3 = moderate infection and 5 = heavy infection. Weighted prevalence was calculated by averaging the intensity of infection of all oysters sampled monthly (Mackin 1962, Ragone Calvo & Burreson 1994). This technique, although less sensitive than the whole-body burden assay described above, is correlated with the body-burden assay and enables processing of high numbers of samples required in field studies (Bushek et al. 1994).

Condition index: The condition index of oysters collected monthly was determined by calculating the ratio of the dry weight of the tissue to the dry weight of shell and multiplying this ration by 100 as recommended by Lucas & Beninger (1985).

Juvenile oyster growth and mortality: Two hundred juvenile oysters (17 mm mean size) were placed in wire mesh cages (5 × 5 mm mesh size) at each sampling site in August 2000. Growth and mortality of 50 randomly selected oysters were determined monthly.

Statistical analyses: Monthly differences for weighted prevalence of *Perkinsus marinus* infection, condition index and growth were tested using a 1-factor ANOVA followed by Tukey's multiple comparison test when significant differences were found ($p < 0.05$). Data were tested for normality and homogeneity of variance and transformed as necessary.

RESULTS

Laboratory experiment

Perkinsus marinus infection intensities

In April, a significant interaction in *Perkinsus marinus* infection intensities was found between the type of treatment (i.e. control and freshet) and the time of sampling (i.e. 7, 14 and 21 d) ($p < 0.0001$). *P. marinus* infection intensities of oysters exposed to the freshet were significantly lower than the infection intensities of control oysters on Days 14 and 21 (Fig. 2). There were significant decreases in infection intensities in oysters exposed to the freshet from Day 7 ($4.70 \times 10^4 \pm 6.00 \times 10^4$ parasites g^{-1} wet tissue) to Day 14 ($1.17 \times 10^4 \pm 1.30 \times 10^5$ parasites g^{-1} wet tissue), and from Day 7 to Day 21 ($4.35 \times 10^3 \pm 9.20 \times 10^3$ parasites g^{-1} wet tissue). Initial infection intensity of oysters sampled prior to the freshet was $2.09 \times 10^5 \pm 1.70 \times 10^5$ parasites g^{-1} wet tissue. The infection intensity of control oysters was significantly higher on Day 14 ($5.04 \times 10^5 \pm 6.5 \times 10^5$ parasites g^{-1} wet tissue) than on Day 7 ($8.84 \times 10^4 \pm 9.8 \times 10^4$ parasites g^{-1} wet tissue) or Day 21 ($3.96 \times 10^5 \pm 4.8 \times 10^5$ parasites g^{-1} wet tissue) (Fig. 2).

