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The Potential for Transmission of Perkinsus Marinus by Fecal Matter from the Eastern Oyster, Crassostrea virginica

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THE POTENTIAL FOR TRANSMISSION OF *PERKINSUS MARINUS* BY FECAL MATTER FROM THE EASTERN OYSTER, *CRASSOSTREA VIRGINICA*

A Thesis

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Master of Science

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by

Christine H. Scanlon

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APPROVAL SHEET

This thesis is submitted in partial fulfillment of
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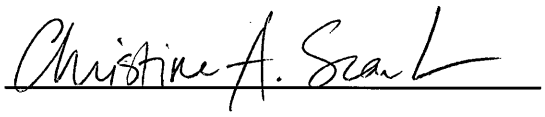

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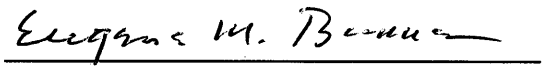

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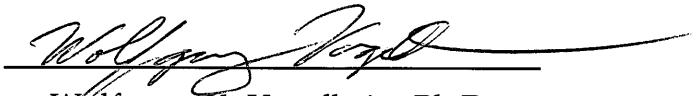

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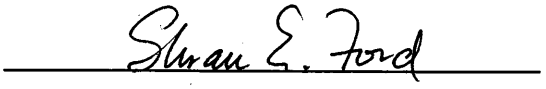

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DEDICATION

To my parents, John and Mary, for their love, support, and foresight enough not to listen to the nuns and to realize that I was more than just mischievous back in kindergarten. Thank you for showing me that there were things just as important as academics for me to participate in while growing up and for the genuine interest you took in both my academics and these everyday activities. I appreciated every instance of this, from long rainy days on a riverbank just to see me row by for a few seconds, to attending my research presentations in college, to wanting to look in the microscope to see what *Perkinsus* cells look like. It gave whatever I did meaning, perspective, and importance. And finally, thank you for giving Patricia and I our educations while simultaneously demonstrating that health, happiness, a sense of humor, love, friends, and family are by far the most important things in life.

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ABSTRACT

Transmission of *Perkinsus marinus*, an important pathogen of the eastern oyster, *Crassostrea virginica*, has been thought to occur via the dispersal of infective *P. marinus* cells upon death and decomposition of infected oysters. However, recent studies have demonstrated the presence of *P. marinus* in fecal matter from live, heavily infected oysters. It has been hypothesized that fecal elimination of *P. marinus* cells may be an important mechanism for transmission, as well as a nondestructive and noninvasive method for estimating infection intensity. The purpose of this project was to examine the role of fecal matter in direct transmission of the parasite. Three experiments were conducted to elucidate this role.

For the first experiment, the infection progression experiment, the abundance of *P. marinus* in the hemolymph and feces of naturally-infected individual oysters was monitored over a period of five months in order to determine the correlation of fecal parasite abundance with infection intensity as estimated from the oyster hemolymph. The mean abundance of *P. marinus* cells per mg feces for each month ranged from 2 to 34 cells. The abundance of *P. marinus* in the feces of infected oysters was positively correlated with *P. marinus* abundance in the hemolymph. A high amount of variability in individual oyster fecal parasite abundance was observed between sampling days. The maximum variability observed in an individual oyster over a 24 hour period was a difference of 80 cells per mg feces.

For the second experiment, the dosing experiment, uninfected oysters were dosed with feces from naturally-infected oysters in order to determine if the fecal matter from *P. marinus*-infected oysters was infective to previously uninfected oysters. This dosing resulted in 100% prevalence of *P. marinus* infections in all exposed oysters with infection intensities ranging from 3 to 128 cells per oyster after 20 days of exposure and a 3 week post-exposure holding period.

For the third experiment, the paired experiment, uninfected oysters were paired with naturally-infected oysters in individual containers in order to determine if infections would result from holding a live, *P. marinus*-infected oyster in close proximity to a previously uninfected oyster. These pairings resulted in 100% prevalence of *P. marinus* infections in all exposed oysters, with infection intensities ranging from 13 to 27,500 cells per oyster after 56 days of exposure and a 7 week post-exposure holding period.

Results from these experiments indicate that fecal elimination of *Perkinsus marinus* results in transmission of the parasite in the laboratory. Further study will be necessary to clearly identify the role that fecal transmission plays relative to the other modes of *P. marinus* transmission in nature. The high amount of variability in fecal parasite abundance observed between sampling days may preclude the use of the fecal assay as a diagnostic tool for *P. marinus* despite its advantages of being both noninvasive and nondestructive.

THE POTENTIAL FOR TRANSMISSION OF *PERKINSUS MARINUS* BY FECAL MATTER FROM THE EASTERN OYSTER, *CRASSOSTREA VIRGINICA*

INTRODUCTION

Historical Perspective and Nomenclature

Perkinsus marinus is a warm-season protistan pathogen that parasitizes eastern oysters, *Crassostrea virginica* (Andrews and Ray 1988). The disease caused by *Perkinsus marinus* was first discovered in the Gulf of Mexico in 1948 (Mackin et al. 1950). Significant mortality of oysters in 1946 in the Gulf of Mexico prompted Louisiana oystermen to file lawsuits against several major oil companies alleging that the mortality was due to in-shore petroleum operations. Several major research groups were commissioned to elucidate the role of the petroleum operations in the oyster mortalities (Ray 1996). Eventually the investigators found that the oil operations were not the cause of the oyster mortalities and in 1950 the causative agent was described as a fungus *Dermocystidium marinum* by Mackin, Owen, and Collier (1950) (Ray 1996). In 1949, several researchers working on this problem, including Mackin, went to Virginia and found the parasite to be present in eastern oysters growing in environments away from oil fields, thus being the first documentation of the parasite in the Chesapeake Bay (Andrews and Hewatt 1957, Andrews 1988). Around this time, Ray's discovery of fluid thioglycollate medium combined with Lugol's iodine stain as an effective diagnostic method for the presence of the parasite greatly reduced the time necessary for diagnosis as compared with the previous histological diagnostic methods (Ray 1952, 1996).

Since its discovery, the parasite has been reclassified twice. First it was reclassified as *Labyrinthomyxa marina* because of the observation of gliding cells similar to those in slime molds (Mackin and Ray 1966). Through

ultrastructural observations, the presence of an apical complex in the biflagellate zoospores produced by the organism was documented (Perkins 1976). This led to the parasite's present classification in the phylum Apicomplexa, class Perkinsasida, genus *Perkinsus* (Levine 1978), despite differences in life cycle characteristics from other members of the phylum (Vivier 1982, Reece et al. 1997).

The phlyogenetic affinities of *P. marinus* were investigated using morphology, 18S-like ribosomal DNA data and actin sequence data by Siddall et al. (1997). Morphological studies indicated that some criteria, such as the trilaminar pellicle and micropores, used to place *Perkinsus* species in the Apicomplexa are common to all alveolates which include the ciliates, dinoflagellates and the apicomplexans. In addition, in all aflagellate apicomplexans, an apical complex is found, but the apical complex is not present in the flagellated microgamete stage. In *Perkinsus*, the conoid structure is found only in the flagellated zoospore stage and not in any aflagellate stage in the life cycle. Siddall et al. (1997) concluded that *Perkinsus* does not have a conoid and does not have an apical complex. When considered separately, 18S-like ribosomal DNA data and actin data sets each support a closer affinity of *P. marinus* with the dinoflagellates than with the Apicomplexans although each data set possesses its own individual biases and weaknesses. Using the phylogenetic principle of total evidence which combines data sets in simultaneous analyses, Siddall et al. (1997) found that a more robust hypothesis could be supported that is stable to both character and taxonomic sampling. The resulting cladogram from this analysis strongly corroborates the placement of *Perkinsus* species as sister group to the Dinoflagellida and not with the Apicomplexans (Siddall et al.

1997). However, *P. marinus* remains classified with the Apicomplexans at this time.

The Parasite in Chesapeake Bay and its Present Geographical Distribution

Perkinsus marinus activity is greatly influenced by salinity within the Chesapeake Bay region, and prevalence and intensity of the pathogen can increase during drought years (Burreson and Ragone Calvo 1996). *Perkinsus marinus* requires a salinity of 12‰ for a full epizootic to occur and the parasite has reduced pathogenicity at salinities below 9 to 10‰ (Ragone and Burreson 1993). Chu et al. (1993) found that oysters held at a salinity of 3‰ acquired light infections when inoculated with a known concentration of parasites isolated from infected oysters, thereby demonstrating the high tolerance of *P. marinus* to low salinities (Ford 1996b). The parasite kills oysters at temperatures above 20°C (Andrews and Hewatt 1957, Andrews 1988). However, *P. marinus* can persist when low temperatures and salinities occur during winter and spring (Andrews 1988). The most important environmental factor affecting the geographic distribution of *P. marinus* is temperature, (Ray and Mackin 1954, Andrews and Hewatt 1957, Quick and Mackin 1971) and it appears that in the Chesapeake Bay region, the pathogen's activity and annual periodicity are largely controlled by seasonal temperature fluctuations (Burreson and Ragone Calvo 1996). Probably more important than either factor acting alone is the interaction of both temperature and salinity (Burreson and Ragone Calvo 1996).

Perkinsus marinus has been the most egregious pathogen of the eastern oyster, *Crassostrea virginica*, since 1987 because of its widespread distribution and persistence in low salinity areas (Burreson and Ragone Calvo 1996). Prior to the late 1980s, the parasite was found only in high

salinity portions of coastal bays and estuaries along the Atlantic coast south of Delaware Bay; however, the seaside bays of the eastern shore of Virginia and Maryland were apparently free of *P. marinus* (Andrews 1988). Until the late 1980's, *P. marinus* had always been responsible for some oyster mortality, but because of the large natural settlements on public beds and good seed-oyster availability for private planters in Virginia, it did not significantly affect the harvest during most years.

The distribution and epizootiology of *P. marinus* in the Chesapeake Bay changed from historical patterns as a result of four consecutive drought years and concomitant warm winters from 1985 to 1988 during which low stream flows resulted in higher than average salinities in upper tributary waters (Burreson and Ragone Calvo 1996). During this time, the parasite spread to all productive oyster grounds in Chesapeake Bay either by natural processes or by transplantation of infected oysters. The pathogen was able to survive in areas that had previously been disease-free because of the elevated salinities and warm winters (Burreson and Ragone Calvo 1996).

In addition to the altered distribution of *P. marinus* in the Chesapeake Bay, a large scale geographic expansion in the parasite range was also observed during this time period. As of late 1995, the range of *P. marinus* is known to be from as far north as the Damariscotta River, Maine (Kleinschuster and Parent 1995, Ford 1996c), south throughout the bays and estuaries along the east coast of the United States, and through the Gulf of Mexico as far south as Tabasco, Mexico (Burreson et al. 1994).

Parasite Background, Infectivity, and Transmission

Although the life cycle of *P. marinus* is not completely understood, three life stages have been identified and described: meront,

prezoosporangia, and biflagellated zoospore (Perkins 1966, Perkins 1988, Chu 1996). Merozoites, or immature meronts are usually found in the phagosomes of oyster hemocytes and are 2-4μm in size. Meronts are 10-20μm in size with an eccentrically located vacuole which often contains a refringent body, the vacuoplast (Chu 1996). This eccentric vacuole forces the nucleus to one edge of the cell, which results in the appearance of the signet ring stage (Ford 1996b, Mackin et al. 1950). An 8 to 32 cell stage enclosed within a cell wall, is a sporangium or schizont of 10-40μm (Chu 1996). The schizonts rupture and release individual merozoites, which develop into meronts and repeat the cycle (Ford 1996b). When placed in fluid thioglycollate medium for 4-5 days, meronts develop into prezoosporangia or hypnospores. These prezoosporangia are observed in dead and moribund oyster tissues and may enlarge to 150μm in fluid thioglycollate media. Zoosporulation, or the production of biflagellated zoospores, usually occurs after incubating thioglycollate-cultured prezoosporangia in sea water for 4-5 days. It is not yet clear whether prezoosporangia released in sea water from moribund and dead oysters would actually undergo zoosporulation in nature (Chu 1996).

Any of the three identified life cycle stages of *P. marinus* are capable of initiating infections in the laboratory (Ray 1954, Andrews 1988, Chu 1996). It is not known, however, which life stage is the principal one for transmitting infections in the field, but recent experiments conducted by Chu (1996) indicate that meronts are most likely the primary transmission agent in nature. Oysters that were inoculated with this life stage had a higher infection prevalence and intensity than oysters inoculated with prezoosporangia (Chu 1996). In addition, it has been reported that 99% of *P. marinus*-like cells found in the water of the upper Chesapeake Bay from

March to October between 1992 and 1993 resembled the meront stage (Dungan and Roberson 1993, Chu 1996). The cells enumerated in the study by Dungan and Roberson (1993) had *Perkinsus marinus*-like morphology and antibody staining characteristics.

The portal of entry for the parasite appears to be through filtration and feeding. Once ingested the parasite crosses the epithelium of the stomach or intestine (Ray 1954, Bushek et al. 1994, Perkins 1994, Chu 1996, Ford 1996b). Zoospores may secrete lytic substances that aid in penetration through the host tissue (Perkins 1976), and the parasites can also be carried through the epithelium via phagocytosis (Mackin 1951, Ford 1996b). The zoospores can also penetrate and encyst in the cells of the gill, labial palp, or mantle epithelium (Perkins 1988). Dungan et al. (1996) challenged uninfected oysters with *P. marinus* and the pathogen cells were routinely observed associated with the external epithelia, as well as within the gut lumina. In laboratory infection experiments conducted by Ragone Calvo and Burreson et al. (1995), few digestive tract infections were observed in contrast with a high proportion of observed parasite cells located in the mantle and gill epithelia. These results suggest that mantle and gill tissue may also serve as primary parasite entry routes in addition to the digestive tract. Once the pathogen is established in the host tissue, an increase in circulating hemocytes is observed and these hemocytes phagocytize and disperse the parasite throughout the entire oyster via the connective tissues and blood sinuses (Ford 1996b, Andrews 1988). Some parasites are destroyed by the phagocytes (LaPeyre et al. 1995), but others continue to develop within the host cells and eventually destroy them (Mackin 1951).

It has been well documented that transmission of *Perkinsus marinus* is direct from oyster to oyster (Ray 1954, Andrews 1988); however, the

natural dynamics of transmission are poorly understood (Burreson and Ragone Calvo 1996). Infection is typically thought to occur through the digestive tract as indicated by the location of foci of infection in the gut epithelium (Mackin 1951). The dose required to initiate infection and the duration of the infection window are also poorly understood (Burreson and Ragone Calvo 1996). Laboratory studies by Chu (1996) estimated that the minimal dose required to initiate a *P. marinus* infection was between 10 and 10^2 meronts or prezoosporangia per oyster. In the same study, the meront stage was found to cause much higher *P. marinus* infection prevalence and intensity in oysters than did prezoosporangia.

Andrews (1988) reported that deaths among oysters are hastened when they are located near disintegrating infected gapers or dead oysters. This phenomenon was observed in tray studies where positions of eastern oysters were fixed and the subsequent transmission of the parasite through the tray was monitored (Andrews 1988). The prevailing conceptual model in the Chesapeake Bay is that transmission occurs via the dispersal of infective *P. marinus* cells upon death and decomposition of infected oysters during periods of high oyster mortality in summer and early autumn (Andrews 1988, Burreson and Ragone Calvo 1996). However, dead, gaping oysters are consumed rapidly by scavengers (Hoese 1964) and most likely do not decompose naturally and release *P. marinus* cells into the water (Burreson and Ragone Calvo 1996). *Perkinsus marinus* can survive passage through a scavenger's gut, (Hoese 1964) but the role that these scavengers play in spreading infections is unclear (Burreson and Ragone Calvo 1996). One vector for *P. marinus* has been identified, the ectoparasitic snail *Boonea impressa*. In laboratory studies, this hemolymph-extracting gastropod

ectoparasite of oysters was capable of transmitting *P. marinus* from one oyster to another (White et al. 1987).

Alternate hypotheses of parasite dissemination include oyster host spawning or excretory activities, alternate host or vector activities, possible heterotrophic environmental proliferation of *P. marinus*, and periodic resuspension of sediment-bound infectious cells (Dungan and Roberson 1993, Ragone Calvo et al. 1995). As infections become more intense, they are often accompanied by sloughing of hemocytes, parasites, and digestive epithelium into the lumen of the stomach and intestine (Mackin 1951, Ford 1996b), which would result in the dissemination of *P. marinus* in fecal matter from live oysters. However, the role of fecal matter in transmission of *P. marinus* has been poorly documented to date.

Fecal material is a common, and usually primary route of transmission for gut parasites of fish and mammals. Unlike *P. marinus*, where fecal transmission is proposed as one of several transmission mechanisms, fecal transmission of gut parasites is the essential and often singular way that these pathogens are transmitted. Coccidians, which are generally parasites of the epithelia that line the alimentary tracts of vertebrates and invertebrates, are an example of such a group where fecal transmission is necessary for the parasite to exit the organism and infect another host (Cheng 1973).