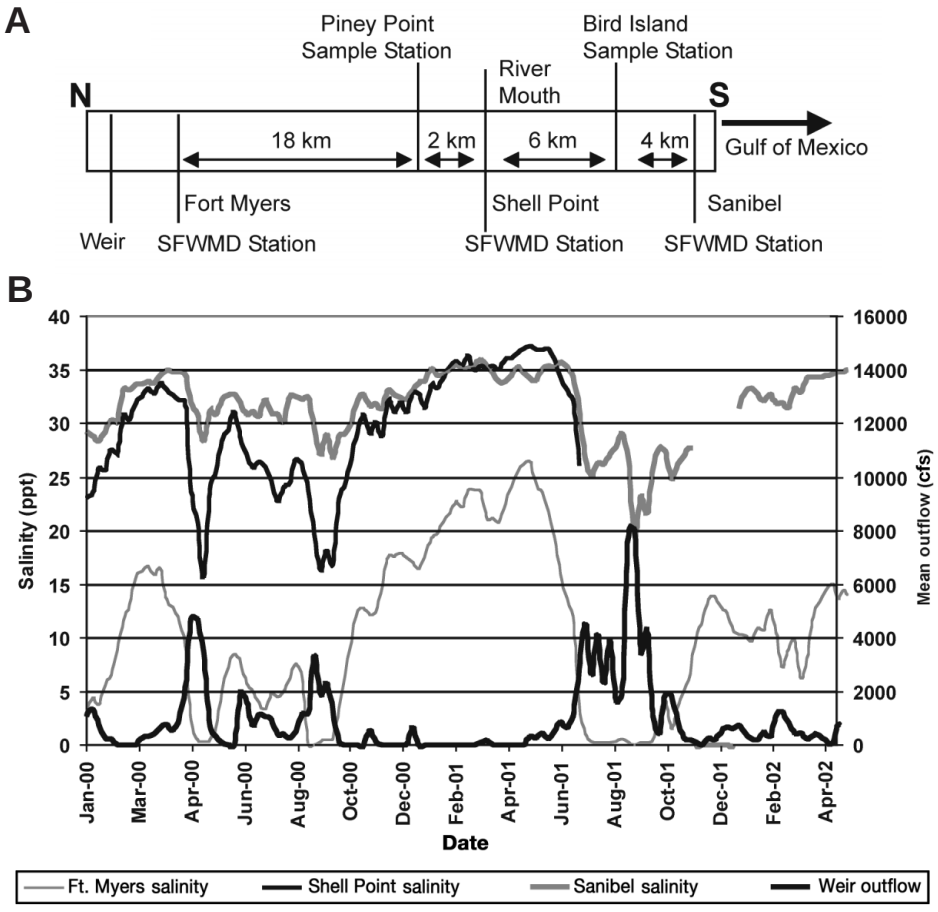


Fig. 1. (A) Stylized map denoting field site and nearby South Florida Water Management District (SFWMD) water quality monitoring stations. (B) Weekly salinities at the Fort Myers (20 km from the mouth of the River), Shell Point (mouth of the River) and Sanibel (10 km from the mouth of the river in San Carlos Bay) SFWMD water quality monitoring stations and freshwater discharge via weir from Lake Okeechobee into the Caloosahatchee River (in cfs [cubic feet per second] = 0.02832 m^3 s^{-1}) from January 2000 to February 2002. Piney Point, our upstream field study site, is 2 km upstream of the river mouth, while Bird Island, our downstream field study site, is 6 km downstream of the river mouth

In July, a significant interaction in *Perkinsus marinus* infection intensities was found between type of treatment and time of sampling ($p < 0.001$). *P. marinus* infection intensities of oysters exposed to the freshet were significantly lower than the infection intensities of control oysters on Days 7, 14 and 21 (Fig. 2). Infection intensities of treatment oysters were significantly reduced from Day 7 ($3.54 \times 10^5 \pm 5.90 \times 10^5$) to Day 14 ($7.22 \times 10^3 \pm 1.10 \times 10^4$ parasites g^{-1} wet tissue) and Day 21 ($2.37 \times 10^4 \pm 5.10 \times 10^4$ parasites g^{-1} wet tissue). Initial infection intensity of oysters sampled prior to the freshet was $7.06 \times 10^5 \pm 1.82 \times 10^6$ parasites g^{-1} wet tissue. The infection intensity of control oysters tended to increase with time from $3.52 \times 10^6 \pm 6.60 \times 10^6$ parasites g^{-1} wet tissue on Day 7 to $4.75 \times 10^6 \pm 8.70 \times 10^6$ parasites g^{-1} wet tissue on Day 14, and $6.95 \times 10^6 \pm 9.70 \times 10^6$ parasites g^{-1} wet tissue on Day 21.

In December, no interaction in *Perkinsus marinus* infection intensities was found between type of treatment and time of sampling. *P. marinus* infection intensities of oysters exposed to the freshet were significantly lower than control oysters ($p < 0.003$) (Fig. 2). No significant effect of time of sampling was found. Initial infection intensities in oysters sampled prior to the freshet was $6.66 \times 10^5 \pm 1.29 \times 10^6$ parasites g^{-1} wet tissue.

Comparison of initial *Perkinsus marinus* infection intensities in oysters sampled prior to the simulated freshet events revealed that they were not significantly different between season ($p = 0.07$), although oysters collected in April had approximately 3-fold less infection intensities ($2.09 \times 10^5 \pm 1.70 \times 10^5$) than oysters collected in July ($7.06 \times 10^5 \pm 1.82 \times 10^6$) or December ($6.66 \times 10^5 \pm 1.29 \times 10^6$).

Oyster mortality

No significant difference in cumulative oyster mortality was found between oysters exposed to freshet and control oysters in April or December (Fig. 3). In July, the cumulative mortality of oysters exposed to the freshet (69%) was significantly higher than that of control oysters (12%) (Fig. 3). There was a significant difference in cumulative mortality between oysters exposed to a freshet in April (1.6%), July (69%) and December (11%). Likewise, there was a significant difference in mortality between control oysters in April (0.8%), July (12%) and December (5%).

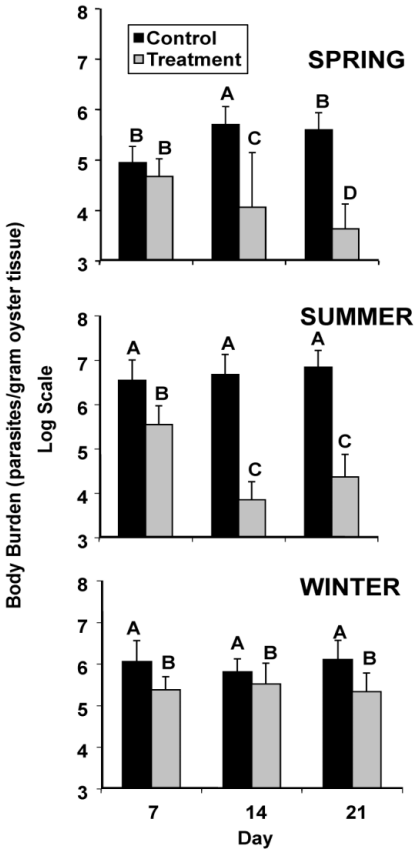


Fig. 2. Infection intensities of control oysters and in oysters exposed to freshet events in the spring, summer and winter, sampled on Days 7, 14, and 21 of the simulated freshet event. Within each seasonal graph, bars with different letters were significantly different ($p < 0.05$). Error bars represent +SD

Oyster condition index

In April, significant interaction in oyster condition index was found between type of treatment and time of sampling ($p < 0.0001$). No significant differences existed in condition index between oysters exposed to the freshet and control oysters until Day 21 (Fig. 4). On Day 21, oysters exposed to the freshet had a high condition index of 2.9 ± 1.00, and control oysters reached a low condition index of 1.9 ± 1.03. Initial condition index of oysters sampled prior to the freshet was 2.67 ± 0.56.

In July, a significant interaction was found between type of treatment and time of sampling for the oyster condition index ($p < 0.0001$). Oysters exposed to the freshet experienced a rapid and significant decline in condition index from Day 7 (2.40 ± 0.99) to Day 14 (1.50 ± 0.58) and from Day 14 to Day 21 (0.8 ± 0.27) (Fig. 4). Release of gametes was observed in the tank (i.e. milky water) in oysters exposed to the freshet. Initial oysters had a mean condition index of 1.82 ± 1.59 from which control oysters did not deviate significantly on Day 7 (2.60 ± 1.01), Day 14 (2.40 ± 0.82) or Day 21 (2.20 ± 0.83).

In December, no significant difference in condition index was found between control oysters and oysters exposed to the freshet (Fig. 4). Condition index in these groups of oysters ranged from 1.54 ± 0.44 to 1.79 ± 0.48, and the initial condition index of oysters sampled prior to the freshet (1.73 ± 0.39) was in this range.

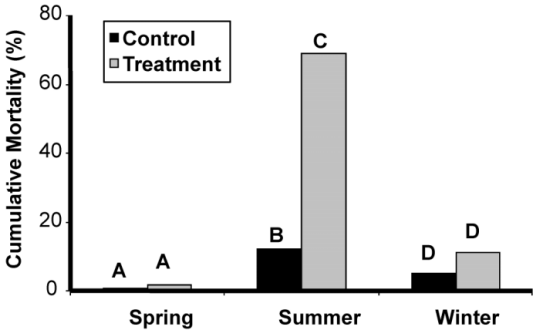


Fig. 3. Cumulative mortality of control oysters and in oysters exposed to a simulated freshet event in the spring, summer and winter. Within each season, bars with different letters were significantly different ($p < 0.05$). Seasonal mortalities were not statistically compared

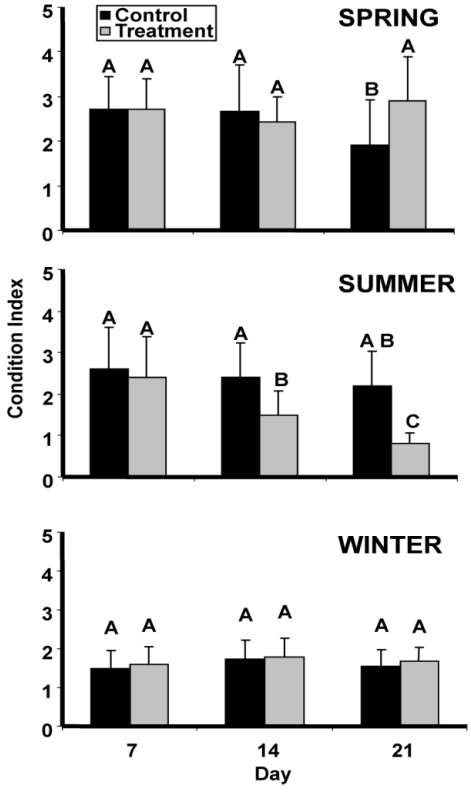


Fig. 4. Condition index of control oysters and in oysters exposed to freshet events in the spring, summer and winter, sampled on Days 7, 14 and 21 of the simulated freshet event. Within each seasonal graph, bars with different letters were significantly different ($p < 0.05$). Error bars represent +SD