Previous Fecal Studies

A recent study by Ford (1996a) focused on whether natural and cultured cells of *P. marinus* are equally pathogenic. As part of this project, the role of feces and pseudofeces in parasite elimination dynamics was investigated. The presence of *P. marinus* in the feces of live, infected oysters

was documented during this study. These results indicated that continuous discharge of *P. marinus* in the feces of infected oysters makes a potentially large contribution to the pool of infective stages found in enzootic waters. Further study indicated that a heavily infected oyster can release 10^3 - 10^4 *P. marinus* cells each day in its feces. This release of parasite cells is an order of magnitude different from that of 10 - 10^2 meronts or prezoosporangia per oyster estimated to be the minimal dose required to initiate a *P. marinus* infection by Chu (1996). A study by Bushek et al. (1997) focusing on infections derived by *in vitro* cultured *P. marinus* resulted in observations of parasites in feces and pseudofeces indicating two potentially important pathways of elimination. As such, fecal elimination provides an additional source of viable, and potentially infective *P. marinus* cells prior to the death of the oyster at which time there is a large release of infective *P. marinus* cells (Ford 1996a). An additional finding in Ford's study is that the abundance of *P. marinus* cells in feces was highly correlated with infection intensity and days to death. This result suggests that the abundance of parasite cells in the fecal matter of an infected oyster as ascertained by fecal sampling could potentially provide an important, nondestructive, noninvasive indicator of infection intensity and days to death.

Objectives and Hypotheses

Objectives. The overall objective of this project was to elucidate the role of fecal matter from live, naturally-infected oysters in transmission of *Perkinsus marinus*. The specific objectives of the project were fourfold: (1) to determine the presence and abundance of *Perkinsus marinus* in the fecal matter of live, naturally-infected oysters and to monitor the fecal parasite abundance as infections progressed; (2) to determine if *P. marinus*

abundance in oyster feces was correlated with infection intensity; (3) to determine if holding a live, naturally-infected oyster with a previously uninfected oyster would result in infection of the naive oyster by the parasite; (4) to determine if the fecal matter from naturally-infected oysters was infective to previously uninfected oysters.

Hypotheses. The four hypotheses for this project were: (1) that *Perkinsus marinus* cells would be present in the fecal matter of live, naturally-infected oysters; (2) that *P. marinus* abundance in oyster feces would be positively correlated with infection intensity; (3) that a previously uninfected oyster would become infected with the parasite when held with a live, naturally-infected oyster; (4) that the fecal matter from naturally-infected oysters would be infective to previously uninfected oysters.

MATERIALS AND METHODS

Oyster Collection and Maintenance

Adult oysters, collected mid-June, 1996 from Point of Shoals, James River, Virginia, were utilized as *Perkinsus marinus*-infected oysters in this study. Adult oysters from the Damariscotta River, Maine, were purchased in mid-June, 1996 from the Pemaquid Oyster Company and were utilized as uninfected oysters in this study. To ascertain whether these Maine oysters were uninfected, 25 oysters were sacrificed and a body burden quantification for *P. marinus* (Choi et al. 1989, Bushek et al. 1994, Fisher and Oliver 1996) was performed.

The oysters that were used in the study were scrubbed and briefly air-dried in order to facilitate labelling. They were then labelled according to a system that incorporated which experiment they were being used in, which number oyster they were in that experiment, and whether they were uninfected or infected. A waterproof marker was used to label the individual containers as well as the oysters.

The oysters were maintained in York River water that was passed through a series of filters: a sand filter, an activated carbon filter, two 10 micron cartridge filters, and finally two 1 micron cartridge filters. This filtration was necessary to reduce the likelihood of any infective stages of the parasite being present in the water. This filtration method has been utilized in previously conducted wet lab *P. marinus* investigations (Ragone and Burreson 1993, Chu and La Peyre 1993). The oysters were kept in individual plastic 1 liter containers. The water in each container was changed every other day, with the exception of the fecal collection periods in Experiments

#1 and #2, when it was changed daily. Water temperature and salinity were recorded immediately after each water change, and reflected that of the ambient York River water. The ambient water temperature ranged from 15.5°C to 27°C over the course of the experiments, and the salinity ranged from 14‰ to 18‰. Once pumped in, water was then equilibrated to and maintained at room temperature, 23-27°C. During the last two months of the study, incoming water temperatures reflected that of the ambient York River water because of problems experienced with the heat exchanger in the wet lab. The incoming water did not equilibrate to room temperature until after the water change rather than before. Salinity was kept at the ambient York River level. The water in each container was aerated using an airstone attached by line to an overhead manifold. The oysters were each individually fed 0.2 grams of *Thalassiosira weissflogii* algal paste daily after any necessary water changes. This algal paste was obtained from the VIMS oyster hatchery and was mixed with filtered York River water and fed to the oysters in the form of a slurry.

Experimental Protocols

Ray's (1952, 1966) fluid thioglycollate culture method involves using a fluid thioglycollate medium that causes *P. marinus* trophozoites in infected oyster tissue to enlarge to sizes that are easily observed by light microscopy after staining with iodine. This fluid thioglycollate culture method was used in the hemolymph assay, the fecal assay, and the total body burden quantification.

Hemolymph assay. A hemolymph assay for the diagnosis of *Perkinsus marinus* in eastern oysters was developed by Gauthier and Fisher (1990). The hemolymph assay is a non-destructive but invasive diagnostic

technique, enabling repeated sampling of individual oysters, while allowing approximately two weeks to elapse between samples in order to minimize the likelihood of stressing the oyster enough to cause mortality.

The protocol used for the hemolymph diagnosis for *Perkinsus marinus* is a modification of the procedure described by Gauthier and Fisher (1990). Oyster shells were notched posterior to the adductor muscle using a lapidary saw, and 300µl of hemolymph were withdrawn from the adductor muscle sinus using a 23 gauge needle and a 3cc disposable syringe. The hemolymph was placed in a microcentrifuge tube and to each tube, 1ml of fluid thioglycollate medium, or FTM, was added and fortified with 50µl of penicllin/streptomycin solution, yielding 500 units of antibiotic per ml of FTM. The samples were incubated at room temperature in the dark for a period that usually lasted 5 to 7 days, but in some instances extended up to 21 days. They were then removed from the incubator and centrifuged at 700xg for 10 minutes. The supernatant was removed by aspiration and the pellet was resuspended in 1ml of 2 molar sodium hydroxide. The samples were incubated for 30 minutes at 60°C, and centrifuged at 700xg for 10 minutes. The supernatant was again removed by aspiration, and the pellets were washed twice in deionized water, centrifuged at 700xg for 5 minutes after each wash, and the supernatant removed via aspiration. After the final wash, the pellets were resuspended in 1ml of a 15-fold aqueous dilution of Lugol's iodine stain and the samples were placed in 24-well tissue culture plates. The stained *P. marinus* cells were then quantified using an inverted light microscope at 50x magnification. If there were fewer than 200-300 cells in the well, the entire sample was viewed and counted. If there were more than 200-300 cells in a sample, three random grid fields

within the well were counted and the final cell count was extrapolated from this.

Fecal Collection and Fecal Assay. The fecal assay is a modification of the hemolymph assay protocol. The fecal assay is both non-destructive and non-invasive and it enables repeated sampling of individual oysters. Since the assay is non-invasive, it is not necessary to allow a recovery period for the oyster after sampling. As such, sampling can be done on a daily basis if necessary.

The water in the individual plastic containers was changed the day before a fecal collection to insure that all feces present at the time of collection had been discharged over the previous 24 hours. The oysters were fed after the water change, and their feces were collected the following morning. The feces can be distinguished from pseudofeces relatively easily based on appearance alone. Oyster feces have a ribbon-like appearance and are often darker in color than pseudofeces, which have a more flocculent appearance. The feces were collected using a long-tipped Pasteur pipet, and the samples were added to previously tared microcentrifuge tubes. The samples were then centrifuged at 400xg for 4.5 minutes, and the resulting supernatant was removed by aspiration. The tubes were weighed again in order to weight-standardize the samples, and 1ml of fluid thioglycollate medium was added to each tube and fortified with 50µl of penicillin/streptomycin solution, yielding 500 units of antibiotic per ml of FTM. The pellet was resuspended in the medium and incubated at room temperature in the dark for a period that usually lasted from 5 to 7 days; however, this incubation period extended to 21 days on some occasions. From this point, the same protocol as that used for the hemolymph assay (Gauthier and Fisher 1990) was followed for the fecal samples.

Body Burden Quantification. A body burden quantification of *P. marinus* (Choi et al. 1989, Bushek et al. 1994, Fisher and Oliver 1996) was used as a means of determining the abundance of *P. marinus* cells in the entire oyster. This assay is destructive to the oyster, in that it necessitates sacrificing the animal and utilizing the entire organism for diagnosis and parasite quantification.

The protocol used for the body burden quantification of *Perkinsus marinus* is a modification of the procedure described by Choi et al. (1989). The oysters were shucked and the tissue removed and weighed. The tissue was then finely minced using a razor blade and the tissue slurry was added to a tube containing 20ml of fluid thioglycollate medium fortified with 1 ml of penicillin/streptomycin solution, yielding 500 units of antibiotic per ml of FTM. The samples were incubated in the dark at room temperature for 7 to 10 days at which time they were removed and centrifuged at 800xg for 10 minutes. The supernatant was removed by aspiration and the pellet was resuspended in 25ml of 2 molar sodium hydroxide. The samples were then incubated for 3 hours at 60°C and upon their removal, were centrifuged at 800xg for 10 minutes. The supernatant was again removed by aspiration, and the pellet was washed three times in deionized water, centrifuged at 1500 x g for 10 minutes after each wash, and the supernatant was removed via aspiration. After the final wash, the pellet was resuspended in a 15-fold aqueous dilution of Lugol's iodine stain to a final volume of 2ml. The samples were placed in 24-well tissue culture plates and the stained *P. marinus* cells were then quantified using an inverted light microscope at 50x magnification. If there were fewer than 200-300 cells in the well, the entire sample was viewed and counted. If there were more than 200-300 cells in a sample, three random grid fields within the well were counted and

the final cell count was extrapolated from this. More heavily infected oyster samples were serially diluted before using the random grid field method to quantify the *P. marinus* cells.

Experimental Design

Experiment #1 - Infection Progression Experiment. The purpose of Experiment #1 was to examine the abundance of *Perkinsus marinus* in oyster feces of naturally-infected oysters and to determine if *P. marinus* fecal abundance was correlated with infection intensity. In Experiment #1, forty naturally-infected oysters were maintained in separate 1 liter containers in the wet lab as the experimental group. Ten uninfected oysters were also maintained in separate containers in the wet lab as a control group. Sampling for Experiment #1 commenced on July 11, 1996. Feces were collected daily from both groups for five consecutive days and the previously described fecal assay was performed on them. After the final fecal sample was collected, a hemolymph assay was performed to determine *P. marinus* infection levels in individuals from both oyster groups. This entire procedure was repeated at 4 weeks, 8 weeks, 12 weeks, and 16 weeks from the starting date. After the final sample was taken on November 15, 1996, a body burden quantification for *P. marinus* was performed. In addition, a body burden quantification for *P. marinus* was performed on any oysters that died over the course of the experiment, prior to the final sample.

Experiment #2 - Dosing Experiment. The purpose of Experiment #2 was to determine if *Perkinsus marinus* cells in oyster feces of naturally-infected oysters were infective. Twenty-five naturally-infected oysters and 25 uninfected oysters were maintained as two separate groups in trays in the

wet lab. These two groups of oysters served as the fecal source for this experiment. Seventeen uninfected oysters were individually maintained and served as the experimental group for the experiment. These were dosed with feces from infected oysters. As a control for this experiment, an additional 17 uninfected oysters were dosed with fecal matter from the group of uninfected oysters. A hemolymph assay was performed at the start of the experiment, October 11, 1996, on 50 of the infected source oysters from Point of Shoals to determine *P. marinus* infection levels and to assist in selection of the 25 oysters with the heaviest infections. A hemolymph assay was performed on the 25 uninfected fecal source oysters as well, to insure that they were uninfected and that all of the fecal source oysters were subjected to the same stresses. Commencing on October 21, 1996, feces were collected from the two source groups using a long-tipped Pasteur pipet, and all fecal matter for each group was pooled. The feces were concentrated by centrifugation and filtered York River water was added to make a slurry. An equal aliquot of the infected source and uninfected source slurry was given to each of the oysters in the experimental and control groups respectively. The oysters were dosed five days a week for 4 weeks, for a total of twenty doses. A subsample of both the infected and uninfected source feces was obtained for each dose and Ray's fluid thioglycollate culture method assays were conducted in order to monitor the amount of *P. marinus* in the doses. After the dosing regime was concluded, the experimental and control oysters were held for 3 weeks to allow the infections to develop. Any pair of oysters that experienced mortality of the source oyster prior to its scheduled removal was eliminated from the experiment to reduce the chance of transmission by *P. marinus* cells arising

from gapers. At the conclusion of the experiment on December 13, 1996, a body burden quantification for *P. marinus* was performed on each oyster.

Experiment #3 - Paired Experiment. The purpose of Experiment #3 was to determine if uninfected oysters held in close proximity to live, *P. marinus*-infected oysters would become infected. Thirty-five naturally-infected oysters were paired with 35 uninfected oysters serving as the experimental group. An additional 50 uninfected oysters were divided into 25 pairs as the control group. Each pair was maintained in a separate plastic container in the wet lab. A hemolymph assay was performed at the start of the experiment, August 8, 1996, on the 35 infected oysters to determine *P. marinus* infection levels. A hemolymph assay was also performed on the same day on the 25 uninfected fecal source oysters to insure that they were uninfected and that all of the fecal source oysters were subjected to the same stresses. The pairings were initiated on August 21, 1996, and maintained for a period of 8 weeks. On October 18, 1996, the fecal source oysters were removed, and body burden quantifications for *P. marinus* were performed. The remaining oysters were held for an additional 7 weeks to let infections develop. At the conclusion of the experiment on December 13, 1996, a body burden quantification for *P. marinus* was performed on all experimental and control oysters.

Data Analysis

All statistical analyses were performed using StatView 4.5 and Statistica 4.1 for Macintosh computers. All fecal and body burden *P. marinus* counts in the three experiments were weight-standardized prior to analysis. All *P. marinus* cell count data in the three experiments were log 10

transformed prior to analysis in order to normalize the data. Residual plots were observed to assess normality and homoscedasticity (Zar 1996).

Experiment #1 - Infection Progression Experiment. For Experiment #1, several analyses were performed. Regression analyses were used to identify specific relationships between: (1) *P. marinus* abundance in hemolymph and time; (2) *P. marinus* abundance in feces and time; (3) *P. marinus* abundance as determined by the hemolymph and fecal assays; (4) *P. marinus* abundance in the feces during the final month of the study and total body burden *P. marinus* abundance. For regression analyses 2, 3, and 4, the mean of the five *P. marinus* fecal data points for each individual for each month was utilized. In addition, for regression analyses 1 and 2, the data set was split into two groups: oysters that did not survive through the end of the study and oysters that did survive through the final sampling period. The data was then re-analyzed for these groups. The significance of the difference in fecal *P. marinus* abundance over the five fecal collection days for each oyster for each month was tested using a repeated measures analysis of variance. The significance of the difference in total body burden *P. marinus* abundance as the oysters died during the course of the study was tested using an analysis of variance.

Experiment #2 - Dosing Experiment. The significance of the difference of the total body burden *Perkinsus marinus* abundance between the control and experimental fecal recipient oysters was not tested statistically because of the clear and obvious quantitative difference between the two groups. The sum, mean and standard deviation of the *P. marinus* abundance in the 20 fecal doses were calculated in order to gauge the level of parasite exposure that the fecal recipient oysters experienced over the dosing regime.

Experiment #3 - Paired Experiment. The significance of the difference of the total body burden *P. marinus* abundance between the control and the experimental fecal recipient oysters was again not tested statistically because of the clear and obvious quantitative difference between the two groups. A correlation and regression analysis of the total body burden abundance of *Perkinsus marinus* cells in the experimental fecal recipient oysters with the abundance of *P. marinus* in the experimental fecal source oyster initial hemolymph sample, as well as with the total body burden abundance of *P. marinus* in the experimental fecal source oysters was performed.

RESULTS

The initial sample of 25 Maine oysters that was sacrificed for body burden assays prior to commencing the experiments were all negative for *P. marinus*. All control oysters in the three experiments that were subjected to hemolymph, fecal, and body burden assays for *P. marinus* were negative for the parasite. All *P. marinus* cell count data have been log transformed unless otherwise noted on the figure. In the regression figures where the months are given as numbers, month 1 corresponds to July; month 2 to August; month 3 to September; month 4 to October; and month 5 to November.

Experiment #1 - Infection Progression Experiment

Hemolymph parasite abundance. The mean *P. marinus* abundance quantified in the oyster hemolymph indicated a progression in infection intensity from July through September (Figure 1). A slight decrease in *P. marinus* hemolymph abundance was observed in October but the parasite abundance increased again in November.

Cumulative mortality. The mortality in the experimental animal group over the five month time period of the study reached 72% in November (Figure 2). The animals were dying from very heavy *P. marinus* infections, as indicated by the body burden assays performed on these oysters as they died over the course of the experiment (Figure 24). The mortality in the control animal group over the five month period of the study was 30%. All control oysters in this experiment were negative for *P. marinus* in all hemolymph, fecal, and body burden assays, and as such, the mortality in this

group was not a result of *P. marinus* infection. This mortality could have perhaps been a result of handling stress or being held in a 1 liter plastic container for an extended period of time.