Plasma osmolality

In July, a significant interaction was found between the type of treatment and the time of sampling for oyster plasma osmolality ($p < 0.0001$). Plasma osmolality of oysters exposed to the freshet was significantly lower than plasma osmolality of control oysters on Days 7, 14 and 21; Fig. 5). There was a significant decrease in plasma osmolality of oysters exposed to the freshet between Day 7 (218 ± 44 mOsm kg^{-1}) and Day 21 (101 ± 29 mOsm kg^{-1}). Initial plasma osmolality of oysters sampled prior to the freshet was 619 ± 116 mOsm kg^{-1}. In the first week after the simulated freshet event, plasma osmolality showed a rapid reduction to 268 ± 8 mOsm kg^{-1} on Day 2 and 196 ± 26 mOsm kg^{-1} by Day 4 (Fig. 6).

In December, no significant interaction in oyster plasma osmolality was found between the type of treatment and the time of sampling. Plasma osmolality of oysters exposed to the freshet was significantly lower than in control oysters ($p < 0.003$) (Fig. 5). Plasma osmolality in oysters exposed to the freshet ranged between 330 ± 127 mOs kg^{-1} and 275 ± 71 mOsm kg^{-1}, and plasma osmolality of control oysters ranged from 679 ± 15 mOsm kg^{-1} to 702 ± 51 mOsm kg^{-1}.

No significant effect of time of sampling was found. Initial oyster plasma osmolality in oysters sampled prior to the freshet was 663 ± 57 mOsm kg^{-1}.

Field study

Water temperature and salinity

The discrete measures of temperature and salinity taken at the collection sites on sample days ranged between 16 and 31°C and 3 and 39 ppt respectively

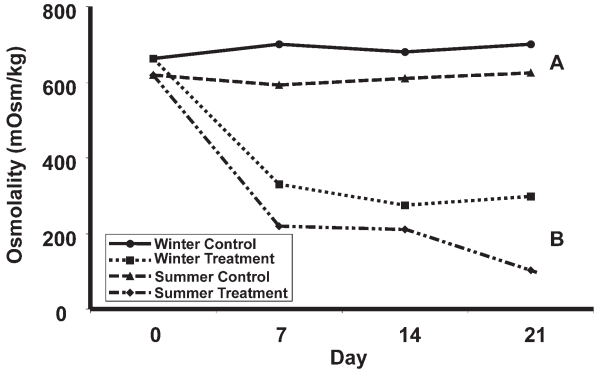


Fig. 5. Osmolality of plasma collected from control oysters and from oysters exposed to freshet events in summer and winter sampled on Days 0, 7, 14 and 21 of the simulated freshet event. Groups of lines with different letters were significantly different ($p < 0.05$)

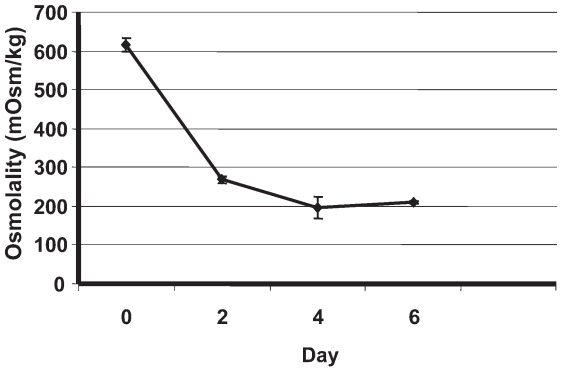


Fig. 6. Plasma osmolality measured prior to the freshet event (Day 0), and at Days 2, 4 and 6 following the freshet event. Plasma osmolality was measured in 10 oysters exposed to the simulated freshet on each day during July 2001. Error bars represent ± SD