Hemolymph parasite abundance & time regression. A logarithmic regression analysis performed on hemolymph data from all oysters was significant ($p<0.05$), and indicated that time accounts for 37% of the variability in hemolymph parasite abundance (Figure 3). The regression plot had some scatter, particularly in the October and November samples. When the logarithmic regression analysis was run on only the oysters that did not survive through the entire experiment, the analysis was not significant ($p<0.95$) (Figure 4). When the logarithmic regression analysis was run only on the oysters that survived the entire experiment, the analysis was significant ($p<0.05$) and indicated that time accounts for 36% of the variability in hemolymph parasite abundance (Figure 5).

Fecal parasite abundance. The monthly mean *P. marinus* abundance quantified in the oyster feces indicated a progression in infection intensity from July through September (Figure 6). After this time, a decrease in *P. marinus* fecal abundance was observed through October and November. The daily mean fecal production of the experimental oysters decreased from July through October, and then increased in November. The daily mean fecal production of the control oysters decreased from July through September, and then increased through October and November (Table 1).

Fecal parasite abundance & time regression. A logarithmic regression analysis performed on fecal data from all oysters was found to be highly significant ($p<0.0001$) and indicated that time accounts for 19% of the variability in fecal parasite abundance (Figure 7). There was a high amount of scatter in the regression plot. When the logarithmic regression analysis

was run on only the oysters that did not survive through the end of the experiment, the analysis was not significant ($p<0.39$) (Figure 8). When the logarithmic regression analysis was run on only the oysters that survived the entire experiment, the analysis was also not significant ($p<0.30$) (Figure 9).

Survivor & non-survivor hemolymph & fecal parasite abundance. The monthly mean hemolymph parasite abundance of the survivor oysters showed an increase from July through November, however, the monthly mean fecal parasite abundance of these oysters showed an increase through October, and then a decrease in November (Figures 10 & 11). The monthly mean hemolymph parasite abundance of the non-survivor oysters showed an increase from July through October, but the monthly fecal parasite abundance of these oysters showed an increase through September and then a decrease in October (Figure 10). (The data from the non-survivor oysters only goes through October, as these oysters had died by the November sampling date.) The monthly hemolymph and mean fecal parasite abundance was plotted individually for each of the 10 survivor oysters (Figures 12-21). Variability in both parasite abundances of these individuals over the course of the experiment was apparent.

Hemolymph & fecal parasite abundance correlation and regression. The results of a correlation analysis demonstrated a highly significant ($p<0.0001$) and strong correlation (Fisher's $r=0.616$) between the hemolymph *P. marinus* abundance and the mean fecal *P. marinus* abundance for individual oysters. A subsequent linear regression analysis was found to be highly significant ($p<0.0001$) as well, and indicated that hemolymph parasite abundance accounts for 38% of the variability in fecal parasite abundance (Figure 22).

Individual sampling day fecal parasite abundance. The mean *P. marinus* abundance for each of the five sampling days in each of the five months showed a high amount of variability between the replicate days, particularly in September and October (Figure 23). Some oysters went from a count of 20 cells one day to a count of 100 cells the next. A repeated measures analysis of variance of the *P. marinus* abundance over the five sampling days for each month in the study indicated that there was a significant difference among both the July and August samples ($p<0.005$), but not for any of the other months in the study (Table 2).

Body burden parasite abundance. The mean body burden abundance of the oysters that died during each month of the experiment did not follow a clear increasing or decreasing trend. The mean body burden abundance of the oysters that survived through the last sampling date of the experiment was lower than the mean body burden abundance for oysters that died during all of the months except July (Figure 24). An analysis of variance and subsequent Fisher's PLSD multiple comparison of the *P. marinus* abundance in oysters that died over the five months and the survivor oysters indicated that there was a significant difference between July and all other months in the study ($p<0.05$), but not a significant difference between oysters that died in July and the survivor oysters. There was also a significant difference between the oysters that died in October and the survivor oysters ($p<0.05$) (Tables 3 & 4).

Final fecal point & body burden parasite abundance correlation and regression. The results of the correlation analysis of the final sampling month mean fecal *P. marinus* abundance with the body burden *P. marinus* abundance was not found to be significant (Fisher's $r=0.236$, $p=0.5253$) (Figure 25).

FIGURE 1. Experiment #1 mean hemolymph *P. marinus* abundance for the five months of the study, July through November. Error bars denote +1 standard error.

Mean Hemolymph Parasite Abundance

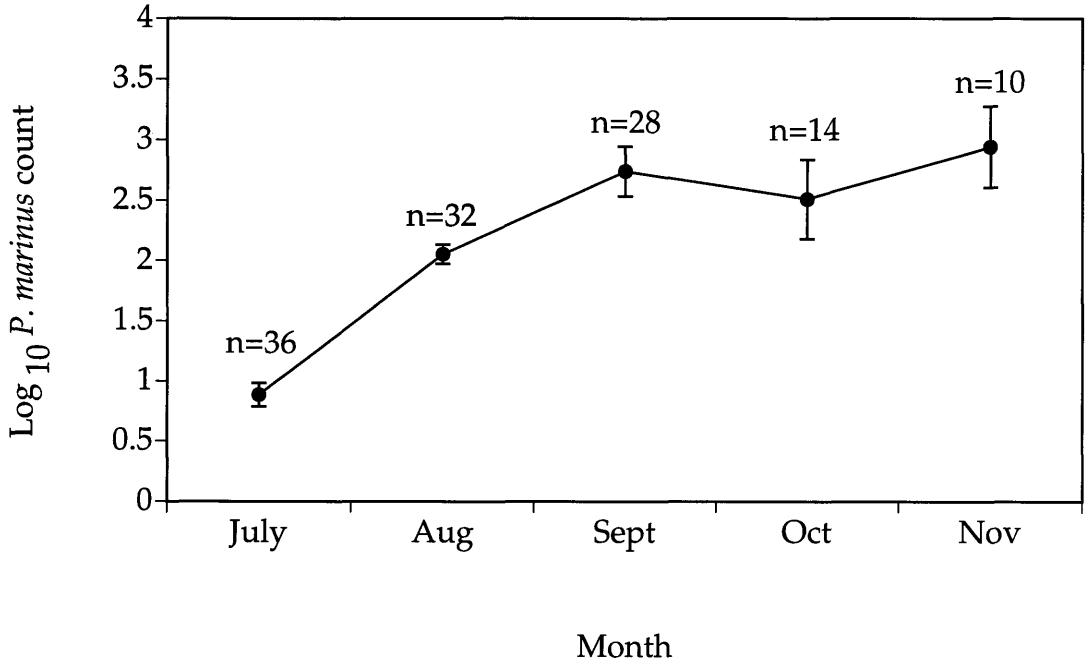


FIGURE 2. Experiment #1 percent cumulative mortality over the five months of the study, July through November.

Percent Cumulative Mortality

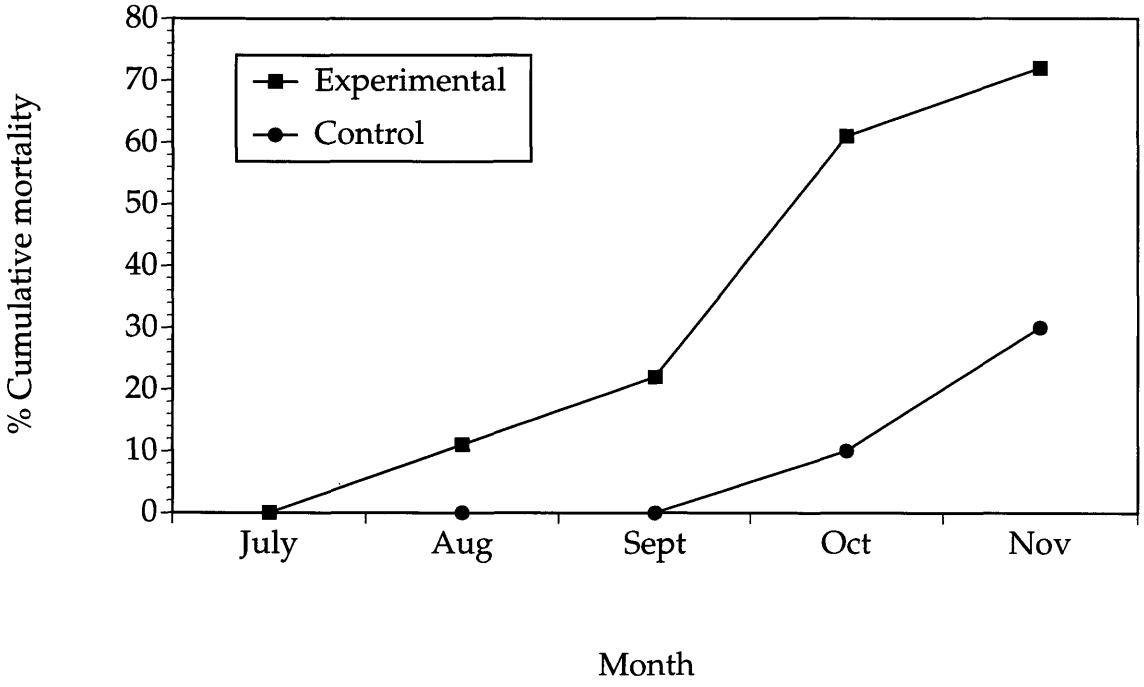
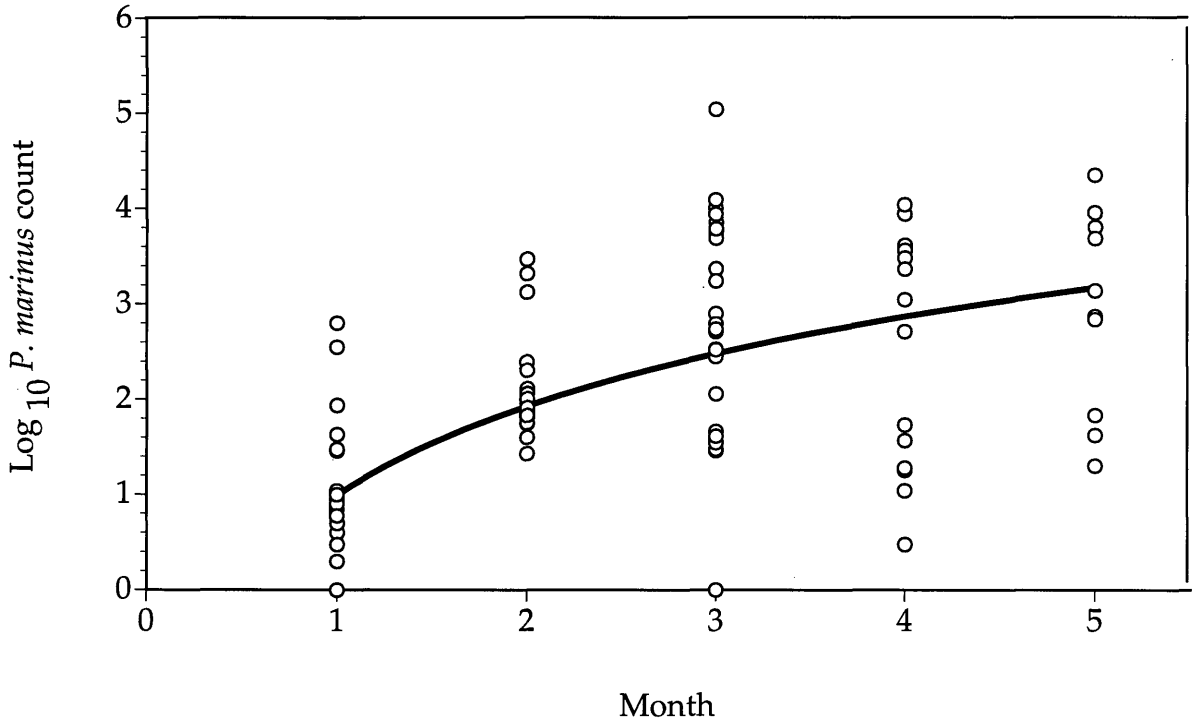


FIGURE 3. Experiment #1 logarithmic regression analysis of hemolymph *P. marinus* abundance through time.

Regression Analysis of Hemolymph Parasite Abundance through Time



Regression: R^2=0.373; P-value<0.05

FIGURE 4. Experiment #1 scatter plot of non-survivor oyster hemolymph *P. marinus* abundance through time.

Scatter Plot of Non-Survivor Hemolymph Parasite Abundance through Time

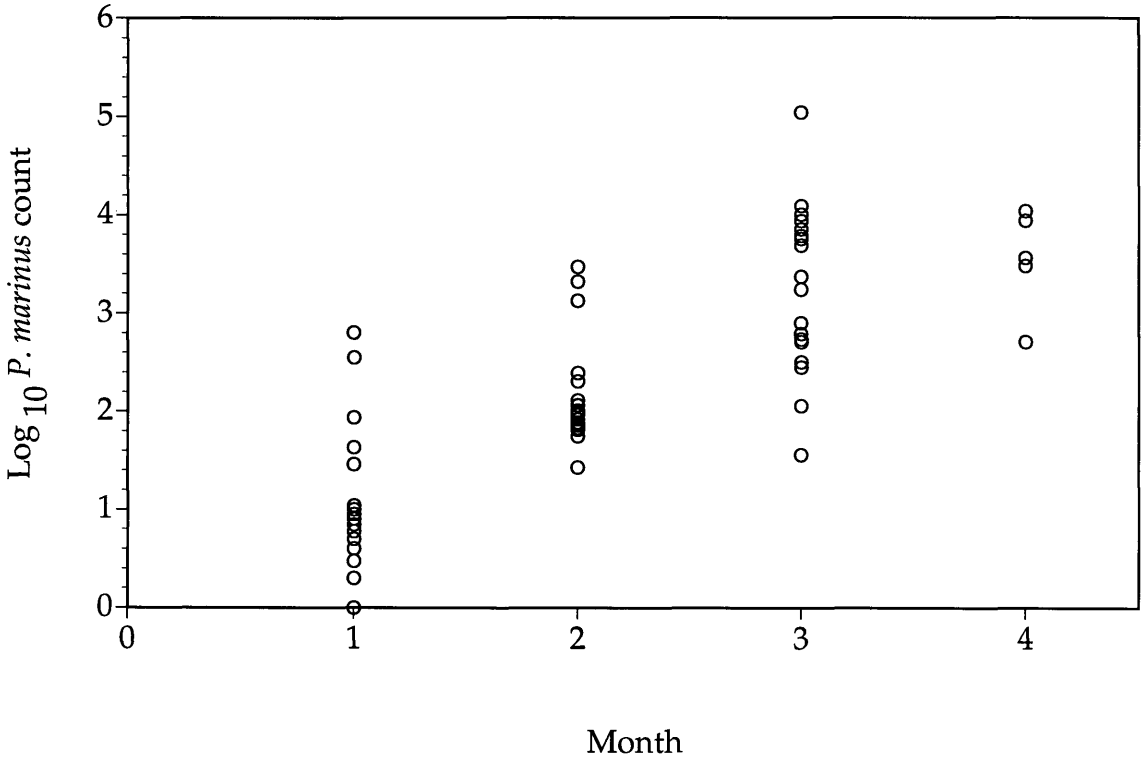
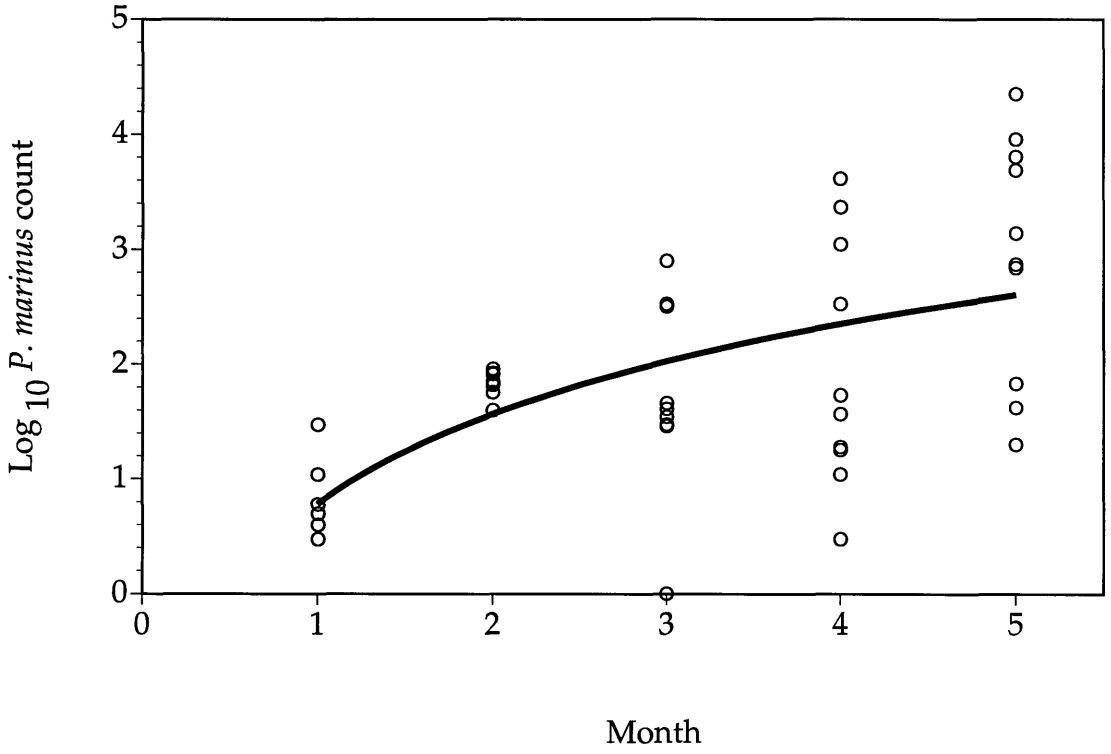


FIGURE 5. Experiment #1 logarithmic regression analysis of survivor oyster hemolymph *P. marinus* abundance through time.

Regression Analysis of Survivor Hemolymph Parasite Abundance through Time



Regression: R^2=0.364; P-value<0.05

FIGURE 6. Experiment #1 mean *P. marinus* abundance (not log transformed) per mg feces for the five months of the study, July through November. Error bars denote +1 standard error.

Mean Monthly Fecal Parasite Abundance

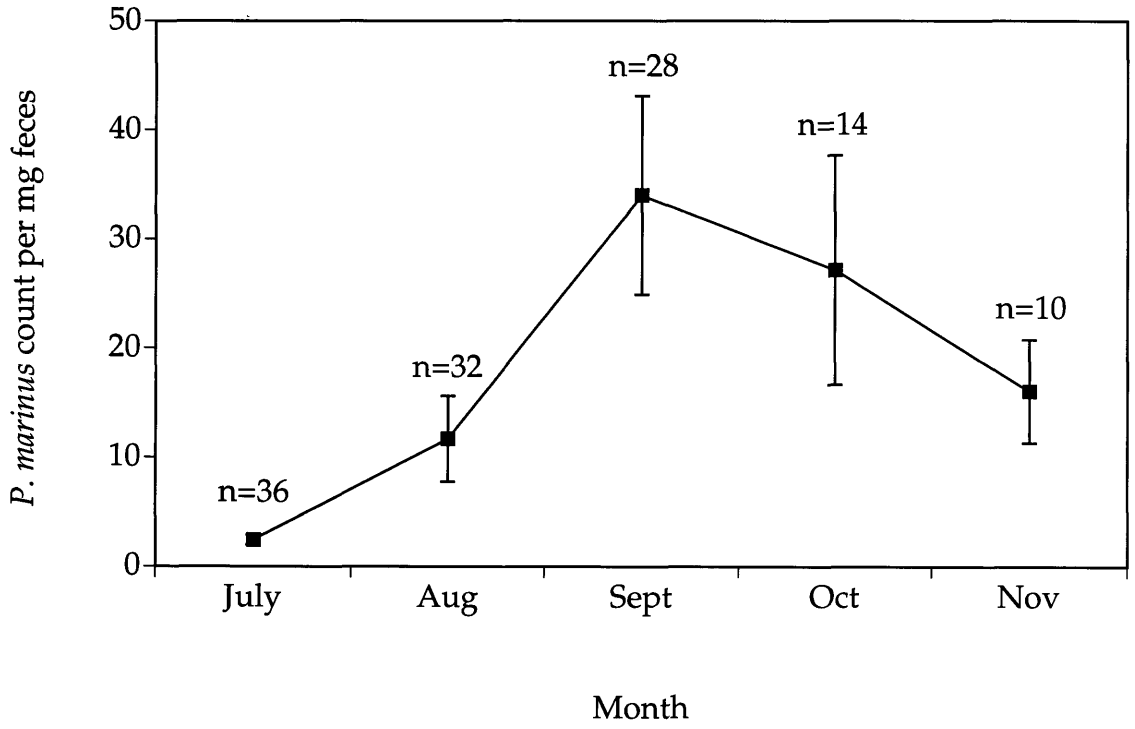
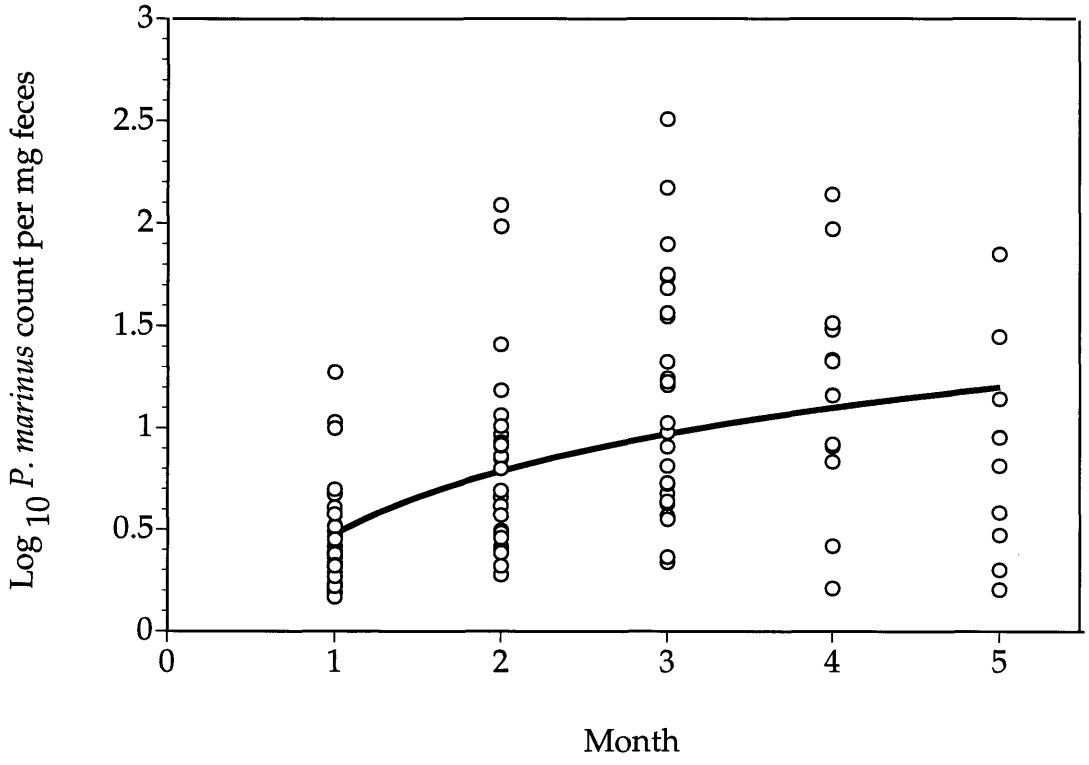


TABLE 1. Mean daily fecal weight produced in Experiment #1.

Month	Mean Experimental Fecal Weight Produced (g)	Mean Control Fecal Weight Produced (g)
July	0.037	0.043
August	0.030	0.030
September	0.026	0.026
October	0.019	0.027
November	0.027	0.033

FIGURE 7. Experiment #1 logarithmic regression analysis of mean *P. marinus* abundance per mg feces for individual oysters through time.

Regression Analysis of Fecal Parasite Abundance through Time



Regression: R^2=0.186; P-value<0.0001

FIGURE 8. Experiment #1 scatter plot of mean *P. marinus* abundance per mg feces for individual non-survivor oysters through time.

Scatter Plot of Non-Survivor Fecal Parasite Abundance through Time

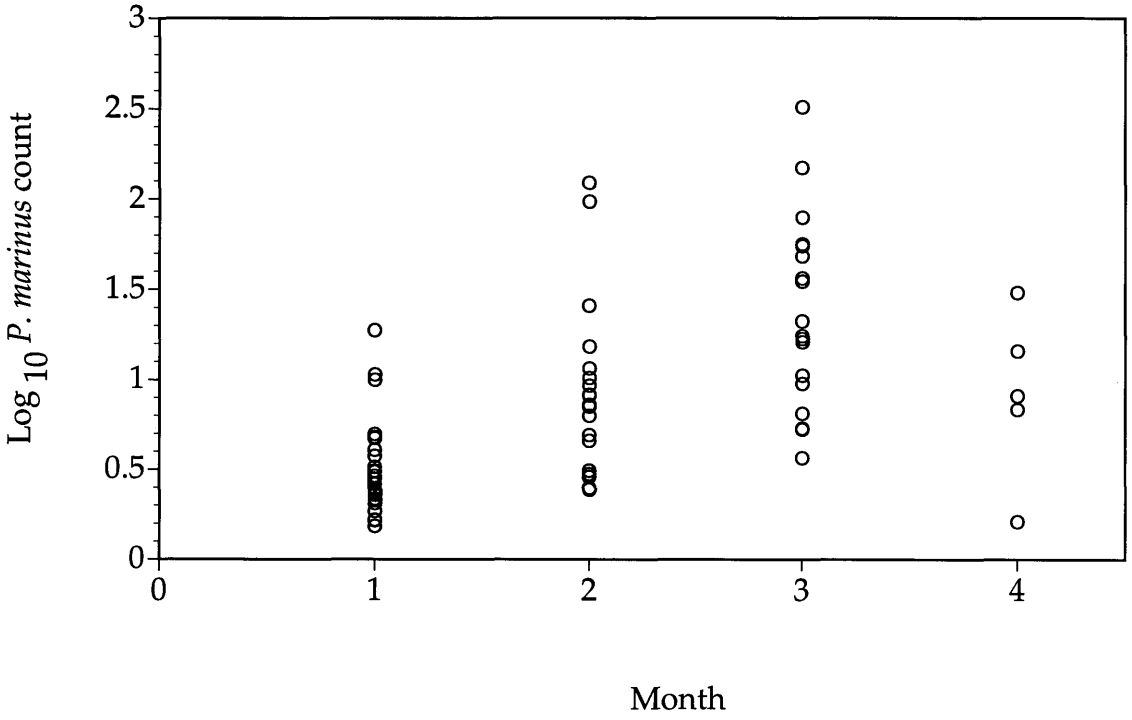


FIGURE 9. Experiment #1 scatter plot of mean *P. marinus* abundance per mg feces for individual survivor oysters through time.

Scatter Plot of Survivor Fecal Parasite Abundance through Time

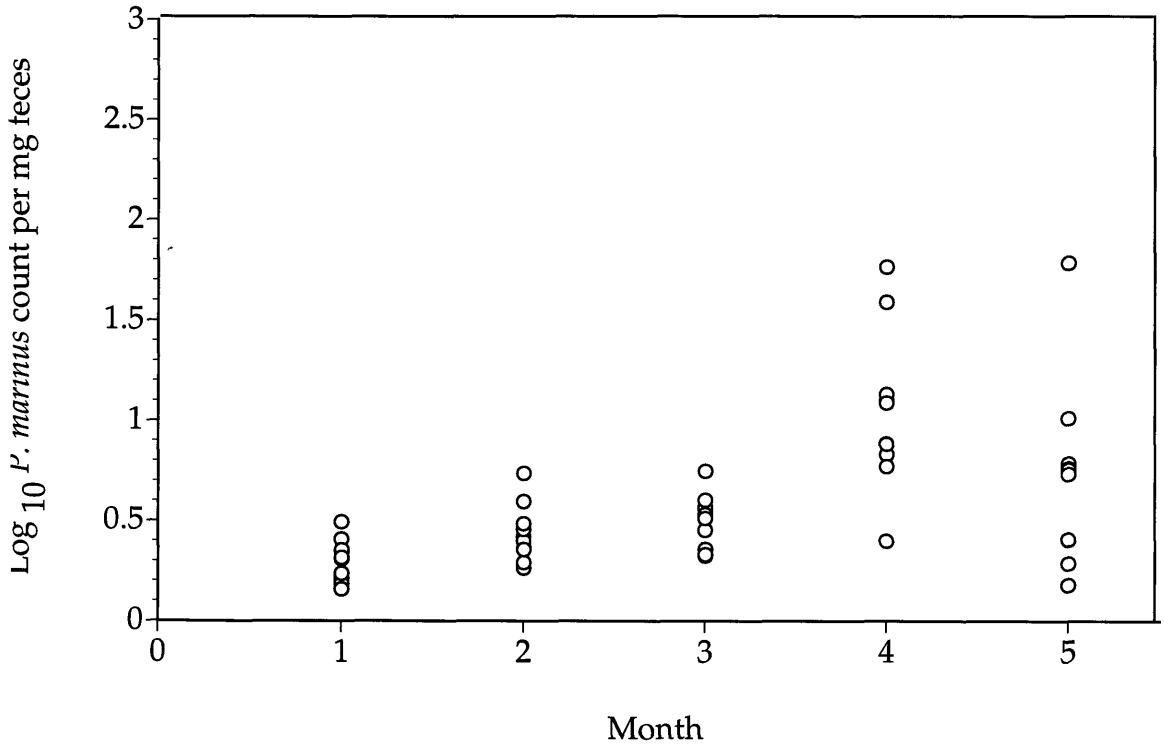


FIGURE 10. Experiment #1 mean *P. marinus* abundance in hemolymph and per mg feces for survivor and non-survivor groups for the five months of the study, July through November.

Survivor and Non-Survivor Mean Monthly Hemolymph and Fecal Parasite Abundance

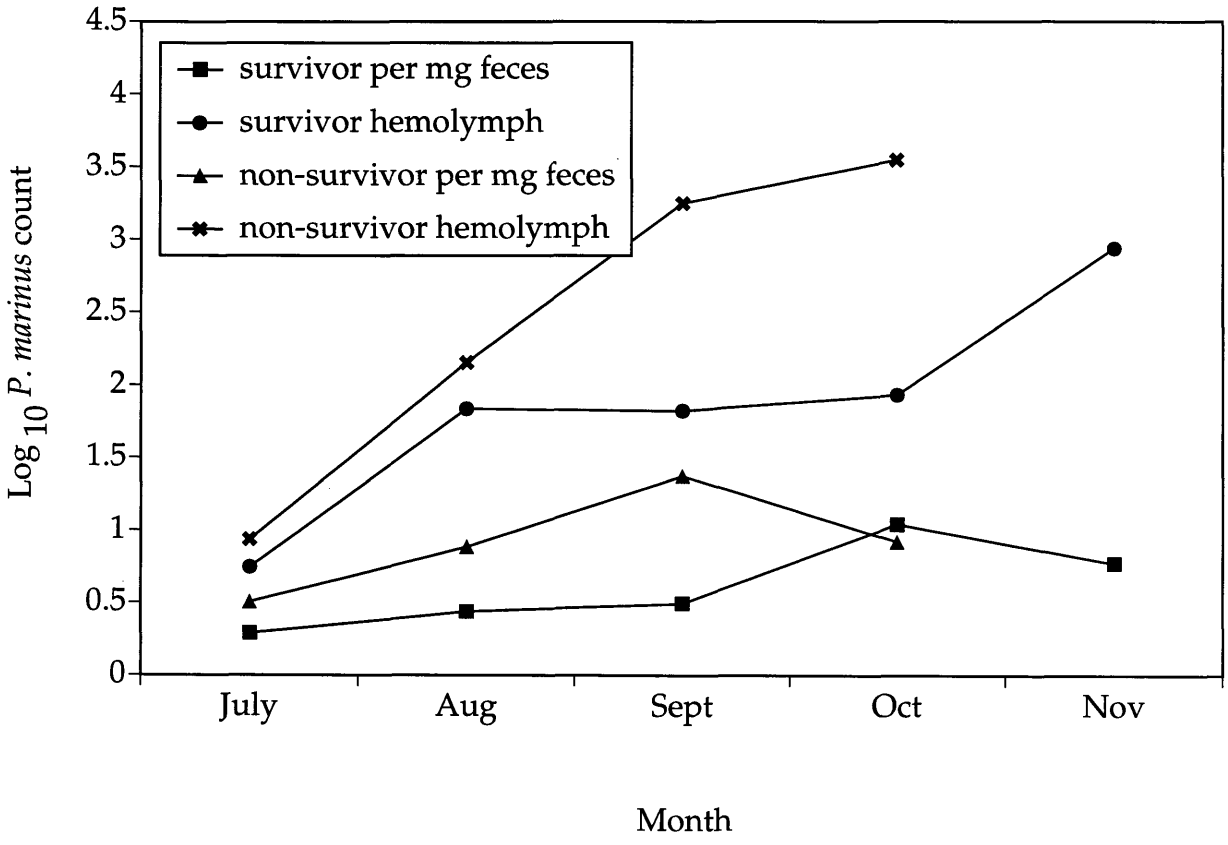


FIGURE 11. Experiment #1 mean *P. marinus* abundance in hemolymph and per mg feces for survivor oysters for the five months of the study, July through November.