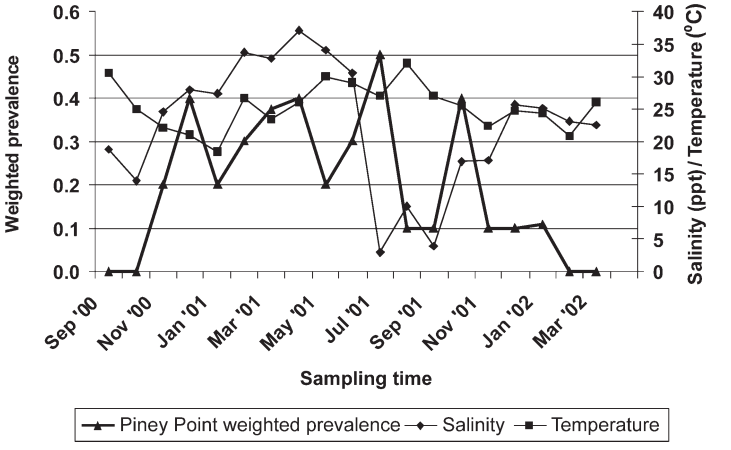
(Fig. 6). Daily salinity data from the closest SFWMD water quality monitoring stations suggest that salinity at Piney Point was lower than 8 ppt and may have reached 0 ppt on certain days between August and November 2001 (Fig. 1). Salinities at the Shell Point monitoring station appear to track closely salinities at the Sanibel monitoring station, but demonstrate a much greater freshening to freshwater releases. Increased freshwater flow at the weir in June 2000 and October 2000 was followed by a decrease in salinity to a low of 12.5 and 10.5 ppt at the Shell point monitoring station, and to a low of 27 and 23.5 ppt at the Sanibel monitoring station. An even greater decrease in salinity was measured at the Sanibel monitoring station from mid-July to mid-October 2001, with a low of 16 ppt recorded in September 2001. Unfortunately, the water quality probe at Shell point monitoring station was not functioning during that period of time. However, following the trend observed before July 2001 between salinities at the Sanibel and Shell Point monitoring stations, it is likely that salinities at the Shell Point monitoring station and at Piney Point sample station, 2 km upstream, would have dropped to near freshwater for at least a few weeks in September 2001. Similarly, it is likely that salinity at Bird Island would have dropped below 10 ppt, and possibly 5 ppt during this time. This contention is supported by a salinity model developed to predict the effects of the weir from Lake Okeechobee on the downstream river and estuary (Bierman 1993). Specifically, the model predicted that moderate mean monthly discharges of only 2000 cfs (cubic feet per second = ca. 57 m^3 s^{-1}) would result in much of the river upstream of Shell Point becoming nearly fresh water, while inflows greater than 4000 cfs (= ca. 113 m^3 s^{-1}) would cause the entire estuary upstream of Shell Point to become fresh water (Bierman 1993).

Perkinsus marinus weighted prevalence

Significant monthly differences were noted in *Perkinsus marinus* weighted prevalence ($p < 0.001$) at both stations, but no seasonal trends were noted. The upstream station, Piney Point, had significantly lower weighted prevalence (mean = 0.20) compared to the downstream station, Bird Island (mean = 0.46) (Fig. 7).

Condition index

Significant differences were observed in monthly oyster condition index ($p < 0.001$). Condition index at both sampling stations was higher during the cooler months into spring (January to May) and decreased from May to October (Fig. 8).



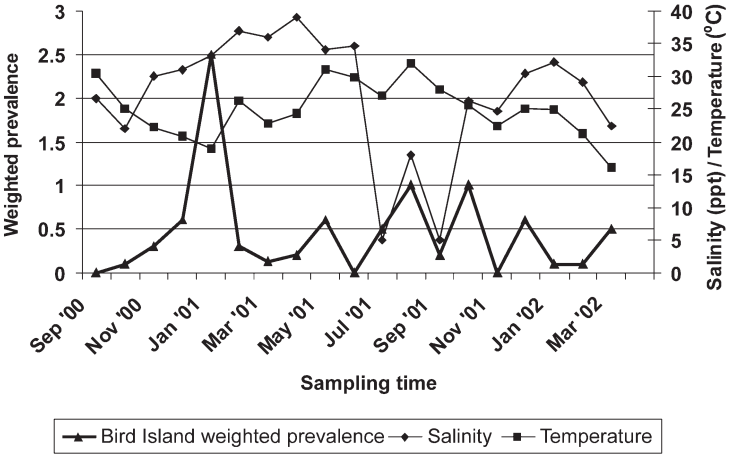


Fig. 7. Monthly *Perkinsus marinus* weighted prevalence, salinity and temperature at Piney Point and Bird Island, the field study sites in the Caloosahatchee estuary, from September 2000 to February 2002. Ten randomly selected oysters were sampled every month at both sites to determine *P. marinus* weighted prevalence. Temperature and salinity values were taken at the surface during sampling

Juvenile oyster mortality and growth

No significant mortality was observed in juvenile oysters between September 2000 and February 2002. Mortality of juvenile oysters during the study period, including after the freshets from July to October 2001, was less than 10%. At the same time, juvenile oysters showed significant growth ($p < 0.0001$) during the study period, increasing from a mean size of 17 mm in August 2000 to a mean size of 63 mm in February 2002 at Piney Point, and a mean size of 54 mm at Bird Island (Fig. 9). The mean growth rate of juvenile oysters at Piney Point was 2.42 ± 8.53 mm mo^{-1} compared to 1.95 ± 8.53 mm mo^{-1} at Bird Island.