Survivor Mean Monthly Hemolymph and Fecal Parasite Abundance

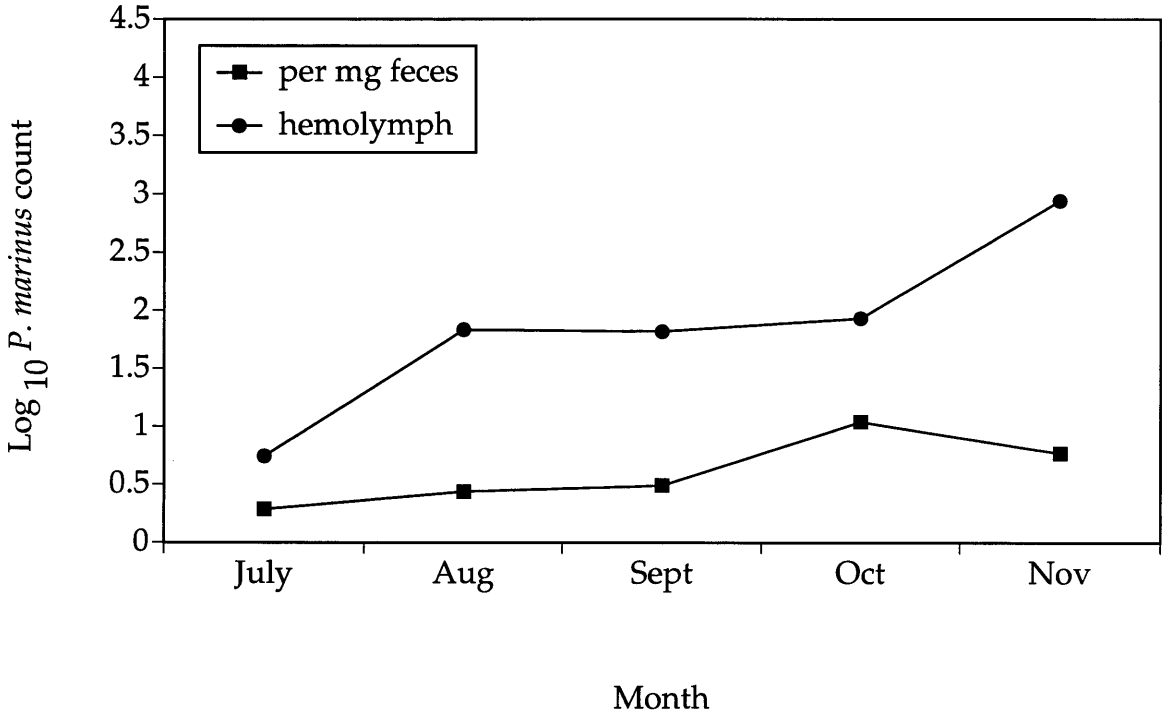


FIGURE 12. Experiment #1 mean *P. marinus* abundance in hemolymph and per mg feces for survivor Oyster #1 for the five months of the study, July through November.

Survivor Oyster #1 Hemolymph and Mean Monthly Fecal Parasite Abundance

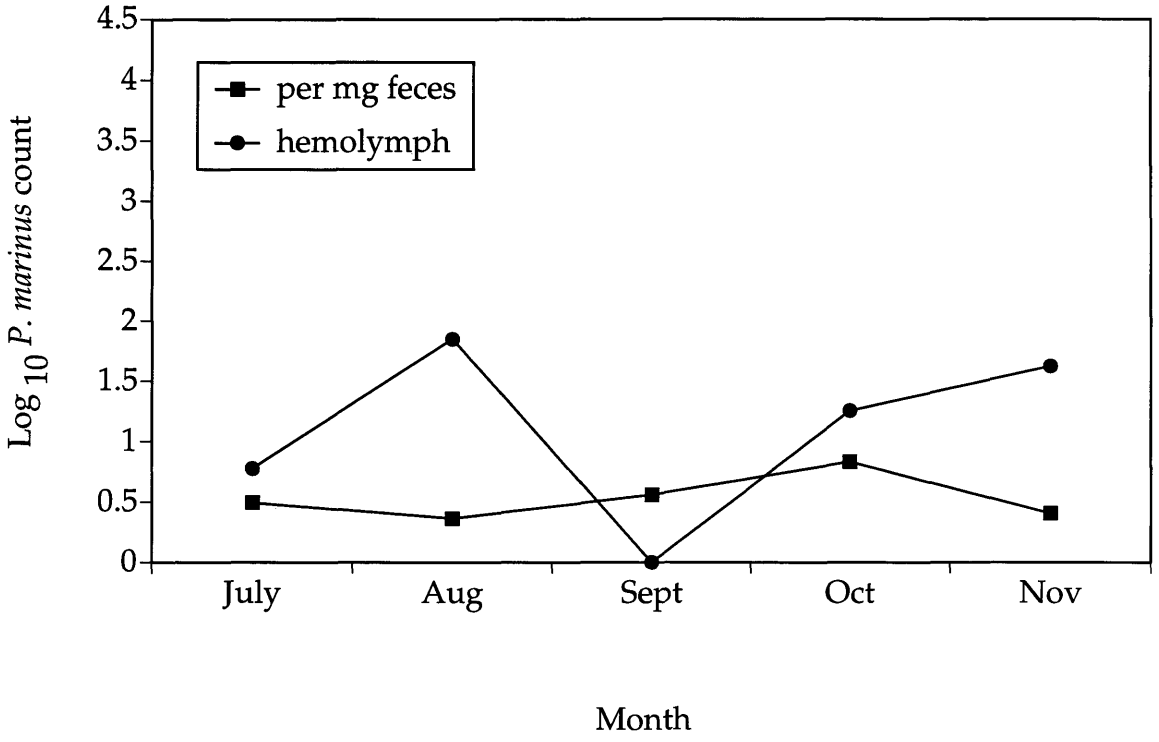


FIGURE 13. Experiment #1 mean *P. marinus* abundance in hemolymph and per mg feces for survivor Oyster #4 for the five months of the study, July through November.

Survivor Oyster #4 Hemolymph and Mean Monthly Fecal Parasite Abundance

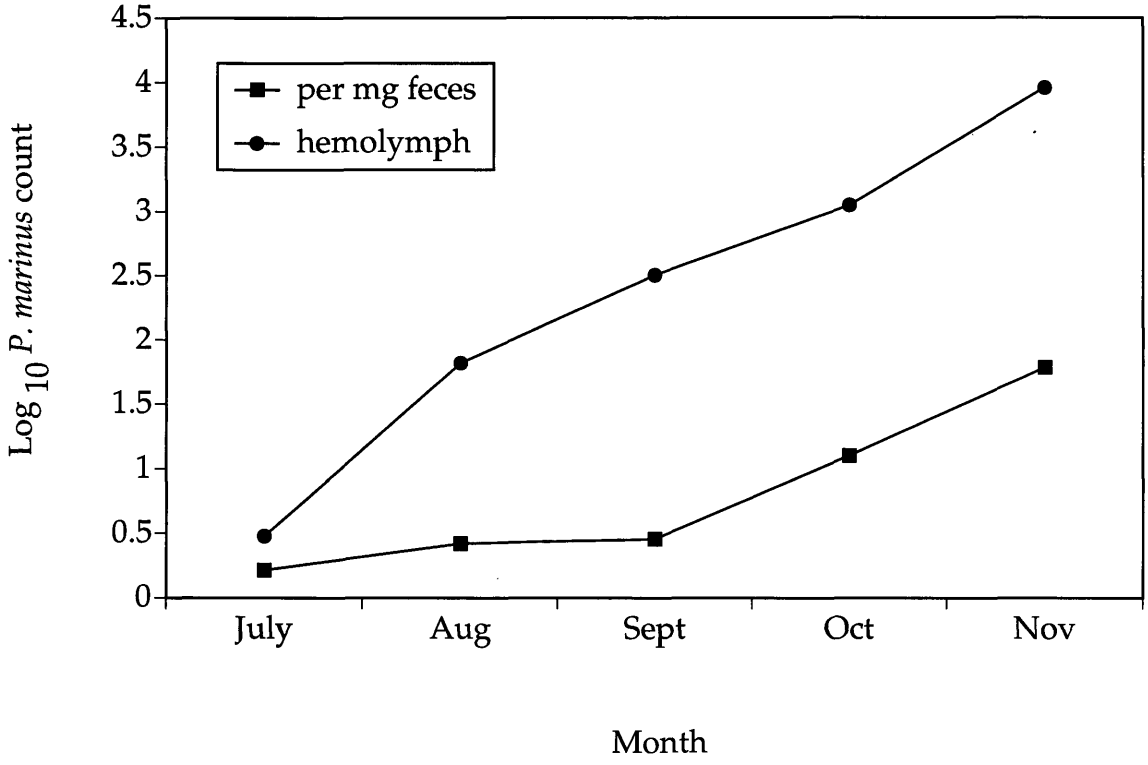


FIGURE 14. Experiment #1 mean *P. marinus* abundance in hemolymph and per mg feces for survivor Oyster #7 for the five months of the study, July through November.

Survivor Oyster #7 Hemolymph and Mean Monthly Fecal Parasite Abundance

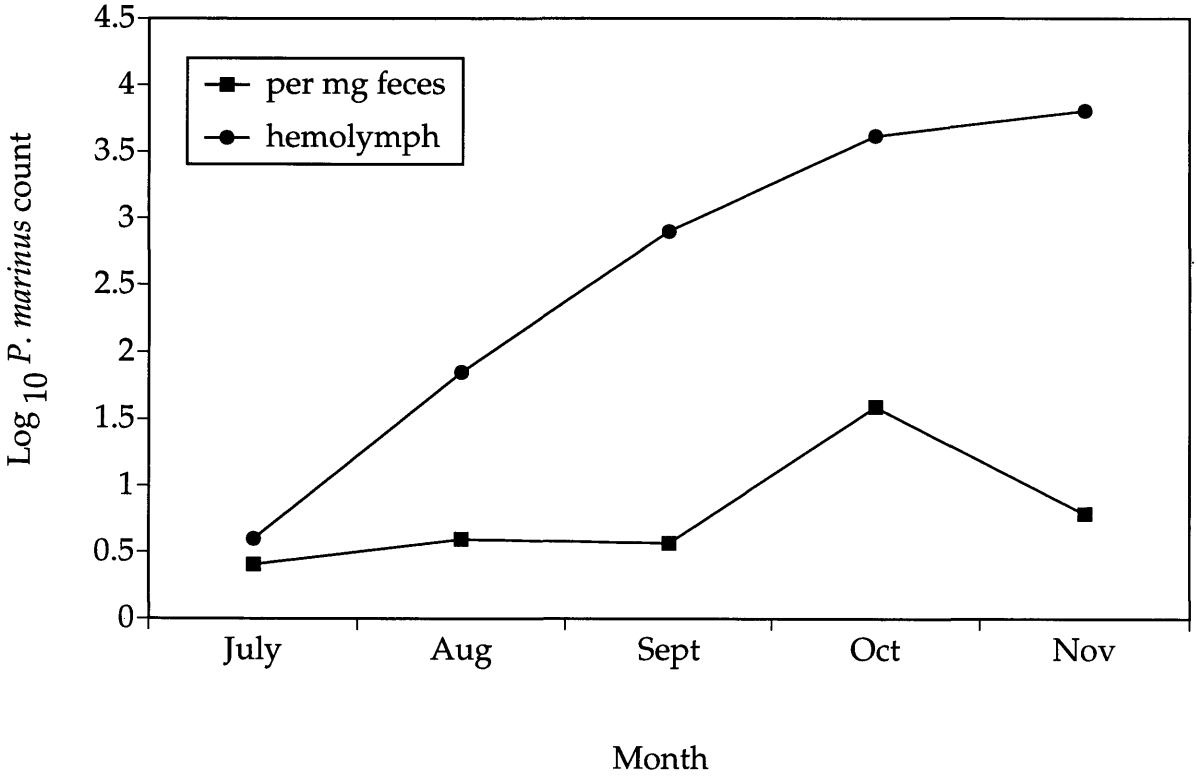


FIGURE 15. Experiment #1 mean *P. marinus* abundance in hemolymph and per mg feces for survivor Oyster #17 for the five months of the study, July through November.

Survivor Oyster #17 Hemolymph and Mean Monthly Fecal Parasite Abundance

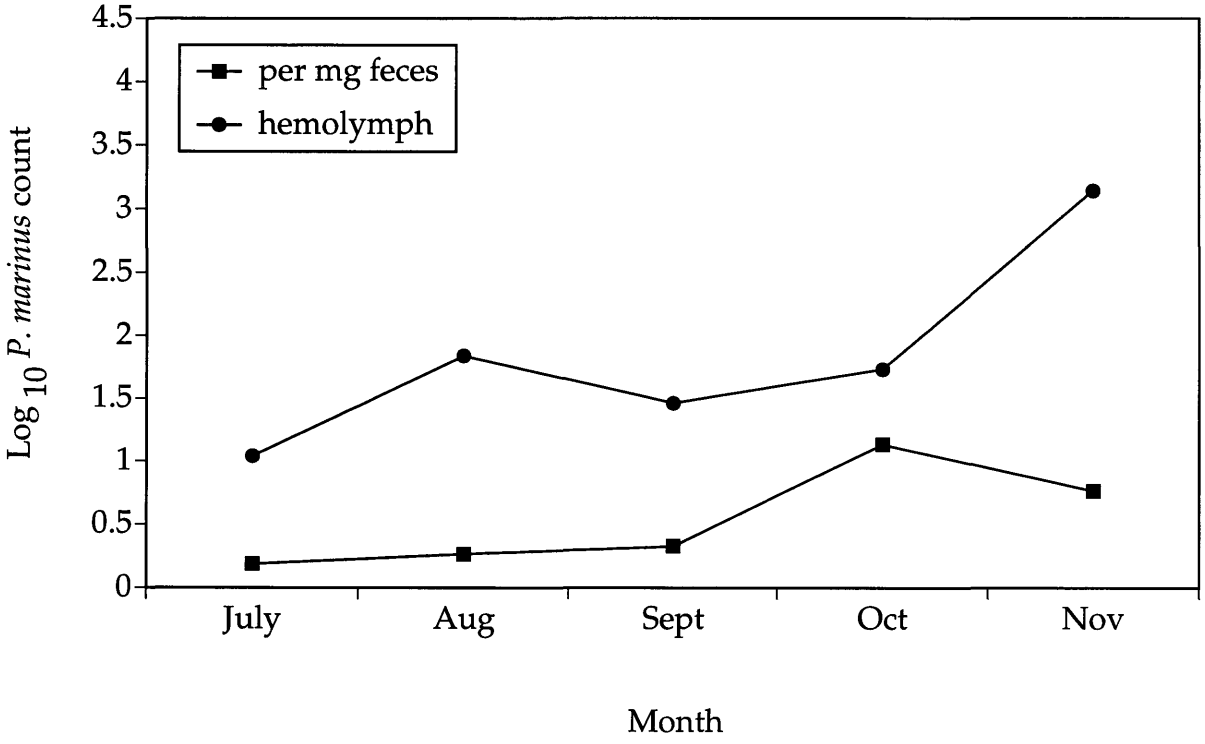


FIGURE 16. Experiment #1 mean *P. marinus* abundance in hemolymph and per mg feces for survivor Oyster #20 for the five months of the study, July through November.

Survivor Oyster #20 Hemolymph and Mean Monthly Fecal Parasite Abundance

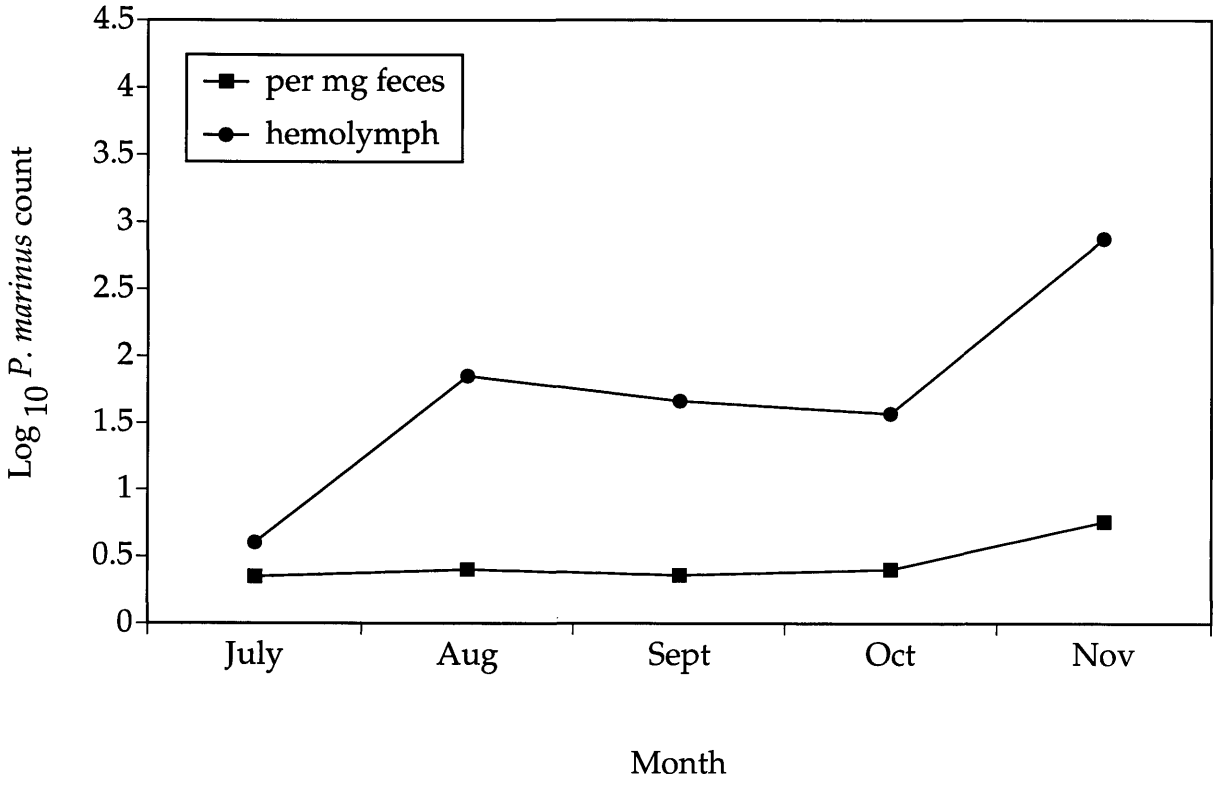


FIGURE 17. Experiment #1 mean *P. marinus* abundance in hemolymph and per mg feces for survivor Oyster #21 for the five months of the study, July through November.

Survivor Oyster #21 Hemolymph and Mean Monthly Fecal Parasite Abundance

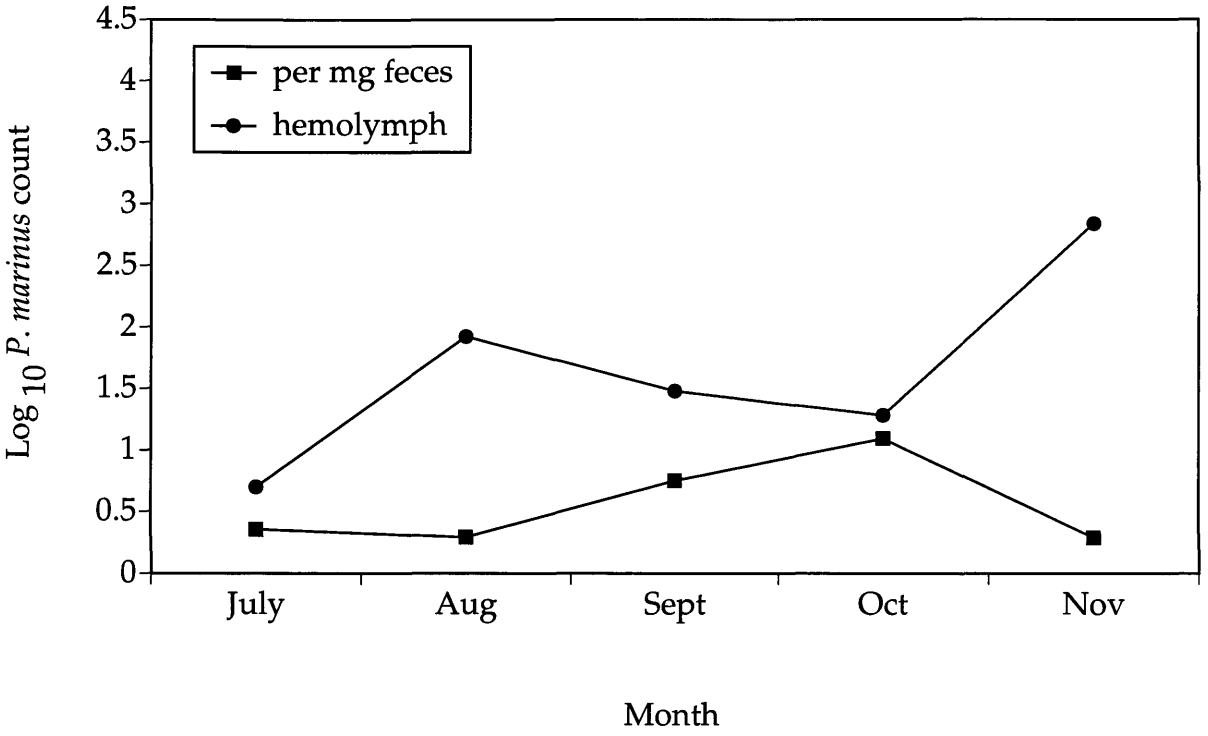


FIGURE 18. Experiment #1 mean *P. marinus* abundance in hemolymph and per mg feces for survivor Oyster #22 for the five months of the study, July through November.

Survivor Oyster #22 Hemolymph and Mean Monthly Fecal Parasite Abundance

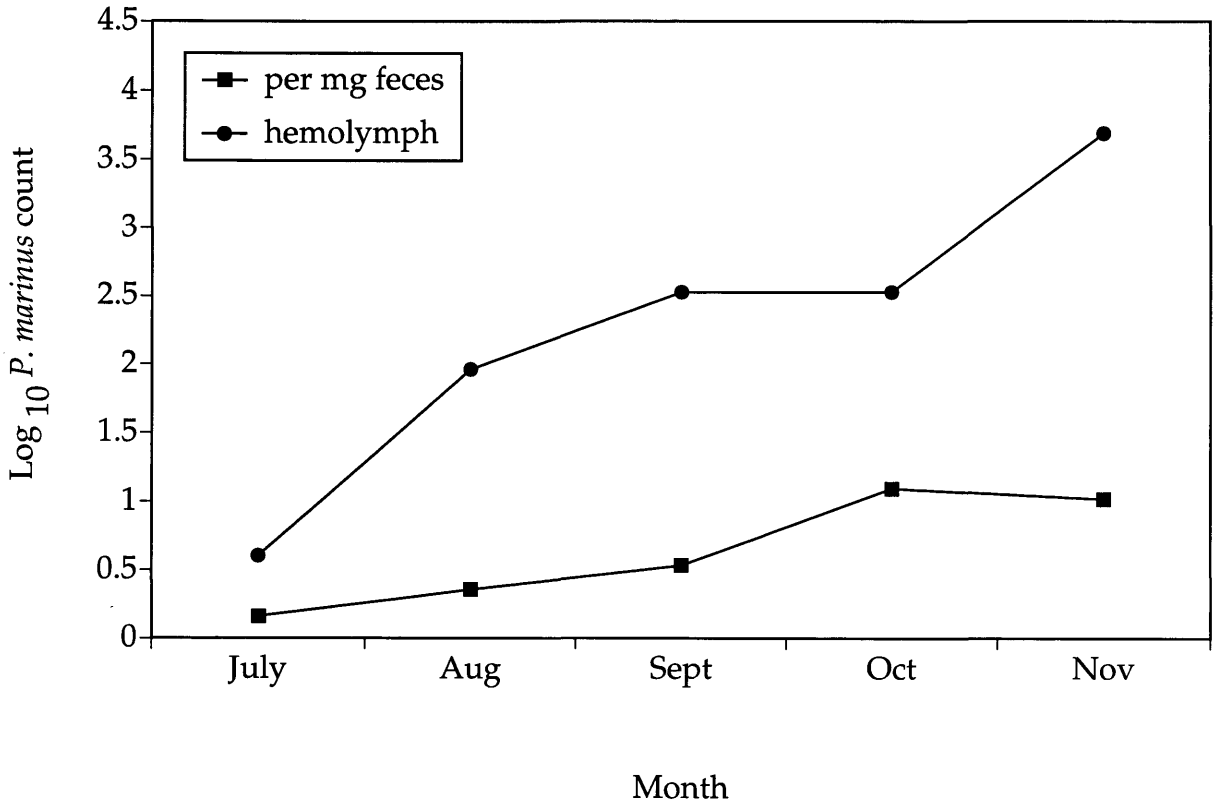


FIGURE 19. Experiment #1 mean *P. marinus* abundance in hemolymph and per mg feces for survivor Oyster #29 for the five months of the study, July through November.

Survivor Oyster #29 Hemolymph and Mean Monthly Fecal Parasite Abundance

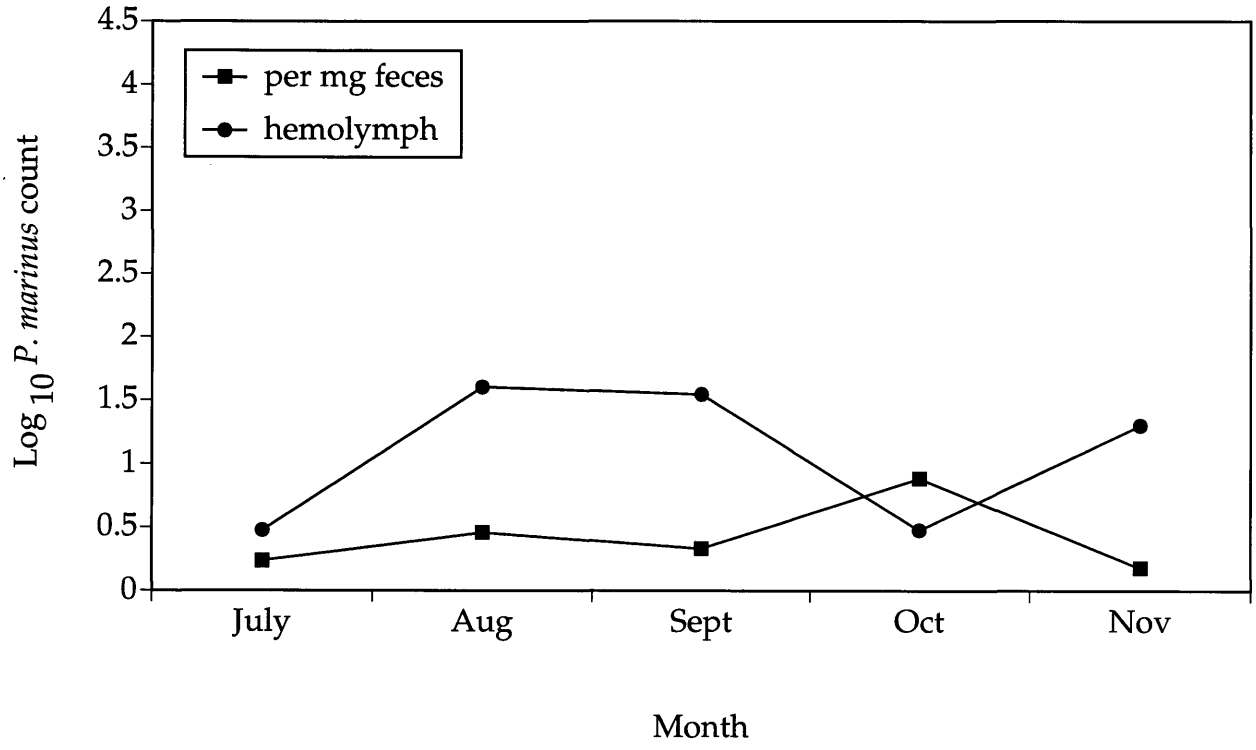


FIGURE 20. Experiment #1 mean *P. marinus* abundance in hemolymph and per mg feces for survivor Oyster #34 for the five months of the study, July through November.

Survivor Oyster #34 Hemolymph and Mean Monthly Fecal Parasite Abundance

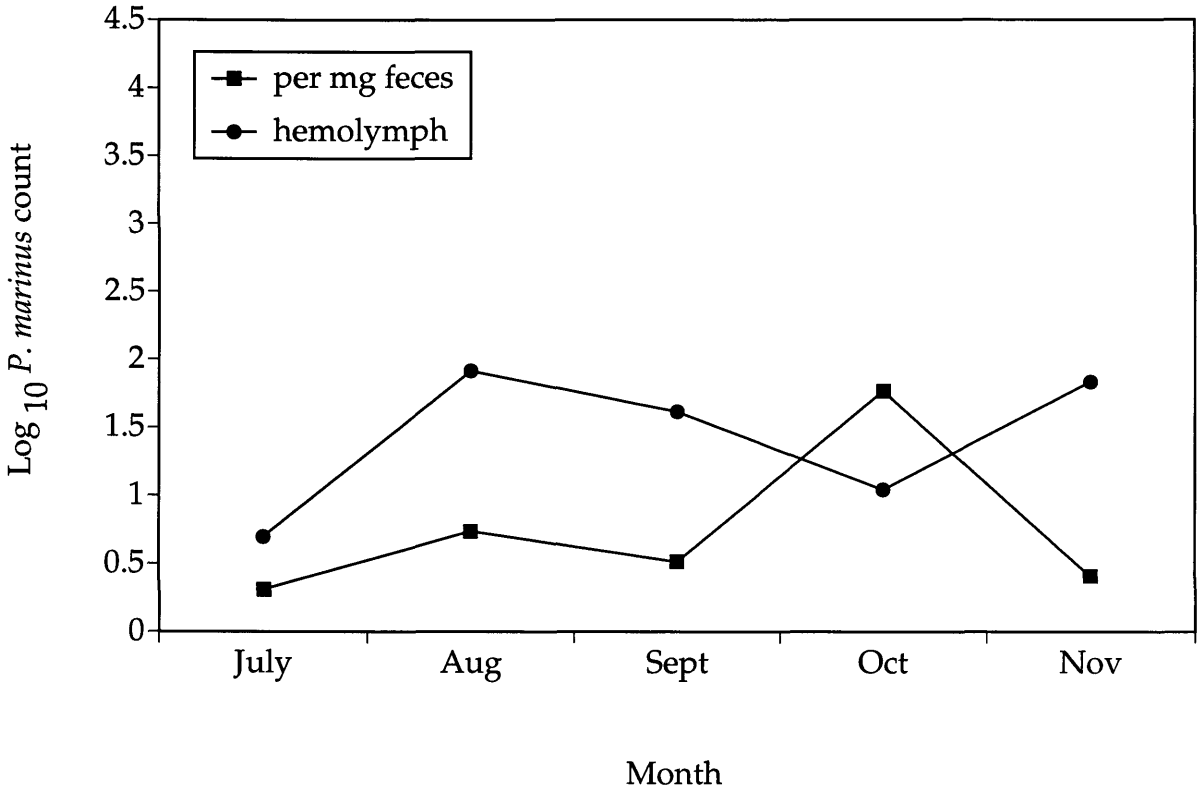


FIGURE 21. Experiment #1 mean *P. marinus* abundance in hemolymph and per mg feces for survivor Oyster #37 for the five months of the study, July through November.

Survivor Oyster #37 Hemolymph and Mean Monthly Fecal Parasite Abundance

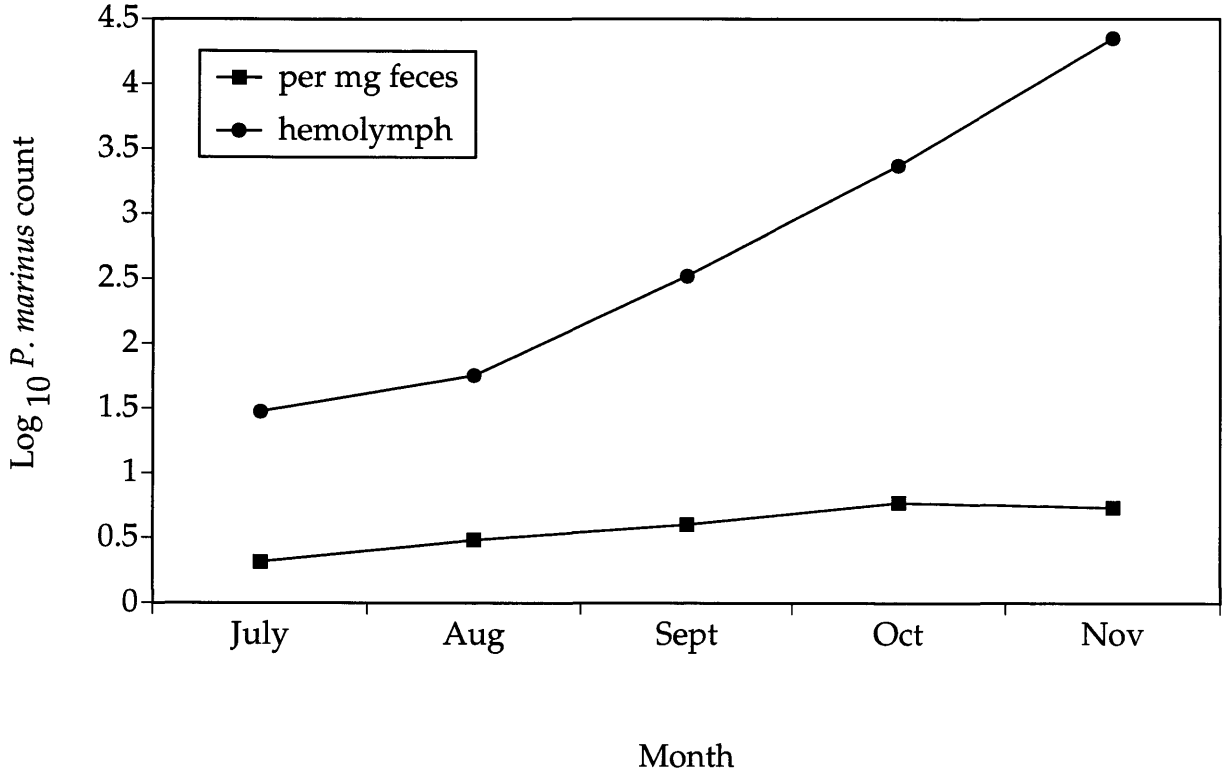
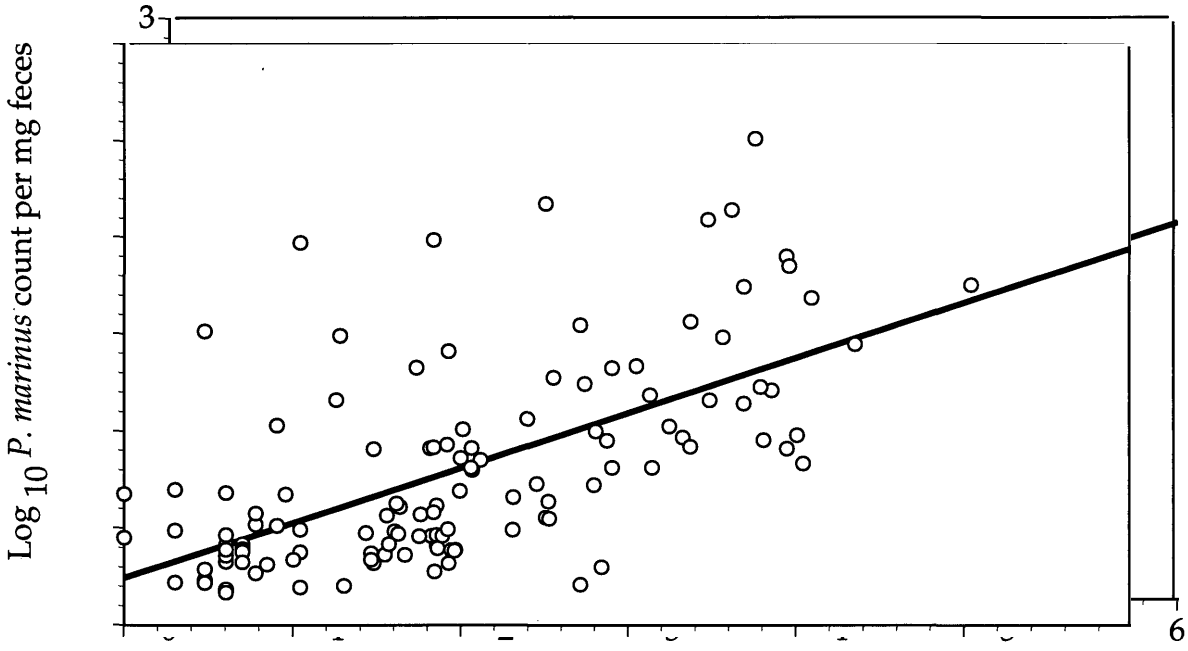


FIGURE 22. Experiment #1 correlation and regression analysis of hemolymph *P. marinus* abundance and mean *P. marinus* abundance per mg feces for individual oysters.