DISCUSSION

Past studies demonstrated that lowered salinities (below 12 ppt) retarded *Perkinsus marinus* disease development in oysters (Ray 1954, Andrews & Hewatt 1957, Chu et al. 1993, Ragone & Burreson 1993). Despite field observations documenting a lack of *P. marinus* infection in areas prone to freshet events, no study, field or laboratory, that we are aware of has explicitly determined experimentally the impact of freshets on *P. marinus* infection intensities in oysters. This controlled quantitative assessment of the effects of freshet events on both *P. marinus* infection intensities and oyster condition in the laboratory provides information on the effect of single freshet events and their potential for use in controlling *P. marinus* infections, while maintaining the viability of oyster cultures. This field study documents the links between a highly variable salinity regime marked by seasonal freshet events, and a maintained low *P. marinus* infection level.

Effects of freshet events on *Perkinsus marinus* infections and oysters

Inherent to a study examining the response of both a host and a parasite to external conditions is the fact that the response of the host can have impacts on the conditions that the parasite experiences. Therefore, despite evidence demonstrating that acute exposure of *in vitro* cultured *Perkinsus marinus*, transferred from 22 to 0 ppt, resulted in >99% mortality (Burreson et al. 1994), the oyster's

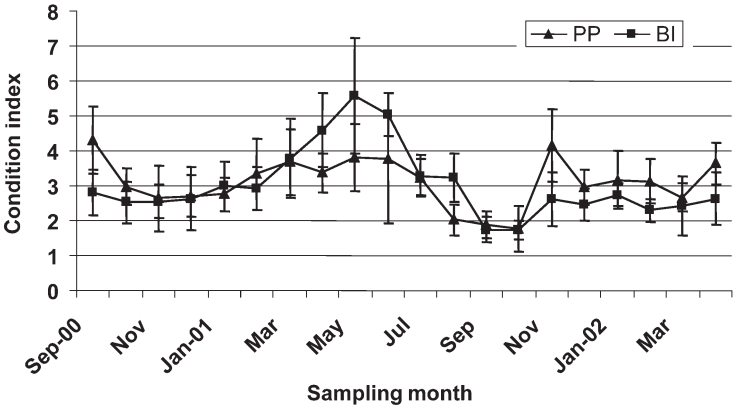


Fig. 8. Condition index (CI) of oysters at our field study sites, Piney Point (PP) and Bird Island (BI), in the Caloosahatchee estuary. Ten oysters from each location were sampled monthly from September 2000 to February 2002 and CI determined according to the procedure of Lucas & Beninger (1985). Results presented are monthly means ± SD

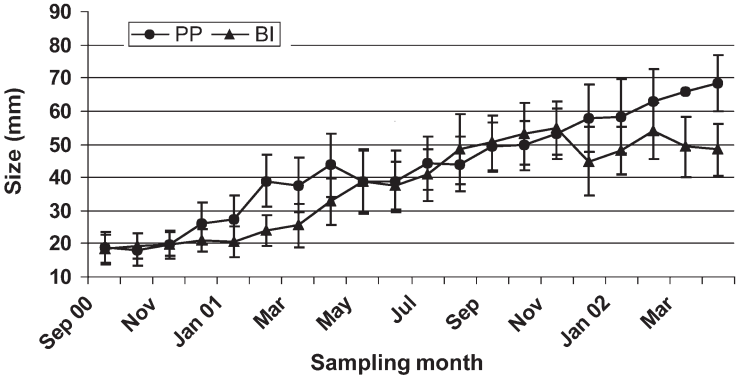


Fig. 9. Mean size (±SD) of juvenile oysters at our sampling sites, Piney Point (PP) and Bird Island (BI), in the Caloosahatchee estuary. Two hundred juvenile oysters were placed in a 0.5 m^2 wire mesh cage (0.5 × 0.5 mm mesh size) at each sampling station. The length of 50 randomly selected oysters was measured monthly at each field site from September 2000 to February 2002

response to the freshet event needed to be examined to understand the conditions that *P. marinus* experienced *in vivo*. Along with oyster mortality rates, 2 clues from the oyster's response that provide further information on the environment that the parasite had to deal with were examined: oyster plasma osmolality and oyster condition index.

The controlled laboratory study indicated that *Perkinsus marinus* infection intensities in eastern oysters were significantly reduced by all 3 simulated freshet events. The simulated freshet events failed, however, to completely eliminate *P. marinus* infections. The lack of complete *P. marinus* elimination was likely due to a combination of factors, including the failure of plasma to reach very low osmolality (<50 mOsm kg^{-1}) and the acclimation of surviving parasites to lowered plasma osmolalities. Mean plasma osmolalities of oysters exposed to freshets ranged between 274 and 330 mOsm kg^{-1} in December and between 218 and 101 mOsm kg^{-1} in July. Interestingly, the largest decrease in *P. marinus* infection intensity (by 99%) following a freshet occurred in July when plasma osmolality was lowest, while *P. marinus* infection intensity was only reduced to 66% in oysters exposed to a freshet in December. The result must be interpreted with caution, however, because it is also possible that the greater mortality of oysters in July could have contributed to the significant decrease in infection intensities if oysters with heavier infection intensities died at a faster rate than oysters with lower infection intensities.