Regression Analysis of Hemolymph and Fecal Parasite Abundance



Regression: $Y=0.244+0.283(X)$; $R^2=0.379$; P-value<0.0001

Correlation: Fisher's r=0.616; P-value<0.0001

FIGURE 23. Experiment #1 mean *P. marinus* abundance (not log transformed) per mg feces for each of the five sampling days in each month of the study, July through November. Error bars denote +1 standard error.

Daily Mean Fecal Parasite Abundance for Sampling Periods

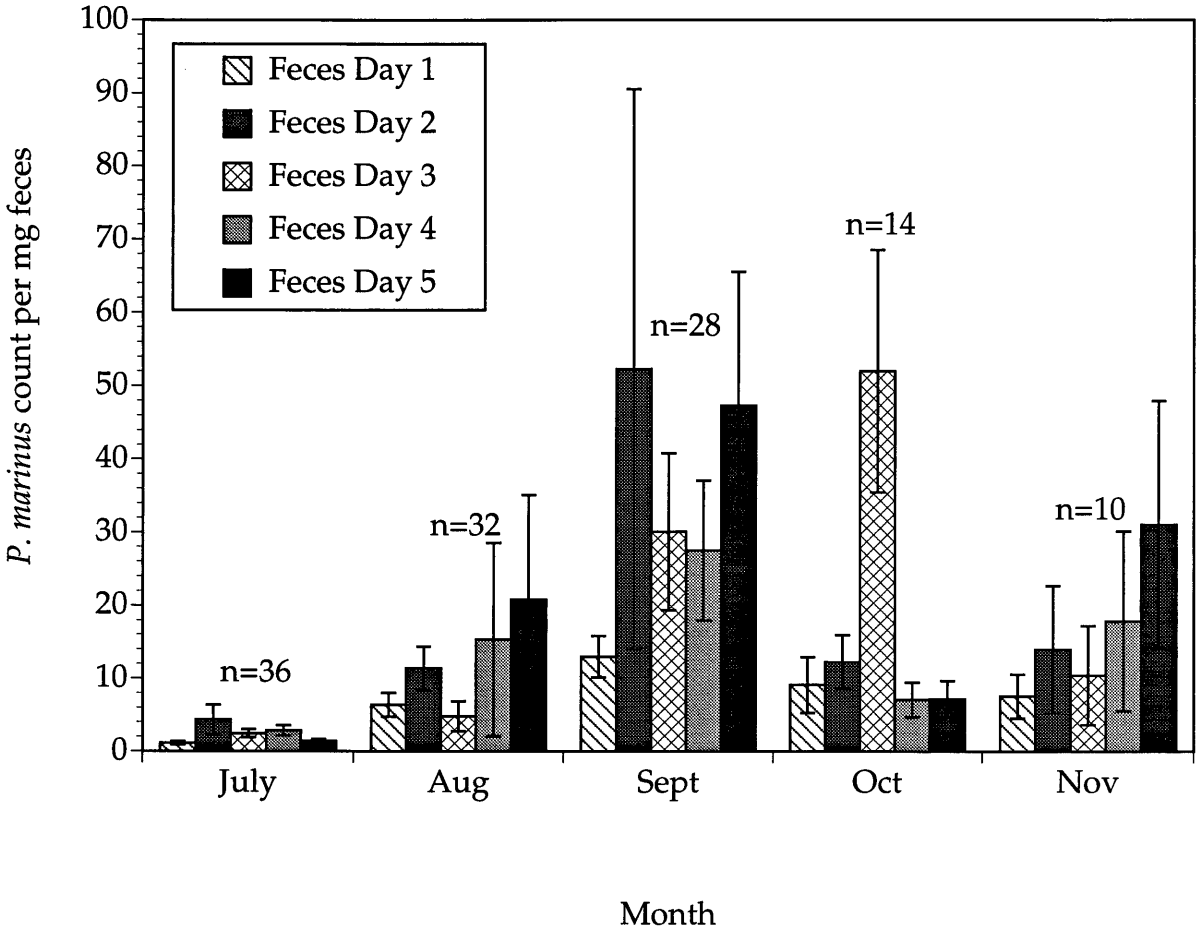


TABLE 2. Results of repeated measures analysis of variance for *P. marinus* fecal abundance on each sample date in Experiment #1.

Month	DF	F Ratio	F Probability
July	4	5.279	0.0006
August	4	6.977	<0.0001
September	4	0.897	0.4699
October	4	1.162	0.3573
November	4	2.780	0.1020

FIGURE 24. Experiment #1 mean *P. marinus* abundance in weight-standardized body burdens for oysters that died during the five months of the study, July through November, and for the survivor oysters at the conclusion of the experiment in November.

Mean Body Burden Parasite Abundance

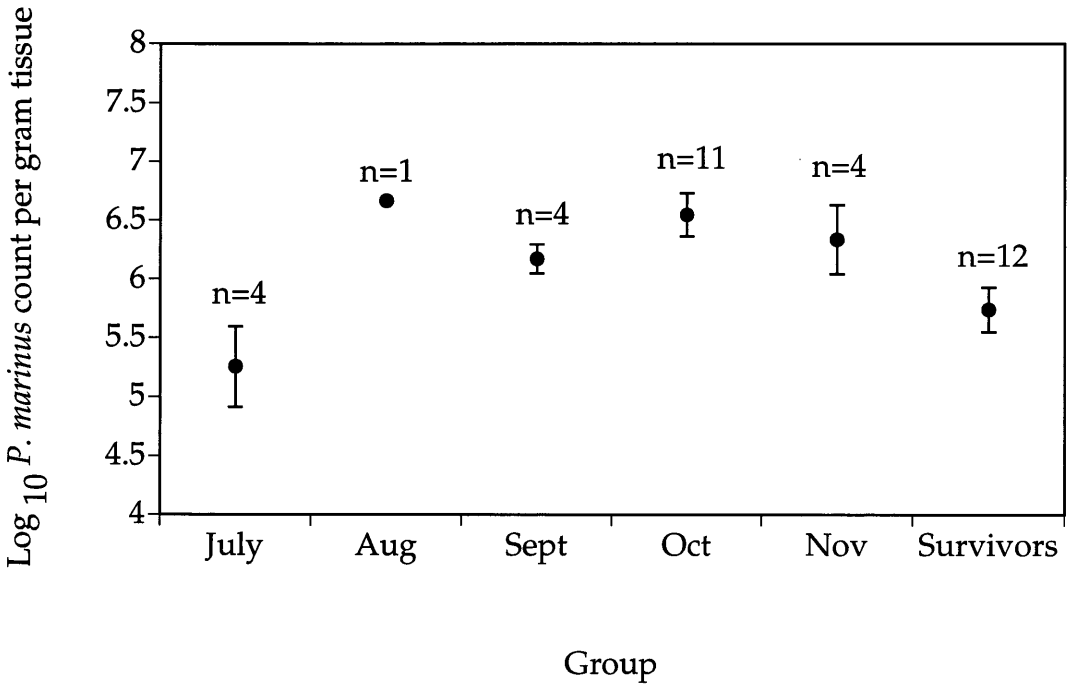


TABLE 3. Results of analysis of variance for *P. marinus* abundance in body burdens from each month in the study and for body burdens of survivor oysters at the conclusion of Experiment #1.

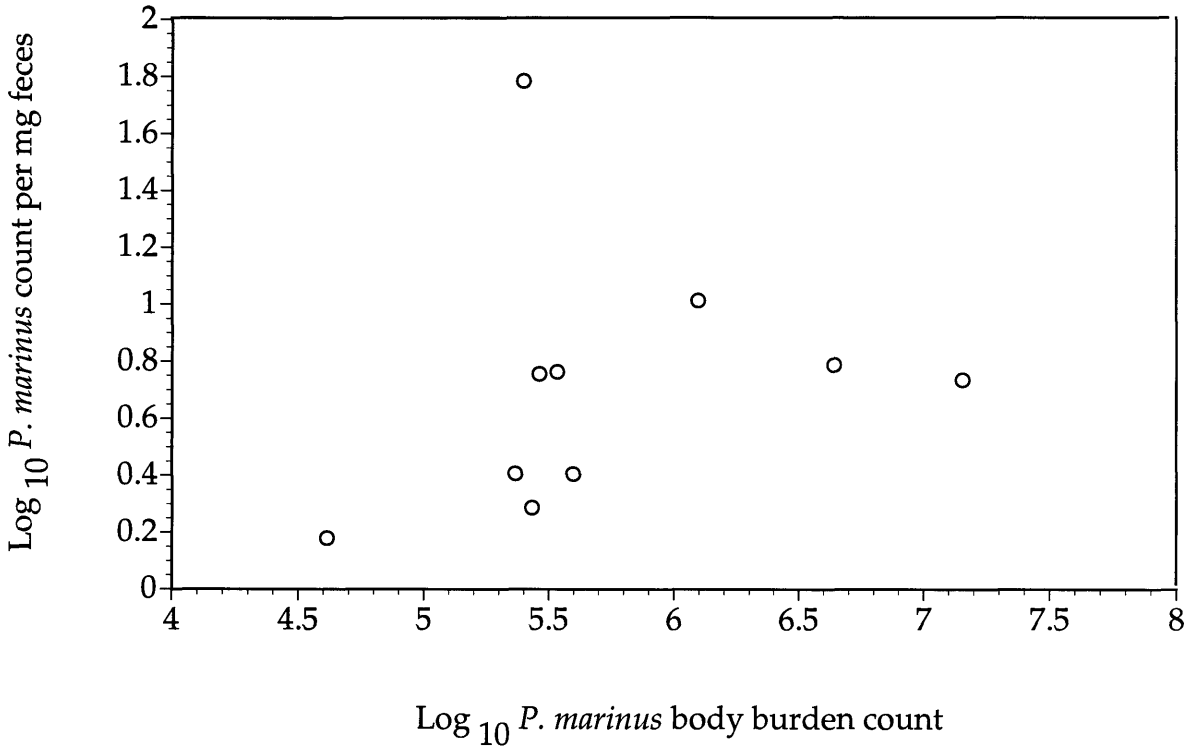
Date	DF	Sum of Squares	Mean Square	F-Value	P-Value
Month	5	7.146	1.429	3.883	0.0078

TABLE 4. Results of Fisher's PLSD multiple comparison test for *P. marinus* abundance in body burdens from each month in the study and for body burdens of survivor oysters at the conclusion of Experiment #1. Means sharing like superscripts do not significantly differ from each other.

Group	Mean	Standard Deviation
July	5.256^{a}	0.679
August	6.667^{bc}	
September	6.171^{bc}	0.245
October	6.548^{b}	0.608
November	6.337^{bc}	0.590
Survivor	5.741^{ac}	0.656

FIGURE 25. Experiment #1 scatter plot of weight-standardized body burden and final sampling month mean *P. marinus* abundance per mg feces for individual oysters.

Scatter Plot of Final Fecal Sample and Body Burden Parasite Abundance



Experiment #2 - Dosing Experiment

Resulting infections. All oysters in the experimental recipient group that were dosed with experimental source oyster feces became infected with the parasite resulting in 100% prevalence, with a range of infection intensities from 3-128 cells/oyster (Table 5).

Doses. The number of *P. marinus* cells that each dosed oyster was exposed to for each day was calculated, along with the sum, mean and standard deviation (Table 6). The *P. marinus* cells in each dose ranged from 94-5648 cells per oyster with the mean dose over the 20 days being 1532 cells/oyster and the total dose over the 20 days being 19569 cells/oyster. There was a high amount of daily variability in dose parasite abundance, much like the variability seen in the Experiment #1 individual sampling day mean fecal parasite abundance in Figure 23.

TABLE 5. Results of Experiment #2 fecal dosed oysters.

Group	n	Prevalence	Infection Intensity
Dosed	16	100%	3-128 cells/oyster
Control	17	0%	0 cells/oyster

TABLE 6. Number, sum, mean and standard deviation of *P. marinus* cells seen by each oyster for each day dosed in Experiment #2.

Dosing Day	*P. marinus* cells/oyster
1	5648
2	558
3	478
4	122
5	790
6	459
7	458
8	227
9	94
10	335
11	367
12	484
13	540
14	5038
15	558
16	900
17	1610
18	307
19	448
20	148
Sum	19569
Mean	978.5
SD	1532.1

Experiment #3 - Paired Experiment

Resulting infections. All oysters in the experimental recipient group paired with the oysters in the experimental source oyster group became infected with the parasite resulting in 100% prevalence with a range of infection intensities from 13-27,500 cells/oyster (Table 7).

Source hemolymph & recipient body burden parasite abundance correlation and regression. The results of the correlation analysis of source oyster hemolymph *P. marinus* abundance at the start of exposure with recipient oyster body burden *P. marinus* abundance at its conclusion were not found to be significant (Fisher's r=0.350, p=0.1876) (Figure 26).

Source & recipient body burden parasite abundance correlation and regression. The results of the correlation analysis of source oyster body burden and recipient oyster body burden *P. marinus* abundance, both at the end of exposure, were not found to be significant (Fisher's r=0.152, p=0.5813) (Figure 27).

TABLE 7. Results of Experiment #3 paired oysters.

Group	n	Prevalence	Infection Intensity
Exposed	17	100%	13-27,500 cells/oyster
Control	15	0%	0 cells/oyster

FIGURE 26. Experiment #3 scatter plot of source oyster hemolymph and recipient oyster weight-standardized body burden *P. marinus* abundance.