Most of the reduction of *Perkinsus marinus* infection intensity is likely due to the rapid decrease in oyster plasma osmolality. *In vitro* studies with cultured *P. marinus* have shown that the viability of parasites measured 24 h after their transfer from a salinity of 22 ppt (~660 mOsm kg^{-1}) to salinities of 9 ppt (~270 mOsm kg^{-1}) was reduced to 57%. Viability was 30% after transfer from 22 to 6 ppt (~180 mOsm kg^{-1}) and 10% after transfer from 22 to 3 ppt (~90 mOsm kg^{-1}). When measured every other day, plasma osmolality was found to decrease rapidly following the freshet event, and remained low throughout the freshet event. This decrease in plasma osmolality, combined with past *in vitro* findings using cultured *P. marinus*, explains the range of reduction of *P. marinus* infection intensities observed in our *in vivo* studies.

The grossest measure of the effects of a simulated freshet event on *Crassostrea virginica* is the mortality rate of oysters. There was a pronounced seasonal effect of the freshet events on oyster mortality in the laboratory experiments. The mortality of oysters exposed to the freshet in April and December remained low throughout each experiment and was not significantly different than the mortality of control oysters. In contrast, oysters exposed to the freshet in July experienced 69% cumulative mortality by Day 21 compared to 12% cumulative mortality of control oysters. It is likely that some confounding factor, such as high temperature, high initial infection intensities and spawning stress of oysters collected in July, led to the high mortality. The laboratory results suggest that oysters with moderate to heavy *Perkinsus marinus* infection intensities in the field would be able to survive freshet events (at least for 21 d) in winter and spring but not in summer. This would agree with

field surveys that have noted that *C. virginica* can survive salinities below 5 ppt, especially when water temperatures are low (Butler 1949, Loosanoff 1953, Andrews et al. 1959, Galtsoff 1964, Austin et al. 1993, Winstead 1995).

In the field study, oysters at our more freshwater dominated sampling station, Piney Point, encountered salinities of less than 10 ppt for at least 3 mo, mid-July through mid-October 2001, and probably salinities less than 3 to 5 ppt for at least 2 to 3 wk in September 2001, with no significant mortalities in deployed juvenile oysters. The overall mortality in deployed juvenile oysters at both sampling stations was less than 10% at the end of the study. While the mortality of wild adult oysters collected at both sites was not measured, it is likely to be less than juvenile oysters, since juvenile oysters are generally more sensitive to freshets and other stress factors than adult oysters. The much lower mortality of Florida oysters in summer 2001, compared to the Louisiana oysters collected in July 2001 and exposed to a simulated freshet in the laboratory, could be due to their much lower initial *Perkinsus marinus* infection intensities.

The laboratory experiment results also indicated that a freshet event of up to 3 wk in length in spring or winter would not adversely affect the condition index of oysters. April- and December-simulated freshets resulted in no significant differences in oyster condition index between control and treatment, except for on Day 21 in April, when control oysters had a lowered condition index. In contrast, a simulated freshet in July may exacerbate already stressed oysters (from high *Perkinsus marinus* infection intensities, high temperatures and spawning) and be detrimental to the oyster populations. Clearly, in July, from Day 7 onwards, there was a reduction in condition index indicating that the oysters were stressed. Part of the reduction in condition index is likely due to spawning, since the release of gametes was observed in the tank following the freshet event. Oysters in Gulf waters have an extended spawning season from April to October in this subtropical region, with gametogenic recycling and sometimes up to 3 spawning events occurring during this period (Hayes & Menzel 1981, Supan & Wilson 2001).

Potential management implications

All natural systems exhibit environmental variability, ranging from predictable seasonal variations to extreme El Niño Southern Oscillation events to intended and unintended human effects. These events, acting on 'primary' environmental variables (i.e. salinity, temperature), have been shown to affect fish stocks (Houde 1997), survival of aquatic organisms (Hobday & Boehlert 2001), habitat use (Peebles & Flannery 1992), as well as impact parasite survival (Bataller & Boghen 2000). Several studies with oysters have found that environmental variability works to eliminate parasites without detrimental effects on the oysters (Haskin & Ford 1982, Ford 1985, Ford & Haskin 1988, Bataller & Boghen 2000), suggesting that managed environmental variability, such as freshet events, has the potential to be a valuable management tool.

In past studies examining the effects of low salinities on *Perkinsus marinus*, results indicated that a reduction of *P. marinus* associated with low salinities was quickly replaced by a rapid proliferation of *P. marinus* once more favorable conditions for the parasite (i.e. higher salinity) returned (Ragone Calvo & Burreson 1994, Fisher & Oliver 1996, Ford 1996, Ford et al. 1999). For freshet events to become a useful management tool in reducing oyster mortality from *P.marinus*, *P. marinus* must either be eliminated in order to prevent its likely rapid proliferation once the freshet event is over, or the freshet events must be repeated in order to maintain it at low levels. Clearly, a single freshet event, similar to our simulated ones, lasting up to 3 wk will not have any significant or lasting effects on the intensity of *P. marinus* infection in an oyster population moderately to heavily infected with the parasite. The use of repeated freshet events may be worth investigating.

A number of investigators have noted that locations characterized by the regular occurrence of freshet events lack significant *Perkinsus marinus* infections (Soniat & Gauthier 1989, Thurston et al. 2001, Volety et al. 2001a,b). Interestingly, higher oyster densities and overall oyster bar growth in Apalachicola Estuary were reported to occur in the vicinity of the confluence of high salinity water and river-dominated low salinity water (Livingston et al. 2000), hence in areas prone to high variation in salinity. In this instance, oyster growth was positively correlated with variation (i.e. standard deviation) in salinity. Further investigation into the actual site characteristics in terms of timing, frequency and length of these freshet events may provide some clues as to the extent of environmental variability that may be correlated, in these instances, with the lack of significant *P. marinus* infections in the oysters.

Based on our discrete monthly samples, depicting an environment characterized by high water temperatures (16 to 31°C) and salinities (3 to 39 ppt) at our sampling sites in the Caloosahatchee estuary, much higher infection intensities in oysters, similar to other studies, were expected (Ragone Calvo & Burreson 1994, Soniat 1996). In the warm water months, from May to October when temperatures exceed 28°C, salinity was variable, and likely reached extreme lows (below 5 ppt at Piney Point, and below 8 ppt at Bird Island) several times due

to the combination of freshwater releases from Lake Okeechobee and heavy rainfall in the basin (Bierman 1993). The numerous freshwater releases from the weir and the high rainfall in the Caloosahatchee River provide a likely explanation for the low *Perkinsus marinus* weighted prevalences we detected, and the lack of a strong seasonal (temperature-related) pattern. *P. marinus* weighted prevalence in oysters at our fresh water site, Piney Point, appeared to follow a trend with decreasing prevalence from 0.5 to 0.1, as salinity was drastically reduced during the summer of 2001. Our more saline site, Bird Island, did not show a significant decline in weighted prevalence, but rather, failed to exhibit any significant peak in weighted prevalence, likely because salinity was drastically reduced in 2001 during the months with warmer temperatures.

Similar decreases in *Perkinsus marinus* weighted prevalence have also been noted to occur concomitantly with decreased salinities in other southwest Florida estuaries (Thurston et al. 2001, Volety et al. 2001a,b). For example, *P. marinus* infection intensities decreased in Blackwater River, Henderson Creek, and Faka-Union estuaries during summer months, a period characterized by heavy rains and the release of freshwater from upstream areas resulting in several freshet events and extremely low salinities. *P. marinus* infections decreased from 0.7, 0.8, and 0.6 to 0.01, 0.08, and 0.05 in Blackwater River, Henderson Creek, and Faka-Union respectively, from July to September 2001.

The overall success of the oyster industry depends on our ability to manage oyster populations in estuaries where the parasite is widespread and abundant. Andrews & Ray (1988) suggested that management measures that support the diversion of fresh water into high salinity areas may be an effective means to control *Perkinsus marinus* in the Gulf of Mexico. Some limited freshwater diversions from the Mississippi River in the 1980s have been cited as being effective in enhancing oyster production, although it is not clear what the impacts were on *P. marinus* (see Andrews & Ray 1988). Recent data from the large Caernarvon diversion water control project in Louisiana demonstrated a significant increase in oyster production associated with increased freshwater flows (Villarubia 1998[1]). Most importantly, data from our field study seem to support the idea that repetitive and well-timed freshet events can prevent infection of oysters with *P. marinus*, or maintain *P. marinus* infection to non-lethal intensities (e.g. $<10^6$ parasites g^{-1} wet tissue) in oyster populations. The use of an adaptive management approach involving control of freshwater inflows could be invaluable to the oyster industry.

[1]Ecosystem response to a freshwater diversion: the Caernarvon experience; available at www.lacoast.gov/programs/Caernarvon/index.htm

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