Scatter Plot of Source Oyster Hemolymph and Recipient Oyster Body Burden Parasite Abundance

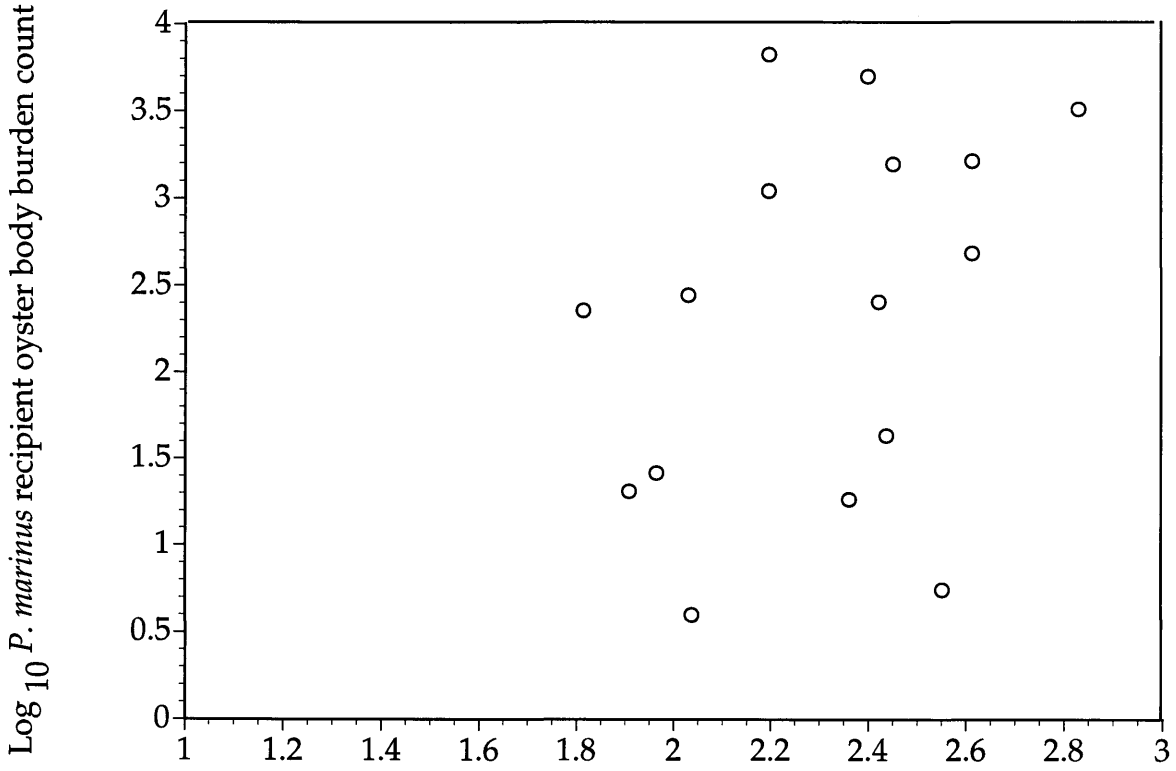
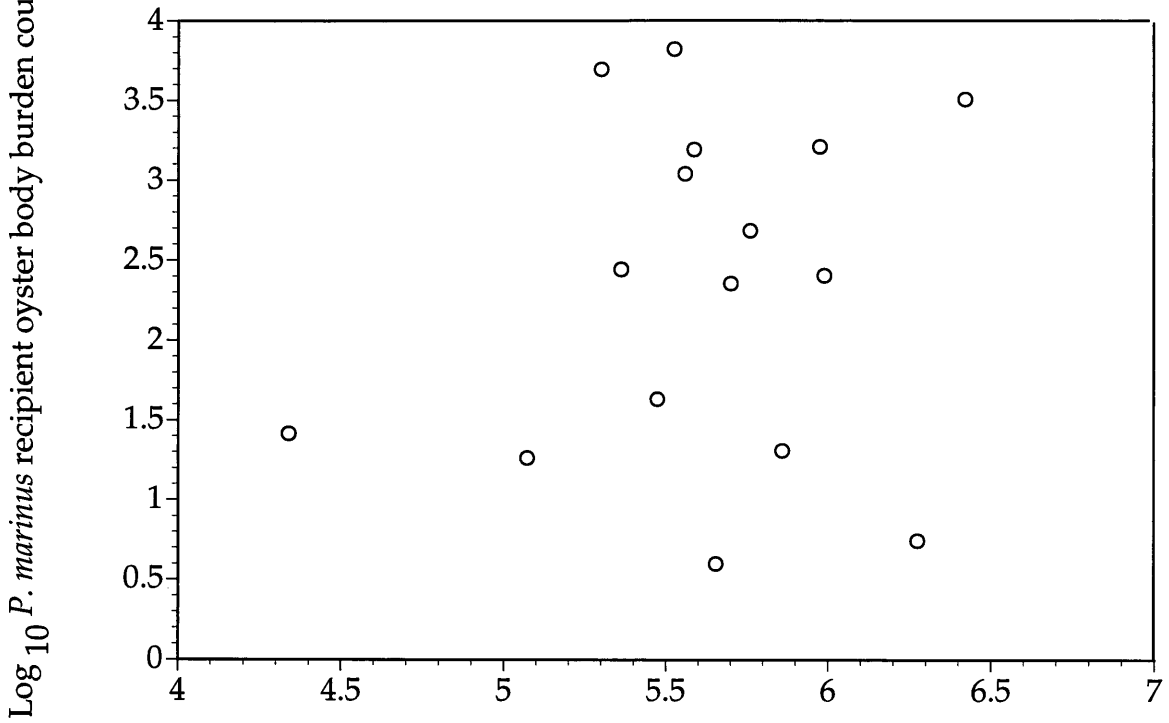


FIGURE 27. Experiment #3 scatter plot of source oyster weight-standardized body burden and recipient oyster weight-standardized body burden *P. marinus* abundance.

Scatter Plot of Source Oyster Body Burden and Recipient Oyster Body Burden Parasite Abundance



DISCUSSION

Previous investigations have shown that *Perkinsus marinus* is present in the fecal matter of infected oysters (Ford 1996a, Bushek et al. 1997) and that an increase in fecal parasite abundance occurs as infections become more intense (Ford 1996a). Fecal parasite abundance was found to be highly correlated with infection intensity and days to death (Ford 1996a). The presence and abundance of *Perkinsus marinus* in eastern oyster fecal matter was again documented in Experiment #1, the infection progression experiment of this study, and the relationship of fecal parasite abundance to infection intensity was investigated again as well.

In Experiment #1, fecal parasite abundance increased through the September sample date as infections became more intense in the experimental animals as estimated by the hemolymph assay. This increase in fecal parasite abundance can be explained by the sloughing of hemocytes, parasites, and digestive epithelium into the lumina of the stomach and intestine that occurs as infections intensify (Mackin 1951, Ford 1996b). The amount of feces being produced by the experimental oysters decreased monthly from July through October and then increased in November. The amount of feces being produced by the control oysters decreased monthly as well from July through September and then increased through October and November.

This decrease in fecal production in *P. marinus*-infected oysters was investigated by Hewatt (1952), who reported that infected Gulf of Mexico oysters often failed to open and feed. Mackin and Ray (1954) found that production of feces and pseudofeces declined in proportion to both the

intensity and duration of infection. Feces and pseudofeces produced by experimentally infected oysters declined by 40% compared to untreated controls over a 3-week period in their study. Oysters with light and moderate to heavy infections produced only 57% and 43% respectively, and as much feces and pseudofeces as did uninfected or very lightly infected oysters (Mackin and Ray 1954). Decreased fecal volumes may also be partly attributed to cold water temperatures experienced by the oysters during the last two months of the study, because of the problems experienced with the heat exchanger in the wet lab explained in the materials and methods section of this document. In October and November of 1996, York River ambient water temperatures went as low as 15.5°C. Although once the water was equilibrated to room temperature it was maintained at 23-27°C, the brief exposure to the much colder water temperatures, most likely no more than 2 hours until the water equilibrated, may have shocked the oysters and caused a decrease in fecal production.

The decrease in fecal parasite abundance in the last two months of the study could have been the result of the destruction of the gut epithelium due to the *P. marinus* infection. This may have led to a decreased ability to sort and discard the parasites by the gills and palps (Ford 1996b) in the most heavily infected oysters, resulting in a lower parasite discard rate via the feces. Conversely, some of the oysters that survived up until and through the October and November sample dates may have been genetically resistant to *P. marinus* infections and as such, had a lower parasite abundance in their tissue and subsequently in their feces. This type of genetic resistance has been previously documented for *Haplosporidium nelsoni* (MSX) disease in eastern oysters (Ford 1988). Between the September and October sampling period, the n was reduced by 50% as many of the more heavily infected

oysters had died. As such, the oysters that were still being sampled may have had less intense infections as a result of resistance to the parasite. These two factors together, the decrease in fecal parasite release by the most heavily infected oysters that were still alive, and the resistant, less heavily infected oysters that had lower parasite burdens, and therefore lower fecal parasite abundances, could have contributed to the reduction in fecal parasite abundance seen in the last two months of the study. The non-survivor oysters exhibited a higher hemolymph and fecal parasite abundance for the entire study as compared with the survivor oysters' hemolymph and fecal parasite burdens.

The increase in the mean fecal weight produced by the experimental oysters in November and the concomitant decrease in mean fecal parasite abundance would not be expected with increasing infection intensities as measured by the hemolymph assay. If the oysters that survived through the November sampling period were genetically resistant to *P. marinus* infections, they perhaps could harbor a greater number of parasite cells without the infection becoming lethal. In essence, the resistant oysters may have had a higher tolerance for high levels of *P. marinus* cells. The survivor oysters did not have *P. marinus* body burdens that significantly differed from any of the months in the study with the exception of October. As such, this higher tolerance for levels of *P. marinus* that might prove lethal to other oysters seems likely. This tolerance concept was employed by Ford (1988) to explain resistance to MSX disease in oysters.

A high amount of variability was observed among the individual fecal sampling days for each month, and a significant amount of variability was present among the July and August samples. Given this high variability, as well as the relatively weak correlation between the

hemolymph and fecal parasite abundances, the use of the fecal assay as a diagnostic tool for *P. marinus* infections may be precluded despite its advantages of being both noninvasive and nondestructive. Perhaps the fecal assay could be used if sampling was conducted over several days in order to counteract this high variability. Another problem with the feasibility of the fecal assay as a reliable diagnostic tool is the labor intensive nature of the assay when used for diagnosing light to moderate infections. This is because the fecal parasite abundance tends to be one order of magnitude less than the hemolymph parasite abundance in the same oyster. As such, fewer *P. marinus* cells are present in the feces of an infected oyster than are in the hemolymph of the same oyster. This greatly increases the microscopy time necessary for diagnosis and gauging of infection intensity. This difference in parasite abundance between the two assays is not what would be expected based on Ford's (1996a) study. In that study, the number of *P. marinus* cells in the feces increased an order of magnitude with each order of magnitude increase in infection level for the oyster. The discrepancy between the fecal parasite abundances found in Ford's 1996 study and this project may have been a result of data analysis. In this project, all fecal data were weight-standardized prior to analysis, but in Ford's study the data were not weight-standardized.

The decrease in fecal production from very heavily infected oysters also becomes a problem with the fecal assay, since sample collection itself is complicated, coupled with the difficulty involved with finding *P. marinus* cells in this substantially decreased fecal volume. Nevertheless, in situations where using a diagnostic tool which is both noninvasive and nondestructive is paramount, the disadvantages of the fecal assay may be outweighed, in light of the alternative methods available. The invasive

nature of the hemolymph assay and the destructive nature of both Ray's tissue assay and the body burden assay would make these methods inappropriate in a situation where leaving the oyster as undisturbed as possible was a priority.

Previous investigations have documented that transmission of *P. marinus* can occur between oysters held in close proximity (Ray 1954, Andrews 1965, 1967). Transmission of *P. marinus* infections between live oysters held in close proximity was again documented in Experiment #3, the paired experiment, of this study. All of the experimental oysters exposed to the parasite acquired *P. marinus* infections, resulting in 100% prevalence. In addition to transmission of *P. marinus* via fecal matter from the source oyster, several other methods of transmission could have contributed to the ensuing infections in the paired experiment. These other possible modes of infection include source oyster excretory activities, source oyster spawning activities (Ragone Calvo and Burreson et al. 1995), sloughing of cells from the source oyster, or release of mantle fluid from the source oyster.

Resulting infections in this experiment were relatively heavy compared with those infections acquired in the fecal dosing experiment, Experiment #2. These heavier infections could have resulted from the paired experiment oysters experiencing greater exposure to the parasite relative to the oysters in the dosing experiment. In the paired experiment, the uninfected oyster was held in the same container with the infected oyster for a prolonged period of time, unlike the oysters in the dosing experiment which were exposed only when dosed. This potentially provided the uninfected oyster with a more constant exposure to the parasite cells, perhaps as a result of other transmission mechanisms such as source oyster excretory or spawn activities. The exposure to fecal *P. marinus*

cells was most likely equivalent for the two groups, as the oysters in the dosing experiment received feces discharged by the source oysters over a 24 hour period. However, the potential for exposure to additional transmission mechanisms is greater for the oysters in the paired experiment.

The transmission of *P. marinus* infections via parasite cells in the fecal matter of infected oysters has not been documented previous to this study. In Experiment #2, the dosing experiment of this project, the transmission of *P. marinus* infections by fecal matter from infected oysters was documented. All of the experimental oysters acquired *P. marinus* infections, resulting in 100% prevalence. Inasmuch as the only exposure that the oysters had to *P. marinus* in this experiment was through fecal matter from infected oysters since they were being held in filtered water and in individual containers, the infections that were acquired by these oysters must have resulted from parasites released in the feces. Ensuing infections in this experiment were fairly light as compared with those infections acquired in the paired oyster experiment. As discussed previously, it is likely that less exposure to the parasite was experienced by the oysters in this experiment relative to the oysters in the paired experiment. This is because in the dosing experiment, the only exposure was from being dosed with feces from infected oysters, and the oysters were not exposed to the alternative methods of transmission that the oysters in the paired experiment were.

Since infections resulted from this dosing, it can be assumed that the minimal dose requirement to cause a *P. marinus* infection was satisfied. In a study by Chu (1996), the minimal dose required to cause an infection was investigated. The lowest dose that initiated a *P. marinus* infection was

between 10 and 10^2 meronts or prezoosporangia per oyster. No mortalities occurred during this study (Chu 1996). This minimal dose was exceeded in every one of the daily doses that were administered to the treated oysters in Experiment #2.

The documentation of fecal transmission of *P. marinus* infections in a laboratory setting has implications for the prevailing conceptual model of *P. marinus* transmission in the Chesapeake Bay. Gapers, although they contain very high numbers of parasites that are mobilized and released into the water column upon death and decomposition of the oyster or predation of the remaining oyster meat, are a one time acute dose of *P. marinus* cells into the water column. Although the feces of live infected oysters contains much lower levels of the parasite than does the oyster meat of a gaper, it is constantly being produced and as such, may serve as a long term, low level dose of *P. marinus* cells. The contribution of *P. marinus* cells released in the feces relative to the contribution of parasites by gapers and other transmission mechanisms such as spawning can only be speculated at this point, as no field studies have been conducted.

Roberson et al. (1995) conducted a flow cytometric enumeration of *P. marinus* cells in Chesapeake Bay waters and observed that water column counts of the parasite did not directly correspond with the death of local oysters. They hypothesized that a source, or sources of ambient *P. marinus* other than the release of the cells from gapers exists. In the absence of release of the parasites by mortality during the winter when little mortality is observed, the presence of *P. marinus* cells in the water column could be explained by an environmental release from another source. They postulated that the major contribution of infectious particles as pre-mortem release may occur while the oyster is living, but heavily infected. Roberson

et al. (1995) concluded that infection of oysters by *P. marinus* cells released from tissues after the death of infected oysters does not appear to adequately explain the infection cycle.

When relating the contribution of fecal transmission to the overall *P. marinus* transmission dynamics in nature, the amount of feces produced by an individual oyster must be considered, along with the number of parasite cells in that feces as well as the fate of the feces. Although the amount of fecal matter produced by an individual may not be substantial enough to make a significant contribution of parasite cells to enzootic waters, the fecal matter produced by an oyster reef as a whole may be making a long term, low level contribution. This fecal parasite contribution may be more of local importance rather than playing a role in geographically wide scale transmission dynamics. In a study by Haven and Morales-Alamo (1966), oyster feces and pseudofeces were labelled with flourescent particles having similar physical characteristics to the biodeposits. Sediment samples were examined at different time intervals after the oysters were placed on the river bottom. These samples showed that a portion of the flourescent particles, and thus the feces and pseudofeces, remained on the bottom while others were progressively incorporated into subsurface layers as demonstrated by the distribution of particles in successive cores. The depth that some particles reached over one month increased to a depth of 7.0cm below the surface sediment. However, many particles remained on the surface after one month had elapsed (Haven and Morales-Alamo 1996). The residence of these particles at or near the surface sediments supports the idea that fecal parasite transmission may be important locally rather than over large geographic distances. Perhaps fecal transmission is a relatively

important transmission mechanism among the oysters in a single reef or between neighboring reefs.

Depending on bottom current speeds, the friction of the water moving over the seafloor may create physical mixing of the bottom water causing a benthic boundary layer. Turbulence in the benthic boundary layer can result in resuspension of bottom sediments and light organic particles will reach maximum concentrations some distance above the bottom (Lalli and Parsons, 1993). In areas where the benthic boundary layers prevail, the bottom currents may resuspend the fecal matter and move it along the bottom thereby moving the parasite cells away from the local area while simultaneously breaking up the fecal pellet. This transport creates the potential for wider scale *P. marinus* fecal transmission.

The actual fate of the *P. marinus* cells within the feces is poorly understood. Eventually the parasite cells most likely become liberated from the feces as the fecal ribbon tends to become more flocculent and less distinct over a time period of about 24 hours. Once this occurs, the parasite cells may behave much like the parasite cells resulting from a gaper and become suspended in the water column. The *P. marinus* cells in the feces used to dose the experimental oysters in the experiment were most likely liberated from the fecal material by the vortexing that was necessary to resuspend the pellet after weighing it. As such, it has not yet been determined whether the *P. marinus* cells are infective when still bound to the feces, or if liberation of the parasite cells from the fecal matter is a necessary step for transmission to occur.

The duration of the infection window of *P. marinus* cells has not been determined, however it is at least a minimum of 24 hours as demonstrated in Experiment #2. In order for fecal transmission to occur,

the feces containing the infective particles must be in proximity to an uninfected oyster within the infection window of the *P. marinus* cells.

In conclusion, this investigation has demonstrated that fecal transmission of *Perkinsus marinus* can occur in oysters. The relative role of fecal transmission compared with other transmission mechanisms is poorly understood. Nevertheless, fecal elimination is a means for the parasite to be continually released over a period of time in order to find and infect another host. The demonstration of the infectivity of feces from *P. marinus*-infected oysters further elucidates the overall transmission dynamics of the disease. The release of the parasites upon death and decomposition of infected oysters as the primary transmission mechanism did not seem likely (Burreson and Ragone Calvo 1996) in light of the rapid predation upon the dead oyster meat by scavengers (Hoese 1964). Although the overall *P. marinus* transmission dynamics are still not completely understood, the documentation of fecal transmission of the parasite clarifies part of the dynamics that were not previously explained. Further study will be necessary in order to clearly identify the role that fecal transmission of the parasite plays relative to other transmission mechanisms in nature.

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